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

Rossana Villa-Rojas [a], Mei-Jun Zhu [b], Narayan C. Paul [c], Peter Gray [b], Jie Xu [a], Devendra H. Shah [c, d, **], Juming Tang [a, *]

[a] Department of Biological and Agricultural Engineering, Washington State University, Pullman, WA, United States
[b] School of Food Science, Washington State University, Pullman, WA, United States
[c] Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA, United States
[d] Paul Allen School for Global Animal Health, Washington State University, Pullman, WA, United States

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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

* Corresponding author. Biological Systems Engineering, Washington State University, P.O Box 646120, Pullman WA 99164-6120, United States.
** Corresponding author. Department of Veterinary Microbiology and Pathology, Washington State University, P. O Box 647040, Pullman WA 99164-6120, United States.
E-mail addresses: dshah@vetmed.wsu.edu (D.H. Shah), jtang@wsu.edu (J. Tang).

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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.