

Modifying Supports Surfaces by Dairy Wastewater Conditioning Film and Relationship with Initial Bacterial Adhesion

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The choice of the best support for microbial adhesion can improve the start-up speed and efficiency of dairy wastewater treatment by biofilm bioreactor. In this study, three substrates were tested: PP (polypropylene), PET (Polyethylene terephthalate), and PVC (polyvinyl chloride). By using contact angle method, the surface's physicochemical characteristics of bacterium, inert substrates and substrates after dairy wastewater (DWW) conditioning film were measured to understand its impact on adhesion as well as the most suitable material to optimize bacterial adhesion. DWW conditioning film affects the physicochemical characteristics of plastic supports, and improves the initial adhesion of bacteria to substrates. Results of initial adhesion tests for untreated and treated supports showed differences in how bacterial cells adhered to substrates. Before treatment, PVC and then PP showed a significant adhesion capacity, double that of PET. After modifying by DWW, initial bacterial adhesion increased by 10^6 (10^5 to 10^{11} CFU/cm²) and PVC demonstrated the highest adhesion capacity, followed by PP and finally PET. Therefore, before the modification of the supports by DWW, PP and PVC are in the same rank for the initial bacterial adhesion and after the modification, PVC seems to be the best for initial bacterial adhesion.

Keywords: Conditioning film; Initial adhesion; Dairy wastewater; Physicochemical properties.

The dairy industry represents an economic and nutritional interest for the sums it generates and dairy products are very important for human nutrition. But this industry generates a lot of wastewater. The quantity of wastewater varies between 0.2 and 10 L for one liter of treated milk¹. Dairy wastewater (DWW) is highly loaded with organic matter².

Treatment of this wastewater is becoming an absolute necessity for environmental protection. There are several methods of treating

DWW to reduce its organic matter loading: electrochemical³, membrane filtration⁴, reverse osmosis⁵, coagulation-flocculation⁶, land treatment⁷, and biological treatments such as anaerobic biofilm reactor⁸. Among the anaerobic bioreactors, it is worth citing the anaerobic digester, which not only produces little sludge but also generates bio-methane used as an energy source. After anaerobic digestion begins, biogas detection only takes place from the second week of start-up⁹ due to the time required to accumulate a sufficient

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amount of biomass to treat the organic matter. Often, when the content is renewed in a bioreactor, some of the biomass is lost through the effluent, which delays the bioprocess¹⁰. The use of supports inside the reactors allows the microorganisms to bind to supportsurfaces, form a biofilm and increase the surface area of contact between the microorganisms and the organic material. Supports, also, can help in maintaining the biomass that may be lost at a reactor outlet and increasing the start-up speed of the bioreactor after the renewal of its contents.

Many different studies have concluded that physicochemical characteristics, roughness, pH, ionic strength, and porosity play an important role in the initial adhesion of bacteria to substrates¹⁰⁻¹⁶. One aspect of microorganisms' adhesion to biocarriers that has received little attention is the conditioning film phenomenon: the deposit of nutrients on material surfaces when immersed in a liquid medium¹⁷. Substances in organic materials such as sugars and proteins can adsorb to surfaces, forming a conditioning film and affecting physicochemical characteristics, roughness, surface charge, and wettability. The conditioning film, in turn, affects the adhesion of bacteria to the surface¹⁷.

To our knowledge, no work has highlighted the effect of DWW conditioning film or, more generally, any organic material on the supports in the bioreactor for wastewater treatment. In this work, our objective is to compare 3 plastic supports before and after treatment with DWW, demonstrate how the DWW conditioning film modifies both the biocarriers' physicochemical characteristics and the bacterium adhesion to substrate surfaces treated and untreated with DWW.

MATERIALS AND METHODS

Bacterial strain and the preparation of a bacterial suspension

The bacterial strain used as a biological model is an optional anaerobic Gram-positive bacterium from a laboratory digester. This bacterium is added to the consortium of microorganisms in the anaerobic digester to increase the quantity of biogas¹⁸. This strain is grown in a liquid Luria Bertani medium (LBL) at 37°C for 24 hours. The bacteria are then collected by centrifugation

(5000 g for 15 min), washed twice with a solution of KNO₃ at 0.1 M of ionic strength. Finally, the bacterial suspension was prepared with a solution of KNO₃ (0.1M).

Plastic supports

Three ordinary plastic carrier materials were selected for their low cost, durability, and availability and because they are commonly used as mobile carrier materials in anaerobic digesters: polyethylene terephthalate (PET:(C₁₀H₈O₄)_n), polypropylene (PP:(C₃H₆)_n), and polyvinyl chloride (PVC:(C₂H₃Cl)_n). Each plastic material was cut into 1.5 cm² square coupons for contact angle measurement experiments, and into 1 cm² squares for adhesion tests. The plastic supports were immersed in ethanol for 15 min to disinfect and remove dirt from their surface and subjected to a sonication bath in sterile distilled water for 10 min. The coupons were rinsed several times with sterile distilled water. Then they were dried in a sterile area before being stored in a sterile condition for later use.

Contact angle measurement

The bacteria

The method for measuring contact angles on bacterial layers has been described by Busscher *et al*¹⁹. Briefly, the prepared bacterial suspension is deposited on a cellulose acetate filter (0.45 μm) using a filtration ramp, producing a bacterial mat whose thickness probably represents 50 to 100 cells. This film is placed on a glass support and allowed to evaporate. The contact angle is then measured. Water (w), formamide (f), and diiodomethane (d) were used as reference solvents. A drop is formed at the end of a syringe to be deposited on the sample surface. A sequence of digital images is immediately acquired (Windrop) using a CCD camera placed on a goniometer (GBX Instruments, France). Three measurements are made for each sample and for each solvent. The experiment is repeated three times. The free surface energies are determined: the Lifshitz-Van der Waals γ^w , electron acceptor γ^+ , and electron donor γ^- using the equation (1) of Van Oss *et al*²⁰. In this approach the contact angles (θ) can be expressed as in Equation (1).

$$\gamma_L(1 + \cos \theta) = 2 \cdot (\sqrt{\gamma_S^w \cdot \gamma_L^w} + \sqrt{\gamma_S^+ \cdot \gamma_L^+} + \sqrt{\gamma_S^- \cdot \gamma_L^-}), \quad \dots(1)$$

And the quantitative hydrophobicity can be estimated by using Equation (2).

$$\Delta G_{iwi} = -2 \left[\left((\gamma_i^{+w})^{\frac{1}{2}} - (\gamma_w^{+i})^{\frac{1}{2}} \right)^2 + 2 \left((\gamma_i^{+i})^{\frac{1}{2}} + (\gamma_w^{+w})^{\frac{1}{2}} - (\gamma_i^{+w})^{\frac{1}{2}} - (\gamma_w^{+i})^{\frac{1}{2}} \right) \right] \dots (2)$$

The supports with and without conditioning film

The effect of DWW conditioning film on supports' physicochemical characteristics was studied by comparing the physicochemical characteristics of these plastic materials before and after their treatment with DWW. Treatment consisted of immersing the materials in DWW for 3 hours at 37°C and then drying in a sterile area.

For untreated supports, the contact angle was measured after cleaning, disinfecting, and drying. For treated supports, the contact angle was measured after treatment with DWW. The basic principle is the same as for bacteria. The contact angles of the supports were measured using the sessile drop technique of the three probe liquids of different polarity and with known surface energy.

Initial adhesion tests

Ten ml of bacterial suspension containing approximately 10^8 CFU/ml was incubated in a petri dish containing PET, PP, PVC coupons (cleaned and disinfected according to the protocol described above) untreated and treated by sterile DWW for 3 hours at 37°C. After incubation, the coupons were then rinsed three times with sterile distilled water to remove non-adherent bacteria. The plastic coupons were immersed in test tubes containing sterile physiological water (NaCl: 9 g/l). The bacterial cells were detached from the inert supports using a sonication bath (ultrasonic) for 5 min²¹. The adhered bacteria were harvested by the sonication method and CFUs were counted using the serial dilution technique of the bacterial suspension (dilution up to 10^{-3} in the case of untreated supports and 10^{-9} for supports treated with DWW).

RESULTS AND DISCUSSION

Surface energy components of the bacterium

Qualitative hydrophobicity θ_w is a measure of the contact angle between a bacterial surface and a drop of water and quantitative

hydrophobicity ΔG_{iwi} is the free energy of interaction between any substance (i) and water (w). The contact angle measurements of the bacterium were taken and then used to determine the surface energy components (Table 1).

In view of the results obtained, our bacterium has a hydrophilic character qualitatively ($\theta_w = 33.8^\circ < 65^\circ$)²². A quantitative approach affirms this result, finding that the strain tested has a positive free surface energy ($\Delta G_{iwi} = 31.1 \text{ mJ/m}^2 > 0$)²⁰. Moreover, this strain has a strong electron donor character ($\gamma^- = 50.62 \text{ mJ/m}^2$), whereas the electron acceptor properties are very low ($\gamma^+ = 0.59 \text{ mJ/m}^2$). In light of these results, Latrache *et al.*²³ have shown that the hydrophobicity measured by the contact angle is directly correlated with the high N / C ratio and inversely correlated with that of O / C ratio and have also shown that the hydrophilicity of *Escherichia coli* is related to the presence of polysaccharides, while hydrophobicity is related to the presence of proteins. Also, the carboxyl and phosphate groups contribute to the negative charges present at *Escherichia coli* cell surface²⁴.

Plastic supports' physicochemical characters

The contact angle measurements for the different plastic supports were taken before and after the supports were treated with DWW and then used to determine the surface energy components (Figure 1).

All the supports (U) are hydrophobic and have a great qualitative hydrophobicity ($\theta_w(\text{PETU}) = 80.9^\circ$; $\theta_w(\text{PPU}) = 77.8^\circ$; $\theta_w(\text{PVCU}) = 73.3^\circ$) (Figure 1.a). Like the qualitative approach (θ_w), the quantitative hydrophobicity (ΔG_{iwi}) of the three supports have a hydrophobic character ($\Delta G_{iwi}(\text{PETU}) = -63.2 \text{ mJ/m}^2$; $\Delta G_{iwi}(\text{PPU}) = -42.3 \text{ mJ/m}^2$; $\Delta G_{iwi}(\text{PVCU}) = -48.5 \text{ mJ/m}^2$) (Figure 1.b). The electron donor γ^- character for the three untreated supports have a feeble valor ($\gamma^-(\text{PETU}) = 4.9 \text{ mJ/m}^2$; $\gamma^-(\text{PPU}) = 4.1 \text{ mJ/m}^2$; $\gamma^-(\text{PVCU}) = 5.3 \text{ mJ/m}^2$) (Figure 1.c) and the valor of electron acceptor character is very feeble ($\gamma^+(\text{PETU}) = 0 \text{ mJ/m}^2$; $\gamma^+(\text{PPU}) = 0.25 \text{ mJ/m}^2$; $\gamma^+(\text{PVCU}) = 1 \text{ mJ/m}^2$) (Figure 1.d).

After treatment with DWW, the support surfaces show a change in their qualitative hydrophobicity θ_w ($\theta_w(\text{PETT}) = 78.97^\circ$; $\theta_w(\text{PPT}) = 100.84^\circ$; $\theta_w(\text{PVCT}) = 93.90^\circ$) (Figure

1.a) and quantitative hydrophobicity ΔGiwi (ΔGiwi (PETT)=-39.08 mJ/m²; ΔGiwi (PPT)=-79.03 mJ/m²; ΔGiwi (PVCT)=-79.03 mJ/m²) (Figure 1.b). The DWW conditioning film increased the valor of the electron donor γ - character for PET (γ -(PETT)= 13.28 mJ/m²) and decreased the valor for PP and PCV (γ -(PPT)= 0.60 mJ/m²; γ -(PVCT)= 2.26 mJ/m²) (Figure 1.c), while the valor of electron acceptor character remained very feeble with a slight increase for PET and small decrease por PP and PVC (γ +(PETT)=0.25 mJ/m²; γ +(PPT)=0.55 mJ/m²; γ +(PVCT)= 0.06 mJ/m²) (Figure 1.d).

Initial adhesion tests of a bacterium to supports treated and untreated with DWW

This section presents results regarding the adhesion power of the selected bacterial strain to several supports that differ by their physicochemical characteristics and by whether they were conditioned in DWW. The bacterial strain's ability to attach to untreated supports compared to those treated with DWW is presented in Figure 2.

In Figure 2.a, results of the adhesion tests show a marked difference among the untreated support materials (PET, PP, and PVC) in their ability to promote initial bacteria adhesion. PET is the substrate with least colonization by the studied bacterial strain, whereas PVC contains the most of adherent bacteria, followed by PP; both PVC and PP had over 2 times more colonization than PET [PVC: 1.58 10⁵ CFU/cm²; PP: 1.48 10⁵ CFU/cm²; PET: 0.72 10⁵ CFU/cm²]. The adhesion on these untreated substrates decreases in this order PVC>PP>PET. Treatment with DWW increases bacteria adhesion across all supports by a factor of 10⁶ (from 10⁵ UFC/cm² for untreated supports to 10¹¹ UFC/cm² for treated supports) (Figure 2.b). Notably, after treatment with DWW, PET is the substrate with least colonization by the studied bacterial strain, whereas PVC contains the higher colonization (56 times more than PET and 4 times more than PP), followed by PP (13.8 times more than PET) [PET: 0.43 10¹¹ CFU/cm²; PP: 5.95 10¹¹ CFU/cm²; PVC: 24.25 10¹¹ CFU/cm²]. The adhesion on these treated substrates decreases in this order PVC>PP>PET.

Microbial adhesion to a surface is quite complex because it involves electrostatic, Van der Waals and acid-base components. Our study consisted in determining the physicochemical

characteristics of the bacterium and the supports treated and untreated with DWW.

Various studies have shown that a conditioning film can be formed by several organic substances such as proteins, polysaccharides, lipids, nucleic acids, and exopolysaccharides^{25,26}. Conditioning film formation is a multi-step phenomenon; as an example, on stainless steel in a marine environment, proteins adsorb first followed by carbohydrates^{27,28}.

It should be noted that DWW has a complex composition. Some authors have mentioned that DWW resembles milk but is diluted, the organic and mineralogical composition differs according to the industrial process (milk, cheese, yogurt and butter) used and that detergents and other products used in the production process is found in trace amounts in the effluent at the outlet of the dairy plant²⁹. It is known that milk is a complex biological fluid consisting of several components including lactose, proteins, fats and calcium phosphate. According to Mittelman³⁰, the adsorption of milk and its components on the substrate surface occurs within 5 to 10 s.

Because of this, other authors put into perspective the role of the medium, especially the conditioning film, on bacterial adhesion^{17,25}.

Treatment with DWW does alter the natural character of the three supports, with remarkable changes in the ΔGiwi and θ_w values (Figure 1.a and b). The ΔGiwi decreased for the PET support after DWW treatment and increased greatly for the PP and PVC supports (Figure 1.b). The electron donor character value increased greatly for the PET (γ -(PETU)= 4.9 mJ/m² to γ -(PETT)= 13.28 mJ/m²) following DWW treatment and decreased greatly for PP (γ -(PPU)= 4.1 mJ/m² to γ -(PPT)= 0.60 mJ/m²); and PVC (γ -(PVCU)= 5.3 mJ/m² to γ -(PVCT)= 2.26 mJ/m²) (Figure 1.c).

The results show that the hydrophobicity, qualitatively and quantitatively, increased after treatment with DWW for PVC and PP supports and remained almost stable qualitatively and increased quantitatively for PET. These results are consistent with those of Hamadi *et al*³¹ who had worked on stainless steel and found that steel coated with milk is more hydrophobic than uncoated steel. The electron donor character for PP and PVC are stronger for untreated supports than for treated

supports with DWW, these results are the same to those found for stainless steel²¹. For PET it is the opposite.

The contact angle method gave very detailed results in terms of hydrophobicity and electron donor/acceptor character for the three supports (PET, PP, PVC) before and after modification with DWW (Figure 2). From a qualitative and quantitative point of view, we found that all the untreated polymer materials have a clearly hydrophobic character. Moreover, all these materials have a low electron donor/acceptor

character. Many different studies have shown the same tendency in the surface physicochemical characteristics for these untreated polymers³²⁻³⁷.

In this study, adhesion tests of the bacterial strain were performed on polyethylene terephthalate (PET), polypropylene (PP), and polyvinyl chloride (PVC). Different results were observed between supports that were modified with DWW and those that were untreated. Microorganism adhesion to surfaces is, as with any inert colloidal particle, largely governed by physicochemical interactions. The sum of these interactions—

Table 1. Contact angle and surface energy components of the bacterium

Surface	Contact angle (°)			Surface energy (mJ/ m ²)				
	θ _d	θ _f	θ _w	γ _{LW}	γ ⁺	γ ⁻	γ _{AB}	ΔG _{iwi}
Bacterium	43.5	38.6	33.8	37.5	0.59	50.62	10.93	31.1
Std. dev.	1.5	2	1.4	0.8	0.1	4.5		

d = diiodomethane, f = formamide and w = water

γ_{LW} surface energy of Lifshitz-Van der Waals, γ⁺ electron acceptor, γ⁻ electron donor, the γ_{AB} Lewis acid–base surface tension and ΔG_{iwi}: the free energy of interaction between two entities of that material when immersed in water

Std. dev.= Standard deviation

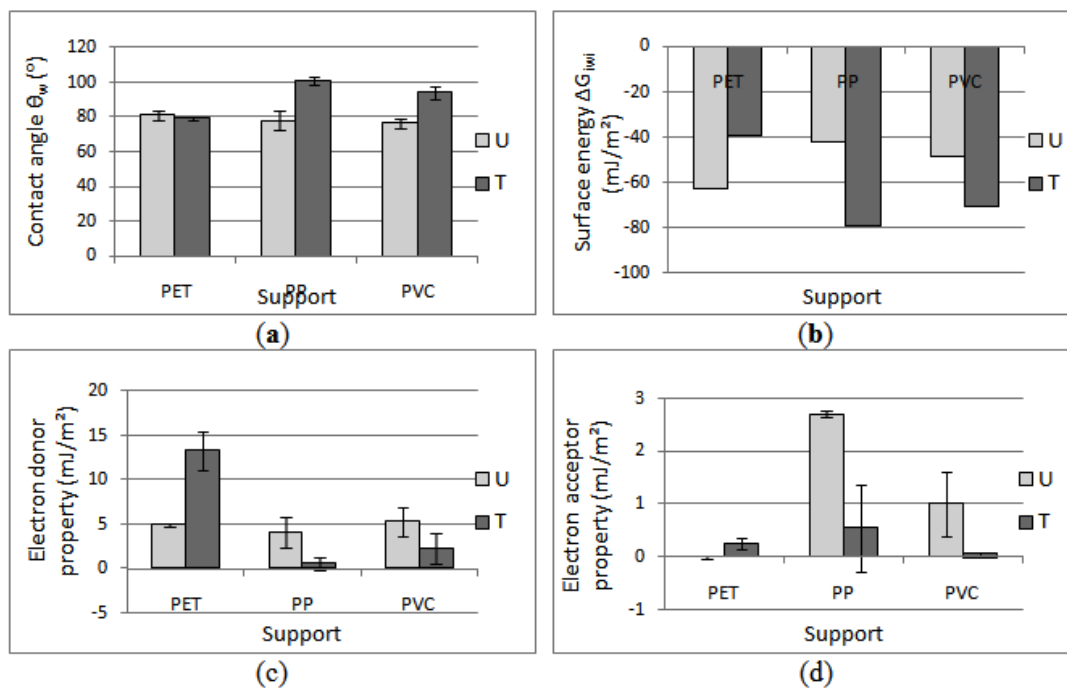


Fig. 1. Physicochemical characteristics of supports (PET, PP, and PVC) untreated (U) and treated (T) with OMWW. (a) Qualitative hydrophobicity; (b) Quantitative hydrophobicity; (c) Electron donor property; (d) Electron acceptor property; U Untreated; T Treated with DWW

including electrostatic, Lifshitz-Van der Waals, and acid-Lewis base interactions—can be attractive or repulsive. These interactions depend on the physicochemical properties of microorganisms' surface, substrate surface, and suspension medium characteristics. These physicochemical properties include hydrophobicity, electrostatic charge, and electron donor/electron acceptor character. All the factors likely to modify the physicochemical surface properties of one of the elements involved in the adhesion phenomenon can thus favor or limit microorganisms' fixation³⁵.

In addition, basic chemistry states that one hydrophilic entity naturally attracts another hydrophilic entity³⁸ and vice versa. Previous research³⁸⁻⁴⁰ have reported that hydrophobicity cannot systematically explain the results of microbial adhesion to a support and that acid-base interactions play a very important role in the adhesion phenomenon^{13,41,42}. According to these assertions, the adhesion of the studied bacterium on the surfaces of supports modified with DWW may be due in part to the acid-base interactions between the strong bacterium's electron-donating character and the weak supports' electron-accepting character, which may also explain the adhesive power of this bacterium on modified and untreated supports with DWW.

Hydrophobicity and electron acceptor/donor characteristics were used here to explain these results. Electrostatic forces were not taken into account because the tests were carried out in a liquid with a high ionic strength⁴³. It is well known that bacteria are usually charged negatively in a liquid medium⁴⁴. To avoid charge interference between the bacteria cells and the DWW, we used high ionic strength of cell suspension.

As mentioned in different studies, polysaccharides can have a hydrophobic or hydrophilic character depending on the state of freedom in solution as well as their three-dimensional conformation, which can influence the physicochemical parameters of the supports treated with DWW^{36,45}. Hamadi *et al*²¹ have shown that physicochemical parameters including hydrophobicity and electron acceptor/donor character of a stainless steel surface can be modified by fatty acid and proteins after conditioning by milk.

In our case, the modification of the three supports' physicochemical characteristics (hydrophobicity and electron donor/acceptor character) is due to DWW properties (carbohydrate, fat content and proteins). The concentration and type of molecules adsorbed on the surface of a material are conditioned by the nature of this material (Δ Giwi, hydrophobicity, electron donor/acceptor character, electrostatic charges, etc.)^{46,47}. This may explain the differences we found concerning hydrophobicity and electron donor/acceptor character between the modified supports.

The more the hydrophobicity decreases (θ_w (PETU) = 80.9°; θ_w (PPU) = 77.8°; θ_w (PVCU) = 76.3°) (Figure 2.a), the more the bacterial adhesion increases for untreated supports [PET: 0.72 10⁵ CFU/cm²; PP:1.48 10⁵ CFU/cm²; PVC:1.58 10⁵ CFU/cm²] (Figure.1a). The work of Pringle and Fletcher⁴⁸ who found a relationship between the contact angle to water (varies from 0° to 110°) and the adhesion of different bacteria on four different surfaces. Also, Absolom *et al*⁴⁹ showed a linear relationship between the contact angle to water of different varieties of polymers (ranging from 58° to 110°) and bacterial adhesion.

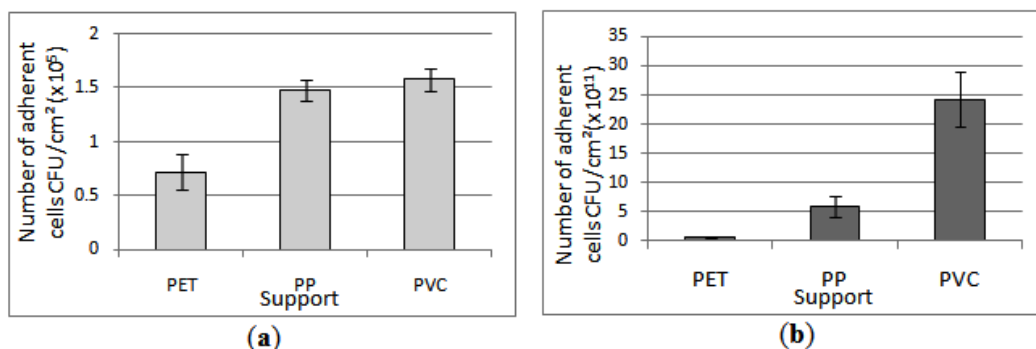


Fig. 2. Number of initial cells adhering to supports. (a) Untreated supports; (b) Treated supports

In our case, the thermodynamic theory (physicochemical proprieties) cannot explain the high number of adhered cells on the treated supports. It must be other factors like specific biological interaction (that include ligand-receptor bond) involved in the bacterial adhesion⁵⁰.

The components of DWW probably adsorb differently on the 3 supports due to difference of plastic chemical composition (more on PVC than PP and less on PET). Thus, DWW conditioning film increase the bacterial adhesion differently, PVC contains the higher colonization (56 times more than PET and 4 times more than PP) followed by PP (13.8 times more than PET).

CONCLUSIONS

The bacterium has a very pronounced hydrophilic character both qualitatively and quantitatively and a strong donor electron character.

Untreated supports with DWW have a hydrophobic character and a very weak electron acceptor/donor character.

After treatment, PET support retained its hydrophobic character with change compared to untreated supports. PVC and PP treated supports have an increase in hydrophobicity and a decrease in the electron donor character.

Bacterial adhesion to untreated supports is affected by hydrophobicity. In fact, the more the hydrophobicity increases, the more the bacterial adhesion increases, and the amount of cell adherence is double for PVC and PP compared to PET for untreated supports. After treatment with DWW, the conditioning film of DWW significantly enhanced the bacterial adhesion for all three supports (from 10^5 UFC/cm² to 10^{11} CFU/cm²).

In conclusion, the choice of support material impacts bacterial adhesion, especially after taking into account the DWW conditioning film, which promotes a high level of bacterial adhesion.

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