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Bacteria co-culture adhesion on different texturized zirconia surfaces

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ABSTRACT

Zirconia is becoming reckoned as a promising solution for different applications, in particular those within the dental implant investigation field. It has been proved to successfully overcome important limitations of the commonly used titanium implants. The adhesion of microorganisms to the implants, in particular of bacteria, may govern the success or the failure of a dental implant, as the accumulation of bacteria on the peri-implant bone may rapidly evolve into periodontitis. However, bacterial adhesion on different zirconia architectures is still considerably unknown. Therefore, the adhesion of *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* to zirconia surfaces with different finishings was evaluated and compared to a titanium surface. The adhesion interaction between *S. aureus* and *P. aeruginosa* was also evaluated using a co-culture since these bacteria are infamous due to their common presence in chronic wound infections. Results showed that different bacterium species possess different properties which influence their propensity to adhere to different roughness levels and architectures. *E. coli* revealed a higher propensity to adhere to zirconia channelled surfaces (7.15×10^6 CFU/mL), whereas *S. aureus* and *P. aeruginosa* adhered more to the titanium control group (1.07×10^5 CFU/mL and 8.43×10^6 CFU/mL, respectively). Moreover, the co-culture denoted significant differences on the adhesion behaviour of bacteria. Despite not having shown an especially better behaviour regarding bacterial adhesion, zirconia surfaces with micro-channels are expected to improve the vascularization around the implants and ultimately enhance osseointegration, thus being a promising solution for dental implants.

1. Introduction

Dental implants are a common practice in the dentistry field and research on their design, materials and techniques has gained a special interest over the past few years (Gaviria et al., 2014). Commercially available dental implants are mostly composed of titanium or its alloys, since this material has demonstrated excellent mechanical properties and biocompatibility (Cionca et al., 2000). However, it possesses poor wear resistance and its appearance comprises a major aesthetic drawback. In addition, it releases metallic ions to the physiologic environment, and thus the development of novel materials and fabrication strategies to overcome these limitations are warranted (Sikora et al., 2018; Souza et al., 2015).

Zirconia, namely tetragonal polycrystalline zirconia stabilized with 3 mol% yttrium oxide (Y-TZP), is being applied in dental applications as an alternative to metallic solutions (Kelly and Denry, 2008; Dantas et al., 2019). This ceramic material is a promising option for such applications mainly due to its opaque white color, low bacterial affinity and good biocompatibility (Cionca et al., 2000). Furthermore, zirconia presents low thermal conductivity, good fracture resistance, suitable flexural strength, high corrosion and wear resistance (Yin et al., 2017). Despite all of these advantages, zirconia is a bioinert material, which means that it does not spontaneously induce osseointegration and bone formation in the vicinity of the implant (Faria et al., 2019). In this sense, efforts are being carried out aiming to improve the zirconia-bone interactions and, consequently, improve the implant osseointegration (Chevalier et al., 2011; Moura et al., 2017). Apart from dental applications, zirconia has also been applied in a wide variety of purposes and industries, namely in the cutlery field. Zirconia knives have been proved to present numerous advantages over common stainless-steel ones: higher sharpness, higher wear resistance, anti-bacterial properties, and also an improved corrosion resistance (Bi et al., 2018).

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Surface roughness and its influence on the implant osseointegration has been a subject of study over the past few years. A recent review performed by Pesce P. et al. evaluated the influence of abutment surface roughness on peri-implant soft tissue behaviour. Results revealed an enhanced connective tissue fibre attachment next to rougher Ti abutments, when compared to machined ones (Pesce et al., 2019). On the other hand, Rezk F., et al. mentioned that abutments with rougher surfaces (above 0.4 μm) increase the affinity of microorganisms and the risk of peri-implant diseases (Al Rezk et al., 2018). Many techniques are already being implemented to control the implants or abutments surface roughness. Sandblasting, acid etching and spray drying represent some examples of these techniques (Depprich et al., 2008; Sennerby et al., 2005). Sandblasting has been applied in dentistry as one of the most effective strategies to increase the bond strength between resin luting materials and restorative materials (Nishigawa et al., 2016). On the other hand, sandblasting followed by an acid etching procedure (SLA) is considered the gold standard surface treatment in the worldwide dental implants market (Roccuzzo et al., 2014; Buser et al., 2012; Costa et al., 2019). Despite being considered a successful surface treatment, the clinical survival of zirconia-based implants with this surface finishing may be compromised as a result of the surface contamination due to residual ceramic particles or even bacterial colonization due to the increased roughness (Faria et al., 2020). A promising alternative to SLA is treating zirconia with purely chemical treatments, namely with hydrofluoric (HF) acid. In fact, zirconia implants treated with this chemical approach are already available in the market and their clinical performance has been proved to be as successful as titanium implants (Bergemann et al., 2015; Oliva and OlivaJ. D, 2010).

In addition to the surface roughness, there are other features and parameters that may influence the implant osseointegration and overall function. An increased surface wettability, for instance, is expected to increase the interactions between the implant surface and the biologic environment (Bornstein et al., 2008). Also, capillarity, a spontaneous movement of a fluid resulting from the cohesive forces between the fluid and the surrounding surface, is crucial to achieve an efficient vascularization around the implant (Jokinen and Franssila, 2008). An implant surface with capillary properties is expected to improve the flow of fluids, namely blood, and thus promote, cell infiltration and proper nutrient supply (Marques et al., 2019). With the aim of enhancing cell and nutrient flow around dental implants, surfaces with interconnected pores and/or micro-channels are also being designed and developed (Marques et al., 2019; Oh et al., 2014). One study performed by the authors research group concluded that the implementation of micro-channels on zirconia surfaces led to high capillarity and hydrophilic behaviours. These behaviours are expected to facilitate biological responses, such as vascularization around the implant, leading to an improved osseointegration and stability (Dantas et al., 2020).

The lack of osseointegration is a well-identified problem. Nevertheless, the adhesion of microorganisms to the implant surface and consequent plaque formation is considered one of the most important factors that influence the primary stability of implants (Costa de Medeiros Dantas et al., 2016). It is known that the quality and quantity of bacterial plaque on the implant surface will be detrimental in dictating whether an implant succeeds or fails (Costa de Medeiros Dantas et al., 2016). This plaque formation is commonly associated with lack of proper oral hygiene but also with the characteristics of the implant components used in the restoration, namely the implant-abutment interface (doNascimento et al., 2014). In fact, the accumulation of bacteria in the implant-abutment assembly is still one of the most critical challenges in dental implantology. An inaccurate fit between these two components may induce the colonization of bacteria that can, in turn, lead to inflammatory reactions with consequent bone resorption (Pedrazzi et al., 2014).

Zirconia is known to have the ability to inhibit the level of bacterial adhesion on its surface when compared to other materials commonly used in dental implant applications (Yin et al., 2017; Manicone et al., 2007). One study performed by Cássio do Nascimento and co-workers evaluated the bacterial adhesion on titanium and zirconia abutment surfaces, depicting a significantly higher total bacterial count in titanium specimen (doNascimento et al., 2014). In another study, bacterial adhesion was investigated on three different materials, under the same surface polishing: resin, titanium and zirconia. Resin specimens exhibited the highest susceptibility to bacteria adhesion when compared to the other two materials (Lee et al., 2011). On the other hand, there was no significant difference between titanium and zirconia specimens. Afya Sahib Diab Al-Radha et al. (2012) also assessed the surface properties of titanium and zirconia surfaces and their influence on bacterial adhesion. Four different surfaces were tested: polished zirconia (PZ), titanium blasted with zirconia (TBZ), titanium blasted with zirconia then acid etched (TBZA), and polished titanium (PT). The overall results suggest that zirconia and titanium blasted with zirconia are more effective in reducing the bacterial adhesion.

There are several studies that indicate that the accumulation of plaque around dental implants is extremely dependent on the surface roughness (Truong et al., 2010; Kang et al., 2017; Riedewald, 2006; Schubert et al., 2019). In fact, rougher surfaces, due to the presence of grooves and pits, are expected to induce a higher bacterial adherence, since, in these surface irregularities, bacteria are protected from the shear stress of salivary flow or other biologic fluids (Costa de Medeiros Dantas et al., 2016). As far as zirconia dental implants are concerned, literature reports that a surface roughness ranging from 130 to 360 nm is crucial to avoid bacterial adhesion (Yin et al., 2017). Apart from the surface roughness, other surface characteristics, such as wettability and surface charge, may also influence the bacterial adhesion to the implants surface (Costa de Medeiros Dantas et al., 2016; Azam et al., 2015). Understanding the phenomenon of bacterial adhesion to zirconia surfaces may also become a useful tool to address the potential microbial cross-contamination that may be induced by the improper use of certain food contact surfaces, including utensils and zirconia cutlery, such as knives and forks (Fink et al., 2017). Many consumers are not aware that domestic food contact surfaces and utensils are considered an important mean of transmission of foodborne diseases (Erickson et al., 2015).

Despite being already in the market, the microbiological response of zirconia implants, in particular when specific geometrical features are intended, such as micro-grooves, for improved osseointegration, still needs to be further investigated. Therefore, in this study, the adhesion of *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* on different zirconia surfaces was assessed. Moreover, a co-culture of *S. aureus* and *P. aeruginosa* was performed aiming to evaluate the interactions between these two bacteria, since they are the most common cause of chronic wound infections and are frequently found together (Alves et al., 2018). The tested specimens were as-sintered zirconia (AS), sandblasted and acid etched zirconia (SbE), zirconia with micro-channels on its surface (MC), zirconia with micro-channels followed by an acid etching treatment (MCE) and titanium with SLA (Ti) as control group, representing the surfaces of commonly used dental implants. Surface parameters such as roughness, wettability and capillarity were also evaluated and discussed.

2. Materials and methods

2.1. Production of titanium specimens

Titanium specimens were cut from a biomedical grade V (Ti–6Al–4V alloy) rod, resulting in specimens with 8 mm diameter and 2 mm of height. Posteriorly, a surface treatment was applied to the specimens' surfaces, aiming to mimic the surface finishing of the commercially available dental implants. This surface modification, referred to as SLA, consists of a sandblasting treatment followed by acid etching, and the procedure is explained in section 2.3 (Scientific Review for Str, 2020; Stafford et al., 2014).

2.2. Production of zirconia green compacts

In the scope of this research work, zirconia green compacts were produced by cold press technique. A 3 mol% yttria-stabilized zirconia spray-dried powder (TZ-3YSBE, Tosoh Corporation, Japan), with a theoretical density of 6.05 g/cm^3 and high purity (99%) was used as starting material. This powder is characterized by spherical agglomerates with an average particle size of 60 μm and crystallites with an average diameter of 36 nm.

A cylindrical steel mold with an internal diameter of 10 mm was used for the compaction of the powder. In a first step, the zirconia powder was introduced in the mold and 200 MPa of pressure was applied, during 30 s. In the second step of this manufacturing technique, the pressure was evenly released and the obtained green compact (with 10 mm of diameter and 5 mm of height) was carefully removed from the mold.

2.3. Surface texturing

Four zirconia surfaces and a titanium surface were tested regarding their propensity for bacterial adhesion. The sintering process to which all zirconia specimens were subjected will be explained in section 2.4 of this work.

Titanium specimens were subjected to an SLA treatment, aiming to mimic the roughness values commonly found in dental implants. White corundum angular particles with a maximum size of 250 μm were projected by a blasting gun with an air pressure of 0.6 MPa and a distance of 10 cm between the nozzle and the treated surface. After being properly cleaned with isopropanol, the specimens were subjected to a 5 min acid-etching process (with 32% HCl, 96% H_2SO_4 and H_2O (2,1,1) (v/v)) at 65 ± 3 °C.

The second group of specimens – AS – correspond to as-sintered zirconia surfaces, which means that these specimens were not subjected to any surface treatment.

The third group of specimens – SbE – was developed mainly for comparison purposes since authors mimicked the treatment used in conventional implants – sandblasting followed by an acid-etching process. This surface treatment was performed after the sintering process and it encompass two steps. In the first step - the sandblasting - white corundum spherical particles with a maximum particle size of 250 μm were projected, during 30 s, by a blasting gun. A constant air pressure of 0.6 MPa was applied and a distance of 10 cm was kept between the nozzle and the treated surface. After this procedure, specimens were ultrasonically cleaned in isopropanol for 5 min. In the second step, the same specimens were subjected to an acid etching treatment. In this stage, the sandblasted surfaces were immersed, for 30 min, in hydrofluoric acid (48% (v/v)), followed by a cleaning procedure in an ultrasonic bath of isopropanol for 5 min.

The fourth group of specimens – MC – correspond to specimens with micro-channels on their surface, aiming to improve the vascularization around the implant. These micro-channels were designed in the pre-sintered zirconia specimens by a Computer Numerical Control (CNC) machining technology. The micro-channels were firstly designed in a CAD software (SolidWorks Corporation, Dassault Systèmes S.A, USA), followed by the development of a file with the code to be read by the machine firmware (DWX – 50 by Roland, Serbia). After this machining process, specimens were sintered. The micro-channels dimensions (200 μm of width and 100 μm of depth) were previously discussed in another study from the authors (Dantas et al., 2020) that aimed to assess which was the best combination of dimensions to promote a proper vascularization.

The fifth and last group of specimens – MCE – correspond to MC specimens with a posterior etching treatment. Following the micro-channels design and the sintering process, specimens were immersed in hydrofluoric acid (48% (v/v)) during 4 h. This fourth group of specimens was developed to assess the influence of the etching treatment on the micro-channels surface roughness and bacterial adhesion.

For a better understanding, in Fig. 1 it is provided a schematic representation of the five different surfaces developed.

2.4. Zirconia green compacts sintering

To sinter the zirconia green compacts developed in the scope of this research work, a high-temperature furnace - Zirkonofen 700, Zirkonzahn, Italy - was used. This process was carried out at 1500 °C with heating and cooling rates of 8 °C/min and a holding time of 2 h.

After the manufacturing process, all specimens were carefully brushed with an RBSTM 50 solution (Sigma-Aldrich, USA) and ultrasonically cleaned with isopropanol for 5 min to remove any loose debris or surface contamination.

2.5. Specimens cleaning procedure

Before the *in vitro* tests, all specimens were subjected to a careful cleaning procedure. First, they were manually cleaned with ethanol for 2 min to remove any grease they could contain. Then, the specimens were sprayed with distilled water and subjected to an ultrasonic bath with isopropanol and RBS^{TM} 50 solution (Sigma-Aldrich, USA) during 30 min. Posteriorly they were rinsed with distilled water and set in an ultrapure water bath for 20 min. Finally, all specimens were subjected to 20 min of UV-C in a laminar flow cabinet.

2.6. Surface analysis

In order to assess whether the applied surface treatments were successful in achieving different surface topographies, surface roughness was evaluated, using a profilometer by Mitutoyo (SJ 210, Japan).

The surface wettability of the as-produced specimens was also assessed, by means of contact angle measurements. An optical goniometer (OCA 15 Plus, Dataphysics, Germany) was used to perform the sessile drop technique. Three droplets of deionized water (at 18.2 Ohm) with a volume of 5 μL and a dosing rate of 1 μL/s were applied to the surface of each tested specimen, and contact angles were measured immediately after the droplet reached the surface (0 s), and after 10 s.

The capillarity of the different tested conditions was assessed through a test in which the specimens were placed in contact with a fluid (a commercial aqueous solution containing phenol red ($C_{19}H_{14}O_5$)) and the fluid rise was observed and recorded by means of photography. This red color pigment was used for the performance of this experimental procedure since the tested materials are characterized by a high opacity and white color.

Scanning electron microscopy (SEM) was performed on the as-produced specimens to analyse their topography and the manufacturing process (JSM- 6390LV; JEOL, Japan). SEM was also used to observe the bacterial interactions with the produced specimens. Only two surface conditions were selected for this analysis – SLA titanium (Ti) and zirconia with micro-channels (MC). The bacterial fixation on the surfaces followed a procedure previously described in the literature (Padrão et al., 2016). Briefly, after removing the culture media, 1 mL of 2.5% (v/v) glutaraldehyde in phosphate buffer saline (PBS) was added to each specimen for 1 h at room temperature to induce the bacteria fixation. Subsequently, a dehydration process with serial ethanol dilutions with increasing ethanol percentage (55, 70, 80, 90, 95, 100% (v/v)) was performed. Specimens were immersed in each solution for 30 min at room temperature and, in the end, the remaining ethanol was left to evaporate. Prior to any SEM analysis specimens were sputter-coated with gold (Model 108 A, Cressington, UK).

2.7. Bacterial adhesion protocol

The bacteria used for the tests were *E. coli* (CIP 110067) and *S. aureus* (CIP 76.25) and *P. aeruginosa* (CIP 76.110). Pre-inocula were prepared by incubation of each bacterium at 37 °C and 120 rpm. *E. coli* and

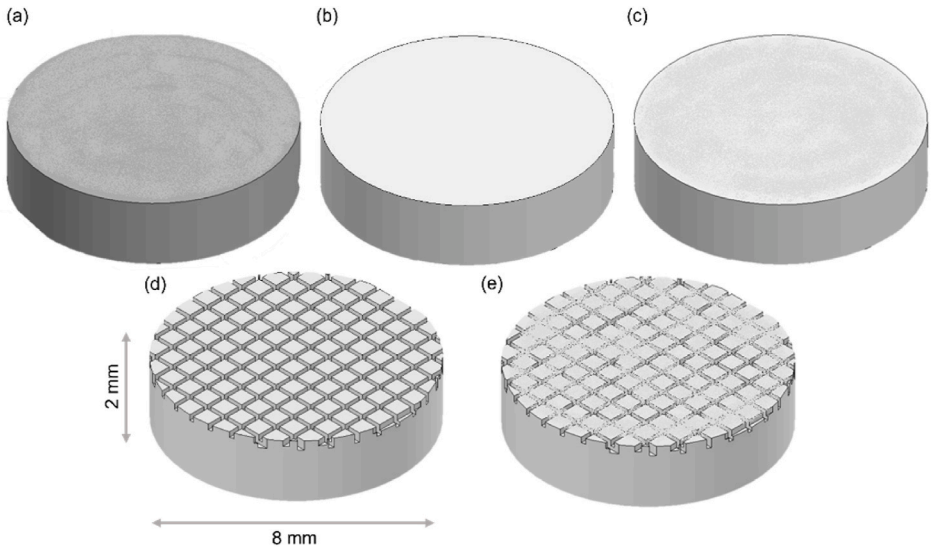

Fig. 1. Schematic representation of the produced specimens: (a) Ti- titanium with SLA; (b) AS- zirconia as-sintered; (c) SbE-zirconia with sandblasting and acid etching; (d) MC- zirconia with micro-channels on the surface; (e) MCE-zirconia with micro-channels and acid etching.

S. aureus were incubated in tryptone soya broth (HiMedia Laboratories Pvt. Ltd., India), whereas *P. aeruginosa* and *S. aureus* (when in co-culture) were cultured in nutrient broth (Condalab, Laboratorios Conda S.A., Spain). In each pre-inocula, bacterial concentration was adjusted by densitometry (Epoch Microplate Spectrophotometer, Biotek, USA) at 600 nm to a McFarland standard optical density corresponding to a bacterial concentration of approximately 1×10^7 colony forming units (CFU)/mL.

Three specimens of each condition were inserted into 24-well plates and incubated with each bacterium for 24 h at 37 °C and 120 rpm. As previously mentioned, the produced specimens have 8 mm diameter and 2 mm of height which corresponds to an apparent exposed area of 100.53 mm^2 (resulting from the sum of the specimen top and lateral areas). Subsequently, the broth was aseptically removed, and each specimen was put into a sterile 15 mL falcon tube with 3 mL PBS solution. To detach the adhered bacterium from the specimens surfaces, each falcon tube was subjected to an ultrasonic bath (50/60 Hz, J.P. Selecta, Spain) for 10 min and then vortexed for 1 min. Afterwards, the CFU/mL of each sample was estimated in Petri dishes incubated at 37 °C for approximately 12 h. This protocol was also performed using a co-culture of *S. aureus* and *P. aeruginosa* containing approximately 1×10^7 CFU/mL of each bacterium for 24 h at 37 °C and 120 rpm. In Fig. 2 it is possible to observe a schematic representation of the developed surfaces and tested bacteria, for a better comprehension.

2.8. Statistical analysis

The bacterial adhesion data was subjected to statistical analysis. Results are presented as the mean ± standard error of the mean. The assumption of normality was verified, and t-tests or one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests were performed. The level of significance was set at $\alpha = 0.05$. This analysis was carried out using GraphPad 6 software (Prism, California).

3. Results and discussion

3.1. Specimens characterization

The different produced specimens were inspected by scanning electron microscopy and the top views are presented in Fig. 3. In Fig. 3(a), which corresponds to the titanium specimen (Ti), it is possible to observe an irregular surface, resulting from the SLA treatment. Fig. 3(b) and (c) correspond to as-sintered zirconia (AS) and zirconia with sandblasting and acid etching (SbE), respectively. By comparing the two micrographs it is easily denotable that the applied surface treatment, as expected, leads to the formation of a more irregular surface, characterized by an increased roughness. As far as the machined surfaces are concerned (Fig. 3(d) and (e) for MC and MCE, respectively) it is possible to observe that the micro-channels bottom is characterized by an

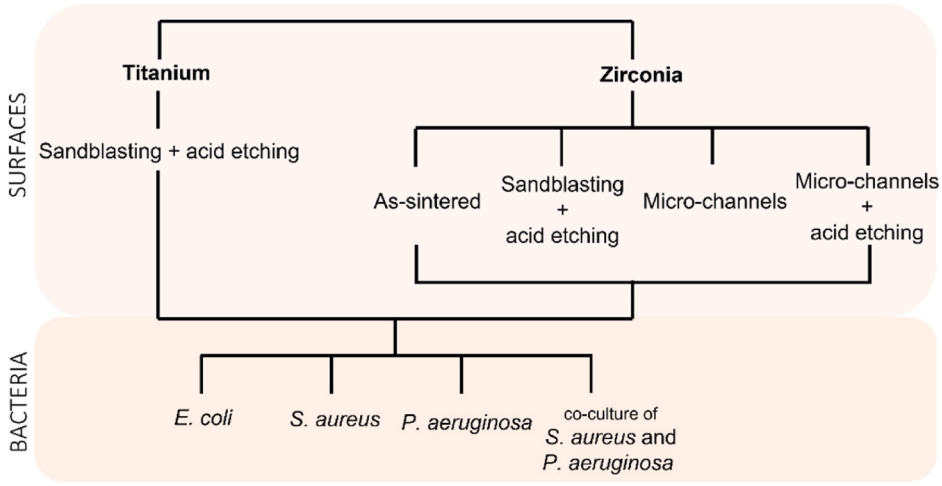

Fig. 2. Schematic representation of the developed surfaces and tested bacteria.

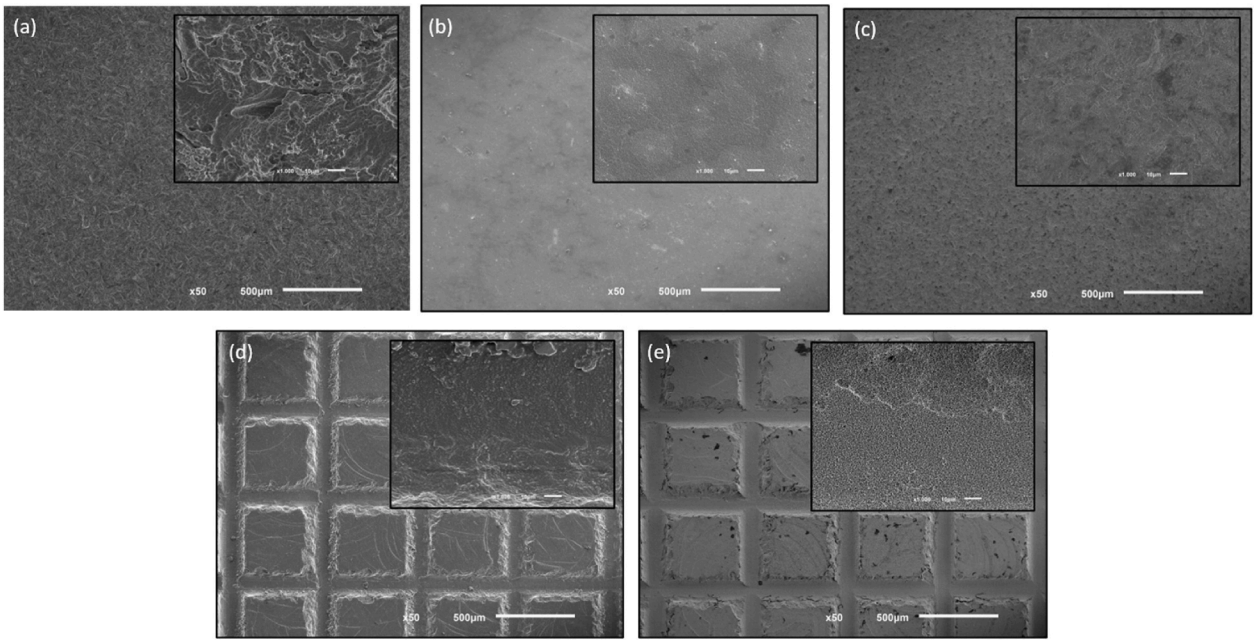

Fig. 3. SEM micrographs of the surface topography of the produced specimens: (a) Ti; (b) AS; (c) SbE; (d) MC; (e) MCE.

irregular topography, that resulted from the milling process. Additionally, in the borders of the channels, it is possible to denote the accumulation of material from the milling process that was not effectively sintered nor removed through the cleaning protocol. This material accumulation, observed in all specimens that were subjected to the machining process, may have influenced the subsequent *in vitro* testing. Again, the surface treatment performed on the MCE specimen seems to have induced an increase in the surface roughness. This increase is not so clear as in the SbE condition that was subjected to both sandblasting and acid etching, whereas MCE underwent only an acid etching treatment.

The results observed in the previous images are corroborated by the roughness measurements present in Table 1.

As seen in Table 1, titanium specimens treated with sandblasting and etching present a mean surface roughness of 1.71 ± 0.4 µm. These results are in accordance with similar studies found in literature, where SLA titanium specimens were characterized (Costa et al., 2019; Chen et al., 2016). On the other hand, and as expected, the average roughness decreased after the acid etching treatment, when compared with sandblasted titanium (Kim et al., 2008). As far as the zirconia specimens are concerned, the obtained average roughness measurements are also in accordance with the literature (He et al., 2014; Ewais et al., 2014). In addition, the sandblasting treatment induced a considerable increase in the average roughness when compared with as-sintered zirconia (2.09 ± 0.23 µm vs. 0.29 ± 0.04 µm, respectively) (He et al., 2014; Kirmali et al., 2014). Surface roughness decreased when an acid etching treatment was applied over the sandblasted surface. When comparing MC with MCE specimens, the surface roughness of the zirconia was greater when the surface was treated with etching, as reported in analogous studies where rough zirconia surfaces were desired for improving the shear bond strength of dental resin cements to zirconia (Lee et al., 2019).

3.2. Wettability and capillarity analysis

Together with surface topography, its hydrophilicity is also expected to have a huge impact on the promotion of proper implant osseointegration. In fact, hydrophilic implant surfaces were proved to promote a faster growth of osteoblast-like cells, when compared with hydrophobic surfaces (Shi et al., 2014). To assess the wettability of the produced specimens, water contact angles (WCA) were measured in static (at the moment the droplet touched the surface) and dynamic mode after 10 s (Fig. 4). Literature reports that a surface is considered hydrophilic if a WCA below 90° is obtained. On the other hand, surfaces with WCA above 90° are designated as hydrophobic. After analysing the results, it is possible to conclude that all specimens present WCA below 90°, denoting a hydrophilic nature (Himma et al., 2019). Additionally, the Ti specimen presents considerably higher WCA when compared with all the zirconia specimens. The WCA obtained for this condition (86.3 ± 4.9° and 70.1 ± 4°, for 0 and 10 s, respectively) are in accordance with previous studies found in literature, which report titanium WCA between 70 and 90° regardless of surface roughness (Ma et al., 2017). Despite these results, there are some studies in the literature that state

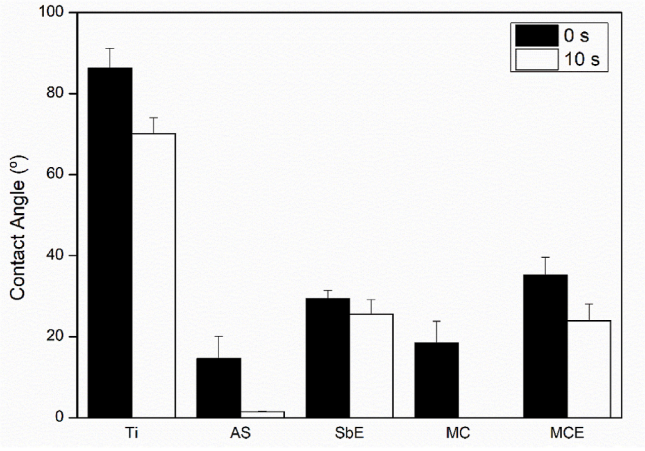

Fig. 4. Contact angles (mean ± SD) of tested materials, for 0 s and 10 s.

Table 1
Mean (SD) of surface roughness of the produced specimens. Roughness of MC and MCE specimens was measured on the top surface.

Specimen	Ti		AS	SbE		MC	MCE
Surface roughness, Ra (µm)	After sandblasting 2.38 (±0.50)	After etching 1.71 (±0.40)	0.29 (±0.04)	After sandblasting 2.09 (±0.23)	After etching 1.59 (±0.19)	0.28 (±0.01)	0.73 (±0.07)

that the WCA of SLA titanium can raise up to 150° (Gittens et al., 2013). These higher WCA found for the SLA condition may be explained by the Wenzel's theory, that states that as the surface becomes rougher, it gets harder for the water to penetrate through the grooves due to the trapped packets of air (de Leon and Advincula, 2015). As far as the zirconia specimens are concerned, despite not existing significant differences between them, an increased surface roughness (SbE and MCE) seems to induce higher WCA when compared to smooth surfaces (AS and MC). These results are, again, corroborated with results found in similar studies that indicate that higher surface roughness led to higher WCA (Dantas et al., 2020; Himma et al., 2017).

Furthermore, it is also possible to conclude that, for all tested specimens, WCA after 10 s is substantially lower than in the initial moment. For the smooth surfaces (AS and MC), after 10 s, the measured CA was approximately 0°, confirming the hydrophilic nature for these specimen conditions.

The main goal of designing micro-channels on the zirconia surface was to induce and facilitate the infiltration and supply of nutrients and fluids around dental implants, consequently improving the vascularization at the implants surface. In Fig. 5 it is possible to observe the tested specimens surface after contact with the red pigment.

In Fig. 5(a), that corresponds to the SLA Ti, it is possible to denote the wetting of the entire surface, which is in accordance with other studies found in the literature that report a capillary nature for this type of material (Bartolomeu et al., 2020; Ou et al., 2016). Fig. 5(b) and (c) - AS and SbE, respectively-indicate that, as expected, zirconia specimens without any microfeature on the surface were not able to promote the desired capillary effect. On the other hand, the incorporation of micro-channels on the zirconia substrate effectively induced the fluid rise and spread. This is evidenced by the reddish color of the entire specimens at the end of the test (Fig. 5(d) and (e)). In fact, this behaviour was already found in another study performed by the same authors of this article, where different micro-channels dimensions were evaluated aiming to find the best combination of depth and width for the promotion of proper vascularization (Dantas et al., 2020). These results suggest that the fluid molecules adhere to the zirconia surface, cohere, and then pull each other through narrow cavities such as the designed channels.

3.3. Bacterial adhesion

a. Mono-cultures

As previously mentioned, in the scope of this research work, three bacteria were tested regarding their adhesion on different surfaces. In Fig. 6 it is possible to observe the obtained results determined from the viable counts.

In Fig. 6(a), which corresponds to *E. coli,* it is obvious that the zirconia specimen with micro-channels (MC) was the one that promoted the greater bacterial adhesion, being the results statistically different from all the other tested conditions. One possible reason for an increased *E. coli* adhesion to the MC specimen could be related to the bacteria shape and charge, to the increased area of exposure, and the rough topography that characterizes the channels walls, as a consequence of the machining process. It also seems that applying an acid etching treatment over the micro-channels surface (MCE) considerably reduced the adhered bacteria, when comparing with the previous specimen. In addition, these results concurred with the findings of Chen, C. et al., where no correlation between surface roughness and *E. coli* bacterial adhesion was found (Chen et al., 2016).

Fig. 6(b) shows that *S. aureus* presents a higher propensity to adhere to the titanium control group (Ti), being the results statistically different from all the other zirconia specimens, except AS. Contrarily to *E. coli,* the micro-channels walls did not favour adhesion of *S. aureus*. Moreover, the etching treatment (MCE) led to an increased bacterial adhesion when comparing to the MC specimen, eventually due to the increased surface roughness.

Fig. 6(c) displays the *P. aeruginosa* results in which, similarly to *S. aureus,* this bacterium seems to adhere more to the SLA Ti specimen when comparing to zirconia surfaces. However, no significant differences are observed between the control group and MCE. When comparing zirconia specimens with and without micro-channels it seems that *P. aeruginosa* shows a higher adherence to channelled surfaces, which may be related to factors inherent to the machining process or the increased exposed area.

Literature reports contradictory outcomes when discussing the influence of surface roughness on bacterial adhesion. While some studies found that there is a clear relationship between these two parameters (Medilanski et al., 2002; Arnold and Bailey, 2000), others did not observe such a correlation (Chen et al., 2016; Verran and Boyd, 2001). In fact, and according to the results found in the present study, no correlation can be established. This lack of consensus regarding the ideal surface roughness is observed in different implant parts, from the implant itself to the implant abutment. Pesce P. et al. in a systematic review mentioned that some authors are in favour of rougher abutment surfaces since they improve the creation of the connective tissue and reduce the epithelial down-growth into the bone-implant interface; on the other hand, others claim that smoother surfaces are expected to reduce plaque formation and, consequently, reduce the risk of

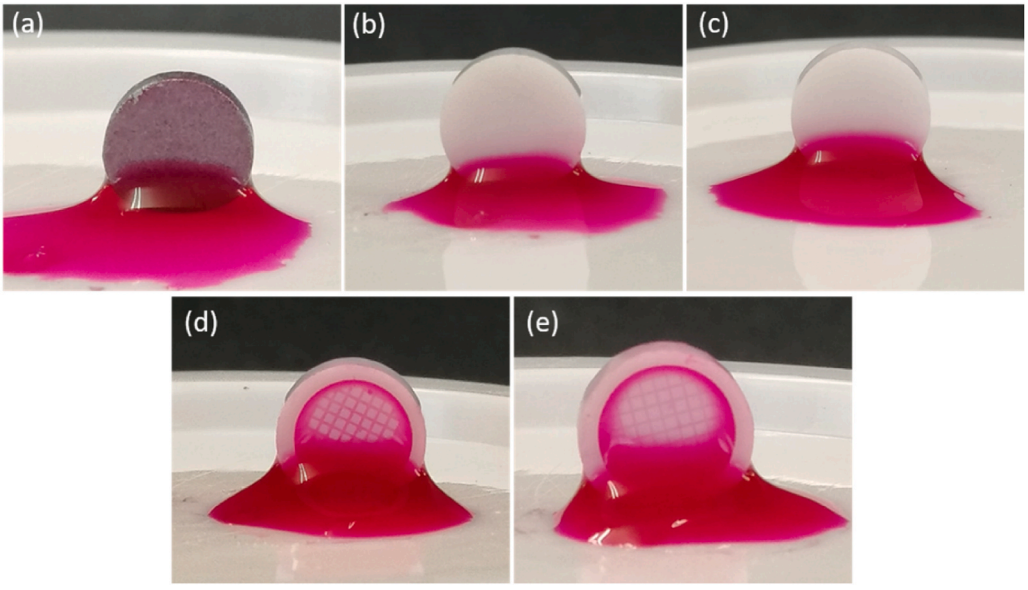

Fig. 5. Representative images of the fluid rise in the capillarity tests: (a) Ti; (b) AS; (c) SbE; (d) MC; (e) MCE.

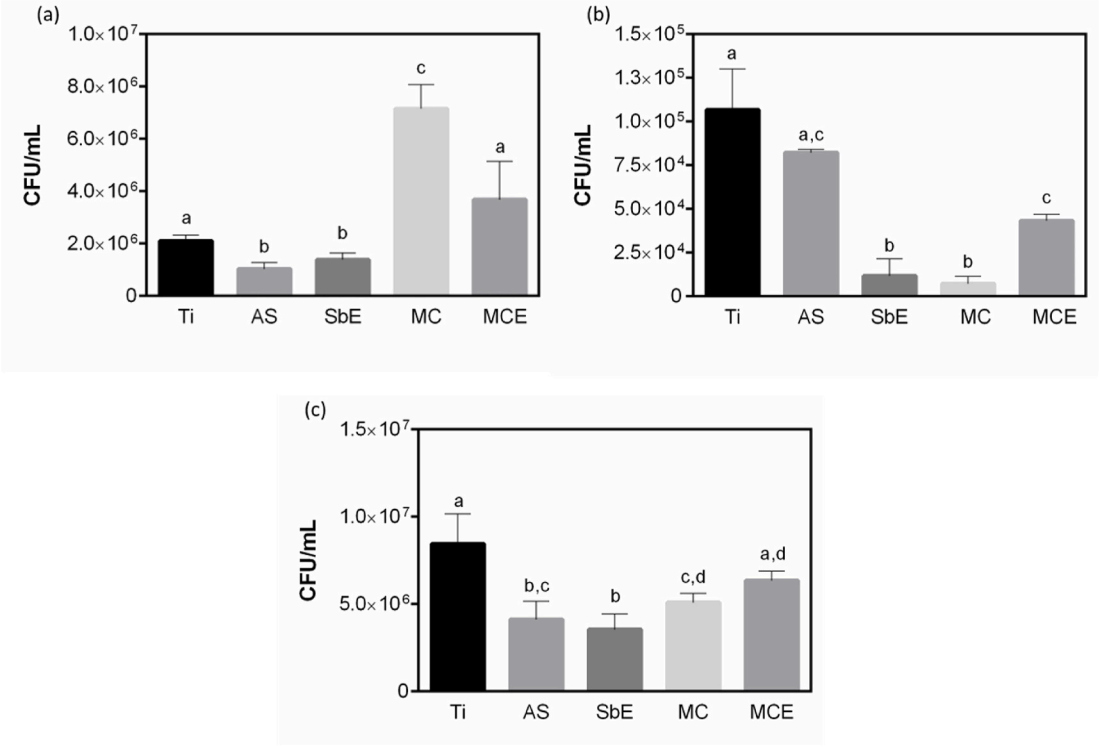

Fig. 6. Bacterial adhesion (CFU/mL) on the tested surfaces: (a) *Escherichia coli*; (b) *Staphylococcus aureus*; (c) *Pseudomonas aeruginosa*. The error bars represent the standard deviation. Different letters between distinct columns denote significant differences using one-way ANOVA with Tukey's multiple comparison test ($p < 0.05$).

periimplantitis (Pesce et al., 2019). Many factors can contribute to the different levels of bacterial adhesion apart from its surface roughness and material. Physiological differences between the tested bacteria, the surface physicochemical properties, and surface wettability are some examples (Cheng et al., 2019), and further studies should be carried out in order to better understand these results.

b. Co-culture of *S. aureus* and *P. aeruginosa*

S. aureus and *P. aeruginosa* are the most common bacteria present in chronic wounds and their virulence and surface proteins have been proved to affect wound healing, namely around dental implants. When these two bacteria co-exist in the same environment, their virulence and antibiotic resistance are even more pronounced, making the infection control a real challenge (Serra et al., 2015). Therefore, the assessment of the influence of the interactions between these two bacteria on the adhesion to the studied specimens is paramount. Fig. 7 displays the adhesion of *S. aureus* and *P. aeruginosa* separately and in co-culture. When comparing *S. aureus* adhesion to the different tested surfaces alone and in co-culture with *P. aeruginosa* (Fig. 7(a)) it is possible to conclude that when in co-culture this bacterium shows significantly higher levels of adhesion for all tested conditions, which indicates that the presence of *P. aeruginosa* stimulates the growth and adhesion of *S. aureus* and eventually promoted biofilm formation. On the other hand, *P. aeruginosa* adhesion shows a slight decrease when this bacterium is in co-culture with *S. aureus* (Fig. 7(b)). These results suggest that the presence of *S. aureus* somehow inhibits the adhesion of *P. aeruginosa*, despite no significant differences were observed for the micro-channelled specimens (MC and MCE).

While there are several studies in the literature reporting the adhesion of these bacteria to implant surfaces inoculated separately, studies reporting the influence of their interaction on the adhesion to implants surfaces are scarce. In fact, most studies found in literature concerning the interactions between *S. aureus* and *P. aeruginosa* comprise studies regarding the biofilm physiology, the treatment of biofilm-related infectious diseases, and their susceptibility to antibiotics (Alves et al., 2018; Trizna et al., 2020). Alves P. and colleagues, for instance, assessed the interaction between *S. aureus* and *P. aeruginosa* in biofilm cultured for 24–72 h, and bacterial aggregates analogous to those observed in early biofilm formation, and interaction with human keratinocytes (Alves et al., 2018). Results showed that *S. aureus* predominated in biofilm and non-attached bacterial aggregates, acting as a

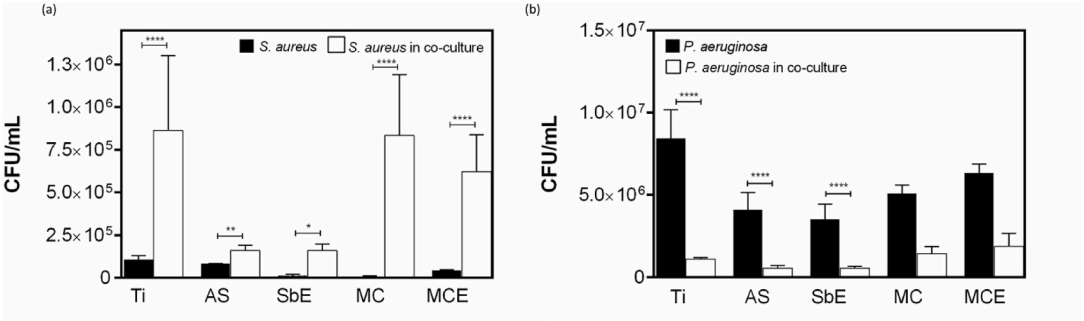

Fig. 7. Bacterial adhesion (CFU/mL) in co-culture: (a) *Staphylococcus aureus*; (b) *Pseudomonas aeruginosa.* The error bars represent the standard deviation and (*) indicates statistically significant differences ($p < 0.05$).

pioneer for the attachment of *P. aeruginosa*. The *in vivo* and *in vitro* interactions between *P. aeruginosa* and *S. aureus* were analysed by Hotterbeekx A. and co-workers and, among other conclusions, authors found that *S. aureus* showed an increased virulence in the presence of *P. aeruginosa* (Hotterbeekx et al., 2017). Authors also state that certain proteins secreted by *S. aureus* inhibit the surface attachment of some *P. aeruginosa* clinical isolates.

Wijesinghe G. and colleagues evaluated the influence of the culture media on *in vitro* growth, adhesion, and biofilm formation of *S. aureus* and *P. aeruginosa* (Wijesinghe et al., 2019). Results depict the huge impact of nutrient composition of the culture media on the biofilm and planktonic growth kinetics of the two bacteria, both in mono and co-culture. Furthermore, the same authors found that *P. aeruginosa* facilitates the microcolony formation of *S. aureus* and outcompetes *S. aureus* in co-culture biofilms. Yang L. and co-workers also evaluated the interactions between these two bacteria and concluded that the extracellular polymeric substances (EPS) secreted by the bacteria when in co-culture facilitates the interspecies interactions by the formation of mixed compact microcolony structures during biofilm formation (Yang et al., 2011). The authors also stated that when in co-culture *P. aeruginosa* is able to protect *S. aureus* from phagocytosis.

To better understand these interactions and the propensity for biofilm formation, specimens with adhered bacteria were inspected by scanning electron microscopy and results can be observed in the next section.

c. Scanning electron microscopy analysis

In Fig. 8 it is possible to observe the SEM images of the adhered bacteria for two different specimens: Ti and MC. Fig. 8(a) and (b) correspond to *E. coli* adhered to Ti and MC, respectively. The surface topography of the Ti specimen makes it difficult to clearly observe the adhered bacteria. On the other hand, on the zirconia micro-channel, it is easily detectable a great amount of adhered *Escherichia coli,* both in the bottom and walls of the channel. These results are in accordance with the bacterial adhesion count (Fig. 6(a)), where this bacterium showed a high value of CFU/mL for this specimen condition. As observed in Fig. 8(b), the irregular topography of the channel walls seems to facilitate *E. coli* adhesion. In Fig. 8(c) and (d), it is observed *S. aureus* adhered to the specimens. Again, it is perceivable the zirconia surface, due to its smoother nature. It is even possible to observe some biofilm formation, characterized by small groups of microorganisms that stick to each other.

Fig. 8(e) and (f), in turn, refer to the adhesion of *P. aeruginosa* to the tested surfaces. Despite being very difficult to detect this bacterium by SEM, after a careful analysis, it was possible to observe some bacteria in both specimens, with no significant differences between them. Authors believe that this type of bacterium adheres more to irregular surfaces and may, probably, be hidden beneath the asperities of the Ti surface.

In Fig. 9 it is shown SEM micrographs of the adhered bacteria when in co-culture (*S. aureus* and *P. aeruginosa)* for the Ti specimen (Fig. 9(a)) and for the zirconia specimen with micro-channels – MC (Fig. 9(b)).

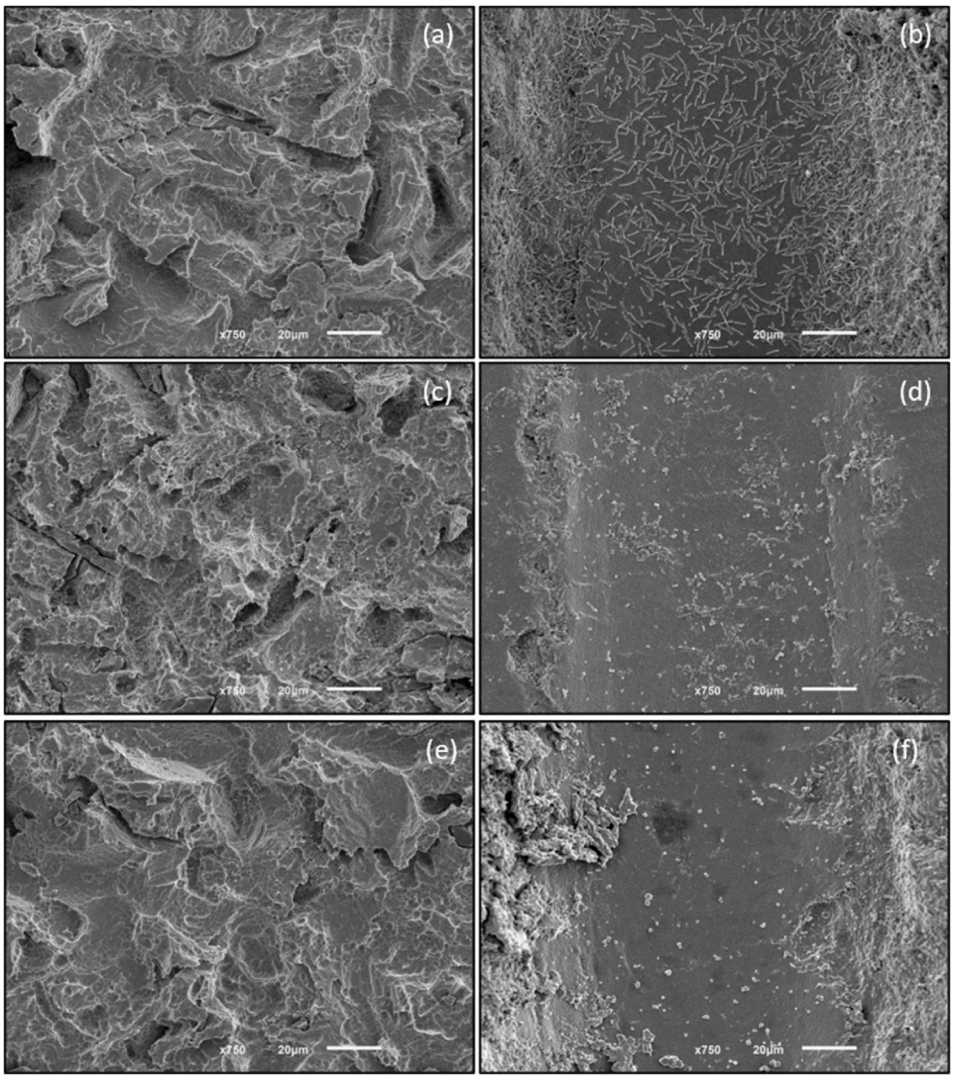

Fig. 8. Representative micrographs of the adhered bacteria: (a) and (b) *E. coli* on Ti and MC, respectively; (c) and (d) *S. aureus* on Ti and MC, respectively; (e) and (f) *P. aeruginosa* on Ti and MC, respectively.

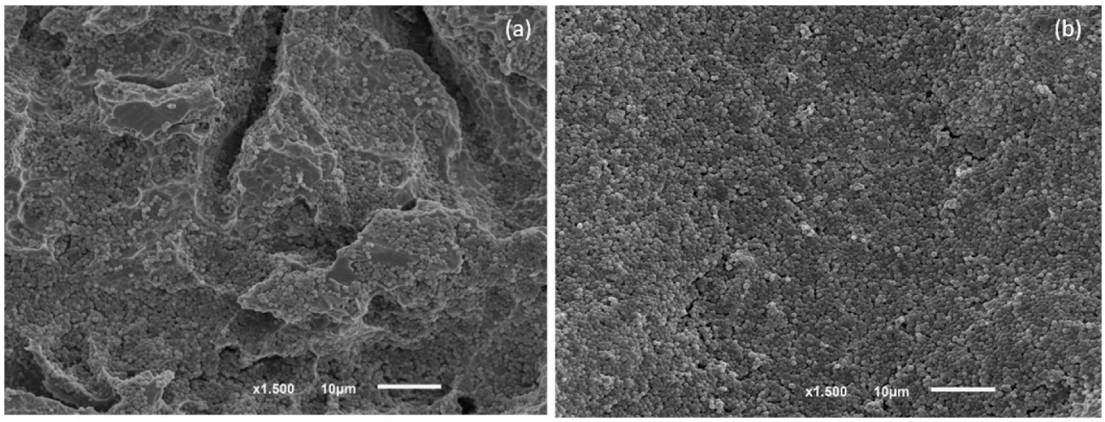

Fig. 9. Representative micrographs of the adhered bacteria in co-culture: (a) Ti; (b) MC.

As expected, and as observed in Figs. 7 and 9, *S. aureus* in co-culture revealed a considerably higher number of adhered bacteria, when comparing to the mono-culture. In fact, when analysing the previous images, it is possible to conclude that both surfaces are completely covered by this bacterium, which corroborates the findings of other authors mentioned before. There are several factors that may be on the basis of these results: the increased virulence of the *S. aureus* when in co-culture with *P. aeruginosa*, the presence of EPS, as well as the fact that *P. aeruginosa* may have facilitated the formation of *S. aureus* microcolonies.

On the other hand, and despite being very difficult to detect *P. aeruginosa* in the previous micrographs, according to Fig. 7, the CFU/mL of each bacterium in co-culture are very similar. This suggests that *P. aeruginosa* may have adhered to the surfaces and be hidden underneath *S. aureus*, making it difficult to detect them in the previous images.

4. Conclusions

Different zirconia surface architectures were successfully produced and tested regarding their bacterial adhesion propensity. Surface roughness and wettability of the zirconia specimens were also assessed. SLA titanium was used as control material, due to its common use in conventional dental implants. The present study supports several conclusions:

- All specimens presented a hydrophilic behaviour, in which an increased surface roughness (Ti, SbE and MCE) led to higher WCA, when comparing to smooth surfaces (AS and MC). After 10 s, WCA decreased substantially for all the tested conditions.
- *E. coli* exhibited a clear preference to adhere to the MC specimen. This behaviour seems to be related to the bacteria shape and charge, as well as the rough topography of the micro-channels walls. Nevertheless, changing the surface with an acid etching treatment considerably reduced *E. coli* adhesion. In addition, this bacterium is not very often found in the mouth environment and was used in this study mainly for comparison purposes, since it is a common model for Gram negative bacterium.
- *S. aureus* revealed a higher propensity to adhere to the SLA Ti specimen. Contrarily to *E. coli*, when a surface treatment is applied to the zirconia channelled specimen, *S. aureus* adhesion increased. This seems to indicate that *S. aureus* prefers rougher surfaces.
- *P. aeruginosa* exhibited an increased adhesion to the Ti control group. When comparing between different zirconia specimens, this bacterium adhered more to channelled surfaces (specially MCE), which may be related to the machining process and surface roughness.
- When in co-culture, the presence of *P. aeruginosa* seems to stimulate the adhesion of *S. aureus* and eventually promote biofilm formation. This is evidenced by the significant increase of bacterial adhesion for all tested conditions (in comparison with the mono-culture). On the other hand, *S. aureus* seems to prevent an extensive adhesion of *P. aeruginosa*.
- Channelled zirconia surfaces did not reveal particular better performance regarding the bacteria adhesion when comparing to the other tested specimens. However, these structures are expected to promote an improved vascularization around the implants and consequent enhanced osseointegration, evidenced by the capillarity analysis. Therefore, combining this effect with the quite satisfactory results found in this research work, channelled surfaces seem to be a promising alternative for dental implants applications.

Given the results obtained in the scope of this study, it is clear that the adhesion of bacteria to a specific surface is a multifactorial mechanism. The interactions between the bacteria and the surface seem to be influenced by the bacteria present, the surface material and roughness, as well as the environment around them, namely the presence or not of other bacteria. This is the major limitation of the present study: no correlation can be established between the adhesion of the different tested bacteria on the developed surfaces. Future studies will comprise 3D dental implants and bacterial communities to assess their efficacy to mitigate bacterial adhesion.

Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

Author statement

Telma Dantas: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Visualization, Funding acquisition.

Jorge Padrão: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Review & Editing, Visualization.

Mariana Rodrigues da Silva: Methodology, Investigation, Writing - Review & Editing.

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Sara Madeira: Methodology, Investigation, Writing - Review & Editing.

Paula Vaz: Supervision.

Andrea Zille: Conceptualization, Methodology, Validation, Writing - Review & Editing, Visualization, Supervision.

Filipe Silva: Conceptualization, Methodology, Validation, Writing - Review & Editing, Visualization, Supervision Funding acquisition, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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