



## Biofilm Forming Ability of Broiler Chicken Meat Associated *Salmonella* spp. on Food Contact Surfaces

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### ABSTRACT

Biofilms in the meat industry pose a significant impact on public health as many foodborne pathogens can form biofilms on equipment and utensils in meat processing plants. Biofilms formed on contact surfaces could be a continuous source of contaminants to food that come in contact with them. In the present study biofilm-forming ability of different *Salmonella* spp. isolated from broiler chicken meat from Sri Lanka was quantified, the presence of biofilm-related genes was investigated, and comparative evaluation on the biofilm formation on plastic, stainless steel, and glass surfaces was performed. It was revealed that the biofilm formation increased as a response to the nutrient stress. Quantification of biofilm formation by the microtitre plate method showed 7 strong (30.4%), 11 moderate (47.8%), and 5 weak (21.7%) biofilm formers at low nutrient media (TSB diluted 1:100), whereas there were only 4 moderate biofilm formers and 19 weak biofilm formers found in the undiluted TSB medium. All biofilm formers exhibited the presence of both *adrA* and *gcpA* regulatory genes. Investigation on the ability of biofilm formation on glass, stainless steel, and plastic at time points of 48, 96, 144, 192, and 240 hrs post incubation with the contamination of  $10^6$  CFU/cm<sup>2</sup> revealed a significant ( $p \leq 0.05$ ) variation among *Salmonella* isolates. All the isolates illustrated the highest ability to form biofilms on plastic, whereas that was minimum on stainless steel.



## INTRODUCTION

Non-typhoidal *Salmonella* spp. are among the most commonly encountered foodborne and waterborne pathogens worldwide, and it is the second most reason for the gastroenteritis after the *Campylobacter* spp. distributed worldwide (Lamas et al., 2018). This spp. gets prime attention as it is an important organism leading to public health issues, and causes a zoonotic bacterial threat in the poultry industry (Merino et al., 2019). The most common route of transmission of *Salmonella* from animals to humans is through contaminated food or foodstuffs, particularly eggs, egg products, poultry meat, and dairy products (EFSA, 2017). One of the most common food contamination routes is food contact surfaces which are used in food processing (Srey et al., 2013).

In addition to invading the host cells and multiplying, *Salmonella* spp. are known to possess the capability of surviving outside the host in a sessile form attaching to surfaces as biofilms (Srey et al., 2013). This phenomenon plays a major role in spreading the organism as biofilms which are much more resistant to disinfectants and chemicals (Bhowmick et al., 2011; Borges et al., 2018; Galie et al., 2018). Biofilms are structured communities of microbial cells and they are complex consortia of microorganisms enmeshed in a self-produced matrix of extracellular polymeric substances (EPS) adherent to inert or living surfaces. Many of the foodborne pathogens have the ability to form biofilms on various surfaces such as plastic, metal, glass, aluminum, wood, and other surfaces in different structures in food processing plants such as floors, pipes, drainage, and walls (Schonewille et al., 2012; Wang et al., 2013; Winkelstroter et al., 2014). Biofilm formation by pathogenic bacteria has a great impact on the food industry, sometimes leading to fatal consequences. Its impact is extended to some other areas such as medicine, dentistry, veterinary medicine, water treatment, all of which directly effect human health and life (Feng et al., 2015). The ability of biofilm formation by different *Salmonella* spp. has shown to be strain-dependent, and related to the properties of cell surface structures. It has also been proven that biofilms have different abilities to resist different sanitizers and antibiotics at different levels (Wang et al., 2017).

The EPS of *Salmonella* biofilm is majorly composed of curli (amyloid fimbriae), cellulose, biofilm-associated protein (Bap), O-antigen capsule and extracellular DNA. The synthesis of these EPS substances is regulated by a cascade of complex regulatory mechanisms in combination with several genes. The main control unit in biofilm

formation is the *csgDEFG* operon. Some genes such as *csgD* of the operon positively regulate *csgBA* and *adrA* expression which is important in biofilm formation of *Salmonella* spp. (Bhowmick et al., 2011; Steenackers et al., 2012). The protein AdrA (coded by the gene *adrA*) controls the levels of cyclic di-GMP which is important in regulating the cellulose production and thereby the biofilm formation (Garcia et al., 2004). GcpA protein, which is the GGDEF domain containing protein A coded by *gcpA* gene, plays a critical role in biofilm formation of *S. typhimurium* under low nutrient conditions (Garcia et al., 2004; Bhowmick et al., 2011).

Biofilm formation in food processing environments is of special importance as it has the potential to act as the chronic source of microbial contamination that may lead to food spoilage or transmission of diseases. The knowledge on *Salmonella* biofilm formation occurring on food contact surfaces is vital to take necessary precautions to avoid possible contaminations of food. In the present study, the biofilm forming ability of different *Salmonella* spp. which were isolated from broiler chicken meat was quantified, and the presence of related genes of biofilm formation were investigated. Further, a comparative evaluation of the biofilm formation on plastic, stainless steel, and glass surfaces was performed.

## METHODOLOGY

### Quantification of biofilm formation using the microtitre plate method

The study accommodated 23 PCR-confirmed *Salmonella* isolates collected from different areas of the country. Biofilm formation was quantified in polystyrene microtitre plates following a method described by Stepanovic et al. (2004). Accordingly, 96 well flat-bottomed polystyrene microtiter plates (Grenier Bio-one, Germany) were filled with 230  $\mu$ L of TSB and 1:100 diluted TSB. Twenty microliters from each of the *Salmonella* isolates cultured for 16 hours was added to each well. The experiment was performed in three replicates. TSB medium without bacterial inoculums was used as the control. The microtitre plates were incubated at 28 °C for 24 hrs. The contents of the well were then discarded and washed thrice with sterile phosphate-buffered saline (PBS) of pH 7.4. Cells attached to the wells of the microtitre plates were fixed with methanol and subsequently stained with 1% crystal violet. Following the staining step, the stained, attached cells were resuspended in 33% (v/v) glacial acetic acid. The Isolates were then classified as strong, moderate, weak, and non-biofilm producers based on the absorbance values measured at 630 nm (OD<sub>630</sub>) in an ELISA reader

(ELx 800, Bio-Tek, 100 Tigan Street, Winooski, VT05404, USA) (Stepanovic *et al.*, 2000). Optical Density (OD) values of the sterile controls were subtracted from the test values to obtain the 'true' biofilm level. Briefly, the cut-off OD ( $OD_c$ ) was defined as three standard deviations above the mean OD of the negative control. Isolates were classified as follows:

$OD \leq OD_c$  = no biofilm producer,

$OD_c < OD \leq (2 \times OD_c)$  = weak biofilm producer,

$(2 \times OD_c) < OD \leq (4 \times OD_c)$  = moderate biofilm producer, and

$(4 \times OD_c) < OD$  = strong biofilm producer.

### Screening for genes responsible for biofilm formation

The presence of some biofilm-associated genes *adrA* and *gcpA* in all 23 *Salmonella* isolates was checked by using polymerase chain reaction (PCR) as described by Bhowmick *et al.* (2011). The genomic DNA was extracted according to Ausubel *et al.* (1992). The purity and concentration of extracted DNA were checked spectrophotometrically by using the NanoDrop spectrophotometer (ND-1000, V3.3.0, Thermo Fisher Scientific, USA).

The PCR was performed in 30  $\mu$ l volumes containing 3  $\mu$ l of 10X buffer (100 mM Tris-HCl (pH 9) 1.5 mM  $MgCl_2$ , 500 mM KCl, 0.1 % Gelatin), 200  $\mu$ M concentrations each of deoxyribonucleotide triphosphates (dATP, dTTP, dGTP and dCTP), 10 picomoles of each primer and 1.0 U of Taq DNA polymerase (Genei™, Merck Bioscience, Bangalore), with 2.0  $\mu$ l of template DNA. The reactions were carried out using a thermal cycler (BioRad, CA, USA). The PCR products were resolved in 1.5% agarose gel, stained with ethidium bromide (5 ng  $ml^{-1}$ ) and bands were visualized and documented using a gel documentation system (Herolab, Wiesloch, Germany).

### Biofilm formation on different surfaces

The ability of *Salmonella* isolates to form biofilm on glass, stainless steel, and plastic food contact surfaces was determined at different post incubation time points (48, 96, 144, 192, and 240 hrs) with  $10^6$  CFU/ $cm^2$  of bacteria. Based on the results of the biofilm quantification experiment, three strong biofilm producers (isolates S4, S6, and S23) and one moderate biofilm producer (isolate S8) were selected for the study. Bacterial cultures were recovered from stock on Luria Bertani Agar

(Himedia, India) and a single colony was used for the study.

All surfaces were properly sterilized before use as described by Joseph *et al.* (2001) and Marques *et al.* (2007). The prepared surfaces (glass, stainless steel, and plastic) were separately placed in Petri dishes and 40 mL of Luria-Bertani medium (LB broth) was added to each petri dish for conditioning.

A single colony from each *Salmonella* isolate was grown in LB broth for 24 h at 37 °C and cells were washed twice in sterile distilled water following incubation. Bacterial cell numbers were determined spectrophotometrically, and  $10^5$  CFU/mL bacterial cells were inoculated to each conditioned Petri plate which contained LB broth and surface materials. After incubation at ambient temperature ( $28 \pm 2$  °C) for 72 hrs, the samples were aseptically removed, washed in sterile phosphate buffer saline (PBS, pH 7.4) in order to remove unattached cells. This procedure was repeated twice in 72-hour time intervals to complete the biofilm formation (Joseph *et al.*, 2001). Experiments were done in three individual replicates. Surface samples without *Salmonella* spp. were used as the uncontaminated control to determine the sterility of surface samples.

Bacterial cells were enumerated at five different time points as 2<sup>nd</sup>, 4<sup>th</sup>, 6<sup>th</sup>, 8<sup>th</sup>, and 10<sup>th</sup> day of the incubation. At each time interval, for the enumeration of attached biofilm cells, the samples were washed with sterile PBS to remove unattached cells and the formed biofilms were removed by scraping with sterile scalpels. The scrapings were collected into 1 mL sterile distilled water, shaken vigorously and serially diluted in sterile distilled water. Enumeration was done by plating the diluted samples on selective medium (Hektoen enteric agar for *Salmonella*) by standard spread plate technique, followed by incubation at 37 °C for 16 hrs. After the incubation period, the number of viable cells was counted, and the results were expressed as log CFU/ $cm^2$ . This procedure was repeated at every 48<sup>th</sup> hour up to 240 hrs. Sterility of the surfaces was checked by incubating the surfaces in Luria Bertani medium without adding the bacterial cultures at each time interval.

### Analysis of data

Biofilm-forming ability was compared by analysing the differences in the degree of biofilm formation using one-way ANOVA and Duncan's multiple range test in SPSS (IBM) software (Version 15, IBM).

## RESULTS AND DISCUSSION

### Quantification of biofilm formation using the microtitre plate method

The ability of different *Salmonella* isolates to form biofilms on microtiter plates was identified by the development of colour intensity in the microtitre plate (Figure 1). The results revealed that the biofilm formation is different in undiluted TSB and 1:100 diluted TSB. The isolates were classified according to the results using the standard classification and found that there were 07 strong biofilm formers, 11 moderate biofilm formers, and 05 weak biofilm formers in diluted medium (TSB diluted 1:100) and there were only 04 moderate biofilm formers and 19 weak biofilm formers found in the TSB medium (Table 1).

Biofilm formation in food processing environments has great importance as it can act as a chronic source of microbial contamination which may lead to food spoilage, and disease transmission (Stepanovic et al., 2004; Souza et al., 2014). There are many factors which affect the formation of biofilm, and many researchers have reported that the nutritional condition in the medium is an important factor for the formation of biofilms (Dewanti et al., 1995; Djordjevic et al., 2002; Stepanovic et al., 2003a, Stepanovic et al., 2003b; Ben Miloud Yahia et al., 2018).

Previous studies have revealed that bacteria grown in nutrients-poor medium exhibit stronger biofilm formation than those grown in nutrients-rich medium (Hood and Zottola, 1997; Joseph et al., 2001; Stepanovic et al., 2004; Lamas et al., 2016;

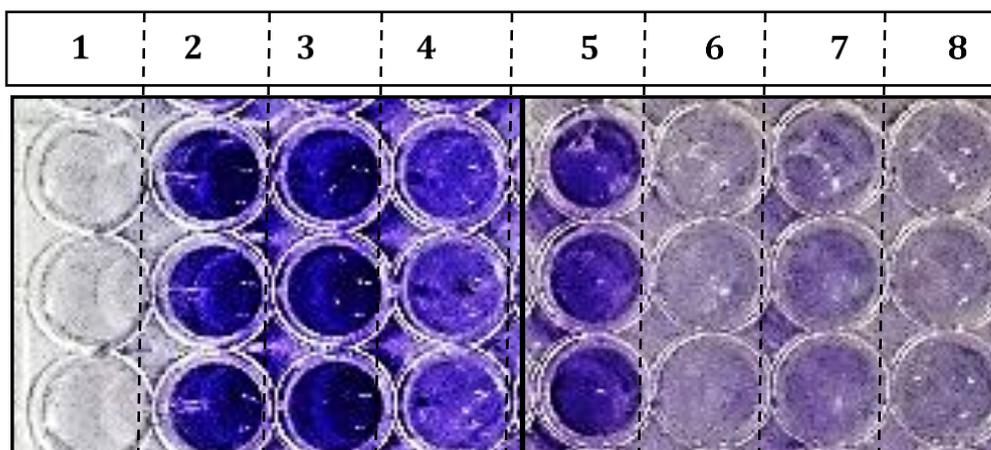
Paytubi et al., 2017; Iniguez-Moreno et al., 2018). The results of the present study also were in line with the previous findings. Therefore, it can be stated that biofilm formation by *Salmonella* isolates may also occur as a response to the nutritional condition of the growth media.

### Presence of genes responsible for biofilm formation

Screening for the presence of the genes *adrA* and *gcpA* (Figure 2) revealed that both genes were present in all the isolates with biofilm formation ability (Table 1). The synthesis of substances involved in biofilm formation by *Salmonella* spp. is regulated by a highly complex regulatory network, and this cascade of mechanisms is controlled by a set of genes. Presence of the genes such as *adrA* and *gcpA* is essential to exhibit biofilm formation by *Salmonella* spp. *adrA* is needed for the cellulose production and biofilm formation in LB complex media, whereas the *gcpA* gene is important in biofilm formation in nutrient deficient environments (Solano et al., 2002; Bhowmick et al., 2011; Seixas et al., 2014; Lamas et al., 2016). As the two genes were present in all isolates it could be further confirmed that the presence of both *adrA* and *gcpA* genes is essential for biofilm formation by *Salmonella* spp.

### Biofilm formation by isolates of *Salmonella* spp. on different surfaces

The biofilm-forming ability of selected isolates at different time points on different surfaces is given in Tables 2, 3 and 4.

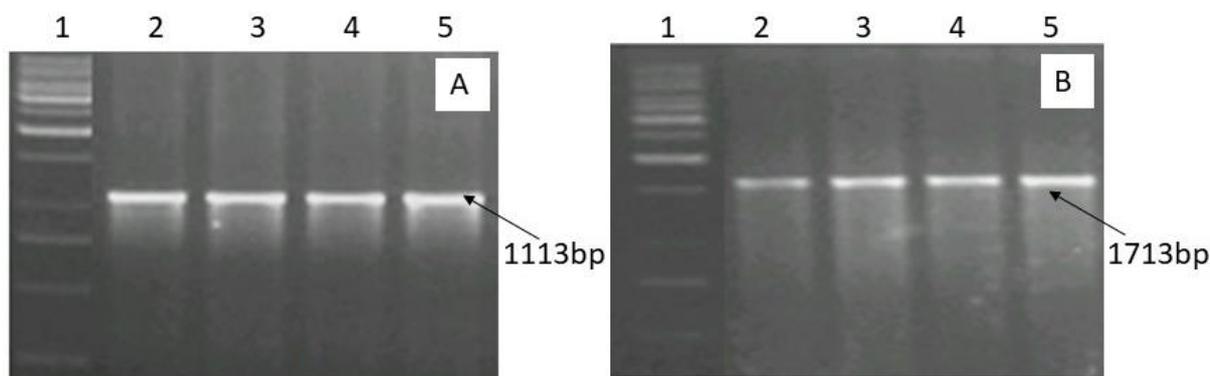


**Figure 1: Quantification of biofilm using the microtitre plate method.**

Note: 1. Negative control; 2 & 3. Strong biofilm formers; 4 & 5. Moderate biofilm formers; 6, 7 & 8: Weak biofilm formers

**Table 1: Quantification of biofilm formation by different *Salmonella* isolates and presence of different genes (*adrA* and *gcpA*) involved in biofilm formation**

Isolate	Mean OD			Presence of biofilm associated genes		
	TSB (1:100)		TSB	<i>AdrA</i>	<i>gcpA</i>	
S1	0.173± 0.016	Moderate	0.081± 0.002	Weak	+	+
S2	0.167± 0.018	Moderate	0.084± 0.003	Weak	+	+
S3	0.134± 0.022	Moderate	0.074± 0.005	Weak	+	+
S4	0.325± 0.032	Strong	0.109± 0.016	Moderate	+	+
S5	0.173± 0.015	Moderate	0.085± 0.006	Weak	+	+
S6	0.280± 0.033	Strong	0.088± 0.003	Weak	+	+
S7	0.183± 0.027	Moderate	0.094± 0.015	Weak	+	+
S8	0.220± 0.022	Moderate	0.092± 0.011	Weak	+	+
S9	0.267± 0.022	Moderate	0.090± 0.006	Weak	+	+
S10	0.167± 0.013	Moderate	0.098± 0.009	Moderate	+	+
S11	0.090± 0.008	Weak	0.073± 0.006	Weak	+	+
S12	0.216± 0.030	Strong	0.100± 0.011	Moderate	+	+
S13	0.213± 0.022	Strong	0.083± 0.008	Weak	+	+
S14	0.070± 0.034	Weak	0.078± 0.036	Weak	+	+
S15	0.232± 0.021	Strong	0.089± 0.008	Weak	+	+
S16	0.182± 0.037	Moderate	0.087± 0.011	Weak	+	+
S17	0.162± 0.014	Moderate	0.089± 0.011	Weak	+	+
S18	0.128± 0.101	Moderate	0.058± 0.031	Weak	+	+
S19	0.040± 0.006	Weak	0.044± 0.001	Weak	+	+
S20	0.038± 0.002	Weak	0.055± 0.011	Weak	+	+
S21	0.055± 0.039	Weak	0.044± 0.002	Weak	+	+
S22	0.244± 0.022	Strong	0.097± 0.004	Moderate	+	+
S23	0.258± 0.102	Strong	0.083± 0.005	Weak	+	+

**Figure 2: Presence of (A) *adrA* gene; (B) *gcpA* gene responsible for biofilm formation in *Salmonella* isolates.**

Note: Lane 1: 1kb DNA ladder, Lane 2-4: *Salmonella* isolates - S1, S2, S3 and S4

**Table 2: Formation of biofilm by four different *Salmonella* isolates on plastic surfaces**

Isolate	Number of biofilm cells (log) at different time intervals				
	48 hours	96 hours	144 hours	192 hours	240 hours
S23	5.19±0.01 <sup>b</sup>	6.19±0.02 <sup>b</sup>	6.41±0.01 <sup>b</sup>	7.53±0.01 <sup>b</sup>	7.43±0.01 <sup>b</sup>
S6	5.20±0.02 <sup>b</sup>	6.21±0.03 <sup>b</sup>	6.39±0.01 <sup>b</sup>	7.53±0.01 <sup>b</sup>	7.45±0.01 <sup>b</sup>
S4	5.29±0.01 <sup>c</sup>	6.28±0.01 <sup>c</sup>	6.48±0.02 <sup>c</sup>	7.56±0.00 <sup>c</sup>	7.54±0.00 <sup>c</sup>
S8	5.09±0.01 <sup>a</sup>	6.10±0.00 <sup>a</sup>	6.30±0.00 <sup>a</sup>	7.39±0.01 <sup>a</sup>	7.41±0.00 <sup>a</sup>

Note: Values in the same columns (differ for each surface) with different superscripts are statistically significantly different at 95% confidence interval

**Table 3: Formation of biofilm by four different *Salmonella* isolates on glass surfaces**

Isolate	Number of biofilm cells (log) at different time intervals				
	48 hours	96 hours	144 hours	192 hours	240 hours
S23	5.12±0.01 <sup>b</sup>	6.11±0.03 <sup>a,b</sup>	6.21±0.03 <sup>b</sup>	7.37±0.01 <sup>b</sup>	7.36±0.02 <sup>b</sup>
S6	5.15±0.00 <sup>c</sup>	6.14±0.03 <sup>b</sup>	6.21±0.01 <sup>b</sup>	7.37±0.01 <sup>b</sup>	7.36±0.01 <sup>b</sup>
S4	5.27±0.00 <sup>d</sup>	6.23±0.02 <sup>d</sup>	6.42±0.02 <sup>c</sup>	7.46±0.01 <sup>c</sup>	7.45±0.01 <sup>c</sup>
S8	5.02±0.01 <sup>a</sup>	6.05±0.01 <sup>b</sup>	6.12±0.00 <sup>a</sup>	7.29±0.01 <sup>a</sup>	7.29±0.01 <sup>a</sup>

Note: Values in the same columns (differ for each surface) with different superscripts are statistically significantly different at 95% confidence interval

**Table 4: Formation of biofilm by four different *Salmonella* isolates on glass surfaces**

Isolate	Number of biofilm cells (log) at different time intervals				
	48 hours	96 hours	144 hours	192 hours	240 hours
S23	5.03±0.01 <sup>b</sup>	6.05±0.02 <sup>a,b</sup>	6.08±0.01 <sup>b</sup>	7.24±0.01 <sup>b</sup>	7.18±0.03 <sup>b</sup>
S6	5.06±0.02 <sup>b</sup>	6.05±0.01 <sup>a,b</sup>	6.09±0.01 <sup>b</sup>	7.25±0.01 <sup>b</sup>	7.20±0.02 <sup>b</sup>
S4	5.18±0.02 <sup>c</sup>	6.11±0.03 <sup>c</sup>	6.29±0.01 <sup>c</sup>	7.31±0.01 <sup>c</sup>	7.32±0.01 <sup>c</sup>
S8	4.96±0.00 <sup>a</sup>	6.01±0.00 <sup>a</sup>	6.01±0.00 <sup>a</sup>	7.07±0.01 <sup>a</sup>	7.08±0.00 <sup>a</sup>

Note: Values in the same columns (differ for each surface) with different superscripts are statistically significantly different at 95% confidence interval

According to the results, it can be stated that the isolate S4 and S8 isolates showed significantly the highest and the lowest ( $p \leq 0.05$ ) biofilm-forming abilities, respectively compared to all other isolates at all-time points on the three surfaces (Tables 2, 3 and 4). Further, it was also revealed that all the tested isolates showed a significantly higher ( $p \leq 0.05$ ) biofilm formation on plastic surfaces (Table 2) than on glass and stainless steel at all the time points (Tables 2, 3 and 4). Formation of biofilm on stainless steel surface was significantly lower ( $p \leq 0.05$ ) than that on other two surfaces (Tables 2, 3 and 4) in all the tested isolates. However, the isolates S8 and S4 showed the lowest

and the highest biofilm formation abilities respectively, on the stainless steel surface too (Tables 2, 3 and 4).

Biofilm formation in food processing environments is of special importance as it has the potential to act as a chronic source of microbial contamination that may lead to food spoilage and/or transmission of diseases. This is especially because the biofilms exhibit enhanced resistance to general cleaning and sanitation practices (Bower and Daeschel, 1999; Joseph *et al.*, 2001). The capability of *Salmonella* spp. in biofilm formation on plastic, metal, glass, or rubber surfaces were reported in

many previous studies (Somers *et al.*, 1994; Dhir and Dodd, 1995; Jones and Bradshaw, 1996; Humphrey *et al.*, 1997; Hood and Zottola, 1997; Wong, 1998; Sommer *et al.*, 1999; Chae and Schraft, 2000; Leriche and Carpentier, 2000; Sinde and Carballo, 2000; Joseph *et al.*, 2001; Ben Miloud Yahia *et al.*, 2018). Similarly, all isolated *Salmonella* spp. formed biofilms on different surfaces such as plastic, glass, and stainless steel in the present study. Biofilm formation by isolates of *Salmonella* spp. on different surfaces was also evident that *Salmonella* isolates S4, S6, and S23 (strong biofilm formers) had significantly higher ( $P < 0.05$ ) biofilm-forming abilities than the isolate S8 (moderate biofilm former) on different surfaces. Furthermore, these bacteria possessed a high capacity of biofilm formation on plastic surfaces than on glass and stainless steel. These results confirm the earlier findings, which showed that biofilm formation by *Salmonella* spp. on plastic surfaces is extensive compared to other surfaces such as stainless steel, glass, and rubber used in the food industry (Romling and Rohde, 1999; Joseph *et al.*, 2001; Mireles *et al.*, 2001; Djordjevic *et al.*, 2002; Stepanovic *et al.*, 2003a,b; Stepanovic *et al.*, 2004; Chia *et al.*, 2009).

In general, it is assumed that glass and stainless steel are hydrophilic materials while plastic is a hydrophobic material (Sinde and Carballo, 2000; Donlan 2002). It has been previously shown that *Salmonella* spp. adhere in high numbers to hydrophobic materials (Cunliffe *et al.*, 1999; Sinde and Carballo, 2000; Donlan 2002). As adhesion is the first step in the complex process of biofilm formation (Donlan 2002), this could be one possible explanation for the high ability of

*Salmonella* spp. to produce biofilm on the plastic surface. Nevertheless, the previous studies have shown that the attachment of bacterial cells to a surface is affected by numerous factors, including the growth medium, motility, growth phase, type and properties of the inert materials, presence of organic materials, temperature, pH, contact time, production of extracellular polysaccharides, and cell-to-cell communication (Chandy and Angles, 2001; Chmielewski and Frank, 2003; Shi and Zhu, 2009; Simoes *et al.*, 2010a, b; Xu *et al.*, 2011).

As plastic, glass, and stainless steel are the commonly used materials to prepare structures in animal slaughterhouses, animal and meat transport structures, and meat processing plants, the knowledge on *Salmonella* spp. biofilm formation occurring on contact surfaces is vital to take necessary precautions to avoid contamination of meat with this highly pathogenic microorganism.

## CONCLUSIONS

The *Salmonella* spp. isolated from broiler meat possessed a high capability of forming biofilm on different surfaces. There were 34.8% strong biofilm forming isolates among them. Plastic surface was more vulnerable to biofilm formation by isolated *Salmonella* spp. than the glass and stainless steel surfaces. Biofilm formation was significantly lower ( $p \leq 0.05$ ) on stainless steel surfaces than on glass surfaces. Thus, serious consideration must be paid in selection of suitable fabrication materials for meat processing, storage and marketing workflows.

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