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Biocontamination in the dairy industry: The effect of raw milk conditioning film on the adhesion of *Escherichia coli*

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Abstract: Conditioning films on surfaces employed in the dairy industry serve as the precursors to the formation of pathogenic biofilms that impact product quality and consumer safety. Conditioning films have been studied from several aspects. However, there has been no study that evaluated the effect of raw milk conditioning film on the adhesion of *Escherichia coli.* This study investigated the adhesion of *E. coli* on glass and stainless-steel surfaces conditioned with raw milk and explored the surface properties potentially influencing this adhesion using the contact angle method. The results showed that after treating surfaces with raw milk, the adhesion of the bacteria on stainless steel and glass was significantly altered. Adhesion increased significantly on stainless steel (from 0.55 $\log_{10}$ to 2.8 $\log_{10}$) but it decreased on glass (from 1.56 $\log_{10}$ to 0.8 $\log_{10}$). Significant alterations were observed in the physicochemical properties of the surfaces. Glass was initially relatively hydrophilic (46.33°), while stainless steel was relatively hydrophobic (82.5°). After treatment, the glass became relatively more hydrophobic (74.6°), and stainless steel became relatively more hydrophilic (69.4°). The electron donor/acceptor components of glass decreased after the treatment, while these components increased for stainless steel. The significant changes in adhesion were hypothesized to be due to the modification of surface properties by the raw milk.

Keywords: dairy processing; bacterial adhesion; physicochemical properties; food safety; bacterial adhesion

Biofilms are communities of microorganisms irreversibly associated with a surface and enclosed in a matrix of extracellular polymeric molecules. Such films pose a significant challenge to the food industry (Carrascosa et al. 2021). Modern food chains and the complexity of manufacturing processes employing glass and stainless steel and the methods of mass production constitute an environment favourable to the biofilm development (Lindsay and von Holy 2006).

In the dairy sector, biofilm formation is recognized as a hazard that compromises product safety and raises the possibility of foodborne illnesses. Thus, biofilms are regarded as an emerging public health risk (Mogha et al. 2014). The dairy industry strives to minimise microbial contamination of its products and to ensure their quality to permanently satisfy the requirements of the customers. Biocontamination in the dairy industry is generally linked to the formation of biofilms on the surfaces of machinery and installations such as milk tanks, pipes, and processing equipment (Bremer et al. 2006). In addition, the negative repercussions of biofilm formation are due to the quality of dairy products (secretion of undesirable enzymes such as proteases or lipases), the economic impacts (increased costs of maintenance and energy consumption) (Khiyami et al. 2006), and equipment damage (corrosion of the surfaces) (George et al. 2012).

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Food contact surfaces encourage the growth of microorganisms (Hood and Zottola 1997). The greater capacity for contamination by microbes is significant given the complexity of the food production chain, like in the case of *Escherichia coli* that continues to threaten the dairy industry (Bodur and Cagri-Mehmetoglu 2012). *E. coli* was used in this study because it is an indicator of hygiene for certain dairy foods such as milk-based cheeses, raw milk butter, and cream, as indicated in the European Regulations 2073/2005.

The process of biofilm formation is dynamic, with several mechanisms contributing to the adhesion, proliferation, and colonisation of microorganisms on surfaces that come into contact with milk (Mogha et al. 2014). Thus, understanding microbial adhesion is crucial to characterise the initial stages of biofilm formation. The bacterial adhesion is a complex process, as it encompasses the physicochemical characteristics of the three interacting entities: the bacteria that adhere, the surface they attach to, and the surrounding liquid medium (Merritt and An 2000). The hydrophobicity of surfaces is an important parameter in determining the attachment of microorganisms (Alsteens et al. 2007). It is necessary to investigate the dynamics of conditioning films adsorbed on submerged surfaces in addition to the physicochemical properties of biofilms to obtain dairy products controlled for microbial contamination (Gademann 2007).

A conditioning film is a thin layer composed of organic and inorganic compounds that forms on the surface of a material when it comes into contact with water or other liquids (Rummel et al. 2021). The conditioning film consists of proteins, polysaccharides, lipids, and minerals that may originate from the surrounding environment or be secreted by microorganisms. The films form rapidly, often within minutes to hours, and their development is affected by various factors, including surface properties, water composition, and the presence of organic substances (Lorite et al. 2011).

The creation of surface conditioning films as the initial stage of biofilm formation has been documented in the literature for other purposes. Lorite et al. (2011) demonstrated the influence of conditioning film on the surface chemistry, adhesion, and biofilm formation of *Xylella fastidiosa*. In addition, Mittelman (1998) examined the roles of protein and polysaccharide components from human physiological fluids such as blood, tears, and urine as conditioning films to explore their impact on the adhesion of bacteria to biomaterials. Loeb and Neihof (1975) found that seawater could rapidly form a conditioning film on a variety of surfaces and that the films with artificial and photo-oxidized seawater reduced the surface energy of platinum. However, to the best of our knowledge, this work is the first attempt to evaluate the role of the conditioning film in *E. coli* adhesion using raw milk.

To achieve this, we investigated the adhesion of *E. coli* to glass and stainless steel during the initial minutes following the raw milk deposition and subsequently we analysed the physicochemical changes on these surfaces, emphasising their impact on *E. coli* attachment.

A key point of this research is the fact that the experimental conditions apply to biocontamination in the dairy industry by minimising the variation that exists both biologically (by using raw milk as the conditioning film and *E. coli* as the indicator bacterium) and practically (by using glass and stainless steel). Furthermore, we hypothesize that the nature and manner of molecular deposition will depend on the physicochemical properties of the material.

MATERIAL AND METHODS

Solid surface and cleaning treatments. A surface area of 2 cm^2 of glass and stainless steel 304L was used in the present work. The substrates were then cleaned to get rid of any organic material that had accumulated by soaking in a 70% (vol/vol) ethanol solution for 15 min and then they were rinsed six times with sterile distilled water and autoclaved at 120 °C for 15 min (Assaidi et al. 2018). Ten millilitres of raw milk were added to a Petri dish that had both glass and stainless-steel surfaces for 10 min at 25 °C. After that, the pieces were washed three times with distilled water, and the contact angles were measured independently in triplicate. In this study we opted to use raw milk to examine the initial stages of milk deposition up to the point of pasteurisation, focusing specifically on evaluating the conditioning film that forms prior to pasteurisation, and to avoid introducing additional variables related to thermal processes.

***Escherichia coli* ATCC 25922 culture conditions and contact angle measurements.** An overnight culture of *E. coli* was carried out on LB (Luria-Bertani) medium at 37 °C, and then the cells were suspended in KNO_3 (0.1 M) and washed by centrifugation at 5 000 × *g* for 15 min. The contact angle measurements followed the protocol of Hamadi et al. (2014). Briefly, the bacterial suspension was filtered under negative pressure using a 0.45 µm *Escherichia coli* cellulose acetate filter. Subsequently, the filters were placed on glass Petri dishes

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to be dried, and the contact angles were measured via triplicate independent replicated tests.

Adhesion experiments. The adhesion protocol of the inert and treated surfaces (glass and stainless steel 304L surfaces) was carried out according to the method of Assaidi et al. (2018) with some modifications. Briefly, 12 mL of *E. coli* suspension with an approximate concentration of 10^8 CFU·mL^{-1} was incubated in Petri dishes with glass and stainless steel 304L surfaces for three hours. Then, the surfaces were cleaned with sterile distilled water to eliminate non-adherent cells. Each surface was placed into a tube containing 10 mL of sterile physiological solution (NaCl: 9 g per L) and sonicated for 2 min using ultrasound (35 kHz). After sonication, a serial dilution was used to determine the number of adherent cells on each surface. The counting of bacterial cells was carried out on solid LB medium after a 24-hour incubation period at 37 °C. Three separate assays were done for each surface.

Characterization of the studied surfaces. The sessile drop method using a goniometer (GBX instruments, France) was employed to determine contact angles. The Lifshitz-Van der Waals (γ^{LW}) and acid-base (γ^{AB}) characters were obtained according to the method of Van Oss et al. (1988). In this approach, the contact angle can be expressed as follows:

$$\begin{aligned}\gamma_{L1}(cos\theta+1)&=2\left[\sqrt{\gamma_S^{LW}\gamma_{L1}^{LW}}+\sqrt{\gamma_S^{+}\gamma_{L1}^{-}}+\sqrt{\gamma_S^{-}\gamma_{L1}^{+}}\right]\\ \gamma_{L2}(cos\theta+1)&=2\left[\sqrt{\gamma_S^{LW}\gamma_{L2}^{LW}}+\sqrt{\gamma_S^{+}\gamma_{L2}^{-}}+\sqrt{\gamma_S^{-}\gamma_{L2}^{+}}\right]\\ \gamma_{Lapolaire}(cos\theta+1)&=2\left[\begin{array}{l}\sqrt{\gamma_S^{LW}\gamma_{Lapolaire}^{LW}}+\sqrt{\gamma_S^{+}\gamma_{Lapolaire}^{-}}+\\ +\sqrt{\gamma_S^{-}\gamma_{Lapolaire}^{+}}\end{array}\right]\end{aligned} \quad (1)$$

where: θ – the measured contact angle; γ^{LW} – the Van der Waals free component; γ^{+} – the electron acceptor component; γ^{-} – the electron donor component.

Equation (1) therefore contains three unknowns, γ_s^{LW}, γ_s^{-}, γ_s^{+}, which can be determined by measuring the contact angle of three standard liquids, two polar *L*1 and *L2* liquids and one nonpolar liquid $L_{apolaire}$, whose surface energy and component values are known as indicated in Table 1.

This study also employed the methodology of Van Oss et al. (1988) to estimate the quantitative hydrophobicity of the surface (ΔG_{iwi}). It was estimated by the free energy interaction (ΔG_{iwi}) between two components of the substance (*i*) when submerged in water (*w*).

Negative or positive surface free energy means that the surface has a hydrophobic or hydrophilic character, respectively, that can be quantified using the following equation.

$$\begin{aligned}\Delta G_{iwi}&=-2\gamma_{iw}=-2[\left(\sqrt{\gamma_i^{LW}}-\sqrt{\gamma_w^{LW}}\right)+\\ &+2\left(\sqrt{\gamma_i^{+}\gamma_i^{-}}+\sqrt{\gamma_w^{+}\gamma_w^{-}}-\sqrt{\gamma_i^{+}\gamma_w^{-}}-\sqrt{\gamma_w^{+}\gamma_i^{-}}\right)]\end{aligned} \quad (2)$$

where: ΔG_{iwi} – surface free energy *i* and *w* – substance submerged in water; γ^{LW} – the Van der Waals free energy component; γ^{+} – the electron acceptor component; γ^{-} – the electron donor component.

RESULTS

The effects of conditioning film on the adhesion of *E. coli* to glass and stainless steel. The conditioning film originating from raw milk significantly influenced the attachment behaviour of *E. coli* ATCC 25922 (Figure 1). This influence was characterised by an increase in attachment to stainless steel from 0.55 $\log_{10}$ (untreated) to 2.8 $\log_{10}$ (treated) and a decrease in glass from 1.56 $\log_{10}$ (untreated) to 0.8 $\log_{10}$ (treated).

Physicochemical properties of dairy surfaces. The contact angle measurements of surfaces treated with raw milk are presented in Table 2. The glass surface was relatively hydrophilic (46.43°), whereas after treatment the substrate became relatively more hydrophobic (74.6°). In contrast, the stainless steel surface was

Table 1. Surface energy of contact angle liquids (mJ·m^{-2})

Liquide	γ^{LW}	γ^{L}	γ^{+}	γ^{-}
Water	21.8	72.8	25.5	25.5
Formamide	39.0	58.0	2.3	39.6
Diiodomethane	50.5	50.8	0.0	0.0

γ^{LW} – the Van der Waals free energy component; γ^{+} – the electron acceptor component; γ^{-} – the electron donor component; γ^{L} – surface tension of the liquid

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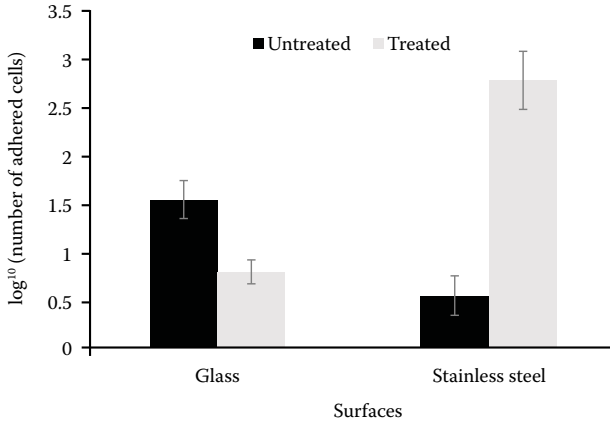

Figure 1. *Escherichia coli* adherence on glass and stainless steel before and after treatment with raw milk

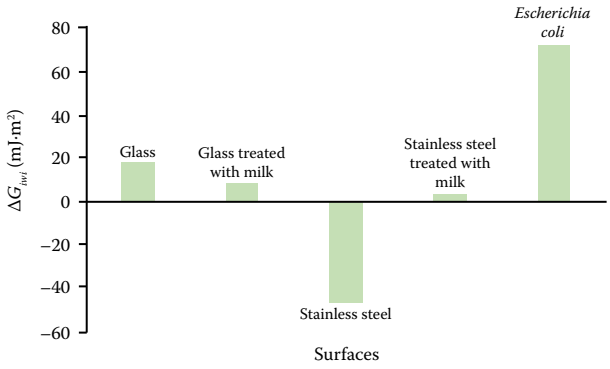

Figure 2. Surface free energy of glass, stainless steel and *Escherichia coli*

ΔG_{iwi} – surface free energy

relatively hydrophobic (82.5°), whereas after treatment it became relatively more hydrophilic (69.4°).

This study also evaluated the effect of the raw milk conditioning film on the acid-base properties of surfaces. The electron donor character (γ^-) decreased for glass from 42.26 mJ·m^{-2} (untreated) to 21.7 mJ·m^{-2} (treated), while the same factor was increased for stainless steel, from 7.1 mJ·m^{-2} (untreated) to 36.2 mJ·m^{-2} (treated). The electron acceptor character (γ^+) of glass decreased from 1.7 mJ·m^{-2} (untreated) to 0.2 mJ·m^{-2} (treated), while for stainless steel the same character increased from 0.5 mJ·m^{-2} (untreated) to 5.6 mJ·m^{-2} (treated).

The results of the physicochemical characterisation of the surface of *E. coli* ATCC 25922 by contact angle (Table 2) revealed that the bacteria were qualitatively (θ_{water} = 29.9°) and quantitatively ($\Delta G_{iwi} > 0$) hydrophilic. In terms of electron donor and acceptor properties, *E. coli* had a strong electron donor character (γ^- = 48.16 mJ·m^{-2}) and a weak electron acceptor character (γ^+= 1.16 mJ·m^{-2}).

In addition, the surface free energy (ΔG_{iwi}) showed a notable modification after treatment of the two surfaces with raw milk (Figure 2). The surface free energy of glass decreased from 18.0 mJ·m^{-2} to 7.9 mJ·m^{-2}; for stainless steel, this property was negative (–47.6 mJ·m^{-2}), while after treatment it became positive (2.9 mJ·m^{-2}).

DISCUSSION

Bacterial biofilms are widely recognised as the primary source of contamination in various dairy products (Bremer et al. 2009). Microbes present in manufacturing facilities contribute to the contamination of the products. This can stem from inadequate cleaning or poor disinfection practices (Lakticova et al. 2020). Preventing biocontamination by controlling the formation of biofilms in the early

Table 2. Contact angle measurements of water (θW), formamide (θF), diiodomethane (θD), Lifshitz-Van der Waals (γ^{LW}) parameters, electron donor (γ^-), acceptor of the two surfaces conditioned with raw milk and of *Escherichia coli* ATCC 25922 strains (γ^+)

Surface	Contact angle (°)			Surface tensions (mJ·m^{-2})		
	θD	θF	θW	γ^{LW}	γ^+	γ^-
Glass	44.3 ± 2.0	28.4 ± 4.0	46.43 ± 1.0	36.3	1.70	42.26
Glass treated with milk	60.7 ± 1.0	73.1 ± 2.0	74.60 ± 3.4	28.2	0.20	21.70
Stainless steel	40.9 ± 1.0	69.6 ± 3.0	82.50 ± 3.0	40.5	0.51	7.10
Stainless steel treated with milk	31.7 ± 2.0	77.4 ± 2.5	69.40 ± 1.6	43.5	5.60	36.20
Escherichia coli	48.4 ± 2.0	30.5 ± 1.0	29.90 ± 1.1	35.1	1.16	48.16

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stages is thus crucial. Our research focused on assessing the biocontamination of dairy product surfaces by studying the adhesion of *E. coli* to surfaces conditioned with raw milk. Initially, *E. coli* adhered better to glass compared to stainless steel. However, conditioning glass and stainless steel with raw milk affected drastically and conversely the adhesion of the bacteria to both surfaces. The changes in adhesion onto the two substrates show that *E. coli* had a greater ability to adhere to stainless steel, whereas adhesion became more difficult on glass.

For glass surfaces, our results agree with the results of other studies (Al-Makhlafi et al. 1994) in which certain milk constituents could minimise bacterial adhesion when used as a conditioning film. Additionally, stainless steel is a common substrate in food processing environments where bacteria can form biofilms (Hamadi et al. 2014), and it has been reported that enhancing surface hydrophobicity through surface conditioning could lead to a reduction in microbial adhesion (Zeraik and Nitschke 2010).

Our study contributes to our understanding of the interaction between *E. coli* and two dairy industry surfaces. An assessment of the physicochemical properties of these surfaces post-treated with raw milk showed notable modifications of these properties. The glass became relatively more hydrophobic, while stainless steel became relatively more hydrophilic. We suppose that the deposition of biochemical molecules from raw milk in the form of proteins, carbohydrates, and lipids would be the main reason for the change in physicochemical properties. The amphiphilic molecules present in raw milk interacted differently with the glass and stainless-steel surfaces (Figure 3). Due to the origin of these molecules having both hydrophilic (water-attracting) and hydrophobic (water-repelling) regions, we expected a selective deposition on the surfaces. Specifically, we hypothesised that the hydrophilic portion of the amphiphilic molecules would be preferentially deposited onto the stainless-steel surface (Figure 3), enhancing its hydrophilicity. Conversely, we supposed that the hydrophobic portion would predominantly adhere to the glass surface (Figure 3), thereby increasing its hydrophobicity. As a result, we predicted that the stainless-steel surface would become relatively hydrophilic, while the glass surface would become relatively hydrophobic.

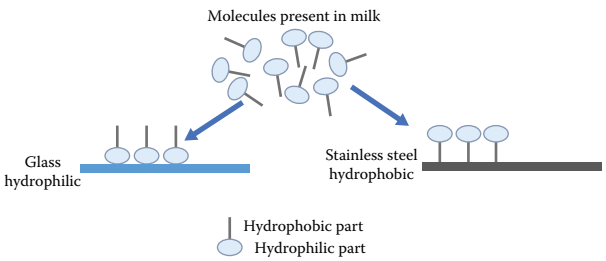

Figure 3. Deposition of biomolecules on the surface of glass and stainless steel

These results agree with another study in which the adsorption of beta-lactoglobulin as a milk compound increased the hydrophobicity and hydrophilicity of several different materials (Yang et al. 1991). Furthermore, Lorite et al. (2011) showed that the periwinkle wilt culture medium as a conditioning film on silicone and glass altered the wetting of these two surfaces after three hours of contact.

In this study, we evaluated the acid-base properties of surfaces after treatment with raw milk. For glass, the electron donor/acceptor components decreased after treatment, while for stainless steel these components increased. The results obtained from the glass surface agree with the results of Szlavik et al. (2012) on glass treated with unhomogenised milk.

CONCLUSION

Examination of the biocontamination of dairy surfaces by *E. coli* attachment to glass and stainless steel showed that the adhesion behaviour was significantly altered after the application of raw milk as a conditioning film. This was accompanied by significant alterations in the physicochemical properties of the treated surfaces. The results provide valuable insights that contribute to our understanding of the factors that influence the adhesion process. Such knowledge is essential for identifying the mechanisms of biofilm formation. Further experimentation by extending the deposition time of the conditioning film could be employed to extend the present findings.

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