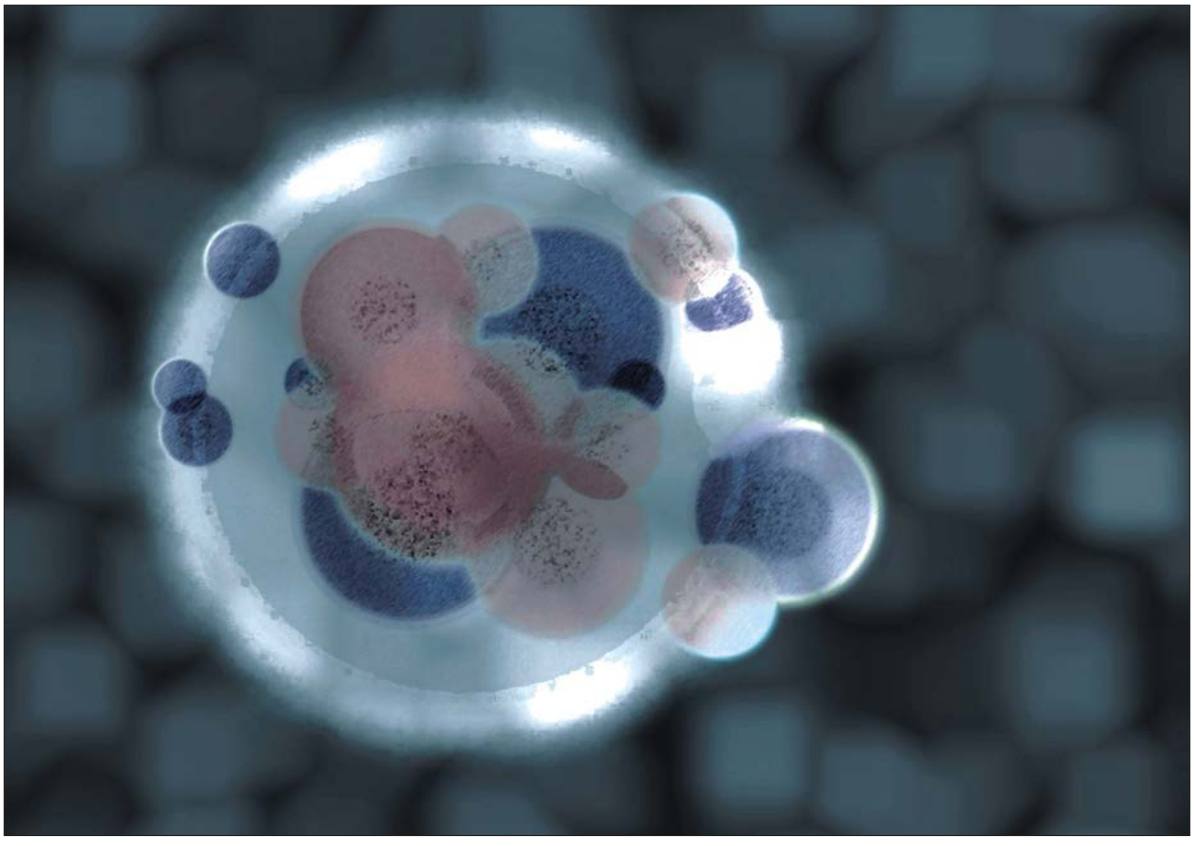

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Biofilm Formation by *Salmonella enteritidis* on Food Contact Surfaces

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Abstract: In this study, initially hydrophobicity of *Salmonella enteritidis* RITTC 1624 was assessed by Microbial Adhesion to Hydrocarbon Method (MATH). *Salmonella enteritidis* is an important foodborne enteric pathogen. This organism is highly resistant in environment and remains an important cause of gastroenteritis in human world-wide. Also *S. enteritidis* forms biofilms on many food contact surface materials. Biofilm formation of bacterium studied at 2, 4, 8, 16 and 20 h after adhesion on surfaces of stainless steel, glass and polyethylene. This organism with 73% hydrophobicity formed biofilm on each of three surfaces but after 2 h biofilm formation was observed on glass and stainless steel more significantly than on polyethylene surface ($p<0.05$). Results indicated that hydrophobicity of bacteria and surface are important criteria in biofilm formation. The type of surface is also play an important role in biofilm formation of various bacteria. These findings in public health and food industry are very valuable.

Key words: Adhesion, biofilm, *Salmonella enteritidis*, hydrophobicity

INTRODUCTION

Salmonella spp. is a food-poisoning organism, which are of considerable significance to the food industry, since they are one of the most important foodborne pathogens (Cox *et al*., 1999). Non-typhoidal salmonellosis is estimated to affect 1.4 million people each year in the United States, while more than 95% of cases of infections caused by these bacteria are foodborne. These infections account for about 30% of deaths resulting from foodborne illnesses (Mead *et al*., 1999). Studies performed so far have lead to the discovery that these bacteria are capable of adhering and forming biofilms on different surfaces (Giaouris and Nychas, 2006; Joseph *et al*., 2001; Sinde and Carballo, 2000). Biofilms can exist on most types of surfaces in food plants ranging from plastic, wood, glass, metals and food products (Trachoo, 2003).

Biofilm formation has serious implications in industrial, environmental, public health and medical situations (Gilbert *et al*., 2003; Hall-Stoodley *et al*., 2004). The occurrence of biofilms in food-processing environments can cause post-processing contamination leading to lowered shelf-life of products and transmission of diseases (Mahdavi *et al*., 2008; Jessen and Lammert, 2003). Biofilms not only present a considerable hygiene risk in the food industry, but also cause economical losses by technical failures in water systems, cooling towers, heat exchangers, etc. Sessile micro-organisms have advantages in that they are more difficult to mechanically remove from food-contact surfaces and are more resistant to disinfectants compared with planktonic forms (Gilbert *et al*., 2001; Trachoo, 2003; Joseph *et al*., 2001). The aim of this study was to assess the ability of *Salmonella enteritidis* to form biofilms on food contact surfaces.

MATERIALS AND METHODS

Bacterial strain and culture conditions: In this study, *S. enteritidis* strain RITCC 1624 was obtained from Razi Institute Tehran, Iran and was cultured on (Xylose Lysine Desoxycholate) XLD agar (Merck) at 30°C and the study was performed during June to November 2006.

Hydrophobicity assessment: Cell surface hydrophobicity was measured by the MATH test. The MATH method developed by Rosenberg *et al*. (1980) gives a cell hydrophobicity index (A% = percentage of adhesion) and is easy to perform. Cell suspension were classified to 0.5 Macfarland standard and 4 mL of cell suspension were transferred to individual test tubes (diameter, 1.7 cm; length, 15 cm), which contained 1 mL of octane. The test tubes were vortexed at full speed for 2 min and then left to stand for 15 min to allow phase separation. The Optical Density $(OD)_{640}$ of the aqueous phase was determined and partitioning of the bacterial suspension was expressed as the percentage of cells adsorbed by the hydrocarbon phase: percentage of partitioning = $[(A_1 - OD_{640}$ of

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aqueous phase)/A_1] × 100. A_1 is primary OD_{640} of cell suspension before adding octane hydrocarbon to it (Mozes and Rouxhet, 1987).

Surface preparation: Three types of material namely Plastic (High Density Polyethylene, HDPE), glass and stainless steel were used to form the biofilm. The stainless steel coupons (type 304, No. 2B) cut to 1/2×5 cm were degreased in acetone to remove grease and were washed in detergent solution, rinsed in deionized water twice and air-dried. Cleaned steel coupons were finally autoclaved in deionized water at 121°C for 15 min prior to use (Chmieiewski and Frank, 2003; Birt and Frank, 2002). The HDPE coupons cutted to 2×5 cm were cleaned with detergent and rinsed with deionized water. Glass slides were cleaned with detergent and then autoclaved in deionized water.

Biofilm formation: *S. enteritidis* strain RITCC 1624 was grown in TSB for 24 h at 37°C and then its cell suspension was classified to 0.5 Macfarland standards. One milliliter of this suspension was added to beakers containing 50 mL TSB and sterile surfaces. After that, beakers were shacked for, 2, 4, 8, 16 and 20 h. This procedure was performed for every three sterile surfaces to form biofilms (Bos *et al*., 1999; Bresford *et al*., 2001).

Enumeration of biofilm cells: To enumerate biofilm cells, the samples were washed with sterile distilled water to remove unattached cells and the biofilm cells were removed by swabbing with sterile cotton swabs. The swabs were transferred to 10 mL physiological saline (0.85% NaCl, w/v prepared in the laboratory) shaken vigorously and enumerated by drop plate method (Herigstad *et al*., 2001). After preparation of sufficient dilution of the sample, sample was expelled in five evenly spaced 10 μL drops onto the quadrant of one of the Petri plates that have been labeled for that particular dilution of the sample. This process was repeated for each tube in the dilution series. After the drops on the agar dried, the Petri plates were incubated at 35°C for 24 h. Finally, Remove plates when colonies have developed and count the dilution which contains 3-30 colonies per 10 μL drops. Viable cell counts were expressed as colony forming units (cfu)/surface area. Calculation was done according to the following formula:

LOG (cfu cm^{-2}) = LOG [(average cfu/drop volume) (dilution counted) (volume scraped into/surface area*)] *scraped slide = LOG [(average of plate counts/0.01 mL) (10 dilution) (10 mL/1.267 cm^2)]

Statistical analysis: The data were analyzed by analysis of variance (ANOVA) and the Tukey-Kramer multiple comparison test. p-values of <0.05 were considered significant

RESULTS

Hydrophobicity of *S. enteritidis* was 73%. *S. enteritidis* formed biofilms on all of three substrates studied, viz. polyethylene, glass and steel. Biofilm formation of *S. enteritidis* on these surfaces had not significant difference ($p>0.05$), except in 2 h adhesion time. At 2 h adhesion time, the cell density of biofilm formed by *S. enteritidis* was highest on glass followed by steel and polyethylene ($p<0.05$). Cell density of biofilm on glass, steel and polyethylene were 1.75×10^4, 1.94×10^3 and 8.33×10^3 cfu cm^{-2}, respectively (Table 1). At 20 h adhesion time, no significant difference was observed ($p>0.05$) and cell density of biofilm on glass, steel and polyethylene were 1.68×10^7, 1.47×10^6 and 2.07×10^7 cfu cm^{-2}, respectively (Table 1).

Table 1 shows the number of cells adhering to all materials following a contact time of 2 and 20 h. There was significant difference in the total number of *S. enteritidis* adhering to each material after 2 h and for each, the number adhering did not increased over 2 h ($p>0.05$). It seems there are no significant difference ($p>0.05$) between the number of *S. enteritidis* that formed biofilms on all surfaces at 2 to 20 h adhesion time (Fig. 1).

As indicated in Fig. 2a, glass slide floating in liquid phases for biofilm formation, shows a little spreading of biofilms. In contrast (Fig. 2b), glass slide placed on interface between air and liquid phases, shows more biofilm formation on the interfacial phases. In general for

Table 1: Biofilm formation of *S. enteritidis* on various surfaces at 2 and 20 h adhesion time

Surface type	Biofilm formation (cfu cm^{-2}) [2 h]	Biofilm formation (cfu cm^{-2}) [20 h]
Stainless steel	$8.33\times10^{+3}$	$1.47\times10^{+6}$
Glass	$1.75\times10^{+4}$	$1.68\times10^{+7}$
Polyethylene	$1.94\times10^{+3}$	$2.07\times10^{+7}$

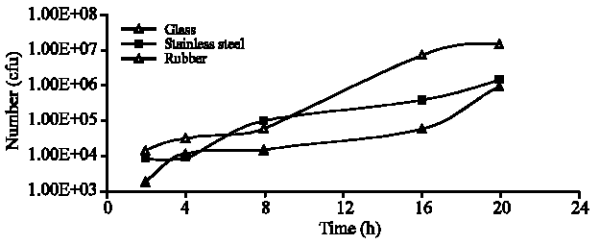

Fig. 1: Biofilm formation of *S. enteritidis* on surfaces

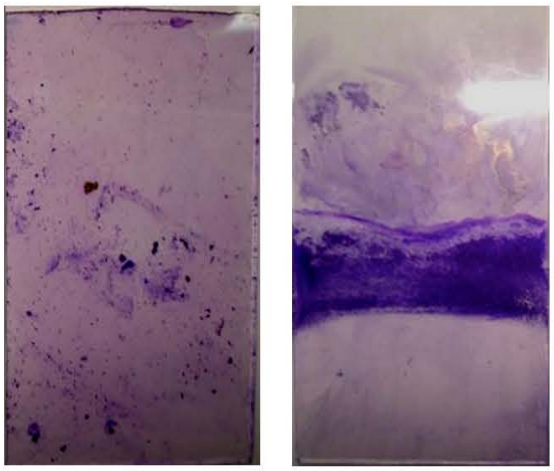

Fig. 2: Biofilm formation of *S. enteritidis* at the air-liquid interface

all tested surfaces, biofilm formation of *S. enteritidis* was observed more on the interface between air and liquid phases.

DISCUSSION

S. enteritidis is an important foodborne bacterium. Its biofilm formation has been investigated due to its role in cross-contamination of foods and disease transmission (Blackman and Frank, 1996; Austin *et al*., 1998; Beuchat, 2002; Stepanovi *et al*., 2003). We demonstrated in this study that *S. enteritidis* readily adheres and forms biofilm on inert food processing surfaces (Stainless steel and Polyethylene). The adhesion of *S. enteritidis* to surfaces occurred rapidly. Similar kinetics has been reported for the attachment of *Listeria monocytogenes* to stainless steel and buna-N rubber (Smoot and Pierson, 1998). *L. monocytogenes* reached maximum attached numbers (10^4 cfu cm^{-2} from a suspension of 10^7 cfu mL^{-1}) in approximately 3 h, whereas the number of adherent *S. enteritidis* increased for almost 8 h. Thus, during a normal shift (8 to 20 h) in a food processing plant, there is ample time for the organism to attach to surfaces (Fig. 1).

Bacterial attachment is influenced by the surface of cells and attachment media as well as other environmental factors (Kumar and Anand, 1998; Frank, 2001). Fletcher and Loeb (1979) noted that large number of bacteria attached to hydrophobic surfaces with little or no surface charge and moderate number attached to hydrophobic metals with positive charge or neutral charge and very few attached to hydrophilic negatively charged substrates. The importance of hydrophobicity in bacterial attachment has been also highlighted by other investigators (Characklis, 1990). Variation in biofilm density of *Vibrio harveyi* depending on surface was also reported by Karunasagar *et al*. (1996). In present study the cell density of *Salmonella* was highest on glass followed by steel and Polyethylene reflecting the surface preference of *Salmonella*. Because of hydrophobic interaction between surface and bacteria make a repellent force, this hydrophobic bacterium with 73% hydrophobicity mostly formed biofilm on hydrophilic surfaces such as glass and stainless steel.

Giaouris and Nychas (2006) reported that the air-liquid interface appears to be very important in enhancing the attachment and biofilm formation of bacteria to surfaces. Biofilm development was found to be sensitive to oxygen availability as it was observed that there was a 2-3 log greater count on partially exposed coupons than similar coupons that were fully submerged. Most bacteria when propagated in static liquid culture grow within the broth phase or quickly sediment to the bottom. However, when propagated for 20 generations or more in a spatially structured environment (e.g., unshaken broth culture in test tube), bacteria (mutants) with an enhanced ability to colonize the air-liquid interface frequently arise (Romling and Rohde, 1999; Solano *et al*., 2002). In present study, as shown in Fig. 3a, attachment and biofilm formation of *S. enteritidis* occurred at the meniscus of the air-liquid interface which provides bacteria with access to both the gaseous (oxygen) and liquid (nutrients).

In conclusion, we have found that *S. enteritidis* is able to attach and form biofilms on food processing surfaces. Formation of biofilm by *Salmonella* on various surfaces has implications for the food processing industry. *Salmonella* strains entering the food processing environment either through contaminated foods or through carriers handling foods, may survive in the premises by forming biofilms on various surfaces. HDPE surfaces are generally available on crates which are used to carry food products. The contact time between foods and HDPE surface may reach to 24-48 h, depending on the processing conditions including design of equipment and cleaning and disinfection regimes. Once a biofilm formed, this could become a source of contamination for foods passing through the same processing line. The results of this study have shown that *Salmonella* spp. can form biofilms on food contact surfaces. Such biofilm cells which are not removed during normal cleaning procedure in a food processing unit could be a source of contamination for foods. The cleaning and disinfection protocols in Iranian food processing units should therefore consider *Salmonella* biofilms.

REFERENCES

Austin, J.W., G. Sanders, W.W. Kay and S.K. Collinson, 1998. Thin aggregative fimbriae enhance *Salmonella enteritidis* biofilm formation. FEMS. Microbiol., 162 (2): 295-301.

Beuchat, L.R., 2002. Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. Microb. Infect., 4 (4): 413-423.

Birt, M.D. and J.F. Frank, 2002. Growth of *Listeria monocytogenes* in a multispecies biofilm formed in a humid environment with soluble protein. M.Sc. Thesis, Georgia University, Athens.

Blackman, I.C. and H.F. Frank, 1996. Growth of *Listeria monocytogenes* as a biofilm on various food processing surfaces. J. Food Prot., 59 (8): 827-831.

Bos, R., H.C. Mei and H.J. Busscher, 1999. Physico-chemistry of initial microbial adhesive interactions-its mechanisms and methods for study. FEMS. Microbiol., 23 (2): 179-230.

Bresford, M.R., P.W. Andrew and G. Shama, 2001. *Listeria monocytogenes* adheres to many materials found in food processing environments. J. Applied Environ. Microbiol., 90 (6): 1000-1005.

Characklis, W.G., 1990. Process Analysis. In: Biofilms, Characklis, W.G. and K.C. Marshall (Ed.). Wiley-Interscience, New York, pp: 17-54.

Chmieiewski, R.A.N. and J.F. Frank, 2003. Heat inactivation of *Listeria monocytogenes* in biofilms. Ph.D Thesis, Georgia University, Athens.

Cox, J., T.S. Hammack and W.H. Andrews, 1999. *Salmonella*. In: Encyclopedia of Food Microbiology, Robinson, R.K., C.A. Batt and P.D. Pates (Eds.). Vol. 3. Academic Press, New York, pp: 1928-1947.

Fletcher, M. and G.I. Loeb, 1979. Influence of substratum characteristics in the attachment of a marine psuedomonad to solid surfaces. Applied Environ. Microbiol., 37 (1): 67-72.

Frank, J.F., 2001. Microbial attachment to food and food contact surfaces. Adv. Food Nutr. Res., 43: 319-370.

Giaouris, D. and E. Nychas, 2006. The adherence of *Salmonella enteritidis* PT4 to stainless steel: The importance of the air-liquid interface and nutrient availability. Food Microbiol., 23 (8): 747-752.

Gilbert, P., J.R. Das, M.V. Jones and D.G. Allison, 2001. Assessment of resistance towards biocides following the attachment of microorganisms to and growth on, surfaces. J. Applied Microbiol., 91: 248-254.

Gilbert, P., A.J. McBain and A.H. Rickard, 2003. Formation of microbial biofilm in hygienic situations: A problem of control. Int. Biodeter. Biodegr., 51: 245-248.

Hall-Stoodley, L., J.W. Costerton and P. Stoodley, 2004. Bacterial biofilms: From the natural environment to infectious diseases. Nat. Microbiol. Rev., 2 (22): 95-108.

Herigstad, B., M. Hamilton and J. Heersink, 2001. How to optimize the drop plate method for enumerating bacteria. J. Microbiol. Methods, 44 (2): 121-129.

Jessen, B. and L. Lammert, 2003. Biofilm and disinfection in meat processing plants. Int. Biodeter. Biodegr., 51: 265-269.

Joseph, B., S.K. Otta and I. Karunasagar, 2001. Biofilm formation by *Salmonella* spp. on food contact surfaces and their sensitivity to samitizers. Int. Food Microbiol., 64: 367-372.

Karunasagar, I., S.K. Otta and I. Karunasagar, 1996. Bioflm formation by *Vibrio harveyi* on surfaces. Aquaculture, 140 (3): 241-245.

Kumar, C.G. and S.K. Anand, 1998. Significance of microbial biofilms in food industry: A review. Int. J. Food Microbiol., 42 (1): 9-27.

Mahdavi, M., M. Jalali and R.K. Kermanshahi, 2008. The assessment of biofilm formation in Iranian meat processing environments. Res. J. Microbiol., 3 (3): 181-186.

Mead, P.S., L. Slutsker, V. Dietz, L.F. McCaig, J.S. Bresee, C. Shapiro, P.M. Grif and R.V. Tauxe, 1999. Food-related illness and death in the United States. Emerg. Infect. Dis., 5 (5): 607-625.

Mozes, N. and P.G. Rouxhet, 1987. Methods for measuring hydrophobicity of microorganisms. J. Microbial. Methods, 6 (2): 99-112.

Romling, U. and M. Rohde, 1999. Flagella modulate the multicellular behavior of *Salmonella typhimurium* on the community level. FEMS. Microbiol. Lett., 180 (1): 91-102.

Rosenberg, M., D. Gutnick and E. Rosenberg, 1980. Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. FEMS. Microbiol. Lett., 9: 29-33.

Sinde, E. and J. Carballo, 2000. Attachment of *Salmonella* spp. and *L. monocytogenes* to stainless steel, rubber and polytetrafuorethylene. J. Food Microbiol., 17 (4): 439-447.

Smoot, L.M. and M.D. Pierson, 1998. Influence of environmental stress on the kinetics and strength of attachment of *Listeria monocytogenes* Scott A to buna-N rubber and stainless steel. J. Food Prot., 61 (7): 1286-1292.

Solano, C., B. Garcia, J. Valle, C. Berasain, J.M. Ghigo, C. Gamazo and I. Lasa, 2002. Genetic analysis of *Salmonella enteritidis* biofilm formation: Critical role of cellulose. Mol. Microbiol., 43 (3): 793-808.

Stepanovi, S., I. Irkovi, V. Mija and M. Vabi-Vlahovi, 2003. Influence of the incubation temperature, atmosphere and dynamic conditions on biofilm formation by *Salmonella* spp. Food Microbiol., 20 (3): 339-343.

Trachoo, N., 2003. Biofilms and the food industry. J. Sci. Technol., 25 (6): 807-815.