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Enterococcus faecium as a *Salmonella* surrogate in the thermal processing of wheat flour: Influence of water activity at high temperatures

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ABSTRACT

This study investigated the influence of temperature-dependent water activity (a_w) on thermal resistances of *Enterococcus faecium* NRRL B-2354 (*E. faecium*) and *Salmonella* Enteritidis PT 30 (*S.* Enteritidis) in wheat flour. The a_w for wheat flour samples at 20, 40, and 60 °C was determined by a vapor sorption analyzer and at 75, 80 and 85 °C using custom-built thermal cells with high temperature humidity sensors. Full-factorial isothermal inactivation studies of both strains in sealed aluminum-test-cells included three temperatures (75, 80, and 85 °C) and three $a_{w,25°C}$ levels (0.30, 0.45 and 0.60 within ±0.02 range, prior to the thermal treatments).

Isotherm results of wheat flour demonstrate a significant increase ($P < 0.05$) of a_w as temperature rises (e.g. $a_{w,25°C} = 0.45 \pm 0.02$ became $a_{w,80°C} = 0.71 \pm 0.02$ in a closed system). Inactivation kinetics of both microorganisms fitted a log-linear model, the yielded D-values varied from 2.7 ± 0.2 min ($D_{85°C}$ of *S.* Enteritidis at $a_{w,25°C}$ 0.60 ± 0.02) to 65.8 ± 2.5 min ($D_{75°C}$ of *E. faecium* at $a_{w,25°C}$ 0.30 ± 0.02). The z_T of *E. faecium* and *S.* Enteritidis decreased from 16.4 and 16.9 °C, respectively, to 10.2 °C with increased moisture content (dry basis) from 10 to 14%.

Under all tested conditions, *E. faecium* exhibited equal or higher (1.0–3.1 times) D- and z_T-values than those of *Salmonella*. Overall, *E. faecium* should be a conservative surrogate for *Salmonella* in thermal processing of wheat flour for control of *Salmonella* over a moisture content of 10–14% and treatment temperatures between 75 and 85 °C.

1. Introduction

Outbreaks of salmonellosis have been associated with consumption of low-moisture foods such as flour (Food Safety, 2008), coated snacks (Sotir et al., 2009), peanut butter (CDC, 2007) and raw almonds (Isaacs et al., 2005). Thermal processing is an effective method for pathogen control in food products. Validation of such processes in manufacturing facilities is critical to eliminating potential *Salmonella* contamination of low-moisture foods (Ceylan and Bautista, 2015). A surrogate, non-pathogenic bacterium with similar characteristics to the target pathogen with equal or higher thermal resistances, is often used to study the fate of the pathogen in thermal processes (FDA, 2015a).

Enterococcus faecium NRRL B-2354 (*E. faecium*) has been identified as a *Salmonella* surrogate in thermal processes of low-moisture foods by the Almond Board of California (ABC, 2014), American Food Industry Association (AFIA, 2010), and American Spice Trade Association (ASTA, 2013). Comparison of thermal resistance parameters of *E. faecium* and the target pathogen *Salmonella* has been reported for various low-moisture foods such as carbohydrate-protein meal in thermal extrusion (Bianchini et al., 2014), pet food products (Ceylan and Bautista, 2015), and wheat flour (Liu et al., 2018). *E. faecium* shows higher thermal resistance than *Salmonella* under tested conditions. But, overly high thermal resistance (17.7 times) of *E. faecium* compared to that of *Salmonella* was also reported (Ma et al., 2007), which reveals limitations of using *E. faecium* as a surrogate for *Salmonella* in process validation. Comprehensive thermal resistance comparison between *E. faecium*

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and *Salmonella* over a wide range of product moisture content and temperature is desirable for developing a proper validation procedure for thermal treatment of a specific food system.

An external but key factor that influences bacterial survivability and thermal resistance in low-moisture foods is water activity (a_w). A_w is defined as the ratio between water vapor pressure in a food matrix and the corresponding vapor pressure of pure water at the same temperature of the food (FDA, 2015b). Due to the limited temperature range of commercial a_w meters (20–60 °C), most published studies fail to estimate the a_w of food matrices at temperatures above 60 °C (Syamaladevi et al., 2016b). Consequently, most previous studies relate bacterial thermal resistance in low-moisture foods to the a_w measured at 25 °C ($a_{w,25°C}$), not at the treatment temperature (Bari et al., 2009; FDA, 2015b; Kataoka et al., 2014; Laroche et al., 2005). Recently, a thermal cell with relative humidity sensors was designed to measure a_w of foods above 60 °C (Syamaladevi et al., 2016a). This study revealed significant changes ($P < 0.05$) of a_w in all-purpose wheat flour and peanut butter when temperature increased from 20 °C to 80 °C. The authors concluded that temperature-induced changes in product a_w cause vast differences in thermal resistance of microorganisms in different food matrices (Syamaladevi et al., 2016a). Therefore, it is critical that we understand how a_w changes in a specific food system at high temperatures and establish relationships between thermal resistance of target pathogens and selected surrogates when developing and validating thermal treatments for control of food pathogens in such food systems.

The overall goal of this study was to compare thermal resistance of *E. faecium* with *S.* Enteritidis in wheat flour at three temperatures (75, 80, and 85 °C) and $a_{w,25°C}$ levels (0.30 ± 0.02, 0.45 ± 0.02, and 0.60 ± 0.02 at 25 °C). *S.* Enteritidis was chosen as the target pathogen because of its high thermal resistance in low a_w systems (Jeong et al., 2009; Smith et al., 2016; Wang et al., 2013), and its association with a raw almond outbreak (CDC, 2004). Specific objectives were to:1) obtain water sorption isotherms of wheat flour at 20–85 °C and evaluate changes of a_w at different temperatures in wheat flour, 2) estimate a_w of wheat flour samples at elevated temperatures, 3) model thermal inactivation kinetics of *E. faecium* and *S.* Enteritidis in wheat flour at designated $a_{w,25°C}$ levels and compare their thermal resistance parameters, and 4) analyze the thermal resistance parameters of both strains with a_w at treatment temperatures and moisture contents.

2. Materials and methods

2.1. Materials

The soft winter organic wheat flour was purchased from Eden Foods (Clinton Township, MI). Bacterial strains (*E. faecium* and *S.* Enteritidis) were acquired from Dr. Linda Harris (University of California, Davis) and kept at −80 °C in tryptic soy broth (TSB) supplemented with 20% (vol/vol) glycerol. Tryptic soy broth (TSB), tryptic soy agar (TSA), yeast extract (YE) and peptone were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ); ammonium iron (III) citrate was purchased from Sigma-Aldrich Corporation (St. Louis, MO); sodium thiosulfate, pentahydrate was from J. T. Baker (Center Valley, PA).

2.2. Wheat flour characterization

Initial a_w was measured with an a_w meter (AQUA PRE, METER group, Pullman, WA). The geometric mean particle size was obtained with an ATM sonic sifter (ATM Corporation, Milwaukee, WI). Chemical composition of wheat flour was analyzed by Silliker, Inc. Northern California Laboratory, Salida, CA, using the analytic methods ash (AOAC 945.46), dietary fiber (AOAC 991.43 Mod.), fat (AOAC 935.38, mojo, acid hydrolysis), moisture (AOAC 927.05), and protein (AOAC 991.20.I). The mesophilic microflora count in wheat flour was enumerated for five random 1 g samples diluted in 9 mL of 0.1% peptone water, plated on TSA and incubated for 48 h at 35 ± 2 °C.

2.3. Water sorption isotherms at elevated temperature

2.3.1. Water isotherm generation at 20, 40, and 60 °C

Water sorption isotherms of wheat flour at 20, 40 and 60 °C were acquired with a vapor sorption analyzer (VSA, Decagon Devices Inc., Pullman, WA) following the dynamic vapor sorption method as previously described (Yu et al., 2007). Briefly, the moisture content of wheat flour was obtained by placing 3–5 g of equilibrated samples in a vacuum oven with 10 kPa pressure inside at 80 °C for 10 h, per AOAC 927.05. A small amount of wheat flour (2–3 g) in a metallic cup was placed in the equipment, and the initial moisture content of the sample was input into the program. Then, the VSA was programmed to change a_w from 0.1 to 0.9 (at 0.1 intervals) and back to 0.1 at 20, 40 or 60 °C. In the VSA, changes in sample weight were registered 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 change data at each equilibration step. All data points were averages of at least two samples of the wheat flour.

2.3.2. Sample preparation of water isotherm generation above 60 °C

The maximum test temperature for the VSA was 60 °C. Thus, custom-made test cells with high temperature relative humidity sensors made by our research group in collaboration with METER group (Pullman, WA) were used to generate water sorption isotherms at temperatures above 60 °C (Syamaladevi et al., 2016a). Prior to the tests, wheat flour samples were either vacuum-dried for 12 h at 10 kPa absolute pressure in a vacuum oven (Yamato Scientific America Inc., CA) set at 50 °C to generate adsorption isotherms, or conditioned in a humidity chamber for 24 h to reach $a_{w,25°C} \approx 1$ for desorption isotherm generation. Conditioned samples were then equilibrated to different $a_{w,25°C}$ in an air-tight container. Different saturated salt solutions (corresponding relative humidity level)—LiCl (11.3%), CH_3COOK (22.5%), $MgCl_2$ (32.8%), K_2CO_3 (43.2%), $MgNO_3$ (52.9%), $NaNO_2$ (65.8%), NaCl (75.3%) or KCl (84.3%) (Fisher Scientific, Houston, TX) —were used in the container to equilibrate samples to $a_{w,25°C}$ of 0.11–0.84, respectively (Greenspan, 1977).

A preconditioned sample (2–3 g, described in section 2.3.2) was transferred into a thermal cell containing a commercial relative humidity sensor (HX15-W, Omega Engineering, Inc.). Sealed thermal cells were placed in a forced air convection oven (Yamato Scientific America Inc., CA, USA) and allowed to slowly reach a set temperature to record the a_w at the set temperature inside the test cells. Then, the sealed cell was removed from the oven and kept at room temperature for approximately 30 min to reach ambient temperature (23 ± 1 °C). The moisture content was obtained as described in section 2.3.1. Water isotherms were generated at 75, 80 and 85 °C, using samples preconditioned to $a_{w,25°C}$ of 0.30 ± 0.02, 0.45 ± 0.02, and 0.60 ± 0.02 (0.02 is a range of $a_{w,25°C}$ acceptable for these experiments).

2.4. Preparation of inoculated wheat flour

The absence of *Salmonella* and *E. faecium* in wheat flour samples was confirmed by diluting 10 random 1 g subsamples in 9 ml 0.1% peptone water, and then plating on TSA supplemented with 0.6% (w/v) YE, 0.03% sodium thiosulfate and 0.05% ammonium ferric

citrate (designated modified TSAYE) or m-*Enterococcus* selective agar (Neogen Inc. Lansing, MI), respectively. All plates were incubated at 35 ± 2 °C for 48 h.

Inoculum preparation: The inoculum was prepared per guidelines published by the Almond Board of California (ABC, 2014) with some modification. Briefly, both microorganisms were subjected to two consecutive transfers (24 h incubation periods at 35 ± 2 °C) in 9.0 ml TSB, and then 1 ml of TSB inoculum was surface-plated onto TSA plates (150 × 15 mm) to obtain bacterial lawns (Hildebrandt et al., 2016). After incubation for 24 h at 35 ± 2 °C, bacterial lawns were harvested with 7 mL of 0.1% peptone water per plate, using a sterile L-shaped glass rod to dislodge bacterial cells. The collected *E. faecium* or *S.* Enteritidis suspension was centrifuged for 15 min at 6000 × g at 4 °C, and the resulting pellet re-suspended in 1/10 volume of 0.1% peptone water to obtain ~10^{10} CFU/ml *E. faecium* or *S.* Enteritidis suspension. One ml of each concentrated bacterial strain was hand mixed into 10 g of flour in a sterile stomacher bag until the pellet was visibly integrated into the sample with no clumps. After mixing, this "seed" flour sample was used to further inoculate 90 g of flour, which was mixed and hand-shaken for approximately 3 min. Ten 1 g samples were randomly collected and plated to confirm the uniform distribution of the inoculum in the flour.

Equilibration: The inoculated samples were spread evenly onto two 150 × 15 mm petri dishes without lids and placed in an equilibration chamber custom-made at Michigan State University (Smith and Marks, 2015). The inoculated samples were maintained in the chamber for a minimum of four days to achieve the targeted $a_{w,25°C}$ of 0.30 ± 0.02, 0.45 ± 0.02, and 0.60 ± 0.02 with corresponding moisture content of 10, 12, and 14 ± 2% (dry basis). The population of *E. faecium* and *S.* Enteritidis across all inoculated samples was 7.5 ± 0.2 log CFU/g after equilibration.

2.5. Thermal treatment and survival enumeration

A full factorial experiment was performed at three inactivation temperatures (75, 80 and 85 °C) and at three constant water activities (0.30 ± 0.02, 0.45 ± 0.02 and 0.60 ± 0.02) measured at 25 °C ($a_{w,25°C}$). All tests were conducted independently in triplicate.

Samples were loaded into thermal-death-time cells (18 mm inner diameter with 4 mm height) designed at Washington State University (Chung et al., 2008), sealed tightly, and immersed in an oil bath (Neslab GP-400, Newington, NH) filled with ethylene glycol (VWR International, Radnor, PA) at 75, 80 or 85 °C. The come-up time for the sample core to reach target temperature ±0.5 °C was approximately 150s, measured with a T-type thermocouple (Omega Engineering, Inc., Stamford, CT) located at the center of a test cell with a non-inoculated sample. Cells were removed at predetermined time intervals and immediately placed in an ice-water bath to stop thermal inactivation (Temperature < 25 °C in ~30 s).

To enumerate survivors, thermally treated wheat flour samples were transferred from test cells into sterile stomacher bags, diluted 1:9 with 0.1% peptone water, and homogenized for 3 min at 260 rpm with a Seward Stomacher (Seward, London, UK) (Harris et al.). Appropriate serial dilutions were plated in duplicate onto TSA (for *E. faecium*) or modified TSAYE (for *S.* Enteritidis). The plates were incubated at 35 ± 2 °C for 48 h, when survivor colonies were counted.

2.6. Modeling of kinetic inactivation

Two primary models are used to describe inactivation curves. The first-order kinetic, or log-linear, model has been primarily used to characterize bacterial inactivation (E.q. 1) along with the Weibull model (E.q. 2) (Gaillard et al., 1998) as:

$$\log\left(N/N_0\right) = -t/D \tag{1}$$

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

where log reduction ($\log\left(N/N_0\right)$) was calculated by dividing survivor counts (N) at treatment time t (min) by the population at time zero (N_0), where D is the time (min) required to reduce the microbial population by 90% at a specified temperature (°C), where δ refers to the time to first decimal reduction, and n describes the general shape of the curve: linear ($\alpha = 1$) or nonlinear ($\alpha \neq 1$) with a decreasing ($\alpha < 1$) or increasing ($\alpha > 1$) inactivation rate with time. The Integrated Pathogen Modeling Program (IPMP) (Huang, 2014) was used to obtain the model parameters.

Data were fitted to the models, and the goodness of fit for each candidate model was quantified by the root mean square error (RMSE) (log CFU/g), accuracy factor (A_f), and bias factor (B_f) (Motulsky and Christopoulos, 2004)

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}\left[\log\left(\frac{N}{N_0}\right)_{data,i} - \log\left(\frac{N}{N_0}\right)_{model,i}\right]^2}{n-p}}, \tag{3}$$

$$A_f = 10^{\frac{\sum_{i=1}^{n}\left|lg\frac{\log\left(\frac{N}{N_0}\right)_{model,i}}{\log\left(\frac{N}{N_0}\right)_{data,i}}\right|}{n}} \tag{4}$$

$$B_f = 10^{\frac{\sum_{i=1}^{n}lg\frac{\log\left(\frac{N}{N_0}\right)_{model,i}}{\log\left(\frac{N}{N_0}\right)_{data,i}}}{n}} \tag{5}$$

where $\log\left(\frac{N}{N_0}\right)_{data,i}$ is the measured log reduction, $\log\left(\frac{N}{N_0}\right)_{model,i}$ is the predicted log reduction from the model, n is the total number of observations, and p is the number of model parameters. All three factors were estimated for three biological independents together. The integrated pathogen modeling program (IPMP) (Huang, 2014) was used for modeling with least RMSE. All three factors, RMSE, A_f, and B_f were used to compare the fitting effect of log-linear and Weibull models. Differences between D-values among samples were evaluated using ANOVA in Minitab 14 (Minitab Inc., State College, PA).

When the log-linear model fits well to microbial inactivation curves, the log of D-values can be plotted against temperature, this is generally referred as the thermal death time curve. The z_T-value required to change the D-value of target microorganisms by 90% (1 log) in specific low-moisture foods may be determined by (Gaillard et al., 1998)

$$z_T = \frac{T_2 - T_1}{\log\left(D_1/D_2\right)}. \tag{6}$$

We consider moisture content as a control parameter in closed systems. Therefore, we plotted the z_T-values against moisture contents of tested samples to understand how z_T-values changed with sample moisture content.

3. Results

3.1. Wheat flour

The initial a_w of the wheat flour sample was 0.30 ± 0.05 (measured at 25 °C). The geometric mean particle size of wheat flour was 144 ± 60 μm. Its chemical composition is given in Table 1. Neither *Salmonella* nor *E. faecium* was detected in the flour before inoculation. The background mesophilic microflora count in wheat flour was 2.20 ± 0.45 log CFU/g, low enough that it shouldn't interfere with the inactivation treatment counts (Villa Rojas, 2015).

3.2. Water sorption isotherms of wheat flour

The isotherms of wheat flour at 20, 40, 60 and 80 °C are presented in Fig. 1 a and b. Both adsorption and desorption curves shifted to the right as temperature increased. Hysteresis, the gap between the adsorption and desorption isotherms (Rockland and Stewart, 2013), was observed in wheat flour at 20 and 40 °C. The degree of hysteresis decreased with increasing temperature: no hysteresis was observed for isotherms of wheat flour measured at 60 or 80 °C (Fig. 1c).

In the thermal inactivation study of microorganisms in wheat flour, inoculated samples were heat treated in sealed aluminum test cells (section 2.5). Thus, the moisture content remained constant. A_w at treatment temperature in wheat flour was estimated from the adsorption isotherm curves in this study (Fig. 1c) and presented in Table 2. For example, at tested a_w at 25 °C ($a_{w,25°C}$) of 0.30 ± 0.02, 0.45 ± 0.02, and 0.60 ± 0.02, the values of a_w at 80 °C ($a_{w,80°C}$) of wheat flour were 0.61, 0.73, and 0.82, respectively.

Table 1
Chemical composition of the wheat flour on a wet basis.

Component	Content % (w/w)[a]
Carbohydrates	78.92 ± 0.16
Total dietary fiber	12.92 ± 0.09
Moisture	8.34 ± 0.12
Protein	5.70 ± 0.00
Fat	3.28 ± 0.09
Ash	1.55 ± 0.03

[a] Data are mean ± standard deviation on replicate measurement (n = 3) on the tested wheat flour.

3.3. Thermal inactivation kinetics of Salmonella and E. faecium in wheat flour

Microbial survival data during thermal inactivation fitted both the log-linear and Weibull models well with a similar RMSE, A_f and B_f (Table 2). Both A_f and B_f of primary models shown in Table 2 were within the acceptable range (A_f = 1.0–1.3, B_f = 0.90–1.05) (Ross et al., 2000). Smaller A_f values and a minor difference between B_f and 1 reflect a lower deviation degree of models. According to Table 2, survival curves of *E. faecium* fit the Weibull model better while those of *S.* Enteritidis conformed better to the log-linear model. Due to one less model parameter, the log-linear model had slightly higher applicability and reliability compared to the Weibull model to describe isothermal inactivation of both strains. Thus, the log-linear model was used for describing thermal resistance parameter D-values and generate z_T-values from Eq. (6). Representative inactivation curves of *Salmonella* and *E. faecium* at 75, 80, and 85 °C are shown in Fig. 2.

3.4. Thermal resistance parameters of Salmonella and E. faecium in wheat flour

The decreased D-values of *Salmonella* and *E. faecium* at increased a_w at treatment temperature are presented in Fig. 3. The ratios of D-values of *E. faecium* and *S.* Enteritidis were smallest at $a_{w,25°C}$ 0.30 ± 0.02 for each treatment temperature. The largest ratio—3.1—was observed at 80 °C at $a_{w,25°C}$ 0.45 ± 0.02. *E. faecium* showed consistently greater D-values than did *S.* Enteritidis for all treatments.

The z_T-values of both microorganisms decreased with increased moisture content. *E. faecium* exhibited equal or lower z_T-values than *S.* Enteritidis at all tested moisture contents (Fig. 4).

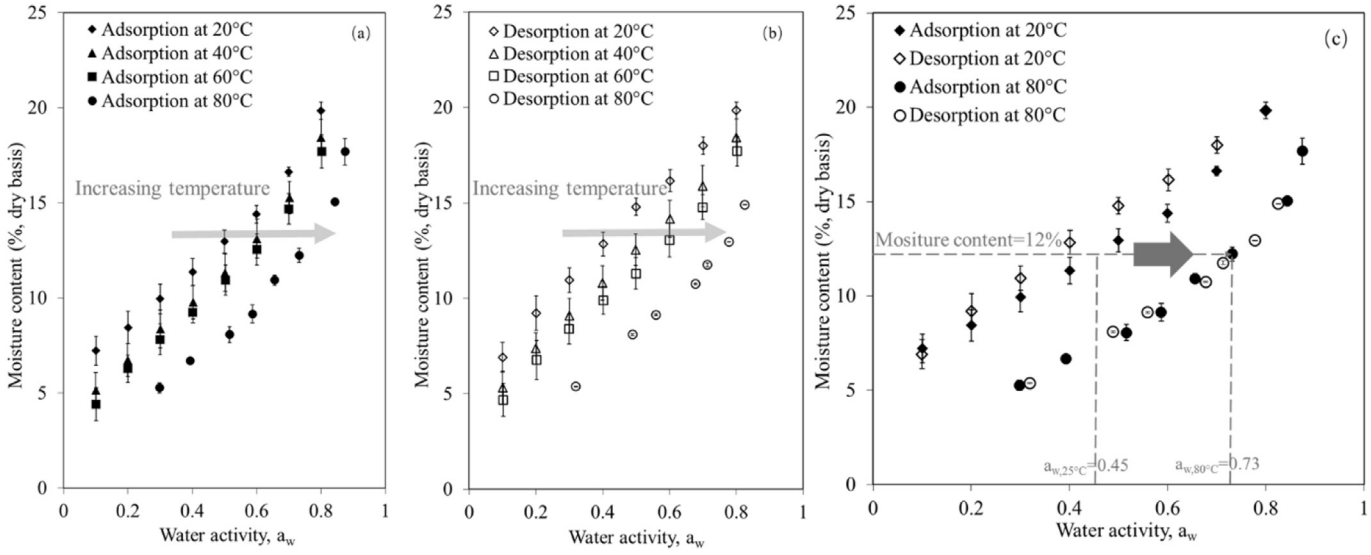

Fig. 1. Adsorption (a) and desorption (b) isotherms of wheat flour. Water content data points are average of at least two samples of the wheat flour used for experiments. Error bars represent standard deviation (n≥2); error bars may be hidden by symbols when small. 1c: adsorption and desorption isotherms of wheat flour at 20 and 80°C presenting an increase of $a_{w,80°C}$ in a closed system (constant moisture content): additional lines indicate that a_w increased from 0.45 to 0.73 when temperature increased from 20 to 80°C at moisture content 12% (dry basis).

Table 2
Parameter estimates for the primary inactivation kinetics models of *E. faecium* and *S.* Enteritidis, as well as the root mean square error (RMSE), accuracy factor (A_f) and bias factor (B_f).

Bacterial species	Moisture content (%, d.b.)	$a_{w,\ 25°C}$ ±0.02	a_w, treatment temperature [a]	T (°C)	Linear model					Weibull Model				
					D (min)	RMSE (log CFU/g)	A_f	B_f	Ratio [b]	δ(min)	α	RMSE (log CFU/g)	A_f	B_f
E. faecium	10	0.30	0.57	75	65.8 ± 2.5	0.19	1.31	1.17	2.7	62.5 ± 7.6	0.96 ± 0.08	0.19	1.26	1.05
			0.61	80	35.4 ± 5.1	0.43	1.19	1.01	3.1	40.4 ± 11.0	1.19 ± 0.41	0.44	1.18	1.02
			0.63	85	15.9 ± 1.0	0.23	1.35	1.18	2.7	9.5 ± 1.7	0.67 ± 0.07	0.17	1.20	0.91
	12	0.45	0.68	75	29.4 ± 1.1	0.18	1.16	1.09	1.7	24.2 ± 3.1	0.87 ± 0.07	0.17	1.10	1.01
			0.73	80	11.8 ± 0.6	0.23	1.08	0.99	1.6	12.9 ± 1.9	1.08 ± 0.13	0.23	1.09	1.01
			0.74	85	4.1 ± 0.2	0.18	1.07	1.00	1.4	4.5 ± 0.5	1.11 ± 0.14	0.19	1.07	1.01
			0.80	75	25.5 ± 1.2	0.21	1.16	1.09	2.1	22.1 ± 3.4	0.89 ± 0.10	0.21	1.10	1.01
	14	0.60	0.82	80	11.4 ± 1.0	0.29	1.08	0.99	2.7	10.8 ± 2.5	0.95 ± 0.20	0.30	1.07	1.01
			0.84	85	2.7 ± 0.2	0.28	1.14	1.03	2.4	3.9 ± 0.4	1.49 ± 0.20	0.23	1.09	0.96
S. Enteritidis	10	0.30	0.57	75	24.5 ± 1.3	0.24	1.11	1.00		17.6 ± 3.2	0.79 ± 0.09	0.21	1.13	0.92
			0.61	80	11.4 ± 0.9	0.28	1.14	1.03		9.5 ± 2.2	0.85 ± 0.15	0.28	1.14	1.02
			0.63	85	5.9 ± 0.5	0.34	1.12	0.99		7.2 ± 1.3	1.26 ± 0.28	0.35	1.13	1.01
	12	0.45	0.68	75	17.7 ± 1.6	0.49	1.17	1.02		18.0 ± 5.5	1.01 ± 0.22	0.50	1.18	1.02
			0.73	80	7.2 ± 0.4	0.19	1.09	1.01	N/A	6.1 ± 0.9	0.87 ± 0.01	0.18	1.08	1.01
			0.74	85	2.9 ± 0.4	0.45	1.14	1.02		2.0 ± 0.9	0.73 ± 0.21	0.44	1.14	1.01
	14	0.60	0.80	75	12.0 ± 1.3	0.35	1.18	1.02		10.7 ± 3.1	0.90 ± 0.22	0.36	1.16	1.02
			0.82	80	4.2 ± 0.4	0.30	1.13	1.02		4.1 ± 0.9	0.96 ± 0.21	0.31	1.13	1.02
			0.84	85	1.1 ± 0.1	0.28	1.10	1.01		1.1 ± 0.2	0.90 ± 0.25	0.29	1.10	1.01

The D-and δ-values shown are the means of three independent trials, expressed as mean ± standard deviation.
[a] a_w at treatment temperature was obtained from the adsorption curves of wheat flour samples.
[b] Ratios between D-value of *E. faecium* and *S.* Enteritidis at tested temperature and moisture content are shown.

4. Discussion

4.1. A_w of wheat flour increases at high temperature

The shape of water sorption isotherm curves of wheat flour samples was typical for flours: the a_w increased significantly with raised temperature in both adsorption and desorption curves at fixed moisture content (Syamaladevi et al., 2016a). The presence of hysteresis at 20 and 40 °C was due to the higher moisture content of wheat flour in the desorption process than that of the adsorption process at the same a_w at low temperatures (Al-Muhtaseb et al., 2002). Hysteresis was reduced at high temperature. Similar reduction of hysteresis at elevated temperature was also reported in apples, rice (Wolf et al., 1972) and potatoes (McLaughlin and Magee, 1998; McMinn and Magee, 1999). The significant difference ($P < 0.05$) between $a_{w,25°C}$ and a_w at treatment temperature of the same wheat flour sample indicates that $a_{w,25°C}$ of food samples cannot represent the real-time a_w during isothermal treatments. Microorganisms in wheat flour were exposed to a more humid environment in the inactivation process than that indicated by the $a_{w,25°C}$ values.

The $a_{w,80°C}$ values of wheat flour corresponding to $a_{w,25°C}$ levels were obtained based on the assumption that no moisture loss or absorbance occurred in wheat flour samples during isothermal inactivation. In contrast, open food systems may continuously exchange moisture with the environment during heat treatment. Therefore, when treating open systems, a different moisture content than that of a closed system may be obtained. Additionally, in food matrices of different composition, the $a_{w,80°C}$ may vary because of diverse equilibrium isotherm curves (Al-Muhtaseb et al., 2002; Syamaladevi et al., 2016a,b). In the present study, we discussed our data with respect to published values at equivalent $a_{w,25°C}$ or moisture content, depending on the information provided in these articles. However, introducing $a_{w,80°C}$ in this study provided additional insight into how bacterial thermal resistance was influenced by relevant process conditions in thermal treatments.

4.2. Thermal reductions of microorganisms in heating and cooling processes

A come-up time of ~150 s was recorded in the development of survival curves (section 2.5). Thermal reduction of microorganisms during the come-up time (so called initial log reduction) varied from 0.02 ± 0.06 (*E. faecium* at 75 °C, $a_{w,25°C} = 0.30 \pm 0.02$) to 1.50 ± 0.35 (*S.* Enteritidis at 85 °C, $a_{w,25°C} = 0.60 \pm 0.02$). In the estimation of D-values, time zero of survival curves was set at the come-up time. That is, the initial log reduction was considered as the starting point of inactivation kinetics and therefore did not interfere with the calculation of the slopes nor the D-values.

At the end of each thermal treatment, ~30 s was recorded to cool the geometric center of wheat flour from the tested temperature to 20 °C. The lethal effects on either strains during cooling from 75 °C should be minimal. Even with the small D-values at 85 °C, particularly at the highest $a_{w,25°C}$ 0.60 ± 0.02, the thermal reductions during cooling were still negligible because the time needed to reduce temperature below the sublethal temperature (60 °C) was 11.5 s. For example, $D_{85°C}$ of *S.* Enteritidis at $a_{w,25°C} = 0.60 \pm 0.02$ was 1.1 ± 0.1 min. Assuming the a_w and temperature did not change within this 11 s, the thermal reduction of *S.* Enteritidis in the first 11.5 s of cooling process was only 1.13*(11.5/60) = 0.22 log CFU/g. With a population error tolerance of 0.2 log CFU/g, thermal reduction of either strains during the cooling process were considered as insignificant either.

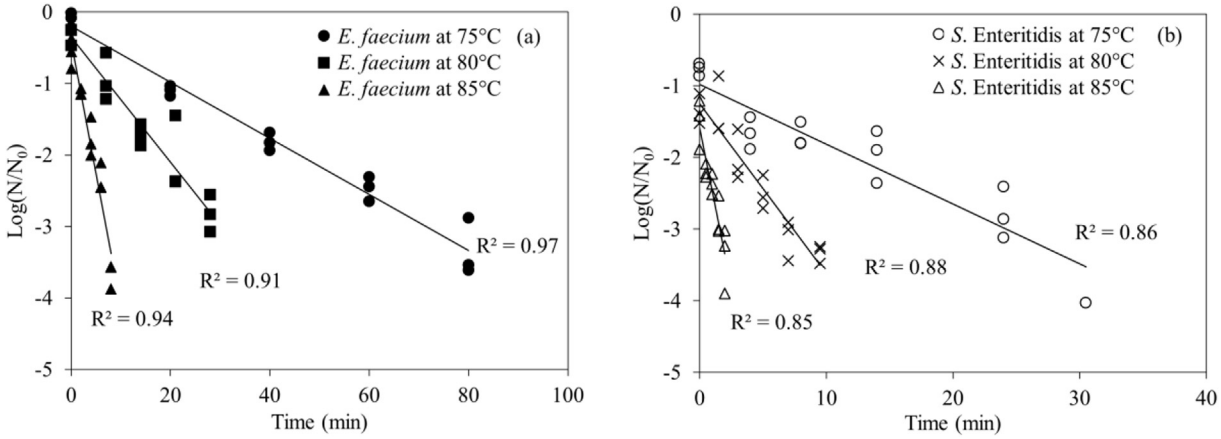

Fig. 2. Thermal inactivation kinetic curves of *E. faecium* (a) and *S.* Enteritidis (b) in wheat flour (moisture content=14%, $a_{w,25°C}$=0.60±0.02) at 75, 80 and 85°C, n=3.

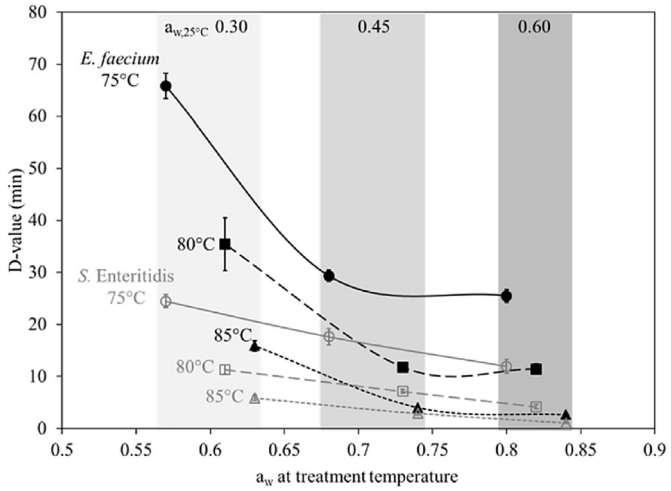

Fig. 3. Decrease of D-values of *E. faecium* and *S.* Enteritidis in wheat flour with increasing a_w and increasing temperatures of treatment. Error bars represent standard deviations (n = 3); error bars may be hidden by symbols when small.

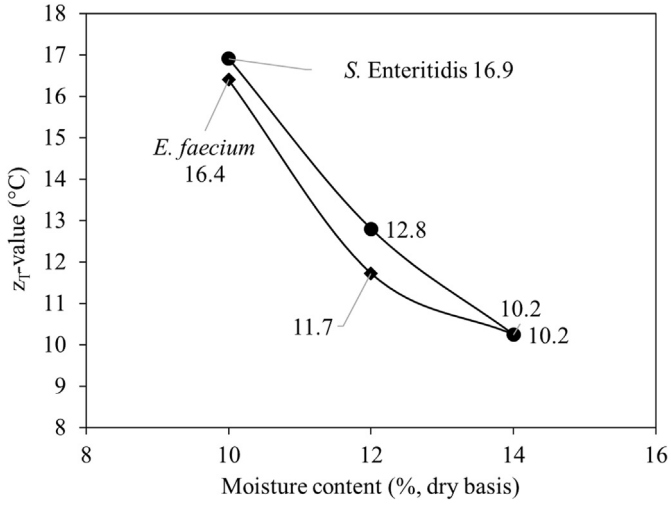

Fig. 4. Decrease of z_T-values of *E. faecium* and *S.* Enteritidis in wheat flour with increasing moisture content (%, dry basis).

4.3. Thermal resistance of E. faecium and S. Enteritidis in wheat flour

S. Enteritidis in wheat flour had thermal resistance comparable to published data. At $a_{w,25°C} = 0.45 \pm 0.02$, the $D_{80°C}$ of *S.* Enteritidis in this study was 7.17 min, which is not significantly different ($P > 0.05$) from that of all-purpose wheat flour (~6.9 min) (Syamaladevi et al., 2016a,b). Compared to *S.* Enteritidis, *E. faecium* had higher D-values under all treatment conditions. Higher thermal resistance of *E. faecium* than that of *S.* Enteritidis was reported in wheat flour (Smith et al., 2016), almonds, (Jeong et al., 2011) and four other low-moisture products (Rachon et al., 2016).

No significant difference ($P > 0.05$) was observed between z_T-values of both microorganisms at tested a_w levels. At $a_{w,25°C} = 0.45 \pm 0.02$, the z_T-values of *E. faecium* and *S.* Enteritidis in wheat flour are in agreement with the published z_T-value of *S.* Enteritidis (16.7 °C) in wheat flour (Smith et al., 2016), but are slightly lower than that of *S.* Weltevreden (19.6 °C) in wheat flour (Archer et al., 1998). The similarities or differences of the z_T-values compared with the literature may be due to biological differences among *Salmonella* serotypes.

4.4. Influence of a_w on D-values of microorganisms

The D-values of both strains sharply decreased with increasing a_w at a selected temperature. The reduction in D-values at increased $a_{w,25°C}$ level has been reported from thermal inactivation studies of *Salmonella* in wheat flour (Laroche et al., 2005; Smith, 2014), low-moisture pet foods (Ceylan and Bautista, 2015), skim milk powder (Laroche et al., 2005) and whey protein powder (Farakos et al., 2013). In thermal processing of low-moisture foods, inactivation efficiency could be enhanced by raising the $a_{w,80°C}$, which reduces D-values and therefore treatment time.

Ratios of D-values of *E. faecium* and *S.* Enteritidis reflect the difference in the thermal resistance parameters between the two strains under the same conditions (Table 2). All the ratios are greater than 1, indicating that *E. faecium* was consistently more thermally resistant than *S.* Enteritidis under tested conditions. The ratios varied from 1.4 to 3.1 depending on moisture content and temperature, suggesting that the two microorganisms had different thermal resistances at various moisture content and treatment temperatures.

4.5. Influence of moisture content on z_T-values of microorganisms

Observed z_T-values of both microorganisms (10.2–16.9 °C) in the present study were higher than z_T-values for *Salmonella* reported in chicken broth (5.8–6.6 °C) (Juneja et al., 2001) and cooked turkey (9.1 °C) (Murphy et al., 2003), *Staphylococcus aureus* in beef (4.8–5.4 °C) (Forsythe, 2000), and *Listeria monocytogenes* in liquid whole egg (7.0 °C) (Doyle et al., 2001; Foegeding and Stanley, 1990). However, *Salmonella* in chocolate (20.4 °C) (Doyle and Mazzotta, 2000; Tamminga et al., 1977), *Listeria monocytogenes* in salted seafoods (10% salt, 9.2 °C) (Doyle and Mazzotta, 2000) and *Escherichia coli* in various foods (10.6 °C) were reported with equal or higher z_T-values.

The z_T-values of both bacteria declined significantly ($P < 0.05$) with increased moisture content. For instance, the z_T-values of *S.* Enteritidis were 16.9 °C and 10.2 °C at 10% and 14% moisture content, respectively. The lower z_T-values at higher moisture content indicate that both strains are more sensitive to temperature in a relatively wet environment. More information is needed to understand this trend. Some responses occurring upon transition of bacterial cells into low-moisture environments were discussed extensively in a recent review (Finn et al., 2013).

5. Conclusions

Isotherm results of wheat flour demonstrate significant increase ($P < 0.05$) of a_w as temperature rises at fixed moisture contents. Both D- and z_T-values of the two strains decreased with increased a_w (or moisture content). Higher D-values and equivalent z_T-values of *E. faecium* compared with those of *S.* Enteritidis demonstrate that *E. faecium* is a conservative surrogate in wheat flour for thermal processes validation. This study suggests that an adequate adjustment of $a_{w,25°C}$ or moisture content may promote inactivation efficiency in the development of thermal processing of wheat flour.

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