International Food Research Journal 18: 31-38 (2011)

Biofilm formation by *Salmonella* Typhi and *Salmonella* Typhimurium on plastic cutting board and its transfer to dragon fruit

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Abstract: Adhesion of microorganism to food contact surface can become a source of microbial contamination. Enhanced resistances to environmental stresses are exhibited by biofilm producers. In this study, biofilm formation by *Salmonella* Typhi and *Salmonella* Typhimurium on plastic cutting board was accessed before the evaluation of the transfer of these two pathogens from plastic cutting board to dragon fruit. By using crystal violet assay, it was found that the adhesion on plastic cutting board by these two pathogens was the greatest at time 12 h. Results showed that *Salmonella* adhesion is strain-dependent and varied with time. The mean transfer rate from contaminated plastic cutting board to dragon fruit was examined to be 0.79 and 0.72 for *Salmonella* Typhi and *Salmonella* Typhimurium, respectively. This indicated that there is a risk of cross-contamination which should be concerned.

Keywords: biofilm, *Salmonella* Typhi, *Salmonella* Typhimurium, plastic cutting board, dragon fruit

Introduction

The incidence of foodborne pathogens on fresh produce gains a great concern in industrialized countries nowadays. *Salmonella* spp. is one of the most commonly isolated pathogens associated with fresh produce where preliminary FoodNet data on the incidence of foodborne illness showed *Salmonella* at the top of the overall incidence in the United States (Penteado and Leitao, 2004). The outbreaks of salmonellosis have been linked to a wide variety of fresh produce including apple, watermelon, mango, tomato, alfalfa sprout, lettuce, cantaloupe, unpasteurized orange juice and parsleys (Lapidot et al., 2006; Pui et al., 2010). In brief, *Salmonella* spp. is enteropathogenic pathogen which mainly transmitted via faecaloral route infecting animals, birds and humans.

It has been well-known that *Salmonella* spp. can contaminate fresh produce at any point from farm to fork. The sources of contamination include improperly composted manure, soil, irrigation or wash water, food contact surfaces and food handlers. The biofilm forming abilities provide an alternative source for *Salmonella* to contaminate fresh produce (Patel and Sharma, 2010). Cross-contamination of fresh fruits by *Salmonella* Typhi and *Salmonella* Typhimurium is likely to occur in domestic kitchens. The bacterial cells persist due to their ability to attach to food contact surfaces. Once attached, they may develop into biofilm which is defined as microbial sessile communities that are attached to a substance, to an interface or to each other (Borucki et al., 2003; Vestby et al., 2009).

It is estimated that 99.9% of the bacteria in nature are attached to a surface in the form of biofilm (Murphy et al., 2002). The biofilm formation on various food contact surfaces such as stainless steel, metal, glass, polyvinyl chloride, polyurethane or rubber surfaces by different foodborne pathogens such as *Bacillus*, *Salmonella*, *Listeria*, *Staphylococcus* and *Escherichia* species have been reported. Out of all these studies, the adhesion of *Salmonella* to food contact surface was the first published report on foodborne bacterial biofilm (Shi and Zhu, 2009). This is due to their role in disease transmission and cross-contamination of food which increase the food safety risk (Silagyi et al., 2009). The studies that had been conducted by

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researchers include *Salmonella* spp. and *Listeria monocytogenes* on plastic surfaces (Stepanovic et al., 2004; Oliveira et al., 2006), *Salmonella* on rubber, glass and cement (Annous et al., 2005), *Escherichia coli* O157:H7 on stainless steel (Ryu et al., 2005), *Salmonella* Enteritidis on stainless steel and polyethylene (Manijeh et al., 2008) as well as *Escherichia coli* and *Salmonella* on stainless steel, high-density polyethylene and granite (Jun et al., 2010).

Biofilm formation develops in four distinct stages: the formation of a conditioning film, adhesion of bacterial cell, extracellular polymer production by the adhering cells and maturation of the biofilm (Gough and Doss, 1998). The bacterial cell adhesion stage is believed to be reversible due to weak interactions between bacteria cell and solid surface. At this stage, bacteria can easily be removed by gentle rinsing and washing. Subsequently, adhesion of bacterial cell turns irreversible due to intracellular adhesion by appendages and production of extracellular polymeric substance (EPS). EPS can be made up of polysaccharides, proteins and DNA depending on the type of bacteria in the biofilm. The removal of bacteria at this stage requires stronger forces such as scrubbing. Thus a comprehensive knowledge on the initial stage of microbial adhesion is important to control the biofilm formation and risk of microbial cross contamination (Hall-Stoodley et al., 2008; Ortega et al., 2010).

Due to the fact that food processors have a zero tolerance level for *Salmonella* spp., a single adherent bacteria and developed biofilm on kitchen surfaces such as cutting boards may compromise food safety and quality. These attached cells are hard to be removed by normal cleaning due to higher tolerance to antibiotics (Hood and Zottola, 1995; Oliveira et al., 2006). This is of concern since approximately 80% of persistent bacterial infections in the United States are associated with biofilm (Janssens et al., 2008). Studies have found that the cross-contamination via food contact surfaces contribute significantly to cross-infection. When microorganisms within a biofilm dislodge from food contact surface, they might attach to the surface of food products. Consequently, there is a constant risk of microbial transfer from these food contact surfaces which might result in foodborne illness and pose a significant health hazard (DeVere and Purchase, 2007).

In our previous study on the prevalence of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium in seven different types of sliced fruits, high incidence of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium were detected in dragon fruit at both hawker stalls and hypermarkets in Malaysia (Pui et al., 2010). Therefore, we used dragon fruit as the fruit model in this study aiming to determine the ability of biofilm formation by *Salmonella* Typhi and *Salmonella* Typhimurium on plastic cutting board used in domestic kitchen and evaluate the transfer of these two pathogens from plastic cutting board to dragon fruit. To our best knowledge, there is no published study conducted to examine the transfer of *Salmonella* Typhi and *Salmonella* Typhimurium biofilm to dragon fruit. The present data is therefore useful in predictive microbiology and quantitative microbial risk assessment to assess the risk management decision regarding foodborne microbial hazards.

Materials and Methods

Bacterial strains and culture conditions

The pure culture of *Salmonella* Typhi and *Salmonella* Typhimurium were obtained from Institute for Medical Research, Malaysia and maintained at -20°C in Tryptic Soy Broth (TSB; Merck, Darmstadt, Germany) containing 20% (v/v) glycerol (Merck, Darmstadt, Germany). They were streaked onto CHROMagar *Salmonella* (CHROMagar Microbiology, Paris, France) at 37°C overnight. A single mauve colony was inoculated into TSB and incubated with shaking at 37°C for 24 h. The overnight cultures were centrifuged and the bacterial pellets were resuspended in 0.85% saline solution (NaCl; Merck, Darmstadt, Germany). The absorbance of the bacteria suspensions at 600 nm were adjusted to a reading of 0.405 for *Salmonella* Typhi and 0.517 for *Salmonella* Typhimurium, which corresponded to about 2.63 x 10^9 CFU/mL and 1.4 x 10^9 CFU/mL, respectively.

Test surfaces

The test surfaces were plastic (polyethylene) cutting boards commonly used in domestic kitchens. The plastic cutting boards were cut into uniform size of 3.5 cm x 4.5 cm for the adhesion experiments. New plastic cutting surfaces were sonicated using commercial detergent (Clorox® Bleach; Oakland, California) in tap water for 30 min. After that, they were thoroughly rinsed by immersion in boiling water to remove any remaining detergent prior to use.

Biofilm formation on plastic cutting board

Biofilm formation assay using crystal violet was adapted from Harvey et al. (2007) and Silagyi et al. (2009) with some modifications. In short, 1 mL of the standardized bacterial suspensions of *Salmonella* Typhi and *Salmonella* Typhimurium were spot-

inoculated evenly onto plastic cutting boards of 3.5 cm x 4.5 cm at the concentration of 10^9 CFU/mL. The negative controls contained sterile saline solution only. The experiment was conducted twice using triplicate samples each time. The plastic cutting boards were incubated in laminar air flow at 28°C for 0, 1, 3, 6, 9, 12, 16, 20 and 24 h for attachment of *Salmonella* Typhi and *Salmonella* Typhimurium on the surfaces.

After post-inoculation, the plastic cutting boards were rinsed three times in 1 mL deionized water to remove loosely attached bacteria. They were then air dried and adherent bacteria were stained with 1 mL of 0.1% (w/v) crystal violet (CV; Merck, Darmstadt, Germany) at 28°C for 20 min. The staining solutions were removed and plastic cutting boards were rinsed three times in 1 mL deionized water. After drying, the crystal violet bound to the biofilm was solubilized with 1 mL of 95% (v/v) ethanol for 20 min. The concentration of crystal violet was determined by measuring the optical density of destaining solution at 570 nm (CV-OD_{570} value). To correct the background staining, the mean CV-OD_{570} value obtained for the controls was subtracted from the mean CV-OD_{570} value obtained for *Salmonella* Typhi and *Salmonella* Typhimurium on plastic cutting board.

Transfer of Salmonella Typhi *and Salmonella* Typhimurium *biofilm from plastic cutting board to dragon fruit*

Transfer of *Salmonella* Typhi and *Salmonella* Typhimurium biofilm from plastic cutting board to dragon fruit was modified from the method described by Peneau et al. (2007) and Silagyi et al. (2009). Intact dragon fruits were bought from hypermarket and cut into uniform size 3.5 cm x 4.5 cm of 10 ± 0.5 g.

For enumeration of the transferred pathogens, the sliced dragon fruits were placed on the surfaces of 12 h *Salmonella* Typhi and *Salmonella* Typhimurium attached plastic cutting board at 28°C. After air-dried in laminar air flow for 5 min, the sliced dragon fruits were removed and the plastic cutting boards were rinsed three times in 1 mL deionized water. Then, the plastic cutting boards were thoroughly swabbed with sterile cotton swabs moistened with 0.1% buffered peptone water (BPW; Merck, Darmstadt, Germany) in three different directions: left to right, top to bottom, and diagonal. The swabs were then soaked in tubes containing 5 mL of 0.1% BPW and the tips were broken into the tube. The tubes and swabs were vortexed vigorously to suspend all removed bacteria into BPW. On the other hand, the removed sliced dragon fruits were pummeled in a stomacher for 60 s with 90 mL of BPW.

The spread plate method was used for the enumeration of viable *Salmonella* Typhi and *Salmonella* Typhimurium. Aseptically, 1 mL of broth was mixed with 9 mL of 0.1 % BPW. Each dilution was plated in triplicates onto CHROMagar *Salmonella* and the plates were incubated at 37°C for 24 h. Finally the mauve colonies on replicate plates were counted and expressed as mean CFU/cm^2. The numbers of CFU/cm^2 were transformed to log for better representation in figures. The appropriate transfer rate was calculated by dividing log *Salmonella* cell count for dragon fruit with log *Salmonella* cell count on plastic cutting board.

Statistical analysis

Normally distributed data for biofilm formation by *Salmonella* Typhi and *Salmonenella* Typhimurium over time was analyzed for differences between groups using two-way analysis of variance (ANOVA) from SPSS software (version 17.0). The level of significance was set at $P<0.05$.

Results and Discussions

The formation of biofilm by *Salmonella* Typhi and *Salmonella* Typhimurium over 24 h was shown in Figures 1 and 2, respectively. Results indicated that biofilm formation by *Salmonella* Typhi and *Salmonella* Typhimurium was significantly different ($F=66.441$, $df=1$, $p<0.05$). Apart from that, the time for the formation of biofilm differed significantly ($F=194.945$, $df=7$, $p<0.05$) when *Salmonella* Typhi and *Salmonella* Typhimurium were inoculated over time. It was observed that there was an increase in the number of attached cells on the plastic cutting board over time, but the increase reached a maximum at time 12 h.

At time 12 h, both *Salmonella* Typhi and *Salmonella* Typhimurium achieved highest saturation coverage with average reading of OD_{570} at (1.082±0.047) and (0.962±0.026), respectively. After 12 h, microbial cells started to detach from the surface of plastic cutting board as they used up carbon source from the polysaccharide film. Within 24 h, the microbial cells decreased to an average reading of OD_{570} at (0.561±0.012) and (0.546±0.003), respectively for *Salmonella* Typhi and *Salmonella* Typhimurium. The main concern here was the formation of homologous biofilm at the same time. When the combined effect of time and bacterial strain was analyzed statistically, it was shown that they affected the formation of biofilm significantly ($F=19.281$, $df=7$, $p<0.05$).

Since the greatest biofilm formation by both *Salmonella* strains were observed at time 12 h, there was an interest to observe the transfer of the biofilm

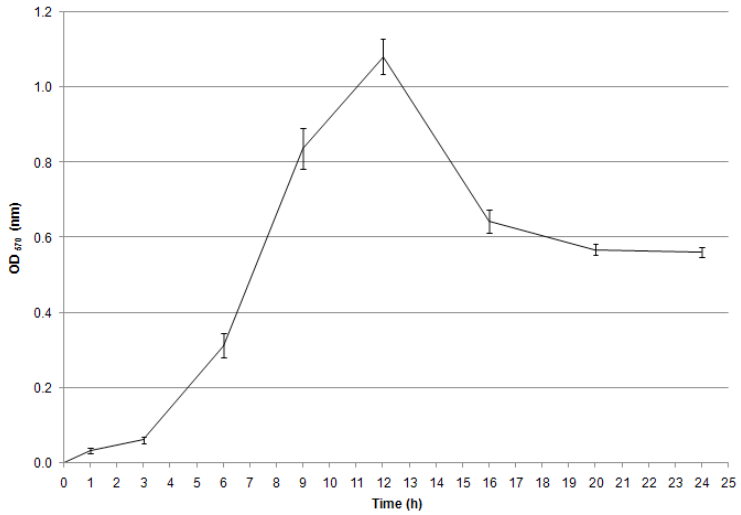

Figure 1. Mean value of biofilm formation by *Salmonella* Typhi on plastic cutting board represented by OD_{570}. Each error bar represents the standard error of mean of triplicate measurements.

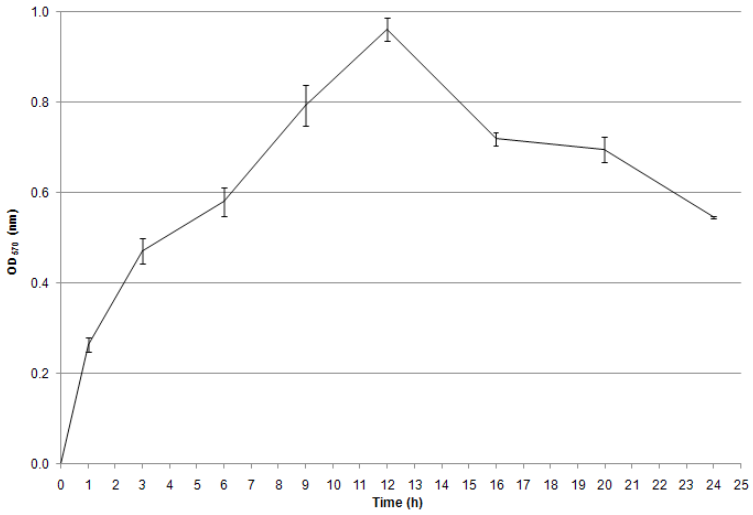

Figure 2. Mean value of biofilm formation by *Salmonella* Typhimurium on plastic cutting board represented by OD_{570}. Each error bar represents the standard error of mean of triplicate measurements.

Table 1. The number of *Salmonella* Typhi and *Salmonella* Typhimurium detected on plastic cutting board and dragon fruit was shown in log CFU/cm^2.

Salmonella	Log CFU/cm^2					
	Plastic cutting board		Dragon fruit		Transfer rate	
	Range	Mean	Range	Mean	Range	Mean
Typhi	5.19-5.28	5.25	4.09-4.18	4.13	0.78-0.81	0.79
Typhimurium	3.65-3.81	3.73	2.48-2.85	2.67	0.68-0.77	0.72

by *Salmonella* Typhi and *Salmonella* Typhimurium to fresh produce at this respective time. Table 1 showed the number of *Salmonella* Typhi and *Salmonella* Typhimurium detected on plastic cutting board and dragon fruit as well as the potential to transfer to dragon fruit. A 5 log CFU/cm^2 of *Salmonella* Typhi was observed on the plastic cutting board whereas 4 log CFU/cm^2 in dragon fruit. The transfer rate was shown to be ranged from 0.78-0.81 with mean transfer rate of 0.79. On the other hand, 3 log CFU/cm^2 of *Salmonella* Typhimurium was observed on the plastic cutting board whereas 2 log CFU/cm^2 in dragon fruit. The transfer rate was shown to be ranged from 0.68-0.77 with mean transfer rate of 0.72. These highlighted that both *Salmonella* Typhi and *Salmonella* Typhimurium biofilm were possible to cross-contaminate dragon fruit.

In this study, plastic cutting board was used as the food contact surface to determine the ability of *Salmonella* Typhi and *Salmonella* Typhimurium to form biofilm and to determine the possibility of the biofilm to contaminate dragon fruit. The popularity of plastic cutting board in domestic kitchen is inevitable even though there is a significant risk of cross-contamination (DeVere and Purchase, 2007). The introduction of plastic cutting board in the 1970s has replaced the use of the traditional cutting board due to the concern that wooden cutting board is more prone to cross-contamination, especially from juices of raw meat and poultry remaining on the surface resulting in the transfer of microorganisms to other foods subsequently prepared on the same surface (Gough and Dodd, 1998).

Adhesion is the first phase of biofilm formation in which the cells attach to a surface within minutes to hours after being exposed to them (Chia et al., 2009). At first, inoculated cells of *Salmonella* Typhi and *Salmonella* Typhimurium grew and migrated to look for secure sites available for attachment on the plastic cutting board. Once introduced to the surface of plastic cutting board, the cells required some time to adjust to the new environmental condition before they diffused to the pre-conditioned substratum. The successfully adsorbed cells surrounded themselves with polysaccharides to bind to the plastic cutting board. They remained on the substratum until completely occupied the sites. The exopolysaccharide kept the cells attached to the surface. Beyond a finite time, in this case, 12 h for *Salmonella* Typhi and *Salmonella* Typhimurium on plastic cutting board, the polysaccharide material disintegrated, making the entire polysaccharide film and its underlying cells dislodged through reversible adsorption (Takhistov and George, 2004). The development of the true biofilm is not necessarily uniform in time for different bacteria and may take days or weeks (Jessen and Lammert, 2003). This was confirmed by the results obtained in this study.

From the data, we suggested that *Salmonella* Typhi needed more time for adaptation than *Salmonella* Typhimurium before adhesion on the plastic cutting board took place. Although similar degree of hydrophobicity might be displayed, *Salmonella* adhesion is strongly strain-dependent. Most bacteria such as *Salmonella* attach to hydrophobic surfaces such as Buna-N rubber and other plastics with little or no surface charge while some bacteria attach to hydrophobic surfaces with positive or neutral charge and very few of them attach to hydrophilic negatively charged surfaces such as stainless steel and glass (Manijeh et al., 2008; Shi and Zhu, 2009). Consequently this constitutes a factor of virulence among the different serotypes (Oliveira et al., 2006). The factors governing the bacteria adhesion to surfaces are still not well understood. In several studies, the adhesion of bacteria partly depends on the nature of the inert surfaces and properties of the bacterial surface. Nevertheless, in some situation it is not easy to establish a relationship between surface properties and the extent of bacterial adhesion (Silva et al., 2008).

The transfer rate of *Salmonella* Typhi and *Salmonella* Typhimurium biofilm to dragon fruit was high because the contaminated plastic cutting board not only transferred the cells but also the slimy biofilm which helped the cells to adhere to the food surface. Many studies reported the attachment of bacteria on the surface of food, for example, attachment of *Salmonella enterica* serotypes to cabbage and lettuce leaves by Patel and Sharma (2010). The transfer efficiency of bacteria from plastic cutting board to dragon fruit was also dependent on the availability of water and nutrient. Fresh produce which contain high water activity and nutrient such as dragon fruit caused *Salmonella* Typhi and *Salmonella* Typhimurium to multiply faster on the cut surface of dragon fruit. Further handling of fresh produce by food handler afforded the opportunity for contamination from food contact surfaces that were previously attached by sessile microorganism. This contributed to cross-contamination by biofilm-forming bacteria (Silagyi et al., 2009).

Detection of biofilm formation can be conducted in various ways, but crystal violet assay which was considered to be the most convenient technique to evaluate bacterial adhesion was employed in this study. It involved the washing, staining and destaining of sessile cells with crystal violet where

the optical density of the stained bacterial biofilm was then determined spectrophotometrically (Chavant et al., 2007; Oh et al., 2007). Chae and Schraft (2001) stated that it is difficult to detect active microorganism within biofilm because there is no single analytical method for the detection of all physiological types of bacteria. This biofilm assay using crystal violet has been used to study biofilm formation by a variety of Gram-positive and Gram-negative bacteria where it yields reproducible result which allows one to study large numbers of strains and conditions at the same time. Furthermore, the method yields quantitative results by measuring the optical density of wells (Murphy et al., 2002).

Swabbing was used for the enumeration of *Salmonella* Typhi and *Salmonella* Typhimurium on plastic cutting board. It is most widely used in hospitals, restaurants, food and dairy industries due to being simple, rapid and inexpensive for the microbiological examination of food contact surfaces. The main concern when examining food contact surfaces for microorganisms is the removal of resident biota as there is no superior method that can recover 100 % of the microorganisms. However, as long as it is realized that not all microorganisms are being recovered, the consistent use on food contact surface can still provide valuable information (Jay et al., 2005).

As a whole, biofilm is attracting interest in many areas in recent years including food safety. Biofilm formation by foodborne pathogens such as *Salmonella* Typhi and *Salmonella* Typhimurium can compromise the sanitation of food contact surface and cause cross-contamination to fresh produce. It would be a great challenge to control biofilm since they can form easily where water is plentiful and cleaning is not performed properly. A regular sanitation programme which includes the removal of gross debris, rinsing, pre-soaking in detergent and final rinsing are effective to eliminate bacteria entrapped in a biofilm (Jessen and Lammert, 2003). Besides, an upgraded HACCP system with biofilm assessment will assist the development of biofilm-free processing systems in the food industry. Lastly, a better understanding regarding the mechanism in biofilm formation will facilitate the development of new strategies to control biofilm formation, thus reducing foodborne outbreaks and economic losses (Shi and Zhu, 2009).

Acknowledgements

This study was supported by Science Fund (project no. 545260) from Ministry of Science, Technology and Innovation, Malaysia.

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