# Effect of hydrophobicity on *in vitro* streptococcal adhesion to dental alloys

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Non-specific interactions such as electrostatic interactions, and surface free energy are of importance in bacterial adhesion to dental surfaces as they determine whether or not bacteria are attracted to the surface. The relationship between adherence of *Streptococcus mitis*, *S. mutans*, *S. oralis* and *S. sanguinis* on precious and non-precious dental alloys, and the bacterial and alloy surface hydrophobicities (a measure of the surface free energy) was studied. The number of adhering bacteria was determined by fluorescence microscopy counts. The hydrophobicity of the bacteria and alloy surfaces were evaluated by adhesion to hexadecane and water contact angles, respectively.

Our results showed that (i) the surfaces of the tested alloys were hydrophobic, (ii) *S. sanguinis*, *S. mutans* and *S. oralis* were hydrophobic, and (iii) *S. mitis* was hydrophilic. *S. oralis*, the more hydrophobic strain, demonstrated the highest adherence on the tested materials, whereas *S. mitis* adhered least on the hydrophobic surfaces. For the tested alloys, bacterial adherence was highest for the high gold content alloy, and lowest for the non-precious alloy.

Our results showed that for the tested bacterial strains, there was a significant correlation between bacterial adhesion and substratum hydrophobicity: hydrophobic metal surfaces favor adhesion of hydrophobic bacteria.


## 1. Introduction

Dental plaque formation, which results from adhesion of oral bacteria to tooth surfaces, can lead to secondary caries, periodontal diseases or alteration of dental restorations [1]. Certain bacterial species, including *Streptococcus sanguinis*, *S. mitis*, *S. oralis*, *S. gordonii* and *S. parasanguinis* [2–5], act as ''pioneers'' in bacterial adhesion to the tooth surfaces.

Bacterial adhesion is governed by non-specific interaction (physico-chemical interactions) and specific interactions (ligand-receptor like interactions). Non-specific interactions comprise van der Waals interactions, electrostatic interactions and acid-base interactions [6–9]. The resultant of these interactions, which plays an important role in the initial bacterial adhesion (DLVO theory) [7, 9] defines the surface free energy [9–11]. Surface free energy can be evaluated by measuring hydrophobicity values [11, 12]. Bacterial and substratum surface hydrophobicities can be evaluated by quantifying adhesion to hexadecane [13, 14] and measuring water contact angles [15, 16], respectively. Specific interactions implicating bacterial adhesions and salivary glycoproteins adsorbed on the tooth surface, play a role in the irreversible adhesion of bacteria to the substratum [5].

Several *in vitro* and *in vivo* studies have shown quantitative and qualitative variations in dental plaque formation between natural teeth and artificial tooth surfaces, and between different dental materials [3, 17, 18]. However, results relating plaque formation and substratum hydrophobicity are conflicting. Thus, certain studies report that dental plaque formation increases on hydrophilic restorative materials such as porcelain and metals when compared to hydrophobic materials such as amalgams and resins [8, 19]. Other studies, however, report more plaque formation on hydrophobic materials [20, 21]. Furthermore, other groups have shown that bacterial hydrophobicity must also be taken into account since converging values for bacterial and substratum surface hydrophobicities facilitate bacterial adhesion [11, 15, 22, 23]. These studies have principally focused on materials used in restorative dentistry and there are few data relating bacterial adherence to metal alloys used in prosthodontics (for review, see [24]).

The aim of the present study was to investigate the effect of alloy and bacterial surface hydrophobicities on bacterial adhesion of three pioneering (*S. oralis*, *S. mitis*, *S. sanguinis*) and one cariogenic (*S. mutans*) strains of streptococci to five metal alloys widely used

in prosthodontics. In order to study the role of the alloy-bacteria non-specific interactions on bacterial adhesion, alloy surfaces uncoated with saliva were used.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

The four streptococci strains used for this study were: *S. mitis* ATCC 49456 (American Type Culture Collection – Rockville, MD, USA), *S. mutans* ATCC 25175, *S. oralis* ATCC 35037 and *S. sanguinis* (emended *S. sanguis* ATCC 10556) [25]. All strains were grown on Columbia agar, supplemented with 5% defibrinated sheep blood (bioMérieux SA, 69280 Marcy I'Etoile, France) in a $CO_2$ enriched atmosphere (Generbox $CO_2$, bioMérieux SA).

### 2.2. Metal alloys

Five metal alloys (four precious and one non-precious) commonly used in prosthodontics were tested (Table I). The samples consisted of 11 mm diameter disks (12 samples for each alloy), each having a test surface that was metallograhically polished with diamond paste by the manufacturer (Table I). Surface roughness was estimated using a Talysurf 10 rugosimeter (Taylor-Hobson, Leicester, UK). For each alloy four measurements were carried out on six randomly chosen disks and mean values were calculated ($n = 24$), (Table I). Prior to use, each disk was briefly polished (ES 200 Polisher, Escil, Chassieux, France), washed in pure acetone, decontaminated in 70% (w/v) alcohol, and rinsed in sterile distilled water.

### 2.3. Bacterial adhesion

The bacteria were incubated in Todd-Hewitt broth (Difco Laboratories, Detroit, USA) for 4 h at 37 °C in a $CO_2$ enriched atmosphere (Generbox $CO_2$, bioMérieux SA, Marcy I'Etoile, France). Bacterial cells were harvested during the exponential growth phase by centrifugation at 4 °C for 15 min, washed twice with 0.15 M PBS buffer (pH = 7) and suspended in the same buffer. To dissociate bacterial chains and aggregates, the suspensions were vortexed for 15 min and sonicated for 10 s at 150 W (Labsonic 1510 Braun, Melsungen, Germany). A final concentration of $5 \times 10^7$ CFU$\cdot$ml$^{-1}$ was obtained as measured by optical densities (OD) of 0.20 for *S. mutans* and 0.25 for the three other test strains, respectively, using a Beckman M24 Spectrophotometer ($\lambda = 550$ nm) (Beckman Instruments SA, Gagny, France). To obtain bacterial deposits, three disks of each alloy were immersed in the bacterial suspensions and continuously stirred for 2 h using a magnetic rod [15]. The disk surfaces were then rinsed with 0.15 M PBS (pH = 7), fixed with 2.5% glutaraldehyde for 30 min at 4 °C (Sigma Chemical Co., St Louis, MO, USA), washed with distilled water and stained with 1% acridine orange (Sigma Chemical Co.) for 30 min. The disks were then rinsed with tap water at room temperature prior to fluorescence microscopy observation (A 1170 microscope, Polyvar-Reichert, Wien, Austria. $\lambda = 546$ nm). For each disk six random fields (0.1 mm$^2$) were chosen and photographed. The photographs (magnification = 1350) were used to enumerate the number of adherent bacteria using a calibrated grid. Each test (three disks of each alloy) was carried out in duplicate, and adhering bacteria means were calculated for each alloy and bacterial strain ($n = 6 \times 3 \times 2 = 36$).

### 2.4. Adhesion to hexadecane

The ability of the bacterial cells to adhere to hexadecane was used as a measure of their hydrophobicity, as described by Rosenberg *et al.* [13]. Bacteria were harvested during the exponential growth phase by centrifugation at 4 °C for 15 min, washed twice with 0.15 M PBS (pH = 7) and suspended in the same buffer. The suspensions were adjusted to an optical density (OD) of 0.85 at 550 nm ($A_0$) (approximately $10^8$ CFU$\cdot$ml$^{-1}$ cell density). Samples (3.0 ml) of the bacterial suspensions were placed in polystyrene tubes ($\phi = 12$ mm) and 400 µl of hexadecane (Sigma Chemical Co.) were added. Control suspensions were prepared without hexadecane. The suspensions were equilibrated in a water bath at 37 °C, mixed using a vortex mixer for two 30-s periods with 5 s in between and allowed to stand until the phases separated. The lower aqueous phase was carefully removed, and its OD was determined at 550 nm ($A_1$). The values were expressed as the percentage of bacteria remaining in the aqueous phase (A) compared with control suspensions as follows: $A = (A_1/A_0) \times 100$. For the four bacterial strains, each test was carried out ten times and the mean values were calculated. Bacteria were considered to be either very hydrophilic ($A = 80 - 100\%$) or very hydrophobic ($A = 0 - 20\%$) as described by Gibbons and Etherden [26].

### 2.5. Alloy contact angle evaluation

As an index of hydrophobicity, the surface contact angles were measured [15, 16]. Briefly, alloy surfaces were cleaned with acetone and dried, five calibrated droplets (0.5 µl) of sterile water were deposited on each disk and contact angles (two measures for each droplet) were

TABLE I Tested metal alloys

| Alloys | Manufacturer | Content (1/1000$^{th}$ total weight) | Ra (µm) |
|---|---|---|---|
| Actazeram$^{TM}$ | ENGELHARD-CLAL | Pd (785), Cu (100), Au (20) | 0.02* (0.01) |
| Diazeram SF$^{TM}$ | Noisy-le-Sec, France | Au (851), Pt (100), Pd (20) | 0.06 (0.01) |
| Palzeram$^{TM}$ | | Pd (615), Ag (265), In (70) | 0.04 (0.02) |
| Qualibond II$^{TM}$ | QUALIDENT Geneva, Switzerland | Au (512), Pd (386), In (86) | 0.03 (0.01) |
| Rexilium III$^{TM}$ | JENERIC GOLD C.O. Stuttgart, Germany | Ni (760), Cr (130) | 0.02 (0.01) |

*Surface roughness means and (SD) were obtained by repeating each test 24 times

immediately measured by horizontal projection technique with a contact angle meter at 20 °C, (G10 Goniometer and G40 software, KRUSS, Germany). For each alloy, four disks were tested and mean values were calculated ($n = 5 \times 2 \times 4 = 40$).

### 2.6. Statistical analysis

All the statistical analyses were carried out with Stat View 4.51.1 software. One way analysis of variance (ANOVA 1) was used to check the reproducibility of each test and Fisher's PLSD test was used to examine paired differences in adherent bacteria counts, percentage of bacterial adherence to hexadecane and alloy roughness.

Two way analysis of variance (ANOVA 2) was used for examination of differences in adherence of the different test bacteria on the different alloys. Regression analysis was used to correlate adherent bacteria counts with contact angle and adhesion to hexadecane values.

For all statistical analyses, the probability of type I error less than or equal to 0.05 was considered as statistically significant.

## 3. Results

### 3.1. Evaluation of bacterial adherence

For each of the test bacterial strains and metal alloys, adherent bacteria counts are shown in Table II and Fig. 1. ANOVA 2 allowed us to show that adherent bacteria counts differed for each of the tested strains ($p = 0.0001$) and metal alloys ($p = 0.0001$). However, since there was a strong interaction ($p = 0.0001$), it was impossible to analyze simultaneously bacterial and alloy related differences. It was therefore necessary to analyze the results of bacterial adherence, (i) in relation to bacterial strains and (ii) in relation to the tested alloys, using Fisher's PLSD test.

Concerning alloy related differences in adherence, our results showed that for *S. mutans*, *S. oralis* and *sanguinis* Diazeram SF™ retained the greatest number of adherent bacterial cells (all paired comparisons were significant at $p < 0.05$), followed by the two high palladium-content alloys (Actazeram™ and Palzeram™) which were not significantly different ($p > 0.05$). It was not possible to establish a clear classification for Qualibond II™ with these three strains. For the non-precious alloy (Rexillium III™) the smallest count of adhering bacteria, irrespective of strains, was obtained (all paired comparisons were significant at $p < 0.05$). A different classification was obtained for *S. mitis* (Fig. 1).

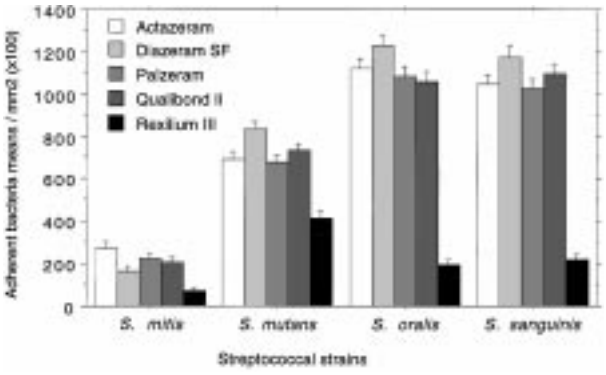


*Figure 1* Adherent bacteria means on the different test alloys and for the different bacterial strains ($n = 36$).

Concerning bacterial strain related differences in adherence, our results showed that for the four precious metal alloys, *S. oralis* and *S. sanguinis* were not significantly different ($p > 0.05$) and showed the greatest adherence (all paired comparisons were significant at $p < 0.05$), *S. mutans* showed intermediate adherence and *S. mitis* showed least adherence (Fig. 1).

Coefficient of variation values ($cv = \frac{SD}{mean}$) for all adherent bacteria counts ranged from 10% (*S. oralis* on Diazeram SF™) to 46% (*S. mitis* on Rexilium III™). The highest values of *cv* were observed with the *S. mitis* strain and with the non-precious metal alloy Rexilium III™ (*cv* values were calculated from Table II).

### 3.2. Bacterial surface analysis

The hydrophobicity of the bacterial surfaces was determined by measuring the percentage of adhesion to hexadecane (Table III). *S. mitis* was found to be highly hydrophilic since this bacterial strain showed 10% adhesion to hexadecane, whereas *S. mutans*, *S. oralis* and *S. sanguinis* were highly hydrophobic, showing 93%, 97% and 95% adhesion to hexadecane, respectively. The bacterial surface of *S. oralis* and *S. sanguinis* showed no significant differences in hydrophobicity ($p > 0.05$).

### 3.3. Metal surface analysis

The surface roughness of alloys was briefly estimated before each adhesion test with a Talysurf rugosimeter

TABLE II Means of adherent bacteria on different alloys, per mm$^2$ (×100)

| | *S. mitis* | *S. mutans* | *S. oralis* | *S. sanguinis* |
|---|---|---|---|---|
| Actazeram™ | 273* (88) | 691 (107)$^{b}$ | 1117 (149)$^{c1}$ | 1045 (131)$^{d1}$ |
| Diazeram SF™ | 166 (62) | 838 (103) | 1232 (122)$^{2}$ | 1176 (142)$^{2}$ |
| Palzeram™ | 225 (64)$^{a}$ | 684 (83)$^{b}$ | 1082 (157)$^{c3}$ | 1031 (125)$^{d3}$ |
| Qualibond II™ | 208 (73)$^{a}$ | 739 (86) | 1059 (150)$^{c4}$ | 1094 (127)$^{4}$ |
| Rexilium III™ | 74 (34) | 417 (82) | 196 (67)$^{5}$ | 216 (83)$^{5}$ |

*Adherent bacteria means and (SD) were obtained by taking six random photographs on triplicate alloy disks, each test was carried out twice ($n = 6 \times 3 \times 2 = 36$).

$^{a,b,c,d}$For each bacterial strain, values with identical superscript letters showed no significant differences in adherence ($p > 0.05$).

$^{1,2,3,4}$For each metal alloy, values with identical superscript numerals showed no significant differences in adherence ($p > 0.05$).

TABLE III Bacterial adhesion to hexadecane

| *S. mitis* | *S. mutans* | *S. oralis* | *S. sanguinis* |
|---|---|---|---|
| 91.0* (3.6) | 6.8 (1.3) | 3.2 (1.7)[a] | 4.6 (0.9)[a] |

*Means and (SD) of bacteria remaining in the aqueous phase were obtained by repeating each test ten times ($n = 10$).
[a]Values with identical superscript letters were not significantly different ($p > 0.05$).

TABLE IV Contact angles of the metal alloy surfaces

| | Actaderam™ | Diazeram SF™ | Palzeram™ | Qualibond II™ | Rexilium III™ |
|---|---|---|---|---|---|
| Contact angles | 71.2* (3.0) | 79.7 (2.9) | 64.3 (2.0)[a] | 68.3 (1.9) | 64.0 (2.1)[a] |

*Contact angle values and (SD) were obtained by repeating each test 40 times.
[a]Values with identical superscript letters were not significantly different ($p > 0.05$).

and compared with previously calculated mean values. Values ranging from 0.02 to 0.06 μm were obtained. Contact angle values ranging from 64.0° to 79.7° were obtained, indicating hydrophobicity of the alloy surfaces (Table IV). Contact angle values for Palzeram™ and Rexilium III™ did not differ significantly ($p > 0.05$).

### 3.4. Bacterial adherence in relation to alloy and bacterial hydrophobicity

No significant linear regression between adherent bacteria counts and alloy contact angle values was observed when the five alloys were analyzed ($p > 0.05$), since Rexillium III™ behaved differently compared to the other test alloys. However, when Rexillium III™ was excluded from the analysis, significant correlations between the adherent bacteria counts and the contact angle values of the metal surfaces were obtained: $r = 0.53$ for *S. mitis*, $r = 0.88$ for *S. mutans*, $r = 0.92$ for *S. oralis*, and $r = 0.87$ for *S. sanguinis* (all regressions were significant at $p \leq 0.05$) (Fig. 2). Positive correlations were obtained for *S. mutans*, *S. oralis* and *S. sanguinis*, and a negative correlation was obtained for *S. mitis* (Fig. 2). Furthermore, the weak slopes of the regression curves could be explained by the narrow range of contact angle values obtained for the test metal alloys (values ranging from 64° to 80°). No significant linear regression between the adherent bacteria counts and the adhesion to hexadecane values was observed ($p > 0.05$). This could be explained by the low bacterial adhesion values found for *S. mitis*.

## 4. Discussion

Accumulation of dental plaque on a restoration can lead to secondary caries, periodontal disease or alteration of the restoration. Numerous studies have focused on bacterial adherence on materials used in conservative dentistry [3, 15, 17, 27], but very few studies have been carried out on materials used in prosthodontics (see [24]) To our knowledge, no study has yet compared bacterial adherence on different metal alloys currently available. Quirynen and Bollen [8] suggested that surface roughness and surface free energy are the main material-linked factors influencing bacterial adhesion. To eliminate the

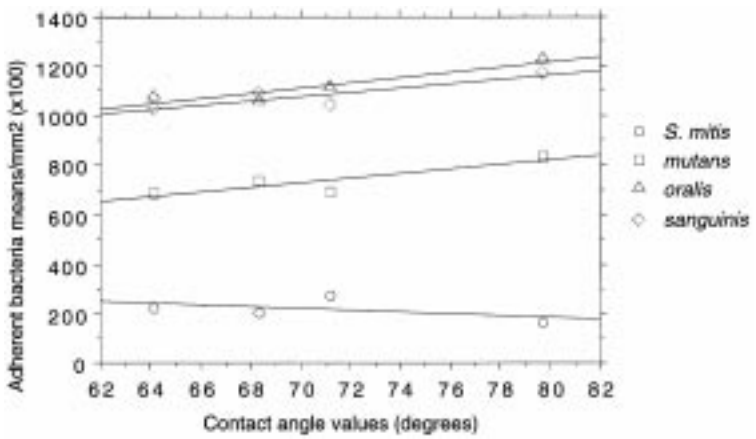


*Figure 2* Relationship between precious alloy contact angles and bacterial adherence. $r = 0.53$ for *S. mitis*, $r = 0.88$ for *S. mutans*, $r = 0.92$ for *S. oralis* and $r = 0.87$ for *S. sanguinis*. All the regressions are significant at $p \leq 0.05$.

effect of surface roughness, five alloys having similar low surface roughness values were selected. The differences in surface roughness of the tested alloys in the present study ranged from 0.02 to 0.06 μm. The influence of such surface roughness values on the bacterial adhesion of our test strains is probably insignificant [8, 28]. Thus, Boulange-Petermann *et al.* [28] found that surface roughness values of stainless steel samples (ranging from 0.02–0.14 μm) were unlikely to have an effect on the adhesion of the bacterial species they tested (*Leuconostoc mesenteroides*). Bollen *et al.* [29] have also shown that a reduction in surface roughness below Ra = 0.2 μm had no further quantitative and qualitative effects on bacterial adhesion. Furthermore, minor variations in roughness do not affect contact angle measurements [8]. In the oral cavity, streptococcal cells adhere directly to the salivary pellicle. To study the bacteria-alloys non-specific interactions and to eliminate the specific interactions closely linked to the salivary pellicle, we have used metal surfaces uncoated with saliva [15, 22, 23].

In order to eliminate the effect of electrostatic interactions, we used 0.15 M PBS (pH = 7) as a buffer since at this high concentration PBS has been shown to minimize these interactions [30, 31]. It seems, therefore, that surface free energy is the only factor that can influence streptococcal adhesion to the alloys tested in our study.

Many authors have used hydrophobicity to predict bacterial adhesion [9–11]. In the present investigation, hydrophobicity of bacterial surfaces was measured by quantifying bacterial adhesion to hexadecane. This method has been shown to be precise and reproducible [14, 32] as long as experimental parameters are carefully defined [20, 26, 30]. Adhesion to hexadecane allowed us to define highly hydrophobic strains (*S. oralis*, *S. sanguinis*, *S. mutans*) and highly hydrophilic strains (*S. mitis*) showing a high and low adhesion to hexadecane, respectively. These results concur with those of other studies using the same protocol, showing that *S. sanguinis*, *S. oralis* and *S. mutans* are highly hydrophobic streptococci [21, 33, 34]. However, Gibbons and Etherden [26] found that *S. mutans* was only slightly hydrophobic and, in contrast to our results, that *S. mitis* was hydrophobic. These differences can be explained by differences in experimental protocols since Van der Mei and Busscher [35] have shown that *S. mitis* hydrophobicity varies with pH, ionic force and the number of subcultures.

Our results agree with those of Satou *et al.* [15] who assessed bacterial surface hydrophobicity by contact angle measurements, but differ from those of Busscher *et al.* [10], Van Pelt *et al.* [36] and Weerkamp *et al.* [37], who calculated the absolute value of surface free energy. These authors found high free energy values for *S. mutans* and *S. sanguinis*, and low free energy values for *S. mitis*, thus reflecting hydrophilic and hydrophobic properties for these bacteria, respectively. These differences could be explained by the different methodologies and bacterial strains used in the studies. Contact angles are also used to measure the hydrophobicity of solid surfaces and the results of the present study concur with those found in the literature [15, 22, 23, 28].

Fluorescence microscopy showed that bacterial strains with similar hydrophobic properties showed similar adhesion patterns to the test alloys. Thus, *S. sanguinis* and *S. oralis* who showed similar hydrophobicity ($p > 0.05$) using the hexadecane adhesion test, also showed comparable adhesion to Actazeram™, Diazeram SF™, Palzeram™, Qualibond II™ and Rexilium III™ ($p > 0.05$). *S. mutans*, shown to be less hydrophobic than *S. sanguinis* and *S. oralis* by the hexadecane adhesion test, was also shown to adhere less to the alloy surfaces. *S. mitis*, whose surface is highly hydrophilic showed minimal adhesion to the five tested alloys. Although our results indicate that bacterial hydrophobicity influences bacterial adhesion to various metal surfaces, further testing on a larger number of bacterial strains is required to confirm these findings.

In our study, the non-precious alloy Rexilium III™ behaved differently from the precious metal alloys. Rexilium III™ showed low bacterial retention despite surface contact angle values close to those of the precious metal alloys. Surface analysis performed by X-ray photoelectron spectroscopy (data not shown), (LTPCM, Electochemistry School of Grenoble, France) revealed that Rexilium III™ was covered by an important negatively charged oxide layer. Electrostatic interactions of this surface layer may be much higher than on precious metal surfaces and could explain the low bacterial adhesion values obtained for Rexilium III™. Our results suggest that positive and negative correlations occur between precious metal alloy surface hydrophobicity and adhesion of hydrophobic and hydrophilic bacteria, respectively. Thus, in the initial stage of bacterial colonization of precious metal alloy surfaces, the surface hydrophobicity of the four tested bacterial strains plays an important role in bacterial adhesion. Concerning the values of adherent bacteria counts, our study has shown reproducible results since coefficient of variation (*cv*) values ranged from 10% to 46%. Interestingly, the highest values are obtained with the hydrophilic strain *S. mitis* and with the non-precious alloy Rexilium III™ which were both characterized by the weakness adhesion properties.

The results of the present study support the theory described by Absolom *et al.* [38] and studies by Van Loosdrecht *et al.* [7], Satou *et al.* [15, 22, 23] and Verran *et al.* [21]. These investigators have used the thermodynamic approach to explain initial bacterial adhesion. Thus, initial adhesion involving bacteria and surfaces with similar hydrophobic properties (*S. oralis*, *S. sanguinis*, *S. mutans*) would be facilitated compared to adhesion involving bacteria and surfaces whose surface properties differ significantly, as shown by *S. mitis*.

We have used a precise and reproducible protocol to quantify bacterial adhesion to different solid supports by using fluorescence microscopy counts of adhering bacteria. This model has allowed us to confirm that the number of adhering bacteria on a metal alloy surface varies with different bacterial species and with the nature of the metal alloy. Our results agree with the theory described by Absolom *et al.* [38], who suggest that there is a relationship between bacterial adhesion and surface hydrophobicity. Hydrophobic bacteria adhere much more readily to hydrophobic supports whereas

hydrophilic bacteria show less adhesion to hydrophobic supports.

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