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The impact of material properties, nutrient load and shear stress on biofouling in food industries

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Abstract:

In the food industry, biofilm formation in pipes, equipment and cooling systems increases maintenance costs, decreases operational efficiencies and is a source of contamination. Shear stress, nutrient load and surface material are important variables affecting the biofilm onset in industry. In this work, the combined impacts of these variables were assessed using three different materials (glass, copper and stainless steel), two nutrient loads (high and low nutrient medium) and two hydrodynamic conditions (static and dynamic). Initial adhesion and biofilm formation were studied in microplates using *Escherichia coli* as a model organism.

Surface material was the factor with the strongest impact and adhesion/biofilm formation were correlated with surface hydrophobicity. However, the impact of this variable was dependent on the nutrient load and imposed shear stress. It was also found that, for the majority of the situations tested, initial attachment performance is a good predictor of biofilm formation behaviour and that the effects observed during attachment are amplified during biofilm maturation. Since shear stress is a major determinant in cell adhesion, the results of this study may find application in industrial systems operating at flow rates between 0.001 and 600 m³ h⁻¹ depending on tube material and diameter.

Keywords: Bacterial adhesion; biofilm formation; surface hydrophobicity; nutrient load; shear stress.

1. Introduction

In industry, microorganisms are more difficult to eradicate when they deposit and adhere to equipment surfaces and piping systems. After this initial step, microorganisms start producing extracellular polymeric substances which protect them from cleaning and

disinfection protocols. This community of microorganisms, known as biofilm, can cause serious problems when it starts to spread in process lines. In the food industry, the formation of this biological fouling in piping, process equipment, cooling towers or heat exchangers can lead to an increase in maintenance costs, decrease of equipment operational efficiencies and can be a source of contamination (Brooks and Flint, 2008). *Escherichia coli* has been reported as one of the most persistent foodborne microorganisms (Dourou et al., 2011; Sagong et al., 2011; Shi and Zhu, 2009). Moreover, it is also a ubiquitous microorganism in natural water systems which are commonly used in industrial cooling systems (Casani et al., 2005). *E. coli* has been found in vegetable process industries, meat industries and ready-to-eat products (Srey et al., 2013) which have different compositions and thus different nutrient loads. Additionally, the water used in the cooling systems of these industries may also have different nutrient compositions. Several authors have been reporting the impact that different nutrient loads may have on biofilm development. Jackson et al. (2002) tested the effect of LB medium (with 0.2% glucose) and colony-forming antigen medium (lacking glucose) on *E. coli* biofilm formation and verified that the presence of glucose in the media inhibited biofilm formation. Gomes et al. (2014a) tested the effect of glucose, peptone and yeast extract load on *E. coli* biofilm formation under static and shaking conditions (0 – 0.07 Pa) and verified that higher glucose concentrations enhanced *E. coli* adhesion in the first 24 h, but variation in peptone and yeast extract concentration had no significant impact on biofilm formation. Teodósio et al. (2011) observed that at higher shear stresses (0.6 Pa) the amount of *E. coli* biofilm formed under two different nutrient loads was similar, concluding that hydrodynamics were probably controlling biofilm formation.

In industrial lines, bacterial adhesion and biofilm growth may be controlled by the shear forces applied on the surface by the fluid flow (Lelièvre et al., 2002). Shear forces can be

controlled by either design of the equipment/pipe diameter or the flow rate used during operation, with hygienic design being preferred due to the cost and limitations of increasing the flow rate (Jensen et al., 2005). The specific hydrodynamic conditions of each system will determine the bacterial concentration on the surface, the structure of biofilm matrix and the nutrient mass transport (Melo and Vieira, 1999). Higher flow velocities (or higher shear forces) are usually preferred in industry since they prevent bacterial adhesion and increase the rate of biofilm detachment (Vieira et al., 1993). However, there are some process lines where lower velocities are necessary and lower velocity zones may also be found in equipment with complex geometries, the so-called dead zones (Walid et al., 2013). Additionally, zones of stagnant flow may be found in wet surfaces and this is also a suitable place for bacterial adhesion and biofilm development (Ganesh and Anand, 1998).

The selection of a material to be used as food contact and processing surface is complex. The material should be non-toxic when in contact with food, suitable for the place where it will be used (pipes, equipment or free surfaces), resistant to corrosion, easily cleanable, etc. (Van Houdt and Michiels, 2010). Stainless steel, glass and copper have been intensively used in food industries (Bonsaglia et al., 2014; Brooks and Flint, 2008; Melo and Flemming, 2010; Shi and Zhu, 2009; Van Houdt and Michiels, 2010). Stainless steel tubing is often used because of its hygienic status (low soiling level and/or high cleanability) (Jullien et al., 2003) and the ability to resist corrosive damage (Flint et al., 2000). Copper and its alloys have desirable properties for industrial applications due to their high thermal conductivity, corrosion resistance and antimicrobial effects (Grass et al., 2011; Wilks et al., 2005) and have been traditionally used, for instance, in heat exchanger tubing in cane and beet sugar refineries (AISI, 1976). Glass is a surface that

has no effect on the final product smell or taste and its transparency is also advantageous (Muller-Steinhagen and Zettler, 2011).

There are several reports in the literature describing the individual effects of nutrient media, hydrodynamics and surface properties on biofilm development, but little is known about the combined impact of these three factors. The aim of the present study was to examine 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. The applicability of the obtained results to industrial settings is also discussed.

2. Material and methods

2.1. Preparation of bacterial strain

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 biofilm reactors operated at different shear stresses (Moreira et al., 2014b, 2013; Teodósio et al., 2012). Additionally, it was shown that the biofilm formation ability of this strain is similar to other *E. coli* strains which are often used for antimicrobial susceptibility and disinfection tests (Gomes et al., 2014a). The strain was grown overnight at 30 °C and 120 rpm in 0.2 L of inoculation medium previously described by Teodósio et al. (2011). This medium consisted of 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), pH 7.0. Then, the cells were centrifuged (3202 g, 10 min, 25 °C) 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 7.6×10^8 cells mL⁻¹, determined by optical density at 610 nm (OD = 1).

2.2. *Surface preparation*

Coupons with dimensions of 1 × 1 cm made from glass (GLA; Vidraria Lousada, Lda, Portugal), stainless steel 316 (SS; F. Ramada, Portugal) and copper (Cu; Neves & Neves, Lda, Portugal) were prepared. SS, Cu and GLA were selected 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).

All 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 (Gomes et al., 2014b). 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 contact angle measurements and for adhesion and biofilm assays.

2.3. *Surface free energy measurements*

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. 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) - ΔG_{iwi} . If the interaction between the two entities is stronger than the interaction of each entity with water ($\Delta G_{iwi} < 0 \text{ mJ m}^{-2}$), the material is considered hydrophobic. Conversely, if $\Delta G_{iwi} > 0 \text{ mJ m}^{-2}$, the material is hydrophilic. ΔG_{iwi} was calculated from the surface tension components of the interacting entities, according to Eq. (1):

$$\Delta G_{iwi} = -2 \left(\sqrt{\gamma_i^{LW}} - \sqrt{\gamma_w^{LW}} \right)^2 + 4 \left(\sqrt{\gamma_i^+ \gamma_w^-} + \sqrt{\gamma_i^- \gamma_w^+} - \sqrt{\gamma_i^+ \gamma_i^-} - \sqrt{\gamma_w^+ \gamma_w^-} \right) \quad (1)$$

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 $\gamma^{AB} = 2\sqrt{\gamma^+ \gamma^-}$.

The surface tension components were estimated by the simultaneous resolution of three equations of the type (2):

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

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

2.4. Cell adhesion and biofilm assays

Two different media were assayed for adhesion and biofilm experiments: a high nutrient medium (HN) and a low nutrient medium (LN). 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).

Mueller-Hinton was selected since it has been used as standard medium for bacterial susceptibility tests to antimicrobial agents (Fernández-Mazarrasa et al., 2009), according to the CLSI guidelines (CLSI, 2007). Additionally, since this medium has approximately 0.2% of nitrogen content (mainly from casein, the main proteinaceous component of milk) (Wang et al., 2013), it was selected to simulate a high nutrient load that can be found in the food industry. Moreover, a medium with a 400-fold lower nitrogen content (from peptone and yeast extract) was also tested to simulate low nutrient conditions. 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 results were expressed as number of attached cells per cm² (Fig. 1). In order to

estimate the number of viable cells, coupons were vortexed in 10 mL of saline solution during 1 min to suspend and homogenize the attached cells as described in Gomes et al. (2014b). Cell culturability was assessed in PCA (Merck, Portugal) after dilution in saline. The number of colony forming units (CFU) was counted for plates with > 10 and < 300 colonies. The total CFU per sample was determined after 24 h of incubation at 30 °C by correcting the colony count for the dilution factor. The result was expressed in percentage of cell culturability, CFU number counts divided by the total cell number obtained by DAPI counts (see Supplementary material, Fig. S1).

2.5. Calculations

The data presented in Fig. 2 originated from the number of attached cells determined by epifluorescence microscopy for each combination of three factors - agitation, nutrient load and material - in both time points - 0.5 h for initial cell adhesion and 6 h for biofilm formation. Regarding the impact of agitation for all material and nutrient load combinations, the ratios between the attached cell number obtained under static and dynamic conditions were 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 and plotted in Fig. 2 (so that the obtained ratio was always equal or higher than 1).

The ratio of each of the 6 possible combinations of material with nutrient load was represented in the radial axes of the top left quadrant of the figure. The inner circle marked in bold is equivalent to ratio = 1.00 (indicating a negligible impact) and each line segment presented in the quadrant has a length equivalent to the value of the calculated ratio (the spacing between two adjacent radial orbits is equivalent to one unit). The same type of calculation was done to graphically display the impact of nutrient load on all material and

agitation conditions (top right quadrant of Fig. 2). To study the impact of 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. Regarding the effect of the material, the number of combinations increased from 6 to 12. A group of 6 combinations concerning the low nutrient load appears on the bottom left section of the graph and the remaining 6 combinations for the high nutrient load are shown on the bottom right section.

Fig. 3 represents the relation between flow velocity and tube diameter to promote a shear stress similar to the one used in this work (0.27 Pa). In order to obtain an equation relating flow velocity with tube diameter for a shear stress of 0.27 Pa, three equations (Teodósio et al., 2013) were combined (3-5). The resulting equation (6) can be used to calculate the flow velocity necessary to obtain a shear stress of 0.27 Pa in standard diameter tubes: steel tube diameters between 1.03 and 72.1 cm, copper tube diameters between 0.16 and 40.6 cm and glass tube diameters between 3.3 and 16.9 cm (Perry and Green, 1997).

The Reynolds number (Re) is defined as:

$$Re = \frac{\rho D v}{\mu} \quad (3)$$

where ρ and μ are the density and viscosity of water, respectively, v the flow velocity and D the tube diameter.

The Blasius equation for smooth pipes and turbulent flow (Batchelor, 1967) is defined as:

$$f = \frac{0.00791}{Re^{0.25}} \quad (4)$$

where f is the friction factor.

The wall shear stress (τ) for a circular tube is given by:

$$\tau = \frac{f \rho v^2}{2} \quad (5)$$

The resulting equation was obtained:

$$v = \sqrt[1.75]{\frac{4.38}{D^{0.25}}} \quad (6)$$

The flow rates necessary to achieve the calculated flow velocities for each range of standard tube diameters were also determined.

2.6. Statistical analysis

Cell adhesion and biofilm quantitation results are averages from three independent experiments performed for each combination of variables (agitation, nutrient load and material). A triplicate set of wells were used for each independent experiment. In order to ascertain the statistical significance, paired *t*-test analysis were performed when appropriate. Results were considered statistically different when a confidence level greater than 95% was reached ($P < 0.05$). Standard deviations (SDs) for three independent experiments are represented by error bars.

3. Results and Discussion

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. Surface properties of the tested materials were first determined and the results are presented in Table 1. From the total free energy results it is possible to observe that GLA is hydrophilic ($\Delta G_{iwi} > 0 \text{ mJ m}^{-2}$) and SS and Cu are hydrophobic ($\Delta G_{iwi} < 0 \text{ mJ m}^{-2}$), being Cu the most hydrophobic surface. Regarding γ^- and γ^+ , results showed that SS is a monopolar surface, being an electron donor and GLA and Cu are polar surfaces, being electron donors and acceptors. Fig. 1 shows the number of attached cells at 0.5 h (initial adhesion) and 6 h (biofilm formation) on each surface

and the results were organized from the most hydrophilic to the most hydrophobic surface. In general, it was possible to observe that the highest bacterial adhesion was obtained in Cu (the most hydrophobic surface) with statistically significant differences in 88% of the cases ($P < 0.05$). In contrast, the lowest number of bacteria was observed in GLA (the most hydrophilic surface) with statistically significant differences in 67% of the combinations ($P < 0.05$). Gomes et al. (2014b) studied the effect of different materials (glass, stainless steel, silicone and polyvinyl chloride) on *E. coli* initial adhesion and biofilm formation. These authors, as well as others (Fonseca et al., 2001; Kochkodan et al., 2008; Treter et al., 2014), also found a correlation between cell attachment and surface hydrophobicity. In this work, this tendency was observed on average for 83% of the obtained results. In a previous study, *E. coli* adhesion to an hydrophilic glass and to an hydrophobic polymer at different shear stresses (between 0.005 and 0.07 Pa) was evaluated (Moreira et al., 2014b). Those results have shown that the effect of surface properties is modulated by shear stress. Therefore, although initial bacterial adhesion can be controlled by the surface hydrophobicity, this effect may be more or less noticeable depending on the hydrodynamic conditions.

Although copper was the surface with a higher number of attached bacteria (at 0.5 and 6 h), a reduced cell culturability was detected when compared with the other materials (see Supplementary material, Fig. S1). Sharifahmadian et al. (2013) studied the effect of surface properties on *E. coli* and *Staphylococcus aureus* adhesion to stainless steel and copper surfaces and they observed an average reduction of 94% in bacterial viability on copper after a 6 h assay. In contrast, the stainless steel surface did not show antibacterial properties. In the present study, similarly to Sharifahmadian et al. (2013) and other previous studies (Chan et al., 2011; Wilks et al., 2005; Xu et al., 2012; Zhang et al., 2011), *E. coli* was rapidly killed on copper regardless of the hydrodynamic condition and nutrient

load. Thus, despite the higher number of adhered cells on this surface, it may be suitable as a surface material for industry since it can reduce the contamination risk.

Fig. 2 was constructed based on the results of Fig. 1 to evaluate the impact of each variable (shear stress, nutrient load and surface material) on initial bacterial adhesion and biofilm formation, when combined with the other two variables. In general, biofilm formation behavior was predicted by initial bacterial adhesion for 63% of the cases. Additionally, it was observed that for 80% of the successfully predicted cases, the impact of the tested variables at 0.5 h (initial adhesion) was further amplified during the biofilm maturation phase (after 6 h). Different studies have shown that lower initial cell adhesion leads to lower amount of biofilm (Cheng et al., 2007; Godoy-Gallardo et al., 2014) whereas others report that these initial events are not always important for biofilm maturation (Bernstein et al., 2014; Cerca et al., 2005).

A biofilm formation time of 6 h was chosen since several industries like the salad washing facilities or the dairy industry commonly use a 4-8 h operational time between sanitation cycles (Lelieveld et al., 2005). Other food industries such as the beverage or condiment industries have longer cleaning intervals (8-16 h and 60-100 h, respectively) (Lelieveld et al., 2005) and for these cases, the obtained results can be even more important in the development of strategies for biofilm control.

Regarding the impact of agitation on the number of attached bacteria (Figure 2, top left quadrant), it is possible to verify that Cu was the surface more sensitive to agitation. In 75% of the analysed cases (including adhesion and biofilm formation), higher values were obtained in static conditions when compared to dynamic conditions (on average, a 60% increase was obtained). These results demonstrate that shear stress is indeed an important factor that affects initial cell adhesion as well as biofilm maturation. Cell adhesion and subsequent biofilm formation can be positively or negatively affected by shear stress since

it is correlated with the fluid velocity and consequently with mass transfer (Moreira et al., 2014a). During the adhesion and maturation phases, a higher fluid velocity will increase the transport of nutrients and cells which has a positive effect on cell adhesion and biofilm formation (Moreira et al., 2014a; 2014b). However, a higher shear stress may prevent cell attachment or promote cell detachment. For the conditions used in this work, it seems that the later situation occurred to a larger extent.

Looking at the top right quadrant of Fig. 2, it can be seen that the impact of the nutrient load on the number of attached bacteria (at 0.5 and 6 h) was slightly more important in dynamic than in static conditions. The impact of the surface type on the number of attached cells at 0.5 and 6 h can be observed on the bottom section of Fig. 2. The type of material was the variable which had the greatest impact on initial bacterial adhesion and biofilm formation and this effect was stronger when the LN medium was used (bottom left section). The results obtained on Cu were very different from the ones obtained on SS and GLA and this difference was higher under low nutrient load. Before cell attachment occurs, a conditioning layer of adsorbed macromolecules such as nutrients from the culture medium is formed on the surface (Chmielewski and Frank, 2003). If the amount of adsorbed molecules is very high, the conditioning layer may shield the incoming cells from the surface properties. This may explain why the impact of the surface properties was higher in low nutrient load conditions where the presence of conditioning molecules in the culture medium is probably reduced.

In this work, it was also observed that the impact of agitation and surface material was higher when a low nutrient load was used. Additionally, it was verified that the impact of the nutrient load became slightly more important in dynamic conditions. Dewanti and Wong (1995) studied the effect of culture conditions on biofilm formation by an *E. coli* strain isolated from ground beef. These authors verified that initial cell adhesion and

biofilm formation in dynamic conditions were higher and faster in low nutrient media. In a previous study (Moreira et al., 2013), the effect of glucose concentration (0.25 and 1 g L⁻¹) and shaking conditions (0.03 and 0.07 Pa) on *E. coli* biofilm formation was evaluated. In that study, it was observed that *E. coli* biofilm formation (up to 12 h) was also promoted by the low glucose concentration under higher shear forces (0.07 Pa). However, under low shear stress, higher amounts of biofilm at 12 h were attained in the high nutrient medium. After 24 h, similar amounts of biofilm were attained on both shaking conditions, but higher values were obtained for the highest glucose concentration. These results show that the impact of the nutrient load on biofilm formation is dependent on the hydrodynamic conditions (Moreira et al., 2013). Peyton (1996) studied the effect of shear stress and substrate loading on *Pseudomonas aeruginosa* biofilm formation and observed that biofilm thickness increased with increasing substrate loading rate. However, he observed that shear stress (between 1.44 and 2.97 Pa) had no significant effect on biofilm thickness. In the present work it was observed that, in general, higher amounts of biofilm were obtained for the high nutrient load conditions (with the exception of Cu). However, an increase in the fluid velocity (from static to dynamic) led to a reduction in the biofilm amount and this was observed, in the majority of cases, for the high as well as for the low nutrient load conditions, confirming that shear stress has an impact on biofilm formation. In a study from Characklis (1981) it was observed that at high substrate loadings, increasing fluid velocity (for shear stresses between 2 and 14 Pa), decreases biofilm formation and that at low substrate loadings, increasing the fluid velocity may decrease biofilm formation or not. Similar results were also obtained on the present study for the HN medium. For the LN medium, a decrease was observed for SS and Cu but not for GLA.

In the food industry, the nutrient load is inherent to each type of product that is being produced. Therefore, this work demonstrates that in food industries (or specific points in the industrial lines, such as the drainage of effluent water from cleaning operations) with lower nutrient loads, the impact of the surface properties and shear stress is more important in the onset of a biofilm. On the other hand, in systems with higher nutrient loads, the impact of surface materials is not so important and thus the use of more expensive materials to inhibit bacterial adhesion may be not justified. Since the nutrient load is characteristic of each type of food industry, only the shear forces operated in industrial lines and the material selected to be used in equipment and pipes can be manipulated in the equipment design phase. In industrial pipe lines, shear forces can be controlled by either the tube diameter or the operated flow rate (Jensen et al., 2005). The usable pipe diameter depends on the available area in the industrial plant and/or on the availability of a particular tube material in these dimensions. A flow rate change may be not possible due to limitations inherent to the production process or it may have a severe impact on the operational costs (Jensen et al., 2005). Therefore, the decision on which parameter to change to control the shear forces operated in the system is not trivial.

In order to translate the experimental results reported here to real systems, it is important to ensure that the same shear stresses (0 or 0.27 Pa) are being generated in the industrial lines since it has been shown that shear stress is a good scale-up parameter between different systems (Moreira et al., 2015; Teodósio et al., 2013). Fig. 3 represents a relation between the flow velocity and tube diameter that is necessary in order to obtain a shear stress similar to the one tested in this work under dynamic conditions (0.27 Pa). Additionally, the corresponding flow rate is indicated. The range of diameters represented concerns the standard tube diameters commercially available (Perry and Green, 1997) for the three different types of materials tested in this work (GLA, SS and Cu). The grey areas

correspond to the range of flow velocities that should be operated in the respective range of tube diameters for each type of material to obtain a shear stress of 0.27 Pa. Therefore, in order to achieve this shear stress in commercial steel tubes with diameters between 1.03 and 72.1 cm, the range of flow rates which should be operated is between 0.1 and 600 m³ h⁻¹; for standard copper tubes with diameters between 0.16 and 40.6 cm, the range of flow rates is between 0.001 and 180 m³ h⁻¹; and for standard glass tubes with diameters between 3.3 and 16.9 cm, flow rates between 1 and 25 m³ h⁻¹ should be used (Perry and Green, 1997). Using this graph, it is possible to verify for a specific industrial line if the operating flow rate in a specific tube (diameter and material) is promoting a shear stress around 0.27 Pa and, in that case, the results and conclusions taken in this work may be extended to those industries. Additionally, in the design phase, these results may be helpful in the selection of the tube material and diameter, and in the selection of the operational flow rate, taking into account the nutrient load in the industrial system.

4. Conclusions

With this work, it was found that surface material is the variable with the highest impact on initial bacterial adhesion and biofilm formation and that there is a correlation between the surface hydrophobicity and initial bacterial attachment. However, the impact of this variable on biofilm growth depends on nutrient load and shear stress. It was also concluded that the impact of the studied variables on initial bacterial adhesion may be amplified on biofilm maturation. This finding suggests that biofilm control in food industries with long operational times can be obtained by reducing initial bacterial adhesion. Using the shear stress as a scale-up factor, the results presented in this work may be applicable to industrial piping with flow rates between 0.1 and 600 m³ h⁻¹ for steel

tubes, between 0.001 and 180 m³ h⁻¹ for copper tubes and between 1 and 25 m³ h⁻¹ for glass tubes.

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Figure and Table captions

Fig. 1 - Number of attached cells on three different materials - GLA, SS and Cu (sorted in order of increasing hydrophobicity) - under static (■) and dynamic (▒) conditions. (a) and (b) present the initial adhesion results, whereas (c) and (d) refer to biofilm formation; (a) and (c) show the amount of attached cells in high nutrient medium, while (b) and (d) present the cell number in low nutrient medium. The means \pm SDs for three independent experiments are illustrated.

Fig. 2 - Impact chart for the analysed variables. Three variables were studied: (represented in highlighted text), *nutrient load* (represented in italics) and **material** (represented in bold). The graphic is divided in three sections (from light to dark grey) and in each of them the impact of a single variable on initial cell adhesion (black) or biofilm formation (yellow) is assessed taking into account all possible combinations of the other two variables. The top left quadrant (light grey) represents the impact of agitation for all material and nutrient load combinations. For each combination, a ratio between the attached cell number obtained in each agitation condition (static vs. dynamic) was calculated and presented in the chart. In the top right quadrant (intermediate grey), 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 chart (dark grey) analyses the material effect. On the bottom left section, low nutrient load was assayed whereas in the bottom right section high nutrient load was presented. 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.

Fig. 3 – Relation between flow velocity and tube diameter to promote a wall shear stress of 0.27 Pa (solid line). The grey areas in the graph represent the range of standard diameter

tubes commercially available for each studied material (glass, copper and stainless steel) and the respective flow velocity range that can be achieved. The flow rate range that should be operated for each tube diameter in order to obtain a wall shear stress of 0.27 Pa is also represented (dashed line).

Table 1 - Contact angles with water (θ_w), formamide (θ_F) and α -bromonaphthalene (θ_B), surface energy parameters (γ^{LW} - Lifshitz-van der Waals component, γ^+ - electron acceptor component, γ^- - electron donor component) and hydrophobicity (ΔG_{iwi}) for the tested materials. Values are means \pm SDs of three independent experiments

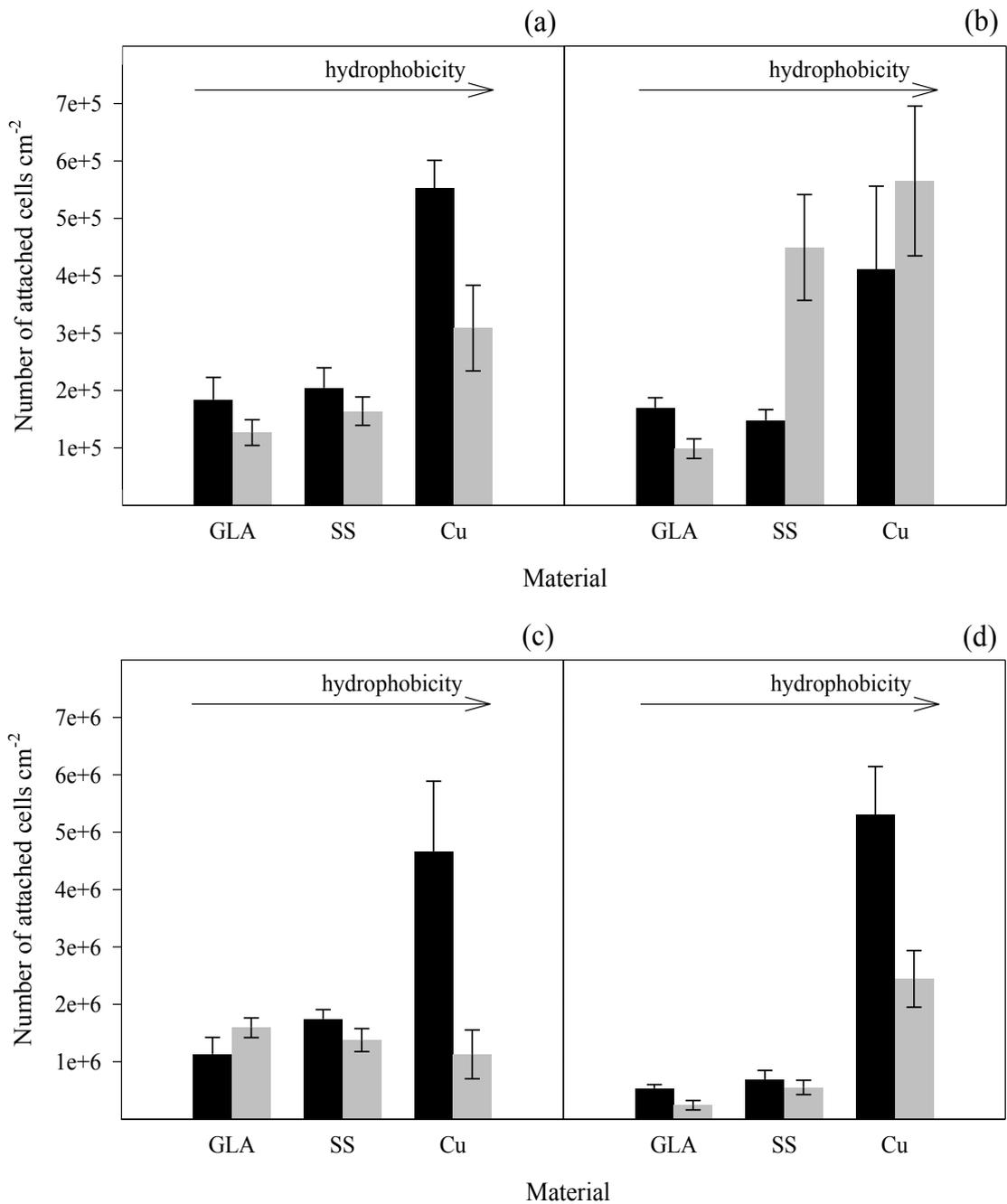


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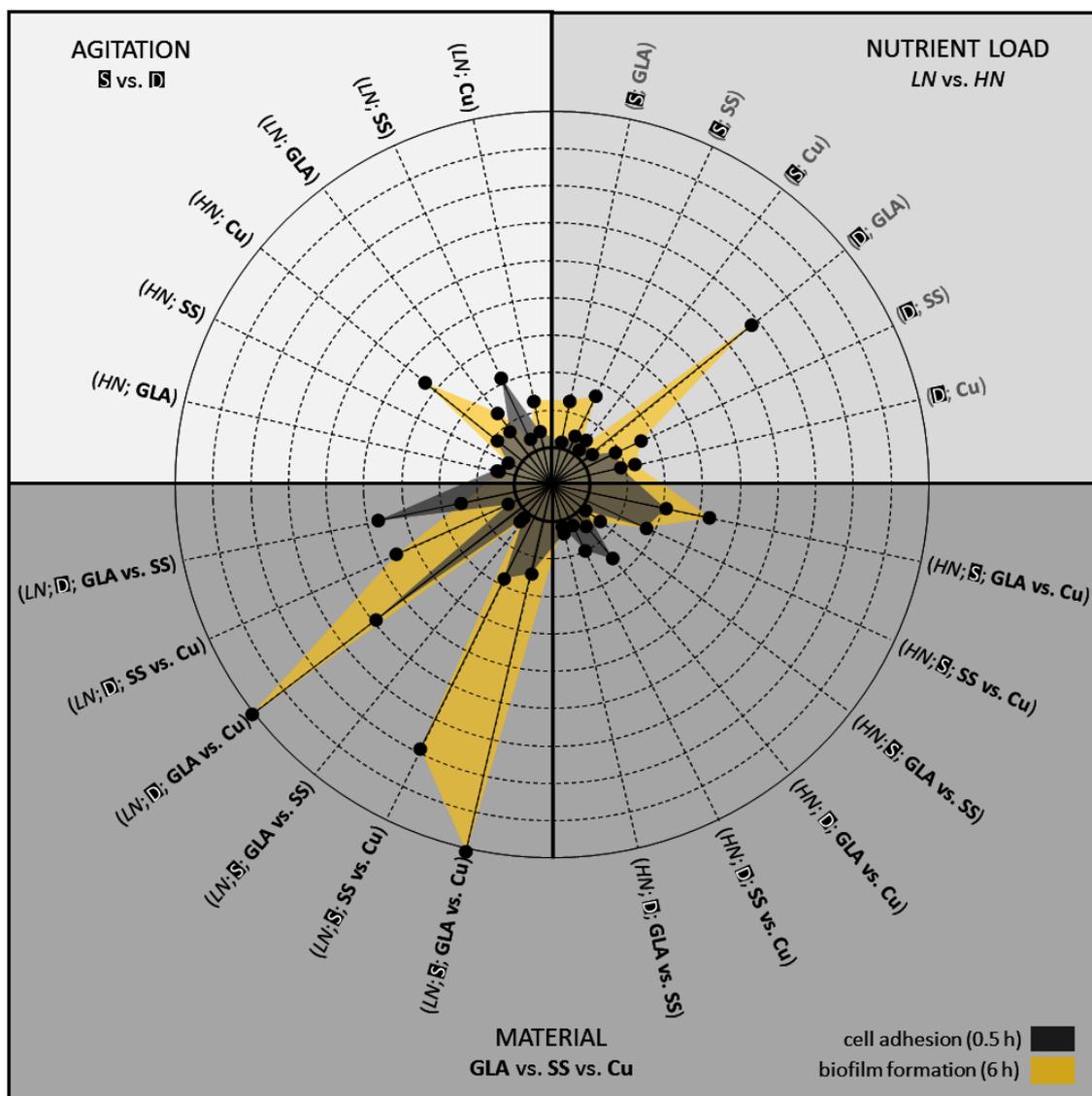


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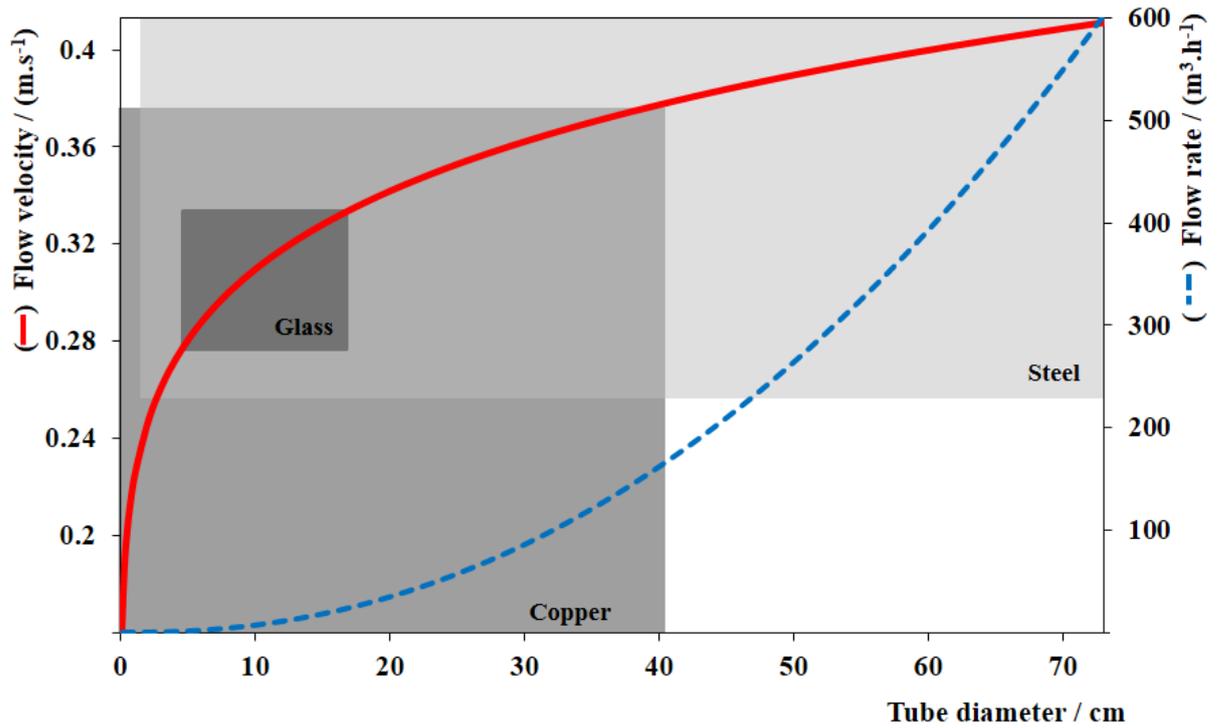


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	Contact angle ($^\circ$)			Surface energy parameters (mJ m^{-2})			Hydrophobicity (mJ m^{-2})
	θ_w	θ_F	θ_B	γ^{LW}	γ^+	γ^-	(ΔG_{iwi})
GLA	47.0 ± 0.4	49.1 ± 0.5	63.4 ± 0.9	23.2	1.7	40.2	19.3
Cu	66.6 ± 7.8	40.0 ± 6.2	24.6 ± 1.8	40.5	1.2	9.7	-36.5
SS	67.0 ± 1.7	60.4 ± 0.4	39.3 ± 0.5	34.9	0.0	21.7	-10.9