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# Evaluation of SICON® surfaces for biofouling mitigation in critical process areas

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**Abstract**

In industrial processes, particularly in the food sector, sustainability is increasingly important. Consumers demand safer food and this is often associated with elevated cleaning costs and high environmental impacts in order to reduce contaminations on equipment and products.

Modified surfaces are seen as a promising strategy for biofouling mitigation and contamination prevention. In this work, the performance of a modified Diamond-Like Carbon (DLC) surface designated by SICON® (a-C:H:Si:O) was compared with stainless steel (316L) regarding bacterial adhesion, biofilm formation and cleanability. Assays were performed at different temperatures using *Escherichia coli*, one of the most persistent foodborne microorganisms and also the natural flora present in the water from an industrial salad washing line. Bacterial adhesion on SICON® and stainless steel were similar and favored at a higher temperature (30 ºC). Biofilm formation was reduced on SICON® (1-2 Log) and this may be explained by the lower ratio between the Lifshitz van der Waals apolar component and the electron donor component ($\gamma^{LW}/\gamma^{-}$) of this surface. It was also shown that after performing a cleaning treatment with chlorine, reduction of viability counts was much higher in SICON® (about 3.5 Log reduction and 15% removal) when compared to stainless steel (1.6 Log reduction and 6% removal). Additionally, it was observed that 18 h after treatment, biofilm values in SICON® were similar to those obtained with stainless steel. Results indicate that for industries with cleaning frequencies of up to 6 h, the use of SICON® on critical areas enables operation at a much higher hygienic level.

**Keywords:** Adhesion, Biofilm, Cleaning, *Escherichia coli,* SICON®, Stainless steel

## 1. Introduction

There is an increasing demand for sustainable manufacturing processes in the food industry (Del Borghi et al., 2014). A sustainable process implies an engagement between high-quality and hygienic products, low environmental impact of the process, reduced costs and lower health risks (Gomes da Cruz et al., 2015; Mauermann et al., 2009). However, this is challenging since elevated cleaning costs are incurred due to the use of disinfectants, water and energy in order to reduce contamination on processing equipment and food products (Gomes da Cruz et al., 2015; Mauermann et al., 2009). Moreover, the use of chemical disinfectants and high water consumption have an elevated environmental impact (Moreira et al., 2014b). The unavoidable attachment of bacterial cells on industrial surfaces and further biofilm development is at the core of the problem (Simões et al., 2010). Bacteria within a biofilm are protected by a self-produced matrix composed by extracellular polymeric substances (EPS). This matrix protects the cells from chemical disinfectants, biocides, surfactants and from mechanical forces promoted by water jets and by scrubbing and scraping actions (Simões et al., 2009). Moreover, in industrial plants, there are critical zones such as crevices, corners, joints, valves, which are difficult to clean due to reduced access and where lower fluid velocities may be found, making these zones suitable niches for biofilm accumulation and growth (Lemos et al., 2015). In these zones, higher amounts of disinfectants and water have to be used in order to achieve recommended cleaning standards and this has environmental and economic impacts.

In food processes such as in the dairy industry, microorganisms may be detected on surfaces after 2 h and values of $8.55\times10^{4}$ cells $cm^{-2}$ can be reached after 5 days (Holah and Kearney, 1992). On a fish filleting process, values of $3.35\times10^{3}$ cells $cm^{-2}$ were found after 6 h of operation and in baked beans transport belts, values higher than $4.30\times10^{7}$ cells $cm^{-2}$ can be achieved after 16 h (Holah and

Kearney, 1992).

*Escherichia coli* is one of the most persistent foodborne microorganisms (Dourou et al., 2011; Sagong et al., 2011; Shi and Zhu, 2009) and its presence on food-contact surfaces has been associated with its ability to attach and form biofilms on these surfaces (Dourou et al., 2011). The most widely used method to detect the presence of the biological contaminants on the equipment surfaces is by swabbing and bacterial cultivation in order to determine the number of cells per $cm^2$ (Sudheesh et al., 2013). Additionally, the microbiological load can also be measured by the adenosine triphosphate (ATP) level (Sudheesh et al., 2013). Both methods only detect viable microorganisms that can grow during the cultivation step or produce ATP. However, it is known that a biofilm is composed by EPS, viable bacteria (the so-called "active layer") and by non-viable bacteria that are usually located at the bottom of the biofilm (Vieira and Melo, 1999). Therefore, the standard methods used to detect attached bacteria on industrial food contact surfaces do not take into account the non-viable bacterial layer and the EPS that may sometimes represent the majority of the biofilm. Thus, after equipment sanitizing, the traditional methods used to determine the cleaning efficiency are not taking into account the non-living biofilm components that may have an important role on biofilm regrowth.

The modification of energetic and topographic surface properties is seen as a good strategy for fouling mitigation (Mauermann et al., 2009) despite the additional costs of surface preparation (Gomes da Cruz et al., 2015). These modifications are excepted to delay bacterial adhesion and/or facilitate the cleaning processes (Mauermann et al., 2009). SICON® is a Diamond-Like Carbon (DLC) coating (a-C:H:Si:O), approved as a food contact surface that has been investigated as alternative to stainless steel in food manufacturing plants due its thermal conductivity, low friction, smoothness, wear resistance and anti-fouling properties (Boxler et al., 2013a). Boxler et al. (2013a,

b) investigated the performance of SICON® and other DLC coatings against milk fouling (whey protein and milk salts). Results showed that surface modification directly affected the formation of deposits, their composition, as well as their adhesive strength and that this was due to the electron donor component of the surface energy. They concluded that a lower deposit mass was formed on SICON® compared to stainless steel and that this surface was easier to clean. Saikhwan (2013) made a preliminary study with DLC coatings in order to evaluate their suitability for biofouling mitigation in building exteriors. Fluid dynamic gauging was used to determine the thickness and the shear stress (between 1.5 and 8 Pa) required to clean biofilms of *Pseudomonas fluorescens* and *Arthronema africanum* formed on the selected surfaces. Although no conclusive results were obtained with SICON®, it was suggested that surface energy had little influence on biofilm formation. Additionally, it was observed that surface roughness affected biofilm formation but had negligible effects on biofilm cleaning.

Despite the beneficial effects of SICON® in the mitigation of abiotic fouling, the preliminary results with biological fouling were inconclusive. Additionally, to the best of our knowledge, this surface has never been evaluated in microbial fouling mitigation in industrial conditions. In this work, the performance of SICON® and stainless steel were compared regarding *E. coli* adhesion, biofilm formation and cleaning. Assays simulated industrial settings using process water from a salad washing line and also tested some extreme operational conditions (higher temperature and nutrient composition) to evaluate if the use of this modified surface in critical areas could be beneficial in maintaining a higher hygienic level in different industrial plants. Biofilm quantification was made by viable plate counting and by biofilm thickness measurement. These methods enabled the determination of the viable biofilm amount and the total biofilm amount. The importance of these measurements on the evaluation of CIP (cleaning in place) efficiency on food

industries is discussed.

## 2. Material and methods

### 2.1 Bacterial and culture conditions

*Escherichia coli* JM109(DE3) from Promega (USA) was used in this study because it has shown a good biofilm forming ability in a variety of *in vitro* platforms operated at different shear stresses (Moreira et al., 2013; 2014a; Teodósio et al., 2012). Additionally, it was shown that its biofilm formation is similar to other *E. coli* strains which are often used for antimicrobial susceptibility and disinfection tests (Gomes et al., 2014). A starter culture was obtained by inoculation of 500 $\mu$L of a glycerol stock (kept at -80 ºC) to a total volume of 200 mL of inoculation medium with 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 $L^{-1}$ $KH_2PO_4$ and 2.60 g $L^{-1}$ $Na_2HPO_4$) at pH 7.0, as described by Teodósio et al. (2011). This culture was grown in a 1 L shake-flask, incubated overnight at 30 ºC with orbital agitation (120 rpm). A volume of 150 mL of this culture was used for the adhesion assays, and a volume of 50 mL was used to inoculate the intermediate fermenter used for the biofilm assays.

### 2.2 Surface preparation

Round coupons (1 cm of diameter) made from electro-polished stainless steel (AISI 316L/ X2CrNiMo17-12-2/1.4404) and SICON® were tested. The coatings were prepared by the Fraunhofer Institute for Surface Engineering and Thin Films (IST) in Braunschweig, Germany using the PECVD method (PECVD: Plasma enhanced Chemical Vapor Deposition). A detailed description of the SICON® preparation method was disclosed before (Corbella et al., 2009; Grischke et al., 1998).

Coupons were first scrubbed and disinfected with ethanol (70%) and were then immersed in a commercial bleach (Continente, Portugal) solution (0.2% v/v) for 20 min under strong agitation. To remove the bleach, coupons were aseptically rinsed and washed again with sterile distilled water under strong agitation, for 20 min.

**2.3 Surface properties determination**

Surface hydrophobicity was evaluated considering the Lifshitz van der Waals acid base approach (van Oss, 1994). The contact angles were determined automatically by the sessile drop method in a contact angle meter (OCA 15 Plus; Dataphysics, Filderstadt, Germany) using water, formamide and α-bromonaphtalene (Sigma) as reference liquids. The surface tension components of the reference liquids were taken from literature (Janczuk et al., 1993). For each surface, measurements with each liquid were performed at 25 ± 2 ºC. Eleven measurements were made with each reference liquid and a maximum deviation of 9% was obtained between them. The model proposed by (van Oss, 1994) indicates that the total surface energy ($\gamma^{Tot}$) of a pure substance is the sum of the Lifshitz van der Waals components of the surface free energy ($\gamma^{LW}$) and Lewis acid-base components ($\gamma^{AB}$):

$$\gamma^{Tot} = \gamma^{\mathrm{LW}} + \gamma^{\mathrm{AB}} \quad (1)$$

The polar AB component comprises the electron acceptor $\gamma^{+}$ and electron donor $\gamma^{-}$ parameters, and is given by:

$$\gamma^{\mathrm{AB}} = 2\sqrt{\gamma^{+}\gamma^{-}} \quad (2)$$

The surface energy components of a solid surface (s) are obtained by measuring the contact angles (θ) with the three different liquids (l) with known surface tension components, followed by the simultaneous resolution of three equations of the type:

$$(1+\cos\theta)\gamma_l = 2\left(\sqrt{\gamma_s^{LW}\gamma_l^{LW}} + \sqrt{\gamma_s^{+}\gamma_l^{-}} + \sqrt{\gamma_s^{-}\gamma_l^{+}}\right) \quad (3)$$

The degree of hydrophobicity of a given surface is expressed as the free energy of interaction ( $\Delta G$ mJ.m$^{-2}$) between two entities of that surface immersed in a polar liquid (such as water (w) as a model solvent). $\Delta G$ was calculated from the surface tension components of the interacting entities, using the equation:

$$\Delta G = -2\left(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 + 4\left(\sqrt{\gamma_s^{+}\gamma_w^{-}} + \sqrt{\gamma_s^{-}\gamma_w^{+}} - \sqrt{\gamma_s^{+}\gamma_s^{-}} - \sqrt{\gamma_w^{+}\gamma_w^{-}}\right); \quad (4)$$

If the interaction between the two entities is stronger than the interaction of each entity with water, $\Delta G < 0$ mJ.m$^{-2}$, the material is considered hydrophobic, if $\Delta G > 0$ mJ.m$^{-2}$, the material is hydrophilic.

The ratio proposed by Liu and Zhao (2011) between the the Lifshitz van der Waals apolar component and the electron donor component ( $\gamma^{LW} / \gamma^{-}$ ) was also calculated for each surface.

**2.4 Adhesion assays**

The adhesion assays were conducted in three different media: 1) an industrial water collected from a salad washing line (with a bacterial load of $3.3\times10^5$ CFU mL$^{-1}$); 2) the same industrial water spiked with *E. coli* JM109(DE3) and 3) a low nutrient medium containing 0.055 g L$^{-1}$ glucose, 0.025 g L$^{-1}$ peptone, 0.0125 g L$^{-1}$ yeast extract in phosphate buffer (1.88 g L$^{-1}$ $KH_2PO_4$ and 2.60 g L$^{-1}$ $Na_2HPO_4$) at pH 7.0 (a 1:100 dilution of the inoculation medium) also spiked with *E. coli* JM109(DE3). For *E. coli*, three aliquots (50 mL) from the overnight grown culture were used to

harvest cells by centrifugation (10 min, 3202 *g*). Pellets were washed twice with saline (8.5% NaCl). One of the pellets was resuspended in the industrial water and the other two in the low nutrient medium (each one to be used at a different assay temperature). For the low nutrient medium, an appropriate volume was used to reach a final optical density (OD) of 0.1 at 610 nm, which corresponds to $7.60\times10^7$ cell $mL^{-1}$. The same volume was used for the industrial water and a total bacterial load of $7.63\times10^7$ cell $mL^{-1}$ was obtained.

To conduct the adhesion assays, a volume of 4 mL of the industrial water and the *E. coli* inoculated suspensions was transferred into separate wells of a sterile 6-well polystyrene, flat-bottomed microtiter plate (VWR International, Portugal) containing a single coupon of the tested materials (stainless steel or SICON®). The microtiter plates were incubated under shaking conditions in order to obtain an average shear stress of 0.25 Pa (Salek et al., 2011). This shear stress can be found in critical zones (corners, valves, angles, pumps, etc) in industrial plants (Cunault et al., 2015; Jensen and Friis, 2005; Lelièvre et al., 2002; Liu et al., 2006). The adhesion assays with industrial water (with and without the *E. coli* spike) were conducted at 5 ºC in order to mimic the industrial conditions found at the salad washing facilities. The low nutrient medium was also used in this work for comparison purposes and, in order to assess the effect of temperature on bacterial adhesion, assays were performed at 5 ºC and 30 ºC.

Coupons were removed from the wells at 0.5, 2 and 6 h and rinsed with sterile saline to remove loosely attached cells. Total cell counts were obtained by direct staining with 4',6-diamidino-2-phenylindole (DAPI), as previously described by Lemos et al. (2015). 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 10 fields were counted and the results were expressed as

logarithm of the number of attached cells per $cm^2$. Three independent experiments were performed for each surface and medium.

**2.5 Biofilm formation assays**

A flow cell system (see supplementary material, Figure S1) was used for these assays and it is composed by a recirculating tank, one vertical flow cell, peristaltic and centrifugal pumps and one intermediate fermenter (Teodósio et al., 2011). This fermenter contained initially 375 mL of sterile inoculation medium and was inoculated with 50 mL of the overnight culture. After inoculation, it was fed (14.5 mL $h^{-1}$) with sterile inoculation medium. The culture was then left to grow under agitation (with a magnetic stirrer) during 4 h at room temperature and aerated using an air pump (air flow rate 250 L $h^{-1}$). After this time, the culture was used to continuously inoculate (0.025 L $h^{-1}$) the recirculating tank of the flow cell system, initially containing 1.5 L of saline solution. The recirculating tank was also fed (300 mL $h^{-1}$) with the sterile low nutrient medium used in the adhesion assay (1:100 dilution of the inoculation medium). Biofilms were formed in SICON® or stainless steel coupons, placed in the vertical flow cell (a semicircular duct). The bacterial suspension was circulated in the system at a flow rate of 300 L $h^{-1}$ in order to obtain a shear stress of 0.25 Pa (Teodósio et al., 2013). Temperature was kept at 30 ºC during operation and, prior to use, the system was properly cleaned with water and diluted bleach (Teodósio et al., 2011). Before inoculation, the bleach solution was completely removed and the system was rinsed with sterile water. Biofilm formation was monitored for 5 days. For biofilm sampling (Teodósio et al., 2011), the system was stopped to allow coupon removal and carefully started again maintaining the flow conditions described above. Coupons were removed daily (24 h step) from the flow cell (and replaced by new ones) and the number of CFU per mL was determined by viable plate counting in

PCA (Plate Count Agar, VWR). The biofilm thickness was determined using a digital micrometer (VS-30H, Mitsubishi Kasei Corporation) (Pereira et al., 2002). Three independent experiments were performed for each surface.

**2.6 Biofilm cleaning and regrowth**

After 5 days of biofilm formation, the intermediate fermenter was disconnected from the recirculating tank and the flow cell system was emptied. A solution of 0.2% commercial bleach (Continente, Portugal) was then applied and recirculated (at 300 L $h^{-1}$) in the system for 20 min. The system was then emptied and filled with sterile water that was recirculated for an additional 20 min in order to remove the disinfectant from the system. The water was then removed and the system was filled with fresh sterile low nutrient medium (1:100 dilution of the inoculation medium) and the recirculation restored maintaining the same flow conditions. The coupons with biofilm formed during 5 days were analyzed immediately after the cleaning process, after 6, 18 and 24 h in order to determine the number of cells per $cm^{-2}$ and the biofilm thickness. Three independent experiments were performed for each surface.

The killing values reported in Table 2 were calculated by the Log difference between the value before treatment (corresponding to a 5-day old biofilm) and immediately after treatment. The biofilm removal values were calculated based on the percentage of biofilm thickness decrease immediately after treatment. The biofilm regrowth was calculated by the Log differences between the values determined at 6 h and at 18 h and the value immediately after treatment. Regrowth was also assessed by the thickness increase percentage at the same time points.

**2.7 Statistical analysis**

Paired *t*-test analyses were performed to evaluate if statistically significant differences were obtained with the two materials. Three independent experiments were performed for each surface. Each time point was evaluated individually using the three independent results obtained with stainless steel and the three individual results obtained with SICON®. Results were considered statistically different for a confidence level greater than 95% ($P < 0.05$). Standard deviation between the 3 values obtained from the independent experiments was also calculated.

**3. Results**

Surface properties of SICON® and stainless steel were first determined and the results are presented in Table 1. From the total free energy results it is possible to observe that both surfaces are hydrophobic ( $\Delta G < 0$ mJ m$^{-2}$), although stainless steel is more hydrophobic. The ratio proposed by Liu and Zhao (2011) between the the Lifshitz van der Waals apolar component and the electron donor component ( $\gamma^{LW} / \gamma^{-}$ ) was also calculated for each surface. Results showed that a higher ratio was obtained for stainless steel. Additionally, it was observed that both surfaces have similar roughness and total surface energy ( $\gamma^{Tot}$ ).

Regarding the bacterial adhesion results, in Figure 1 it is possible to observe that similar adhesion values were obtained on both surfaces at 5 ºC using industrial water (Figure 1a). Moreover, adhesion did not increase with time (from 0.5 to 6 h). Similar results ($P > 0.05$) were also obtained using industrial water spiked with *E. coli* (Figure 1b) and in the low nutrient medium with *E. coli* (Figure 1c) at 5 ºC. Regarding the results obtained at 30 ºC (Figure 1d), similar adhesion was observed on both surfaces ($P > 0.05$) and these values did not increase with time (from 0.5 to 6 h). However, a higher bacterial adhesion was obtained at 30 ºC (Figure 1d) than at 5 ºC (Figure 1c) ($P < 0.05$).

Biofilm growth on SICON® and stainless steel was followed for 5 days and the results are depicted in Figure 2. In general, the number of viable cells (Figure 2a) and the biofilm thickness (Figure 2b) was lower (on average 1 Log and 18 %, respectively) on SICON® than on stainless steel. The lowest amount of biofilm formed was observed on SICON® on day 1 (2 Log difference and 28% thinner than in stainless steel). Regarding the number of viable cells, an increase of 1.3 and 2.5 Log was observed on stainless steel and SICON®, respectively from day 1 to day 3. From day 3 onwards the number of viable cells remained almost constant. Biofilm thickness on stainless steel was almost constant during the 5-day assay.

A cleaning protocol was applied on the 5-day old biofilms (Figure 3). The number of viable cells and biofilm thickness were measured immediately after the treatment (1 h time point) and after 6, 18 and 24 h after treatment. In Figure 3 it is possible to observe that before the treatment, the number of viable cells (Figure 3a) was less than 1 Log lower on SICON® than on stainless steel and that after biofilm treatment this gap increased to 2.6 Log. Regarding biofilm thickness (Figure 3b), values obtained with SICON® were always lower than those obtained with stainless steel (although the differences were not statistically significant).

The killing and removal efficiencies obtained immediately after treatment and after 18 h are indicated in Table 2 along with the biofilm regrowth. Values show that immediately after treatment, the number of killed bacteria was higher on SICON® than on stainless steel (1.9 Log) and the percentage of biofilm removed was also higher (9%). The viability results showed a small regrowth on stainless steel and almost a full recovery of the biofilm in SICON® after 18 h.

## 4. Discussion

The results obtained in this work show that the natural microbial flora present in the industrial water adhered similarly to SICON® and stainless steel. Moreover, addition of another microorganism to this water in significant amounts (a 2.3 Log increase in bacterial load) or changing the culture medium did not affect adhesion. Thus, it seems that under the conditions tested, cell adhesion was not strongly influenced by planktonic cell concentration, surface type or composition of the culture medium. However, bacterial adhesion was significantly higher at the highest temperature tested (30 ºC). It has been reported that temperatures above 18 ºC and below 55 ºC can potentiate microbial development and food deterioration (Garayoa et al., 2014; Kim et al., 2013; Kuo and Chen, 2010). Additionally, 30 ºC may also be a critical temperature as it is the optimal growth temperature for many mesophilic bacteria, including pathogenic *E. coli*, *Salmonella spp*., and *Bacillus cereus* (Kim et al., 2013). Despite the higher bacterial adhesion observed at the higher temperature, it was again verified that similar adhesion results were obtained on both surfaces. From the surface properties analysis it was possible to verify that both surfaces are hydrophobic and have a similar roughness. According to the thermodynamic theory, a higher bacterial adhesion was expected on stainless steel, the most hydrophobic surface ((Van Oss and Giese, 1995; Wang et al., 2011). However, this was not observed indicating that other factors affected adhesion. Although it has been suggested that surface roughness may affect bacterial adhesion, it has also been shown that high roughness values (in the order of bacterial size), increase the contact area (and hence the binding potential) whereas surfaces with roughness features smaller than cells have little effect (Whitehead et al., 2006). Within the food processing industry, regulations stipulate that hygienic surfaces have a roughness value of $\leq 0.8$ μm (Flint et al., 1997). Both surfaces tested in this work have a small roughness (0.1 μm) when compared to the size of the microorganism present in the tested media (> 0.5 μm, as observed by microscopy) and with

the hygienic roughness value specified in the industrial regulations. Thus, it seems natural that surface roughness was not controlling bacterial adhesion. In a previous work using this strain, it was verified that shear stress can affect cell adhesion and can even modulate the effects of the surface properties (Moreira et al., 2014a). Thus, it is also possible that the hydrodynamic conditions were controlling bacterial adhesion in the present study and that this effect was more significant than other factors such as bacterial composition, bacterial concentration, medium composition or surface properties.

Since a higher bacterial adhesion was obtained in the low nutrient medium at 30 ºC, these conditions were selected to conduct the biofilm formation and cleaning assays in order to test the surfaces under the worst possible scenario. Results showed that lower amounts of biofilm were obtained on SICON®.

Although a correlation between surface properties and bacterial adhesion has not been found, possibly due to the predominant effect of the hydrodynamic conditions, surface properties had some effect on biofilm formation. A higher biofilm formation was observed on stainless steel, the most hydrophobic surface as expected according to the thermodynamic theory (Van Oss and Giese, 1995; Wang et al., 2011) and the surface with the highest $\gamma^{LW}/\gamma^{-}$ ratio. In a previous work (Moreira et al., 2015), a correlation between the $\gamma^{LW}/\gamma^{-}$ ratio of metallic and polymeric surfaces and biofouling formation was observed. In that work, it was concluded that biofouling establishment can be reduced in surfaces with lower $\gamma^{LW}/\gamma^{-}$ as it happened with SICON®. These results demonstrated that, for the particular conditions tested on this work, the use of SICON® enables operation at a higher hygienic status (1.1 Log difference). Additionally, for an operational time frame of 24 h, a reduction of 2.1 Log can be obtained if SICON® is used instead of stainless steel.

Regarding the biofilm cleaning process, it was verified that the treatment led to a reduction in the biofilm viability and thickness on both surfaces. However, a higher log reduction was observed on SICON®, and although a similar biofilm thickness reduction has been attained after 6 h of the cleaning on both surfaces, this effect was immediate on SICON® and delayed on stainless steel. In the study made by Boxler et al. (2013a), it was found that protein deposits were easier to clean from SICON® than from stainless steel. Additionally, it was observed that on surfaces such as SICON® the foulant was almost completely removed by the flow within the first five minutes of the experiment due to the high $\gamma^-$. It was concluded that the $\gamma^-$ component affects the adhesive and cohesive strength of the deposit and consequently the force required to clean the surface. In the present work, it also seems that a higher $\gamma^-$ (or a lower $\gamma^{LW} / \gamma^-$) in SICON® affected the biofilm adhesive and cohesive strength and this may have facilitated biofilm removal by the fluid flow.

No biofilm regrowth on stainless steel was observed after treatment during the 24 h assay. However, a rapid bacterial growth was observed on SICON® after an induction period of 6 hours. This result seems to indicate that the treatment was efficient both on killing and removal of the biofilm formed on stainless steel. Regarding the SICON® surface, although a bacterial regrowth has been detected by the viability test, the same increase was not observed by thickness. It is possible that the treatment only affected the top biofilm layer, killing part of the active bacteria although it appears that these dead cells were not totally removed. Thus, it is possible that during the regrowth phase, cells that have resisted treatment started to multiply, originating new cells which replaced the dead cells on the top layer. Interestingly, these results showed that viable cell determination as the sole indicator for evaluating the performance of CIP operations may not be sufficient to assess the true extent of biofilm reduction. The results obtained in the cleaning process demonstrated that, for the particular conditions tested in this work, there is a time window of more

than 6 h in which operation can proceed at a much higher hygienic status if SICON® is used (2 Log difference). This is particularly important for food industries like the salad washing industry which have cleaning cycles every 6-8 hours.

## 5. Conclusions

In this work, it was shown that bacterial adhesion on SICON® and stainless steel were similar. Bacterial adhesion increased at a higher temperature demonstrating that this parameter has influence on biofouling establishment under the tested conditions. It was also shown that the use of SICON® enables biofilm reduction and, after cleaning, biofilm inactivation is higher than on stainless steel. These results may be explained by the lower $\gamma^{LW} / \gamma^{-}$ value that may have affected the biofilm structure. An operational time window exceeding 6 hours was identified for the conditions used in this work where a higher hygienic status can be attained if SICON® is used instead of stainless steel. The duration of this time window as well as the magnitude of this difference are likely to depend on the process conditions and need to be evaluated for each individual case. Additionally, it was also observed that the standard methods used in food industry to detect the presence of the biological contaminants on the equipment surfaces (viable counts or ATP level) may not be a good indicators of the cleaning efficiency if used on their own.

The results obtained in this work showed that by using SICON®, a more sustainable manufacturing process can be implemented. If a satisfactory hygienic level is already attained with stainless steel, using SICON® may extend the operational time by reducing the frequency of cleaning or the duration of the cleaning period which may reduce the global production costs. Additionally, the same hygienic level may also be attained by lowering the concentration or the amount of cleaning agent leading not only to a reduction in costs but also a reduction in the environmental impact.

Moreover, there is also a potential for cleaning water saving. However, the replacement of existing materials or the production of equipment with modified surfaces usually has a high cost. Therefore, the application of modified surfaces would be more suitable for critical areas such as corners, valves or other types of areas which are shielded from the main flow, where bacterial attachment is more likely to occur and where cleaning is particularly difficult. It is possible that the economic savings and the benefit of implementing a more sustainable manufacturing process compensate for the additional capital investment necessary to use SICON® surfaces instead of stainless steel in critical areas of the process line.

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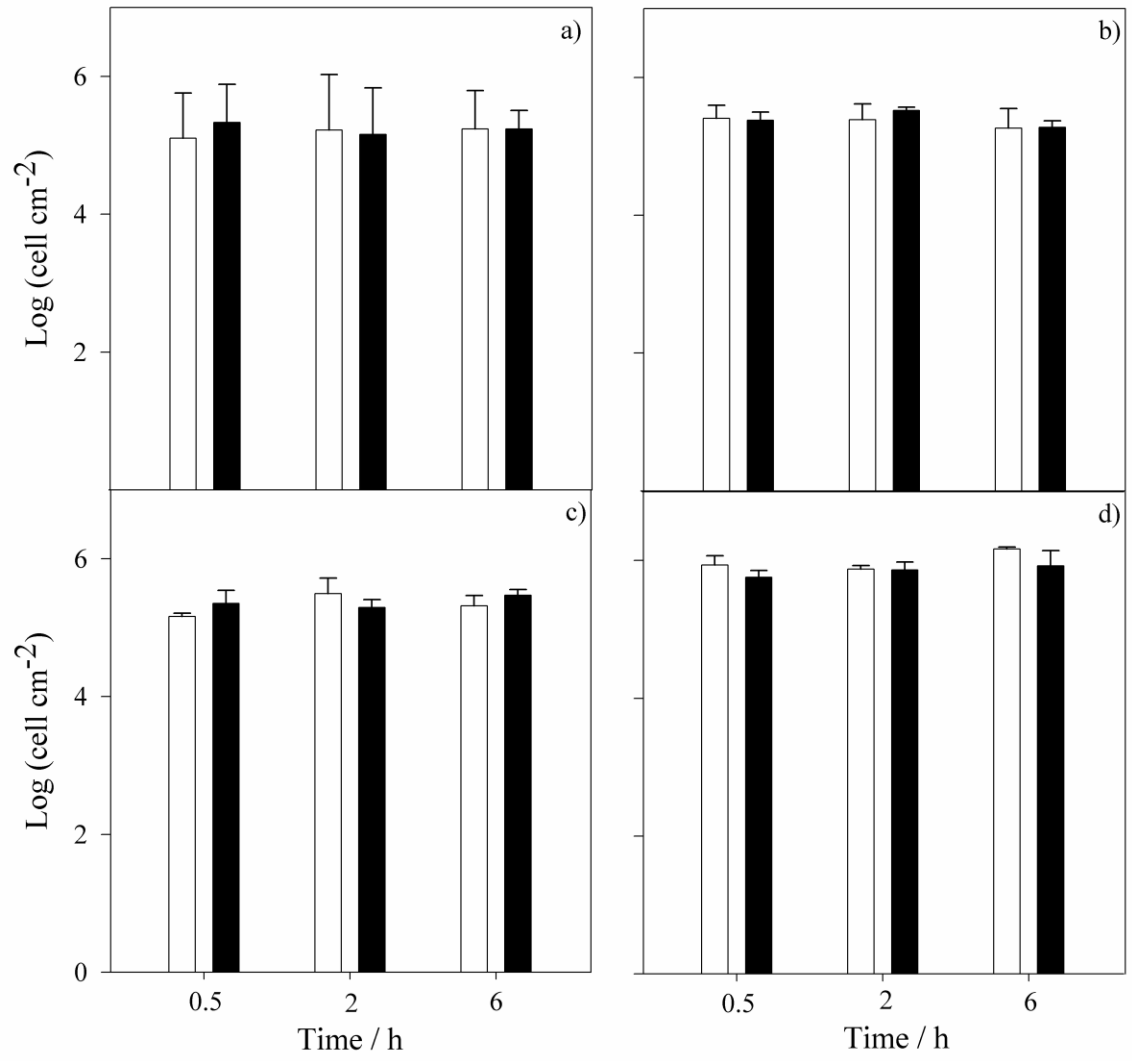
**Figure captions**

Figure 1 Number of attached cells evaluated at three time points (0.5, 2 and 6 h) in SICON® (white bar) and stainless steel (black bar) surfaces using a) industrial water at 5 ºC, b) industrial water with spiked with *E. coli* at 5 ºC, c) medium with *E. coli* at 5 ºC and d) medium with *E. coli* at 30 ºC. Error bars shown for each surface, at each time point, represent the standard deviation from three independent experiments.

Figure 2 Time-course evolution of biofilm development: a) number of viable cells in the biofilm and b) biofilm thickness. Closed symbols – biofilm formed on stainless steel, open symbols – biofilm formed on SICON®. Statistical analysis corresponding to each time point is represented with a star for a confidence level greater than 95% ($P < 0.05$). Error bars shown for each surface, at each time point, represent the standard deviation from three independent experiments.

Figure 3 Time-course evolution of biofilm regrowth after cleaning: a) number of cells in the biofilm and b) biofilm thickness. Closed symbols – biofilm regrowth on stainless steel, open symbols – biofilm regrowth on SICON®. The points at 0 h are the results of the biofilm formed before cleaning (biofilm formed during 5 days), after this point a cleaning protocol was applied and the first measurement was made immediately after cleaning (at 1 h). Statistical analysis corresponding to each time point is represented with a star for a confidence level greater than 95% ($P < 0.05$). Error bars shown for each surface, at each time point, represent the standard deviation from three independent experiments.

Figure S1 Representation of the flow cell system used.

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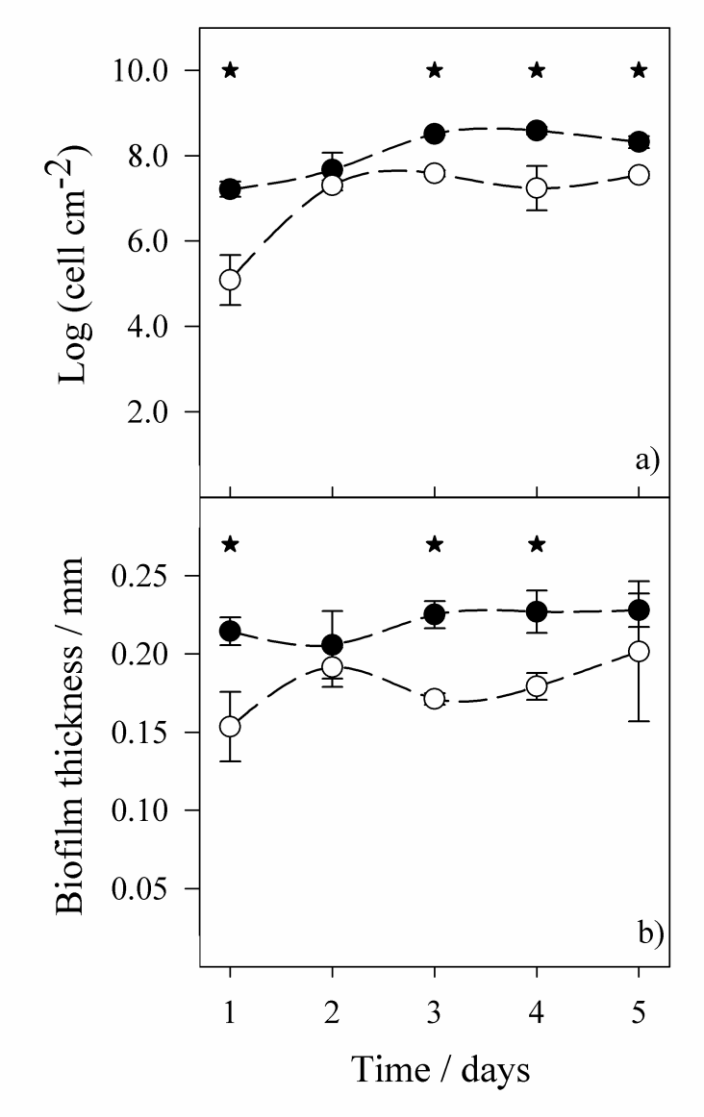


Figure 2 Time-course evolution of biofilm formation: a) number of viable cells in the biofilm and b) biofilm thickness. Closed symbols – biofilm formed on stainless steel, open symbols – biofilm formed on SICON®. Statistical analysis corresponding to each time point is represented with a star for a confidence level greater than 95% ($P < 0.05$). Error bars shown for each surface, at each time point, represent the standard deviation from three independent experiments.

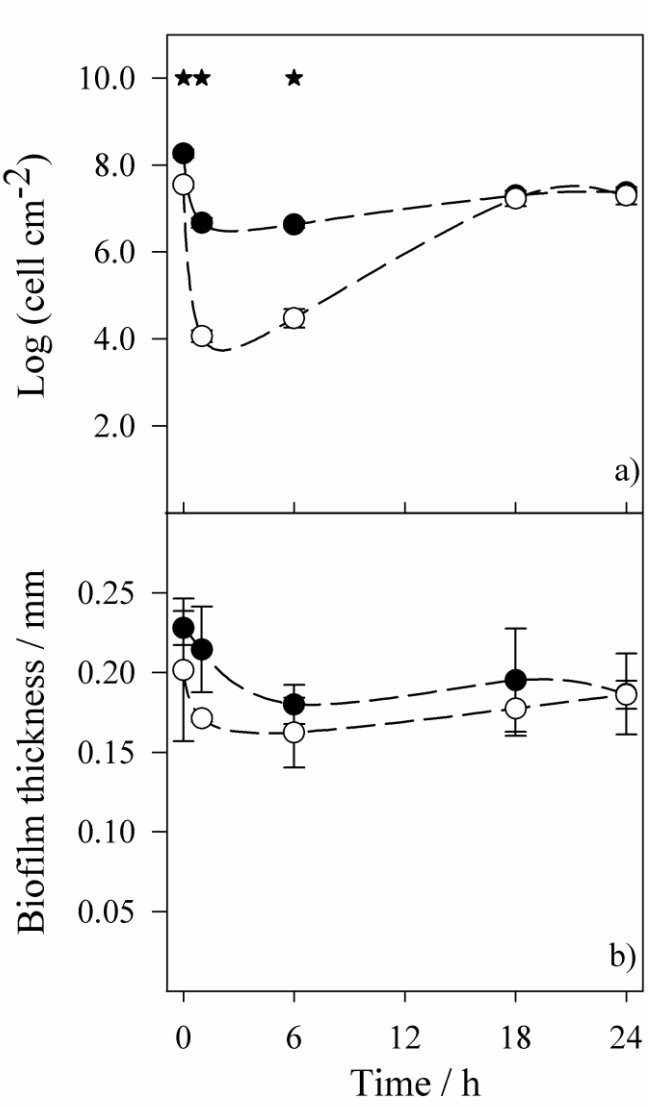


Figure 3 Time-course evolution of biofilm regrowth after cleaning: a) number of cells in the biofilm and b) biofilm thickness. Closed symbols – biofilm regrowth on stainless steel, open symbols – biofilm regrowth on SICON®. The points at 0 h are the results of the biofilm formed before cleaning (biofilm formed during 5 days), after this point a cleaning protocol was applied and the first measurement was made immediately after cleaning (at 1 h). Statistical analysis corresponding to each time point is represented with a star for a confidence level greater than 95% ($P < 0.05$). Error bars shown for each surface, at each time point, represent the standard deviation from three independent experiments.

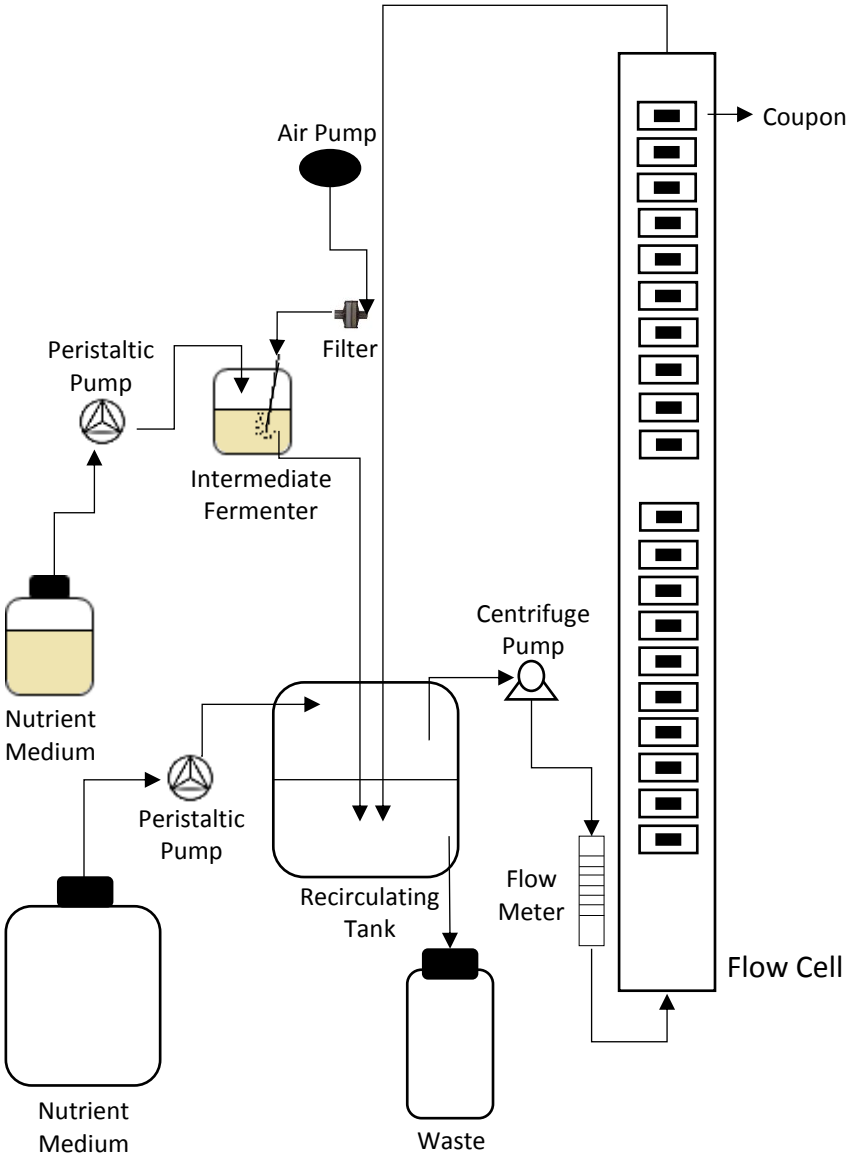


Figure S1 Representation of the flow cell system used.

Table 1 The total surface energy ( $\gamma^{Tot}$ ), the ratio between the Lifshitz van der Waals apolar component and the electron donor component ( $\gamma^{LW} / \gamma^{-}$ ), the hydrophobicity ( $\Delta G$ ) and roughness of stainless steel and SICON®.

| **Surface** | $\gamma^{Tot}$ / (mJ.m$^{-2}$) | ($\gamma^{LW} / \gamma^{-}$) / (mJ.m$^{-2}$) | $\Delta G$ / (mJ.m$^{-2}$) | Roughness[a]/ µm |
|---|---|---|---|---|
| **Stainless Steel** | 36.8 | 4.13 | -46.6 | 0.10±0.05 |
| **SICON®** | 33.4 | 2.75 | -34.1 | 0.11±0.0 |

[a] Mean roughness ($R_a$) values adapted from (Boxler et al., 2013a)

Table 2 Killing and removal efficiencies immediately after treatment, removal and regrowth 6 h after biofilm treatment and biofilm regrowth between 18 to 24 h after treatment.

| Parameter | Surface | Immediately after treatment | | 6 h after treatment | | 18-24 h after treatment |
|---|---|---|---|---|---|---|
| | | Removal | Killing | Removal | Regrowth | Regrowth |
| Log (CFU $cm^{-2}$) difference | Stainless steel | - | 1.6 | - | 0.0 | 0.6 |
| | SICON® | - | 3.5 | - | 0.4 | 3.2 |
| Thickness / % | Stainless steel | 5.9 | - | 21 | 0.0 | 0.0 |
| | SICON® | 15 | - | 19 | 0.0 | 3.5 |