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Biofilm forming *Salmonella* strains exhibit enhanced thermal resistance in wheat flour

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ABSTRACT

The biofilm lifestyle of bacteria confers a remarkably increased tolerance to antimicrobial interventions and environmental stresses, however little is known about influence of biofilms on thermal resistance of *Salmonella* in low-moisture foods. This study was aimed to assess the correlation between the ability of biofilm formation of *Salmonella* Enteritidis (*S.* Enteritidis) strains and their capacity to survive desiccation and thermal treatment in wheat flour as a model for low-moisture food. The production of the biofilm in *S.* Enteritidis strains was analyzed qualitatively and quantitatively using calcofluor fluorescence, congo-red binding, pellicle formation and microtiter-plate test. Subsequently, three biofilm-forming and four non-forming *S.* Enteritidis strains were selected. Survival after desiccation was evaluated by population counts before and after equilibration for 4–5 days at 45% RH. Thermal resistance ($D_{80\ ^\circ C,\ 0.45\ aw}$) of *S.* Enteritidis in wheat flour was evaluated by fitting the thermal inactivation kinetic data with the first order kinetics model. The biofilm forming ability was not associated with resistance to desiccation. However, thermal resistance ($D_{80\ ^\circ C,\ 0.45\ aw}$) and pre-formed biofilm amount ($OD_{492\ nm}$) showed a linear correlation (Spearmen correlation $\rho = 0.8$, $p < 0.05$), indicating more biofilm production confers more thermal resistance. Average thermal resistance ($D_{80\ ^\circ C,\ 0.45\ aw}$) was significantly ($p < 0.05$) higher among biofilm formers (14.1 ± 0.6 min) when compared with non-formers (6.0 ± 0.2 min). This study shows that the amount of biofilm produced by *Salmonella* on congo red-calcofluor media is linearly correlated with the thermal resistance of *Salmonella* in wheat flour. The findings reinforce the necessity of appropriate management in sanitation and biofilm removal in plants that process low-moisture foods.

1. Introduction

Salmonellosis is one of the leading food-borne illnesses resulting in >1 million cases each year (Batz, Hoffmann, & Morris, 2012). A variety of low and high moisture foods have been implicated as risk factors for human infection (CDC, 2015; Scott et al., 2009). In high moisture foods that have water activity (a_w) > 0.96, *Salmonella* is relatively sensitive to thermal killing with $D_{70\ ^\circ C}$ values ranging from 0.06 to 0.2 min (Schmidt & Fontana, 2007; Silva & Gibbs, 2012). However, in low-moisture foods with a_w<0.60, the thermal resistance of *Salmonella* increases dramatically. Archer, Jervis, Bird, and Gaze (1998) reported that in wheat flour with a_w between 0.5 and 0.6, *S.* Weltevreden showed a $D_{69-71\ ^\circ C}$ of 80 min. Similarly, *S.* Typhimurium in molten chocolate showed a $D_{70\ ^\circ C}$ of 816 min (Goepfert & Biggie, 1968), *S.* Enteritidis PT30 in almond meal at a_w = 0.60 showed a $D_{70\ ^\circ C}$ of 15 min (Villa-Rojas et al., 2013), and *S.* Orianienburg showed a $D_{75\ ^\circ C}$ of 10.4 and 7.7 min in hazelnuts and cocoa, respectively (Izurieta & Komitopoulou, 2012). Although these reports indicate increased thermal resistance of *Salmonella* in low a_w foods, the underlying mechanism remains elusive.

Published studies have shown *Salmonella* may utilize complex defense mechanisms to cope with harsh conditions such as desiccation by producing compatible solutes and by forming biofilm

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(Finn, Condell, McClure, Amézquita, & Fanning, 2013; Kumar & Anand, 1998; Steenackers, Hermans, Vanderleyden, & De Keersmaecker, 2012). Biofilms form when stressed bacterial cells start attaching to a surface and secrete a protective layer constituted by extracellular polysaccharides (EPS), proteins and nucleic acids; this layer encases the bacterial community and provides both protection and a means to interact with their environment (Billi & Potts, 2002; Braune & Sanke, 1979; Potts, 1994). Recent studies have shown *Salmonella* can attach to the surface of foods such as cantaloupes, melons (Annous, Solomon, Cooke, & Burke, 2005), tomatoes (Iturriaga, Tamplin, & Escartín, 2007), almonds (Suehr, Jeong, & Marks, 2015) and grains (Cui, Walcott, & Chen, 2015), as well as food contact surfaces (Joseph, Otta, & Karunasagar, 2001). Once attached, *Salmonella* can produce Tafi or curli thin aggregative fimbriae (AgfD, AgfB) and cellulose (AdrA) which are important indicator of biofilm production. The thin aggregative fimbriae allow the bacterial cells to attach and colonize surfaces (Ambalam, Kondepudi, Nilsson, Wadström, & Ljungh, 2012; Uhlich, Cooke, & Solomon, 2006). The production of fimbriae confers a rough morphology to the colonies and can be detected on laboratory media because Congo red dye included in the medium attaches to the fimbriae and/or extracellular material producing a red colored colony (Branda, Vik, Friedman, & Kolter, 2005). The biofilm forming ability, amount and composition of the biofilm may vary depending on the *Salmonella* strain and the environment (Shi & Zhu, 2009). This presents a considerable challenge in the food industry, because biofilms confer significant resistance to sanitizers, antibiotics and other environmental stresses (Steenackers et al., 2012). For example, White, Gibson, Kim, Kay, and Surette (2006) reported that survival of wild-type *S.* Typhimurium is 1000-fold higher upon exposure to sodium hypoclorite when compared with its mutants lacking the ability to form biofilm. Production of curli fimbriae was also shown to enhance long-term desiccation survival of different *Salmonella* serotypes (Vestby, Møretrø, Ballance, Langsrud, & Nesse, 2009). However, the current knowledge on whether biofilm influences thermal resistance is not only limited but also contradictory. It is believed that increase in heat tolerance of *Salmonella* is partly driven by intrinsic and extrinsic properties of food and types of food products (Finn et al., 2013). Dhir and Dodd (1995) reported that *S.* Enteritidis biofilms harvested from either glass coverslips or stainless steel coupons showed increased thermal resistance when compared to their planktonic cells counterparts. In contrast, Scher, Romling, and Yaron (2005) reported that *S.* Typhimurium biofilm grown in the form of a pellicle from a 24-h broth culture was more sensitive to thermal treatment when compared with their planktonic counterparts.

The objective of this study was to examine the relationship between the amount of pre-formed biofilm obtained from different *S.* Enteritidis strains and their resistance to short-term desiccation survival and thermal inactivation in wheat flour as a model for low-moisture foods.

2. Material and methods

2.1. Salmonella strains

Six genetically distinguishable, but closely related (Fig. 1) strains of *S.* Enteritidis (G3, MD4, UK1, G2, MD9 and P97) were used as model organisms for this study. These strains were specifically chosen for this study because they display unique differences in biofilm formation ability and serve as a model to study effect of biofilms on thermal resistance of *Salmonella* (see below). In addition, a curli or thin aggregative fimbriae deficient *S.* Enteritidis G1 (*ΔcsgB*:Tn5) strain was included as a non-biofilm producing control strain for comparison (Shah et al., 2011, Shah, Zhou, Kim, , Call, & Guard, 2012). The bacterial strains were routinely grown in Luria Bertani (LB) medium (Difco, BD Diagnostic Systems, Spark, MD, USA) at 37 °C overnight (16 h) with shaking at 200 rpm. Genetic relatedness of the strains was determined by *Xba*I-pulse field gel electrophoresis (PFGE) following protocol described previously (CDC, 2013).

2.2. Biofilm production assays

Qualitative and quantitative biofilm production assays were performed to assess biofilm forming ability of all *S.* Enteritidis strains. Biofilm production was initially assessed based on formation of pellicle at the air–broth interface as described previously (Solano et al., 2002) with minor modifications. Briefly, *Salmonella* strains were grown in 5 mL LB no-salt at 28 °C with shaking (120 rpm) in borosilicate tubes for 48 h. It is expected that these growth conditions should induce all forms of biofilms produced by any of the strain included in this study (Scher et al., 2005). Strains that formed a slimy layer at the air–broth interface were considered as biofilm formers.

Production of thin aggregative fimbriae and/or cellulose was determined qualitatively following previously described methods (Römling, Sierralta, Eriksson, & Normark, 1998). Briefly, overnight cultures were streaked onto LB no-salt agar plate supplemented with Congo red (40 μg/mL, Sigma-Aldrich, St. Louis, MO, USA) and coomassie brilliant blue G (20 μg/mL,Sigma-Aldrich) followed by incubation at 28 °C for 48 h. Cells producing only thin aggregative fimbriae formed brown, dry, and rough colonies (*bdar* morphotype), and those producing both thin aggregative fimbriae and cellulose formed red, dry, and rough colonies (*rdar* morphotype). Cells producing only cellulose formed pink, dry, and rough colonies (*pdar* morphotype), and those producing neither thin aggregative fimbriae nor cellulose formed smooth and white or pink colonies (*saw* or *sap* morphotype).

Cellulose produced by *Salmonella* is an important component of biofilms (Branda et al., 2005). Binding of cellulose produced by *Salmonella* cells to calcofluor results in fluorescence upon exposure to shining UV light (Harrington & Hageage, 2003). Cellulose production was determined qualitatively as previously described (Solano et al., 2002). Briefly, *Salmonella* cultures were plated on LB no-salt agar (Difco, BD) plates supplemented with 200 μg/mL calcofluor white stain (fluorescent brightener 28, Sigma-Aldrich) and incubated at 28 °C for 48 h. Fluorescent stain bound to the cellulose produced by *Salmonella* cells was observed under UV light (365_{nm}). Strains that showed fluorescence under UV light were considered as cellulose positive.

The quantity of biofilm produced by each strain was determined in 96-well flat-bottom polystyrene plates using safranin staining as described previously with minor modifications (Shah et al., 2011). Briefly, individual isolates were grown overnight (16 h) at 37 °C in 1 mL LB in a 96 well -block (Greiner bio-one, NC, USA). Subsequently, bacterial cultures (1:100 dilution) were transferred into

Fig. 1. Macrorestricition enzyme- pulsed field gel electrophoresis (MRP-PFGE) profiles of *Xba*I-digested DNA of *S.* Enteritidis strains.

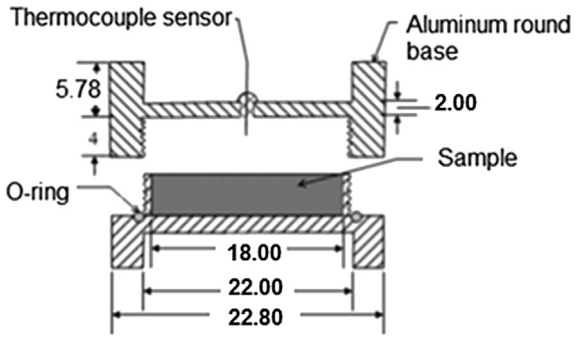

Fig. 2. Schematic representation of the cross-section of the thermal death treatment (TDT) test cell, dimensions in mm (Chung et al., 2008).

96-well plates (Evergreen Scientific, CA, USA) containing 200 μL LB no-salt via a metal pronged replicator (Boekel Scientific, PA, USA), incubated at 25 °C for 72 h without shaking. Next, the medium from each well was decanted and washed three times with 250 μL of sterile 1X PBS to remove any loosely associated bacteria. Plates were air dried for 20 min followed by addition of 250 μL of 0.1% safranin (Sigma-Aldrich) and incubated for 15 min at room temperature. Wells were washed once with 250 μl of 1XPBS and bound safranin was dissolved by addition of 250 μL of 30% acetic acid (J. T. Baker, Center Valley, PA, USA). Finally, $OD_{492\ nm}$ was measured using ELISA plate reader (Multiskan MCC, Fisher Scientific, USA). Each strain was tested in triplicates in three independent experiments. To normalize the data, the average $OD_{492\ nm}$ value of a uninoculated media control was subtracted from all initial $OD_{492\ nm}$ values. The mean ± SEM $OD_{492\ nm}$ value of all biological replicates was reported for each strain.

2.3. Inoculum preparation, sample inoculation and survival to desiccation

The premise of this study was to show how *Salmonella* biofilm increased thermal resistance in low moisture food. The first approach was to inoculate the wheat flour with liquid inoculum and then re-equilibrate the sample to a lower a_w (0.45) to naturally induce stressful conditions that would trigger the biofilm response. However, micrographies obtained with Scanning Electron Microscopy (SEM, data not shown) were not able to unequivocally show that biofilms were present in the flour due to the interference of particle agglomeration and dough formation after SEM sample preparation. Therefore, to ensure the presence of biofilm in wheat flour, biofilms were induced by growing on agar (referred to as preformed biofilm) then inoculated into the sample.

For the purpose of inducing and harvesting biofilms, the *Salmonella* strains were streaked on LB no- salt agar supplemented with combination of calcofluor (200 μg/mL), Congo red (40 μg/mL) and brilliant blue G (20 μg/mL) followed by incubation at 28 °C for 48 h. Biofilm production under these growth conditions has been well documented (Jonas et al., 2007; Latasa et al., 2005; Römling, Rohde, Olsén, Normark, & Reinköster, 2000; Solano et al., 2002; Uhlich et al., 2006; Zogaj, Nimtz, Rohde, Bokranz, & Römling, 2001). This pre-formed biofilm with bacterial community was then harvested from the above plate in maximum recovery diluent (MRD, Fisher Scientific, Pittsburg, PA), pelletized, re-suspended in 3 mL of MRD and 100 μL were used to inoculate 100 g of organic soft white wheat pastry flour (Eden Foods, Clinton, MI) to obtain an initial population between $10^7 - 10^8$ CFU/g. Inoculated samples were equilibrated to $a_w = 0.45$ for approximately 4–5 days in a small glove box (EW-34788-00, Cole Parmer, Vernon Hills, IL) with a humidity control system costume designed at Michigan State University. The bacterial population within the harvested biofilms was enumerated immediately after mixing with wheat flour and also after the flour reached the desired a_w (0.45) to evaluate the survival after desiccation.

2.4. Thermal treatments

For thermal death inactivation kinetics, the thermal death treatment (TDT) aluminum test cells (Fig. 2) designed at Washington State University were used (Chung, Birla, & Tang, 2008). To measure heat penetration and come up time (CUT), which is the time necessary to reach the target temperature, a TDT cell embedded with a T-type thermocouple at the geometrical center was used. The thermocouple was attached to a thermometer (Digiense DuaLogR 99100-50, Cole-Parmer Instruments Co., Vernon Hills, IL) and time - temperature history was recorded in triplicate; the resulting CUT was 2.20 min. The TDT cells were filled with 0.7 g of wheat flour pre-inoculated with different *Salmonella* strains and equilibrated to the target a_w, followed by isothermal treatment at 80 °C in an oil bath (Isotemp 5150 H11, Fisher Scientific, Inc., PA, USA). Representative cells from respective treatments were collected in triplicates at different time points, then immediately cooled in an ice-water bath for 30 s. The flour samples within each TDT cell were mixed with 6.3 mL of MRD, stomached for 3 min at 230 rpm in a Stomacher® (400, Seward, West Sussex, UK), serially 10-fold diluted and plated on TSA plates supplemented with 0.6% (w/v) yeast extract, 0.05% (w/v) ferric ammonium citrate, and 0.03% (w/v) sodium thiosulfate. Plates were incubated for 48 h at 37 °C before counting characteristic *Salmonella* colonies with a dark black center.

Table 1
The biofilm forming ability of the selected *S.* Enteritidis strains.

Strain	Calcofluor fluorescence[a]	Congo-red agar morphotype[b]	Pellicle formation	Microplate titration (OD_{492nm})[c,d]
Non-biofilm formers (bf−)				**0.11 ± 0.00***
P97	−	saw	Clear surface	0.03 ± 0.01^{A}
MD9	−	saw	Clear surface	0.08 ± 0.03^{B}
G1 *Δcsgb*::Tn5	±	sap	Clear surface	0.08 ± 0.04^{B}
G2 (PT13a)	+	pdar	Clear surface	0.20 ± 0.07^{C}
Biofilm formers (bf+)				**0.68 ± 0.00**
G3 (PT13a)	++	rdar	Slimy layer (++)	0.59 ± 0.12^{D}
UK1 (PT4)	++	rdar	Slimy layer (++)	$0.67 \pm 0.15^{D,E}$
MD4	++	rdar	Slimy layer (++)	0.79 ± 0.24^{E}

[a] +, ± or − non-former; ++ former.
[b] saw, smooth and white; sap, smooth and pink; pdar, pink, dry and rough; rdar, red, dry and rough.
[c] Significantly different means ($p < 0.05$) in a column have different letters.
[d] * indicates there's significant difference ($p < 0.05$) between OD_{492nm} means of biofilm groups.

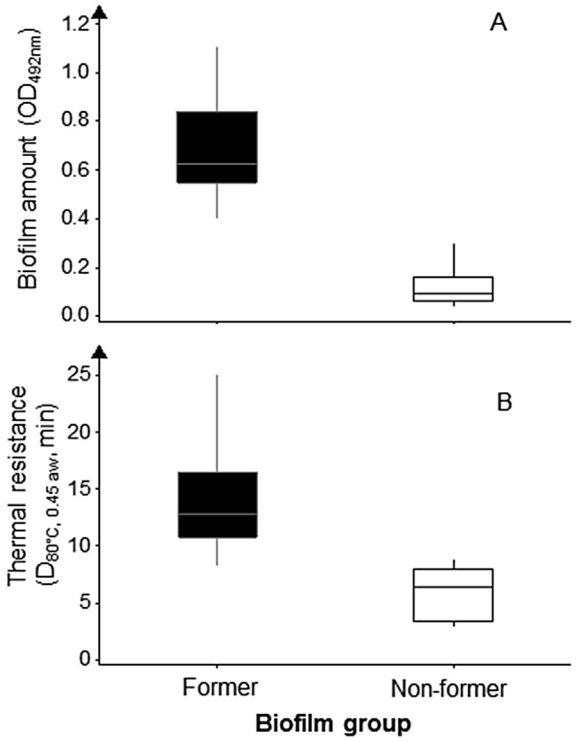

Fig. 3. Comparison between non-biofilm formers (bf−, □) and biofilm formers (bf+, ■) on: (A) group average biofilm forming ability ($OD_{492\ nm}$) and (B) group average thermal resistance ($D_{80\ °C,\ 0.45\ a_w}$). At least three replicates per strain were tested.

2.5. Modeling and statistical analysis

Thermal resistance (D-values) were calculated using the first order kinetics model defined by the following equation (Peleg, 2006):

$$\text{Log } S(t) = -t/D \quad (1)$$

$$\text{for } S(t) = N/N_0 \quad (2)$$

N: population at time t (CFU/g)
N_0: initial population (CFU/g)
t: time of isothermal treatment (min)
D: time required to reduce the microbial population by 90% at a determined temperature (°C) in min.

The linear regression and goodness of fit (R^2 and RMSE) were calculated in Minitab 14. A student's t-test was used to compare group averages of D and $OD_{492\ nm}$ values from biofilm non-formers and formers ($p = 0.05$) in Minitab 14. Significant differences ($p = 0.05$) between strain $D_{80\ °C,\ 0.45\ aw}$ and $OD_{492\ nm}$ values were evaluated using Friedman's test, while linear correlation was evaluated with Spearman coefficient in SAS.

3. Results and discussion

3.1. Biofilm production of Salmonella strains

The differences in the quantity and quality of biofilm production of seven genetically distinguishable, but closely related *S.* Enteritidis strains (Fig. 1) are shown in Table 1. Strains G3, UK1 and MD4 produced a typical slimy layer at the air-broth interface in the tube pellicle formation assay which is similar to what was reported earlier for biofilm producing *S.* Enteritidis strains (Solano et al., 2002). Consequently, these strains were identified and designated as bf+ or 'biofilm formers'. In contrast, no slime layer was detected at the air-broth interface for strains P97, MD9, G2 and G1 Δ*csgB::Tn5* (Table 1), suggesting that these strains were bf− or 'biofilm non-formers'. Subsequent quantitative assay using Safranin staining revealed that bf+ strains had significantly higher ($p < 0.05$) $OD_{492\ nm}$ values (range = 0.59–0.79, mean = 0.68 ± 0.00) than the bf− (range = 0.03–0.20, mean = 0.11 ± 0.00) (Table 1). The average $OD_{492\ nm}$ of bf− strains was significantly lower than that of bf+ strains (Fig. 3A). As expected, all bf+ strains exhibited strong fluorescence on calcofluor medium suggesting that all bf+ strains produced cellulose, one of the important components of biofilms. In contrast, two bf− strains (P97 and MD9) showed no fluorescence whereas the remaining two bf− strains (G1 Δ*csgB::Tn5* and G2) showed very weak fluorescence (Table 1), suggesting that the latter strains are likely weak cellulose producers. Consistent with Safranin staining and calcofluor fluorescence, all bf+ strains (G3, UK1 and MD4) produced colony morphotype characterized by red, dry and rough (*rdar*) morphotype, suggesting that in addition to cellulose, these strains also produced thin aggregative fimbriae, another important component of biofilms (Table 1). In contrast, two bf− strains (P97 and MD9) produced the smooth and white (*saw*) colony morphotype, suggesting that these strains do not produce thin aggregative fimbriae or cellulose and therefore do not form

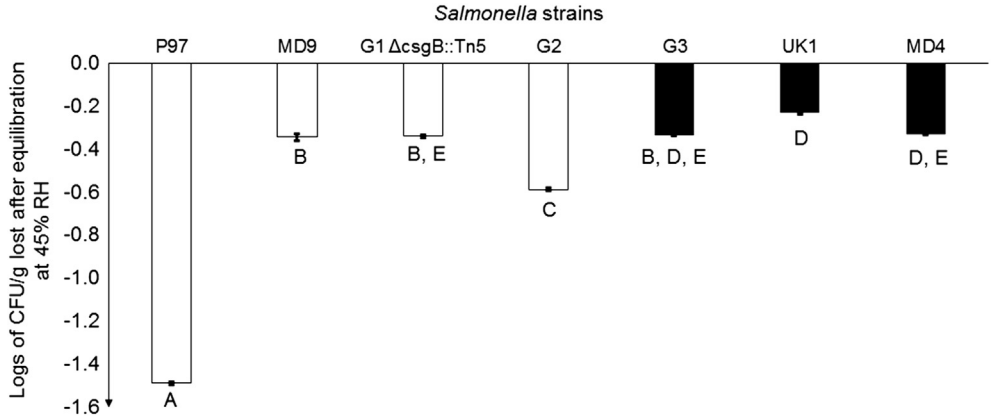

Fig. 4. Log reduction of *Salmonella* after equilibration at 45%RH. □: non-biofilm former (bf−) strains; ■: biofilm former (bf+) strains. Significantly different ($p < 0.05$) means have different letters. Bars represent standard error.

biofilms under inducible conditions used in this study. Strain G2 produced the pink, rough and dry (*pdar*) colony morphotype whereas strain G1 Δ*csgB::Tn5* produced the smooth and pink (saw) colony morphotype suggesting that these strains are, at best, weakly cellulose positive as detected by the calcoflour assay. Collectively, these results suggested that P97, MD9, G1 Δ*csgB::Tn5* and G2 strains do not produce biofilms (i.e. bf−), whereas G3, UK1 and MD4 strains produce strong biofilms (i.e. bf+) when grown under inducible conditions used in this study (Table 1).

3.2. Survival after desiccation

The initial inoculation level was similar for all strains, but the viable counts significantly declined ($p < 0.05$) after equilibration to a RH of 45% in approximately 4–5 days (Fig. 4). Although two non-biofilm formers (P97 and G2) showed significant drops in population after desiccation (−1.49 ± 0.01 and −0.59 ± 0.01 Log CFU/g, respectively), there was no evident advantage associated with the presence or amount of biofilm and resistance to desiccation because the viable counts of all other strains declined by less than 0.5 log after equilibration (Fig. 4). These results suggest that biofilms afforded no clear resistance of *Salmonella* to desiccation. In support of our results, Dancer, Mah, Rhee, Hwang, and Kang (2009) reported that biofilm formation afforded no clear advantage to *E. sakazakii* under similar conditions at low a_w. Barron and Forsythe (2007) also encountered a similar situation when comparing 10 different bacteria belonging to the family Enterobacteriaceae including *E. coli* and *Salmonella*. These authors desiccated the bacteria at 25 °C in infant formula and assessed resistance to desiccation and persistence to storage at 25 °C for 2.5 years. They reported a decline in population numbers after desiccation for all bacteria, with no clear advantage of capsulation. Aviles, Klotz, Eifert, Williams, and Ponder (2013) studied the influence of physiological state of *S.* Tennessee (planktonic vs biofilm) during desiccation in milk powder and subsequent storage for 30 days at 25 °C. They concluded that there was no clear advantage afforded by biofilms in resisting desiccation. Thus, our results corroborate with published studies and suggest that biofilm formation may not influence the ability of *Salmonella* to resist desiccation (Kumar & Anand, 1998).

3.3. Correlation between pre-formed biofilm and thermal inactivation kinetics

The next step was to determine if the pre-formed biofilm affected the thermal resistance of *Salmonella* strains. First, the inactivation kinetics at 80 °C was evaluated by constructing the survivor curve, Log N/N_0 vs treatment time. As expected, all strains showed a Log linear trend (Fig. 5), suggesting that inactivation rate is constant and a first order kinetic model could properly describe the inactivation kinetics. Next, the $D_{80\ °C,\ 0.45\ aw}$ was calculated with the first order kinetic model which had a good fit represented by low RMSE (0.17–0.49) and relatively high R^2 (0.78–0.95,Table 2), followed by a pair-wise comparison among strains and compared the average D-value of the bf+ and bf− strains. The bf+ strains had higher $D_{80\ °C,\ 0.45\ aw}$ (10.2–18.9 min) when compared with the bf− strains (3.1–8.0 min). The average group D-values of bf+ strains were significantly higher than those of bf− strains ($p < 0.05$) (Table 2, Fig. 3B).

Table 2
Calculated $D_{80\ °C,\ 0.45\ aw}$ for *S.* Enteritidis strains in organic wheat flour.

Strain (replicas)	D- value ± SE (min)[a, b]	R^2 ± SE	RSME ± SE
Non-formers (bf−)	**6.0 ± 0.2***	**NC**	**NC**
P97 (3)	NC[c]	NC	NC
MD9 (3)	**3.1 ± 0.2**^{D}	0.95 ± 0.01	0.17 ± 0.01
G1 *Δcsgb*::Tn5 (3)	**6.1 ± 0.4**^{D}	0.91 ± 0.03	0.19 ± 0.04
G2 (4)	**8.0 ± 0.4**^{C}	0.87 ± 0.02	0.33 ± 0.02
Biofilm formers (bf+)	**14.1 ± 0.6**	**NC**	**NC**
G3 (4)	**10.2 ± 0.7**^{B}	0.89 ± 0.03	0.26 ± 0.01
UK1 (3)	**12.9 ± 1.2**^{B}	0.78 ± 0.05	0.49 ± 0.08
MD4 (4)	**21.7 ± 3.5**^{A}	0.86 ± 0.03	0.29 ± 0.04

[a] Significantly different means ($p < 0.05$) in a column have different letters.
[b] * indicates significant difference ($p < 0.05$) between biofilm group mean D-values. Group means are in bold.
[c] NC, not calculated.

In order to determine if the $D_{80\ °C,\ 0.45\ aw}$ is influenced by biofilm formation, the average values of each strain $D_{80\ °C,\ 0.45\ aw}$ were plotted against the mean quantity of biofilm ($OD_{492\ nm}$) to observe the trend in the relationship (Fig. 6). It revealed that thermal resistance ($D_{80\ °C,\ 0.45\ aw}$) and the amount of pre-formed biofilm ($OD_{492\ nm}$) had a strong linear relationship (Spearman correlation $\rho = 0.85$, $p < 0.05$), suggesting that biofilm forming ability directly influences thermal resistance of *Salmonella*. To the best of the authors' knowledge the current study is the first showing that biofilm produced by *Salmonella* enhances thermal resistance in low moisture foods such as wheat flour. It is noteworthy that throughout the current study, *Salmonella* cultures were induced for biofilm production prior to harvesting and thermal treatment which ensured that biofilm was present at the time of thermal treatment and allowed for any correlations to be

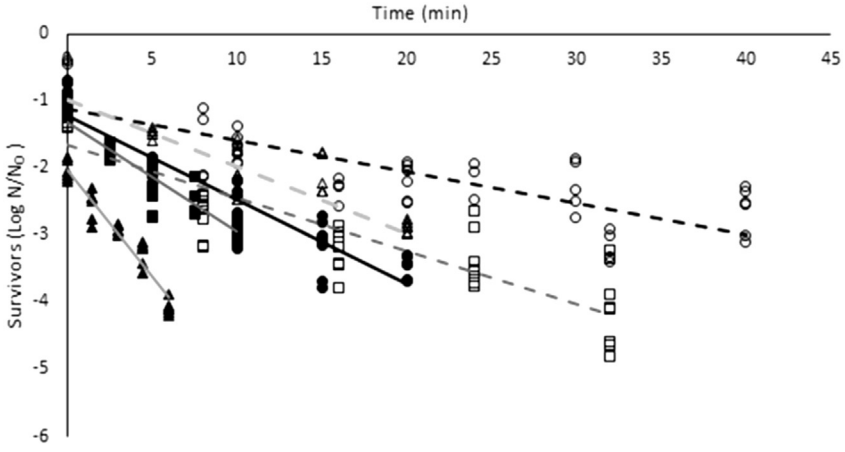

Fig. 5. Survivor curves for *Salmonella* strains: non-biofilm former (bf−) strains (G2 ●—, G1 *Δcsgb*:Tn5 ■ –, MD9 ▲ –) and former (bf+) strains (MD4 ○–, UK1 □–, G3 Δ –).

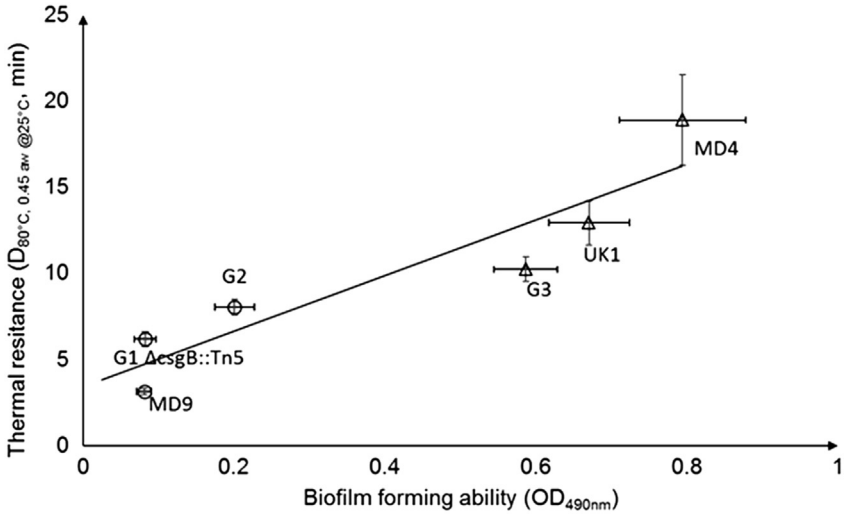

Fig. 6. Relationship between thermal resistance ($D_{80\ °C,\ 0.45\ a_w}$) of *S.* Enteritidis strains inoculated in organic wheat flour and the pre-formed biofilm amount produced ($OD_{492\ nm}$). ○ = non-biofilm former (bf−) strains, △ = biofilm former (bf+) strains; bars represent standard error for each value of min 3 replicates.

established. This is in contrast to the previously published reports in which the pellicles obtained from bf+ and bf− *S.* Typhimurium strains did not differ in their inactivation curves at 70 °C (Scher et al., 2005). However, unlike the current study, these authors tested thermal inactivation kinetics of biofilms in normal saline which is a high moisture environment. Similarly, Al-Holy, Lin, Abu-Ghoush, Al-Qadiri, and Rasco (2009) compared the thermal resistance of planktonic cultures of two bf+ and two bf− *E. sakazakii* strains in a high moisture environment (i.e. reconstituted infant formula) and found no clear advantage of the biofilm forming strains over their counterparts. It is also important to note that the current study utilized biofilms grown on a solid surface, however these authors used planktonic cultures of *E. sakazakii* without priming for biofilm production prior to thermal treatment in reconstituted infant formula. In line with our data, Dhir and Dodd (1995) reported that when *S.* Enteritidis pSB311 planktonic cells were heated to 52 °C in a phosphate-buffered saline solution, these cells had half the D-value when compared with glass or stainless steel attached cells (biofilm) heated in a humid environment to the same temperature. In addition, colonies detached from the biofilm maintained their thermal resistance (Dhir & Dodd, 1995). This along with the results of our study suggests that growth conditions of bacterial strains may influence biofilm production and in turn their thermal resistance. It is likely due to differences in the composition and properties of biofilms produced in different environments (Branda et al., 2005). Thus, the use of pre-formed biofilms, as used in the current study appears to be an appropriate approach for studying the thermal resistance in low-moisture foods.

In conclusion, preformed biofilm influenced thermal resistance of *Salmonella* in low moisture foods such as wheat flour. The results of the current study also raise an important question: how does biofilm formation trigger thermal stress coping mechanisms of *Salmonella*? Thus, further research is needed to develop better understanding of the mechanisms underlying thermal resistance conferred by biofilms.

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