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Stability of *Listeria monocytogenes* in wheat flour during extended storage and isothermal treatment

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ABSTRACT

Foodborne pathogens including *Salmonella* have been implicated in recent recalls of low-water activity (a_w) foods, such as peanut butter, almond flour, wheat, flour and dry milk powder, and are primary concerns for the microbiological safety of dry food products. Although there are an increasing number of studies on *Salmonella* thermal resistance conducted in low-moisture foods, little information is available on *Listeria monocytogenes* thermal resistance in those products. This study evaluated the survival of *L. monocytogenes* in wheat flour during long-term storage as well as its thermal resistance in wheat flour equilibrated to a_w 0.30, 0.45, and 0.60. *L. monocytogenes* survived in wheat flour at both a_w 0.31 and 0.56 during 6 months of storage at room temperature, with populations decreasing about 2.52 and 6.27 logs at a_w 0.31 and 0.56, respectively. Equilibration in low-a_w flour enabled *L. monocytogenes* to become more resistant to thermal treatment. At treatment temperature between 70 and 80 °C, D-values increased with decreasing a_w. For a_w 0.30. 0.45, and 0.60 (measured at room temperature), respectively, D-value (in min) ranges for 70–80 °C were 37.10–7.08, 17.44–3.13, and 16.85–1.59. The z-values were 12.9, 14.2, and 9.9 °C for a_w 0.30, 0.45, and 0.60, respectively. These data highlight the need for vigilance when processing dry foods, and provide valuable information for the industry to validate thermal processing for control of *L. monocytogenes* in low-moisture foods. This study also offers insight into the development of thermal inactivation strategies to control *L. monocytogenes* and other foodborne pathogens in foods with similar matrices.

1. Introduction

Listeria monocytogenes (*Lm*) has one of the highest mortalities among foodborne pathogens, with a mortality rate for vulnerable populations (e.g., the elderly, neonates, and the immunocompromised) between 20 and 30% (Scallan et al., 2011). Recent worldwide estimates of *Lm* infection rates are ~23,000 infections and ~5400 deaths per year. These numbers have held steady since 2010 (de Noordhout et al., 2014). For the United States, yearly averages are 1600 infections and 260 deaths (CDC, 2016). The Foodborne Outbreak Online Database (FOODTool) of the Centers for Disease Control and Prevention (CDC) shows that, from 1998 to 2015, there have been 61 outbreaks of listeriosis associated with 818 illnesses, 578 hospitalizations, and 121 deaths (CDC, 2016). The high mortality rate from *Lm* infection highlights the need for continued vigilance in the food processing sector.

In contrast to the danger posed by the potential presence of *Lm* in foods with higher water activity (a_w) ($\geq$ 0.60), little or no documented evidence exists regarding its safety in low-a_w foods (La_wF) ($a_w < 0.60$) (Koseki, Nakamura, & Shiina, 2015). Most studies have focused on *Salmonella* and Shiga toxin-producing *Escherichia coli* (STEC), especially since outbreaks involving these microorganisms in La_wF have increased in the past five years (Anderson et al., 2017; Martinez, Stratton, Bianchini, Wegulo, & Weaver, 2015). However, *Lm* can be stable in La_wF, such as milk powder, instant cereals, and flour-based dry products (Kenney & Beuchat, 2004; Rachon, Peñaloza, & Gibbs, 2016). The U.S. Food and Drug Administration (FDA) has attempted to streamline compliance with *Lm* policy and make transparency a central priority (FDA, 2017a). Certainly a risk-based approach (Todd, 2011) for *Lm* management, based on dose-response models and concordant with worldwide *Lm* policies, is a viable strategy for companies manufacturing La_wF. While tolerances on *Lm* levels in several foods that do not support its growth are generally agreed upon worldwide (de Noordhout

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et al., 2014), harmonized policies between various food processing sectors will need to be implemented as more research data emerge on the viability and heat resistance of *Lm* in La_wF (Farber, Kozak, & Duquette, 2011).

Microorganisms will not grow in low-a_w environments but can still survive, with some organisms reverting into a viable but non-culturable (VBNC) state (Besnard, Federighi, Declerq, Jugiau, & Cappelier, 2002; Cunningham, O'Byrne, & Oliver, 2009; Syamaladevi et al., 2016). Recent occurrences of pathogenic bacteria in raw flour have highlighted the need for increased attention to the safety of La_wF (Entis, 2017). Research has found that wheat flour harbors pathogenic bacteria such as *Salmonella* spp., STEC, and *Bacillus cereus*, as well as mycotoxin producers such as *Aspergillus flavus* (Sabillón & Bianchini, 2015). Additionally, *Lm* was found in flour and dried nuts and seeds in Portugal (Mena et al., 2004), and in trace amounts in buckwheat flour (Losio et al., 2017). With the increased number of recalls involving *Lm* in various risk categories (FDA, 2017a, 2017b), the food industry will most likely begin requesting data on *Lm* in La_wF to be able to substantiate risk assessment, which is now a regulatory requirement. Recent outbreaks of *Salmonella* and STEC related to flour and other La_wF further stress the importance of assessing the risk of *Lm* in these products. This study examined the long-term stability and heat resistance of a three-strain cocktail of *Lm* in wheat flour equilibrated to target a_w during extended storage and thermal treatments.

2. Materials and methods

2.1. Bacteria strains and preparation of bacterial lawns

Two *Lm* outbreak strains, NRRL B-57618 (1/2a) and NRRL B-33053 (4b), and one processing plant *Lm* isolate, NRRL B-33466 (1/2b), were used to prepare a 3-strain cocktail inoculum. These strains were obtained from the culture collection of the National Center for Agricultural Utilization Research (NRRL), USDA Agricultural Research Service (Peoria, IL), and stored in a stock solution of trypticase soy broth supplemented with 0.6% (w/v) yeast extract (TSBYE) (Hardy Diagnostics, Santa Maria, CA) and 20% glycerol at −80 °C until used. The cultures were individually activated twice in TSBYE by consecutively culturing at 35 ± 2 °C for 24 h. The *Lm* lawn was plated onto sterile tryptic soy agar with 0.6% yeast extract (TSAYE) (Hardy Diagnostics) in a 100 × 15 mm plate and incubated at 35 ± 2 °C for 24 h. Each *Lm* lawn was collected from TSAYE using a "hockey stick" steel spreader and 5 mL of sterilized phosphate buffered saline (PBS, pH7.4), and centrifuged at 8000 × g, 4 °C for 15 min (Centrifuge 5810 R®, Eppendorf North America, Hauppauge, NY). The resulting pellet was re-suspended in sterile PBS to achieve ~1×10^{10} CFU/ml, then combined in equal volumes to obtain the 3-strain cocktail. The population of the inoculum was confirmed by enumeration.

2.2. Inoculation of flour

Organic soft white wheat pastry flour was obtained from Eden Foods (Clinton, MI). We chose wheat flour as the representative food matrix because it is a staple food and has a uniform texture, which facilitated this work. Forty grams of the flour was inoculated with 400 μL of a 3-strain *Lm* cocktail (~1×10^{10} CFU/mL) inside a stomacher bag (Fischer Scientific, Pittsburgh, PA), and then vigorously hand-mixed until homogenized to achieve ~10^8 CFU/g flour. Detection of background flora was performed by plating appropriate serial dilutions on TSAYE and then incubating at 35 ± 2 °C for 24 h before enumeration.

2.3. Inoculated flour equilibration

The above inoculated flours were divided into two 150 mm Petri dishes (Fisher Scientific), placed in a a_w-equilibration chamber (Custom-designed at Michigan State University) (Smith, Hildebrandt, Casulli, Dolan, & Marks, 2016), set at target a_w values of 0.30, 0.45, and 0.60, and equilibrated for a minimum of 4 days at 22 °C. The a_w of the respective wheat flour was monitored in triplicate with an Aquameter (Aqualab Series 3, Decagon Devices, Inc., Pullman, WA). Samples with a targeted $a_w \pm 0.02$ were used for thermal inactivation.

Listeria was enumerated in the inoculated flour directly after inoculation and 4 days after equilibration. One gram of inoculated flour was added to 9.0 ml of PBS, homogenized for 2 min at 220 rpm in a stomacher (Seward Stomacher® Circulator 400), followed by serial 10-fold dilutions in PBS. The appropriate serial dilutions were then plated in duplicate onto TSAYE and incubated at 35 ± 2 °C for 48 h.

2.4. Heat treatment of Lm in wheat flour

After 4-day equilibration at the target a_w, inoculated and equilibrated flour (0.60 ± 0.02 g) was loaded into aluminum thermal death-treatment (TDT) cells designed by Washington State University with a cavity capacity of one ml (Chung, Birla, & Tang, 2008). TDT cells loaded with inoculated samples at all three a_w values were submerged in an ethylene glycol bath (Isotemp®, Fischer Scientific) at 70, 75, and 80 °C. The temperature of glycol bath was calibrated using an Omega Precision RTD temperature recorder (OM-CP-RTDTemp2000, Omega Engineering Inc., Norwalk, CT). TDT cells with T-type thermocouples at the geometric center were used to measure heat penetration and come-up time (CUT), or the time needed to reach within 0.5 °C of the target temperature. The thermocouple was attached to a digital thermometer and time-temperature history was recorded in triplicate. In our study, we employed a CUT of 90 s, which is close to the CUT of 68 s used in thermal inactivation of *Salmonella* Enteritidis PT30 in wheat flour (Smith et al., 2016). For each heat treatment, triplicate samples were collected at 5 sampling points – 0 min (actually 1.5 min, in consideration of CUT), and four others that varied based on a_w and temperature. TDT cells were withdrawn for each sampling point and immediately cooled in an ice-water bath for 1.5 min. All tests were conducted three times independently.

2.5. Enumeration of Listeria survivors in wheat flour

The content of each TDT cell was placed inside a Whirl-Pak® bag (Nasco, Ft. Atkinson, WI) and diluted 1:10 with PBS, then agitated for 2 min in a stomacher (Seward Stomacher® Circulator 400). The recovered *Lm* suspensions were 10-fold serially diluted, after which appropriate dilutions were plated on Luria-Bertani (LB) in duplicate and incubated at 35 ± 2 °C for 48 h.

2.6. Survival of Lm on wheat flour (a_w 0.31 and a_w 0.56) during storage

Inoculated flours were prepared and equilibrated as described above. Wheat flour equilibrated at a_w 0.31 ± 0.03 or a_w 0.56 ± 0.03 was subjected to long-term storage. The equilibrated and inoculated wheat flour was sealed in a moisture barrier bag (Dri-Shield 3000®, Desco Industries, Inc) with a 6 mm width, and then stored at 22 °C for up to 210 days. For each storage condition (a_w), two sets of biologically independent inoculated flour were each prepared in triplicate 100 g batches. For each independent set, we had three samples at each storage sampling time. The a_w of samples inside

each moisture barrier bag was monitored at each sampling day. Survival was analyzed bi-weekly or monthly per the above described method.

2.7. D-values and z-values estimation

The USDA Integrated Pathogen Modeling Program (IPMP) (Huang, 2014) was used for data analyses. The linear regression was used for estimation of D-values per the equation below:

$$\log (N_t) - \log (N_o) = -t/D \quad (1)$$

where t = time (min), log N_t is the log number of bacteria at time t, log (N_o) is the original log number in the sample, and D is the D-value in min.

The z-value was estimated by the following equation

$$\log (D) - \log (D_{ref}) = -1/z\,(T - T_{ref}) \quad (2)$$

where D is the D-value in min at temperature (T,°C), D_{ref} is the D-value in min at reference temperature (T_{ref},°C), T is the heating temperature, and T_{ref} is the reference heating temperature (°C).

3. Results

3.1. Long-term storage of Lm in wheat flour

Lm was able to survive in flour stored at 22 °C for 6 months. For *Lm* in flour at a_w 0.31, the bacterial population dropped by 2.52 ± 0.16 log CFU/g after 210 days of storage at 22 °C (Fig. 1A). For *Lm* in flour with a_w 0.56, the population of *Lm* dropped by ~6.27 ± 0.51 log CFU/g after 190 days of storage at 22 °C (Fig. 1B). *Lm* equilibrated and held at a_w 0.31 showed much higher stability than *Lm* at a_w 0.56. At both a_w values, *Lm* populations remained stable during the first two weeks of storage, after which the levels of *Lm* started to decrease, with the population of *Lm* at a_w 0.56 flour decreasing more rapidly.

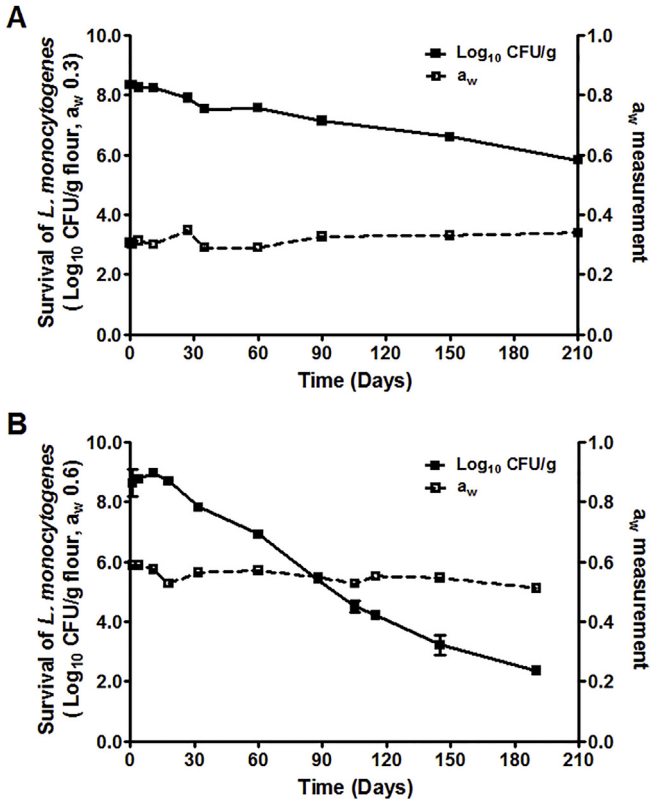

Fig. 1. Survival of *Lm* in wheat flour at different water activity (a_w) values during 210 days of storage at 22 °C. (A) a_w 0.31 ± 0.03; (B) a_w 0.56 ± 0.03. Mean ± SEM (n = 3). Experiments were repeated independently twice.

3.2. Thermal inactivation of Lm in wheat flour

At each selected temperature, the thermal inactivation curves showed a log-linear trend for *Lm* in flour at different a_w values, meaning that the inactivation rates were constant ($R^2 > 0.93$, Fig. 2A–C). At all three temperatures used for heat inactivation (70, 75, and 80 °C), *Lm* showed the highest thermal resistance in wheat

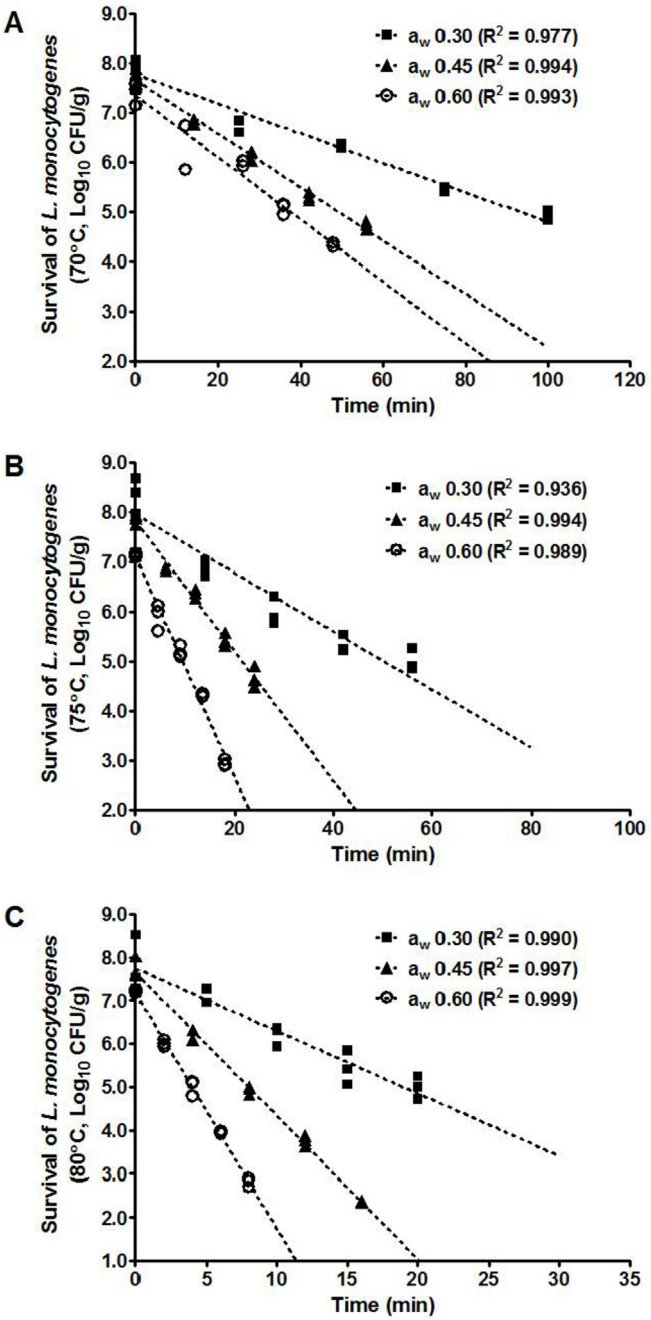

Fig. 2. Representative death curves for *Lm* in wheat flour equilibrated to a_w 0.30, 0.45, and 0.60 at (A) 70 °C, (B) 75 °C, and (C) 80 °C. Experiments were independently repeated thrice.

flour equilibrated to at a_w 0.30. Fig. 2A–C indicate that a gap existed between a_w 0.30 and the other two a_w, 0.45 and 0.60, in terms of the trend of overall heat inactivation. Also, the inactivation curves for a_w 0.45 and 0.60 became closer in slope as the temperature dropped from 80 to 70 °C (Fig. 2).

The D_{70}-values of *Lm* were 37.10 ± 2.77, 17.44 ± 0.77, and 16.85 ± 0.58 min in samples at a_w of 0.30, 0.45, and 0.60, respectively (Table 1). The D_{70}-value at a_w 0.30 was more than twice that at a_w 0.45. The D_{75}-values were 19.94 ± 1.29, 7.73 ± 0.37, and 4.61 ± 0.19 min at a_w 0.30, 0.45, and 0.60, respectively (Table 1). At 75 °C, the D-value gap between a_w 0.60 and a_w 0.45 was larger than that at 70 °C, while the D_{75}-value at a_w 0.30 flour was about 2.5 times that at a_w 0.45 (Table 1). The D_{80}-values were 7.08 ± 0.44, 3.13 ± 0.22, and 1.59 ± 0.07 min, at a_w 0.30, 0.45, and 0.60, respectively (Table 1). The D_{80}-value for a_w 0.30 was approximately twice as high as that at a_w 0.45 and followed the same trend as for the other temperatures.

3.3. z-values

The z-values of *Lm* were 12.86 ± 1.04; 14.18 ± 0.88, and 9.92 ± 0.46 °C in flour samples with a_w of 0.30, 0.45, and 0.60, respectively (Table 1). The z-values at both a_w 0.30 and 0.45 were higher than that at a_w 0.60 (Fig. 3).

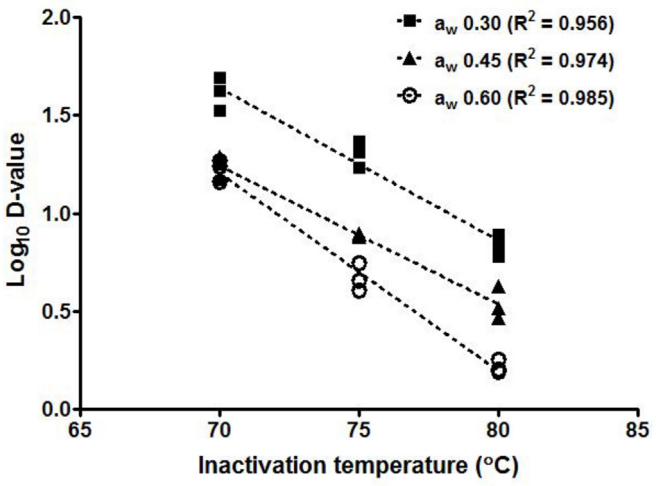

Fig. 3. z-values for *Lm* in wheat flour at a_w 0.30, 0.45, and 0.60.

4. Discussion

The focus of this study was to determine the effect of desiccation and thermal treatment of *Lm* in flour at different a_w values. In this study, a_w values were chosen to mimic a_w of low-moisture foods in common environments and settings. In terms of applicability to industry, there is evidence that heating flour for as little as 20 min at 80 °C can have negative effects on various matrix properties, such as a decrease in gluten functionality (Mann, Schiedt, Baumann, Conde-Petit, & Vilgis, 2014). This study reinforces the complexity of developing effective pathogen control strategies for *Lm* in wheat flour as well as other La_wF. Based on our data, 80 °C, 20 min thermal treatments should provide more than a 6-log reduction of *Lm* in wheat flour with a room temperature a_w of 0.60. A practical challenge is how to deliver thermal energy effectively to bulk flour.

4.1. Comparative studies of Salmonella Enteritidis PT-30 and Lm

Recent studies reported that D_{80}-values for *S.* Enteritidis PT30 in wheat flour at ~ a_w 0.30 were 10.3 min (Smith et al., 2016) and 11.4 min (Liu, Rojas, Gray, Zhu, & Tang, 2018), which was higher than the D_{80} -value for *Lm* obtained in this study using the same matrix at the same a_w. The D_{75}-values for *S.* Enteritidis PT30 in wheat flour of ~a_w 0.30 were 14.5 min (Smith et al., 2016) and 24.5min (Liu, Rojas, et al., 2018), comparable to the D_{75}-value for *Lm* in wheat flour obtained in this study at a similar a_w. Also, the D_{80}- values for *S.* Enteritidis PT30 in wheat flour (a_w 0.58-0.60) were 1.3-4.2 min (Liu, Rojas, et al., 2018; Smith et al., 2016), compared to the D_{80} -value of 1.6 min for *Lm* in wheat flour (a_w 0.60). These data suggest that, in wheat flour, *Lm* has a comparable or slightly lower heat resistance compared to *S.* Enteritidis PT30 depending on a_w and treatment temperatures. Concordantly, *Lm* has similar D_{80}-values as *Salmonella* in culinary seasoning (a_w 0.66) and pet food (a_w 0.65), but much smaller D_{80}-values or less heat resistance in high sugar confectionery (a_w 0.57) and chicken meat powder (a_w 0.38) (Rachon et al., 2016). These data imply that thermal inactivation may be less differentiating for bacteria at a higher matrix a_w, which reinforces previous findings (Laroche, Fine, & Gervais, 2005; Murrell & Scott, 1966). Research has indicated that a_w values of 0.20–0.50 are the optimum range for maximal heat resistance of both *Saccharomyces cerevisiae* and *Lactobacillus plantarum* in wheat four, with greatest resistance for *L. plantarum* at a_w 0.35 and *S. cerevisiae* at a_w 0.42 (Laroche et al., 2005).

Higher z-values of *Lm* in wheat flour at a_w 0.30 and 0.45 as compared to the z-value at a_w 0.60 were observed in this study. This is consistent with the observation of *S.* Typhimurium in glucose-enhanced nutrient broth, where a higher a_w value yielded a lower z-value (Aljarallah & Adams, 2007), although other research has found no specific trend regarding a_w levels and z-values (Acosta, Usaga, Churey, Worobo, & Padilla-Zakour, 2017).

4.2. Factors influence Lm thermal resistance

In a recent study, the mean D_{80}-values of *Lm* in confectionery (a_w 0.57), culinary seasoning (a_w 0.66), pet food (a_w 0.65), and chicken meat powder (a_w 0.38) were reported as 0.94, 1.80. 0.62,

Table 1
Thermal inactivation data for *Lm* in wheat flour based on linear model.

A_w	Temp (°c)	D-value (min)	95% CI upper limit fro D-value	RMSE	z-value (°c)
0.30	70	37.10 ± 2.77	42.69	0.52	12.86 ± 1.04
	75	19.94 ± 1.29	22.55	0.44	
	80	7.08 ± 0.44	7.96	0.37	
0.45	70	17.44 ± 0.77	19.00	0.32	14.18 ± 0.88
	75	7.73 ± 0.37	8.48	0.32	
	80	3.13 ± 0.22	3.58	0.52	
0.60	70	16.85 ± 0.58	18.58	0.35	9.92 ± 0.46
	75	4.61 ± 0.19	4.99	0.38	
	80	1.59 ± 0.07	1.73	0.37	

Mean ± SEM. CI:confidence interval; RMSE: the root mean square error; A_w: water activity.

and 2.00 min based on first order kinetics, respectively (Rachon et al., 2016). These D_{80}-values are at the low end of D_{80}-values (1.6–7.1 min) obtained in wheat flour with a similar a_w range (0.30–6.0). However, in the Rachon study employing a different food matrix under variable a_w (Rachon et al., 2016), it is difficult to see the relationship between D_{80}-values and a_w. Using a high-carbohydrate food matrix under controlled water activities, the current study showed a negative relationship between D_{80}-values and a_w. *Lm* exhibited much higher resistance at low a_w (0.30) than that at high a_w (0.60). Due to the different a_w values and food matrices used, D_{80}-values in the current study were not directly comparable to those from Rachon et al. (2016). Both studies, however, indicated that heat resistance depends upon the matrix composition, with the current study showing a direct inverse relationship between a_w level and heat resistance. This negative relationship between D-values and initial a_w levels was also observed in *S*. Enteritidis PT-30 in wheat flour (Smith et al., 2016; Liu, Rojas, et al., 2018), as well as in an inert carrier, silicon dioxide granules (Liu, Tang, Tadapaneni, Yang, & Zhu, 2018).

Research also indicates that the composition of inoculated matrices affects the D-values of pathogenic bacteria. *Lm* exhibited D_{60}-values of 26.0 and 37.5 min, respectively, in peanut butter (a_w 0.32) and chocolate-peanut spread (a_w 0.46) (Kenney & Beuchat, 2004). *S*. Typhimurium (Juneja & Eblen, 2000), *E. coli* O157:H7 (Line et al., 1991), and *L. monocytogenes* (Fain et al., 1991) showed a higher heat resistance in beef with higher fat levels. Additionally, each type of food matrix behaves differently during heat treatment, and a_w of a selected food matrix changes to various extents depending on treatment temperature and initial moisture content (Tadapaneni, Yang, Carter, & Tang, 2017).

Our findings broaden the horizon for research into the behavior of *Lm* in La_wF and give food processors reference points to consider when implementing preventive control measures. Along with assisting the industry, studies on *Lm* behavior in La_wF should aid in research efforts to understand the mechanisms of foodborne pathogen survival at reduced a_w. During desiccation, the down-regulation of *Lm* flagellar proteins ostensibly redirects cellular energy resources and increases the ratio of saturated-to-unsaturated fatty acids in membranes (Hingston, Piercey, & Hansen, 2015). Trehalose uptake and subsequent conversion to glucose appear to aid *Lm* to become more resistant to heat and osmotic stress by producing higher solute concentrations (Ells & Hansen, 2011).

4.3. The stability of Lm in flour during long-term storage at room temperature

Lm was stable in wheat flour during 6 months storage at 22 °C. Concordantly, *Lm* remained at detectable levels in inoculated peanut butter and chocolate-peanut spread during 24 weeks of storage at 22 °C (Kenney & Beuchat, 2004). In almonds stored at 4 °C *Lm* showed no appreciable decrease over a 12-month storage period (Kimber, Kaur, Wang, Danyluk, & Harris, 2012). Our storage study demonstrates the heightened stability of *Lm* at a_w 0.30 as compared to a_w 0.56, which corresponds to higher heat resistance at a_w 0.30 than at a_w 0.45 or 0.60. The storage data could help processors in validation studies aiming to test thermal resistance of any possible bacteria at various storage stages. The data also complement each other by showing how handling conditions can influence populations of *Lm*. The observed *Lm* behavior in La_wF mirrors tests done in dry (<10% moisture) soils, in which *Lm* was found to survive for more than 6 months (Dowe, Jackson, Mori, & Bell, 1997; Locatelli, Spor, Jolivet, Piveteau, & Hartmann, 2013).

4.4. Conclusion

Lm was stable in wheat flour during room temperature storage for up to 210 days. Its survival in wheat flour during storage depended on the a_w of flour. There was only a 2.5 log CFU/g reduction of the *Lm* population in flour of a_w 0.31 during 210 days of storage at 22 °C. When equilibrated in low-a_w flour, *Lm* demonstrated higher heat resistance. Our findings provided technical information for the industry in validating thermal processes for the control of *Lm* in La_wF and the development of other thermal inactivation strategies to eliminate *Lm* in similar matrices.

Competing financial interest

The authors declare no competing financial interest.

Conflicts of interest

The authors have no known conflicts of interest.

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