

THE IMPORTANCE OF MATERIAL PROPERTIES, NUTRIENT LOAD AND SHEAR STRESS ON BIOFILM FORMATION

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ABSTRACT

Inorganic and biological fouling reduce the performance of heat exchangers leading to frequent process interruptions for equipment cleaning. In order to identify the key parameters affecting bacterial adhesion and biofilm formation, different surfaces and different nutrient loads were tested under static and dynamic conditions. Copper, stainless steel and glass surfaces were assayed under high or low nutrient loads in static conditions or under agitation (shear stress of 0.27 Pa) using *Escherichia coli* as a model organism.

Results show that nutrient load had a higher effect on biofilm maturation than on cell adhesion. It was also found that shear stress is particularly important when copper surfaces are used. For this surface, higher biofilm amounts were obtained in static conditions especially under high nutrient load (4-fold increase when compared to dynamic conditions). This trend was observed since the initial adhesion step.

Collectively, results show that for all the tested conditions, surface properties have the most significant effect on cell adhesion and biofilm formation. This effect is more pronounced under low nutrient load and, in most cases, a correlation with surface hydrophobicity was found.

INTRODUCTION

In industry, microorganisms can deposit and adhere to equipment surfaces and piping systems and form a biofilm. Biofilm cells are protected from cleaning and disinfection agents by a matrix of self-produced extracellular polymeric substances, making them more difficult to eradicate.

In the food industry, the formation of this biological fouling can cause serious problems when it starts to spread to process lines leading to an increase in maintenance costs, decrease in equipment operational efficiencies and product contamination (Brooks and Flint, 2008).

Escherichia coli is an ubiquitous microorganism in the natural water used in industrial cooling systems (Casani et al., 2005) and it has also been reported as one of the most persistent foodborne microorganisms (Dourou et al., 2011, Sagong et al., 2011, Shi and Zhu, 2009).

Process water in different industries may have different compositions and thus different nutrient loads. Several reports have shown the impact of different nutrient loads on biofilm development (Gomes et al., 2014a, Jackson et al., 2002, Teodósio et al., 2011). Other important factor affecting bacterial adhesion and biofilm formation is the specific hydrodynamics of each industrial system since they will determine the shear forces and the nutrient mass transport (Melo and Vieira, 1999). Higher flow velocities are common in industry to prevent bacterial adhesion and

increase the rate of biofilm detachment (Vieira et al., 1993). However, lower flow velocity zones or even stagnate flow may also be found in equipment with complex geometries (Walid et al., 2013) making these areas suitable places for bacterial adhesion and biofilm development (Ganesh and Anand, 1998).

In order to control the development of biofilms and avoid contamination spread, different materials have been used in the industrial lines. Stainless steel is one of the most used materials due to its hygienic status (low soiling level and/or high cleanability) (Jullien et al., 2003) and ability to resist corrosive damage (Flint et al., 2000). Copper has also been used for industrial applications due to its antimicrobial effects (Grass et al., 2011, Wilks et al., 2005). Glass is a surface that may be used in different applications since it has no effect on the final product smell or taste and its transparency is also an important feature (Muller-Steinhagen and Zettler, 2011).

Different authors have reported the individual effects of nutrient media, hydrodynamic conditions and surface properties on bacterial adhesion and biofilm formation. However, little is known about the combined effect of these three factors. Therefore, the aim of this work was to explore the combined effect of two nutrient loads (high and low nutrient medium), two hydrodynamic conditions (static and dynamic) and three materials typically used in food industry (glass, copper and stainless steel) on *E. coli* adhesion and biofilm formation.

MATERIALS AND METHODS

Culture conditions

Culture conditions were similar to those previously described by Teodósio et al. (2011). Briefly, *Escherichia coli* JM109(DE3) was grown overnight at 30 °C and 120 rpm in 0.2 L of a culture medium containing 5.5 g L⁻¹ glucose, 2.5 g L⁻¹ peptone, 1.25 g L⁻¹ yeast extract in phosphate buffer (1.88 g L⁻¹ KH₂PO₄ and 2.60 g L⁻¹ Na₂HPO₄) at pH = 7.0.

Then, the cells were centrifuged (3202 g, 10 min) and washed twice with saline solution (8.5 g L⁻¹ NaCl in distilled water). The pellet was resuspended and the cellular suspension was adjusted to a final concentration of approximately 7.6 × 10⁸ cells mL⁻¹, determined by optical density at 610 nm (OD = 1).

Surface preparation

Stainless steel (SS, F. Ramada, Portugal), copper (CU, Neves & Neves, Lda, Portugal) and glass (GLA, Vidraria Lousada, Lda, Portugal) were selected as materials to be used in this work because of their common use in heat exchange

equipment and pipes in food processing lines (Bonsaglia et al., 2014, Brooks and Flint, 2008, Melo and Flemming, 2010, Shi and Zhu, 2009, Van Houdt and Michiels, 2010). These materials were cut in square coupons with dimensions of 1x1 cm and cleaned according to the procedure described by Gomes et al. (2014b). The materials were immersed in a solution of 5% (v/v) commercial detergent (Sonasol Pril, Henkel Ibérica S.A.) for 30 min with gentle shaking (Azevedo et al., 2006). To remove any remaining detergent, coupons were rinsed in ultrapure water and immersed in 96% (v/v) ethanol for 30 min (b). After being rinsed again with ultrapure water and air-dried, all coupons were autoclaved for 15 min at 121 °C (Gomes et al., 2014b) before being used in the assays.

Surface properties determination

The surface energy components of the tested materials (GLA, SS and Cu) were determined after measuring the contact angles of the surfaces by the sessile drop method using a contact angle meter (OCA 15 Plus, Dataphysics, Germany). These measurements were carried out at room temperature (25 ± 2 °C) with three pure liquids: water, formamide and α-bromonaphthalene (Sigma-Aldrich Co., Portugal). Reference values for surface tension components were obtained from the literature (Janczuk et al., 1993). Contact angle data were obtained from at least 25 determinations for each liquid and surface (Table 1). Afterwards, the hydrophobicity of the surfaces was evaluated by the method of van Oss et al. (1988). In this approach, the degree of hydrophobicity of a given material (i) is expressed as the free energy of interaction between two entities of that material immersed in water (w) - Δ*G*_{iw*i*}. If the interaction between the two entities is stronger than the interaction of each entity with water (Δ*G*_{iw*i*} < 0 mJ m⁻²), the material is considered hydrophobic. Conversely, if Δ*G*_{iw*i*} > 0 mJ m⁻², the material is hydrophilic. Δ*G*_{iw*i*} was calculated from the surface tension components of the interacting entities:

$$\Delta G_{iwi} = -2 \left(\sqrt{\gamma_i^{LW}} - \sqrt{\gamma_w^{LW}} \right)^2 + 4 \left(\sqrt{\gamma_i^+ \gamma_w^-} + \sqrt{\gamma_i^- \gamma_w^+} - \sqrt{\gamma_i^+ \gamma_i^-} - \sqrt{\gamma_w^+ \gamma_w^-} \right)$$

where γ^{LW} accounts for the Lifshitz-van der Waals component of the surface free energy and γ⁺ and γ⁻ are the electron acceptor and electron donor parameters, respectively, of the Lewis acid-base component (γ^{AB}), with γ^{AB} = 2√γ⁺γ⁻.

The surface tension components were estimated by the simultaneous resolution of three equations of the type:

$$(1 + \cos \theta) \gamma_i^{TOT} = 2 \left(\sqrt{\gamma_s^{LW} \gamma_i^{LW}} + \sqrt{\gamma_s^+ \gamma_i^-} + \sqrt{\gamma_s^- \gamma_i^+} \right)$$

where θ is the contact angle and γ^{TOT} = γ^{LW} + γ^{AB}.

Bacterial adhesion and biofilm formation assays

A high nutrient medium (HN) and a low nutrient medium (LN) were assayed for adhesion and biofilm experiments. The HN corresponds to the Mueller-Hinton broth (Merck, Germany) and the LN is a 1:100 dilution of the inoculation medium in phosphate buffer (1.88 g L⁻¹ KH₂PO₄ and 2.60 g L⁻¹ Na₂HPO₄) (Simões et al., 2007). A total of 0.4 mL of the cell suspension previously prepared was transferred into each well of a sterile 6-well polystyrene, flat-bottomed microtiter plate (Orange Scientific, USA) containing a single coupon of the selected material (GLA or SS or Cu) and 3.6 mL of growth medium (HN or LN). The microtiter plates were incubated (AGITORB 200, Aralab, Portugal) at 30 °C in static (0 rpm, 0 Pa) and shaking conditions (115 rpm, 0.27 Pa, Reynolds number of 2400) (Salek et al., 2011). It is known that in agitated vessel systems, flow may become turbulent at Reynolds numbers as low as 100 (Nauman, 2008). Shear stresses similar to the one tested in this work can be found in different industrial equipment, pipes and accessories (Ahmed et al., 2011, Jensen and Friis, 2005, Konuklar and Gunasekaran, 2002, Lelièvre et al., 2002, Liu et al., 2006). At different sampling times, 0.5 h for adhesion and 6 h for biofilm studies, coupons were removed from the microwells and rinsed with sterile saline to remove loosely attached cells. Total bacterial counts were obtained by direct staining with 4',6-diamidino-2-phenylindole (DAPI), as previously described by Lemos et al. (2014). Cells were visualized under an epifluorescence microscope (Eclipse LV100, Nikon, Japan) equipped with a filter block sensitive to DAPI fluorescence (359-nm excitation filter in combination with a 461-nm emission filter). For each coupon, a minimum of 20 fields were counted and the number of attached cells per cm² determined. Three independent experiments were performed for each surface, each of them with a triplicate set of wells.

Calculations

The number of attached cells during initial adhesion (0.5 h) and biofilm formation (0.6 h) for each combination of 3 factors (agitation, nutrient load and material) was calculated. In order to assess the impact of each of these 3 factors, the relevant ratios of attached cell numbers obtained in each condition were calculated (the number of cells obtained in the first condition was divided by the number of cells obtained on the second condition). These ratios are presented in Figure 1 using a grey-scale to highlight the most significant factor combinations. Thus, in order to assess the impact of agitation for all materials and nutrient load combinations, the ratios between the attached cell number obtained under static (S) and dynamic (D) conditions were calculated. The same type of calculation was done to evaluate the impact of nutrient load on all materials and agitation conditions. To study the impact of the surface material on all nutrient and agitation conditions, the ratio between the number of attached cells for each pair of materials (GLA vs. Cu, SS vs. Cu and GLA vs. SS) was calculated. Since the aim of this study is to assess the

impact of each variable (in combination with the other two), whenever the ratio between two conditions was lower than 1, the inverse of the ratio was calculated (to obtain values equal or higher than 1).

RESULTS

In this work, the combined impact of three materials (GLA, SS and Cu), two nutrient loads (HN and LN) and two hydrodynamic conditions (S and D) on bacterial adhesion and biofilm formation was evaluated.

Table 1 contains the results from the contact angle measurements as well as the hydrophobicity calculations. It is possible to observe that GLA is a hydrophilic surface ($\Delta G_{iwi} > 0 \text{ mJ m}^{-2}$) whereas SS and Cu are hydrophobic ($\Delta G_{iwi} < 0 \text{ mJ m}^{-2}$), being Cu the most hydrophobic surface tested.

Figure 1 was constructed to evaluate the impact of each tested variable (shear stress, nutrient load and surface material) on initial bacterial adhesion and biofilm formation when combined with the other two variables. The higher (and darker) the ratio for a given condition, the stronger the impact of that variable on bacterial adhesion and/or biofilm formation. Regarding the impact of agitation on the number of attached bacteria, it is possible to verify that Cu had the highest variation whereas SS had the lowest. Additionally, higher cell adhesion and biofilm formation were obtained in static conditions when compared to dynamic conditions.

Table 1. Contact angles with water (θ_w), formamide (θ_F) and α -bromonaphthalene (θ_B), and hydrophobicity (ΔG_{iwi}) for the tested materials.

	Contact angle (°)			Hydrophobicity (mJ m^{-2})
	θ_w	θ_F	θ_B	(ΔG_{iwi})
GLA	47.0 ± 0.4	49.1 ± 0.5	63.4 ± 0.9	19.3
Cu	66.6 ± 7.8	40.0 ± 6.2	24.6 ± 1.8	-36.5
SS	67.0 ± 1.7	60.4 ± 0.4	39.3 ± 0.5	-10.9

In what concerns the nutrient load impact on the number of attached bacteria (at 0.5 and 6 h), this factor was more important in dynamic than in static conditions. Nutrient load was particularly important for biofilm development in GLA under dynamic conditions, where a 6.5 fold increase was found in high nutrient conditions when compared to a low nutrient load. In general, higher biofilm formation was achieved in the presence of greater amounts of nutrients.

The type of surface was the most important factor affecting cell adhesion and biofilm formation. This effect was more pronounced under low nutrient conditions. Under these conditions, Cu was the surface with the highest cell adhesion and biofilm values. Cell adhesion and biofilm formation values were similar in SS and GLA particularly in high nutrient conditions.

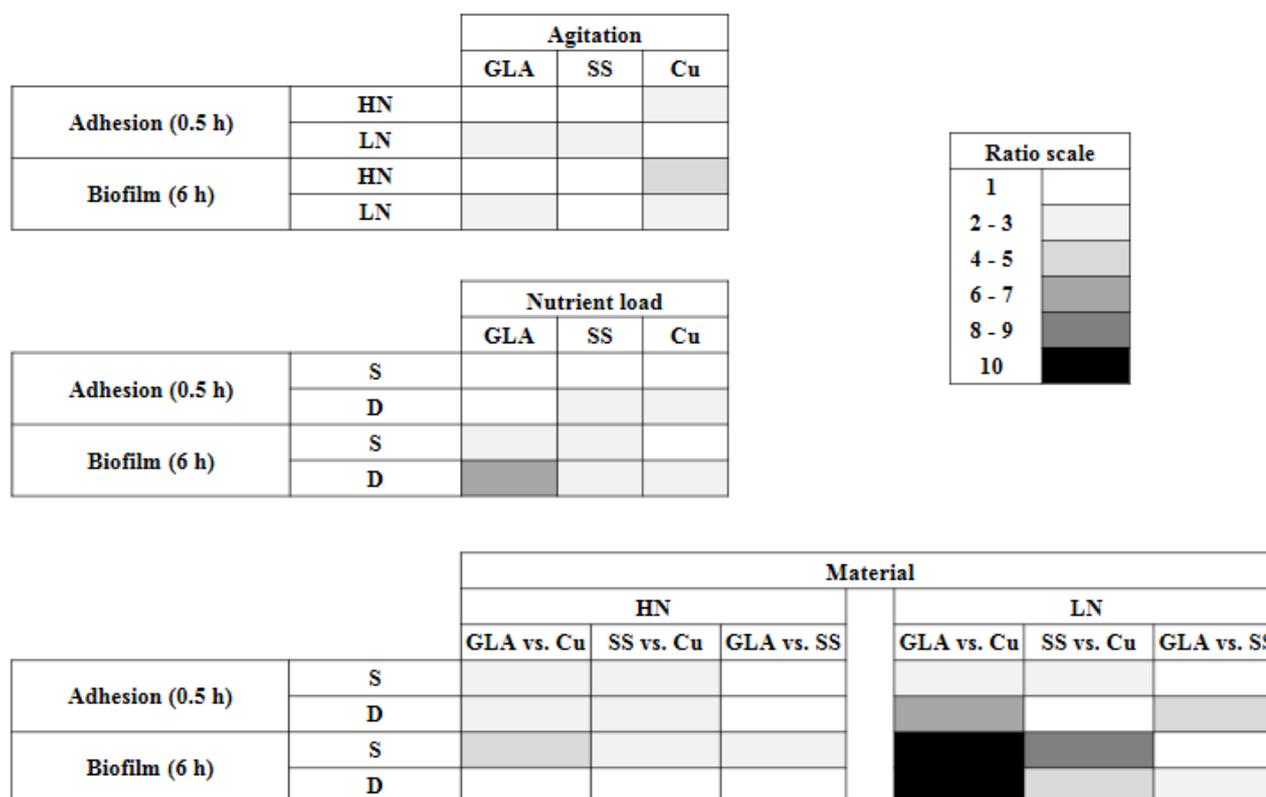


Fig. 1 Impact of the analyzed variables on *E. coli* adhesion (0.5 h) and biofilm formation (6 h). Three variables were studied: agitation, nutrient load and material. For the impact of agitation, a ratio between the attached cell number obtained in each agitation condition (static vs. dynamic) was calculated. The effect of nutrient load was assessed as before and ratios were calculated for low nutrient vs. high nutrient conditions. The bottom part of the figure analyses the material effect at high nutrient load (HN) and low nutrient load (LN). Ratios were calculated for all combinations of the remaining variables (agitation and nutrient load). Abbreviations: S - static; D - dynamic; LN - low nutrient; HN - high nutrient; GLA - glass; SS - stainless steel; Cu - copper.

DISCUSSION

E. coli adhesion and biofilm formation was correlated with surface hydrophobicity in most cases. This observation is consistent with previous reports from Gomes et al. (2014b) and Treter et al. (2014) who also found a correlation between cell attachment and surface hydrophobicity.

Cell adhesion and biofilm formation was enhanced in static conditions when compared to dynamic conditions. Although a higher fluid velocity will increase the transport of nutrients and cells with positive effects on cell adhesion and biofilm formation (Moreira et al., 2014a, Moreira et al., 2014b), a higher shear stress may prevent cell attachment or promote cell detachment, as it may have happened in the present study.

By analyzing the effect of surface material, it is possible to conclude that this is the factor with the highest impact in biofilm formation, particularly in low nutrient conditions. It is probable that in high nutrient conditions, a denser conditioning layer may be formed at the surface, thus masking the effects of the original surface properties.

CONCLUSIONS

In this study, it was shown that for the majority of the situations tested, *E. coli* adhesion and biofilm formation could be correlated with surface hydrophobicity. It was also shown that hydrodynamic conditions have a strong influence on cell attachment. Under the conditions tested, the type of surface was the factor that had the most significant impact on cell adhesion and biofilm formation. This impact was stronger under low nutrient conditions, possibly because a denser conditioning layer is formed under high nutrient conditions thus masking the original surface properties.

This study also shows that analyzing the impact of a single factor at a time on biofilm development may not be the best approach but rather the combined analysis of the most significant factors may yield more robust conclusions about the biofilm development process.

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REFERENCES

Ahmed, S., Seraji, M.T., Jahedi, J. and Hashib, M.A.,

2011, CFD simulation of turbulence promoters in a tubular membrane channel, *Desalination*, Vol. 276, pp. 191-198.

Azevedo, N.F., Pinto, A.R., Reis, N.M., Vieira, M.J. and Keevil, C.W., 2006, Shear stress, temperature, and inoculation concentration influence the adhesion of water-stressed *Helicobacter pylori* to stainless steel 304 and polypropylene, *Appl Environ Microbiol*, Vol. 72, pp. 2936-2941.

Bonsaglia, E.C.R., Silva, N.C.C., Fernandes, J.A., Araújo, J.J.P., Tsunemi, M.H. and Rall, V.L.M., 2014, Production of biofilm by *Listeria monocytogenes* in different materials and temperatures, *Food Control*, Vol. 35, pp. 386-391.

Brooks, J.D. and Flint, S.H., 2008, Biofilms in the food industry: problems and potential solutions, *Int J Food Sci Technol*, Vol. 43, pp. 2163-2176.

Casani, S., Rouhany, M. and Knøchel, S., 2005, A discussion paper on challenges and limitations to water reuse and hygiene in the food industry, *Water Res*, Vol. 39, pp. 1134-1146.

Dourou, D., Beauchamp, C.S., Yoon, Y., Geornaras, I., Belk, K.E., Smith, G.C., Nychas, G.-J.E. and Sofos, J.N., 2011, Attachment and biofilm formation by *Escherichia coli* O157:H7 at different temperatures, on various food-contact surfaces encountered in beef processing, *Int J Food Microbiol*, Vol. 149, pp. 262-268.

Flint, S.H., Brooks, J.D. and Bremer, P.J., 2000, Properties of the stainless steel substrate, influencing the adhesion of thermo-resistant streptococci, *J Food Eng*, Vol. 43, pp. 235-242.

Ganesh, K.C. and Anand, S.K., 1998, Significance of microbial biofilms in food industry: a review, *Int J Food Microbiol*, Vol. 42, pp. 9-27.

Gomes, L.C., Moreira, J.M.R., Teodósio, J.S., Araújo, J.D.P., Miranda, J.M., Simões, M., Melo, L.F. and Mergulhão, F.J., 2014a, 96-well microtiter plates for biofouling simulation in biomedical settings, *Biofouling*, Vol. 30, pp. 1-12.

Gomes, L.C., Silva, L.N., Simões, M., Melo, L.F. and Mergulhão, F.J., 2014b, *Escherichia coli* adhesion, biofilm development and antibiotic susceptibility on biomedical materials, *J Biomed Mater Res Part A*, Vol. 103, pp 1414-1423.

Grass, G., Rensing, C. and Solioz, M., 2011, Metallic copper as an antimicrobial surface, *Appl Environ Microbiol*, Vol. 77, pp. 1541-1547.

Jackson, D.W., Simecka, J.W. and Romeo, T., 2002, Catabolite repression of *Escherichia coli* biofilm formation, *J Bacteriol*, Vol. 184, pp. 3406-3410.

Janczuk, B., Chibowski, E., Bruque, J.M., Kerkeb, M.L. and Caballero, F.G., 1993, On the consistency of surface free energy components as calculated from contact angles of different liquids: an application to the cholesterol surface, *J. Colloid Interface Sci.*, Vol. 159, pp. 421-428.

Jensen, B.B.B. and Friis, A., 2005, Predicting the cleanability of mix-proof valves by use of wall shear stress, *J Food Process Eng*, Vol. 28, pp. 89-106.

Jullien, C., Bénézech, T., Carpentier, B., Lebret, V. and Faille, C., 2003, Identification of surface characteristics relevant to the hygienic status of stainless steel for the food

industry, *J Food Eng*, Vol. 56, pp. 77-87.

Konuklar, G. and Gunasekaran, S., 2002, Rennet-induced milk coagulation by continuous steady shear stress, *J Colloid Interface Sci*, Vol. 250, pp. 149-158.

Lelièvre, C., Legentilhomme, P., Gaucher, C., Legrand, J., Faille, C. and Bénézech, T., 2002, Cleaning in place: effect of local wall shear stress variation on bacterial removal from stainless steel equipment, *Chem Eng Sci*, Vol. 57, pp. 1287-1297.

Lemos, M., Gomes, I., Mergulhão, F., Melo, L. and Simões, M., 2014, The effects of surface type on the removal of *Bacillus cereus* and *Pseudomonas fluorescens* single and dual species biofilms, *Food Bioprod Process*, Vol. 93, pp. 234-241.

Liu, Z., Lin, Y.E., Stout, J.E., Hwang, C.C., Vidic, R.D. and Yu, V.L., 2006, Effect of flow regimes on the presence of *Legionella* within the biofilm of a model plumbing system, *J Appl Microbiol*, Vol. 101, pp. 437-442.

Melo, L. and Flemming, H., Yea. *The science and technology of industrial water treatment*, CRC Press, USA, New York.

Melo, L.F. and Vieira, M.J., 1999, Physical stability and biological activity of biofilms under turbulent flow and low substrate concentration, *Bioprocess Eng*, Vol. 20, pp. 363-368.

Moreira, J.M.R., Araújo, J.D.P., Miranda, J.M., Simões, M., Melo, L.F. and Mergulhão, F.J., 2014a, The effects of surface properties on *Escherichia coli* adhesion are modulated by shear stress, *Colloids Surf B*, Vol. 123, pp. 1-7.

Moreira, J.M.R., Simões, M., Melo, L.F. and Mergulhão, F.J., 2014b, The combined effects of shear stress and mass transfer on the balance between biofilm and suspended cell dynamics, *Desalin Water Treat*, pp. 1-7.

Muller-Steinhagen, H. and Zettler, H.U., Yea. *Heat exchanger fouling mitigation and cleaning technologies*, PP Publico Publications, Germany.

Nauman, E.B., Yea. *Chemical reactor design, optimization, and scaleup*, Wiley, New Jersey.

Sagong, H.-G., Lee, S.-Y., Chang, P.-S., Heu, S., Ryu, S., Choi, Y.-J. and Kang, D.-H., 2011, Combined effect of ultrasound and organic acids to reduce *Escherichia coli* O157:H7, *Salmonella Typhimurium*, and *Listeria monocytogenes* on organic fresh lettuce, *Int J Food Microbiol*, Vol. 145, pp. 287-292.

Salek, M.M., Sattari, P. and Martinuzzi, R.J., 2011, Analysis of fluid flow and wall shear stress patterns inside partially filled agitated culture well plates, *Ann Biomed Eng*, Vol. 40, pp. 707-728.

Shi, X. and Zhu, X., 2009, Biofilm formation and food safety in food industries, *Trends Food Sci Technol*, Vol. 20, pp. 407-413.

Simões, M., Pereira, M.O., Sillankorva, S., Azeredo, J. and Vieira, M.J., 2007, The effect of hydrodynamic conditions on the phenotype of *Pseudomonas fluorescens* biofilms, *Biofouling*, Vol. 23, pp. 249-258.

Teodósio, J.S., Simões, M., Melo, L.F. and Mergulhão, F.J., 2011, Flow cell hydrodynamics and their effects on *E. coli* biofilm formation under different nutrient conditions and turbulent flow, *Biofouling*, Vol. 27, pp. 1-11.

Treter, J., Bonatto, F., Krug, C., Soares, G.V., Baumvol, I.J.R. and Macedo, A.J., 2014, Washing-resistant surfactant coated surface is able to inhibit pathogenic bacteria adhesion, *Appl Surf Sci*, Vol. 303, pp. 147-154.

Van Houdt, R. and Michiels, C.W., 2010, Biofilm formation and the food industry, a focus on the bacterial outer surface, *J Appl Microbiol*, Vol. 109, pp. 1117-1131.

van Oss, C.J., Chaudhury, M.K. and Good, R.J., 1988, Interfacial Lifshitz-van der Waals and polar interactions in macroscopic systems, *Chem Rev*, Vol. 88, pp. 927-941.

Vieira, M.J., Melo, L.F. and Pinheiro, M.M., 1993, Biofilm formation: hydrodynamic effects on internal diffusion and structure, *Biofouling*, Vol. 7, pp. 67-80.

Walid, B., Patrick, L., Thierry, B. and Francine, F., 2013, Cleanability study of a Scraped Surface Heat Exchanger, *Food Bioprod. Process*, Vol. 91, pp. 95-102.

Wilks, S.A., Michels, H. and Keevil, C.W., 2005, The survival of *Escherichia coli* O157 on a range of metal surfaces, *Int J Food Microbiol*, Vol. 105, pp. 445-454.