International Food Research Journal 19(1): 71-76 (2012)

Biofilm forming ability and time course study of growth of *Salmonella* Typhi on fresh produce surfaces

[1]Tang, P. L., [1*]Pui, C. F., [1]Wong, W. C., [1,2]Noorlis, A. and [1]Son, R.

[1]Center of Excellence for Food Safety Research, 1Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia
[2]Universiti Teknologi MARA Pahang, 26400 Bandar Tun Abdul Razak Jengka, Pahang Darul Makmur, Malaysia

Abstract: This study aimed to determine the biofilm formation ability by *Salmonella* Typhi on cucumber, mango and guava surface, as well as to determine the relationship between time contact and biofilm formation. Crystal violet assay was performed to quantify the biofilm formation based on the value of optical density at 570 nm of the destaining crystal violet at the specific interval time. The result showed that the attachment of the bacterial cells on the fresh produce surface increased with the contact time. The readings of OD_{570} at time 12 h for cucumber, mango and guava surfaces were 0.824, 0.683 and 0.598, respectively, indicating that the biofilm formation by *Salmonella* Typhi on different fresh produce surface varied with time. Since the result showed that *Salmonella* Typhi formed biofilm on fresh produce surfaces, hygienic practice from farm to fork including handling, processing, distribution and storage of the fresh produce should be of concern.

Keywords: Biofilm, *Salmonella* Typhi, cucumber, mango, guava

Introduction

The consumption trend of fresh produce increases greatly nowadays. The main reason for this phenomenon is the awareness of consumers of their body health. Unfortunately, the increased consumption of minimally processed fruits and vegetables had led to an increase in the number of salmonellosis outbreaks associated with these fresh produce (Sewell and Farber, 2001; Pui *et al*., 2011a). Fresh produce including alfalfa sprout, lettuce, fennel, cilantro, cantaloupe, unpasteurized orange juice, tomatoes, melon, mango, celery and parsley have been associated with salmonellosis outbreak (Lapidot *et al*., 2006). This means that consumers are at risk because bacteria can grow and form biofilm on fresh produce surfaces.

Salmonella contamination can occur at any point during production, harvest, processing, and transport (Lapidot *et al.*, 2006). Soil, raw or improperly composted manure, irrigation and wash water can cause contamination at plantation stage (Kroupitski *et al*., 2009). *Salmonella* are present naturally in soil and manure where they can subsequently go into the irrigation system and wash water, and finally contaminate the fresh produce. Furthermore, feces of infected humans or animals can enter the water system through different sources such as sewage overflows, sewage systems that are not working properly, polluted storm water runoff and contaminated agricultural runoff (Centers for Disease Control and Prevention, 2009). To remove those contaminants such as insects, pesticide residues and visible soils, fruits and vegetables are usually submerged in a rinse tank during harvest (Sivapalasingam *et al*., 2003; Duffy *et al*., 2005). Pathogens which are present in the rinse water previously have the opportunity to adhere to the plant tissue (Lapidot *et al*., 2006), and if the contact time is long enough, biofilm can be formed. Furthermore, handling of fresh produce by food handler creates another pathway to contaminate the fresh produce (Pui *et al*., 2011b). Food handler must take care of their personal hygiene especially for those who contact the fresh produce directly. Besides, containers which are used during harvest, transport and display are often not effectively cleaned and sanitized, and this can lead to the formation of biofilm (Beuchat, 2002).

Biofilm is formed when bacterial cells attach to one another and/or adhere to a living or inert contact surface. The attached bacterial cells are enclosed in a self-produced polymeric matrix. Biofilm are very dangerous biological structure because they can become a persistence source of contamination (Costerton *et al*., 1999; Houdt and Michiels, 2010). The organisms can increase their ability to colonize and survive in a harsh condition if they are able to form this biofilm (Monier and Lindow, 2003).

Once biofilm forms on the fresh produce surface, they not only can cause cross contamination to the equipment surfaces in industry, they also result in a potent health hazard to consumer. The objectives of this study were therefore to determine the biofilm formation ability by *Salmonella* Typhi on cucumber,

*Corresponding author.
Email: *chaifung_85@yahoo.com*

mango and guava surface and to determine the relationship between contact time and biofilm formation by *Salmonella* Typhi.

Materials and Methods

Bacterial strains and culture conditions

The pure culture of *Salmonella* Typhi was obtained from Institute for Medical Research, Malaysia. A 1 ml of the pure culture was inoculated into 9 ml of TSB and incubated at 37°C in an incubator shaker for 24 h. The overnight cultures were centrifuged at 10 000 rpm for 3 min, and the bacterial pellets were resuspended in 0.85% saline solution (NaCl, Merck, Darmstadt, Germany). The absorbance of the bacteria suspension at 600 nm was adjusted to a reading of 0.461 corresponding to 9 log CFU/mL of cells.

Test surfaces

The test surfaces were cucumber (*Cucumis sativus*), mango (*Mangifera Indica*) and guava (*Psidium guajava* L.) surface purchased from hypermarket. They were thoroughly rinsed under running water to carry out a general cleaning which resembles practiced by consumers in domestic kitchen. After cleaning, they were cut into uniform size of 5 cm x 2 cm for bacterial cells attachment. The cut surfaces were then placed onto petri dish prior to use. All the apparatus which were used during this cutting process were sterilized using UV light and the study was carried out in a laminar air flow.

Biofilm formation on test surfaces

For inoculation, 1 ml of the standardized *Salmonella* Typhi suspension was inoculated on the 5 cm x 2 cm fresh produce surfaces. For the negative controls, 1 ml of sterile saline solution was used to substitute the 1 ml of *Salmonella* Typhi suspension. The surfaces were then incubated in laminar air flow at 28°C for 0, 3, 6, 9 and 12 hours for adhesion of *Salmonella* Typhi on the surfaces.

Biofilm quantification

Crystal violet assay was used to quantify the biofilm formation. This assay was adapted from Peeters *et al*. (2008), Silagyi *et al*. (2009) and Pui *et al*. (2011b) with some modifications. After a particular incubation time, the fresh produce surfaces were rinsed three time using 1 ml deionized water. This step was used to rinse off loosely attached bacterial cells. Then, they were air dried and the attached bacterial cells were stained with 1 ml of 0.1% (w/v) crystal violet (CV, Merck, Darmstadt, Germany) for 20 min. The crystal violet solutions were removed from the produce surfaces and rinsed with 1 ml deionized water thrice and air dried. After drying, the attached crystal violet was solubilized with 1 ml of 95% (v/v) ethanol for 20 min. The crystal violet concentration was then determined by measuring the optical density of the destaining ethanol solution at 570 nm.

Results

In this study, cucumber, mango and guava surfaces were used to test the biofilm formation ability of *Salmonella* Typhi. Previous study by Pui *et al*. (2011b) showed that the number of attached *Salmonella* Typhi on plastic cutting board surface reached a maximum production of biofilm at time 12 h. Since the biofilm formation by *Salmonella* Typhi was observed at time 12 h, the longest incubation time used in this study was 12 h. For biofilm formation by *Salmonella* Typhi on cucumber surface, the OD value increased during this 12 h period, as shown in Figure 1. From 0 to 3 h, the OD value increased significantly from 0 to 0.640. After that, the OD value continued to increase from 0.640 to 0.824 at time 3 to 12 h. For biofilm formation by *Salmonella* Typhi on mango surface, the OD value increased from 0 to 0.310 during the first 3 h of incubation, followed by 0.310 to 0.428 from time 3 to 9 h and finally 0.428 to 0.683 from time 9 to 12 h. Overall the OD value increased with contact time as shown in Figure 2. According to Figure 3, the biofilm formation by *Salmonella* Typhi on guava surface, the OD value increased from 0 to 0.219 during the first 3 h of incubation. There was a slightly decrease of the OD value at time 6 h where the value was found to be 0.168, followed by an increased to 0.177 at time 9 h and to 0.598 from time 9 to 12 h. As shown in Figure 4, there was an obvious and a significant difference among three sets of OD values. For the cucumber surface, the OD values were larger than the OD values for mango and guava test surfaces, with the guava test surface giving the smallest OD values.

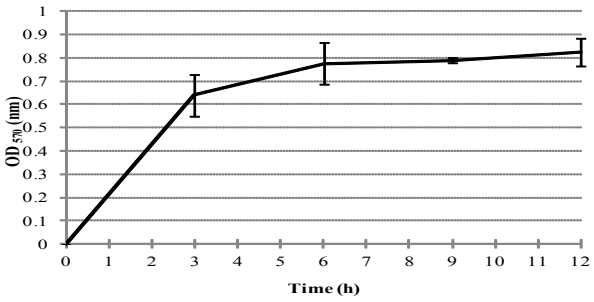

Figure 1. Mean value of biofilm formation by *Salmonella* Typhi on cucumber surface represented by OD_{570}. Each error bar represents the standard error of mean of triplicate measurements.

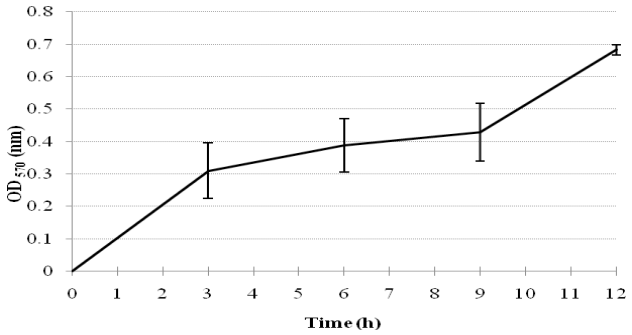

Figure 2. Mean value of biofilm formation by *Salmonella* Typhi on mango surface represented by OD_{570}. Each error bar represents the standard error of mean of triplicate measurements.

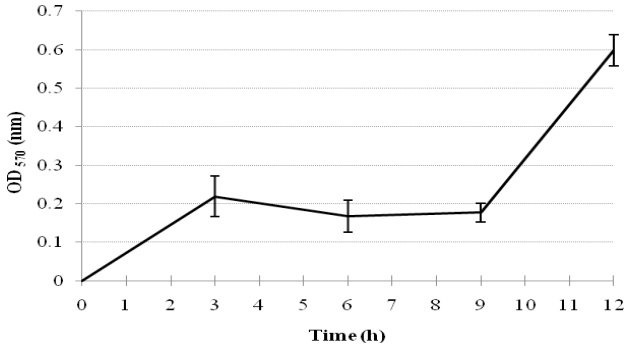

Figure 3. Mean value of biofilm formation by *Salmonella* Typhi on guava surface represented by OD_{570}. Each error bar represents the standard error of mean of triplicate measurements.

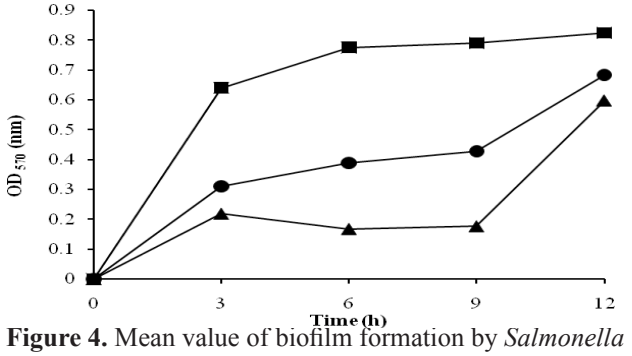

Figure 4. Mean value of biofilm formation by *Salmonella* Typhi on cucumber (■), mango (●) and guava (▲) surfaces represented by OD_{570}.

Discussion

For all the tested fresh produce surfaces, the OD value which represented the quantity of biofilm formation was 0 at time 0 h. This indicated that there was no biofilm formation by *Salmonella* Typhi on the fresh produce surfaces. The reason behind was that the cells need time to adapt to the new environment conditions when the bacterial cells are newly transferred onto the fresh produce surfaces. Besides, the inoculated bacterial cells needed time to migrate on the produce surface to seek for suitable secure sites for adhesion (Pui *et al*., 2011b). Once they attached to the fresh produce surface, they surrounded themselves with polysaccharides. These exopolysaccharides enabled the bacterial cells to attach to the fresh produce surface and also among them (Costerton *et al*., 1999; Pui *et al*., 2011b). Apart from that, the attachment of bacterial cells to fresh produce surface or among them is a physicochemical process which is determined by Van der Waals, Lewis acid–base and electrostatic interactions (Houdt and Michiels, 2010; Strevett and Chen, 2003).

From the result, the OD value increased with the increased incubation time. Incubation time here can be known as contact time which is the time for the bacterial cells to contact with the fresh produce surface. The attachment strength of *Salmonella* Typhi is directly related to the contact time of the bacterial cells to the fresh produce surface. When the contact time increases, more bacterial cells have enough time to attach to the surface and form biofilm. When more attached bacterial cells are found on the fresh produce surface, this indicates that more interaction forces can be formed between the biofilm and the fresh produce surface. Thus, it is proven that the attachment strength of *Salmonella* Typhi on fresh produce surface increases with the increase of contact time (Ukuku and Fett, 2002). As the contact time increases, the biofilm is not easy to be rinsed off during the rinsing process even in variable hydrodynamic shear. In an earlier study being conducted by Pui *et al*. (2011b), the data showed that the number of attached cells on the plastic cutting board increased over incubation time, and at time 12 h, the number of attached cells was the highest.

The quantity of biofilm formation can be represented by the OD values. These OD values can be affected by variable hydrodynamic shear that normally occurs with the changes in the flow rate. For biofilm which is grown under low shear or static condition, the attached bacteria cells dislodge more easily when the flow rate changes within a system (Stoodley *et al*., 1999; Hall-Stoodley and Stoodley, 2005). In this study, the inoculated bacteria were under static condition, thus they can be rinsed off more easily during the rinsing process. This was the reason why the OD value decreased slightly at time 6 h for guava surface. However, there is a drawback of the crystal violet assay used in this study to quantify the biofilm formation, as the crystal violet stains not only cells, but also any attached material on the surface of the fresh produce. As a result, crystal violet staining may overestimate the number of adherent bacteria of the biofilm (Merritt *et al*., 2005).

The properties of the attachment surface are important factors to determine the biofilm formation potential. The properties such as surface roughness, cleanability, disinfectability, wetability (determined

by hydrophobicity) and vulnerability to wear influence the ability of bacterial cells to adhere to a particular surface (Houdt and Michiels, 2010). In this study, surface roughness of the fresh produce, was not directly affecting the adhesion of the bacterial cells on the fresh produce surface, but it affected the retention of the bacterial cells during the rinsing process. The rougher is the surface, the more deep crevices or polish lines present on the surface. The high retention of the bacterial cells during rinsing process may be due to the possible entrapment of microbial cells in crevices of the surface, because these crevices provide refuge to the adherent bacterial cells from shear force (Ortega *et al*., 2010). As a result, the biofilm are not easy to be rinsed off using simple washing step. According to the data obtained, the OD values for the cucumber surface were the highest comparing with the OD values for the mango and guava surfaces. This may indicate that cucumber surface was the roughest attachment surface.

Surface roughness of the attachment surface is an important factor which can affect the removal of bacterial cells. According to Barnes *et al*. (1999), the amount of adherent *S. aureus* on 2B stainless finish (R_a = 0.412 µm) was greater if compared to the No. 8 mirror finish (R_a = 0.035 µm). The surface of 2B stainless finish was rougher than the surface of No. 8 mirror finish. The R_a value represents the surface roughness. The larger is the R_a value, the rougher is the surface. Besides, Ortega *et al*. (2008) showed increased adhesion and decreased removability of *S. epidermidis* for a rough stainless steel surface (R_a = 1.37 µm) compared with smoother surface ($R_a \leq 0.14$ µm). Furthermore, a positive correlation between cleanability and increased surface smoothness in the removal of biofilms was demonstrated by Leclercq-Perlat and Lalande (1994) and Wirtanen *et al*. (1995). Besides, a recent study showed that the roughest surface of stainless steel (R_a = 1.37 µm) had high retention of the adherent *E. coli* cells compared with the smoother surface ($R_a \leq 0.14$ µm) after the whirlpool rinsing (Ortega *et al*., 2010).

Hydrophobicity of bacteria and attachment surface are vital criteria in biofilm formation. Hydrophobicity of attachment surface can be influenced by the surface roughness. The surface hydrophobicity increases with the surface roughness due to Cassie effect. This Cassie effect occurs when the surface tension of a water droplet is supported by the rough bumps beneath it (Naha *et al*., 2007). On the other hand, many previous studies showed that microorganisms attached to hydrophobic nonpolar surface more rapidly than to hydrophilic surface (Fletcher and Loeb, 1979; Pringle and Fletcher, 1983; Bendinger *et al*., 1993; Donlan, 2002). Karunasagar and Otta (1996) reported that the biofilm density of *Vibrio harveyi* was affected by the nature of the attachment surface. It should be noted that the bacterial cell surface hydrophobicity can influence the adhesion process. An earlier study showed that the hydrophobicity of *Salmonella enteritidis* was 73% and this hydrophobic bacterium formed biofilm more readily on hydrophilic surface due to the repellent force between hydrophobic attachment surface and hydrophobic bacteria (Manijeh *et al*., 2008).

There are various methods that can be used to detect and quantify the formation of biofilm. Crystal violet assay is one of the methods which can be considered as the most convenient technique to examine the biofilm formation (Pui *et al*., 2011b). Washing, staining and destaining, and spectrophotometrically determine the optical density of the stained biofilm cells are the main steps in this assay (Chavant *et al*., 2007; Oh *et al*., 2007). This crystal violet assay gives a cheap, simple and straightforward way for the scientist to evaluate the biofilm formation by various types of gram-positive and gram-negative bacteria. Thus, this assay can give reproducible result at which a scientist can study large numbers of strains and conditions at the same time (Pui *et al*., 2011). Furthermore, the repeatability of this assay is high since there is only a minor difference that can be observed (Peeters *et al*., 2008).

For fresh produce, contamination can occur from farm to fork, but in this study, biofilm formation is shown to be a potent factor which contributes to the cross-contamination where it can become a persistent source of cross contamination. In conclusion, hygienic practice during handling, processing, distribution and storage of the fresh produce is an important public health concern.

Acknowledgements

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

References

Barnes, L. M., Lo, M. F., Adams, M. R. and Chamberlain, A. H. 1999. Effect of milk proteins on adhesion of bacteria to stainless steel surfaces. Applied and Environmental Microbiology 65: 4543-4548.

Bendinger, B., Rijnaarts, H.H.M., Altendorf, K. and Zehnder, A.J.B. 1993. Physicochemical cell surface and adhesive properties of coryneform bacteria related to the presence and chain-length of mycolic acids. Applied and Environmental Microbiology 59:

3973–3977.

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

Centers for Disease Control and Prevention. 2009. From *Salmonella* and Drinking Water from Private Wells. Retrieved from *http://www.cdc.gov/healthywater/drinking/private/wells/disease/salmonella.html,* March 24, 2011.

Chavant, P., Gaillard-Martinie, B., Talon, R., Hebraud, M. and Bernardi, T. 2007. A new device for rapid evaluation of biofilm formation potential by bacteria. Journal of Microbiological Methods 68: 605-612.

Costerton, J., Stewart, P. and Greenberg, E. 1999. Bacterial biofilms: a common cause of persistent infections. Science 284: 1318-1322.

Donlan, R.M. 2002. Biofilms: Microbial life on surfaces. Emerging Infectious Diseases 8: 881–890.

Duffy, E., Cisneros-Zevallos, L., Castillo, A., Pillai, S., Ricke, S. and Acuff, G. 2005. Survival of *Salmonella* transformed to express green fluorescent protein on Italian parsley as affected by processing and storage. Journal of Food Protection 68: 687-695.

Fletcher, M., and Loeb, G.I. 1979. Influence of substratum characteristics on the attachment of a marine pseudomonad to solid-surfaces. Applied and Environmental Microbiology 37: 67–72.

Hall-Stoodley, L. and Stoodley, P. 2005. Biofilm formation and dispersal and the transmission of human pathogens. Trends in Microbiology 13: 7-10.

Houdt, R. V. and Michiels, C. 2010. Review Article: Biofilm formation and the food industry, a focus on the. Journal of Applied Microbiology 109: 1117-1131.

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

Kroupitski, Y., Pinto, R., Brandl, M., Belausov, E. and Sela, S. (2009) Interactions of *Salmonella enterica* with lettuce leaves. Journal of Applied Microbiology 106: 1876-1885.

Lapidot, A., Romling, U. and Yaron, S. 2006. Biofilm formation and the survival of *Salmonella* Typhimurium on parsley. International Journal of Food Microbiology 109: 229-233.

Leclercq-Perlat, M. N. and Lalande, M. 1994. Cleanability in relation to surface chemical composition and surface finishing of some materials commonly used in food industries. Journal of Food Engineering 23: 501-517.

Manijeh, M., Mohammad, J. and Roha, K. K. 2008. Biofilm formation by *Salmonella enteritidis* on food contact surfaces. Journal of Biological Sciences 8: 502-505.

Merritt, J. H., Kadouri, D. E. and Toole, G. A. 2005. Growing and Analyzing Static Biofilms. Current Protocols in Microbiology 1B.1,1- 1B. 1, 17.

Monier, J. M. and Lindow, S. E. 2003. Differential survival of solitary and aggregated baterial cells promates aggregate formation on leaf surfaces. Proceedings of the National Academy of Sciences, USA 100: 15977-15982.

Naha, S., Sen, S. and Puri, I.K. 2007 Flame synthesis of super-hydrophobic amorphous carbon surfaces. Carbon 45: 1702–1706.

Oh, S., Chen, P. and Kang, D. 2007. Biofilm formation by *Enterobacter sakazakii* grown in artificial broth and infant milk formula on plastic surface. Journal of Rapid Methods and Automation in Microbiology 15: 311-319.

Ortega, M. P., Hagiwara, T., Watanabe, H. and Sakiyama, T. 2008. Factors affecting adhesion of *Staphylococcus epidermidis* to stainless steel surface. Japan Journal of Food Engineering 9: 251-259.

Ortega, M. P., Hagiwara, T., Watanabe, H. and Sakiyama, T. 2010. Adhesion behavior and removability of *Escherichia coli* on stainless steel surface. Food Control 21: 573-578.

Peeters, E., Nelis, H. J. and Coenye, T. 2008. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. Journal of Microbiological Methods 72: 157-165.

Pringle, J.H. and Fletcher, M. 1983. Influence of substratum wettability on attachment of fresh-water bacteria to solid-surfaces. Applied and Environmental Microbiology 45: 811– 817.

Pui, C. F., Wong, W. C., Chai, L. C., Lee, H. Y., Nillian, E., Ghazali F. M., Cheah, Y. K., Nakaguchi, Y., Nishibuchi, M. and Radu, S. 2011a. Simultaneous detection of *Salmonella* spp*., Salmonella* Typhi and S*almonella* Typhimurium in sliced fruits using multiplex PCR. Food Control 22: 337-342.

Pui, C. F., Wong, W. C., Chai, L. C., Lee, H. Y., Tang, J. Y. H., Noorlis, A., Farinazleen, M. G., Cheah, Y. K. and Son, R. 2011b. Biofilm formation by *Salmonella* Typhi and *Salmonella* Typhimurium on plastic cutting board and its transfer to dragon fruit. International Food Research Journal 18: 31-38.

Sewell, A. M. and Farber, J. M. 2001. Foodborne outbreaks in Canada linked to produce. Journal of Food Protection 64: 1863-1877.

Silagyi, K., Kim, S.-H., Lo, Y. M. and Wei, C. I. 2009. Production of biofilm and quorum sensing by *Escherichia coli* O157:H7 and its transfer from contact surfaces to meat, poultry, ready-to-eat deli, and produce products. Food Microbiology 26: 514-519.

Sivapalasingam, S. B., Van Duyne, S., De Witt, W., Ying, M., Frisch, A., Phan, Q., Gould, E., Shillam, P., Reddy, V., Cooper, T., Hoekstra, M., Higgins, C., Sanders, J. P., Tauxe, R. V. and Slutsker, L. 2003. A multistate outbreak of *Salmonella enterica* Serotype Newport infection linked to mango consumption: impact of water-dip disinfestation technology. Clinical and Infectious Diseases 37: 1585-1590.

Stoodley, P., Dodds, I., Boyle J. D. and Lappin-Scott, H. 1999. Influence of hydrodynamics and nutrients on biofilm structure. Journal of Applied Microbiology 85: 19S-28S.

Strevett, K. A. and Chen, G. 2003. Microbial surface thermodynamics and applications. Research in Microbiology 154: 329-335.

Ukuku, D. O. and Fett, W. F. 2002. Relationship of cell

surface charge and hydrophobicity to strength of attachment of bacteria to cantaloupe rind. Journal of Food Protection 65: 1093-1099.

Wirtanen, G., Ahola, H. and Mattila-Sandholm, T. 1995. Evaluation of cleaning procedures in elimination of biofilm from stainless steel surfaces in open process equipment. Transactions of the Institution of Chemical Engineering 73C: 1-9.