1 **Biofilm forming Salmonella strains exhibit enhanced thermal resistance in wheat**
2 **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 ($D_{80^{\circ}\text{C}, 0.45a_w}$) 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 ($D_{80^{\circ}\text{C}, 0.45a_w}$) and pre-formed biofilm amount ($\text{OD}_{492\text{nm}}$) showed a linear correlation (Spearman correlation $p=0.8$, $p<0.05$), indicating more biofilm production confers more thermal resistance. Average thermal resistance ($D_{80^{\circ}\text{C}, 0.45a_w}$) 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.

Keywords: *Salmonella*, biofilm, thermal resistance, wheat flour.

1 Introduction

Salmonellosis is one of the leading food-borne illnesses resulting in >1 million cases each year (Batz et al., 2012). 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 $D_{70^\circ\text{C}}$ values ranging from 0.06 to 0.2 min (Schmidt and Fontana, 2007; Silva and Gibbs, 2012). However, in low-moisture foods with a_w <0.60, the thermal resistance of *Salmonella* increases dramatically. Archer et al. (1998) reported that in wheat flour with a_w between 0.5-0.6, *S. Weltevreden* showed a $D_{69-71^\circ\text{C}}$ of 80 min. Similarly, *S. Typhimurium* in molten chocolate showed a $D_{70^\circ\text{C}}$ of 816 min (Goepfert and Biggie, 1968), *S. Enteritidis* PT30 in almond meal at a_w 0.60 showed a $D_{70^\circ\text{C}}$ of 15 min (Villa-Rojas et al., 2013), and *S. Orianienburg* showed a $D_{75^\circ\text{C}}$ of 10.4 and 7.7 min in hazelnuts and cocoa, respectively (Izurieta and 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 (Finn et al., 2013; Kumar and Anand, 1998; Steenackers et al., 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 and Potts, 2002; Braune and Sanke,

1979; Potts, 1994). Recent studies have shown *Salmonella* can attach to the surface of foods such as cantaloupes, melons (Annous et al., 2005), tomatoes (Iturriaga et al., 2007), almonds (Suehr et al., 2015) and grains (Cui et al., 2015); as well as food contact surfaces (Joseph et al., 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 et al., 2012; Uhlich et al., 2006). The production of fimbriae confers a rough morphology to the colonies and can be detected on laboratory media because the Congo red dye included in the media attaches to the fimbriae and/or extracellular material producing a red colored colony (Branda et al., 2005). The biofilm forming ability, amount and composition of the biofilm may vary depending on the *Salmonella* strain and the environment (Shi and 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 et al. (2006) reported that survival of wild-type *S. Typhimurium* is 1000-fold higher upon exposure to sodium hypochlorite 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 et al., 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 et al. (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 (Figure 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 ($\Delta csgB::Tn5$) strain was included as a non-biofilm producing control strain for comparison (Shah et al., 2011, 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 XbaI-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 slimy layer at air–broth interface were considered as biofilm formers.

Production of thin aggregative fimbriae and/or cellulose was determined qualitatively following previously described methods (Romling et al., 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 and Hageage, 2003). The 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 96 well -block (Greiner bio-one, NC, USA). Subsequently, bacterial cultures (1:100 dilution) were transferred into 96-well plates (Evergreen Scientific, CA, USA) containing 200 µL LB no-salt via 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 thrice 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 250µl of 1XPBS and the bounded safranin was dissolved by addition of 250 µL of 30% acetic acid (J. T. Baker, Center Valley, PA, USA). Finally, OD_{492nm} 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_{492nm} value of un-inoculated media control was subtracted from all initial OD_{492nm} values. The mean ± SEM OD_{492nm} value of all biological replicates was reported for each strain.

2.3 Inoculum preparation, sample inoculation and survival to desiccation

The premises of this study were 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 show, beyond the shadow of a doubt, biofilms were present in the flour due to the interference of particle agglomeration and dough formation after SEM sample preparation. Therefore, the presence of biofilm in the wheat flour, it was grown in agar (referred to as pre-formed biofilm) and 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 $\mu\text{g/mL}$), Congo red (40 $\mu\text{g/mL}$) and brilliant blue G (20 $\mu\text{g/mL}$) 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; Römlig et al., 2000; Solano et al., 2002; Uhlich et al., 2006; Zogaj et al., 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 right 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 (Figure 2) designed at Washington State University were used (Chung et al., 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), serial 10-fold diluted and plated on TSA plate 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.

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):

203 $\text{Log } S(t) = -t/D$ (1)

204 for $S(t) = N/N_0$ (2)

205 N: population at time t (CFU/g)

206 N_0 : initial population (CFU/g)

207 t: time of isothermal treatment (min)

208 D: time required to reduce the microbial population by 90% at a determined temperature
209 ($^{\circ}\text{C}$) in min.

210 The linear regression and goodness of fit (R^2 and RMSE) were calculated in Minitab 14.

211 A student's t-test was used to compare group averages of D and $\text{OD}_{492\text{nm}}$ values from

212 non-formers and formers ($p=0.05$) in Minitab 14. Significant differences ($p=0.05$)

213 between strain $D_{80^{\circ}\text{C}, 0.45\text{aw}}$ and $\text{OD}_{492\text{nm}}$ values were evaluated using Friedman's test,

214 while linear correlation was evaluated with Spearman coefficient in SAS.

215 **3 Results and discussion**

216 **3.1 Biofilm production of *Salmonella* strains**

217 The differences in the quantity and quality of biofilm production of seven genetically
218 distinguishable, but closely related *S. Enteritidis* strains (Figure 1) are shown in Table I.

219 Strains G3, UK1 and MD4 produced a typical slimy layer at the air-broth interface in

220 tube pellicle formation assay which is similar to what was reported earlier for biofilm

221 producing *S. Enteritidis* strains (Solano et al., 2002). **Consequently**, these strains were

222 **identified and** designated as bf+ or 'biofilm formers'. In contrast, no slime layer was

223 detected at air-broth interface for strains P97, MD9, G2 and G1 $\Delta\text{csgB}::Tn5$ (Table I),

224 suggesting that these strains were bf- or 'biofilm non-formers'. Subsequent quantitative
225 assay using Safranin staining revealed that bf+ strains had significantly higher ($p < 0.05$)
226 OD_{492nm} values (range=0.59-0.79, mean= 0.68 ± 0.00) than the bf- (range=0.03-0.20,
227 mean= 0.11 ± 0.00) (Table 1). The average OD_{492nm} of bf- strains was significantly lower
228 than that of bf+ strains (Figure 3A). As expected, all bf+ strains exhibited strong
229 fluorescence on calcofluor medium suggesting that all bf+ strains produced cellulose,
230 one of the important component of biofilms. In contrast, two bf- strains (P97 and MD9)
231 showed no fluorescence whereas the remaining two bf- strains (G1 $\Delta csgB::Tn5$ and G2)
232 showed very weak fluorescence (Table I), suggesting that the latter strains are likely
233 weak cellulose producers. Consistent with Safranin staining and calcofluor
234 fluorescence, all bf+ strains (G3, UK1 and MD4) produced colony morphotype
235 characterized by red, dry and rough (*rdar*) morphotype, suggesting that in addition to
236 cellulose, these strains also produced thin aggregative fimbriae, **another important**
237 **component of biofilms** (Table I). In contrast, two bf- strains (P97 and MD9) produced
238 smooth and white (*saw*) colony morphotype, suggesting that these strains do not
239 produce thin aggregative fimbriae or cellulose **and therefore do not form biofilms under**
240 **inducible conditions used in this study**. Strain G2 produced pink, rough and dry (*pdar*)
241 colony morphotype whereas strain G1 $\Delta csgB::Tn5$ produced pink and smooth (*pas*)
242 colony morphotype suggesting that these strains are, at best, weak cellulose positive as
243 detected by calcofluor assay. Collectively, these results suggested that P97, MD9, G1
244 $\Delta csgB::Tn5$ and G2 strains **do not produce biofilms (i.e. bf-)**, whereas G3, UK1 and
245 MD4 strains **produce strong biofilms (i.e. bf+) when grown under inducible conditions**
246 used in this study (Table I).

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 (Figure 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 less than 0.5 log after equilibration (Figure 4). These results suggest that biofilms afforded no clear resistance of *Salmonella* to desiccation. In support of our results, Dancer et al. (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 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 et al., (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 to desiccation (Kumar and 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/N₀ vs the time of treatment. As expected, all strains showed a Log linear trend (Figure 5), suggesting the inactivation rate is constant and a first order kinetic model could properly describe the inactivation kinetics. Next, the D_{80°C, 0.45aw} was calculated with the first order kinetic model which had a good fit represented by low RMSE (0.17 to 0.49) and relatively high R² (0.78 to 0.95, Table II), 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, 0.45aw} (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 II, Figure 3B).

In order to determine if the D_{80°C, 0.45aw} is influenced by the biofilm formation, the average values of each strain D_{80°C, 0.45aw} were plotted against the mean quantity of biofilm (OD_{492nm}) to observe the trend in the relationship (Figure 6). It revealed that thermal resistance (D_{80°C, 0.45aw}) and the amount of pre-formed biofilm (OD_{492nm}) had a strong linear relationship (Spearman correlation $\rho = 0.85$, $p < 0.05$), suggesting that the biofilm forming ability directly influences thermal resistance of *Salmonella*. To the best of authors' knowledge the current study is the first study showing that the biofilm produced by *Salmonella* enhances thermal resistance in low moisture food 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 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 et al. (2009) compared the thermal resistance of planktonic cultures of two bf+ and two bf- *E. sakazakii* strains in 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, unlike this current study which utilized biofilms grown on solid surface, 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, detached colonies from the biofilm maintained their thermal resistance (Dhir and 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 the 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 biofilm formation triggers 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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461

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

Strain	Calcofluor fluorescence ¹	Congo-red agar morphotype ²	Pellicle formation	Microplate titration (OD _{492nm}) ^{3,4}
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				
$\Delta csgb::Tn$	+/-	sap	Clear surface	0.08 ± 0.04 ^B
5				
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 ± 0.15 ^{D,E}
MD4	++	rdar	Slimy layer (++)	0.79 ± 0.24 ^E

¹ +, +/- or - non-former; ++ former

² saw, smooth and white; sap, smooth and pink; daw, dry and white; rdar, red, dry and rough

³Significantly different means ($p < 0.05$) in a column have different letters.

⁴ * indicates there's significance ($p < 0.05$) between OD_{492nm} means of biofilm groups.

Table II. Calculated $D_{80\text{ }^{\circ}\text{C}, 0.45\text{ aw}}$ for *S. Enteritidis* strains in organic wheat flour.

Strain (replicas)	D- value $\pm$ SE (min) ^{5, 6}	R ² $\pm$ SE	RSME $\pm$ SE
Non-formers (bf-)	6.0 $\pm$ 0.2*	NC	NC
P97 (3)	NC ⁷	NC	NC
MD9 (3)	3.1 $\pm$ 0.2^D	0.95 $\pm$ 0.01	0.17 $\pm$ 0.01
G1 Δ csgb::Tn5 (3)	6.1 $\pm$ 0.4^D	0.91 $\pm$ 0.03	0.19 $\pm$ 0.04
G2 (4)	8.0 $\pm$ 0.4^C	0.87 $\pm$ 0.02	0.33 $\pm$ 0.02
Biofilm formers (bf+)	14.1 $\pm$ 0.6	NC	NC
G3 (4)	10.2 $\pm$ 0.7^B	0.89 $\pm$ 0.03	0.26 $\pm$ 0.01
UK1 (3)	12.9 $\pm$ 1.2^B	0.78 $\pm$ 0.05	0.49 $\pm$ 0.08
MD4 (4)	21.7 $\pm$ 3.5^A	0.86 $\pm$ 0.03	0.29 $\pm$ 0.04

⁵ Significantly different means ($p < 0.05$) in a column have different letters.

⁶ * indicates significant difference ($p < 0.05$) between biofilm group mean D-values.

⁷ NC, not calculated

Figure legends

Figure 1. Macrorestriction enzyme- pulsed field gel electrophoresis (MRP-PFGE) profiles of XbaI-digested DNA of *S. Enteritidis* strains.

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

Figure 3. Comparison between non-biofilm formers (bf-, □) and biofilm formers (bf+, ■) on: (A) group average biofilm forming ability (OD_{492nm}) and (B) group average thermal resistance ($D_{80\text{ }^{\circ}C, 0.45\text{ aw}}$). At least three replicas per strain.

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

Figure 5. Survivor curves for *Salmonella* strains: non-biofilm formers (bf-) strains (G2 ●—, G1 $\Delta csgb::Tn5$ ■ —, MD9 ▲ —) and formers (bf+) strains (MD4 ○—, UK1 □—, G3 Δ —).

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

Time (min)