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Biofilm-producing abilities of *Salmonella* strains isolated from Turkey

Basar Karaca[1], Nefise Akcelik[2] & Mustafa Akcelik[1]*

[1]*Department of Biology, Faculty of Science, Ankara University, 06100 Tandogan, Ankara, Turkey; e-mail: akcelik@science.ankara.edu.tr*
[2]*Biotechnology Institute, Ankara University, 06100 Tandogan, Ankara, Turkey*

Abstract: In the present study the biofilm-forming characteristics of 99 serotyped (DMC strains) and 41 genus level-identified (IS strains) *Salmonella* strains originating from Turkey were investigated. The strains were selected based on their ability to show the biofilm morphotype on Congo red agar plates. In addition, all strains were evaluated with regard to properties related to forming pellicle structures, physical differences of pellicles, any changes in the media associated with the formation of pellicles, and the presence of cellulose within the formed biofilm matrix as determined using 366 nm UV light. The *Salmonella* Typhimurium DMC4 strain was the best producer of biofilm grown on polystyrene microtiter plates (optical density at 595 nm: 3.418). In subsequent experiments industrial process conditions were used to investigate different morphotyped *Salmonella* strains' biofilm-forming capability on stainless steel, a commonly preferred surface for the food industries, and on polystyrene surfaces. The effect of other important industrial conditions, such as temperature (5, 20, 37°C), pH (4.5, 5.5, 6.5, 7.4) and NaCl concentration (0.5, 1.5, 5.5, 10.5%) on the production of biofilm of the different morphotyped *Salmonella* strains (DMC4; red, dry and rough morphotyped *S.* Typhimurium, DMC12; brown, dry and rough morphotyped *S.* Infantis, DMC13; pink, dry and rough morphotyped *S.* subsp. Roughform) were also assessed. On the other hand, pH values exhibited variable effects on biofilm-forming features for different *Salmonella* strains on both polystyrene and stainless steel surfaces.

Key words: *Salmonella*; biofilm; stainless steel; polystyrene.

Abbreviations: bdar, brown, dry and rough; cfu, colony forming unit; CR, Congo red; LB, Luria Bertani; OD, optical density; pdar, pink, dry and rough; rdar, red, dry and rough; saw, smooth and white; sbam, smooth, brown and mucoid.

Introduction

Salmonella is a Gram-negative, facultative anaerobe and rod-shaped bacterium belonging to the family Enterobacteriacea. Some *Salmonella* serovars, often foodborne, are capable of becoming invasive (Agbaje et al. 2011). Salmonellosis, a disease of humans and animals caused by *Salmonella* spp., is one of the most common and widely distributed foodborne disease in humans (Vestby et al. 2009). *Salmonella* contamination of food and food supplements poses a significant health risk all over the world, and this case increases infection risks with animals and consumers of animal food products (Crump et al. 2002). Despite the large amount of resources by food manufacturing factories put into fighting *Salmonella*, it has been reported that some clones are able to persist in the factory environment for several years (Kumar et al. 1998; Vestby et al. 2009). Furthermore the biofilm-forming abilities of these clones make it difficult to remove biofilms efficiently from surfaces using disinfectants (Moretro et al. 2003).

Bacterial biofilms are defined as microorganism communities that attach to abiotic and/or biotic surfaces and constitute a protected mode of growth within extracellular matrix substances (Costerton 1995). The bacterial cells in the biofilm, embedded within the matrix structure, function like a chemical and physical barrier against various environmental factors, and because biofilms can accumulate several noxious compounds within their matrix, they may cause major problems associated with food industry. Biofilms are responsible for equipment damage, process down time and losses of efficiency (Giaouris et al. 2005). In medicine, biofilm-associated microorganisms are less susceptible to antimicrobial agents and treatments. Sessile biofilm communities can be up to 1.000-fold more resistant to antibacterial agents compared with communities existing in the planktonic form (Gilbert et al. 2002; Scher et al. 2004).

Biofilms are identified as an assemblage of surface-associated microbial cells that are enclosed in hydrated extracellular polymeric substances (Sauer et al. 2007; Shi & Zhu 2009). The exopolymeric substances produced by microrganisms has significant influence on the

* Corresponding author

initiation of adhesion and immobilization of bacteria to solid surfaces (Kumar et al. 1998). Mature biofilms are highly organized ecosystems in which water channels are dispersed and can provide passages for exchange of nutrients, metabolites and waste products (Sauer et al. 2007; Shi & Zhu 2009). The nutrient transfer is also more rapid in biofilms than for bacterial cells existing outside the biofilms (Kumar et al. 1998).

Bacterial attachment to surfaces is influenced not only by the bacterial strains that form the biofilm, but also by the material of the surface, conditions for growth and the environment. Numerous studies have shown that *Salmonella* species is capable of adhering to and forming biofilm on surfaces, such as polystyrene, glass and stainless steel, which are commonly used in the food processing industry, kitchens, and bathrooms (Hood et al. 1997; Woodward et al. 2000). The most frequently used material for food-processing surfaces is stainless steel. Stainless steel is moderately hydrophilic, with a net negative charge surface, whereas polystyrene is hydrophobic (Ryu et al. 2005).

Pathogenic biofilm formation also depends on properties of the substrate and bacterial cells (hydrophobicity, flagellar formation and motility), in addition to environmental factors including pH, temperature and salinity (Shi & Zhu 2009). The pH and temperature of the contact surface have an influence also on the degree of adhesion of microorganisms (Kumar et al. 1998). These environmental factors play an important role in the phenotypic change from planktonic cells to the sessile form (Shi & Zhu 2009).

The main objective of this study was to evaluate the biofilm-forming abilities of 140 *Salmonella* strains. It is also to examine the influence of incubation temperatures (5, 20 and 37 ℃), pH (4.5, 5.5, 6.5 and 7.4) and NaCl concentrations (0.5, 1.5, 5.5 and 10.5%) on biofilm formation by four different morphotyped *Salmonella* strains on stainless steel and polystyrene. These two materials were selected because stainless steel has hydrophilic characteristics, whereas polystyrene possesses hydrophobic characteristics.

Material and methods

Bacterial strains and culture conditions

Ninety-nine *Salmonella* strains isolated from various beef products and forty-one strains from raw chicken products found in Turkey were obtained from the Ankara University Prokaryotic Genetics Laboratory. The 99 *Salmonella* strains were typed as follows: serovar Agona ($n = 5$), serovar Anatum ($n = 3$), serovar Bispebjerg ($n = 1$), serovar Corvallis ($n = 4$), serovar Enteritidis ($n = 16$), serovar Group C1 ($n = 8$), serovar Infantis ($n = 13$), serovar Kentucky ($n = 11$), serovar Montevideo ($n = 4$), serovar Nchanga ($n = 2$), serovar Rauform ($n = 3$), serovar Roughform ($n = 1$), serovar Salford ($n = 1$), serovar Senftenberg ($n = 1$), serovar Telaviv ($n = 2$), serovar Thompson ($n = 6$), serovar Typhimurium ($n = 2$), serovar Virchow ($n = 16$). These and the 41 *Salmonella* spp. strains were used in this study to examine biofilm-forming capabilities. *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 strain was screened for biofilm formation as a control.

All strains were stored at −80 ℃ in Luria Bertani (LB) broth (Merck, Germany). The bacterial cultures were then inoculated into LB broth and incubated at 37 ℃ for 18 h with shaking at 200 rpm. This process was repeated at least twice before experiments. LB broth without NaCl containing 10 g/L bacto-tyrptone (Fluka, France) and 5 g/L yeast extract (Merck, Germany) was used as test broth in the biofilm assays (Römling et al. 1998).

Screening of morphotypes

All *Salmonella* strains were evaluated on Congo red (CR) agar plates according to morphological colony characteristics to determine biofilm morphotype for each strain. Morphotype monitoring assay was performed according to the method by Römling & Rohde (1999) with slight modifications. Briefly, 10 µL of overnight cultures were dropped onto LB agar without NaCl (yeast extract 5 g/L, bacto-tyrptone 10 g/L, routine agar 15 g/L) containing 40 µg/mL Congo red (Sigma-Aldrich, USA) and 20 µg/mL Coomassie Brillant Blue R250 (Sigma-Aldrich, USA), and incubated at 20 ℃ for up to one week. Following the incubation CR agar plates were visually examined with a dissection microscope (Leica, Germany) under 1X magnification and isolates were grouped into three distinct morphotypes: (i) rdar (red, dry and rough) – indicating curli fimbriae and cellulose; (ii) bdar (brown, dry and rough) – indicating only curli fimbriae; and (iii) pdar (pink, dry and rough) – indicating only cellulose. The saw (smooth and wet) morphotype, indicating neither cellulose nor fimbriae, was not detected. Experiments were performed in duplicate.

Pellicle formation in liquid-air interface

The pellicle is a biofilm structure that is observed in a liquid-air interface. The 0.5 mL of overnight cultures were inoculated into 4.5 mL LB without NaCl broth, and incubated statically at 20 ℃ for eight days. The strains were visually examined every day and classified according to their formation of a pellicle structure, the physical differences of the pellicle and any changes in the media related to pellicle formation (Solano et al. 2002). Experiments were performed in duplicate.

Calcofluor binding assay

All *Salmonella* strains were screened on LB agar without NaCl containing 200 µg/mL calcofluor, a fluorescent brightener (Sigma-Aldrich, China). Calcofluor binding assay was performed as described previously (Vestby et al. 2009) with some modifications. Briefly, overnight cultures were transferred onto LB agar containing calcofluor, and then agar plates were incubated for seven days at 20 ℃. Fluorescing colonies on agar plates when exposed under 366 nm UV light indicated the presence of cellulose (Kodak Gel Logic 200 Imaging System). Experiments were performed in duplicate.

Microtiter plate assay

The assay was based on the method described by Woodward et al. (2002) with the modifications of Stepanovic et al. (2000). Overnight cultures were diluted in LB without NaCl broth to optical density (OD) at 595 nm equal to 0.2, i.e. 10^8 colony forming units (cfu) per mL for all strains (Shimadzu spectrophotometer, Japan). Thirty µL of this suspension were transferred to each well of 96 wells of a polystyrene microtiter plates previously filled with 100 µL LB without NaCl broth, and then the microtiter plates were incubated statically for two days (48 h) at 20 ℃. Negative

control wells contained broth only. Following the incubation, plates were washed once with sterile distilled water to remove loosely attached and planktonic cells. The plates were dried at room temperature before addition of 130 µL of 98% methanol (Merck, Germany). After 10 min incubation, the methanol was removed and plates were dried again. One hundred and thirty µL of 1% crystal violet used for Gram staining (Merck, Germany) was transferred into the wells and stained for 30 min. Excess stain was removed by washing three-times with sterile distilled water and wells were dried at room temperature. Finally, 130 µL of 33% glacial acetic acid (Sigma-Aldrich, Germany) were added to each well and bound dye was solubilized from the adherent cells. The OD of each well was measured at 595 nm (Shimadzu, Japan). The result for each strain was calculated by substracting the median OD_{595} of the three parallels of the control (LB without NaCl broth only) from the median OD_{595} of the three parallels of sample. Experiments were performed in triplicate.

Effects of different NaCl concentrations, incubation temperatures and pH values on biofilm-forming capabilities in polystyrene microtiter plates

To elucidate important environmental parameters (temperature values: 5, 20 and 37℃; pH values: 4.5, 5.5, 6.5, and 7.4; NaCl concentration values: 0.5, 1.5, 5.5, and 10.5%) on biofilm formation in microtiter plates, three different morphotyped *Salmonella* strains were compared. NaCl concentrations were adjusted by adding the appropriate amount of NaCl (Merck, Germany) to the LB without NaCl broth. pH values were adjusted with 1 M NaOH or 1 M HCl before autoclaving. For all tests but the temperature treatment, microtiter plates were incubated at 20℃ for two days. The crystal binding assay was performed at the end of the first and second incubation days. After the incubation, the method was performed as described above. The experiments were performed in triplicate with three parallels.

Effects of different NaCl concentrations, incubation temperatures and pH values on biofilm-forming capabilities on stainless steel surfaces

Stainless steel was chosen for the experiments since it is the most frequently used surface for food-processing. The stainless steel coupons (0.1, 0.8 and 2.5 cm) were prepared for treatment assays as previously described (Giaouris et al. 2005). Temperature, NaCl concentration and pH treatments were performed as described for the polystyrene microtiter plate assay.

Stainless steel coupons were placed individually in 3.5 mL of LB without NaCl broth media, and test tubes with the coupons inside them were autoclaved at 121℃ for 15 min. The same pH and NaCl concentration values, which affect adhesion capabilities of the stainless steel surface, were evaluated as described above. Overnight cultures were adjusted to an OD_{595} of 0.2 (10^8 cfu/mL), and these dilutions were inoculated to test tubes. The test tubes were incubated for seven days.

After the incubation, each coupon was removed with sterile forceps and transferred to an empty sterile tube, where it was left for 5 min to dry at room temperature. The coupon was rinsed twice with 5 mL of 0.85 % sterile physological saline solution to remove loosely attached cells. Following the second washing and a drying step, each coupon was transferred to a new tube that contains 4.5 mL of sterile physological saline solution and five glass beads (3 mm diameter). The coupon was then vortexed for 1 min at maximum intensity (IKA Genius Vortex 3, Germany).

Table 1. Distribution of different biofilm morphotypes among *Salmonella* strains.[a]

Biofilm morphotype	DMC	IS	Total
rdar	65	14	79
bdar	31	26	57
pdar	2	1	3
sbam	–	–	–
saw	–	–	–
Unidentified	1	–	1
Total	99	41	140

[a] DMC coded strains are serotyped, IS coded strains are identified at genus level.

Quantification of biofilm production was performed using an agar plating method. One mL of suspension was taken from each tube, and ten-fold serial dilutions were prepared in SP solution. Bacterial colonies on LB agar plates were counted after incubation at 37℃ for 18 h. The count of cfu per square centimeter were transformed to a logarithmic value for better representation.

Statistical analysis

All statistical analysis was performed by Minitab statistics software, version 15 (USA). Experiments were performed in triplicate. Results were given as mean standard error values. Chi-Square and Fisher's exact tests were performed for frequency of morphotype distribution among strains and relationships between pellicle structure characteristics and various morphotypes. One-way ANOVA test was preferred for microtiter plate assay data.

Results

Biofilm morphotype

Three different colony morphotypes were detected among the 140 *Salmonella* isolates. The rdar morphotype was observed in 79 strains (65 DMC and 14 IS strains), the bdar morphotype in 57 strains (31 DMC and 26 IS strains), and the pdar morphotype was identified in only 3 strains (2 DMC and 1 IS). In one strain we found a morphotype that had not been described previously (Table 1).

When screening biofilm morphotype distribution among *Salmonella* serotypes, it was found that the rdar morphotype was commonly expressed by *S.* Anatum (100%), *S.* Bispebjerg (100%), *S.* Corvallis (100%), *S.* Enteritidis (75%), *S.* Group C1 (100%), *S.* Montevideo (75%), *S.* Nchanga (100%), *S.* Salford (100%), *S.* Senftenberg (100%), *S.* Thompson (100%), *S.* Tyhimurium (100%) and *S.* Virhow (93,75%). The bdar morphotype was found as the dominant morphotype in *S.* Agona (80%), *S.* Infantis (100%), *S.* Kentucky (54,5%) serovars. The pdar morphotype was only detected in one Kentucky and Roughform serovars and in one *Salmonella* spp. strain (Table 2).

Pellicle formation in liquid

All strains were tested for their pellicle forming ability. Also 31 DMC strains, containing strong rdar morphotype on Congo red agar plates, were selected in

Table 2. Biofilm morphotype frequencies of the different *Salmonella* serotypes.

Serotype	bdar	pdar	rdar	saw	sbam	Total
Agona[a]	4 (80%)	–	–	–	–	5
Anatum	–	–	3 (100%)	–	–	3
Bispebjerg	–	–	1 (100%)	–	–	1
Corvallis	–	–	4 (100%)	–	–	4
Enteritidis	4 (25%)	–	12 (75%)	–	–	16
Group C1	–	–	8 (100%)	–	–	8
Infantis	13 (100%)	–	–	–	–	13
Kentucky	4 (36.4%)	1 (9.1%)	6 (54.5%)	–	–	11
Montevideo	1 (25%)	–	3 (75%)	–	–	4
Nchanga	–	–	2 (100%)	–	–	2
Rauform	2 (66.6%)	–	1 (33.4%)	–	–	3
Roughform	–	1 (100%)	–	–	–	1
Salford	–	–	1 (100%)	–	–	1
Senftenberg	–	–	1 (100%)	–	–	1
Telaviv	2 (100%)	–	–	–	–	2
Thompson	–	–	6 (100%)	–	–	6
Typhimurium	–	–	2 (100%)	–	–	2
Virchow	1 (6.25%)	–	15 (93.75%)	–	–	16
Total	31	2	65			99

[a] *S.* Agona strain contains unidentified morphotype.

Table 3. Evaluation of *Salmonella* strains that contain different morphotypes according to the pellicle existence at the air-liquid interface.

Morphotype	Presence of pellicle	Absence of pellicle	Total
bdar	41	16	57
rdar	72	7	79
Total	113	23	136

order to learn if there is any correlation between biofilm-producing ability and pellicle formation rate at the air-liquid interface. All members of the tested serotypes formed pellicles at the liquid-air interface except the members of *S.* Enteritidis (86.6%), *S.* Rauform (33.3%), *S.* Kentucky (90.9%), *S.* Infantis (84.6%), *S.* Agona (80%) and *S.* Virchow (93.8%) serovars.

Salmonella strains were evaluated according to their pellicle-forming abilities along with alterations in media (turbidity and pellet at the bottom) and physical features of pellicle structures (rigid, fragile, and elastic), characteristics which were visually examined daily over the course of eight days. High frequencies of pellicle formation were found among the rdar and bdar morphotypes, 91.1% and 72%, respectively, while none of the pdar morphotypes and one identified strain formed any pellicle. Frequence differences are given in Table 3 (Chi square $p < 0.01$). The rdar morphotype formed rigid pellicle [94.6% rigid ($n = 68$), 5.6% fragile ($n = 4$)], while the bdar morphotype formed pellicle with fragile structure [97.6% fragile ($n = 1$), 2.4% rigid ($n = 40$); $p < 0.001$ by Fisher's exact test] (Table 4a). At the end of the eight-day incubation, the test tubes containing the "bdar" morphotyped pellicle (turbid-clear; $n = 29$, $n = 28$) had a much greater turbidity than the test tubes containing "rdar" morphotyped pellicle [turbid-clear; $n = 17$, $n = 62$; $p < 0.01$ by Chi-square test] (Table 4b).

Table 4. Evaluation of *Salmonella* strains according to their pellicles' physical characteristics depending on pellicle structure (a) and turbidity of growth medium (b).

(a)	Morphotype	Rigid	Fragile	Total
	bdar	1	40	41
	rdar	68	4	72
	Total	69	44	113
(b)	Morphotype	Turbidity (turbid)	Turbidity (clear)	Total
	bdar	29	28	57
	rdar	17	62	79
	Total	46	90	136

To compare pellicle formation at the air-liquid interface of serovars during the eight-day incubation period, 31 strains examined using on Congo red agar plates showed a strong rdar morphotype, the dominant morphtype among serotypes, and were selected for further workup. These are Anatum, $n = 2$; Bispebjerg $n = 1$; Corvallis, $n = 4$; Enteritidis, $n = 3$; Group C1, $n = 4$; Kentucky, $n = 4$; Montevideo, $n = 1$; Nchanga, $n = 2$; Salford, $n = 1$; Senftenberg, $n = 1$; Thompson, $n = 1$; Typhimurium, $n = 2$; Virchow, $n = 5$; total:

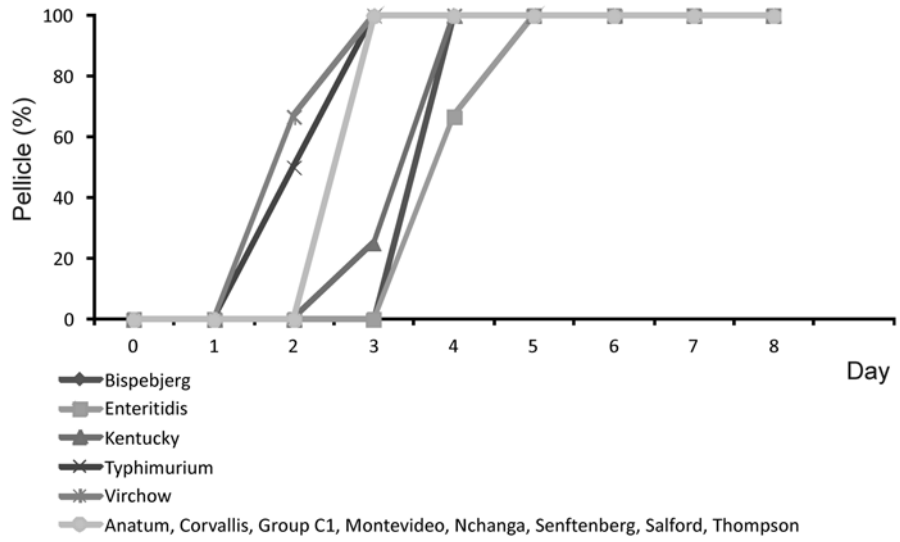

Fig. 1. Percentage of strains within each *Salmonella* serovar with a pellicle in standing liquid at different incubation days.

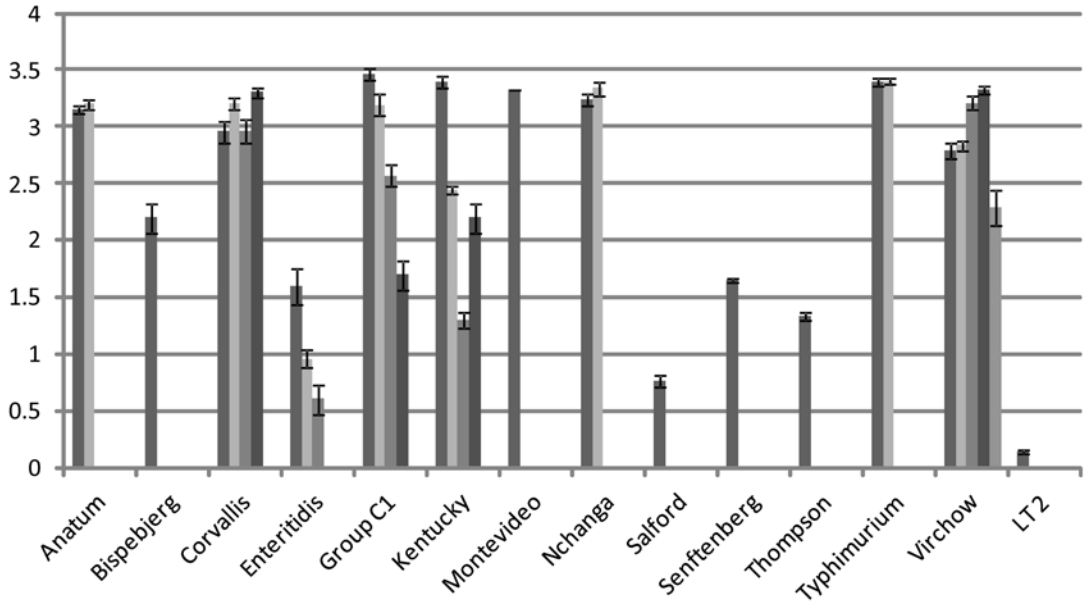

Fig. 2. Comparative biofilm production on microtiter plates among selected different *Salmonella* serovars. All bars represent standard errors.

31. The rate of pellicle formation varied between the serovars tested. Also a negative correlation was found between rates of pellicle production and of the biofilm quantity on polystyrene microtiter plates ($r = -0.606$ by Pearson correlation, $p < 0.01$) (Fig. 1 and Fig. 2).

Calcofluor binding

Screening of all 140 isolates on calcofluor LB agar revealed that all strains classified as rdar and pdar always bound to calcofluor and exhibited flourescence properties under UV light, identified as positive for cellulose. One strain classified as an unidentified morphotype (*S.* Agona) and 57 strains classified as bdar did not bind calcofluor and were found to be cellulose-negative. The results of the calcofluor experiments were consistent with results of pellicle formation and Congo red assays (Fig. 3).

Biofilm on microtiter plate

In the screening of the 31 *Salmonella* strains with the strong rdar morphotype selected for biofilm characteriziation, the growth in microtiter plates showed OD_{595} values ranging from 0.8077 (*S.* Salford) to 3.435 (*S.* Typhimurium). Statistically significant differences between serovars were observed (means were compared by one-way ANOVA; $p < 0.05$). The strongest biofilm-producing ability was observed in *S.* Typhimurium serovar. The two *Salmonella* Typhimurium strains DMC 4 and DMC 95 produced more biofilm in microtiter plates compared to the LT2 control strain (3.398, 3.435, and 0.15, respectively) (Fig. 2).

Effect of temperature, pH and NaCl on biofilm production on microtiter plate

To investigate the effect of different temperatures (5, 20

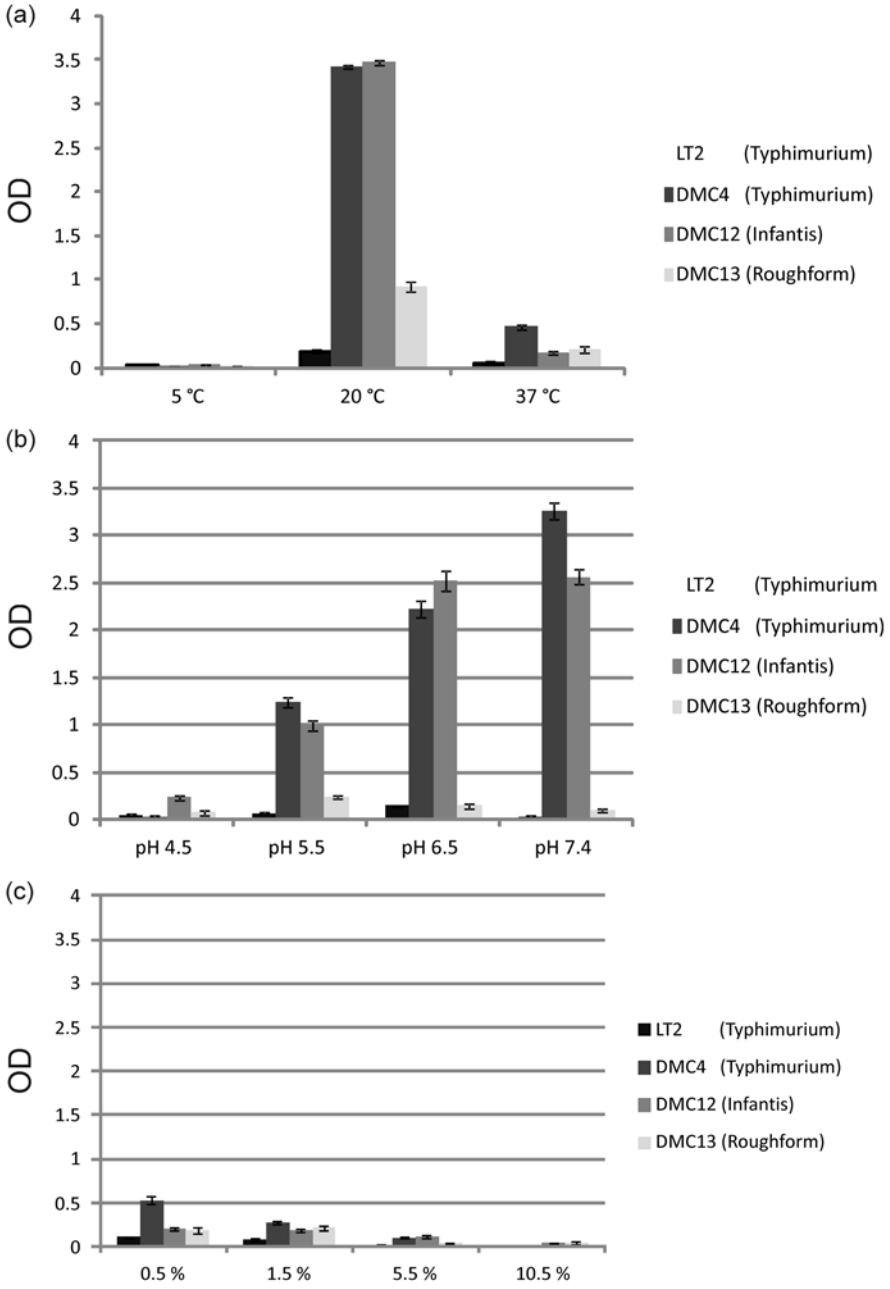

Fig. 3. Impact of different incubation temperatures (a), pH values (b), and NaCl concentrations (c) on biofilm production at the end of the second incubation day (48 h). LT2, *S.* Typhimurium as a control strain; DMC4, rdar morphotyped *S.* Typhimurium; DMC12, bdar morphotyped *S.* Infantis; DMC13, pdar morphotyped *S.* subsp. Roughform. All bars represent standard errors.

and 37 °C), pH values (4.5, 5.5, 6.5 and 7.4) and NaCl concentrations (0.5, 1.5, 5.5 and 10.5%) on biofilm production abilities of *Salomenella* strains on microtiter plates, three strains were chosen to represent each morphotype. *Salmonella* Typhimurium LT2 was used as a control strain.

The effect of incubation temperatures on biofilm production values of different morphotypes on microtiter plates at 5, 20 and 37 °C is shown in Figure 3a. Biofilm was produced at higher levels on the second day of incubation. It was concluded that a 48-h incubation period is critical for biofilm production. The highest amount of biofilm was formed by all tested strains in microtiter wells at 20 °C, but no production was observed at 5 °C. At 20 °C, biofilm was produced at similar levels by *S.* Typhimurium (rdar, OD_{595}: 3.418) and *S.* Infantis (bdar, OD_{595}: 3.478) strains. Despite *S.* Roughform (pdar) being the strongest biofilm producer following the 24-h incubation, its biofilm amount was dramatically decreased 3.39-fold by the end of the 48-h incubation at 37 °C (0.88).

Effects of different pH values were recorded at the end of the second incubation day and it was found that for many serovars having a pH of 4.5, their biofilm production was quite low. The *S.* Infantis serovar, however, produced much more biofilm at this pH. With higher pH conditions there were increases in biofilm production of *S.* Typhimurium and *S.* Infantis serovars. The

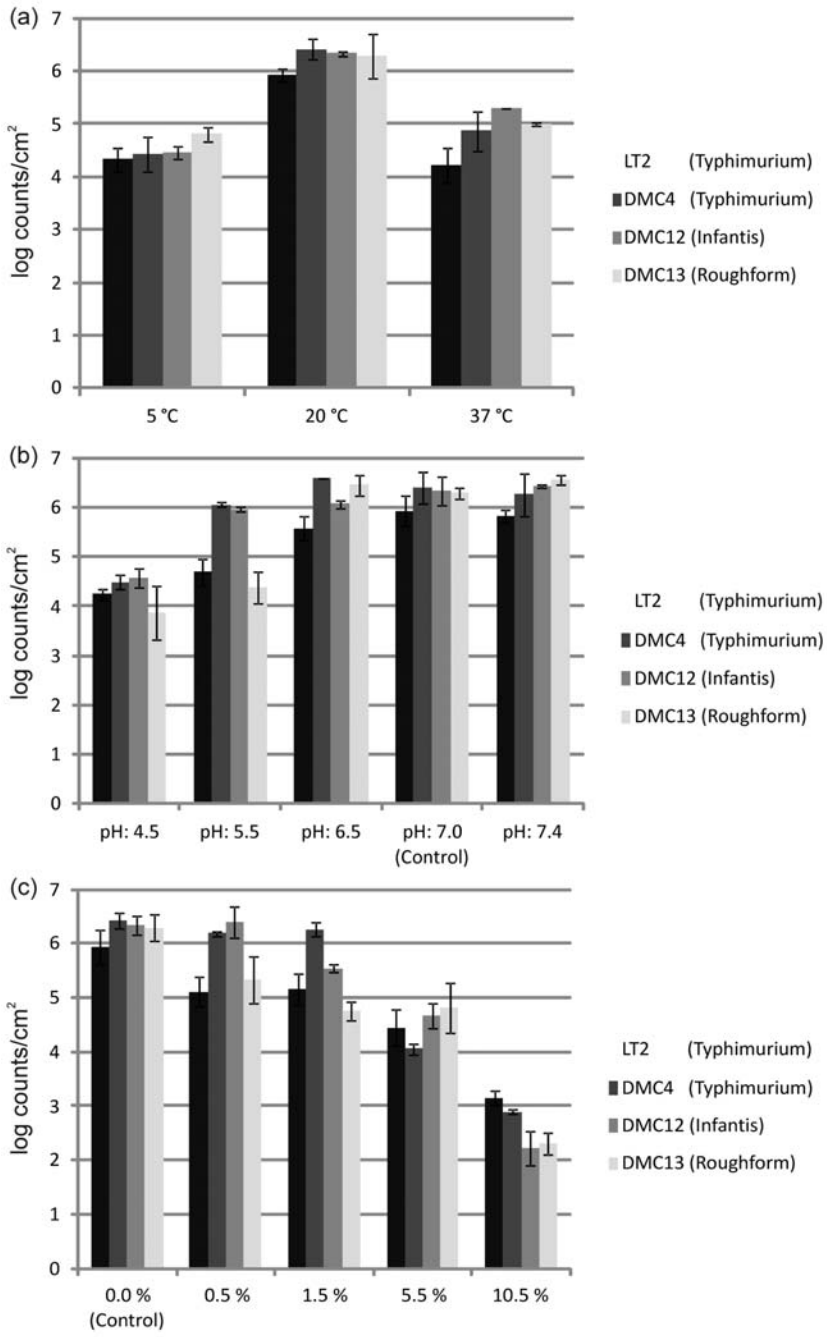

Fig. 4. Evaluating biofilm forming abilities on stainless steel surfaces under different incubation temperature (a), pH (b), and NaCl concentration (c) conditions. All bars represent standard errors.

S. Infantis (bdar morphotype) biofilm yields were found to be similar at pH 6.5 and 7.4, and it was concluded that a pH 5.5 environment was optimal for *S.* Roughform. Analysis of the results of measured pH values and the biofilms produced by various morphotypes at 20 ℃ at pH 7.0 suggests that optimal biofilm formation on polystyrene microtiter plate is pH 7.0 (Fig. 3b).

Increases in NaCl concentrations from 0.5% to 10.5% resulted in complete inhibition of bacterial adhesion to microtiter plate wells, which likely means that biofilm production was affected adversely to enable adhesion. NaCl concentration of 0.5% gave the highest biofilm production (Fig. 3c).

Effect of temperature, pH and NaCl on biofilm production on stainless steel surfaces

Biofilm production and adhesion on stainless steel surfaces were assessed using the same strains with microtiter plate experiments. Moreover, the same stress parameters were investigated as described above. The results of heat treatments are shown in Figure 4a. As shown in the results of the microtiter plate experiments, a 20 ℃ temperature is optimal for biofilm production on stainless steel surfaces. The optimum pH level for adhesion and biofilm production varies between pH 6.5–7.4 according to tested strains (Fig. 4b). The amount of biofilm produced for tested strains except *S.*

Typhimurium (rdar) was not affected significantly by incubation at 0.5, 1.5 and 5.5% NaCl concentrations (Fig. 4c). Treatment with 10.5% NaCl killed all cells.

Discussion

The "bdar" and "rdar" morphotyped *Salmonella* strains have different survival capabilities. It is known that the "rdar" morphotyped strains can survive longer at unfavourable environmental conditions and are more resistant to the various chemical agents in comparison with the "bdar" morphotyped strains (Vestby et al. 2009). The "rdar" morphotype was detected as dominant and common (at 56.43%) among *Salmonella* strains originating in Turkey, and it was observed that disinfection and sterilization procedures must be applied more vigorously in food manufacturing processes compared to other locales. The failure to make this vigorous effort at disinfection and sterilization could account at least in some aspect for why salmonellosis cases are common in Turkey.

Many bacteria form aggregates at the bottom of containers and attach to the container surfaces in liquid media. However, some bacteria such as *Salmonella enterica* serovars, *Escherichia coli*, *Pseudomonas fluorescens*, and *Vibrio cholerae* produce rigid or fragile pellicle structures at air-liquid interfaces, and these structures overlie all of the liquid surface (Rainey & Travisano 1998; Yildiz & Schoolnik 1998; Römling & Rohde 1999; Anriany et al. 2001; Zogaj et al. 2001; Solano et al. 2002; Spiers et al. 2002). Also biofilm production at the air-liquid interface by colonization of the air-liquid surface can facilitate and contribute to gas exchange, while enabling the acquisition of nutrients and water from the liquid phase (Spiers et al. 2003). The biofilms at air-liquid and solid-air interfaces can cause serious problems in industrial water systems (Scher et al. 2004). It should be noted as well that the *Salmonella* strains of Turkish origin form a pellicle, which can make up 85%, a high percentage, of the biofilm structure.

It is hypothesized that the persistence of *Salmonella* strains is related to their ability to form biofilms in many environments, and support for this comes from results showing that the persistent strains clearly were better biofilm-producers than the non-persistent strains (Vestby et al. 2009). Two *Salmonella* strains of Turkish origin have been shown to have extremely good biofilm-forming abilities on polystyrene surfaces; observations show that this biofilm production is not dependent upon serovar. This observation also can be seen in each of the serovar groups. It is important to emphasize that strict criteria must be chosen for classifying strains as persistent or non-persistent, and that comparisons are made in accordance with similar studies performed with *Listeria monocytogenes* (Lunden et al. 2000; Borucki et al. 2003).

Three important incubation temperature values (5, 20 and 37 °C) were performed to understand biofilm-forming abilities; as it was an objective to evaluate different morphotyped strain characteristics, particularly their biofilms under these conditions. The 5 °C temperature is a routine condition for the storage of several kinds of food. The temperature 20 °C mimics standard room and factory conditions. The 37 °C temperature represents an optimum for *Salmonella* growth. Our results show that 20 °C is the optimum temperature for *Salmonella* biofilm formation. It is well known that two divergent *csg* operons are highly expressed at 20 °C (Römling & Rohde 1999). This temperature condition can contribute to the survival abilities of *Salmonella* under environmental conditions, in which the bacteria are free-living (outside a host it may have infected). The 20 °C temperature was shown to be a suitable temperature for each strain, especially for the "bdar" and "rdar" morphotyped strains. The "rdar" and "bdar" morphotyped strains have similar biofilm-producing capability at 20 °C, but the "rdar" morphotyped strain produced greater amounts of biofilm than did the other strains at 37 °C.

Biofilm formation was increased among all tested strains at neutral pH values. All of these results may support the hypothesis that biofilm-forming capability is not dependent upon biofilm morphotype because all tested serovars show a similar trend at optimal pH and temperature conditions, although biofilm quantities were different for each serovar.

During food processing, adjustments to pH and salt levels can be used to prevent food spoilage and food-borne diseases and to prolong food shelf life. A long-time brining (use of salt as a preservative) has been a traditional technique to eliminate and to prevent germination of food spoilage microorganisms or of food-borne pathogens (Doyle & Glass 2010). Our studies indicate that the presence of biofilm-forming *Salmonella* can be prevented by adjusting salt and pH levels similarly to the conditions characteristic of those traditional procedures, and that these can contribute to food safety.

In order to bolster and expand upon the results we found using the microtiter plate assay, examining the effects of various environmental stress conditions to biofilm formation on abiotic surfaces, our experiments included studies of a stainless steel surface. Stainless steel surfaces are favourable to the formation of biofilm for *Salmonella* A particular difficulty with stainless steel, however, is that its surface can feature crevices and zones of corrosion, which can prevent an effective cell count. But more troubling is that these surface crevices can make it difficult to remove biofilms (Holah et al. 1990; Dhaliwal et al. 1992; Flint et al. 1996).

When evaluating different stress conditions that were described for the microtiter plate assay for experiments with stainless steel surfaces, a 20 °C temperature was found again to be optimal for the biofilm-forming capability of each serovar. In addition, neutral pH conditions and the absence of a low pH (pH 4.5) were optimal. A surprising result is that each serovar has similar biofilm-producing capability at both 5 °C and 37 °C; results shown for the microtiter plate assay.

Our results demonstrate that greater attention

must be given to the choice of potential contact surfaces and cleaning procedures when considering the efficient removal of biofilms. It is well known that biofilm elimination from surfaces with routine disinfection steps is quite difficult (Knight & Craven 2010). As for the dynamics of biofilm production of *Salmonella* serovars of Turkish origin, a model system should be designed that mimics factory conditions so as to evaluate biofilm-forming capabilities on various surfaces (e.g., stainless steel) and for the prevention of biofilm formation and the elimination of existing biofilms.

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