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Research article/Araştırma makalesi

Influence of surface kind on biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* from food-contact surfaces

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Abstract

Salmonella spp. and *Listeria monocytogenes* are important pathogenic bacteria, which are transmitted by food. It is known that both microorganisms may produce biofilm on biotic or abiotic surfaces. Bacteria in biofilms exhibit enhanced resistance to cleaning and sanitation. In this study, we investigated the biofilm producing ability of 8 *Salmonella* spp. and 6 *L. monocytogenes* isolates by microtiter plate and tube adherence method. All tested *Salmonella* spp. and *L. monocytogenes* strains produced biofilm but strains of *L. monocytogenes* exhibited a higher ability of biofilm formation. Concominantly with these two methods, adhesion and biofilm formation of selected strains to six different industrial surface was also assessed by scanning electron microscope (SEM). In addition, biofilm formation and development of selected two strains were also evaluated on granite surfaces and at five incubation periods (2th, 4th, 6th, 24th and 48th hours). Mature biofilm formation was determined after 24 and 48 hours. Granite, marble, wood and glass surfaces presented higher intensity of biofilm, compared to the steel and plastic surfaces. Especially granite and marble are the surfaces in which we found to be the most convenient for the biofilm formation.

Key words: Biofilm, *Salmonella, Listeria*, Surface Kind

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Besin temas yüzeylerinden elde edilen *Salmonella* spp. ve *Listeria monocytogenes* izolatlarının biyofilm oluşumu üzerine yüzey çeşidinin etkisi

Özet

Salmonella spp. ve *Listeria monocytogenes* besin yoluyla taşınan önemli patojenik bakterilerdendir. Her iki mikroorganizmanın da canlı ve cansız yüzeyler üzerinde biyofilm oluşturabildikleri bilinmektedir. Biyofilmde bulunan bakteriler temizleme ve sanitasyon işlemlerine artan bir direnç gösterirler. Biz bu çalışmada 8 *Salmonella* spp. ve 6 *Listeria monocytogenes* izolatının biyofilm oluşturma yeteneğini mikrotitre plaka ve tüp aderans metodlarıyla araştırdık. Test edilen tüm *Salmonella* spp. ve *L. monocytogenes* izolatları biyofilm oluşturmuş ancak *L. monocytogenes* daha yüksek derecede biyofilm oluşumu göstermiştir. Bu iki teste ilave olarak seçilen izolatların altı farklı endüstriyel yüzey üzerindeki adezyon ve biyofilm oluşumları da taramalı elektron mikroskopla tayin edilmiştir (SEM). Ayrıca seçilen iki izolatın biyofilm oluşum ve gelişimleri granit yüzeyler üzerinde ve beş farklı inkübasyon periyodunda değerlendirilmiştir (2., 4., 6., 24. ve 48. saatler). Olgun biyofilm oluşumu 24. ve 48. saatler sonrasında tespit edilmiştir. Granit, mermer, tahta ve cam yüzeyler, çelik ve plastik yüzeylere kıyasla daha yoğun biyofilm oluşturmuşlardır. Özellikle granit ve mermer biyofilm oluşumu için daha elverişli yüzeyler olarak bulunmuştur.

Anahtar kelimeler: biyofilm, *Salmonella*, *Listeria*, yüzey çeşidi

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1. Introduction

Biofilms are commonly defined as communities of microorganisms attached to a surface or interface and producing an extracellular matrix in which these microorganisms are embedded (Costerton et al., 1995). It is a dynamic and complex process which requires an extensive variation of gene expression and, consequently, major physiological changes (Davey et al., 2000). Briefly, biofilm formation has two key steps: (i) The reversible attachment of freely moving bacteria to a surface is followed by irreversible binding to the surface, growth of microcolonies and production of a polymer matrix. (ii) The maturation of the biofilm in a three dimentional structure often showing water filled channels and mushroom like structures (Botticella et al., 2013). Once they develop on any kind of surface, it is hard to eradicate them and they have the capability of spreading to other areas to form new colonies (Dag et al., 2014). In the food industry, pathogenic biofilms have been of considerable interest in the context of food safety. Because it allows microorganisms to persist in the environment and resist desiccation, UV light, and treatment with antimicrobial and sanitizing agent (Borucki et al., 2003). *Salmonella* spp. and *L. monocytogenes* are pathogenic bacteria capable of developing biofilms on food processing surfaces, a pathway leading to cross contamination of foods. Exposure of these pathogens to surfaces may take place either by direct contact with contaminated materials or indirectly through airborne microflora (Ciccio et al., 2015).

The major components of biofilm formation are the bacterial cells, the attachment surface and the surrounding medium. The surface properties are important for attachment and biofilm formation. The choice of surface material has significant influence in designing food contact and processing surfaces. Factors such as cleanability, roughness, wettability or disinfectability affect the adherence ability of cells and perform the hygienic property of material (Houdt and Michiels, 2010). The association of microorganisms to surfaces has been mainly analyzed in the laboratory. However, such studies still need to be standardized since they are difficult to carry out *in situ*, in food processing environments. The investigation of different experimental models allows the study of biofilms under defined and controlled conditions and are necessary fort he execution of reproducible experiments (Oliveria et al., 2010).

In order to control the *Salmonella* spp. and *L. monocytogenes* biofilm in the food industry, the greater understanding of the interactions between microorganisms and food processing equipment is required. Regarding these aspects, this study was carried out with the aim of evaluating the ability of *Salmonella* spp. and *L. monocytogenes* strains isolated from variety food samples, to form biofilms on six different surfaces (plastic, steel, marble, wood, granite and glass) at five different time periods (2. 4. 6. 24. and 48. hours) by Scanning Electron Microscope (SEM). The biofilm formations of strains were also suggested with microbiological methods.

2. Materials and methods

2.1 Organisms and growth medium

L. monocytogenes and *Salmonella* spp. isolates obtained from an accredited laboratory for this species (Eskisehir Food Control Laboratory). 490 several food sources samples screened for *Salmonella* spp. and 113 samples also tested for *L. monocytogenes*. Total of 14 strains, isolated from several food sources were used in this study; 8 *Salmonella* spp. and 6 *L. monocytogenes* strains. Obtained isolates were deposited in TSB (tyrpcase soy broth) with 12.5 % glyserol for stock. Organisms were cultivated in TSB at 35 °C for incubation and biofilm studies. *L. monocytogenes* ATCC7644 was used to control microorganisms as highly biofilm producer.

2.2. Biofilm formation

Biofilm producing ability of isolates were tested by microtiter plate and qualitative tube adherence method. In addition, adhesion and biofilm formation of selected strains to different surfaces was also assessed by scanning electron microscope (SEM).

2.2.1. Tube test

The qualitative method for biofilm formation was studied according to tube test described by Christensen et al (Christensen et al., 1982). A loopful of pure culture of strains were inoculated in test tube filled with 10 ml TSB containing 1% glucose. Tubes were incubated for 24 h at 35°C. After overnight incubation tubes were carefully drained and dried. The solution of crystal violet 0,1% was added to these tubes. After the staining, the solution was removed and washed and than placed upside to dry.

Positive results were determined as the presence of stained material adhered to the inner wall of the tube. The adherence was evaluated as no biyofilm (0), weak (+), moderate (++), or strong (+++). The observation of stained ring at the liquid–air interface was not considered to be positive. The tests were carried in triplicate for each isolate.

2.2.2. Microtiter –plate test

The method for assessment of biofilm formation on polystyrene microtiter plates was based on the techniques described by Stepanovic et al (Stepanovic et al., 2000). The wells of a sterile 96-well flat- bottomed polystyrene microplate, were added with 230 µl of the TSB medium. The negative controls contained only medium. Each strain was tested in triplicate. Wells were inoculated with 20 µl of overnight bacterial culture. After incubation for 24 h at 35°C the plate contents were discarded and rinsed three times with 300 µl of sterile distilled water. The content of the each well were fixed with 250 µl of methanol for 15 min , aspirated and air dried.The plates were stained for 5 min with 250 µl per well of Crystal violet (Gram-colour staining set for microscopy; Merck). The plates were washed with tap water to remove the excess of the stain. Later the plates were air dried, the dye bound to the adherent cells was resolubilized with 250 µl of 33% (v/v) glacial acetic acid each well. By using an automated ChroMate 4300 reader, the optical density (O.D.) of each well was measured at 450/630 nm. According to the values of optical density, isolates were categorized as no biofilm producers, weak, moderate or strong biofilm producers, as previously described (Stepanovic et al. 2004). Optical density cut-off (ODc) was determined. It is defined as average OD of negative control + 3× standard deviation (SD) of negative control. The strains were classified as follows: Absorbance/ COV (cut off value) < 1.125 non produced.

1.125 < Abs/ COV <2.00 weak
2.00< Abs/COV < 4.00 modarete
4.00 < Abs/COV < 6.00 high
All tests were carried out in triplicate and the results were averaged.

2.2.3. Scanning electron microscopy (SEM)

Granite, marble, wood, plastic, steel and glass surface samples were used in attachment and biofilm studies. They were cutted as surface area of each piece was 1cm^2 coupons and cleaned by 70% ethanol 10 min. They were washed with sterilized distilled water, dried for 2 hours at 60 ºC and autoclaved at 121 ºC for 15 minutes.

For scanning electron microscobic analysis, surface samples were placed in 2.5% glutaraldehyde (prepared in 0.1 M phosphate buffer, pH 7.4) for 24 hours at 4°C as a prefixation step. They were then rinsed twice with 0.1 M phosphate buffer (pH 7.4), postfixed using 1% osmium tetroxide for 1 hour at room temperature, and finally rinsed with distilled water. Next, the specimens were dehydrated using graduated concentrations of ethyl alcohol (30%, 50%, 70%, 90%, and 96%) for 15 minutes each followed by absolute alcohol for 30 minutes. All samples were air dried and coated with gold with a Polaron SC7620 Sputter Coater was used. Finally, each specimen was examined using a JEOL scanning electron microscope (JEOL JSM-5600LV).

3. Results

In our study, 8 *Salmonella* spp. and 6 *L. monocytogenes* strains were assayed for biofilm formation by using a microtiter plate assay and tube adherence assay. *L. monocytogenes* ATCC 7644 formed the strongest biofilm by two experiments and was used as quality control strains. Value of absorbance at 450/630 nm was 6.25 of control isolate. The results of the tube adherence test are presented in Table 1. In our study, all *Salmonella* spp. isolates showed the weak biofilm 8 (%100). For *L. monocytogenes* isolates, the number of strong biofilm producers were 4 (%67) and moderate were 2 (%33). Tube adherence method showed good correlation with the microtiter plate method for strongly biofilm forming *L. monocytogenes* isolates and total 3 (% 50) isolates were picked up as strong and 3(%50) were moderate biofilm producers. By microtiter plate method, 1 (12.5%) *Salmonella* spp. isolate were determined as moderate and 7 (87.5%) isolates were observed as weak biofilm producers. However, it was difficult to discriminate between moderate and weakly biofilm producing isolates by tube adherence method.

Table 1. Percent of isolates formed biofilm by tube adherence test

Isolates (n)	Adherence	Isolate number/percent values
Salmonella spp. (8)	strong	0 (%0)
	moderate	0 (%0)
	Weak/non biofilm producer	8 (%100)
Listeria monocytogenes (7)	strong	4 (%67)
	moderate	2 (%33)
	Weak/non biofilm producer	0 (%0)

In control assays, surface properties of samples were investigated by SEM. Based on the obtained results, each type of surface showed different degrees of roughness. The control micrographs of granite, steel, marble and wood surfaces showed some crevices. Plastic and glass surfaces were also more smooth (Fig. 1).

Then, isolates were allowed to form a biofilm on these surfaces. *Salmonella* spp. S8 and *L. monocytogenes* 13L isolates were also taken to study for SEM analysis. Figure 2 demonstrated that the biofilm formation of *Salmonella* spp. S8 isolate on different six surfaces at 24 hours. As also shown in figure; granite, marble, wood and glass surfaces presented higher intensity of biofilm, compared to the steel and plastic surfaces. This isolate clearly produced a dense, three-dimentional composite of cells on marble, wood, granite and glass surfaces (Fig. 2 c, d, e, f). The image in Figure 2e shows the EPS-embedded bacteria colonies on granite surface. However, it was observed that sparse aggregates of cells on steel and plastic surfaces (Fig 2a, b). Figure 3 demonstrated that the biofilm formation of *L. monocytogenes* 13L isolate on different surfaces at 24 hours. Similarly, three dimentional biofilm and EPS structures were determined on granite, marble, wood and glass surfaces. Colonization was less on plastic and steel surfaces. Figure 3F shows attachment and biofilm formation by *L. monocytogenes* cells.

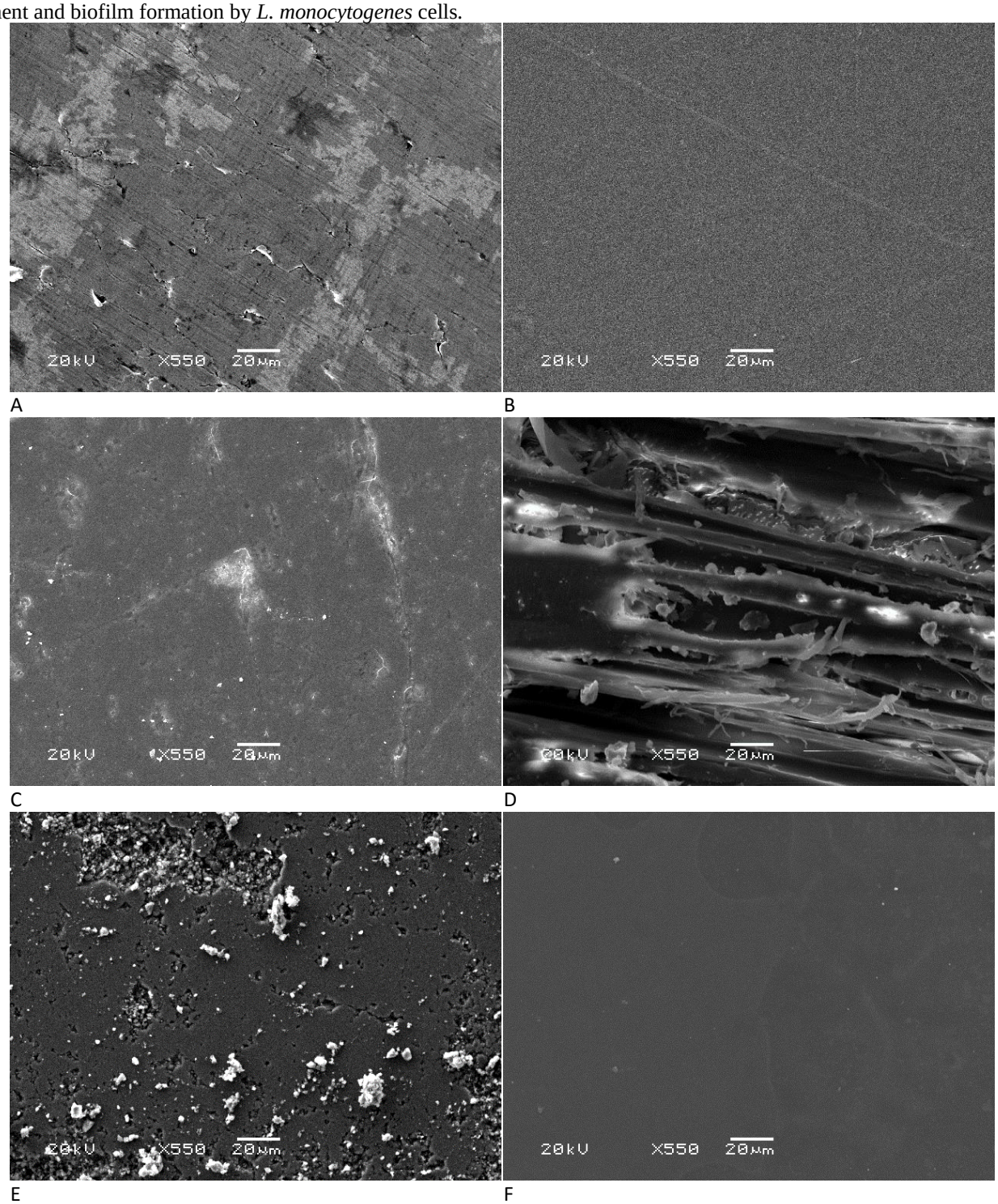

Figure 1. Control micrographs of all surfaces. steel (A), plastic (B), marble (C), wood (D), granite (E) and glass (F) surfaces. (Mag x550)

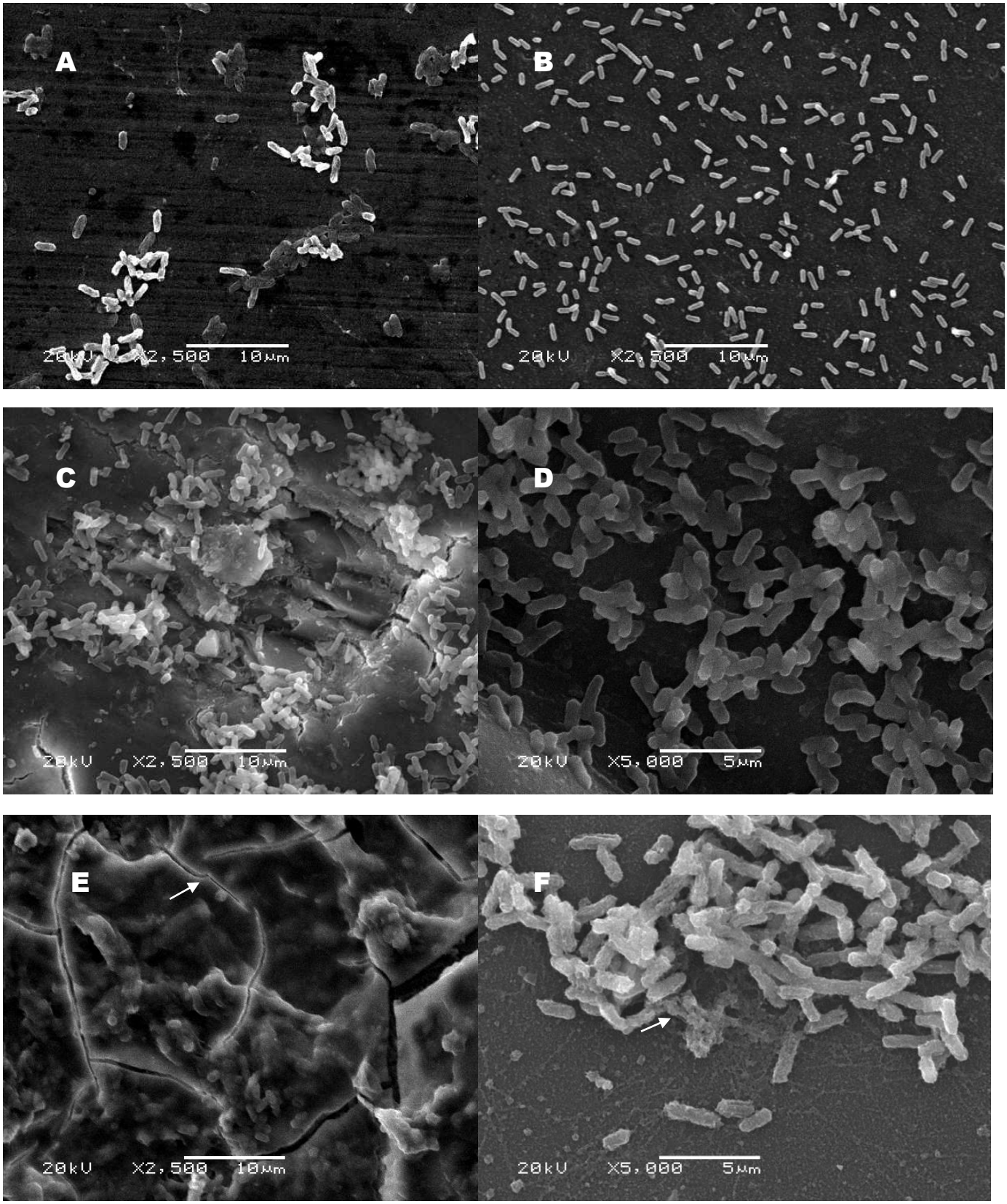

Figure 2. SEM images of strain *Salmonella* spp. S8 on steel (A), plastic (B), marble (C), wood (D), granite (E) and glass (F) surfaces. (A, B, C, E Mag x2500; D-F Mag x5000)

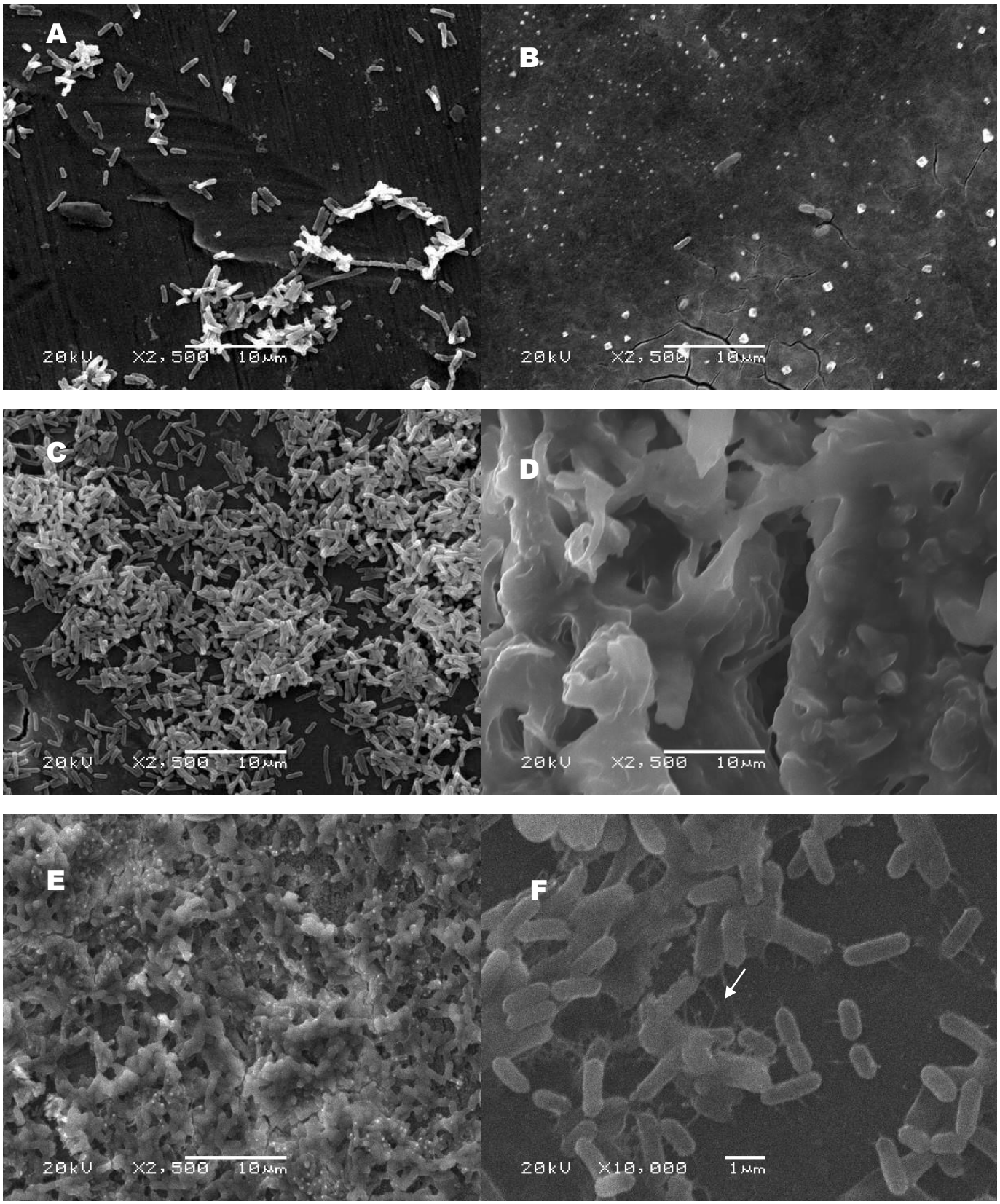

Figure 3. SEM images of strain *L. monocytogenes* 13L on steel (A), plastic (B), marble (C), wood (D), granite (E) and glass (F) surfaces. (A, B, C, D, E Mag x2500; F Mag x10000)

In the study, the adherence, biofilm formation and development of the test isolates on granite surfaces was assessed in five different incubation temperatures, 2., 4., 6., 24. and 48.h. Granite surface was chosen due to the their high biofilm performing capacity. As observed by SEM, *L. monocytogenes* 13L and *Salmonella* spp. S8 adhered to the

granite surface after 6 hours of contact (Figure 4, 5). Strain *Salmonella* spp. S8 showed distinctly more adherence than the *L. monocytogenes* 13L after 6 hours of contact, however, the distribution of the surface adhered cells occurred irregularly. Although in some areas several cells were adhered to surface, in some places, the bacterial adherence observed was not so evident. However mature biofilm formation was determined after 24 and 48 hours for both two isolates.

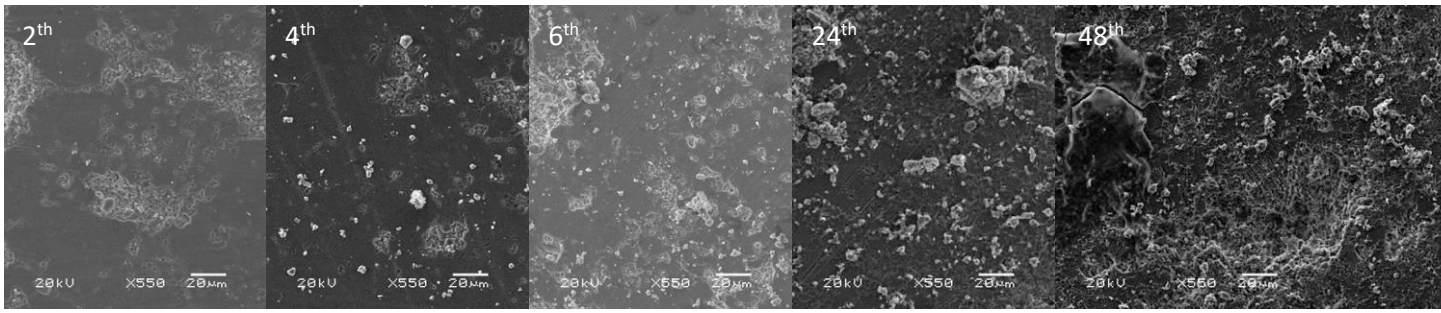

Figure 4. SEM images of the biofilm formation of *Salmonella* spp. S8 isolate on granite surfaces at five time point (Mag x550)

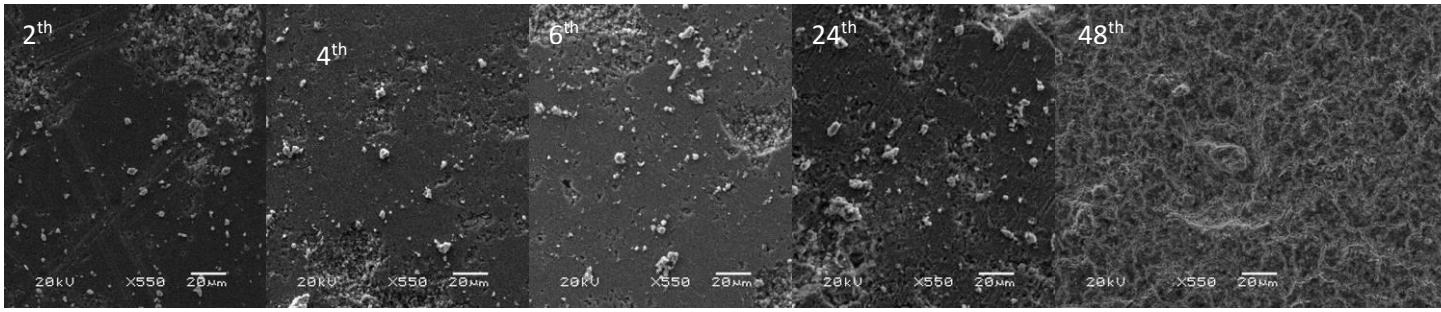

Figure 5. SEM images of the biofilm formation of *L. monocytogenes* 13 L isolate on granite surfaces at five time point (Mag x550)

4. Conclusions and discussion

The ability of many bacteria to adhere to surfaces and to form biofilms has major implications in the food industry, where biofilms create a persistent source of contamination. If the microorganisms from food contact surfaces that are not completely removed, they may lead to biofilm formation and also increase the biotransfer potential. The formation and development of biofilms is affected by many factors, including the spesific bacteria strain, material surface properties and variety environmental parameters. Biofilm cells are more resistant to antimicrobial agents than planctonic bacteria (Srey et al., 2013).

Owing to *Salmonella* spp and *L. monocytogenes* can be quickly transferred from biofilm to food, they have great concern for food safety (Botticella et al., 2013). Several studies reported that *L. monocytogenes* and *Salmonella* spp. may produce biofilms on variety food processing surfaces (Bonaventura et al., 2008). However differences in the degree of attachment and biofilm formation by these pathogens affected by various types of food-contact surfaces. Bacterial adhesion mostly depended on the nature of the inert surface and the bacterial surface property. However, in some situations, it has been difficult to explain the interactions between surface properties and the bacterial adhesion (Silva et al., 2008).

The present study represents our attempt to assess biofilm formation and development by *L. monocytogenes* and *Salmonella* spp. on six different industrial surfaces. In our study, the biofilm degrees of isolates were evaluated based on adherence ability and absorbance values of quality control isolate *L. monocytogenes* ATCC 7644. To detect biofilm forming ability on different surfaces, only selected isolates were examined by SEM. The results of the research indicated that all isolates used in the study were capable of forming biofilms. These findings were consistent with those of some of the earlier observations (Hood and Zottola, 1997; Wong, 1998; Chae and Schraft, 2000). Earlier studies have suggested that the topography of bacterial contact surface plays an important role in facilitating bacteria adhesion and biofilm formation (Simoes et al., 2010). Characklis et al. noted that the extent of microbial colonization appears to increase as the surface roughness increases (Characklis et al., 1990). Howell and Behrends noticed that the extent of microbial attachment is correlated with surface roughness (Howell and Behrends, 2006). This is because surface tension is diminished, and surface area is larger on rougher surfaces. Our study also shows, rough surfaces including granite,

marble and wood showed higher biofilm formation than the other surfaces. Actually in our study, each type of surface (wood > granite > marble > steel > plastic > glass) had different degrees of roughness by the control micrographs obtained from SEM. Especially wood showed to be the roughest surface, with many pores and deep crevices. Granite showed many small pores and crevices. Steel and marble had many oblique, straight line, and narrow crevices on the surface. Plastic and glass surfaces was remained without crevices. However in our study, each type of surface showed different degrees of biofilms (granite > marble > wood> glass> steel > plastic) surfaces. Although the wood had the roughest surface in our study, the most biofilm formation was observed in granite and marble surfaces. Silva et al demostrated that the adhesion ability of 10 isolates *L. monocytogenes* in eight materials commonly used in kitchens and evaluated the viability of the cells to adhere to different surfaces. Similarly, to the our findings, they showed all isolates adhered to surfaces. *L. monocytogenes* adhered most strongly to marble and granite, followed by stainless steel, glass, silestones and finally polypropylene surfaces (Silva et al., 2008).

On the other hand, the biofilm formation is related both the nature of the attachment and the properties of the environmental factors and bacterial cell (Houdt and Michiels, 2010). So, general predictions for the degree of biofilm formation on a particular material can not be made. For example, depending on the hydrophobicity of material, bacteria adhere differently to surfaces with different hydrophobicities. When Adetunji and Isola compared the biofilm formation by *L. monocytogenes* on glass, steel and wooden surfaces immersed in nutrient broth, biofilm formation was strongest on wooden surfaces, followed by steel and glass. It was theorized that *L. monocytogenes* might attach to wooden surfaces more readily because of its higher hydrophobicity (Adetunji and Isola, 2011). Because surfaces with high free surface energy, such as stainless steel and glass, are more hydrophilic. Araujo et al also investigated that the adhesion of *Bacillus cereus* strains isolated from dairy plants, to stainless steel, granite and glass surfaces. They showed the adhesion to the hydrophobic surfaces of granite and stainless steel was greater than adhesion to glass, which was classified as a hydrophilic surface. We also observed strong adhesion and biofilm formation in hydrophobic surfaces such as wood or steel. On the other hand, all plastics are naturally hydrophobic but plasticizer reagents are used to change the plastics' surface character and make them hydrophilic. In our study, we used the hydrophilic coated polypropilen (PP5) plastic and glass materials. Our data also suggested that the hydrophobicity of the surface material influence the initial adhesion and consequently the biofilm formation.

According to the our SEM results, there was an initial attachment of isolates after 6 hours. Fully mature biofilms, are produced after incubation for 24-48 hours. Leonhard et al showed that the initial microbial adhesion might be promoted by rough surfaces but it does not have an impact on later phases of biofilm formation. Also they reported that the surface character did not effect long term biofilm formation (Leonhard et al., 2014). In our research, we have investigated that the biofilm formation and developments until 48hours. But even at 48 hours, smooth surfaces such as plastic and glass showed the weaker biofilm formation compared to the rough surfaces.

Bacterial appendages such as flagella and fimbriae are responsible for bacterial motility, thus contributing to the growth and spread of biofilm development along surfaces. They also showed the hydrophobicity contributed the bacterial cell surfaces. As known, flagellar motility is critical for *L. monocytogenes* biofilm formation (Houdt and Michiels, 2010). In also our study, *L. monocytogenes* produced the stronger biofilm activity by tube adherence and microtiter plate assays than *Salmonella* spp. On the other hand, we have also observed the biofilm formation was less on glass and plastic surfaces than on other materials tested. Although there is some controversy about the effect of surface materials on biofilm formation, we think that bacterial appendages such as flagella and fimbriae may also provide an additional hydrophobicity to bacteria.

The adhesion and biofilm formation of *L. monocytogenes* and *Salmonella* spp. to abietic surfaces is a multifactorial phenomenon. Biofilm formation by these bacteria was affected from by factors such as type of surface, incubation time and hydrophobicity. Granite and marble, although widely used as in the food processing area or for domestic use, are highly convenient surfaces for biofilm formation. For this reason, they may not be preferable for areas where effective disinfection opportunities are not available. Whereas, usage of plastic and glass surfaces may promise a safer environment. Although usage of plastics has negative effect to environmental protection and global warming, our data showed less surface adhesion and biofilm formation in plastic surfaces. However, more studies are needs to better understanding of the microbiological and physicochemical factors in biofilm formation and development.

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