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In Vitro Study Comparative of Bacterial Adhesion on Dental Surfaces, on Stainless-Steel Surfaces and on Titanium Surfaces

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Abstract

Adhesion is a phenomenon of general significance that governs the evolution of microorganisms and their interaction in all the environments in which they occur, that is to say in the whole of the biosphere. The elucidation of the mechanisms at the molecular level of bacterial adhesion to solid surfaces has not been fully accomplished. The oral cavity is part of these environments, the bacterial adhesion is interested in different structures in the mouth: dental, mucosal structures and structures of therapeutic interest (composite, brackets, orthodontic wire, ceramic, stainless steel, titanium…). The adhesion phenomenon involves nonspecific factors of different types (ionic, dipolar, hydrophobic, hydrogen bonding) between the macromolecules on the surface of the microorganisms and those of the support and other specific factors. Stereochemical order involves interactive complementary chemical groups. **Objective**: Our study aims to observe the behavior of bacteria from the oral flora in contact with dental surfaces and titanium to understand the adhesion mechanism. Specifically, our study seeks to: Observe the ability of these germs to adhere to dental surfaces, titanium, and stainless steel; Quantitatively evaluate the potential for adhesion and proliferation of these germs when in contact with these surfaces; Compare their behavior across the three different surfaces. **Method:** We studied in vitro the behavior of Staphylococcus aureus Métis, streptococcus in-termedius and haemophilus influenza, to understand the bacterial adhesion and pro-liferation mechanisms using counting methods (by culture and by optical assay). The biomaterials used were Implants in titanium alloy Ti6Al4V (90% Titanium, 6% Aluminum, 4% Vanadium), cylinders in Stainless steel and dental fragments, all were dis-infected for 15 minutes and then sterilized in a humid autoclave at 120˚C for 30 min. **Results:** The results of our work have shown that the three bacteria exhibited different behaviors and adhesion capacities vis-à-vis the two surfaces. In contact with the tooth surfaces the three seeds were able to adhere and proliferate. However, when in contact with stainless steel and titanium, an inhibition of adhesion was observed. The bacteria required more time to adhere and proliferate on these surfaces, indicating that surface characteristics, particularly surface roughness, play a crucial role in bacterial adhesion. **Conclusion:** The study concludes that surface properties, especially roughness, significantly influence bacterial adhesion.

Subject Areas

Dentistry

Keywords

Microbial Adhesion, Biomaterial Surface Interaction, Oral Biofilm, Tooth Surface, Stainless-Steel, Titanium

1. Introduction

The oral cavity is home to one of the most complex bacterial ecosystems as it is along with the colon the most septic part of the human body. Several hundred species of microorganisms coexist in the oral environment: bacteria, yeasts, protozoa and viruses. Many authors have tried to quantify this population: a milligram of plaque contains approximately 100 million bacteria; 1 milliliter of saliva contains an average number of 750 million bacteria (including 100 million bacteria that can be cultivated on culture medium).

The complexity of this environment is further amplified by the presence of fixed orthodontic appliances, which are composed of various biomaterials such as stainless steel and titanium.

In the oral cavity, metallic biomaterials are exposed to many factors such as saliva, bacterial microflora, food, temperature fluctuations, and mechanical forces [1]. It may lead to ecological changes in the oral environment and increase Streptococcus mutans count in the saliva and dental plaque [2].

biofilm formation in the oral cavity is a gradated process consisting of four distinct stages: acquired pellicle formation, primary colonization, secondary colonization/co-aggregation, and finally, the establishment of a mature biofilm.

The accumulation of dental plaque, which can lead to white spot lesions (WSL) and gingival inflammation, represents a significant challenge to excellence in clinical orthodontics [3]. Enamel demineralization and the development of white spots around orthodontic brackets are among the most important complications resulting from orthodontic treatments [2].

The process of oral biofilm creation proceeds at the dental and biomaterial interface, where saliva plays an important role. Glycoproteins and phosphoproteins present in saliva, such as mucins and proline-rich proteins adhere to the bacteria-free surfaces of teeth, oral mucosa and biomaterials through ionic Van der Waals and hydrophobic interactions in a highly selective process [4]-[7].

Adsorption of proteins electrochemically changes tooth and biomaterial surfaces, which mediates interactions with the microbe-rich oral environment. In effect, microorganisms interact directly with built-in film forming molecules which have an influence on the further colonization of adsorbed microbes [8] [9].

1.1. Biofilm

After adsorption of microorganisms to the surface of the pellicle, faster, adhesion of microorganisms together with glycoproteins and phosphoproteins is observed (**Figure 1**). Witch then form dental and denture plaque. Saliva plays an important role in the process of biofilm growth, it serves as a transporter of nutrients for persistent microorganisms in root canals, and a carrier of antimicrobial compounds: lysozyme, lactoferrin, sialo peroxidase, histatin, statherin and bacteriocin [10].

The extracellular matrix (EPS) produced during biofilm formation serves as a scaffold, providing structural support to the microbial community and offering protection against external environmental factors [11] [12]. The constant flow of saliva in the oral cavity presents a challenge to microbial colonization, yet mucins and other glycoproteins also contributes to this process trough aggregation of bacteria this process is part of a protective mechanism against pathogenic organisms, thereby facilitating biofilm development [13] [14].

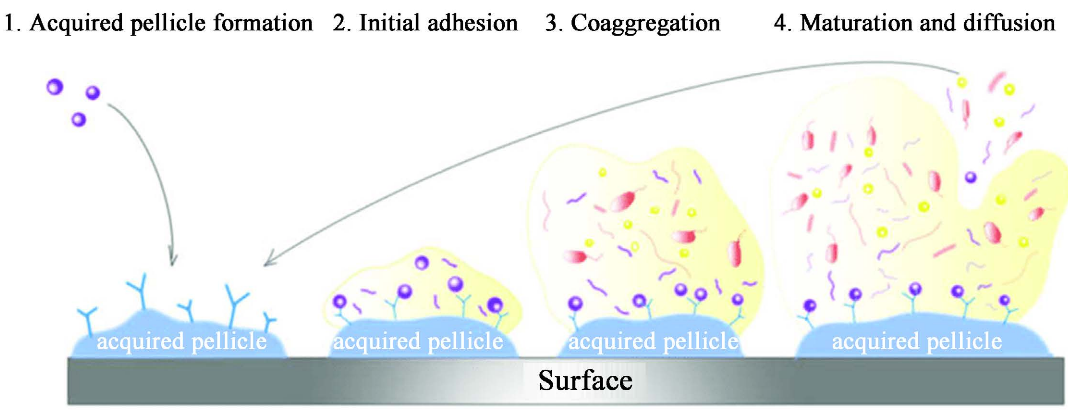


Figure 1. The process of biofilm formation in the oral cavity [15].

Human saliva provides the nutrients necessary for microorganisms to adhere to surfaces, forming a thin, heterogeneous, acellular pellicle (5 - 10 µm thick), known as the acquired pellicle or conditioning film, that enables early colonizers like (*S. oralis*, *S. gordonii*, *S. mitis*, *Actinomyces naeslundii*, *Capnocytophaga ochracea*, *S. mutans*, *and S. sobrinus*) to adhere and co-aggregate on surfaces. These microorganisms comprising 60% - 80% of primary colonizers, start biofilm formation through quorum sensing [16].

Secondary colonization begins 3 to 5 days after pellicle formation, leading to biofilm structural organization and maturation within 2 to 3 weeks [17].

The biofilm formation on materials for oral rehabilitation also depends on oxygen, nutriments and Ph [18] [19].

1.2. Quorum Sensing

Bacteria and micro-colonies within the biofilm communicate through quorum sensing, a process where gene expression of the bacteria is regulated by the accumulation of signaling compounds known as auto inducers of specific genes that mediates intercellular communication. Activated by a response of cell density, quorum-sensing system is found Gram-positive and Gram-negative micro-organisms [20] [21].

The severity of the adhesion occurs when biofilm which is an assemblage of microbial cells that is irreversibly and often embedded in a matrix of extracellular polymeric substances (EPS) complicates the eradication of the pathogen making it difficult, time-consuming and expensive [22]. Thus, many studies have been performed focuses on factors influencing bacterial adhesion and also all the possible methods for avoiding the formation of biofilm on certain surfaces. Factors, including bacterial characteristic (e.g., hydrophobicity, surface charge), surface properties (e.g., roughness, wettability) and environment condition (e.g., pH, temperature) which involve the physicochemical and also the molecular interactions [23]-[25].

Our study is used to observe the behavior of bacteria in contact with dental surfaces and titanium, in order to understand the adhesion mechanism. Aiming to study *in vitro* the behavior of certain bacteria of the oral flora in contact with a dental surface and stainless-steel to:

-Observe the ability of these germs to adhere to dental surfaces and to stainless-steel surfaces.

-Quantitatively assess the adhesion and proliferation potential of these germs in contact with dental surfaces and titanium.

-Compare their behavior on contact with these 2 surfaces.

2. Materials and Methods

2.1. Biomaterials

Implants in titanium alloy Ti6Al4V (90% Titanium, 6% Aluminum, 4% Vanadium) were machined using a disc placed on a mandrel carried by a handpiece, in different samples of 5 mm in length and 3 mm wide and 2 mm thick.

Stainless-steel cylinders, commonly called stainless-steel or inox, is an alloy of steel (containing less than 1.2% carbon) with more than 10.5% chromium, the property of which is to be insensitive to corrosion and not to degrade into rust. These cylinders were machined using a disc placed on a mandrel carried by a handpiece, in different samples of 5 mm in length and 3 mm in width and 2 mm in thickness.

Freshly extracted permanent natural teeth were cut longitudinally into different samples of 5 mm in length and 3 mm in width and 2 mm in thickness, consisting essentially of enamel, the outer layer of the crown of the tooth.

The dental fragments, steel fragments and titanium fragments were disinfected with (Hexanios G+R, Anios Laboratory) for 15 minutes then sterilized in a humid autoclave (Tau Clave 3000, Vacuum) at 120˚C for 30 min.

2.2. Bacterial Strains

Three reference bacterial strains were used in this study:

-Staphylococcus aureus Meti S ATCC 29213.

-Streptococcus intermedius ATCC 27335.

-Haemophilus influenzae ATCC 49247.

The strains were preserved as an aliquot in a medium composed of brain heart broth (BHI) supplemented with 10% glycerol and frozen at −20˚C.

2.3. Reactivation of Germs

The reference strains used are cultured, by placing them in a liquid enrichment medium (BHI) then incubated in the oven at 37˚C for 2 to 4 hours. Then a drop of this broth incubated with a sterile loop, the media suitable for the culture of these strains:

-Chocolate agar composed of Columbia agar with cooked blood supplemented with polyvitamin supplements allowing the growth of all bacterial strains and especially deficient bacteria.

-Fresh blood agar with added nalidixic acid and colistin, selective for streptococci.

-Chapman agar (hypersaline medium with 7% Nacl) selective for staphylococci.

The inoculated boxes are incubated in the oven at 37˚C, for 18 to 24 hours under 5% CO_2.

2.4. Preparation of the Media Necessary for Bacterial Adhesion to Biomaterials

We used two pairs of plates of each of the following media: MHS (Mueller Hinton = Mueller-Hinton + Fresh blood), MH (Mueller Hinton) and chocolate agar:

-The first pair of dishes each contains two dental fragments embedded in the agar so that the enamel surface is on the same plane as the surface of the agar, we chose the enamel surface because it is the first surface in contact with the bacteria in the oral cavity.

-The second pair of dishes each contains 2 stainless-steel fragments embedded in the agar with an accessible surface and on the same plane as the agar.

-The third pair of dishes each contains 2 titanium fragments embedded in the agar with an accessible surface and on the same plane as the agar.

The fourth pair of dishes each contains 2 titanium fragments embedded in the agar with an accessible surface and on the same plane as the agar.

2.5. Bacterial Strain Confirmation Tests

<u>-Confirmation by sowing on Chapmen medium:</u>

Chapman agar is the selective medium for halophilic bacteria and more particularly fermenting red mannitol. It is a semi-synthetic medium. It is used for the isolation of Staphylococcus.

For the preparation of a liter of medium we need:

-Peptone:... 10.0 g;

-Beef extract:.......................... ....1.0 g;

-Sodium chloride:... 75.0 g;

-Mannitol:... 10.0 g;

-Phenol red:... 0.025 g;

-Agar-Agar:... 15.0 g;

-Distilled water:...1 L.

111 g/l of medium: Classic autoclaving at 120˚C for 20 minutes.

This environment is characterized by:

-An ordinary nutritional base.

-A high NaCl content which allows the selection of halophilic bacteria (such as Staphylococcus) and inhibits the vast majority of other bacteria.

-A differentiation criterion: the fermentation of mannitol revealed thanks to the change in the colored pH indicator: phenol red which allows orientation towards certain species (such as the Staphylococcus aureus species)

In a Chapmen tube, a few colonies of staphylococcus aureus ATCC 29213 were seeded using a plastic handle on the slope of the agar. The observation was made after 24 hours of incubation at 37˚C.

-Confirmation by biochemical catalase test

This test is the basis for the identification of Gram + bacteria. On a clean slide, a few colonies were deposited by a loop with a few drops of the catalase reagent in order to observe the behavior of the bacteria.

-Confirmation by Gram staining

Gram stain (developed by Christian Gram) is a basic stain in bacteriology. It is a "double coloring", which makes it possible to differentiate bacteria:

-According to their shape

-According to their affinity for dyes.

+Preparation of the smear

A new blade was used. On the perfectly cold slide a drop of culture or microbial emulsion was placed using a plastic handle, and spread carefully so that the germs are evenly distributed without forming a clump and allowed to dry in the free air the smear thus obtained.

When the smear is perfectly dry, it is fixed by heat.

+GRAM coloring

-The smear was covered with gentian violet, for 1 minute.

-Gentian violet has been washed and replaced with potassium iodide for 30 seconds contact.

-After rejecting the potassium iodide solution, the slide was discolored by dropping the alcohol drop by drop on the surface, keeping it in an oblique position.

-When the alcohol flows colorless, the slide was quickly washed under running water.

-Recolouring with Fushine was carried out leaving it to act for 5 minutes then the slide was rinsed with water and allowed to air dry.

+Microscopic observation

Examine at the ×100 objective, immersion (with a drop of oil), with significant lighting (open diaphragm).

2.6. Seeding and Cultivation of Biomaterials

From a bacterial suspension which corresponds to a turbidity of 0.5 McFarland of each bacteria studied (≈106 CFU/ml), we seeded the surfaces of the 2 pairs of blood agars using a sterile swab. (MHS) encrusted with fragments to be studied.

For Staphylococcus aureus is seeded on Mueller Hinton agar (HD). One dish in each pair was incubated for 6 hours at 37˚C and 5% CO_2, the other dish in each pair was incubated for 24 hours at 37˚C and 5% CO_2.

2.7. Macroscopic and Microscopic Observations of the Culture Dishes

Macroscopic observation and a binocular magnifying glass (V.M.Z. 1 to 4 Japan, Olympus) of bacterial proliferation was performed regularly after 6 h and 24 h of incubation.

An optical microscope observation based on GRAM staining allowed to visualize the presence or absence of bacteria and to differentiate them.

2.8. Confirmation Test for Bacterial Fixation on Biomaterials by Transplanting on Culture Media

We scraped a portion of the culture on the surface of each substrate (tooth, titanium and stainless steel) with a calibrated loop of 1 μl and suspended with physiological saline.

This suspension was readjusted to a concentration of 0.5 MacFarland and was subjected to different dilutions 1/10, 1/100 and 1/1000.

From each suspension prepared, we seeded 1 μl on different media which are specific to them. Interpretive cultures were read after incubation in an oven at 37˚C for 24 hours.

2.9. Bacterial Adhesion

Adhesion of Haemophilus influenzae, Streptococcus intermedius and Staphylococcus aureus Meti on dental fragments was compared to that obtained on stainless-steel fragments and titanium fragments. For example, the number of bacteria adhered as a function of the number of bacteria inoculated (CFU/ml) was reported for the two pre-incubated samples. The slopes of the lines obtained make it possible to determine the percentages of adhesion. Counting bacteria in culture was carried out by two different methods:

-Culture on Congo red medium

The medium was prepared by adding 0.8 g of Congo red and 36 g of sucrose to 1 liter of brain heart agar, then autoclaved at 115˚C for ten minutes. (50). In each sterile tube containing 0.5 ml of 1× phosphate buffered saline (PBS), introduce the cultured material (tooth, stainless-steel or titanium), vortex for 5 minutes to release the bacteria attached to the substrates. We adjusted the bacterial suspension with physiological saline to prepare a suspension of 0.5 Mc Farland. Then we seeded on each box of red Congo medium, 1 µl of a suspension of 0.5 McFarland (a colony in 20 ml of distilled water). The reading was made after 24 hours of incubation at 37˚C. The adhesive strains gave black colonies with a rough surface and red colonies with a smooth surface for non-adhesive strains.

-Bacterial enumeration by optical assay:

-Cultivated tooth and titanium fragments are washed with physiological saline to remove any non-stick bacteria.

-In each sterile tube containing 0.5 ml of 1× phosphate buffered saline (PBS), introduce the cultured material (tooth, stainless-steel or titan), vortex for 5 minutes to release the bacteria attached to the substrates.

-Following a procedure based on the protocols described by Christensen *et al.* [26]. The bacterial suspension was adjusted to an optical density (OD) of 0.5 to 610 nm. A 1:10, 1:100 and 1:1000 dilution were prepared with the adjustment of the bacterial suspension in tryptic soy broth (TBS). The contents of each tube were gently aspirated for quantification of adhesive bacteria. The OD of the resulting solution was measured at 560 nm.

-Optical dosage: relies on the Beer-Lambert law: The form used is as follows:

A: absorbance of the solution without unit.

ε: molar extinction coefficient in $L \times mol^{-1} \times cm^{-1}$ (sometimes noted with a ξ).

l: length of the tank crossed by the light in cm.

C: molar concentration in $mol \times L^{-1}$.

The measurement of the absorbance is given by a spectrophotometer which measures the optical density. The more a solution is concentrated, the more light is difficult to pass through the medium, which leads to an increase in the absorbance of the solution, the measurements obtained are expressed in OD (optical density), the solutions used in the optical assay are the same dilutions and the mother solution used in the inoculation of the dishes in the first method (bacterial count per culture).

3. Results

3.1. Confirmatory Tests for the Bacterial Strain

-Culture on Chapmen:

After 24 hours of incubation, a bacterial layer was observed all along the s lope.

The consumption of mannitol by the strain allowed acidification of the medium and by therefore the colored indicator turns from red to yellow, which confirms the characteristics of Staphylococci aureus (**Figure 2**).

-Biochemical catalase test:

Bubbles appeared, which allowed us to conclude that staphylococci aureus are catalase positive (Figure 3).

-Gram staining:

Figure 2. Culture of Staphylococci aureus ATCC 29213 on Chapmen medium after 24 h of incubation.

Figure 3. Appearance of the positive catalase test.

Observation of the slide by an optical microscope (Leica microsystems DM1000) at the × 100 objective, showed cocci in clusters stained in purple (Gram+).

3.2. Observations with the Naked Eye of Bacterial Cultures on Biomaterials after 6 Hours and 24 Hours of Incubation

-After 6 hours of incubation:

No bacterial cultures (the three strains) were observed on the dishes encrusted with dental fragments, stainless-steel fragments and titanium fragments (Figure 4(a)-(c)).

-After 24 hours of incubation:

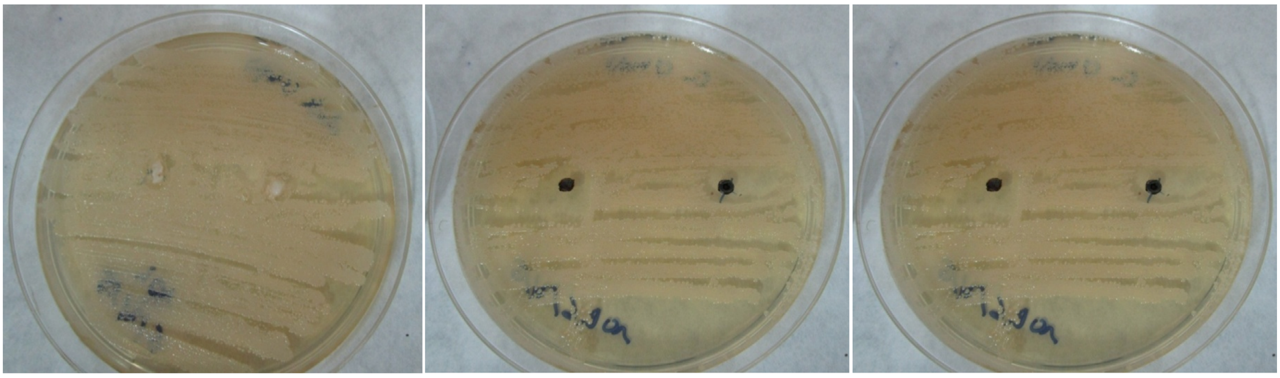
-Staphylococcus aureus Meti S. ATCC 29213

The dishes seeded with Staphylococcus aureus showed the formation of a bacterial carpet which covers the entire box including the dental fragments (Figure

4(a)). While at the level of the second box we observed a reduction in the proliferation around the steel fragments and the titanium fragments with the appearance of a 1 mm inhibition zone (Figure 4(b) and Figure 4(c)).

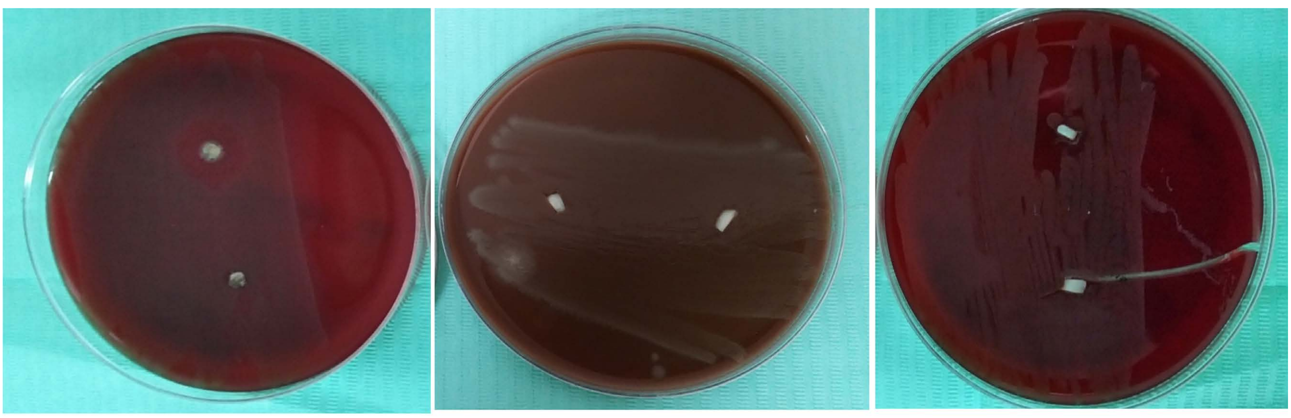
-Streptococcus intermedius ATCC 27335

Figure 5(a) showed a total invasion of streptococcus intermedius on both culture dishes and even on the surface of the dental fragments. On the other hand, in the culture dish in the presence of steel fragments and titanium fragments, we observed an inhibition zone of 2 mm around the 2 substrates (Figure 5(b) and Figure 5(c)).



(a) (b) (c)

Figure 4. Culture of Staphylococcus aureus Meti S. ATCC 29213 on MH agar encrusted with dental fragments, steel fragments and titanium fragments after 24 hours of incubation.



(a) (b) (c)

Figure 5. Culture of Streptococcus intermedius ATCC 27335 on MH agar encrusted with dental fragments, steel fragments and titanium fragments after 24 hours of incubation.

-Haemophilus influenzae ATCC 49247

The plates seeded with haemophilus influenzae showed the formation of a bacterial carpet which covers the entire seeded agar and the surface of the dental fragments (Figure 6(a)), on the other hand around the steel fragments and titanium fragments appears an inhibition zone of 1mm in diameter (Figure 6(b) and Figure 6(c)).

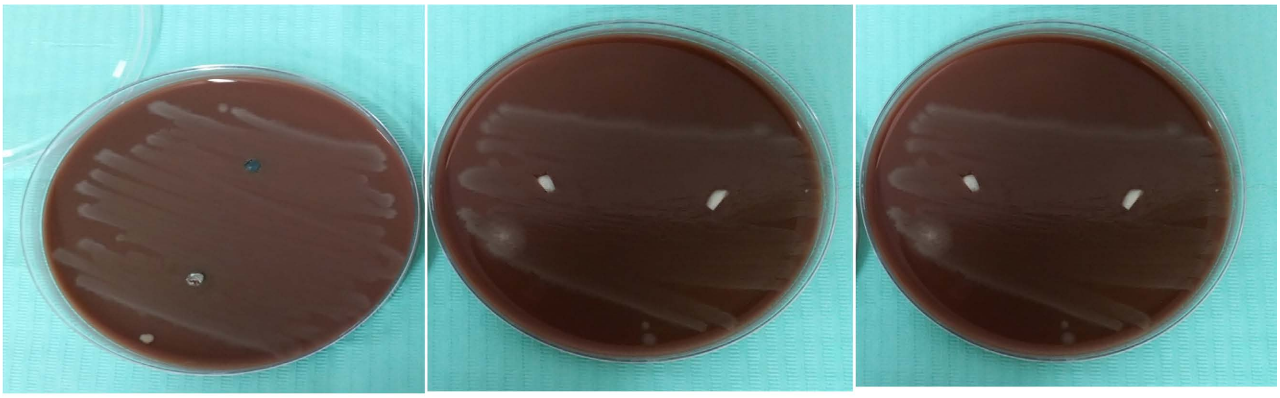


Figure 6. Culture of Haemophilus influenzae ATCC 49247 on MH agar encrusted with dental fragments, steel fragments and titanium fragments after 24 hours of incubation.

3.3. Confirmation Test of Bacterial Fixation on Biomaterials by Transplanting onto Culture Media

-The culture of Staphylococcus aureus Méti S. ATCC 29213 diluted 1/100 on MH medium showed:

-After 6 hours of incubation, the entire box was submerged by bacterial growth, even the surface of the dental fragments. On the other hand, there is no bacterial proliferation on the agar which contains steel fragments and titanium fragments.

-After 24 hours of incubation, growth was noted on the entire surface of the agar containing dental fragments but we noted the presence of only a few colonies on the agar encrusting steel fragments and titanium fragments.

The results also showed that with the other dilutions there were no bacterial growths on the agar plates containing steel fragments and titanium fragments after 6 hours or after 24 hours of incubation, but on the surfaces of the fragments in steel and titanium fragments there was no adhesion of these bacteria on the contrary they were inhibited.

Therefore, Staphylococcus aureus adheres and proliferates more easily on dental fragments than on agar with the same dilution.

Whereas Staphylococcus aureus does not adhere and therefore does not proliferate in the presence of steel fragments and titanium fragments even with the stock solution (Figure 7).

-The culture of Streptococcus intermedius ATCC 27335 diluted 1/100 on MHS medium showed:

-After 6 hours of incubation, the entire box was submerged by bacterial growth, even the surface of the dental fragments. On the other hand, we did not have any bacterial growth on the agar which contains steel fragments and titanium fragments.

-After 24 hours of incubation, we noted a growth on the entire surface of the agar containing dental fragments but we noted the total absence of shoots on the encrusting agar; we did not have bacterial growths and titanium fragments.

-After 24 hours of incubation we have a small number of bacterial colonies in the culture dishes with a dilution of 1/100 in the presence of dental fragments and in the presence of agar.

In the presence of steel fragments and titanium fragments, the results showed the absence of bacterial colonies after 24 hours of incubation with the different dilutions and even with the stock solution. At a dilution of 1/1000 we had no culture in all the dishes.

Therefore, Streptococcus intermedius has a low capacity to adhere and proliferate not only in the presence of dental fragments but also on agar with the same dilutions.

While Streptococcus intermedius did not adhere and therefore did not proliferate in the presence of steel fragments and titanium fragments with all diluted solutions (Figure 8).

-The culture of Haemophilus influenzae ATCC 49247 diluted 1/100 on chocolate medium showed:

-After 6 hours of incubation, we have the same number of colonies in the culture dishes in the presence of dental fragments and agar with dilutions: 1/10, 1/100, and 1/1000 however we do not have no colonies in the presence of steel fragments and titanium fragments with the different dilutions.

-After 24 hours of incubation, we have a significant number of bacterial colonies in the culture dishes in the presence of dental fragments and agar with a dilution of 1/100, on the other hand we have fewer colonies in the presence of steel fragments and titanium fragments and with the same dilution.

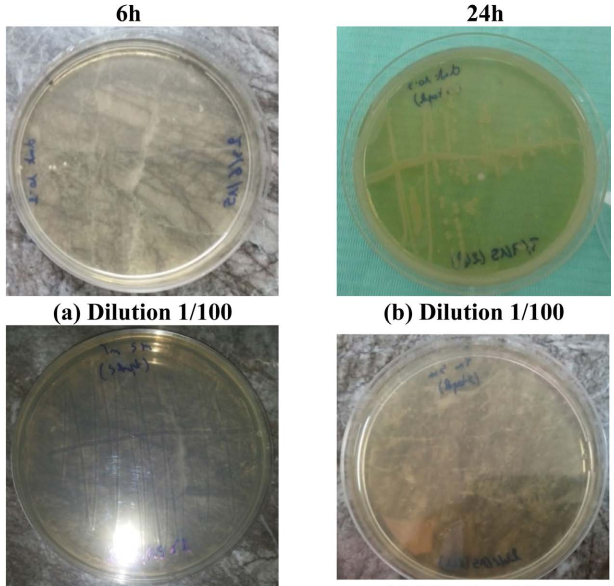
Therefore, Haemophilus influenzae needs more time to adhere and proliferate in the presence of titanium fragments. Whereas in the presence of dental fragments and agar this type of bacteria adhered and proliferated much more quickly (Figure 9).

-Staphylococcus aureus Meti S. ATCC 29213

6h 24h

TOUTH

STEEL



(a) Dilution 1/100 (b) Dilution 1/100

(c) Dilution 1/100 (d) Dilution 1/100

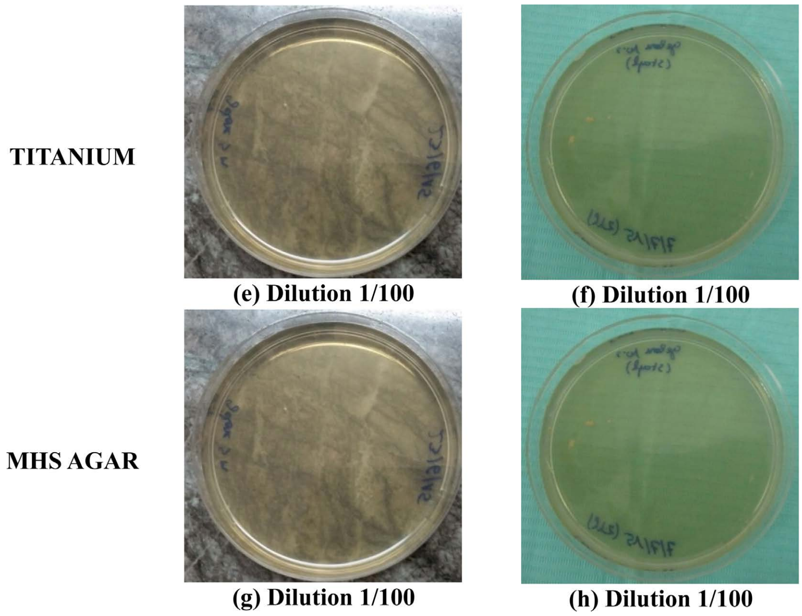
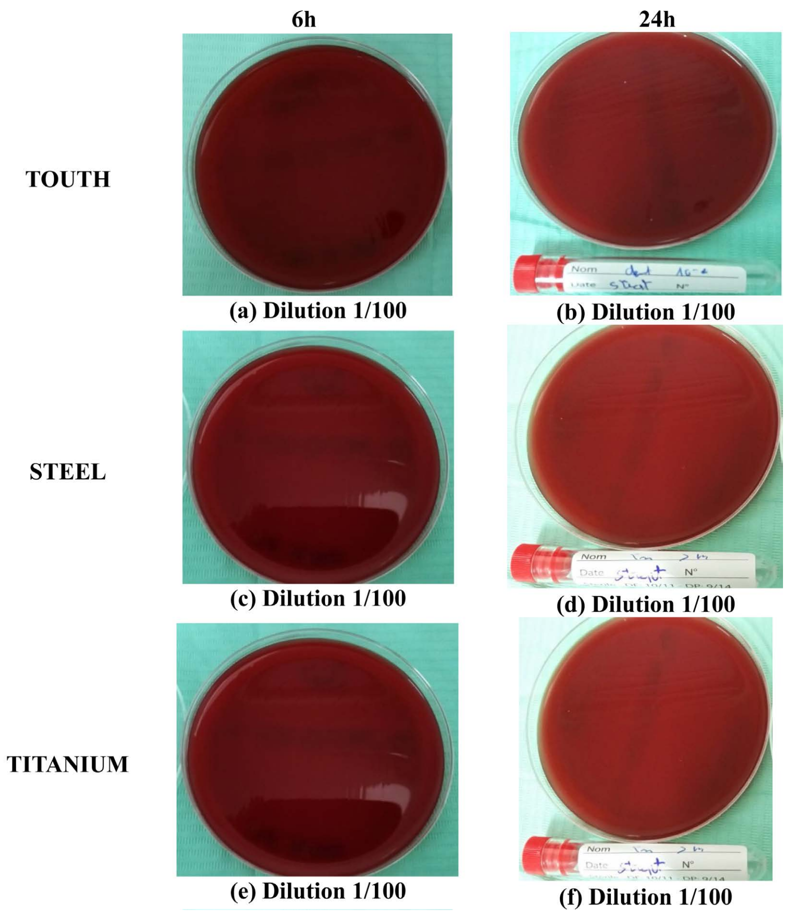
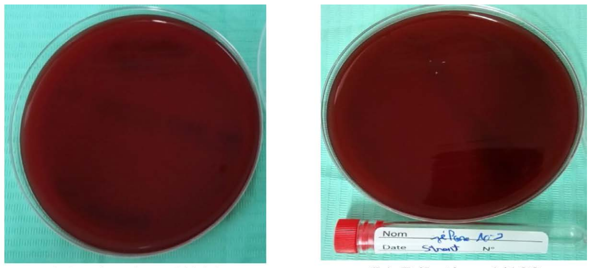


Figure 7. Results of cultures with different dilutions after 6 h and 24 h incubation of Staphylococcus aureus Meti S. ATCC 29213 in the presence of dental fragments, steel fragments, titanium fragments, and agar.

-Streptococcus intermédius ATCC 27335



MHS AGAR



(g) Dilution 1/100 (h) Dilution 1/100

Figure 8. Results of cultures with different dilutions after 6 h and 24 h incubation of Streptococcus intermedius ATCC 27335 in the presence of dental fragments, steel fragments, titanium fragments, and agar.

-Haemophilus influenzae ATCC 49247

6h 24h

TOUTH

STEEL

TITANIUM

MHS AGAR

(a) Dilution 1/100 (b) Dilution 1/100

(c) Dilution 1/100 (d) Dilution 1/100

(e) Dilution 1/100 (f) Dilution 1/100

(g) Dilution 1/100 (h) Dilution 1/100

Figure 9. Results of cultures with different dilutions after 6 h and 24 h incubation of Haemophilus influenzae ATCC 49247 in the presence of dental fragments, steel fragments, titanium fragments, and agar.

3.4. Evaluation of Adhesion Capacity by Enumeration by Culture on Congo Red Medium

The bacteria cultivated on the biomaterials are resuspended in distilled water readjusted to a turbidity of 0.5 Mc Farland, then we inoculated the Congo red medium with 1 µl of the suspension.

After incubation we counted the number of colonies that grew on the medium, which phenotypically marks adhesive colonies as black colonies with a rough surface versus red colonies with a smooth surface for the non-adhesive strains.

In order to determine the time necessary for the bacteria to be in exponential phase, proliferation kinetics of S. aureus, S. intermedius and H. influenzae in suspension was carried out for 24 hours. The results expressed in CFU/ml for two culture times have been reported in Table 1.

They showed that the culture time necessary for the bacteria to be in exponential phase is 24 hours. This period was chosen to study adhesion on steel and titanium and dental surfaces. Indeed, it is during this growth phase that bacterial adhesins are most expressed but the concentration differs from one strain to another and from one support to another.

Table 1. Bacterial enumeration (colonies/1µl) by culture on red Congo medium, stock solutions after 6 h and 24 h incubation in the presence of dental fragments, steel fragments, titanium fragments and agar.

	S. aureus **Number of colonies**				***S. intermedius*** **Number of colonies**				***H. influenzae*** **Number of colonies**			
	6 h		**24 h**		**6 h**		**24 h**		**6 h**		**24 h**	
	CB	CR	**CB**	CR	**CB**	CR	**CB**	CR	**CB**	CR	**CB**	CR
TOUTH	80	110	300	450	10	30	150	400	40	80	100	220
STEEL	0	40	20	150	0	10	15	85	0	80	25	120
TITANIUM	0	0	25	60	0	0	5	15	0	0	10	36
MH AGAR	200	440	600	1400	20	60	300	750	100	321	300	410

CB: Black colony; CR: Red colony.

The results showed:

-Staphylococcus aureus:

+ In the presence of dental fragments:

- After 6 hours of incubation, we obtained 80 colonies.
- After 24 incubations, we obtained 300 colonies.

+ In the presence of steel fragments:

- After 6 hours of incubation, we obtained 0 colonies.
- After 24 incubations, we obtained 20 colonies.

+ In the presence of titanium fragments:

- After 6 hours of incubation, the number of colonies is zero.
- After 24 hours of incubation, we obtained 25 colonies.

+ In the presence of agar:

- After 6 hours of incubation, we obtained 200 colonies.

- After 24 hours of incubation, we obtained 600 colonies.

-Streptococcus intermedius:

+ In the presence of dental fragments:

- After 6 hours of incubation, we obtained 10 colonies.
- After 24 incubations, we obtained 150 colonies.

+ **In the presence of steel fragments:**

- After 6 hours of incubation, we obtained 0 colonies.
- After 24 incubations, we obtained 15 colonies.

+ In the presence of titanium fragments:

- After 6 hours of incubation, the number of colonies is zero.
- After 24 hours of incubation, the number of colonies is 5.

+ In the presence of agar:

- After 6 hours of incubation, we obtained 20 colonies.
- After 24 hours of incubation, we obtained 300 colonies.

-Haemophilus influenza:

+ In the presence of dental fragments:

- After 6 hours of incubation, we obtained 40 colonies.
- After 24 incubations, we obtained 100 colonies.

+ In the presence of steel fragments:

- After 6 hours of incubation, we obtained 0 colonies.
- After 24 incubations, we obtained 25 colonies.

+ In the presence of the titanium fragment:

- After 6 hours of incubation, we obtained 0 colonies.
- After 24 incubations, we obtained 10 colonies.

+ In the presence of agar:

- After 6 hours of incubation, we obtained 100 colonies.
- After 24 hours of incubation, we obtained 300 colonies.

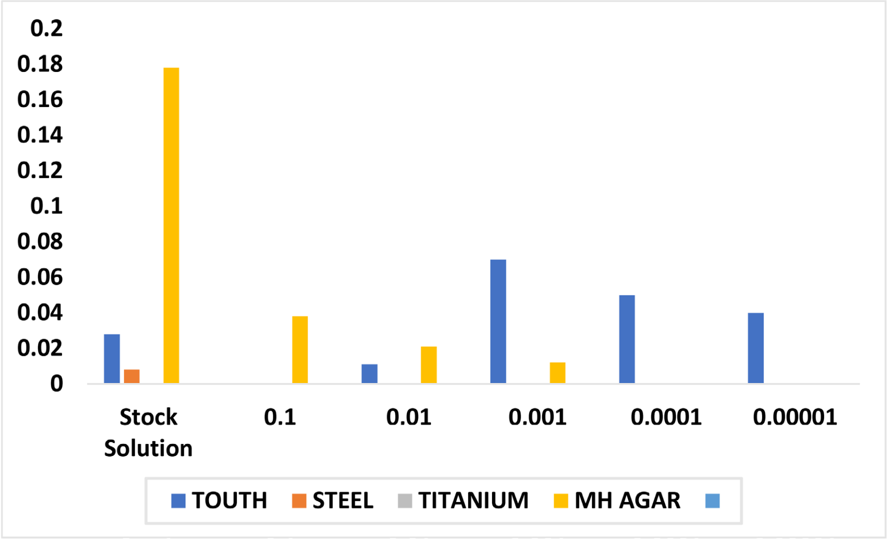
3.5. Evaluation of Adhesion Potential by Optical Dosage

-Evaluation of the adhesion potential by optical dosage depending on the different types of substrates and the seeding density (Table 2).

Evaluation of the adhesion potential by optical assay after 6 hours of incubation of Streptococcus intermedius showed zero results on dental fragments, steel fragments, titanium fragments and on agar.

Table 2. Optical dosage of the stock solution and the different dilutions prepared after 24 hours of incubation of Streptococcus intermedius in the presence of dental fragments, steel fragments, titanium fragments and MH agar.

DO	Stock Solution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Dental fragments	0.028	0.019	0.011	0.07	0.05	0.04
Steel fragments	0.008	0	0	0	0	0
Titanium fragments	0	0	0	0	0	0
MH AGAR	0.178	0.038	0.021	0.012	0	0



Graph 1. Optical dosage of the stock solution and the different dilutions prepared after 24 hours of incubation of Streptococcus intermedius in the presence of dental fragments, steel fragments, titanium fragments and MH agar.

Table 3. Optical dosage of the stock solutions after 6 hours and 24 hours of incubation of the three germs in the presence of dental fragments, steel fragments, titanium fragments and agar.

	TOUTH		STEEL		TITANIUM		MHS AGAR	
	6 h	24 h	6 h	24 h	6 h	24 h	6 h	24 h
S. aureus	0.011	0.053	0	0.010	0	0	0.012	0.179
S. intermeduis	0	0.028	0	0.008	0	0	0	0.178
H. inf	0.026	0.048	0	0.026	0	0.050	0.008	0.11

-Evaluation of the adhesion potential by optical dosage depending on the types of substrates and the different types of germs (Table 3).

-Staphylococcus aureus:

+ In the presence of dental fragments:

- After 6 hours of incubation we obtained an OD of 0.011.
- After 24 incubations we obtained an OD of 0.053.

+ In the presence of steel fragments:

- After 6 hours of incubation we obtained an OD of 0.
- After 24 incubations we obtained an OD of 0.010.

+ In the presence of titanium fragments:

- After 6 hours of incubation we obtained an OD of 0.
- After 24 incubations we obtained an OD of 0.

+ In the presence of agar:

- After 6 hours of incubation we obtained an OD of 0.012.
- After 24 hours of incubation we obtained an OD of 0.179.

-Streptococcus intermedius:

+ In the presence of dental fragments:

- After 6 hours of incubation, the optical density is zero.
- After 24 incubations we obtained an OD of 0.028.

+ In the presence of steel fragments:

- After 6 hours of incubation we obtained an OD of 0.
- After 24 incubations we obtained an OD of 0.008.

+ In the presence of titanium fragments:

- After 6 hours of incubation we obtained an OD of 0.
- After 24 incubations we obtained an OD of 0.

+ In the presence of agar:

- After 6 hours of incubation, the optical density is zero.
- After 24 hours of incubation we obtained an OD of 0.178.

-Haemophilus influenza:

+ In the presence of dental fragments:

- After 6 hours of incubation we obtained an OD of 0.026.
- After 24 incubations we obtained an OD of 0.048.

+ In the presence of steel fragments:

- After 6 hours of incubation we obtained an OD of 0.0.
- After 24 incubations we obtained an OD of 0.026.

+ In the presence of titanium fragments:

- After 6 hours of incubation, the optical density is zero.
- After 24 incubations we obtained an OD of 0.050.

+ In the presence of agar:

- After 6 hours of incubation we obtained an OD of 0.008.
- After 24 hours of incubation we obtained an OD of 0.11.

3.6. Comparison between Enumeration Results from Cultures and Dosage Optics

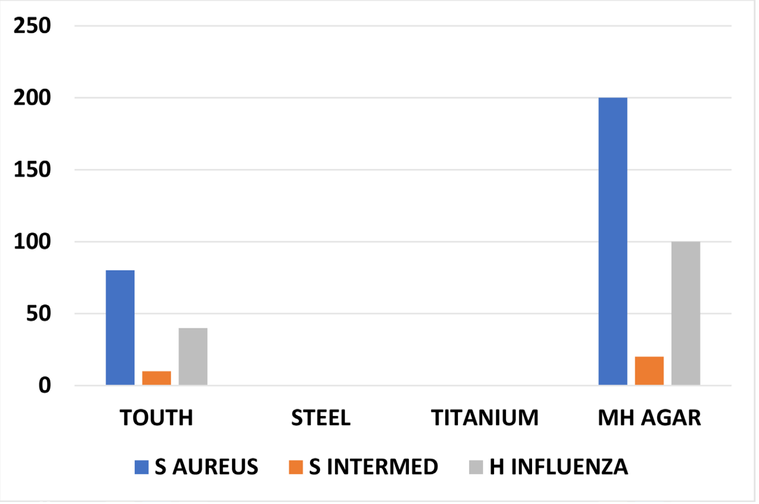
The two enumeration methods showed similar and correlated results for the three germs after 6 hours and 24 hours of incubation in the presence of dental fragments, steel fragments and titanium fragments and agar (**Graphs 2-5**).

Enumeration by both methods showed that the number of the three bacteria increased with incubation time in the presence of dental fragments, steel fragments, titanium fragments and agar.

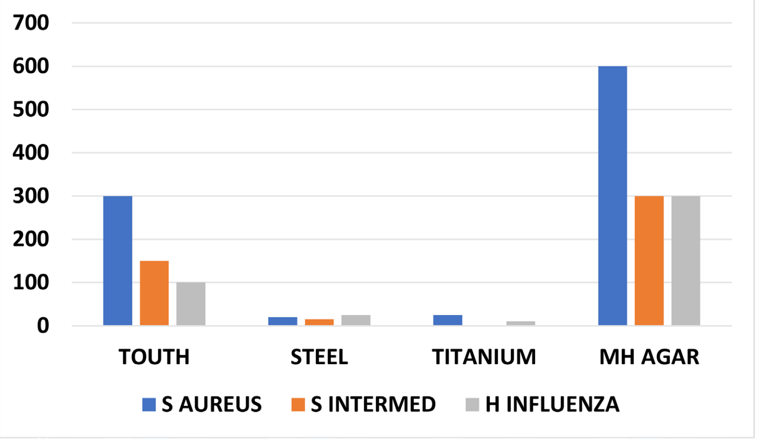
The adhesion and proliferation of Streptococcus intermedius are lower than those of Staphylococcus aureus and Haemophilus influenzae after 6 hours and 24 hours of incubation in the presence of dental fragments, steel fragments, titanium fragments and agar.

The results also showed that the count of the three bacteria after 6 hours of incubation and in the presence of steel fragments and titanium fragments was zero, which confirms the primary results that we obtained.

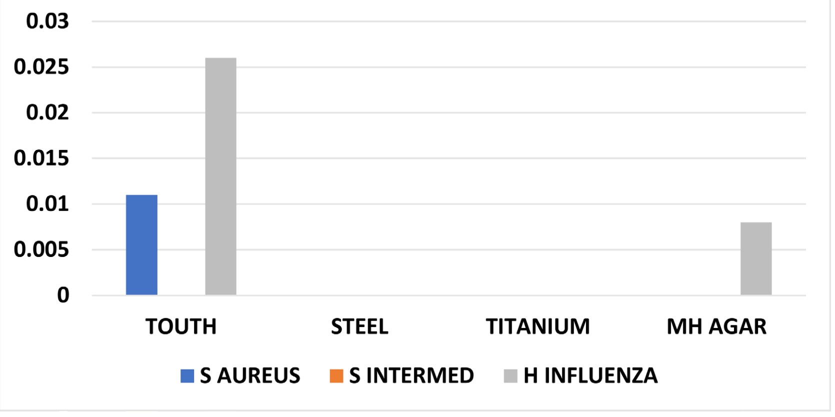
After 24 hours of incubation, we observed that the number of bacteria increased in the presence of dental fragments and agar. On the other hand, the number of bacteria increases only with Haemophilus influenzae in the presence of steel



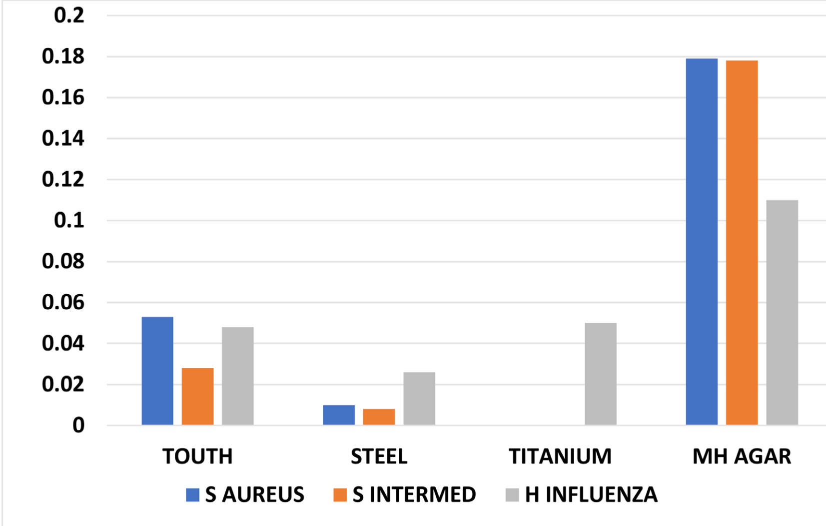
Graph 2. Bacterial enumeration (colonies/1µl) by culture on Congo red medium, stock solutions after 6 hours of incubation in the presence of dental fragments, steel fragments, titanium fragments and agar.



Graph 3. Bacterial enumeration (colonies/1µl) by culture on Congo red medium, stock solutions 24 hours of incubation in the presence of dental fragments, steel fragments, titanium fragments and agar.



Graph 4. Optical dosage of the stock solutions after 6 hours of incubation in the presence of dental fragments, steel fragments, titanium fragments and agar.



Graph 5. Optical dosage of the stock solutions after 24 hours of incubation in the presence of dental fragments, steel fragments, titanium fragments and agar.

fragments and titanium fragments, whereas the count is zero with Streptococcus intermedius and Staphylococcus aureus.

4. Discussion

In order to understand the mechanisms of bacterial adhesion on surfaces, we have cultured germs in contact with dental surfaces, stainless-steel surfaces and titanium fragments. This allowed us to understand the bacterial adhesion and proliferation mechanisms using counting methods (by culture and by optical assay).

The bacteria we have chosen is present in the oral flora and was also available at the bacteriology laboratory at the University Hospital Center of Casablanca. The evaluation of the adhesion capacity of these germs was carried out by a bacterial count after 6 h and 24 h incubation.

We used two counting methods:

-A count by culture on a special medium by pigmentation of adherent bacteria and non-adherent bacteria,

-Enumeration by optical dosage of bacteria adhering to the substrates; for this count we used decreasing dilution solutions of the mother solution prepared for the 1/1000 dilution.

4.1. Observations with the Naked Eye of Bacterial Cultures on Biomaterials after 6 Hours and 24 Hours of Incubation

-After 6 hours of incubation:

No bacterial cultures (the three strains) were observed on the plates encrusted with dental fragments, stainless-steel fragments and titanium fragments.

-After 24 hours of incubation:

-Staphylococcus aureus Meti S. ATCC 29213

The dishes inoculated with Staphylococcus aureus showed the formation of a bacterial which covers the entire dish including the dental fragments. While in the second dish, a decrease in proliferation around the stainless-steel fragments and the titanium fragments were observed with the appearance of a zone of inhibition of 2 to 3 mm.

-Streptococcus intermedius ATCC 27335

Figure 5(a) showed a total invasion of streptococcus intermedius on both culture dishes and even on the surface of the dental fragments. On the other hand, in the culture dish in the presence of steel fragments and titanium fragments, we observed an inhibition zone of 2 mm around the 2 substrates (Figure 5(b) and Figure 5(c)).

-Haemophilus influenzae ATCC 49247

The plates seeded with haemophilus influenzae showed the formation of a bacterial carpet which covers the entire seeded agar and the surface of the dental fragments (Figure 6(a)), on the other hand around the steel fragments and titanium fragments appears an inhibition zone of 1 mm in diameter (Figure 6(b) and Figure 6(c)).

Similar studies by Elagli A. [27] have worked on the effects of titanium powder on seven bacteria generally found in dental plaque or in the gingival sulcus, they have shown that the titanium alloy has no inhibitory or stimulating on bacterial adhesion. The adhesion of bacteria to the material depends on the physicochemical and topographic surface properties of it.

The same study has showed that the culture dishes inoculated with Staphylococcus aureus, showed the formation of a bacterial carpet that covers the entire box including dental fragments. Whereas at the level of the second box a decrease in proliferation around the titanium fragments was observed with the appearance of a 1 mm inhibition zone. The same zone was observed in the present study around the stainless-steel fragments (2 to 3 mm) and the titanium fragments [26].

4.2. Test to Confirm Bacterial Attachment to Biomaterials by Subculturing onto Culture Media

-The culture of Staphylococcus aureus Meti S. ATCC 29213 diluted 1/100 on MH medium showed:

-After 6 hours of incubation, the entire box was submerged by bacterial growth, even the surface of the dental fragments. On the other hand, there is no bacterial proliferation on the agar which contains steel fragments and titanium fragments.

-After 24 hours of incubation, growth was noted on the entire surface of the agar containing dental fragments but we noted the presence of only a few colonies on the agar encrusting steel fragments and titanium fragments.

The results also showed that with the other dilutions there were no bacterial growths on the agar plates containing steel fragments and titanium fragments after 6 hours or after 24 hours of incubation, but on the surfaces of the fragments in steel and titanium fragments there was no adhesion of these bacteria on the contrary they were inhibited.

Therefore, Staphylococcus aureus adheres and proliferates more easily on dental fragments than on agar with the same dilution.

Whereas Staphylococcus aureus does not adhere and therefore does not proliferate in the presence of steel fragments and titanium fragments even with the stock solution (**Figure 7**).

-The culture of Streptococcus intermedius ATCC 27335 diluted 1/100 on MHS medium showed:

-After 6 hours of incubation, the entire box was submerged by bacterial growth, even the surface of the dental fragments. On the other hand, we did not have any bacterial growth on the agar which contains steel fragments and titanium fragments.

-After 24 hours of incubation, we noted a growth on the entire surface of the agar containing dental fragments but we noted the total absence of shoots on the encrusting agar; we did not have bacterial growths and titanium fragments.

-After 24 hours of incubation we have a small number of bacterial colonies in the culture dishes with a dilution of 1/100 in the presence of dental fragments and in the presence of agar.

In the presence of steel fragments and titanium fragments, the results showed the absence of bacterial colonies after 24 hours of incubation with the different dilutions and even with the stock solution. At a dilution of 1/1000 we had no culture in all the dishes.

Therefore, Streptococcus intermedius has a low capacity to adhere and proliferate not only in the presence of dental fragments but also on agar with the same dilutions.

While Streptococcus intermedius did not adhere and therefore did not proliferate in the presence of steel fragments and titanium fragments with all diluted solutions (**Figure 8**).

-The culture of Haemophilus influenzae ATCC 49247 diluted 1/100 on chocolate medium showed:

- After 6 hours of incubation, we have the same number of colonies in the culture dishes in the presence of dental fragments and agar with dilutions: 1/10, 1/100, and 1/1000 however we do not have no colonies in the presence of steel fragments and titanium fragments with the different dilutions.

- After 24 hours of incubation, we have a significant number of bacterial colonies in the culture dishes in the presence of dental fragments and agar with a dilution of 1/100, on the other hand we have fewer colonies in the presence of steel fragments and titanium fragments and with the same dilution.

Therefore, Haemophilus influenzae needs more time to adhere and proliferate in the presence of titanium fragments. Whereas in the presence of dental fragments and agar this type of bacteria adhered and proliferated much more quickly.

Similar studies [1] have shown that culturing cells on biomaterials *in vitro* after 6 hours of incubation is not sufficient for cells to adhere and proliferate on their substrates. Other studies have shown that the density of seeding influences the phenomena of adhesion and proliferation [2].

A study that aimed to compare bacterial adhesion to dental fragment and Titanium fragment have showed that after 6 hours of incubation with Staphylococcus aureus, the whole box has been submerged by bacterial growth of the same surface of the dental fragments. On the other hand, on the agar which contains titanium fragments, there was no bacterial proliferation. After 24 hours of incubation, a growth was observed on the entire agar surface containing dental fragments, but only a few colonies were noted on the agar inlaid with titanium fragments. The results also showed that with the other dilutions there were no bacterial colonies on the agars containing titanium fragments, after 6h and after 24h incubation, but on the surfaces of the titanium fragments there was no adhesion of these bacteria instead they were inhibited. The study concluded that Staphylococcus aureus adheres and proliferates more easily on dental fragments on agar with the same dilution. While it does not adhere and therefore does not proliferate in the presence of titanium fragments even with the stock solution [27] [28].

This disparity in results depends on the composition of the substrates and the nature of its surface state since several studies have shown the influence of these components on cell adhesion and proliferation phenomena [29] [30].

Optical microscopic examination of the bacteria showed from the first hours of culture that the presence of dental fragments and agar did not affect cell adhesion and spreading capacity. Numerous studies have shown that the porous or fibrillar structure of a material [31]-[33], its topography or its roughness [29] [30] [33] and its physicochemical properties [34]-[36] play a determining role in the phenomena of migration, adhesion and synthesis of the extracellular matrix.

The results obtained with the cultures of the germs in the presence of the dental fragments can be explained by the roughness of its surface state resembling that of the trabecular bone. Indeed, many *in vivo* studies have shown that rough surfaces allow better bone integration than smooth surfaces [31] [37] [38].

The dental fragments used in our study have rough surfaces and sometimes have reliefs, which could explain the proliferation of bacteria around the dental fragments contrary to the results obtained with the stainless-steel fragments. Similar results have been obtained with stainless-steel having a smooth surface, a significant inhibition of the proliferation of Streptococcus aureus has been observed [39].

Generally speaking, dental plaque buildup is much greater on rough surfaces than on smooth surfaces, such as metal alloys [40] [41]. Dental plaque not only adheres in larger quantities, but is also more difficult to remove when the surface of the material is uneven [42] [43]. Indeed, the grooves and other surface defects lead to an increase in the potential surface to be colonized, and are places favorable to the creation of microbial niches [43].

The chemical composition of stainless-steel is a factor that influences bacterial growth and adhesion. Indeed, stainless steel, commonly called stainless-steel or stainless steel, is a steel alloy (containing less than 1.2% carbon) with more than 10.5% chromium. This alloy has a very different adhesion sensitivity than that of agar seeded by bacteria. Thus, the chemical composition of the material can influence the adhesion and colonization of bacteria on the adhesion surface.

Indeed, several researches are currently focused on chemical and topographic surface modifications of materials in order to develop surfaces with properties inhibiting bacterial adhesion [44]-[46].

The result obtained from another study shows that, increasing the average laser power is leading to an enhanced S. aureus adhesion while *E. coli* adhesion is reduced which is due to the hydrophobicity interaction and difference in surface texture. Meanwhile, the laser treatment

showed significant reduction of the bacterial adhesion on its surface compared to the polished surfaces. Thus, ultrafast laser texturing can be suggested as a promising method to reduce the bacterial adhesion, which reduced the adhesion of >80% for *E. coli* and >20% for S. aureus [47].

Biophysical modification of the material surfaces to prevent or reduce bacteria adhesion is an attractive alternative to antibiotic treatment. Since stainless-steel is a widely used material for implants and in hospital settings, in this work, we used stainless steel to investigate the effect of the material surface topographies on bacterial adhesion and early biofilm formation [48].

In contrast, clusters of the bacterial cells (microcolonies) were observed on electropolished smooth surfaces. Our study demonstrates that nanoscale surface roughness can play an important role in restraining bacterial adhesion and formation of microcolonies [48].

4.3. Evaluation of the Adhesion Potential by Optical Assay:

-Evaluation of the adhesion potential by optical dosage depending on the different types of substrates and the seeding density (Table 4).

Evaluation of the adhesion potential by optical assay after 6 hours of incubation of Streptococcus intermedius showed zero results on dental fragments, steel fragments, titanium fragments and on agar.

Table 4. Optical dosage of the stock solution and the different dilutions prepared after 24 hours of incubation of Streptococcus intermedius in the presence of dental fragments, steel fragments, titanium fragments and MH agar.

DO	Solution mère	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Dental fragments	0.028	0.019	0.011	0.07	0.05	0.04
Steel fragments	0.008	0	0	0	0	0
Titanium fragments	0	0	0	0	0	0
MH AGAR	0.178	0.038	0.021	0.012	0	0

-Evaluation of the adhesion potential by optical dosage depending on the types of substrates and the different types of germs (Table 5).

Table 5. Optical dosage of the stock solutions after 6 hours and 24 hours of incubation of the three germs in the presence of dental fragments, steel fragments, titanium fragments and agar.

	TOUTH		STEEL		TITANIUM		MHS AGAR	
	6 h	24 h	6 h	24 h	6 h	24 h	6 h	24 h
S. aureus	0.011	0.053	0	0.010	0	0	0.012	0.179
S. intermeduis	0	0.028	0	0.008	0	0	0	0.178
H. inf	0.026	0.048	0	0.026	0	0.050	0.008	0.11

4.4. Comparison between the Results of Enumeration from Cultures and Optical Assay

The two enumeration methods showed similar and correlated results for the three germs after 6 hours and 24 hours of incubation in the presence of dental fragments, steel fragments and titanium fragments and agar (**Graphs 2-5**).

Enumeration by both methods showed that the number of the three bacteria increased with incubation time in the presence of dental fragments, steel fragments, titanium fragments and agar.

The adhesion and proliferation of Streptococcus intermedius are lower than those of Staphylococcus aureus and Haemophilus influenzae after 6 hours and 24 hours of incubation in the presence of dental fragments, steel fragments, titanium fragments and agar.

The results also showed that the count of the three bacteria after 6 hours of incubation and in the presence of steel fragments and titanium fragments was zero, which confirms the primary results that we obtained.

After 24 hours of incubation, we observed that the number of bacteria increased in the presence of dental fragments and agar. On the other hand, the number of bacteria increases only with Haemophilus influenzae in the presence of steel fragments and titanium fragments, whereas the count is zero with Streptococcus intermedius and Staphylococcus aureus

The adhesion and proliferation of bacteria depend on the types of bacteria, the conditions of cell culture but also on the properties of the biomaterials used. The surface topography of a biomaterial affects not only adhesion but also the migration and proliferation of bacterial strains [49] [50].

An *in vitro* study that aimed to evaluate the ability of Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumoniae, and Pseudomonas aeruginosa to adhere to and to form biofilms on the surface of five orthopedic biomaterials, viz., cobalt and chromium, highly cross-linked polyethylene, stainless steel, trabecular metal, and titanium alloy, have shown that the highest level of adherence was observed on highly cross-linked polyethylene, followed by titanium, stainless steel, and trabecular metal, with the lowest occurring on the cobalt-chromium alloy. Among the bacterial strains tested, the ability for high adherence was observed with S. epidermidis and K. pneumoniae followed by P. aeruginosa and *E. coli*, whereas S. aureus showed the least adherence [29].

Stainless-steel (316L) is a metallic biomaterial, with a sensible biocompatibility and simple to machine; consequently, it is broadly utilized for orthopedic, cardiovascular and craniofacial applications because of its good corrosion resistance and formability [51].

The adhesion of bacteria on material surfaces is governed by many factors, including bacterial characteristic (e.g., hydrophobicity, surface charge), surface properties (e.g., roughness, wettability) and environment condition (e.g., pH, temperature) which involve the physicochemical and the molecular interactions [52].

Studies have showed that many factors affect the initial attachment of organisms to inert substrata, and their subsequent retention or removal/detachment, including the physical and chemical nature and location of the substratum, the type of organic material and microorganisms potentially fouling the surface, and the nature of the interface (solid-liquid in the body; solid-air on environmental surfaces) [53].

The composition and structure of surface biofilm depend on the biomaterial's localization. Moreover, the flow of saliva is also an important factor which may change the quantity and quality of biofilm. During saliva flow, the components of EPS can be changed, which may influence the adhesiveness of the biofilm to the surface. Biomaterials exposed to a high flow of saliva (where high shear forces occur) are less susceptible to biofilm formation [54]. On the other hand, surfaces with locally high nutrient content for microorganisms are more susceptible to colonization for example, between denture elements. The presence of biofilm on dental metallic biomaterials in the oral cavity is related to numerous processes of its surface destruction, such as corrosion and friction. In the case of the second phenomenon (friction), the wear process of teeth and biomaterial [21] [55].

The experimental results showed also that the bacterial attachments are in increasing order when the surface roughness of biomaterials increases but at the same time the bacterial inhibitions are in decreasing order. Which can explain the difference of adhesion between dental and stainless-steel surfaces in our study [51].

Rough surfaces of orthodontic archwires for example provide opportunities for bacterial adhesion by increasing the surface area, providing suitable niches for bacteria and impairing bacterial colony dislodgment.

Where the biofilm first develops within the valleys of uneven surfaces by irreversible attachment of planktonic pioneer bacteria, smoothing the rough regions [56].

In addition, changes in Surface Roughness of greater than 0.1 mm influence the contact angle, there by changing the surface free energy values, which comprise the second surface characteristic affecting bacterial adhesion to orthodontic materials [57].

5. Conclusions

Bacterial adhesion to dental surfaces and to the surfaces of biomaterials used in orthodontics constitutes an essential step in the formation and maturation of the biofilm responsible for pathological processes such as dental caries and periodontal disease. Consequently, a broad interest has been shown for many years in the study of the adhesion mechanisms of bacteria on dental surfaces and on metal alloys and in particular the study of modifications in the surface state of these substrates.

Reducing this adhesion on these surfaces has been approached by several methods, such as chemical or immunological blocking of adhesion sites and mechanical removal of bacteria through biofilm disorganization and surface treatment of metallic biomaterials. Many studies on the mechanisms of oral bacterial adhesion have been carried out on powder with hydroxyapatite as substrate, but only a few have been carried out on metallic substrates. In our study we used metal surfaces to try to understand the mechanisms of bacterial adhesion in conditions similar to the oral cavity.

Conflicts of Interest

The authors declare no conflicts of interest.

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