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Quantitative Adhesion of *Staphylococcus aureus* on Stainless Steel Coated with Milk

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ABSTRACT

The surface energy characteristics of uncoated (clean) and coated stainless steel with UHT milk at various contact time (5 min, 30 min, 1 hours, 3 hours, 6 hours, 24 hours) were determined using contact angle measurement. Whatever the contact time, the clean stainless steel coupons became more hydrophobic and more electron acceptor when they are coated by milk. Inversely, the electron donor character seems to decreasing in this condition. The calculated surface energy component of coated stainless steel was found to vary with contact time. Its hydrophobicity and its electron acceptor were minimal after 3 hours of contact, but its electron donor was minimal after 1 hours of contact. Adhesion experiments of *Staphylococcus aureus* were carried out on uncoated and coated stainless steels at various contact times. For all contact times, the adhesion results show that milk reduce *S. aureus* adhesion, and the level of this reduction depend on contact time. This reduction was lower and higher after 1 hour, 5 min and 30 min of contact respectively.

Keywords: Surface Energy Characteristics; *S. aureus*; Adhesion; Milk; Stainless Steel

1. Introduction

The formation of biofilm creates major problems in the food industry since it may represent an important source of contamination for materials or foodstuffs coming into contact with them, so leading to food spoilage or transmission of diseases. Biofilms are of interest in the dairy industry, as bacteria within biofilms are more difficult to eliminate than plank tonic cells, and bacteria detached from biofilms can contaminate milk and milk products [1]. This biotransfer may affect hygiene and the commercial value of the product. To control these problems, it has been recognized that a greater understanding of the interactions between microorganisms and food—processing surface is required [2-4].

The adhesion of bacteria to surface is the first and essential stage in the formation of biofilm. This adhesion depends on both physicochemical properties of cell surface and solid surface, and also on characteristics of the surrounding medium.

Stainless steel is the most frequently used material for food processing equipment because of its high importance related to food safety reasons. There are many circumstances in dairies where substratum surface is either continuously or periodically in contact with liquids that contain microorganisms. These conditions could affect the substratum surface properties and consequently the adhesion process.

Staphylococcus aureus is a gram positive bacterium, which is an important food-borne pathogen [5-7]. In food industry this organism could be able to attach and to form biofilms on the food-processing surface [5,7,8]. *S. aureus* was studied here because little information [5-7] is available of its adhesion behaviour in dairy industry in comparison with other organisms such as *Listeria monocytognes* and bacillus [4,9-12].

The aim of this study was therefore to investigate the surface properties of stainless steel at various times of contact with milk. The adhesion of *S. aureus* to stainless steel was also examined and discussed in terms of physicochemical properties of cell surface and substratum surface.

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2. Materials and Methods

2.1. Bacterial Strains, Growth Conditions and Preparation of Microbial Suspension

The bacterial strain used in this study was *Staphylococcus aureus* ATCC 25923. The strain was cultured in Luria Burtani broth at 37˚C for 24 h after culture, the cells were harvested by centrifugation for 15 min at 8400 xg and were washed twice with and resuspended in KNO_3 solution with ionic strength 0.1 M. The physicochemical properties of this strain were measured by contact angle measurements. The results are presented in **Table 1** [13].

2.2. Cleaning of Stainless Steel Coupons

The solid support selected for this study was stainless steel 304. Before being coated with milk, the steel was cut into 1cmx1cm coupons and cleaned by soaking for 15 min in ethanol solution. The coupons were then rinsed with distilled water and autoclaved at 120˚C for 15 min.

2.3. Treatment of Stainless Steel Coupons with UHT-Milk

The cleaned stainless steel coupons were placed into a Petri dish and 10 ml of ultrahigh-temperature (UHT)-treated milk was added. The steel was allowed to contact milk for 5 min, 30 min, 1 h, 3 h, 6 h and 24 h at 4˚C. After each contact time, the coupons of steel were rinsed three times with distilled water.

2.4. Contact Angle Measurements

Contact angle measurements were performed using a goniometer (GBX instruments, France) by the sessile drop method. One drop of a liquid was deposited onto a dry stainless steel uncoated and coated by milk at different contact times. Three to six contact angle measurements were made on substratum surface for all probe liquids including water, formamide and diiodomethane. The Lifshitz-Van der Waals (γ^{LW}), electron donor (γ^{-}) and electron acceptor (γ^{+}) components of the surface tension of bacteria and for stainless steel were estimated from the approach proposed by Van Oss *et al.* (1988) [14]. In this approach the contact angles (θ) can be expressed as:

$$\cos\theta = -1 + 2\frac{\sqrt{\gamma_S^{LW}\gamma_L^{LW}}}{\gamma_L} + 2\frac{\sqrt{\gamma_S^{+}\gamma_L^{-}}}{\gamma_L} + 2\frac{\sqrt{\gamma_S^{-}\gamma_L^{+}}}{\gamma_L}$$

The Lewis acid-base surface tension component is defined by:

$$\gamma_S^{AB} = 2\sqrt{\gamma_S^{-}\gamma_S^{+}}$$

The surface hydrophobicity was evaluated through contact angle measurements and by the approach of Van Oss [14,15]. 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 when immersed in water (w): ΔG_{iwi}. If the interaction between the two entities is stronger than the interaction of each entity with water, the material is considered hydrophobic ($\Delta G_{iwi} < 0$); conversely, for a hydrophilic material, $\Delta G_{iwi} > 0$. ΔG_{iwi} is calculated through the surface tension components of the interacting entities, according to the following formula:

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

2.5. Adhesion Experiments

Ten millimetres of bacterial suspension containing 10^8 $CFU.ml^{-1}$ was incubated in a Petri dish containing stainless steel coupons treated by milk for 3 h at 4˚C. After 3 h of incubation, the coupons were then rinsed three times with distilled water to remove the nonadhering bacteria. The stainless steel coupons were immersed in a test tube containing physiological water (Nacl: 9 g/l). Bacterial cells were detached from the inert support by using a sonication bath (ultrasonic) for 5 min. CFUs were counted by using the serial dilution technique of the bacterial suspension obtained after sonication. Counts were determined on Luria Burtani agar after incubation for 24 h at 37˚C. Each experiment was performed in duplicate.

3. Results and Discussion

3.1. Surface Free Energy Characteristics of Stainless Steel Uncoated and Coated with Milk at Various Contact Times

Contact angles were measured on stainless steel surface before and after coating with milk using the three test liquids: water, formamide and diidomethane (**Table 2**).

Table 1. Contact angle values, surface energy and their components of *S. aureus* [13].

Contact angle			Surface free energy components (mj/m^2)					
Water	Formamide	Diidomethane	γ^{LW}	γ^{-}	γ^{+}	γ^{AB}	γ^{t}	ΔG_{iwi}
26.45 (1)	30.4 (1)	59.45 (2)	28.85 (0)	51.3 (0.99)	2.4 (0)	21.9 (3)	50.75 (2.4)	28.57

Standard deviation is given in parentheses.

Table 2. Contact angle values, surface energy and their components of uncoated (control) and coated stainless steel at different contact times.

Contact time	Contact angle			Surface free energy components (mj/m^2)					
	Water	Formamide	Diidomethane	γ^{LW}	γ^-	γ^+	γ^{AB}	γ^t	ΔG_{iwi}
Control	64 (2)	68 (1.8)	60 (5)	28.1 (2.6)	32.5 (2)	0.1 (0)	3.16 (2)	31 (5)	11.5
5 min	127 (5)	104 (1.13)	79 (2)	18 (3)	0.5 (0)	0.7 (0)	0.75 (0.3)	18.8 (4)	−73.53
30 min	129 (2)	107 (4)	75 (4)	19.65 (2.1)	0.45 (0)	1.15 (0.6)	0.85 (1)	20.5 (3)	−69.76
1 h	129 (0.7)	112 (0.9)	72 (4)	21.45 (2)	0.05 (0)	3.25 (0.4)	0.4 (0)	21.9 (1.9)	−62.68
3 h	115 (0.45)	72 (0.9)	51 (0.6)	33.3 (0.3)	3.9 (0.1)	0.6 (0.1)	3 (0.37)	36.5 (0.6)	−42.10
6 h	127 (4)	96 (4)	63 (2)	26.6 (1.1)	1.6 (1)	1.11 (0.2)	2.3 (1.2)	28.9 (2.4)	−67.60
24 h	115 (1.7)	73 (3)	56 (0.7)	31.19 (0.6)	3.1 (0.7)	0.7 (0.4)	2.9 (1.1)	34.13 (1.8)	−64.46

Standard deviation is given in parentheses.

The contact angle data were then used to calculate the surface energy components of all samples (**Table 2**). The results show that uncoated stainless steel surface was hydrophilic with ΔG_{iwi} = 11.50 mj/m^2. Regardless of contact time, stainless steel coated with milk alters significantly its surface hydrophobicity. The uncoated stainless steel surface hydrophobycity ranged from hydrophilic character (positive value of ΔG_{iwi}) to hydrophobic character (negative value of ΔG_{iwi}). It is known that milk is a complex biological fluid composed by several components including proteins, fats and calcium phosphate. According to Mittelman (1998) [16], the adsorption of milk and its components on substratum surface occurs within 5 s to 10 s.

The effect of proteins hydrophobicity of solid surface is reported by some works [17,18]. Yang *et al*. (1991) [17] have found that the adsorption of *β*-lactoglobulin onto substratum surface could render hydrophilic surfaces more hydrophobic and hydrophobic surfaces more hydrophilic. Barnes *et al*. (1999) [5] reported that the fat components are likely to interact with hydrophobic surface of stainless steel. Other works [19], have reported that surface energy characteristics of a solid surface influence the extent and rate of protein adsorption. In our work the observed increasing hydrophobicity of coated stainless steel could be due to the adsorption of proteins and/or fat components to substratum surface. The order of deposition of milk components should be related to initial surface energy characteristic of substratum.

Harnett *et al*. (2006) [20] have calculated the surface energy of various materials coating a series of proteins of collagen, and fibroncetin and they found that these proteins affect significantly the electron donor and the electron acceptor of some substratum surfaces. To our knowledge, the effect of proteins or other components of milk on electron donor/electron acceptor properties of substratum surface were not examined previously. Stainless steel coated with milk has a very lower electron donor compared to stainless steel uncoated with very high electron donor property (**Table 2**). In opposite, the electron acceptor of stainless steel was not markedly affected by the presence of milk (**Table 2**). The variation of hydrophobicity, electron donor and electron acceptor properties of stainless steel pretreated with milk as a function of contact time are presented in **Figure 1**.

The surface hydrophobicity decreases from 5 min to 3 h and increases from 3 h to 24 h (**Figure 1(a)**). This hydrophobicity achieved the minimum at 3 h of contact. **Figures 1(b)** and **(c)** show that contact time affect markedly the electron donor and electron acceptor properties of coated stainless steel. The electron donor and electron acceptor properties achieved the maximum at 3 h of contact and 1h of contact respectively.

Kim and Lund (1997) [21] have found that the adsorption process for *β*-lactoglobulin on stainless steel was very rapid in the first 5 min and essentially reached equilibrium within 10 min. These authors have also reported that the precipitation of calcium phosphate onto the stainless steel surface was very slow compared to monolayer deposition of *β*-lactoglobulin. Addesso and Lund (1997) [19] show that protein adsorption onto a surface depends on protein concentration. The random observed variation of physicochemical properties of stainless steel as a function of contact time should be related to a nature and an amount of milk components adsorbed onto substratum surface and its kinetic deposition.

3.2. Adhesion of *S. aureus* to Stainless Steel Treated with Milk under Different Contact Time. Kinetic Evolution of *S. aureus* Adhesion on Stainless Steel Pretreated by Milk

Several works [4,5,11,12,22-24] have studied the effect of milk or proteins milk on bacterial adhesion. In this study, we are interested to examine the adhesion kinetic of *S. aureus* to stainless steel coated with UHT milk. The results of *S. aureus* adhesion on coated and uncoated stainless steel are presented in **Figure 2**.

Coated stainless steel with UHT milk was shown to reduce the attachment of *S. aureus* whatever contact time.

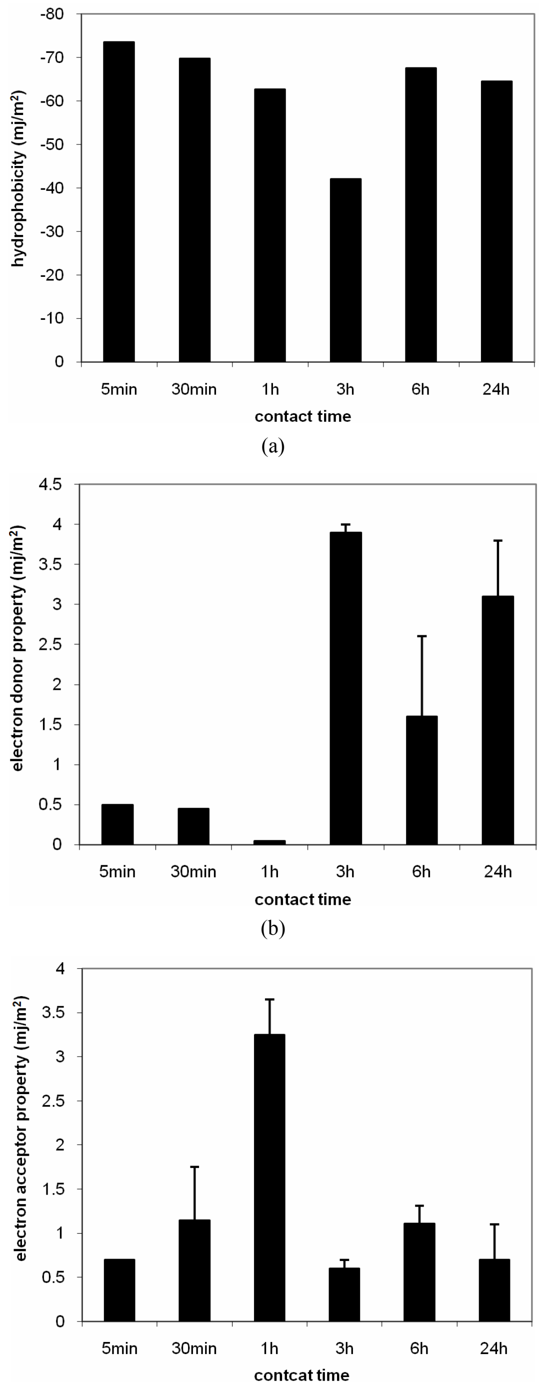

Figure 1. Variation of physicochemical properties of stainless steel coated with milk as a function of contact time. (a) Hydrophobicity; (b) Electron donor property; (c) Electron acceptor property.

The role of milk or components of milk in inhibiting bacterial adhesion was reported previously by several works. Barnes *et al*. (1999) [5] have reported that the pretreatment of stainless steel with skim milk was found to reduce *S. aureus* adhesion. According to Hood and

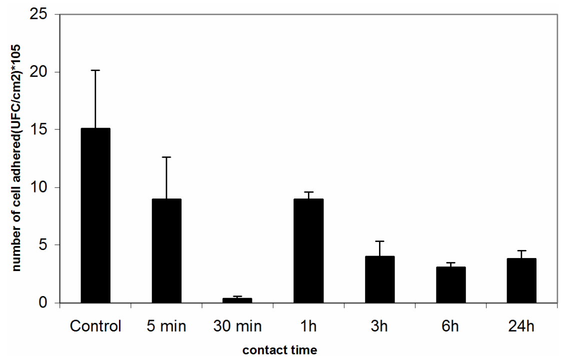

Figure 2. Number of *S. aureus* cells adhered to uncoated (control) and coated stainless steel at different contact times.

Zottola (1997) [4], the attachment of *Listeria monocytogenes* and *Salmonella typhimurium* to stainless steel was inhibited by preconditioning with whole and chocolate milk and was enhanced when using diluted milk.

To our knowledge, the surface physicochemical properties have not been considered in interpreting the effect of milk on bacterial adhesion results despite the clear change in substratum surface physicochemical properties after contact with milk or milk components.

The adhesion results obtained here were discussed and interpreted in terms of hydrophobicity and electron donor/electron acceptor properties of both surfaces (cell surface, stainless steel surface). The electrostatic interactions were neglected since our experience was performed in a solution with high ionic strength [25,26]. Since *S. aureus* is hydrophilic (**Table 1**) and uncoated stainless steel surface is also hydrophilic (**Table 2**), the *S. aureus* adhesion on this substratum was increased. In the other hand, the adhesion of hydrophilic *S. aureus* was reduced on hydrophobic stainless steel coated with milk. These results are in accord with the hypothesis that the hydrophobic cells tend to attach to a hydrophobic substrate and the hydrophilic cells tend to attach to a hydrophilic substrate. On the other hand, the difference in level adhesion between stainless uncoated and coated stainless steel could be related to the contribution of acid-base interactions; these interactions seem to be lower in the case of uncoated stainless steel since its electron donor was very low in comparison with the electron donor of coated stainless steel.

From **Figure 2**, we also observe that the level of *S. aureus* adhesion changes as a function of contact time. The *S. aureus* adhesion was much reduced at 30 min comparatively for other times of contact. This variation is not completely explained by physicochemical interactions. However, others interactions between cell surface and milk or milk components adsorbed on surface could be contribute in bacterial adhesion at different contact times. The difference in nature of proteins adsorbed for each contact time and the faster conformational rearrangement undergone by one protein at surface relative to that of other proteins could be the origin of the variation observed in adhesion results. Barnes *et al*. (1999) [5] have found that the pre-treatment of stainless steel with the individual milk proteins α-, β- and κ casein and α-lactalbumin at equal concentration reduce attachment of *S. aureus* and this reduction was marked with β casein. McEldowney and Fletcher (1987) [27] observed that hydrated layers of polymers and proteins that form on inert surfaces can either facilitate or reduce bacterial adhesion. Al Makhlafi *et al*. (1994) [22] examined the effect of competitive adsorption of bovine serum albumin (BSA) and β-lactoglobulin on Listeria monocytogenes adhesion to silica, and they found that the film formed by the adsorption of β-lactoglobulin followed by BSA encouraged adhesion more than the film formed by the adsorption of BSA followed by β-lactoglobulin.

4. Conclusion

The results obtained here show that the physicochemical properties including hydrophobicity and electron donor-electron acceptor properties of stainless steel surface were markedly affected by treatment by milk. The adhesion results show that whatever the contact time, the pre-treatment of substratum by milk reduce the adhesion level. This reduction is random with the contact time. This research suggests that it is very important to take into account the contact time between the substratum and milk in the cleaning and sanitizing process.

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