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Biofilm formation of *Salmonella* Enteritidis under food-related environmental stress conditions and its subsequent resistance to chlorine treatment

Yishan Yang [a], Marta Mikš-Krajnik [a, b], Qianwang Zheng [a], Sang-Bong Lee [c], Seung-Cheol Lee [d], Hyun-Gyun Yuk [a, *]

[a] Food Science & Technology Programme, Department of Chemistry, National University of Singapore, Science Drive 4, 117543, Singapore
[b] Chair of Industrial and Food Microbiology, Faculty of Food Science, University of Warmia and Mazury in Olsztyn, Plac Cieszyński 1, 10-726, Olsztyn, Poland
[c] Department of Food Science and Biotechnology, Dongguk University, Seoul, 100-715, Republic of Korea
[d] Department of Food Science and Biotechnology, Kyungnam University, Changwon, Gyeongnam, 631-701, Republic of Korea

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ABSTRACT

This study determined the effects of temperature (4 and 25 °C), pH (5.3, 7.3, and 8.3), and nutrient availability (TSB and 20 times diluted TSB (1/20 TSB)) on *Salmonella* Enteritidis biofilm formation and its resistance to chlorine treatment (pH 6.8, 50 ppm for 1 min). The results showed that biofilm density was significantly higher ($P < 0.05$) at 25 °C or in 1/20 TSB, regardless of pH and bacterial strains. Moreover, 1/20 TSB significantly enhanced the chlorine resistance of biofilms formed at 25 °C, especially for *S.* Enteritidis with rdar morphotype, with an average reduction of 1.52 log CFU/cm^2 compared to that of biofilm in TSB with 4.07 log reduction. All biofilms formed at 4 °C were very sensitive to chlorine treatment. In most cases, acidic pH sensitized biofilms to chlorine treatment compared with neutral and alkaline pHs. The further analysis of cellulose production of biofilms indicated that it had a positive impact on biofilm resistance to chlorine treatment. This study suggests that environmental stress conditions encountered in food processing plant might alter *S.* Enteritidis biofilm resistance to sanitizer treatment possibly by acting on the cellulose production.

1. Introduction

Salmonella Enteritidis is the most common serotype responsible for salmonellosis in many countries. In 2013, a total of 82,694 laboratory-confirmed cases of salmonellosis were reported in Europe, of which 39.5% were caused by this pathogen (EFSA and ECDC, 2015). Similarly, 540 cases out of 1735 laboratory-confirmed cases of non-typhoidal salmonellosis in 2013 were caused by *S.* Enteritidis in Singapore (MOH, 2014). One possible cause of *Salmonella* outbreaks might be linked to its capability to attach and form biofilm on the surface of food processing facilities that subsequently cross-contaminate the final food products (Wang et al., 2013).

Biofilm is an aggregation of microbial cells on a surface that are embedded within an extracellular polymeric substance (EPS) matrix (Vu et al., 2009). The formation of biofilm renders bacteria greater resistance to extreme temperature and pH, desiccation, ultraviolet radiation, oxidative stress, and sanitizing agents, which might contribute to the persistence of foodborne pathogens in food processing plants (Annous et al., 2009). As biofilm matures, cells can detach and initiate attachment on another food contact surface or even the surface of food products (Annous et al., 2009; Rodríguez and McLandsborough, 2007), providing a potential transmission route of foodborne pathogens.

Adequate cleaning and sanitizing procedures are essential to control and remove biofilm in the food industry. To date, several types of chemical sanitizers have been approved by the United States Food and Drug Administration (FDA) for use on the food contact surface (FDA, 2014), among which chlorine-based sanitizers are the most widely used due to its low price and broad-spectrum bactericidal activities (Liu et al., 2006). To achieve a safe and effective sanitizing effect, chlorine is recommended to be used at a concentration of 50–200 ppm with pH 6.0–7.5 (Parish et al., 2003).

* Corresponding author.
E-mail address: chmyukhg@nus.edu.sg (H.-G. Yuk).

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It has been known that environmental factors, such as temperature, pH, nutrients, osmolarity, and oxygen levels, play significant roles in biofilm formation (Giaouris et al., 2012). In addition, a few studies also demonstrated that culture conditions could affect the resistance of bacterial biofilms to sanitizing agents (Abdallah et al., 2014, 2015; Belessi et al., 2011; Nguyen and Yuk, 2013). In food processing plant, *Salmonella* might experience different kinds of stresses during food processing and storage, such as low temperature, acidic and alkaline antimicrobials, and starvation. However, little is known about how these environmental stresses would affect *S*. Enteritidis biofilm formation and its resistance to sanitizing agents. Moreover, the mechanisms behind the altered resistance of biofilm after exposure to different stresses are still unclear. A deeper understanding of *S*. Enteritidis biofilm formation under different food-related environmental stress conditions and its subsequent resistance to sanitizer would be conducive to propose better strategies to reduce and eliminate biofilms in food industry.

Therefore, the objective of this study was to investigate the effects of temperature (4 and 25 °C), pH (5.3, 7.3, and 8.3), and nutrient availability (TSB and 20 times diluted TSB) on the biofilm formation of three different *S*. Enteritidis strains, as well as on the resistance of *S*. Enteritidis biofilms to chlorine treatment. In addition, the capability of different *S*. Enteritidis strains to produce curli fimbriae and cellulose, along with the cellulose production of biofilms formed under different environmental conditions were investigated for a better understanding of resistance of *S*. Enteritidis biofilm during sanitizer treatment.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Three *S*. Enteritidis strains were used in this study. *S*. Enteritidis ATCC13076 was purchased from the American Type Culture Collection (Manassas, VA, USA), while *S*. Enteritidis 124 (phage type 8, Maryland Department of Health and Mental Hygiene, MD, USA) and 125 (phage type 13A, U.S. Department of Agriculture, Washington DC, USA) were obtained from Dr. Kunho Seo of Konkuk University in Republic of Korea. All strains were stored at − 80 °C in separate cryoinstant vials with porous beads (DeltaLab, Barcelona, Spain). Before use, one bead was transferred into 10 mL of tryptic soy broth (TSB) (Oxoid, Basingstoke, UK) and incubated at 37 °C for 18 h. After two consecutive transfers at 37 °C for 18 h, cells were concentrated by centrifugation at 3500 × *g*, 4 °C for 10 min and washed twice using phosphate buffered saline (PBS) (Vivantis Inc., CA, USA). Cell suspension was prepared by suspending the pelleted cells with PBS to make an initial concentration of 10^{10} CFU/mL.

2.2. Biofilm formation

Stainless steel (grade 304) coupons (2.5 cm × 1 cm × 0.2 cm) were used as the tested surface. Coupons were washed with a detergent solution for 30 min followed by 70% (v/v) ethanol for 15 min in an ultrasonic bath (57H, Ney Dental International, CT, USA). Subsequently, coupons were rinsed with deionized (DI) water, air-dried and autoclaved at 121 °C for 15 min (Yang et al., 2015).

Biofilms were formed in either TSB or 20 times diluted TSB (1/20 TSB) at different temperature (4 or 25 °C) and pH (5.3, 7.3, or 8.3) conditions. The pH of the media was adjusted with either lactic acid (Sigma, St Louis, MO, USA) or trisodium phosphate (TSP) (Merck, Darmstadt, Germany), which are widely used to decontaminate poultry products (FSIS, 2013). Cell suspension was added to each medium to achieve a final concentration around 10^8 CFU/mL. Twenty-five milliliters of the inoculated media were dispensed into each sterile petri dish (Φ 90 mm) containing four sterile coupons, of which two coupons were used as control and the other two were treated with sanitizer. The petri dishes were incubated for 2, 4, or 7 days under static conditions.

2.3. Chlorine treatment

Chlorine solution was freshly prepared by diluting the sodium hypochlorite solution (Bleach, Hygold Chemical Supplies, Singapore) to 50 ppm with potassium phosphate buffer solution (0.05 mol/L, pH 6.8). The concentrations of free chlorine in the solution were confirmed using an RQflex® 10 reflectometer (Merck) according to the manufacturer's instructions. To determine the resistance of biofilms formed under different conditions to chlorine treatment, coupons were carefully taken out of the petri dish with sterile forceps and gently washed three times with PBS to remove any loosely attached cells. A coupon was subsequently placed in a sterile plastic tube containing 5 mL of chlorine solution and treated for 1 min. After chlorine treatment, the coupon was immediately put in a centrifuge tube with 5 mL of D/E neutralizing broth (Acumedia, lansing, MI, USA) to neutralize the residual chlorine solution.

2.4. Enumeration of planktonic and attached cells

The number of planktonic cells in the petri dish was monitored at day 2, 4, and 7. Briefly, 1 mL of cell suspension was withdrawn and diluted with 0.1% (w/v) peptone water (Oxoid). Fifty microliters of cell suspensions with appropriate dilutions were then spiral plated (WASP 2, Don Whitley Scientific Ltd., West Yorkshire, UK) on tryptic soy agar (TSA) (Oxoid) plates and the plates were incubated at 37 °C for 24 h. Enumeration of cells was conducted by an automated colony counter (aCOLyte, Synbiosis, Frederick, MD, USA) and the detection limit was 20 CFU/mL. To enumerate the number of attached cells on a coupon without chlorine treatment, the coupon was carefully washed three times with PBS and transferred into a sterile centrifuge tube containing 5 mL of 0.1% peptone water. The centrifuge tube was then placed into the ultrasonic bath and sonicated at 48 kHz for 3 min, followed by vigorously vortexed for 30 s to remove and disaggregate the biofilm cells from the coupon. The method was confirmed to be sufficient for the removal of attached cells by staining the coupons with LIVE/DEAD® *Bac*Light Bacterial Viability Kit (Molecular Probes, Eugene, OR, USA) as previously described (Yang et al., 2015) after treatment (data not shown). Meanwhile, the enumeration of attached cells on a coupon after chlorine treatment was performed by directly subjecting the centrifuge tube with 5 mL of D/E neutralizing broth to sonication and vortex. The cell suspension in the centrifuge tube was diluted, spiral plated, and the cell number was counted after incubation as described above. If a low cell number was expected after chlorine treatment, pour plating method was performed with 1 mL of undiluted cell suspension and the detection limit was 1 CFU/mL.

2.5. Screening of morphotype on congo red and calcofluor agar plates

All three strains were analyzed on congo red and calcofluor agar plates to examine their capabilities to produce curli fimbriae and cellulose according to the previously described method (Castelijn et al., 2012) with a slight modification. In brief, 5 μL of overnight culture was spot-inoculated onto Luria–Bertani (LB) agar plates without NaCl (tryptone 10 g/L, yeast extract 5 g/L, and agar 15 g/L) supplemented with 40 μg/mL of congo red (Sigma) and 20 μg/mL of Coomassie brilliant blue (AppliChem, Darmstadt, Germany). The cellulose production was further investigated with LB agar plates without NaCl containing 200 μg/mL of calcofluor (Fluorescent

Brighter 28, Sigma). All plates were incubated at 25 °C for 48 h. The morphotypes on congo red agar plates were categorized as rdar (express curli fimbriae and cellulose), pdar (express cellulose), bdar (express curli fimbriae), and saw (express neither cellulose nor curli fimbriae) (Römling et al., 2003). Calcofluor binding was examined under UV light and fluorescence of colonies indicated cellulose production (Römling et al., 2003).

2.6. Calcofluor staining of S. Enteritidis biofilms

The cellulose production of biofilms was evaluated by staining the coupons with the calcofluor dye. Each biofilm sample was rinsed three times with DI water and stained with 200 μL of calcofluor dye (250 μg/mL) for 30 min in dark. After incubation, samples were gently washed and air-dried. The biofilms were observed using an Olympus BX51 fluorescence microscope (Olympus corporation, Tokyo, Japan) with an appropriate filter cube for calcofluor (WU, DM400, BP330-385, BA420). All images were taken by an attached Olympus DP71 camera (Olympus corporation).

2.7. Statistical analysis

Mean values were calculated from three independent experiments with duplicate samples (n = 6). Statistical analysis was conducted by one-way analysis of variance (ANOVA) and means were compared by Duncan's multiple range test using SPSS software (Statistical Package for the Social Sciences, version 18.0, IBM, NY, USA). The difference was considered as significant if P value is less than 0.05. Principal component analysis (PCA) was performed using STATISTICA 10.0 software (StatSoft, Kraków, Poland) to discriminate the influence of different factors (temperature, pH, nutrients availability, time, and strain) on planktonic cells, biofilm formation, and biofilm resistance to sanitizer treatments.

3. Results

3.1. Effect of environmental temperature, pH and nutrient availability on the number of planktonic cells

The number of planktonic cells was monitored at day 2, 4, and 7. In general, incubation time did not significantly ($P > 0.05$) affect the number of planktonic cells, unless otherwise stated. Therefore, only the number of planktonic cells after 2 days of incubation is shown in Fig. 1 as an example. *S.* Enteritidis could not multiply at 4 °C and thus the number of planktonic cells remained unchanged during storage, with an average number of 7.71 log CFU/mL (Fig. 1A). However, the number of planktonic cells in TSB at 25 °C increased to 9 log CFU/mL after 2 days of incubation, regardless of pH and bacterial strain (Fig. 1B). Interestingly, high cell density (9 log CFU/mL) in 1/20 TSB was only observed for *S.* Enteritidis ATCC 13076, while those of *S.* Enteritidis 124 and 125 were significantly ($P < 0.05$) lower, with an average number of 7.27 log CFU/mL for all tested pH conditions, except for *S.* Enteritidis 125 at pH 5.3 (8.16 log CFU/mL). Moreover, a decrease (0.42–0.81 log reduction) in the cell population was found at 25 °C in TSB (pH 7.3 or 8.3) at a later stage (day 4 or day 7) (data not shown).

3.2. Effect of environmental temperature, pH and nutrient availability on S. Enteritidis biofilm formation

Biofilm densities at day 4 were similar to those at either day 2 or day 7. Therefore, only biofilm densities at day 2 and day 7, which represented the early and late stage of biofilm formation in this study, are shown in Fig. 2. *S.* Enteritidis formed significantly

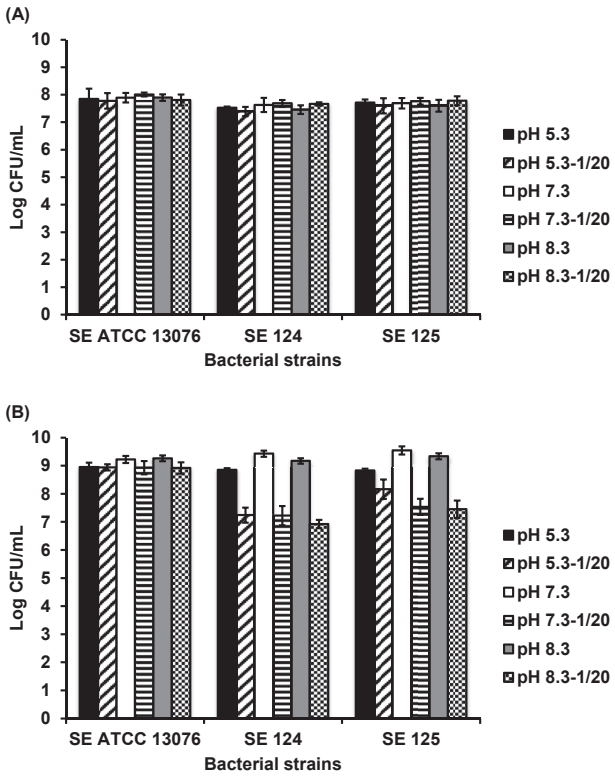

Fig. 1. The number of planktonic *Salmonella* Enteritidis (SE) cells suspended in tryptic soy broth (TSB) or diluted TSB (1/20) with different pHs at 4 °C (A) or 25 °C (B) for 2 days.

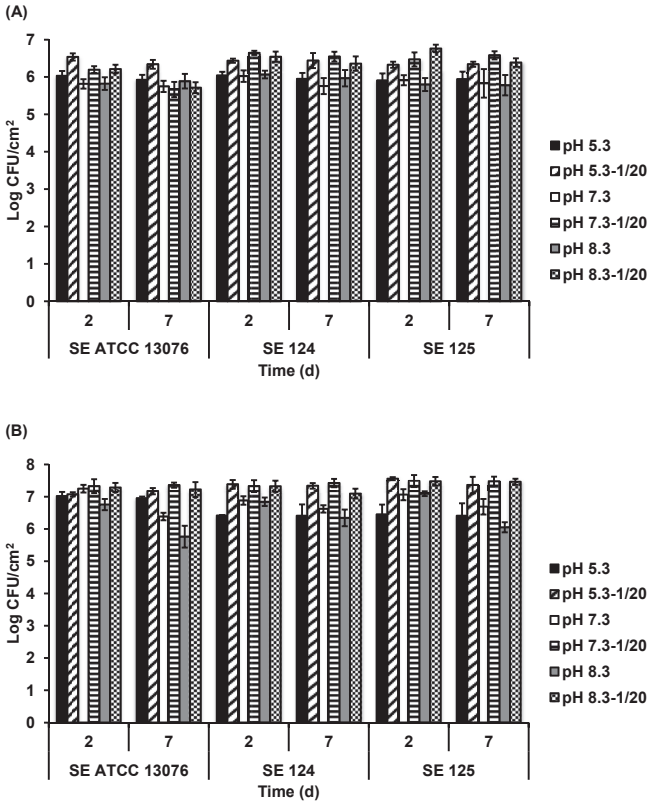

Fig. 2. Densities of *S.* Enteritidis biofilms formed in TSB or diluted TSB (1/20) with different pHs at 4 °C (A) or 25 °C (B) for 2 and 7 days.

($P < 0.05$) denser biofilms (5.76–7.56 log CFU/cm^2) at 25 °C, which were 0.85 log CFU/cm^2 higher, compared to those formed at 4 °C (5.67–6.76 log CFU/cm^2). In addition, significantly ($P < 0.05$) higher biofilm densities were observed in 1/20 TSB than TSB, with average differences of 0.46 and 0.78 log CFU/cm^2 at 4 and 25 °C, respectively. Although pH significantly ($P < 0.05$) affected cell population in a few cases, no consistent trend was found. Moreover, as biofilm aged, cell population of *S.* Enteritidis ATCC 13076 at pH 7.3 and 8.3 decreased by 0.55 and 0.50 log CFU/cm^2 in 1/20 TSB at 4 °C, and by 0.87 and 1 log CFU/cm^2 in TSB at 25 °C, respectively. Also, 0.50 and 1.04 log reductions were observed for *S.* Enteritidis 124 and 125 biofilms in TSB at pH 8.3, 25 °C. The capabilities of different strains to form biofilm were also compared under all tested conditions (24 in total for each strain, excluding the data at day 4). The statistical analysis indicated that *S.* Enteritidis ATCC 13076 had lower biofilm densities compared to the other two strains under 4 conditions (4 °C, pH 7.3, 1/20 TSB, day 7; 4 °C, pH 8.3, 1/20 TSB, day 2; 4 °C, pH 8.3, 1/20 TSB, day 7; 25 °C, pH 5.3, 1/20 TSB, day 2), while it had denser biofilms under 2 conditions (25 °C, pH 5.3, TSB, day 2; 25 °C, pH 5.3, TSB, day 7). No significant difference was found under the other 18 conditions ($P > 0.05$).

3.3. Effect of environmental temperature, pH and nutrient availability on S. Enteritidis biofilm resistance to chlorine treatment

The log reductions of biofilms formed at different environmental conditions to 50 ppm chlorine (pH 6.8) treatment for 1 min are shown in Fig. 3. The increase in environmental temperature significantly ($P < 0.05$) enhanced the resistance of biofilm formed in 1/20 TSB to chlorine treatment, with an average log reduction of 2.21 log CFU/cm^2 at 25 °C compared to that of 4.78 log CFU/cm^2 at 4 °C. However, higher temperature did not generally confer biofilms formed in TSB greater resistance, with an average log reduction of 4.08 log CFU/cm^2 at 25 °C compared to that of 4.43 log CFU/cm^2 at 4 °C. No significant ($P > 0.05$) difference was found in biofilm resistance between low and high nutrient condition at 4 °C. However, biofilms formed in 1/20 TSB at 25 °C were more resistant to chlorine treatment than those in TSB, with some exceptions. Acidic pH condition (pH 5.3) seemed to be a factor to sensitize biofilm resistance to chlorine because biofilms formed at pH 5.3 were significantly ($P < 0.05$) weaker than those formed at either pH 7.3 or pH 8.3 under 16 out of 24 tested conditions. The differences were most obvious for *S.* Enteritidis 125 in 1/20 TSB at 25 °C, with an average log reduction of 3.81, 0.96, and 0.94 for biofilms formed at pH 5.3, 7.3, and 8.3, respectively. Overall, biofilm resistance was unchanged as biofilm aged. The comparison among different strains demonstrated that biofilms formed by *S.* Enteritidis ATCC 13076 were more sensitive to chlorine treatment than the other two strains under all tested conditions at 4 °C, and in 1/20 TSB at pH 7.3 and 8.3, 25 °C, whereas it was stronger in TSB (pH 7.3) at day 2. No significant difference was observed between *S.* Enteritidis 124 and 125, except that biofilms of *S.* Enteritidis 125 grown in 1/20 TSB at pH 5.3 and 25 °C were weaker but that incubated in TSB at pH 8.3 and 25 °C was stronger at day 7.

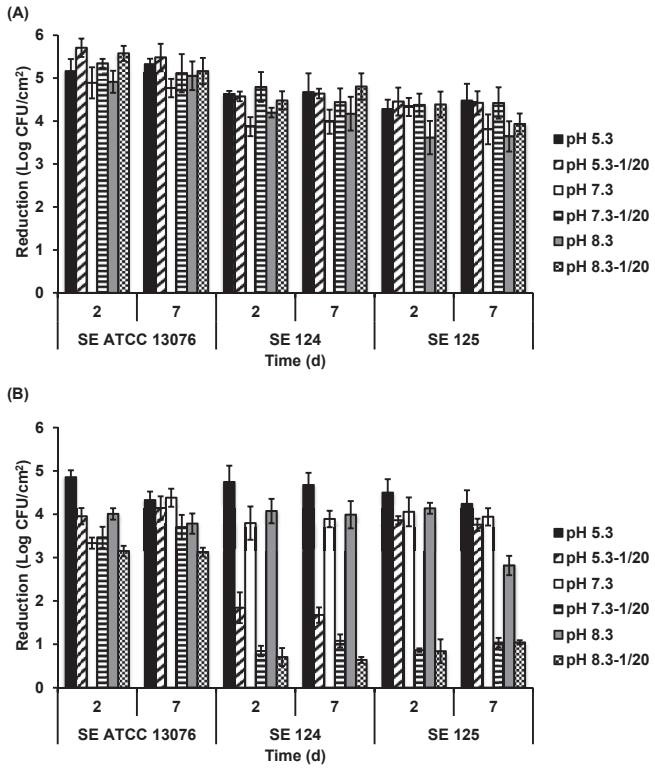

Fig. 3. Log reductions of *S.* Enteritidis biofilms formed in TSB or diluted TSB (1/20) with different pHs at 4 °C (A) or 25 °C (B) for 2 and 7 days to 50 ppm chlorine (pH 6.8) treatment for 1 min.

3.4. Principal component analysis

Principal components analysis (PCA) was performed in order to evaluate the influence of different factors, namely, temperature, pH, nutrient availability, time, and strain on the planktonic cells, biofilm formation and biofilm resistance. A PCA of all raw data (972 data points) resulted in the PCA score plot with two principal components (PCs), which explained 88% of the total variance (54% and 34% for PC1 and PC2). The biofilm formation and biofilm resistance showed high positive (0.83) and negative loading coefficient (−0.93), respectively, with PC1, while planktonic cells showed a high negative loading coefficient (−0.93) with PC2 (Fig. S1).

Four clusters (C1–C4), which were separated mainly due to the difference in environmental temperature and nutrient availability, were observed in PCA diagram (Fig. 4). The first cluster (C1) was formed by all tested strains at 4 °C, regardless of pH and nutrient

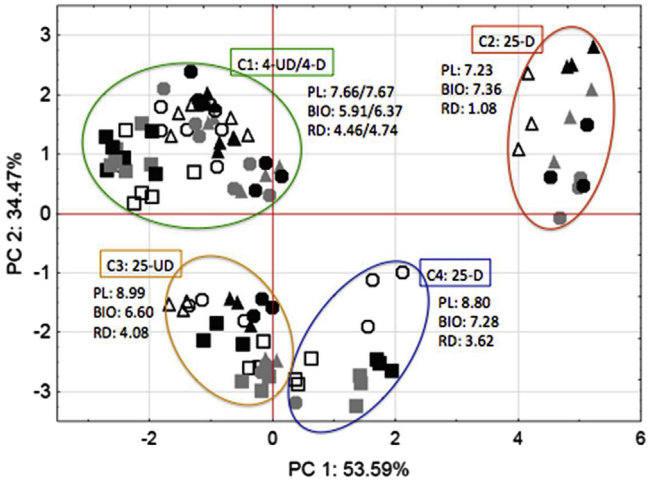

Fig. 4. A principal component analysis (PCA) diagram showing the discrimination of mean score factors for planktonic cells (PL), biofilm formation (BIO), and biofilm resistance (RD) of *S.* Enteritidis strains (Δ-124, ○-125, □-13076) incubated in diluted (D) and undiluted (UD) TSB at different temperature (4–4 °C, 25–25 °C) and pH (5.3-white, 7.3-grey, 8.3-black) conditions. The values are representing the average counts (log CFU/mL or log CFU/cm^2) of *S.* Enteritidis clustered (C1–C4) together on PCA biplot.

availability, which had less planktonic cells, the least biofilm densities and weakest sanitizer resistance. The other three clusters (C2–C4) were formed at 25 °C due to the difference in nutrient availability and bacterial strains. C2 (including mainly *S.* Enteritidis 124 and 125 in 1/20 TSB) had the least planktonic cells but the highest biofilm densities and greatest sanitizer resistance. Although both C2 and C4 included biofilms developed in 1/20 TSB at 25 °C and did not differ in biofilm formation, C4 (including *S.* Enteritidis 13076 and 125 at pH 5.3) had more planktonic cells and relatively weaker resistance than C2. C3 were separated from C2 and C4 due to nutrient availability, and biofilms formed in TSB at 25 °C showed lower densities and weaker resistance. Subsequently, the set of data from C1 (4 °C) was subjected to separate PCA (Fig. S2), which revealed that biofilm development at 4 °C was determined mainly by nutrient availability, while biofilm resistance was shown to be strain-dependent.

These results indicated that environmental temperature and nutrient availability were the dominant factors influencing the number of planktonic cells, biofilm formation and biofilm resistance, although bacterial strains and pH might affect the number of planktonic cells and biofilm resistance at certain conditions.

3.5. Curli fimbriae and cellulose production on agar plates

The capabilities of three *S.* Enteritidis strains to produce curli fimbriae and cellulose were evaluated using congo red and calcofluor agar plates. The results showed that both *S.* Enteritidis 124 and 125 strains expressed the red, dry, and rough (rdar) morphotype on congo red agar plate at 25 °C (Fig. 5A), indicating that these two strains can produce both curli fimbriae and cellulose. However, *S.* Enteritidis ATCC 13076 strain expressed the brown (in the web version), dry and rough morphotype (bdar), indicating that it can produce curli fimbriae only. The cellulose production was further confirmed using agar plates containing calcofluor. Similarly, intense fluorescence was observed for colonies of *S.* Enteritidis 124 and 125 strains under UV light, while no fluorescence was observed for *S.* Enteritidis ATCC 13076 strain (Fig. 5B).

3.6. Cellulose production of S. Enteritidis biofilms

The biofilm cells formed on stainless coupons were stained with calcofluor dye to examine the cellulose production under different environmental conditions (Fig. 6). At 25 °C, large clusters of calcofluor fluorescence were observed for biofilm formed in 1/20 TSB, indicating that large amount of cellulose was present in the extracellular matrix of the biofilm (Fig. 6A). In addition, small spots of calcofluor fluorescence were found in biofilm formed in TSB, demonstrating that a small amount of cellulose was also produced under such a condition. However, almost no fluorescence was detected at 4 °C, even though biofilm formed in 1/20 TSB had a dense layer of cells.

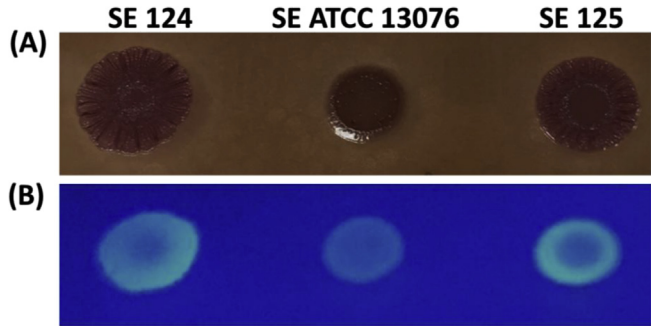

Fig. 5. Morphotypes of different *S.* Enteritidis strains on LB (Luria–Bertani) agar plates without NaCl supplemented with congo red (A) and calcofluor (B) at 25 °C for 2 days.

To investigate if cellulose production was pH- or strain-dependent, calcofluor staining was also performed for biofilms formed either by *S.* Enteritidis 125 in 1/20 TSB at different pH (5.3, 7.3, and 8.3) conditions, or by different strains in 1/20 TSB (pH 7.3) at 25 °C for 7 days. The images showed that a large subpopulation of biofilm cells at pH 7.3 and 8.3 were stained with calcofluor, while only a small subpopulation of biofilm cells were stained at pH 5.3 (Fig. 6B). In addition, fluorescence intensity of biofilms formed by *S.* Enteritidis 124 and 125 was substantially higher than that by *S.* Enteritidis ATCC 13076 (Fig. 6C). These results exhibited that higher amounts of cellulose were produced at neutral or alkaline pHs than acidic pH, as well as by *S.* Enteritidis 124 and 125 than *S.* Enteritidis ATCC 13076.

4. Discussion

This study investigated the effects of temperature, pH, and nutrient availability on the biofilm formation of three different *S.* Enteritidis strains and their biofilm resistance to chlorine treatment. Three strains were selected in this study because *Salmonella* biofilm forming behavior is strain-dependent (Lianou and Koutsoumanis, 2012). In addition, although *S.* Enteritidis can grow over a wide range of pH conditions (pH 5.3–9.0) (Yang et al., 2014), alkaline effect was studied using pH 8.3 instead of pH 9.0 because biofilm density at pH 9.0 was too lower to compare biofilm resistance among different pH conditions (data not shown). Moreover, 1/20 TSB was used as the growth medium to simulate the condition in food industry (Castelijn et al., 2012; Stepanović et al., 2004). Besides, multivariate statistical analysis, namely, principal component analysis (PCA) was applied in order to evaluate the effect of different environmental factors on biofilm development.

The number of planktonic cells was monitored to investigate whether the differences in biofilm formation was due to the differences in growth capabilities. As expected, the number of planktonic cells at 4 °C was relatively constant over time and was close to the initial level. On the other hand, the number of planktonic cells at 25 °C increased by approximately 1 log CFU/ml after 2 days of incubation, however, there were some minor exceptions. The lower planktonic cell counts in diluted TSB might not be due to the low nutrient availability since *Salmonella* cell counts reached 8.5 log CFU/mL when inoculated (8 log CFU/mL) into glass test tubes containing 1/20 TSB without coupons (data not shown). Interestingly, a thin film was observed at the bottom of the petri dish as well as on the coupons under the conditions with lower numbers of planktonic cells (Fig. S3). Once the film was recovered from the bottom, the total cell suspension was also around 8.5 log CFU/mL. Thus, this finding indicates that low nutrient condition (1/20 TSB) might promote the attachment of planktonic cells to the surface at 25 °C.

Biofilm formation was investigated on coupons of stainless steel, which is the most commonly used contact material in food processing environment (Olszewska, 2013). The present study showed that higher cell counts of biofilms were obtained at 25 °C than at 4 °C, indicating that environmental temperature might have an effect on *S.* Enteritidis biofilm formation. These results are in agreement with those reported by Lianou and Koutsoumanis (2012) that various serotypes of *Salmonella enterica* strains generated more biofilms at 25 °C compared to 4 °C. In addition, nutrient availability played a significant role in *S.* Enteritidis biofilm formation in this study since higher biofilm density was observed in 1/20 TSB compared to TSB at both temperatures. Similarly, Stepanović et al. (2004) also reported that 1/20 TSB was more effective in promoting *Salmonella* biofilm formation than TSB at 35 °C.

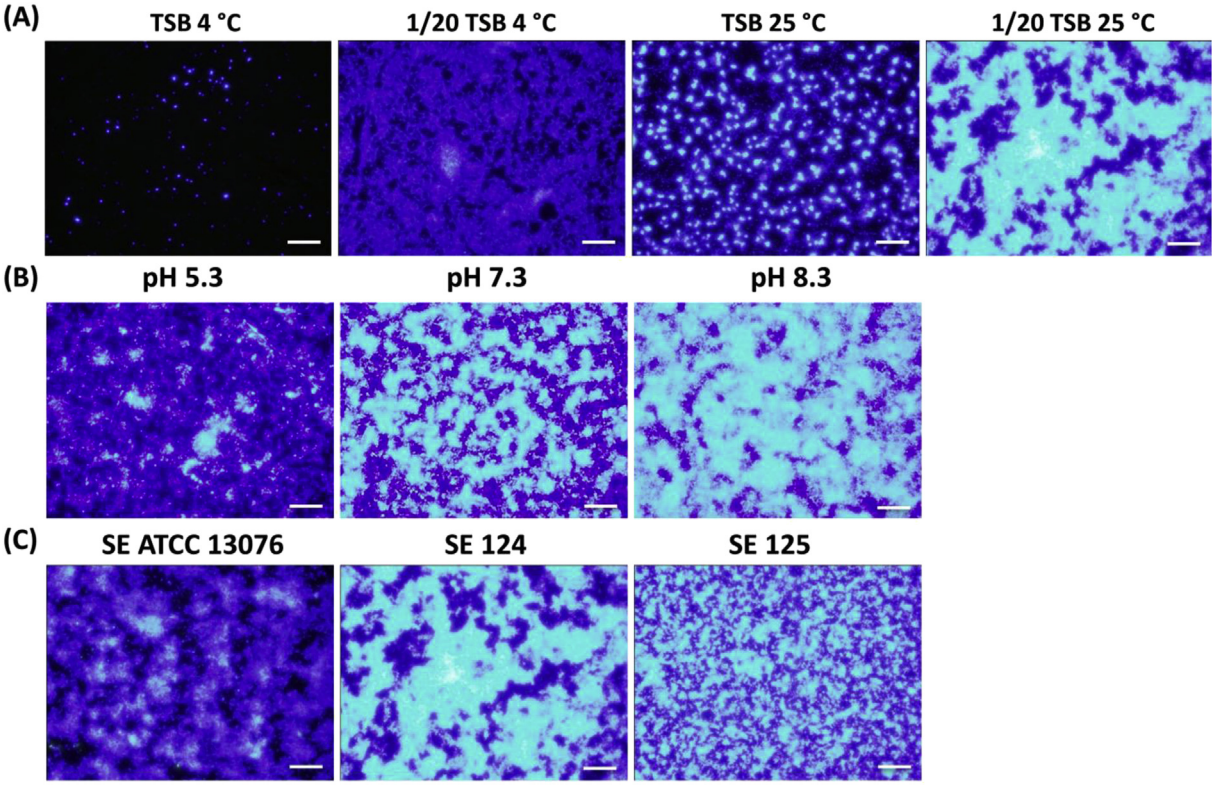

Fig. 6. Calcofluor staining of biofilms formed by *S.* Enteritidis 124 under different combinations of temperature and nutrient availability at pH 7.3 (A), by *S.* Enteritidis 125 under different pH conditions (B) and by different strains at pH 7.3 (C) in 1/20 TSB at 25 °C for 7 days. Scale bars represent 50 μm.

However, these results are contradictory to the previous report by Castelijn et al. (2012) who found that 24 out of 26 *S.* Typhimurium strains isolated from the clinical samples or outbreak-associated foods produced denser biofilms in TSB rather than 1/20 TSB at 25 and 37 °C, while the opposite effect was only found in the other two strains at 25 °C. Moreover, no significant difference in biofilm formation between TSB and 1/20 TSB was also observed for several *Salmonella* serotypes at 30 °C (Kroupitski et al., 2009). The different effects of low nutrient medium on biofilm formation might be due to different serotypes and temperatures being tested in the studies.

In general, biofilm formation of *S.* Enteritidis in this study was not affected by environmental pH and biofilm age, regardless of bacterial strains. Similar results have also been obtained by Karaca et al. (2013) that no significant difference in biofilm production of *S.* Typhimurium was observed between pH 5.5 and 7.4. However, biofilm formation of *S.* Infantis and *S.* Roughform at pH 7.4 was higher than that at pH 5.5 (Karaca et al., 2013). By contrast, Lianou and Koutsoumanis (2012) reported that most *S. enterica* strains exhibited greater biofilm-forming capability at pH 5.5 than at pH 7.0. The variable effects of environmental pHs on *Salmonella* biofilm formation might result from the different genetic backgrounds of bacterial strains. Although some studies demonstrated that biofilm densities of *S. enterica* increased as biofilm aged (Corcoran et al., 2014; Giaouris and Nychas, 2006), others suggested that the cell densities of biofilms remained stable and might decrease at a later stage (Giaouris and Nychas, 2006; Wong et al., 2010). The discrepancy could be explained by the fact that refreshment of medium was applied in the former studies but not in the latter ones.

The resistance of biofilm formed at different environmental conditions to 50 ppm chlorine solution was analyzed. The results indicate that higher temperature (25 °C) and lower nutrient availability (1/20 TSB) enhanced the resistance of biofilm against chlorine treatment. In addition, pH and bacterial strains, under certain environmental conditions, also had great impacts on the resistance of *S.* Enteritidis biofilm. These results are in accordance with those observed in *Listeria monocytogenes* that biofilms formed at a low temperature (5 °C) or an acidic pH (pH 5.0) were more sensitive to sanitizers than those formed at a high temperature (20 °C) or a neutral pH (pH 7.0) (Belessi et al., 2011). However, to the best of our knowledge, no attempt has yet been made to investigate the influences of nutrient availability on bacterial biofilm resistance. In addition, the different resistance of biofilm among bacterial strains observed in this study suggests that it is important to use multiple strains to evaluate the sanitizer efficiency against biofilm.

PCA is a useful and powerful mathematical tool to simplify and describe the multivariate structure of data. In present study, PCA was applied to find out whether environmental factors (pH, temperature and nutrient availability), tested strains, or biofilm age contributed to the differences in the number of planktonic cells, biofilm formation and sanitizer resistance. In general, the PCA clustering clearly indicates the importance of temperature and nutrient availability over pH, biofilm age and bacterial strains in biofilm formation and resistance. Furthermore, biofilm development at 4 °C was found to be promoted by low nutrient content, while its resistance to chlorine treatment was strain-dependent. This study indicates the usefulness of PCA to evaluate the importance of environmental stress factors affecting bacterial biofilm formation and its resistance.

The mechanisms of biofilm resistance to sanitizers have not been fully understood, however, EPS production might increase biofilm resistance as it could hinder the penetration of sanitizer into biofilm (Olszewska, 2013; Van Houdt and Michiels, 2010). The

major components of the extracellular matrix of *Salmonella* biofilms are curli fimbriae and cellulose (Castelijn et al., 2012), therefore, they were investigated in this study. The microscopic examination of biofilms showed that higher cellulose production was observed at higher temperature, especially in low nutrient medium. The observation is supported by the findings of Castelijn et al. (2012) that the expression of genes involved in cellulose production was induced in *S.* Typhimurium biofilm that formed in 1/20 TSB at 25 °C. Therefore, the greater biofilm resistance in 1/20 TSB at 25 °C was probably the result of the higher cellulose content, which protected the cells deep within the biofilm from being attacked by sanitizer.

The cellulose production of biofilms formed under different pH conditions was also determined using calcofluor staining to investigate whether environmental pH affects biofilm resistance to sanitizer treatment by acting on cellulose production. The investigation was carried out for *S.* Enteritidis 125 in 1/20 TSB at 25 °C due to the noticeable pH effect on biofilm resistance. The lower cellulose amount present in the biofilm at pH 5.3 was well correlated to its poor resistance to sanitizer treatment. Therefore, the lower cellulose production might be one of the reasons for the weaker sanitizer resistance of biofilm under an acidic pH condition at 25 °C. On the other hand, no cellulose was produced under all tested pHs at 4 °C, but biofilms formed at pH 5.3 were still more sensitive to chlorine treatment compared to pH 7.3 or 8.3. Thus, this finding indicates that factors other than cellulose production may play important roles in biofilm resistance.

To understand the mechanism of different biofilm resistance among *S.* Enteritidis strains, curli fimbriae and cellulose production were investigated. The results showed that the morphotypes of strain 124 and 125 were rdar while that of ATCC strain was bdar, suggesting that all three strains can produce curli fimbriae but only *S.* Enteritidis 124 and 125 have the ability to produce cellulose. The capabilities of different strains to produce cellulose were further tested by directly staining the biofilms on coupons. The microscope analysis revealed that *S.* Enteritidis 124 and 125 produced greater amounts of cellulose than *S.* Enteritidis ATCC 13076 and this might explain the reason why *S.* Enteritidis 124 and 125 had stronger sanitizer resistance than *S.* Enteritidis ATCC 13076. Interestingly, a low intensity of fluorescence was also observed in biofilms of *S.* Enteritidis ATCC 13076, indicating that a small amount of cellulose might be present, which was opposite to the results obtained using congo red and calcofluor plates. One of the possible explanations for the difference is that the morphotype on agar plates was investigated after 2 days while the cellulose production within biofilms was examined after 7 days, and thus *S.* Enteritidis ATCC 13076 might be a slow cellulose producer. Besides, it was also notably that different media (LB without NaCl and 1/20 TSB) used to analyze the cellulose production could affect cellulose production due to the difference in nutrient sources (Chawla et al., 2009). Similar to pH effect, the weaker resistance of *S.* Enteritidis ATCC 13076 at 4 °C might be attributed to other unknown factors involved in biofilm resistance.

It was found that biofilm of *S.* Enteritidis 125 was more sensitive to sanitizer treatment compared to that of *S.* Enteritidis 124 in 1/20 TSB at pH 5.3, 25 °C, although both of them had rdar morphotype. The variation might be due to that acidic condition dramatically reduced cellulose production of *S.* Enteritidis 125 as discussed above while it had a less effect on that of *S.* Enteritidis 124 (data not shown). Therefore, these results demonstrate that *S.* Enteritidis with the same morphotype might have different biofilm resistance.

5. Conclusion

This is the first study to investigate the effects of food-related environmental stress factors on the resistance of *S.* Enteritidis biofilms to sanitizer treatment and correlate their resistance with cellulose production. The results showed that higher temperature (25 °C) or low nutrient condition (1/20 TSB) promoted *S.* Enteritidis biofilm formation, while greater biofilm resistance was only found under low nutrient condition at 25 °C. In addition, biofilms formed at an acidic condition or by *S.* Enteritidis with bdar morphotype were generally more sensitive to chlorine treatment than those formed at neutral or alkaline conditions, or by strains with rdar morphotype, although environmental pH and bacterial strains did not affect biofilm formation. The multivariate biofilm data were well described using PCA, specifying that environmental temperature and nutrient availability might be the dominant factors affecting biofilm formation and resistance. Moreover, cellulose production was found to be positively correlated with biofilm resistance to chlorine treatment. Therefore, this study indicates that environmental stress conditions found in food processing plant could have a profound impact on biofilm resistance to sanitizer treatment, possibly due to their positive or negative effects on the cellulose production.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2015.10.010.

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