Evaluation of *Enterococcus faecium* NRRL B-2354 as a surrogate for *Salmonella* during cocoa powder thermal processing

Hsieh-Chin Tsai\(^a\), Kenneth F. Ballom\(^a\), Song Xia\(^a\), Juming Tang\(^b\), Bradley P. Marks\(^c\), Mei-Jun Zhu\(^a,\)*

\(^a\) School of Food Science, Washington State University, Pullman, WA 99164, USA
\(^b\) Department of Biological Systems Engineering, Washington State University, Pullman, WA, 99164, USA
\(^c\) Department of Biosystems and Agricultural Engineering, Michigan State University, East Lansing, MI, 48824, USA

**ABSTRACT**

*Salmonella* is capable of surviving in a low moisture environment for long periods. Once adapted to the xeric conditions, the thermal resistance of *Salmonella* is enhanced. Cocoa powder is a low water activity (aw) food (Laaw) that is an essential component in a wide variety of desserts and ready-to-eat (RTE) foods and drinks. The aim of this study was to evaluate the desiccation and thermal resistance of *Salmonella* in cocoa powder, as well as to examine the suitability of *Enterococcus faecium* NRRL B-2354 as a surrogate for *Salmonella* during cocoa powder thermal processing. Natural unsweetened cocoa powder was inoculated with a 3-strain *Salmonella* cocktail or *E. faecium* and was equilibrated to aw 0.30 and 0.45 at room temperature, then subjected to isothermal treatments at 70–80 °C or 12-month storage at RT (room temperature, 22.0 ± 0.5 °C, aw 0.30). At 70 and 80 °C, D-values of both *Salmonella* and *E. faecium* increased with decreasing aw. D-values of *Salmonella* at aw 0.30 cocoa powder were 46.2 ± 4.7, 20.5 ± 1.7, and 11.5 ± 0.9 min at 70, 75 and 80 °C, respectively. Higher heat resistance of *E. faecium* in aw 0.30 cocoa powder was observed with D-values of 59.9 ± 5.0, 28.9 ± 1.8, and 16.1 ± 1.4 min at 70, 75, and 80 °C, respectively. However, *E. faecium* demonstrated less heat resistance than *Salmonella* when aw 0.30 was increased to 0.45. D-values for *Salmonella* at aw 0.45 were 31.6–7.0 min at 70–80 °C compared to 25.8–4.7 min for *E. faecium*. During 12 months of storage at RT, surviving *E. faecium* population in aw 0.30 cocoa powder was higher than that of the *Salmonella* cocktail; the population decreased by 1.39 and 3.75 logs, respectively. These findings indicate that the suitability of *E. faecium* as a surrogate organism for *Salmonella* is influenced by aw of cocoa powder. The aw correlates with thermal inactivation rates in both *Salmonella* and *E. faecium*, and should be considered as a significant contributor to the thermal resistance of *Salmonella* in cocoa powder.

1. Introduction

*Salmonella* is one of the most challenging foodborne pathogens in the food industry, due to its virulence and thermal resistance in low moisture foods. Infections by this pathogen have led to numerous illnesses and deaths (Fabrega and Vila, 2013). According to the United States Centers for Disease Control and Prevention (CDC, 2016), between 1998 and 2016, there were 3260 reported outbreaks of salmonellosis resulting in 78,158 illnesses and 113 deaths (CDC, 2016). Combined with non-reported food outbreaks and illnesses, it has been estimated that *Salmonella* is responsible for approximately 19,000 hospitalizations and 380 deaths annually in the U.S. alone (Scallan et al., 2011). In Canada, 50% of bacterial foodborne outbreaks were directly associated with *Salmonella* infection (Kozak et al., 2013). The number of outbreaks and recalls due to *Salmonella* contamination has increased over last two decades (CDC, 2013; Crim et al., 2014; Huang et al., 2016; Marder et al., 2017).

Low water activity (aw) foods (Laaw) and food ingredients are in characterised by aw < 0.60, a aw low enough to inhibit the growth of most microbes. However, low aw doesn't guarantee microbial safety of food products. Outbreaks of salmonellosis caused by consumption of Laaw foods such as chocolate (Craven et al., 1975; D'Aoust, 1977; D'Aoust et al., 1975), tree nuts and their products (CDC, 2007; Kirk et al., 2004), powdered milk (CDC, 1993), cereal (CDC, 1998; Rushdy et al., 1998), and other foods (Imanishi et al., 2014; Koch et al., 2005) have occurred all over the world. *Salmonella* can survive in a low-aw environment for...
several years, which is equivalent to or in excess of the recommended shelf-life of many L.a.-F (Abd et al., 2012; Blessington et al., 2013; Kimber et al., 2012; Santillana Farakos et al., 2013; Usugi et al., 2006). The adaptation of Salmonella in response to xeric stress yields an increased propensity for survival when exposed to thermal treatment. As a result, Salmonella has been recognized as a major threat to L.a.-F microbial safety. The U.S. Food and Drug Administration (FDA) has requested data and models on survival of Salmonella to develop a quantitative assessment of the risk of human salmonellosis in tree nuts (Santillana Farakos et al., 2016).

Cocoa is the fermented dried seed of the cocoa tree, Theobroma cacao (McShea et al., 2009). Cocoa powder is rich in polyphenols with known health benefits and inherent antimicrobial properties (Calatayud et al., 2013; Kajiya et al., 2004). Cocoa powder is a key ingredient in chocolate confections and drink mixes. Salmonella might be introduced during processing, storage or transportation steps of cocoa powder production (Lima et al., 2012). Salmonella survives in the chocolate filling of cookie and cracker sandwiches (Beuchat and Mann, 2015) or chocolate (Tamminga et al., 1977) stored at ambient temperature for long durations. The D-values of Salmonella in molten milk chocolate products at 70 °C were up to 17.5 h (Goepfert and Biggie, 1968), highlighting the need to understand the inactivation kinetics of Salmonella in cocoa powder.

Salmonella contamination is a significant concern for cocoa powder due to the ability of this organism to survive in L.a.-F as well as an increase in the number of salmonellosis outbreaks linked to L.a.-F, including chocolate products. Surrogate organisms, which are similar but avirulent (Sinclair et al., 2012), are commonly utilized to evaluate the effectiveness of thermal processing. Enterococcus faecium NRRL B-2554 (E. faecium is used hereafter) has been widely used as a surrogate for Salmonella in validation of L.a.-F thermal processing, such as thermal treatments for cocoa powder (Tamminga et al., 1977) stored at ambient temperature for long periods. The TDT test cells were hermetically sealed and subjected to isothermal treatments (70, 75, or 80 °C) in an ethylene glycol bath. The temperature of TDT test cells designed by Washington State University (Chung et al., 2012), and incubated at 35 ± 2 °C for 24 h. The resulting pellet was suspended in sterile PBS to achieve ∼10^11 CFU/g of cocoa powder inside a stomacher bag (Fisher Scientific), which was then mixed by hand vigorously to ensure homogenous dispersion of inoculum.

Inoculated cocoa powder was divided into two 150 mm petri dishes (Fisher Scientific), in an even layer before being placed in aw-equilibration chamber (Custom-designed at Michigan State University) set at the target a_w (0.30 and 0.45), and equilibrated for a minimum of 4 days at room temperature (RT, 22.0 ± 0.5 °C). The a_w of the respective cocoa powder samples was measured by a a_w meter (Aqualab Series 3, Decagon Devices, Inc., Pullman, WA). Samples were used for thermal inactivation after reaching the target a_w ± 0.02. All aw was measured at RT.

Cocoa powders inoculated with individual E. faecium or 3-strain Salmonella cocktails were enumerated immediately following inoculation and a 4 days post-equilibration. Enumeration was achieved by combining 1.0 g of inoculated cocoa powder, in a 2 oz. Whirl-Pak® bag (Nasco, Ft. Atkinson, WI), with 9.0 ml sterile PBS, homogenizing for 2 min at 230 rpm in a stomacher (Seward Stomacher® Circulator 400, Worthing, UK), and serially diluting 10-fold in sterile PBS. The appropriate serial dilutions were then plated in duplicate onto TSAYE plate and incubated at 35 ± 2 °C for 24–48 h.

2.2. Cocoa powder inoculation and water activity (a_w) equilibration

Natural unsweetened 100% cocoa powder (The HERSHEY Company, USA) was purchased from a local grocery store, which contains 57.36 ± 0.68% carbohydrate, 25.91 ± 0.09% protein, 6.50 ± 0.48% fat, 6.22 ± 0.03% ash and 4.00 ± 0.62% moisture as analyzed per AOAC standard methods. Detection of cocoa background flora was performed by taking three random 1.0 g samples diluted in 9.0 ml of sterile PBS, plating appropriate serial dilutions on TSAYE then incubating at 35 ± 2 °C for 48 h. Forty grams of cocoa powder was inoculated with 400 μl of a 3-strain Salmonella cocktail or E. faecium strain to achieve ∼10^11 CFU/g of cocoa powder inside a stomacher bag (Fisher Scientific), which was then mixed by hand vigorously to ensure homogenous dispersion of inoculum.

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2.3. Thermal inactivation

Following equilibration to target a_w, 0.40 ± 0.02 g of inoculated cocoa powder was loaded into thermal death treatment (TDT) aluminum test cells designed by Washington State University (Chung et al., 2008). The TDT test cells were hermetically sealed and subjected to isothermal treatments (70, 75, or 80 °C) in an ethylene glycol bath (Isotemp Heat Bath Circulator, Fisher Scientific). The temperature of the ethylene glycol bath was recorded by Omega Precision RTD temperature recorder (OM-CP-RTDTemp2000, Omega Engineering Inc., Norwalk, CT). TDT test cells with T-type thermocouples at the geometrical center were used to measure heat penetration and come up time (CUT, the time required for sample core temperature to reach with 0.5 °C of the set temperature). The resulting CUT for cocoa powder was 1.5 min, and heat treatment timing started after reaching CUT. Test cells from respective treatments were collected at the selected time points, then immediately chilled in an ice-water bath for 2 min. All tests were conducted in triplicate and repeated three times independently.

2.4. Enumeration of viable bacteria

Heat-treated cocoa powder was transferred from TDT test cells to a
Following four days post-inoculation at RT, 0.68, 0.75, 0.58 and 0.56 strains and *Salmonella* extended storage

3.1. Stability of *Salmonella* and *E. faecium* during equilibration and extended storage

To evaluate strain variability, the survival of three individual *Salmonella* strains and *E. faecium* during desiccation was determined. Following four days post-inoculation at RT, 0.68, 0.75, 0.58 and 0.56 Log10 CFU/g reductions were determined for *S. Enteritidis* PT30, *S. Tennessee* K4643, *S. Agona* 447967 and *E. faecium*, respectively, indicating these strains had a similar stability in aw 0.30 cocoa powder during 4 days of equilibration in the moisture chamber (Fig. 1). During 4-day equilibration at aw 0.30, there were 0.87 and 0.29 Log CFU/g reductions of *Salmonella* cocktail and *E. faecium*, respectively (Fig. 2A). The population of *Salmonella* cocktail and *E. faecium* remained at similar levels as the initial inoculation level during the first month of RT storage. Both *Salmonella* cocktail and *E. faecium* survived in aw 0.30 cocoa powder during 1-year RT storage with ~1.39 Log...
at a w 0.30, calculated from the slope of the trend lines of 70, 75, and 80 °C were 31.6 ± 3.3, 16.9 ± 2.0, and 7.0 ± 0.9 min, respectively (Fig. 3B), compared to the D-values of 25.8 ± 1.3, 13.7 ± 0.6, and 4.7 ± 0.6 min for E. faecium, respectively (Fig. 3B). At all three temperatures used for heat inactivation, both Salmonella and E. faecium showed a higher thermal resistance in cocoa powder equilibrated at a_w 0.30 than those equilibrated at a_w 0.45.

The z-values for Salmonella in cocoa powder at a_w 0.30 to 0.45 were 16.9 ± 1.9 and 17.5 ± 2.7 °C, respectively (Table 1, Fig. 4). The z-values of E. faecium decreased from 15.2 ± 2.5 to 13.0 ± 1.3 °C as a_w increased from 0.30 to 0.45 (Table 1, Fig. 4).

4. Discussion

La_w,F are traditionally considered safe, yet there are significant occurrences of foodborne pathogen outbreaks linked to La_w,F. According to CDC reports, from 2006 to 2017, there were 59 Salmonella outbreaks linked to a variety of fruits, shelled eggs, sprouts, tree nuts, organic shakes and meals, peanut butter products, frozen and raw fish, chicken, turkey, cheese, ground beef, pork, and cereals (CDC, 2017). Amongst these outbreaks, approximately one third of reported Salmonella outbreaks were associated with La_w,F (CDC, 2017). Multiple Salmonella strains, including S. Eastbourne (Graven et al., 1975), S. Napoli (Gill et al., 1983), S. Nima (Hockin et al., 1989), S. Typhimurium (Kapperud et al., 1990), and S. Oranienburg (Werber et al., 2005), were linked to chocolate outbreaks worldwide, clearly demonstrating a need to evaluate and validate thermal processing of cocoa powder against potential Salmonella contamination and outbreaks.

Pathogen reduction procedures for the manufacture of cocoa powder includes alkalization, heating up to 100 °C, and roasting of whole cocoa beans or nibs for 5–35 min at temperatures between 110 and 140 °C, which are considered the main kill steps for Salmonella (Beckett, 2008; ICMSF, 2005; Lima et al., 2012). However, pathogenic bacteria including Salmonella might survive during roasting, or be introduced post process, during storage or transportation steps of cocoa powder production. In current industry practice, after roasting, no other heat treatment steps are applied to cocoa powder until it is used as an ingredient for chocolate, chocolate drinks, and other formulated products containing chocolate (Beckett, 2008; Lima et al., 2012). Conching at 50–70 °C during chocolate manufacturing alone is not sufficient to eliminate Salmonella contamination (Krapf and Gantenbein-Demarchi, 2010; Nascimento Mda et al., 2012). To sufficiently develop and implement appropriate procedures as part of a risk-based preventative control measure, it is key to understand the thermal inactivation kinetics of Salmonella at common thermal processing conditions and further validate E. faecium as a surrogate for thermal processing of cocoa powder.

4.1. Salmonella thermal resistance in cocoa powder

The survival of Salmonella in cocoa powder during thermal inactivation is inversely related to a_w. Similar phenomena were reported in previous studies on the thermal inactivation kinetics of Salmonella in La_w,F such as wheat flour (Liu et al., 2018a), pet foods (Ceylan and Bautista, 2015), skim milk powder (Laroche et al., 2005), and peanut paste (Kataoka et al., 2014). A similar relationship between a_w and thermal resistance has also been observed in Listeria monocytogenes in wheat flour (Taylor et al., 2018). The underlying mechanisms of the increased thermal resistance of Salmonella, once adapted to low a_w environments, have yet to be elucidated.

Besides a_w, thermal resistance of bacteria is also affected by many other factors including bacteria strain (Goepfert and Biggie, 1968), bacterial growth stage (Ngl et al., 1969; Rolfe et al., 2012), food physical structure (Mogollon et al., 2009), fat content (Juneja and Eblen, 2000), and carbohydrates (He et al., 2011). D-values of S. Typhimurium in molten milk chocolate at 70–90 °C were nearly 2-fold of those of S.
Table 1
Thermal inactivation data for Salmonella and E. faecium NRRL B-2354 in cocoa powder.

<table>
<thead>
<tr>
<th>Initial sample a&lt;sub&gt;0&lt;/sub&gt;</th>
<th>Log-Linear Model</th>
<th>Weibull Model</th>
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<tbody>
<tr>
<td></td>
<td>Temp (°C)</td>
<td>D-value (min)</td>
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<tr>
<td>0.30</td>
<td>Salmonella</td>
<td>70</td>
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<td>75</td>
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<td></td>
<td>E. faecium</td>
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RMSE: the root mean square error; a<sub>0</sub>: water activity. Upper limit of the 95% prediction interval (PI) for the D-value was calculated from the regression line equation + 2 RMSE. Mean ± SEM, n = 3.

Senftenberg 775 W (Goepfert and Biggie, 1968). D<sub>70</sub> and D<sub>80</sub> values of Salmonella in molten chocolate were much higher than respective D-values of Salmonella strains in cocoa powder obtained in this study (Goepfert and Biggie, 1968). The observed difference could be due to combination of strain, food matrix, a<sub>0</sub>, and food physical state. E. faecium showed a much higher resistance in wheat flour compared to cocoa powder at both a<sub>0</sub> 0.30 and 0.45; D<sub>70</sub>- and D<sub>80</sub>- values for Salmonella in cocoa powder were comparable to those in wheat flour (Liu et al., 2018a). During 1-year RT storage in aw 0.30 cocoa powder, a 3.75 log reduction of Salmonella in cocoa powder during storage was observed.

4.2. Suitability of E. faecium as a surrogate for Salmonella

The Food Safety Modernization Act final rule for Preventive Controls for Human Food (FDA, 2015) mandates that a food company must implement and validate interventions to prevent or control identified significant hazards, such as Salmonella in many L<sub>a</sub>F. Due to its non-pathogenic characteristics and higher heat and acid resistance (Kopit et al., 2014), E. faecium has been used extensively as a surrogate for Salmonella in L<sub>a</sub>F thermal validation. When compared to Salmonella, E. faecium exhibits a higher heat resistance during the processing of various L<sub>a</sub>F, such as the extrusion processing of carbohydrate-protein meal (Bianchini et al., 2014), thermal heating of low-a<sub>0</sub> pet food (Ceylan and Bautista, 2015) and peanut paste (Kataoka et al., 2014), and radio-frequency processing (Liu et al., 2018b) or thermal treatment of wheat flour (Liu et al., 2018a).

In cocoa powder with a<sub>0</sub> 0.30, D-values of E. faecium at all test temperatures were higher than those of Salmonella. Ratios of D-values of E. faecium and Salmonella ranged between 1.3 and 1.4, indicating E. faecium is a suitable surrogate for Salmonella during thermal processing of cocoa powder at a<sub>0</sub> 0.30. However, when the initial a<sub>0</sub> is increased to 0.45, the D-value of Salmonella exhibited a higher resistance than those of E. faecium in cocoa powder at all target temperatures, with the ratios of D-value of E. faecium and S. Enteritidis at 0.67–0.81. This indicates that E. faecium may not be an ideal surrogate for Salmonella when a<sub>0</sub> is increased beyond a certain level in cocoa powder. D<sub>60</sub>-values of Salmonella in a confectionery formulation (87.5% carbohydrate, 1% fat and 3% protein) with a<sub>0</sub> 0.57 were higher than that of E. faecium (Rachon et al., 2016). In the Log<sub>10</sub>-D versus temperature plot of this study, the line of Salmonella intersects with the line of E. faecium at approximately 70 °C at a<sub>0</sub> 0.45, indicating the point at which the suitability of E. faecium as bacterial surrogate changes. Temperatures below this point such as 60 °C, showed a higher thermal resistance in E. faecium (data not shown).

4.3. Salmonella stability in unsweetened cocoa powder

During 1-year RT storage in a<sub>0</sub> 0.30 cocoa powder, a 3.75 log reduction of Salmonella in cocoa powder during storage was observed. Similarly, during a 12-month RT storage, Salmonella inoculated on almond kernels and in shell pistachios at a<sub>0</sub> 0.40 decreased approximately 2.4 and 1.8 log, respectively (Kimber et al., 2012). A 2.5 log reduction of Salmonella also occurred on inoculated almond kernel stored at RT for 1 year (Uesugi et al., 2006). In peanut paste with 47% fat and a<sub>0</sub> 0.30, a 1.3 to 1.8 log reduction of Salmonella was observed.
over 12-month RT storage dependent on *Salmonella* strain (Kataoka et al., 2014). However, a 7-week 25 °C storage resulted in a 6.0-log reduction of *S. Montevideo* or *S. Heidelberg* inoculated on *a*₀ 0.43 cocoa powder using slant-grown inoculum (Juven et al., 1984), much less stable than *Salmonella* cocktail in *a*₀ 0.30 cocoa powder determined in this study. These data highlight important roles of *a*₀ in *Salmonella* survival in cocoa powder. Additionally, strain variability, inoculation method, food matrix, and possibly cocoa powder source might also contribute to the difference in desiccation stability of *Salmonella* in cocoa powder during prolonged ambient storage. *Salmonella* was more stable in pine nuts compared to pecan and hazelnuts at each *a*₀ and storage temperature combination (Farakos et al., 2017). Lawn-grown *Salmonella* displayed much higher stability in cocoa butter oil than those inoculated with liquid inoculum; within the same inoculation, S. Oranienburg was more stable than S. Enteritidis PT30 in cocoa butter oil over 21 day storage at both 5 and 21 °C (Komitopoulos and Penalosa, 2009). The ability of *Salmonella* to survive in *La*₀,F is also dependent on storage temperature. *Salmonella* survived better in sucrose stored at 5 °C than those stored at 25 °C regardless of *a*₀ (Beuchat et al., 2017). *Salmonella* were more stable in pine nuts, pecan, and hazelnuts stored at 4 and 10 °C than those stored at 25 °C, and there was less than 1.5-log CFU/g reduction over 1-year storage at 4 and 10 °C (Farakos et al., 2017). *Salmonella* retains a higher viability in dried fruits (Beuchat and Mann, 2014), almond (Kimber et al., 2012; Usugui et al., 2006), pistachios (Kimber et al., 2012), infant formula (Koseki et al., 2015) stored at 4 or 5 °C compared to ambient storage temperature.

Similarly, Shiga toxin producing *E. coli* retains a higher viability in chocolate (~ *a*₀ 0.40) stored at 10 °C compared to RT storage (Baylis et al., 2004). Our data suggest that *E. faecium* is an appropriate surrogate for *Salmonella* in cocoa powder during RT storage; there was a 3.75 and 1.39 Log reduction of *Salmonella* and *E. faecium* in *a*₀ 0.30 cocoa powder, respectively, over 1-year RT storage.

5. Conclusions

Both *Salmonella* and *E. faecium* survived in *a*₀ 0.30 cocoa powder over 1-year RT storage. *E. faecium* had a higher desiccation stability in *a*₀ 0.30 cocoa powder compared to *Salmonella*. There was a 1.39 log reduction of *E. faecium* during 12 months of storage at RT compared to a 3.75 log reduction of the *Salmonella* cocktail stored at the same condition and environment. Thermal resistance, as indicated by the D-values, of both *Salmonella* and *E. faecium* increased as sample *a*₀ decreased from 0.45 to 0.30. However, *E. faecium* showed higher D-values in *a*₀ 0.30 cocoa powder compared to *Salmonella*, but at *a*₀ 0.45 cocoa powder, *E. faecium* had lower D-values than *Salmonella*. These data provide technical information for *Salmonella* inactivation validation during cocoa powder production. Data also highlight that precaution is needed when adopting *E. faecium* as a *Salmonella* surrogate for *La*₀,F thermal process validation.

Conflicts of interest

The authors have no known conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.01.005.