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Evaluation of SICAN performance for biofouling mitigation in the food industry

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Abstract

Biological fouling in food industry leads to an increase in maintenance costs, decreases operational efficiencies and promotes food contamination leading to economic losses and the dissemination of foodborne pathogens. In order to maintain production efficiency and hygienic standards, cleaning in place (CIP) procedures are required. However, the existence of critical zones shielded from the main flow carrying the CIP disinfectants requires new strategies for reducing biofilm buildup and/or easy to clean surfaces. In this work, a Diamond-Like Carbon (DLC) coating modified by incorporation of silicon (a-C:H:Si or SICAN), was evaluated regarding bacterial adhesion, biofilm formation and cleanability. Assays included the natural flora present in industrial water (from a salad washing line) and *Escherichia coli*, one of the most persistent foodborne microorganisms. Results show that bacterial adhesion and biofilm formation on SICAN and stainless steel were similar, thus surface modification was not able to prevent biological fouling development. However, it was verified that after performing a cleaning protocol with chlorine, reduction of bacterial counts was much higher in SICAN (about 3.3 Log reduction) when compared to stainless steel (1.7 Log reduction). Although full biofilm recovery was observed on both surfaces 18 h after treatment, an operational window was identified for which processes with cleaning intervals of about 6 h could potentially use SICAN surfaces on critical areas (such as dead zones, crevices, corners, joints) and therefore operate at a much higher hygienic level than the one attained with stainless steel.

Keywords: Adhesion, Biofilm, Cleaning, *Escherichia coli,* SICAN, Stainless steel

1. Introduction

Biological fouling establishment in industrial piping, equipment and cooling systems is a serious problem in the food industry (Brooks & Flint, 2008). The formation of these biological deposits, starts with the interaction between planktonic (free floating) bacteria and the industrial surfaces (Srey, Jahid, & Ha, 2013). After a first contact, bacteria adhere to the surface and start producing extracellular polymeric substances. Further cell growth leads to biofilm formation (Costerton, Stewart, & Greenberg, 1999). Biofilms can cause equipment damage through corrosion, local clogging and heat transfer resistance, leading to increased maintenance costs and decreased equipment operational efficiencies (Characklis, 1981). Additionally, they can cause contamination of product and staff leading to economic losses and health incidents (Agle, 2007; Bott, 2011; Shi & Zhu, 2009).

Escherichia coli has been reported as one of the most persistent foodborne microorganisms (Dourou, et al., 2011; Sagong, et al., 2011; Shi & Zhu, 2009) that can be found in vegetable process industries, meat industries and ready-to-eat products (Srey, et al., 2013). The presence of *E. coli* on food-contact surfaces is well documented and has been associated with the ability of this bacterium to attach and form biofilms on these surfaces (Dourou, et al., 2011). In a biofilm, bacteria are more resistant to biocides becoming more difficult to eradicate (Simões, Simões, & Vieira, 2010). Sodium hypochlorite is the most widely used industrial disinfectant (Lomander, Schreuders, Russek-Cohen, & Ali, 2004). Despite its proven efficiency against planktonic bacteria, care must be taken when dealing with adhered bacteria (Luppens, Reij, van der Heijden, Rombouts, & Abee, 2002). Rossoni and Gaylarde (2000) isolated *E. coli*, *Pseudomonas fluorescens* and *Staphylococcus aureus* from chicken carcasses and studied the efficiency of sodium hypochlorite (100 and 200 ppm from a 10% active chlorine formulation) and peracetic acid (250 and 1000 ppm from a 14% active ingredient formulation) on the killing and removal of

those bacteria adhered on a stainless steel surface. In all cases, sodium hypochlorite was more effective than peracetic acid and 1.5 to 2 Log reductions were obtained with *E. coli* (lower reductions were obtained for the other species).

In order to maintain the production efficiency and meet hygienic standards, regular and intensive cleaning in place (CIP) procedures have been implemented in industry (Shi & Zhu, 2009). Cleaning times in some food industries can represent up to 15% of the total production time (Mauermann, Eschenhagen, Bley, & Majschak, 2009) and some typical cleaning frequencies are indicated in Table 1. These frequent stops for cleaning, as well as the energy and chemical products used for disinfection translate into elevated costs (Mauermann, et al., 2009). Despite the progress in the field of industrial CIP, this operation is still difficult and depends on many factors like the nature and age of the biological fouling layer, the cleaning agent composition and concentration, the degree of turbulence of the cleaning solution and the characteristics of the surface (Bremer, Fillery, & McQuillan, 2006; Pogiatzis, Vassiliadis, Mergulhão, & Wilson, 2014). In industrial plants it is common to find critical zones such as crevices, corners, joints, valves, which are difficult to clean due to difficult access and where lower fluid velocities may be found, making these zones suitable niches for biofilm accumulation and growth (Lemos, Mergulhão, Melo, & Simões, 2015). One of the approaches to reduce biological fouling and to increase the removal of formed deposits in these critical zones is the modification of the energetic and topographic surface properties (Mauermann, et al., 2009) in order to reduce bacterial adhesion and to improve cleanability (Boxler, Augustin, & Scholl, 2013b). Diamond-like carbon (DLC) coatings, approved as food contact surfaces, have been investigated as alternative to stainless steel in food manufacturing plants due to their thermal conductivity, low friction, smoothness, wear resistance and anti-fouling properties (Boxler, Augustin, & Scholl, 2013a). A modification of DLC coatings

by incorporating silicon (a-C:H:Si) leads to some additional characteristics such as increased optical transmittance and thermal resistance (Corbella, Bialuch, Kleinschmidt, & Bewilogua, 2009). Some reports have shown the benefits of using modified DLC coatings on abiotic fouling mitigation in the food industry. Augustin, Geddert, and Scholl (2007) investigated the influence of aluminum, copper, DLC coatings, and modified DLC coatings (a-C:H:Si/SICAN and a-C:H:Si:O/SICON®) on the induction period of whey protein fouling deposition. These authors observed that the fouling induction period may be extended in an electro-polished stainless steel surface coated with SICAN. In other study (Geddert, Bialuch, Augustin, & Scholl, 2007), it was observed that this surface also extended the induction period of $CaSO_4$ crystallization. Boxler, et al. (2013b) investigated the influence of SICAN and SICON® coatings, against milk fouling. Results showed that surface modification directly affected the formation of deposits, their composition, as well as their adhesive strength. They concluded that SICAN was effective for abiotic fouling mitigation. Later, these authors validated their results in a pilot-scale plate heat exchanger and concluded that SICAN is a good alternative to stainless steel to be applied on heat transfer surfaces in the food industry (Boxler, Augustin, & Scholl, 2014). Additionally, Boxler, et al. (2013a) also verified in another study that cleaning of whey protein and milk salts is easier on SICAN than on stainless steel.

Despite these promising evidences showing the beneficial effects of SICAN in the mitigation of abiotic fouling, no studies were performed concerning bacterial fouling in industrial conditions. In this work, the behavior of a-C:H:Si/SICAN coatings and stainless steel were compared regarding *E. coli* adhesion, biofilm formation and cleaning. Assays tried to replicate industrial settings using process water from a salad washing line and also testing some extreme operational conditions (higher temperature and contact times) to evaluate if the use of this modified surface in critical

118 areas could be beneficial in maintaining a higher hygienic level in industrial plants.

119

120 **2. Material and methods**

121 **2.1. Bacteria and culture conditions**

122 *Escherichia coli* JM109(DE3) from Promega (USA) was used in this study because it has shown
123 a good biofilm forming ability in a variety of *in vitro* platforms operated at different shear stresses
124 (Moreira, et al., 2014; Moreira, et al., 2013; Teodósio, Simões, Alves, Melo, & Mergulhão, 2012).
125 Additionally, it was shown that its biofilm formation is similar to other *E. coli* strains which are
126 often used for antimicrobial susceptibility and disinfection tests (Gomes, et al., 2014). A starter
127 culture was obtained by inoculation of 500 μL of a glycerol stock (kept at -80 ºC) to a total volume
128 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
129 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
130 Teodósio et al. (2011). This culture was grown in a 1 L shake-flask, incubated overnight at 30 ºC
131 with orbital agitation (120 rpm). A volume of 100 mL of this culture was used for the adhesion
132 assays described in section 2.4. A volume of 50 mL of this culture was used to inoculate the
133 intermediate fermenter used for the biofilm assays described in section 2.5.

134

135 **2.2. Surface preparation**

136 Round coupons (1 cm of diameter) made from electro-polished stainless steel (AISI 316L/
137 X2CrNiMo17-12-2/1.4404) and SICAN coated coupons were tested. The coatings were prepared
138 by the Fraunhofer Institute for Surface Engineering and Thin Films (IST) in Braunschweig,
139 Germany and a detailed description of the SICAN preparation method was disclosed before
140 (Corbella, et al., 2009; Grischke, Hieke, Morgenweck, & Dimigen, 1998).

141 Surfaces were cleaned with ethanol and then immersed in a commercial bleach (Continente,
142 Portugal) solution (0.2% v/v) for 20 min under strong agitation. To remove the bleach, coupons
143 were aseptically rinsed and washed again with sterile distilled water under strong agitation, for 20
144 min.
145
146 **2.3. Surface characterization**
147 Surface hydrophobicity was evaluated considering the Lifshitz-van der Waals acid base approach
148 (van Oss, 1994). The contact angles were determined automatically by the sessile drop method in
149 a contact angle meter (OCA 15 Plus; Dataphysics, Filderstadt, Germany) using water, formamide
150 and α-bromonaphtalene (Sigma) as reference liquids. The surface tension components of the
151 reference liquids were taken from literature (Janczuk, Chibowski, Bruque, Kerkeb, & Gonzales-
152 Caballero, 1993). For each surface, measurements with each liquid were performed at 25 ± 2 ºC.
153 The model proposed by van Oss (1994) indicates that the total surface energy (γ^{Tot}) of a pure
154 substance is the sum of the Lifshitz-van der Waals components of the surface free energy (γ^{LW})
155 and Lewis acid-base components (γ^{AB}):

156 $$\gamma^{Tot} = \gamma^{\mathrm{LW}} + \gamma^{\mathrm{AB}} \quad (1)$$

157 The polar AB component comprises the electron acceptor γ^{+} and electron donor γ^{-} parameters,
158 and is given by:

159 $$\gamma^{\mathrm{AB}} = 2\sqrt{\gamma^{+}\gamma^{-}} \quad (2)$$

160 The surface energy components of a solid surface (s) are obtained by measuring the contact angles
161 (θ) with the three different liquids (l) with known surface tension components, followed by the
162 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 (ΔG mJ.m^{-2}) between two entities of that surface immersed in a polar liquid (such as water (w) as a model solvent). ΔG 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.

2.4. Adhesion assays

The adhesion assays were made in three different media, an industrial water collected from a salad washing line (with a bacterial load of 3.3x10^5 CFU mL^{-1} and a Chemical Oxygen Demand below 26 mgO_2 L^{-1}), the same industrial water spiked with *E. coli* JM109(DE3) and 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*, two aliquots (50 ml) from the overnight grown culture (described in section 2.1) were used to harvest cells by centrifugation (10 min, 3,202 *g*). Cells were washed twice and then resuspended in the final medium. 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 7.60x10^7 cell mL^{-1}. The same volume was used for the industrial water and a total bacterial load of 7.63x10^7 cell mL^{-1} was obtained.

185 To conduct the adhesion assays, a volume of 4 mL of the industrial water and the *E. coli* inoculated
186 suspensions was transferred into separate wells of a sterile 6-well polystyrene, flat-bottomed
187 microtiter plate (VWR Internacional, Portugal) containing a single coupon of the tested materials
188 (stainless steel or SICAN). The microtiter plates were incubated under shaking conditions in order
189 to obtain an average shear stress of 0.25 Pa (Salek, Sattari, & Martinuzzi, 2011). This shear stress
190 can be found in critical zones (corners, valves, angles, pumps, etc) in industrial plants (Cunault, et
191 al., 2015; Jensen & Friis, 2005; Lelièvre, et al., 2002; Liu, et al., 2006). The adhesion assays with
192 the industrial water (with and without the *E. coli* spike) were conducted at 5 ºC in order to mimic
193 the industrial conditions found at the salad washing facilities (Figure 1). The low nutrient medium
194 was also used in this work for comparison purposes and, in order to assess the effect of temperature
195 on bacterial adhesion, assays were performed at 5 ºC and 30 ºC. It was found that *E. coli* adhesion
196 (during 2 h) was similar at these temperatures on both surfaces (see supplementary material, Figure
197 S1). However, it has been reported that temperatures above 18 ºC and below 55ºC can potentiate
198 microbiological development and food deterioration, (Garayoa, Díez-Leturia, Bes-Rastrollo,
199 García-Jalón, & Vitas, 2014; Kim, Yun, Lee, Hwang, & Rhee, 2013; Kuo & Chen, 2010).
200 Therefore, a temperature of 30 ºC was selected to conduct the biofilm formation and cleaning
201 assays (Figure 2) in order to test these surfaces under the worst possible conditions.
202 At different sampling times, between 0.5 and 6 h, coupons were removed from the wells and rinsed
203 with sterile saline to remove loosely attached cells. Total cell counts were obtained by direct
204 staining with 4',6-diamidino-2-phenylindole (DAPI) due to the high sensitivity of the method, as
205 previously described by Lemos et al. (2014). Cells were visualized under an epifluorescence
206 microscope (Eclipse LV100, Nikon, Japan) equipped with a filter block sensitive to DAPI
207 fluorescence (359-nm excitation filter in combination with a 461-nm emission filter). For each

208 coupon, a minimum of 10 fields were counted and the results were expressed as logarithm of the
209 number of attached cells per cm^2. Three independent experiments were performed for each surface
210 and medium.
211
212 **2.5. Biofilm formation and sampling**
213 A flow cell system (see supplementary material, Figure S2) was used for these assays and it is
214 composed by a recirculating tank, one vertical flow cell, peristaltic and centrifuge pumps and one
215 intermediate fermenter (Teodósio, et al., 2011). This fermenter contained initially 375 mL of sterile
216 inoculation medium and was inoculated with 50 mL of the overnight culture (described in section
217 2.1). After inoculation, it was fed (14.5 mL h^{-1}) with sterile inoculation medium. The culture was
218 then left to grow under agitation (with a magnetic stirrer) during 4 h at room temperature and
219 aerated using an air pump (air flow rate 250 L h^{-1}). After this time, the culture was used to
220 continuously inoculate (0.025 L h^{-1}) the recirculating tank of the flow cell system, initially
221 containing 1.5 L of saline solution (8.5 g L^{-1} NaCl). The recirculating tank was also fed (300 mL
222 h^{-1}) with the sterile low nutrient medium used in the adhesion assay described in section 2.4 (1:100
223 dilution of the inoculation medium). Biofilms were formed on SICAN or stainless steel coupons,
224 cleaned as described in section 2.2 and placed in the vertical flow cell. The bacterial suspension
225 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
226 (Teodósio, et al., 2013). Temperature was kept at 30 ºC and biofilm formation was monitored for
227 five days. For biofilm sampling (Teodósio, et al., 2011), the system was stopped to allow coupon
228 removal and carefully started again maintaining the flow conditions described above. Coupons
229 were removed daily (24 h step) from the flow cell (and replaced by new ones to seal the system)

and the number of CFU per mL was determined by viable plate counting using PCA (plate counting agar). Three independent experiments were performed for each surface.

2.6. Cleaning and regrowth

After the five days of biofilm formation, the intermediate fermenter was disconnected from the recirculating tank and the flow cell system was emptied. A disinfection 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 disinfection process and after 6 h, 18 h and 24 h in order to determine the number of CFU per mL. The amounts of removed biofilm were assayed by wet weight determination by weighing the coupons containing biofilms prior and after disinfection as described before (Teodósio, et al., 2011). Three independent experiments were performed for each surface.

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 and medium. Each time point was evaluated individually using the three independent results obtained with stainless steel and the three individual results obtained with SICAN. Results were considered statistically different for a confidence level greater than 95% ($P < 0.05$). Standard

253 deviation between the 3 values obtained from the independent experiments was also calculated.

254

255 **3. Results and Discussion**

256 Surface properties of SICAN and stainless steel were first determined and the results are presented
257 in Table 2. From the total free energy results it is possible to observe that both surfaces are
258 hydrophobic ($\Delta G < 0$ mJ m^{-2}). Regarding γ^- and γ^+, results showed that both surfaces are
259 monopolar electron donors. Additionally, it can be observed that the Lifshitz-van der Waals
260 component γ^{LW} contributed more significantly to the total surface energy γ^{Tot} than the acid-base
261 polar component γ^{AB}. It was also possible to verify from the literature (Boxler, et al. 2013a) that
262 both surfaces have a similar roughness.

263 Figure 1 shows the cell adhesion results on both surfaces at 5 ºC. Similar adhesion results were
264 obtained on both surfaces using industrial water (figure 1a). Moreover, adhesion did not increase
265 with time (from 0.5 to 6 h). These results showed that microorganisms belonging to the natural
266 flora present in the industrial water are capable of adhering to both surfaces equally. Furthermore,
267 addition of another microorganism in significant amounts (2.3 Log difference) did not potentiate
268 cell adhesion (figure 1b) indicating that adhesion was not affected by planktonic cell concentration
269 in any of the surfaces ($P > 0.05$). This was further confirmed by the results in the low nutrient
270 medium (figure 1c) which were similar to the ones obtained in the previous conditions ($P > 0.05$).
271 Thus, it seems that under the conditions tested, cell adhesion was not strongly influenced by
272 planktonic cell concentration, surface type and composition of the culture medium (Figure 1) and
273 also assayed temperature (Figure S1). In a study by Azevedo, Pinto, Reis, Vieira, & Keevil (2006)
274 with *Helicobacter pylori*, temperatures between 4 ºC and 37 ºC were tested and it was also
275 observed that there was no effect of the temperature on *H. pylori* adhesion to stainless steel. The

276 similar adhesion values observed on both surfaces may be explained by the effect of the surface
277 properties. Both surfaces are hydrophobic and have similar roughness (table 2) and it is known
278 that these two parameters have an important role on microbiological adhesion (Goulter, Gentle, &
279 Dykes, 2009; Schlisselberg & Yaron, 2013), thus in this work it seems that adhesion is being
280 controlled by these two parameters. Boxler, et al. (2013a) performed a study with several surfaces
281 for milk soil fouling mitigation. They also observed that a similar deposit mass was obtained on
282 the electro-polished stainless steel and on the SICAN coating. In the present work, it was also
283 observed that an increase in planktonic cell load did not lead to an increase in the number of
284 adhered cells and that after initial adhesion (30 min), a cellular adhesion plateau was attained. In
285 the study made by Azevedo, et al. (2006) the effect of the inoculum concentration on the adhesion
286 of *H. pylori* to stainless steel was also evaluated. They observed that there was a maximum number
287 of bacterial cells that could adhere to a surface after a certain elapsed time and therefore increasing
288 the initial cellular concentration did not lead to a higher *H. pylori* adhesion. Cerca, Pier, Oliveira
289 & Azeredo (2004) reached a similar conclusion when studying coagulase-negative staphylococci
290 adhesion by a static and dynamic method. They observed that bacterial adhesion increased from
291 30 min to 120 min but from this time onwards the number of adhered bacteria remained constant.
292 In a previous study with the same strain, it was found that hydrodynamics (shear stress) was
293 controlling the thickness of an *E. coli* biofilm grown at two different substrate loading rates
294 (Teodósio, et al., 2011). It was also recently shown for this strain that shear stress can affect cell
295 adhesion and can even modulate the effects of the surface properties (Moreira, et al., 2014). Thus,
296 it is likely that the hydrodynamic conditions were also controlling bacterial adhesion in the present
297 study and their effect was stronger than the bacterial composition, bacterial concentration, medium
298 composition, surface properties and assay temperature. Although it has been reported that

299 temperatures above 18 ºC and bellow 55 ºC can potentiate microbiological development and food
300 deterioration, (Garayoa, et al., 2014; Kim, et al., 2013; Kuo & Chen, 2010), in the present study it
301 was verified that *E. coli* adhesion was not influenced by temperature.
302 Figure 2 depicts the bacterial adhesion values obtained at 30 ºC as well as the biofilm formation
303 and cleaning results. Since cleaning intervals in the food industry vary (Table 1), biofilm formation
304 was allowed to occur for 5 days prior to cleaning. In this figure it is possible to verify that bacterial
305 adhesion (until 6 h) was constant with time and similar on both surfaces for all time points tested
306 ($P > 0.05$). Regarding biofilm formation (from 24 until 120 h), it was also observed that similar
307 values were obtained on both surfaces ($P > 0.05$). A slight increase in the number of attached cells
308 was observed for both surfaces up to 72 h and after that the number of cells stabilized. It is well
309 known that after the bacterial adhesion phase, the biofilm starts to grow from the adhered cells and
310 a biofilm increase is observed until a pseudo steady state is reached. At this point, the biofilm
311 sloughing rate equals the growth rate and the steady state is attained (Melo & Bott, 1997). In this
312 work, this pseudo steady state was achieved at 72 h.
313 After 5 days of biofilm formation (120 h), the biofilm was treated with a 0.2% chlorine solution
314 (about 100 ppm) for 20 min and after this treatment it was possible to observe that the number of
315 viable cells (1 h after the beginning of the treatment) decreased approximately 1.7 Log on stainless
316 steel and 3.3 Log on SICAN (Figure 2). Besides viability, the amount of biofilm assayed by wet
317 weight determination showed that there was a 20% reduction after treatment in stainless steel
318 whereas a 40% reduction was obtained with SICAN (data not shown). An induction period of 6 h
319 was observed for both surfaces with a rapid bacterial growth for SICAN after this period. For the
320 particular conditions tested on this work, there was a time window of more than 6 h (Figure 2) for
321 which operation can proceed at a much higher hygienic status if SICAN is used (1.6 Log

322 difference). At 18 h after treatment, both surfaces recovered the bacterial colonization levels
323 attained during the first 24 h of biofilm formation. Some authors (Bang, et al., 2014; Lomander, et
324 al., 2004; Schlisselberg & Yaron, 2013) investigated the effect of surface properties on biofilm
325 susceptibility to chlorine. These authors verified that disinfection was more effective in smoother
326 surfaces. They suggested that in rougher surfaces, bacteria may hide and get protect from chlorine
327 and thus have a higher chance to survive. The surfaces tested in the present work have similar
328 surface energy and roughness, therefore, it seems that other parameters such as chemical
329 composition or surface charge may have affected biofilm topology or the properties of the biofilm
330 matrix resulting in a different biofilm sensitivity to chlorination.
331
332 **4. Conclusions**
333 In this work, it was shown that bacterial adhesion and biofilm formation on SICAN and stainless
334 steel were similar, thus the surface coating was not able to prevent biological fouling. It was also
335 shown that biofilm inactivation was higher in SICAN. An operational time window exceeding 6
336 hours was identified for the conditions used in this work where a higher hygienic status can be
337 attained if SICAN is used instead of stainless steel. The duration of this time window and also the
338 magnitude of this difference are likely to depend on the process conditions and need to be evaluated
339 for each individual case. Also, if a satisfactory hygienic level is already attained with stainless
340 steel, using SICAN may extend the operational time by reducing the frequency of cleaning or the
341 duration of the cleaning period. The optimization of a cleaning schedule is not a trivial task
342 (Pogiatzis, et al., 2014) but the same hygienic level may also be attained by lowering the
343 concentration or the amount of cleaning agent. Additionally, there is also a potential for cleaning
344 water saving. The implementation of these strategies will result in a more eco-friendly process

particularly in food industries with frequent CIP operations (Table 1).

There has been some interest in the application of modified surfaces for biofilm reduction in the food industry, as the ability to modify operational parameters (flow rates, equipment geometries, temperatures,) is often limited (Gomes da Cruz, et al., 2015). However, due to the low profit margin of some products, there is often limited capital resources to replace existing materials or to produce equipment with modified surfaces which usually have a higher cost (Gomes da Cruz, et al., 2015). 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 obtained from reducing water and disinfectants consumption or extension of the operational time may compensate for the capital investment necessary to use SICAN surfaces instead of stainless steel in critical areas of the process line.

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References

Agle, M. E. (2007). Biofilms in the food industry. In H. P. Blaschek, H. H. Wang & M. E. Agle (Eds.), *Biofilms in the food environment* (pp. 3-17). Iowa, USA: Blackwell publishing.

368 Augustin, W., Geddert, T., & Scholl, S. (2007). Surface treatment for the mitigation of whey
369 protein fouling. In H. Müller-Steinhagen, M. Reza Malayeri & A. P. Watkinson (Eds.),
370 *Heat Exchanger Fouling and Cleaning VII -Challenges and Opportunities*. (pp. 206-214).
371 Portugal: ECI Symposium Series.
372 Azevedo, N. F., Pinto, A. R., Reis, N. M., Vieira, M. J., & Keevil, C. W. (2006). Shear stress,
373 temperature, and inoculation concentration influence the adhesion of water-stressed
374 *Helicobacter pylori* to stainless steel 304 and polypropylene. *Appl Environ Microbiol,*
375 *72*(4), 2936-2941.
376 Bang, J., Hong, A., Kim, H., Beuchat, L. R., Rhee, M. S., Kim, Y., & Ryu, J.-H. (2014).
377 Inactivation of *Escherichia coli* O157:H7 in biofilm on food-contact surfaces by sequential
378 treatments of aqueous chlorine dioxide and drying. *Int J Food Microbiol, 191*(0), 129-134.
379 Bott, T. R. (2011). Biofilms in Industry. In T. R. Bott (Ed.), *Industrial Biofouling* (pp. 181-201).
380 Amsterdam: Elsevier.
381 Boxler, C., Augustin, W., & Scholl, S. (2013a). Cleaning of whey protein and milk salts soiled on
382 DLC coated surfaces at high-temperature. *J Food Eng, 114*(1), 29-38.
383 Boxler, C., Augustin, W., & Scholl, S. (2013b). Fouling of milk components on DLC coated
384 surfaces at pasteurization and UHT temperatures. *Food Bioprod Process, 91*(4), 336-347.
385 Boxler, C., Augustin, W., & Scholl, S. (2014). Influence of surface modification on the
386 composition of a calcium phosphate-rich whey protein deposit in a plate heat exchanger.
387 *Dairy Sci Technol, 94*(1), 17-31.
388 Bremer, P. J., Fillery, S., & McQuillan, A. J. (2006). Laboratory scale Clean-In-Place (CIP) studies
389 on the effectiveness of different caustic and acid wash steps on the removal of dairy
390 biofilms. *Int J Food Microbiol, 106*(3), 254-262.

391 Brooks, J. D., & Flint, S. H. (2008). Biofilms in the food industry: problems and potential
392 solutions. *International Journal of Food Science & Technology, 43*(12), 2163-2176.
393 Cerca, N., Pier, G. B., Oliveira, R., & Azeredo, J. (2004). Comparative evaluation of coagulase-
394 negative staphylococci (CoNS) adherence to acrylic by a static method and a parallel-plate
395 flow dynamic method. *Res Microbiol, 155*(9), 755-760.
396 Characklis, W. G. (1981). Bioengineering report: Fouling biofilm development: A process
397 analysis. *Biotechnol Bioeng, 23*(9), 1923-1960.
398 Corbella, C., Bialuch, I., Kleinschmidt, M., & Bewilogua, K. (2009). Up-scaling the production of
399 modified a-C:H coatings in the framework of plasma polymerization processes. *Solid State*
400 *Sci, 11*(10), 1768-1772.
401 Costerton, J. W., Stewart, P. S., & Greenberg, E. P. (1999). Bacterial biofilms: a common cause
402 of persistent infections. *Science, 284*(5418), 1318-1322.
403 Cunault, C., Faille, C., Bouvier, L., Föste, H., Augustin, W., Scholl, S., Debreyne, P., & Benezech,
404 T. (2015). A novel set-up and a CFD approach to study the biofilm dynamics as a function
405 of local flow conditions encountered in fresh-cut food processing equipments. *Food*
406 *Bioprod Process, 93*(0), 217-223.
407 Dourou, D., Beauchamp, C. S., Yoon, Y., Geornaras, I., Belk, K. E., Smith, G. C., Nychas, G.-J.
408 E., & Sofos, J. N. (2011). Attachment and biofilm formation by *Escherichia coli* O157:H7
409 at different temperatures, on various food-contact surfaces encountered in beef processing.
410 *Int J Food Microbiol, 149*(3), 262-268.
411 Garayoa, R., Díez-Leturia, M., Bes-Rastrollo, M., García-Jalón, I., & Vitas, A. I. (2014). Catering
412 services and HACCP: Temperature assessment and surface hygiene control before and
413 after audits and a specific training session. *Food Control, 43*(0), 193-198.

414 Geddert, T., Bialuch, I., Augustin, W., & Scholl, S. (2007). Extending the induction period of
415 crystallization fouling through surface coating. In Hans Müller-Steinhagen, M. Reza
416 Malayeri & A. P. Watkinson (Eds.), *Heat Exchanger Fouling and Cleaning VII -*
417 *Challenges and Opportunities* (Vol. RP5, pp. 221-228). Portugal: ECI Symposium Series.
418 Gomes da Cruz, L., Ishiyama, E. M., Boxler, C., Augustin, W., Scholl, S., & Wilson, D. I. (2015).
419 Value pricing of surface coatings for mitigating heat exchanger fouling. *Food Bioprod*
420 *Process, 93*(0), 343-363.
421 Gomes, L. C., Moreira, J. M. R., Teodósio, J. S., Araújo, J. D. P., Miranda, J. M., Simões, M.,
422 Melo, L. F., & Mergulhão, F. J. (2014). 96-well microtiter plates for biofouling simulation
423 in biomedical settings. *Biofouling, 30*(5), 1-12.
424 Goulter, R. M., Gentle, I. R., & Dykes, G. A. (2009). Issues in determining factors influencing
425 bacterial attachment: a review using the attachment of *Escherichia coli* to abiotic surfaces
426 as an example. *Lett Appl Microbiol, 49*(1), 1-7.
427 Grischke, M., Hieke, A., Morgenweck, F., & Dimigen, H. (1998). Variation of the wettability of
428 DLC-coatings by network modification using silicon and oxygen. *Diam Relat Mat, 7*(2–
429 5), 454-458.
430 Janczuk, B., Chibowski, E., Bruque, J. M., Kerkeb, M. L., & Gonzales-Caballero, F. J. (1993). On
431 the consistency of surface free energy components as calculated from contact angle of
432 different liquids: an application to the cholesterol surfaces. *J Colloid Interface Sci, 159*(2),
433 421-428.
434 Jensen, B. B. B., & Friis, A. (2005). Predicting the cleanability of mix-proof valves by use of wall
435 shear stress. *J Food Process Eng, 28*(2), 89-106.
436 Kim, S. A., Yun, S. J., Lee, S. H., Hwang, I. G., & Rhee, M. S. (2013). Temperature increase of

foods in car trunk and the potential hazard for microbial growth. *Food Control, 29*(1), 66-70.

Kuo, J.-C., & Chen, M.-C. (2010). Developing an advanced multi-temperature joint distribution system for the food cold chain. *Food Control, 21*(4), 559-566.

Lelieveld, H. L. M., Mostert, M. A., & Holah, J. (2005). *Handbook of hygiene control in the food industry*. England: Woodhead publishing limited.

Lelièvre, C., Legentilhomme, P., Gaucher, C., Legrand, J., Faille, C., & Bénézech, T. (2002). Cleaning in place: effect of local wall shear stress variation on bacterial removal from stainless steel equipment. *Chem Eng Sci, 57*(8), 1287-1297.

Lemos, M., Gomes, I., Mergulhão, F., Melo, L., & 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*, (in press).

Lemos, M., Mergulhão, F., Melo, L., & Simões, M. (2015). The effect of shear stress on the formation and removal of *Bacillus cereus* biofilms. *Food Bioprod Process, 93*(0), 242-248.

Liu, Z., Lin, Y. E., Stout, J. E., Hwang, C. C., Vidic, R. D., & Yu, V. L. (2006). Effect of flow regimes on the presence of *Legionella* within the biofilm of a model plumbing system. *J Appl Microbiol 101*(2), 437-442.

Lomander, A., Schreuders, P., Russek-Cohen, E., & Ali, L. (2004). Evaluation of chlorines' impact on biofilms on scratched stainless steel surfaces. *Bioresour Technol, 94*(3), 275-283.

Luppens, S. B. I., Reij, M. W., van der Heijden, R. W. L., Rombouts, F. M., & Abee, T. (2002). Development of a standard test to assess the resistance of *Staphylococcus aureus* biofilm cells to disinfectants. *Appl Environ Microbiol, 68*(9), 4194-4200.

Mauermann, M., Eschenhagen, U., Bley, T., & Majschak, J. P. (2009). Surface modifications –

Application potential for the reduction of cleaning costs in the food processing industry. *Trends Food Sci Technol, 20, Supplement 1*(0), S9-S15.

Melo, L. F., & Bott, T. R. (1997). Bifouling in water Systems. *Exp Therm Fluid Sci, 14*, 375-381.

Moreira, J. M. R., Araújo, J. D. P., Miranda, J. M., Simões, M., Melo, L. F., & Mergulhão, F. J. (2014). The effects of surface properties on *Escherichia coli* adhesion are modulated by shear stress *Colloid Surface B, 123*, 1-7.

Moreira, J. M. R., Gomes, L. C., Araújo, J. D. P., Miranda, J. M., Simões, M., Melo, L. F., & Mergulhão, F. J. (2013). The effect of glucose concentration and shaking conditions on *Escherichia coli* biofilm formation in microtiter plates. *Chem Eng Sci, 94*(0), 192-199.

Pogiatzis, T. A., Vassiliadis, V. S., Mergulhão, F. J., & Wilson, D. I. (2014). Choosing When to Clean and How to Clean Biofilms in Heat Exchangers. *Heat Transf Eng, 36*(7-8), 676-684.

Rossoni, E. M. M., & Gaylarde, C. C. (2000). Comparison of sodium hypochlorite and peracetic acid as sanitising agents for stainless steel food processing surfaces using epifluorescence microscopy. *Int J Food Microbiol, 61*(1), 81-85.

Sagong, H.-G., Lee, S.-Y., Chang, P.-S., Heu, S., Ryu, S., Choi, Y.-J., & 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, 145*(1), 287-292.

Salek, M. M., Sattari, P., & Martinuzzi, R. J. (2011). Analysis of fluid flow and wall shear stress patterns inside partially filled agitated culture well plates. *Ann Biomed Eng, 40*(3), 707-728.

Schlisselberg, D. B., & Yaron, S. (2013). The effects of stainless steel finish on *Salmonella Typhimurium* attachment, biofilm formation and sensitivity to chlorine. *Food Microbiol,*

35(1), 65-72.

Shi, X., & Zhu, X. (2009). Biofilm formation and food safety in food industries. *Trends Food Sci Technol, 20*(9), 407-413.

Simões, M., Simões, L. C., & Vieira, M. J. (2010). A review of current and emergent biofilm control strategies. *LWT - Food Sci Technol, 43*(4), 573-583.

Srey, S., Jahid, I. K., & Ha, S.-D. (2013). Biofilm formation in food industries: A food safety concern. *Food Control, 31*(2), 572-585.

Teodósio, J. S., Silva, F. C., Moreira, J. M. R., Simões, M., Melo, L., & Mergulhão, F. J. (2013). Flow cells as quasi Ideal systems for biofouling simulation of industrial piping systems. *Biofouling, 29*(8), 953-966.

Teodósio, J. S., Simões, M., Alves, M. A., Melo, L., & Mergulhão, F. (2012). Setup and validation of flow cell systems for biofouling simulation in industrial settings. *The Scientific World Journal ID 361496*.

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

van Oss, C. (1994). *Interfacial Forces in Aqueous Media*. New York, USA.: Marcel Dekker Inc.

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518 **Figure captions**

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520 Figure 1 Attached cells at three time points (0.5, 2 and 6 h) in stainless steel (black bar) and SICAN
521 (grey bar) surfaces at 5 ºC in: a) industrial water, b) industrial water with *E. coli* and c) medium
522 with *E. coli*. Error bars shown for each surface, at each time point, represent the standard deviation
523 from three independent experiments.

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525 Figure 2 *E. coli* adhesion phase (until 6 h), biofilm growth phase (between 24 and 120 h), biofilm
526 cleaning phase (between 120 and 121 h) and biofilm regrowth phase (between 121 and 144 h) in
527 stainless steel (black line) and SICAN (grey line) surfaces. The interruption in the graph (between
528 6 and 24 h) represents the division between the results obtained for bacterial adhesion phase

assayed in microtiter plates and the biofilm assays performed in a flow cell. Both assays were made at the same shear stress (0.25 Pa), temperature (30 ºC) and using the same culture medium. The disinfection time point is indicated with an arrow and the shaded area represents the time interval were a higher hygienic level can be achieved with SICAN. Error bars shown for each surface, at each time point, represent the standard deviation from three independent experiments.

Table 1 Typical cleaning times in the food industry

Process/ Industry	**Cleaning Schedule**
Salad washing	6-8 h
Milk pasteurization	4-8 h
Ice-cream production	24-62 h
Condiment industry	8-16 h
Artisan bread production	12-24h
Minced meat production	24 h
Meat preparation (cutting boards)	4 h
Refrigerated or RTE* frozen products	< 24 h
Wine production	24 h
Beer production	36-120h
Beverage industry	60-100 h

543 * RTE – Ready To Eat. Cleaning schedule depends on the temperature of the room where the

544 surface/equipment is located (from 4 h above 13ºC up to 24 h for temperatures lower than 5ºC)

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554 Table 2 The apolar (γ^{LW}) and the surface polar parameters (γ^{+} and γ^{-}), the hydrophobicity (ΔG) and roughness of two surfaces (stainless steel and SICAN)

Surface	γ^{LW} / (mJ.m^{-2})	γ^{+} / (mJ.m^{-2})	γ^{-} / (mJ.m^{-2})	ΔG / (mJ.m^{-2})	Roughness[a]/ µm
Stainless Steel	36.8	0	8.90	-46.6	0.10±0.05
SICAN	36.1	0	6.52	-58.2	0.11±0.01

[a] values adapted from Boxler, et al. (2013a)

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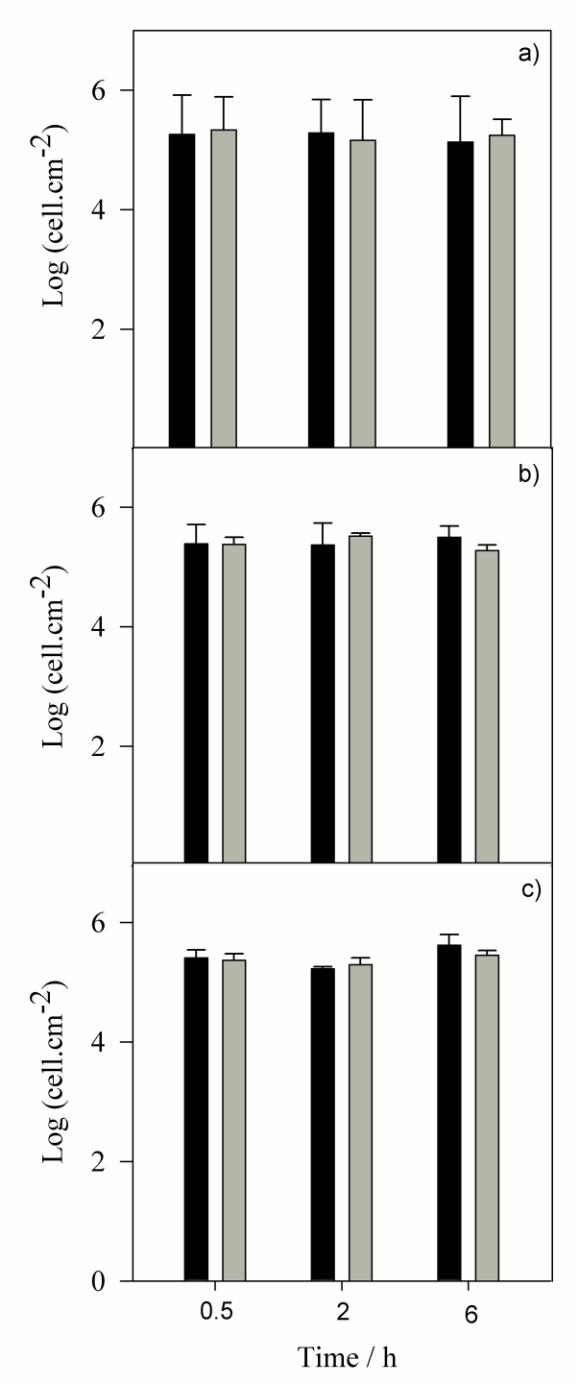

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575 Figure 1 Attached cells at three time points (0.5, 2 and 6 h) in stainless steel (black bar) and SICAN
576 (grey bar) surfaces at 5 ºC in: a) industrial water, b) industrial water with *E. coli* and c) low nutrient
577 medium with *E. coli*. Error bars shown for each surface, at each time point, represent the standard
578 deviation from three independent experiments.

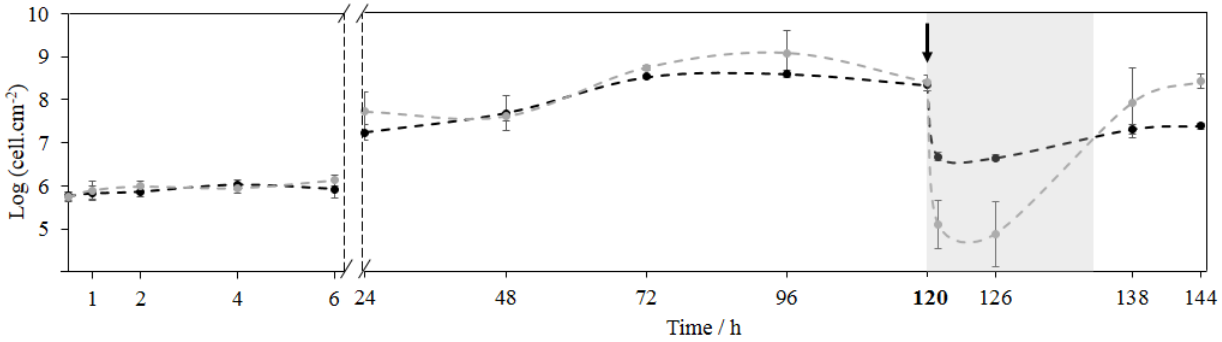
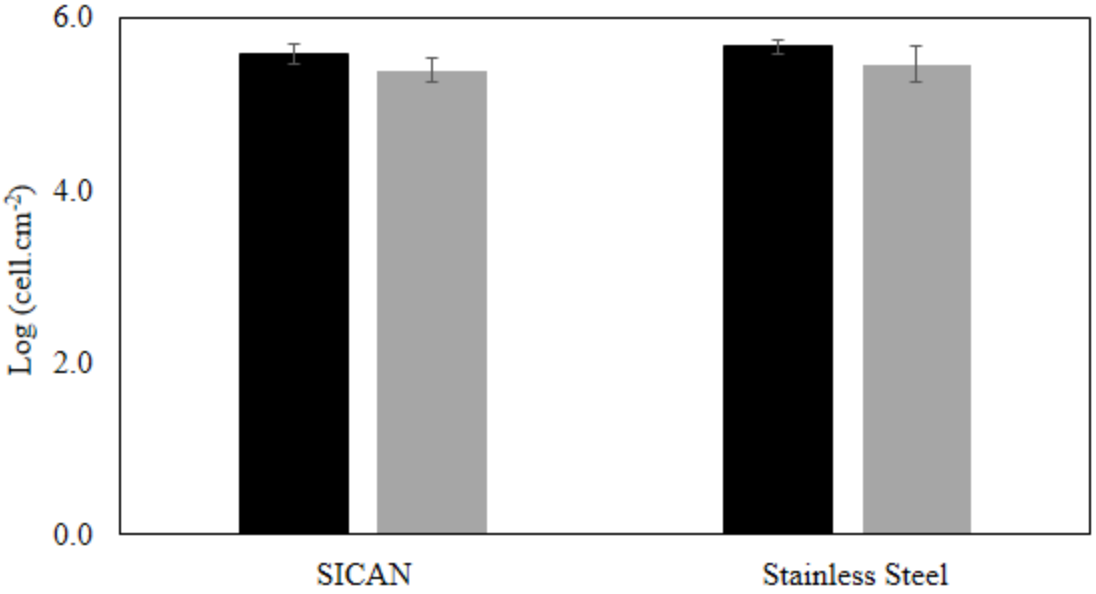


Figure 2 *E. coli* adhesion phase (until 6 h), biofilm growth phase (between 24 and 120 h), biofilm cleaning phase (between 120 and 121 h) and biofilm regrowth phase (between 121 and 144 h) in stainless steel (black line) and SICAN (grey line) surfaces. The interruption in the graph (between 6 and 24h) represents the division between the results obtained for bacterial adhesion phase assayed in microtiter plates and the biofilm assays performed in a flow cell. Both assays were made at the same shear stress (0.25 Pa), temperature (30 ºC) and using the same culture medium. The disinfection time point is indicated with an arrow and the shaded area represents the time interval were a higher hygienic level can be achieved with SICAN. Error bars shown for each surface, at each time point, represent the standard deviation from three independent experiments.

Supplementary material:



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599 Figure S1 Bacterial adhesion (2 h) in SICAN and stainless steel at 30ºC (black bars) and 5ºC (grey

600 bars).

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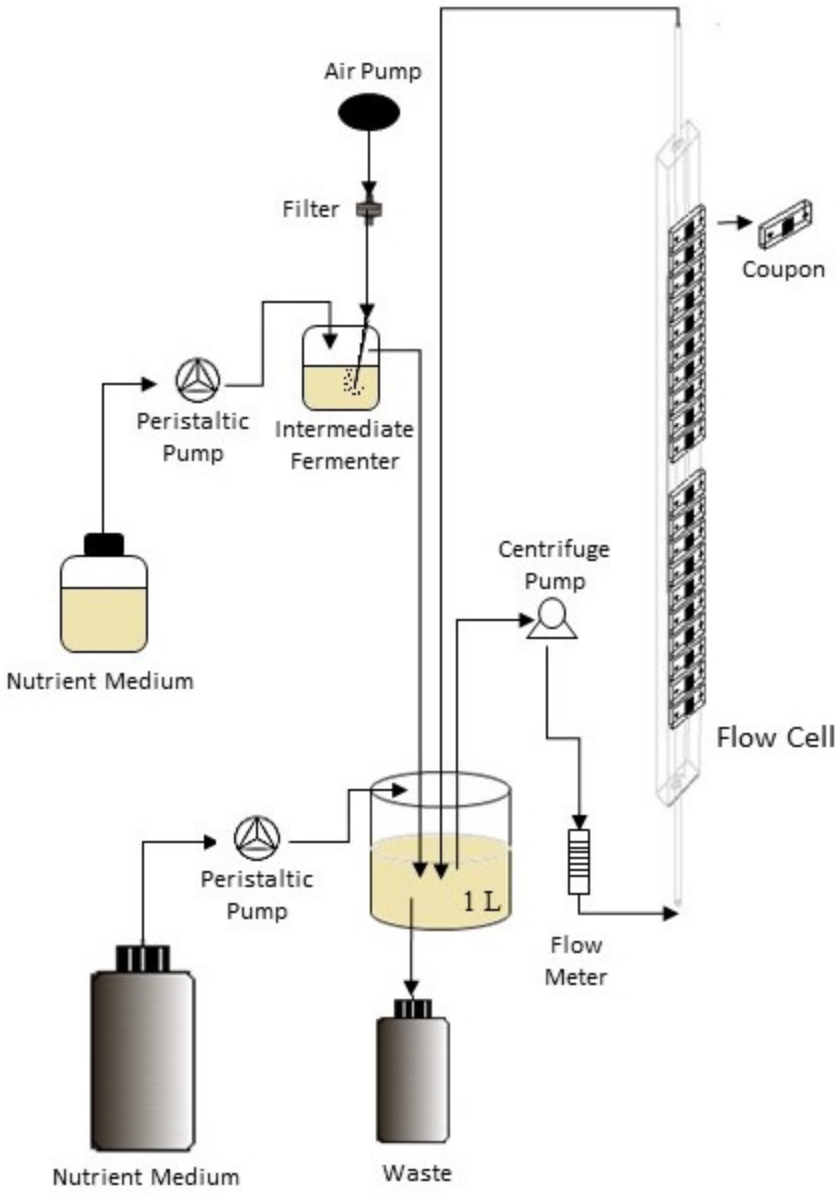

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609 Figure S2 Representation of the flow cell system used.