UNIVERSITÀ DEL PIEMONTE ORIENTALE

SCHOOL OF MEDICINE

# MASTER DEGREE IN MEDICAL BIOTECHNOLOGIES

Master Thesis

## Gallium-doped Zirconia Coatings Modulate Microbiological Outcomes in Dental Implant Surfaces


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# ABSTRACT

Despite the considerable advancements in manufacturing materials that support advanced therapies, peri-implantitis remains a significant complication.

In this study, we utilized a sol-gel process to effortlessly create antibacterial zirconia coatings on bulk zirconia, which is increasingly favored for dental implant abutments. The physical and chemical properties of the coatings were analyzed using XRD and SEM-EDS investigations, the stability and wettability of the coatings were assessed through scratch resistance and static contact angle measurements.

We successfully obtained uniform tetragonal zirconia coatings doped with gallium, which exhibited optimal mechanical stability and hydrophilic behavior. Biological investigations included cytocompatibility assessment towards human gingival fibroblasts (HGF), antibacterial properties evaluation against a pathogenic bacterial strain (*A. actinomycetemcomitans*) and against a commensal bacterial strains (*S. salivarius*). Moreover, the materials in exam have been put in contact with an oral plaque collected from 3 healthy volunteers, in order to evaluate a possible shift in oral microbiota. Results revealed cytocompatibility of the materials, as well as an antibacterial effect toward the pathogenic bacteria taken into consideration. Furthermore, the viability of the commensal was preserved.

Additionally, proteomic analyses revealed that the presence of gallium did not disrupt the normal oral microbiota. Interestingly, it resulted in a 17% decrease in the presence of *Fusobacterium nucleatum*, a Gram-negative, strictly anaerobic bacteria naturally found in the gastrointestinal tract.

Overall, this approach represents a promising starting point for developing coatings that can easily enhance the performance of zirconia dental Despite significant advancements in manufacturing materials that support advanced therapies.

# INTRODUCTION

## 1.1 THE DEFINITION OF MICROBIOTA

The microbiota consists of a wide variety of bacteria, viruses, fungi, and other microorganisms present in a singular environment in a specific period of time, meanwhile, the microbiome is defined as the collective genetic information contained within the microbiota residing in and on the human body. [1]

This symbiotic relationship benefits microbes and their hosts as long as the body is in a healthy state and the composition of the human microbiota can greatly vary between people. Different parts of the body will also host different microbial communities, for example, the oral cavity, skin, gastrointestinal system and respiratory tract. [2]

Microorganisms are crucial to human life and carry out various vital functions. Indeed, they help train the immune system, help the digestion of nutrients, and fight systemic diseases. [3]

## 1.2 THE COMPOSITION OF ORAL MICROBIOTA

The oral cavity contains one of the most diverse and unique microbiota consisting of over 700 different species of bacteria but also includes fungi, viruses, archaea, and other microorganisms. The oral cavity encompasses various environments, such as the teeth, gingival sulcus, tongue, cheeks, saliva, and tonsils, each hosting distinct populations of microorganisms. These microbial communities in the mouth comprise a diverse array of bacterial species, both Gram-positive and Gram-negative, including both aerobic and anaerobic organisms. The advent of culture-independent techniques has contributed to a broader identification and categorization of microbial species within these communities. [4]

Modern methodologies like biomarker sequencing and shotgun metagenomics have become widely adopted in studying the human microbiome, allowing researchers to investigate its composition and genetic characteristics. Additionally, [5] other “omics” technologies, including proteomics and metabolomics, complement these approaches by providing insights into the underlying mechanisms and pathways linking microbial activity to various states of health and disease. [6-7]

Since Antonie van Leeuwenhoek first discovered the existence of microbes in the 1700s while analyzing dental plaque under a microscope, the composition of oral microbial communities has been extensively studied. [8]

More than 250 species originating from the oral cavity have been successfully studied in culture, among which *Fusobacterium nucleatum*, *Streptococcus mutans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Aggregatibacter actinomycetemcomitans*, known to contribute to the development of dental caries and periodontal disease. [9-10-11]

The unique conditions present in the oral cavity play a crucial role in determining which microbes can establish and persist in this environment. Selective pressures in the oral cavity foster distinct relationships between microorganisms and their host.

Moreover, the oral microbial ecosystem is consistently exposed to external substances from the environment. [12]

Among the initial colonizers of the oral cavity, pioneer microbes like *Streptococcus mitis*, *Streptococcus sanguinis*, *Streptococcus gordonii*, and *Streptococcus salivarius* possess specific characteristics that make them well-adapted to this particular niche. These microbes can selectively bind to cells on the tongue and cheeks even before the teeth emerge, giving them a competitive advantage over other microbial species. [13]

The composition of the oral microbiota can be influenced by various factors, including oral hygiene practices, diet, genetics, age, and systemic health conditions. The disruptions in the balance of the oral microbiota, referred to dysbiosis, can lead to oral diseases and infections. [14]

Understanding the oral microbiota and its interactions with the host can lead to the development of targeted interventions and treatments for oral diseases, as well as provide insights into the potential links between oral health and systemic conditions such as cardiovascular disease, diabetes, and respiratory infections. [14]

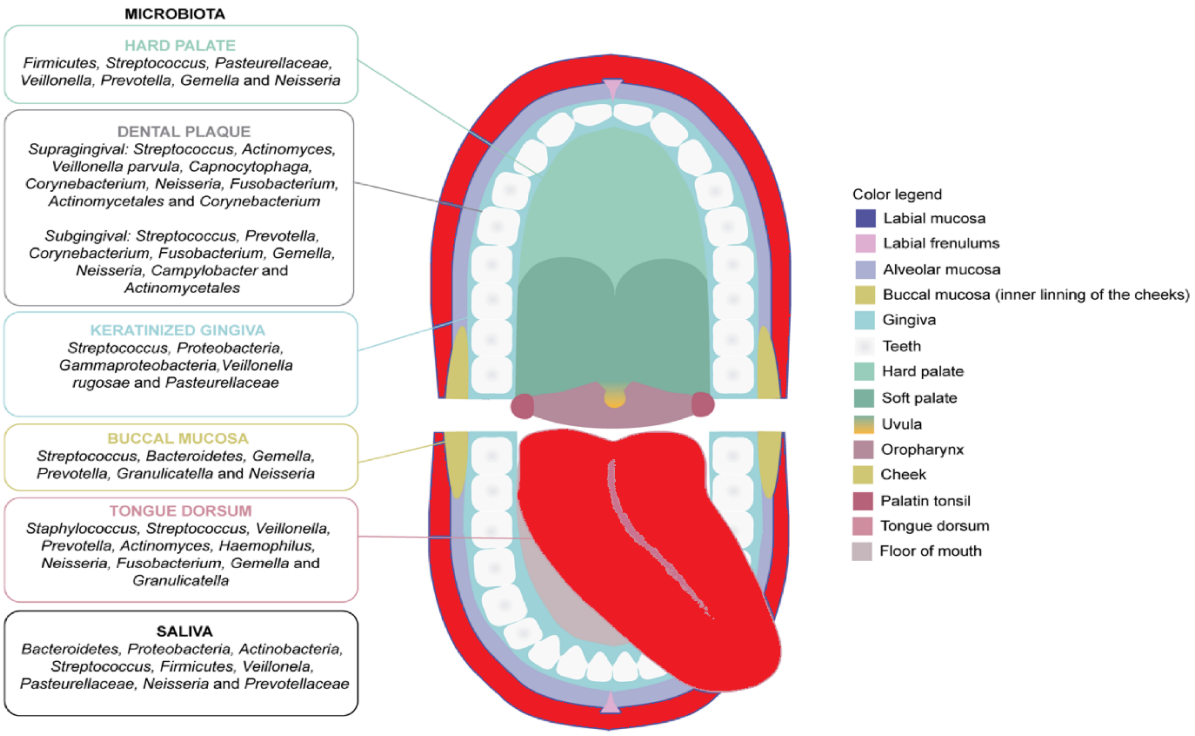


**Figure 1.** The picture shows the diverse microbiota populations that form the oral microbiota throughout the different oral cavity regions. [15]

## 1.3 GINGIVITIS AND PERIODONTITIS

The subgingival crevice, which is the space between the gums and the teeth, contains a diverse range of microbial communities. Changes in the composition of these communities are observed as gingivitis and periodontitis develop, representing stages of deteriorating periodontal health. [16]

Gingivitis is characterized by inflammation of the gum tissue, often caused by the accumulation of dental plaque, during this stage, there is an increase in the abundance of certain bacterial species, such as *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*. The increase in the quantity of these bacteria are considered to be associated with the shift from gingivitis to periodontitis and are commonly found in higher numbers in individuals with periodontitis. [17-18]

Gingivitis-associated microbial communities tend to be more diverse compared to those found in individuals with periodontitis. This suggests that as the disease progresses and dysbiosis occurs when a certain bacterial species become dominant and contribute to the pathogenesis of periodontitis. [19-20]

The shift in the composition of the oral microbiota community with the increase in the abundance of specific bacteria associating with the inflammation and the dominance of specific bacterial species in periodontitis suggests that they may play a significant role in tissue damage and disease progression. [21]

These bacteria have the potential to perturb the equilibrium of the oral microbial community, thereby initiating inflammation and tissue degradation within the periodontal tissues. Notably, the process of bone destruction commences from the depths of the gingival crevice, triggered by the inflammatory response that activates osteoclasts. As a consequence, the affected bone surrounding the tooth undergoes resorption, leading to diminished support for the tooth. Consequently, tooth mobility ensues due to the compromised integrity of the supporting periodontal tissues, ultimately culminating in tooth loss. [22]

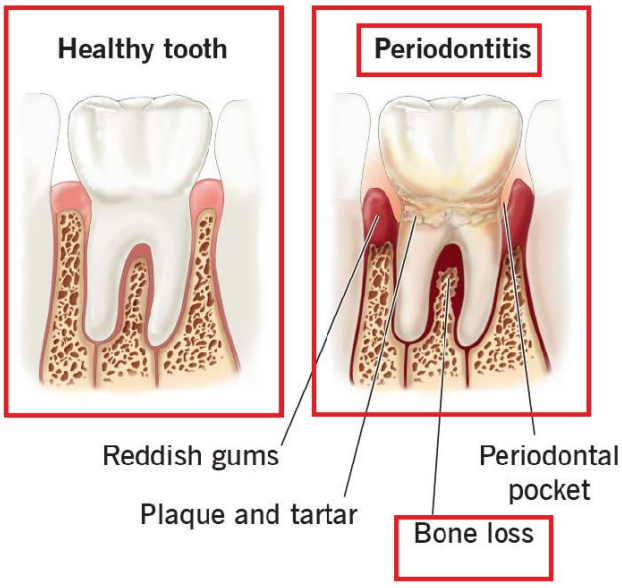


**Figure 2.** The picture shows the Plaque, bone loss and red, bleeding gums are all hallmark symptoms of periodontitis. [22]

## 1.4 IMPLANTS

The purpose of using dental implants is to restore the functional and esthetical properties by replacing the missing teeth. They provide a success rate of approximately 90- 95% over 10 years. They can serve as replacements for missing teeth by effectively replacing the roots of the teeth, in this process, the crown. Which is the visible part of the tooth, is then replaced by a prosthetic restoration. When a dental implant is placed, its surface directly interfaces with the bone, without the presence of a fibrous intermediary that is typically observed when foreign materials are introduced into an organism. [23]

This unique quality of a biomaterial is known as "Osteoconductivity" which essentially enables bone to develop in direct contact with the surface of a dental implant or a bone graft/substitute. Implants lost may be caused by peri-implantitis which has been linked to a Gram-negative anaerobic microbiota, which is similar to the microbiota found in cases of severe periodontitis around natural teeth. [25]

Peri-implantitis is characterized by the presence of peri-mucositis along with the loss of osseous support. While some degree of natural bone remodeling is expected after implant placement, if an implant shows ongoing changes in the level of bone support despite being stable, it is considered indicative of peri-implantitis. [25]

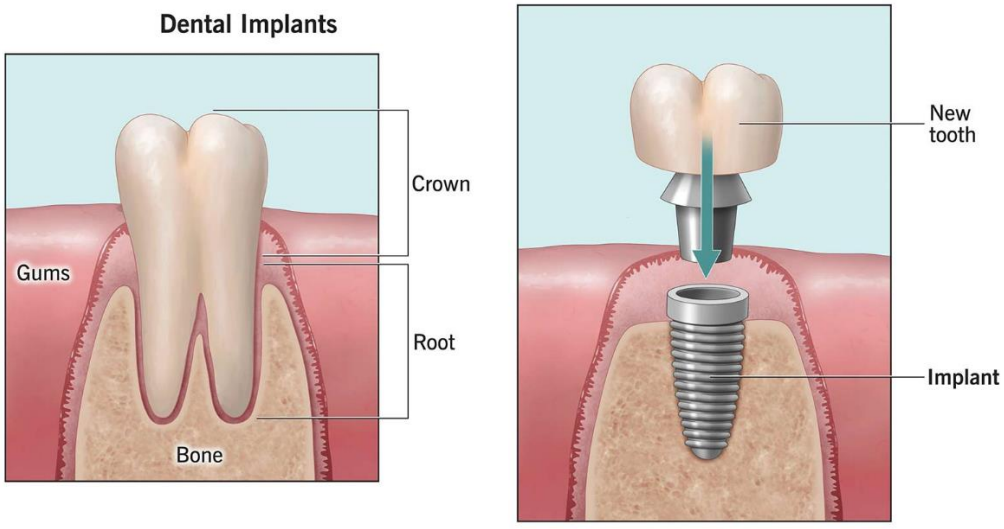


**Figure 3.** The picture displays a dental implant, and the crown on top to restore function and appearance. [26]

## 1.5 THE FORMATION OF BACTERIAL BIOFILMS ON THE SURFACE OF IMPLANTS.

Peri-implantitis can manifest at varying levels of severity, with approximately 10% of cases resulting in implant loss within a 5- year timeframe. A previous study has reported that peri-implantitis occurs in 11.3- 47.1%of dental implants. [27]

It is commonly associated with periodontal pathogens and is often treated with antibiotics. The primary causative factor of peri-implantitis is believed to be bacterial biofilm, which is a highly organized community of bacteria enclosed within a matrix, existing in a sessile state. Biofilm formation is a characteristic feature of bacterial growth, enabling them to evade host cells and compete with other microbial communities. [28]

The process of biofilm formation involves several steps:

(1) initial adhesion of planktonic bacteria to the surface;

(2) establishment of a stable attachment to the surface;

(3) Co-aggregation with other bacteria to enhance the structural integrity of the biofilm;

(4) nutrient absorption from the environment to support growth until maturation;

(5) detachment of portions of the biofilm to invade other susceptible sites. [28]

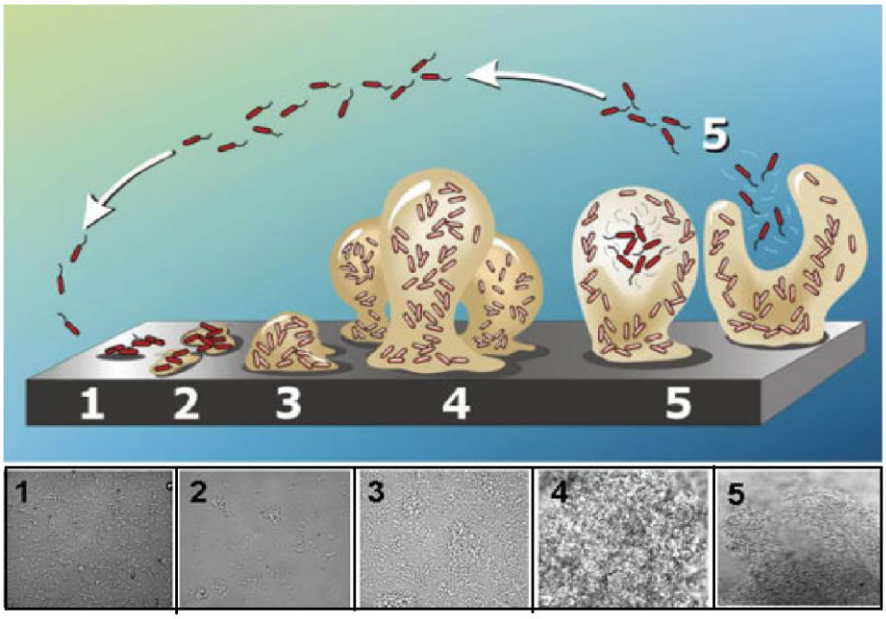


**Figure 4.** the picture shows the stages of Biofilm development. **(1)** initial adhesion **(2)** stable attachment **(3)** Co-aggregation **(4)** Growth **(5)** detachment and invading susceptible sites. [29]

Apart from surface roughness, the choice of materials and surface treatments also play a significant role in the initial attachment of bacteria. Hence, it is crucial to evaluate the influence of different implant surfaces on the attachment of both early and late colonizing bacteria. [30]

*S. aureus, P. gingivalis, S. sanguinis, and S. mutans* are known to be associated with peri-implantitis. They have been found on the surfaces of implants, they can also be present in the saliva of healthy adults and can transform into opportunistic pathogens, becoming the primary culprits behind various oral diseases. [31]

## 1.6 MATERIALS OF DENTAL IMPLANTS

Dental implant materials need to possess certain characteristics to ensure compatibility with the oral environment and provide long- term stability.

The most commonly used materials for dental implants include titanium and its alloys, zirconia, and ceramic materials.

### 1.6.1 TITANUIM IMPLANTS

The initial documentation of commercially pure titanium for medical purposes occurred in 1940, illustrating its remarkable bone compatibility in an animal trial. [32]

Subsequently, studies highlighted its compatibility with both bone and soft tissue in rabbits, its non-cytotoxicity attributed to exceptional corrosion resistance in biological settings, and its outstanding biocompatibility. By the latter part of the 1940s, a large-scale industrial production process for titanium was established, facilitating extensive research for medical use due to its favorable biocompatibility evident in long-term animal assessments. Consequently, the efficacy of commercially pure titanium became widely acknowledged by the latter part of the 1960s, validated through clinical assessments and it has been effectively utilized in dentistry as dental implants since 1965. [33]

But over the years, the use of materials from the titanium family for abutments has been a subject of debate, primarily because of the unsightly grey coloration that tends to develop around the soft tissues, to address this aesthetic concern other materials such as Alumina and Zirconia have been considered to be used. [34]

The difference between titanium grades 1 to 5 lies in their composition, mechanical properties, and intended applications. Here's a breakdown of the key characteristics: [35]

Titanium Grade 1 is the purest form of titanium, containing the least amount of alloying elements. It possesses low stress-to-strain ratio and low temperature tolerance. For those reasons an alternative alloys of titanium have been developed in order to pass those limitations. Titanium Grade 2 is also relatively pure, with slightly higher oxygen content compared to Grade 1. It offers higher corrosion resistance, and higher stress-to-strain ratio.

Grade 5 titanium (Ti-6Al-4V) which is an alloy composed of titanium, aluminum, and vanadium. It exhibits high strength, good corrosion resistance, and excellent biocompatibility. It is widely used in medical implants. [36]

## 1.6.2 ALUMINA IMPLANTS

Alumina, also known as aluminum oxide, is a widely used material in the production of ceramic products and components, it possesses desirable characteristics such as hardness, high temperature resistance, and low electrical conductivity, due to its high density, alumina is commonly used as a refractory material. [37]

When used as a micrometer grain size material, it exhibits excellent wear resistance and insulation properties. Instead of directly bonding with the host tissue, alumina implants are encapsulated by a thin layer of fibrous tissue after being implanted. Alumina with a purity level greater than 99.5% is particularly valued for implant applications due to its biocompatibility with adjacent tissue, favorable wear and friction properties, and aesthetic appeal. [37]

*In vitro* biocompatibility tests conducted that alumina exhibited the highest cell growth percentage of 93.05% and demonstrated to be not cytotoxic. Furthermore, alumina implants have good aesthetic characteristics as their color matches that of natural teeth. [38]

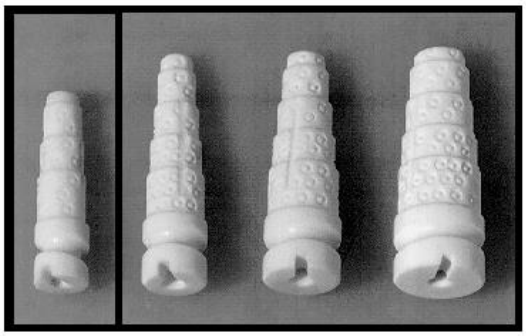

**Figure 5.** The image shows aluminum oxide implants with specific micro-irregularities on the surface. [39]

## 1.6.3 ZIRCONIA IMPLANTS

Zirconia dental implants have emerged as a newer option in dentistry and have gained popularity in recent years. They are made from a synthetic material called zirconia, which has been used in dentistry since the 1990s. One of the advantages of zirconia implants is that they are suitable for individuals with metal allergies or those who may experience difficulties with titanium implants. [38]

Zirconia implants can be customized to match the color of natural teeth, offering better aesthetic results compared to traditional materials like Titanium. The use of Zirconia in dentistry offers several advantages:

Zirconia exhibits excellent chemical stability and possesses high mechanical properties. It can promote ''osteogenesis'' which is referred as the formation of new bone with the implant surface, enhancing the stability and longevity of the implant. [40]

Zirconia implants improve aesthetics in the area surrounding the gingival soft tissues. This is particularly important in dental procedures where a natural and esthetically pleasing appearance is desired. Zirconia naturally reduces bacterial contamination, while the antimicrobial properties of zirconia may vary depending on the bacterial species, [41] *In vitro* studies have generally shown that zirconia is superior to titanium in inhibiting bacterial adhesion. However, it has also been demonstrated that *S. mutans*, a bacterium associated with dental caries, can better colonize polished zirconia compared to titanium. [42-43]

*In vivo* studies provide more consistent results, showing a decrease in bacterial adhesion and colonization on zirconia compared to titanium implants, this is beneficial considering that dental materials come into contact with numerous bacterial species, both pathogenic and commensal, from the oral microbiota. [44]

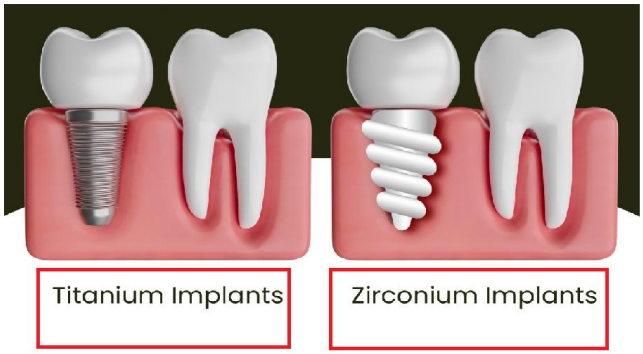


**Figure 6.** The figure shows two types of implants: titanium implant and zirconium implant. [45]

## 1.7 DENTAL IMPLANTS COATING

To address the presence of pathogenic bacteria while maintaining the commensal species, it is crucial to functionalize the material. Functionalization techniques can be employed to reduce the presence of pathogenic bacteria and create a more favorable environment for the growth of beneficial bacteria, enhancing the overall oral health of the patient. Numerous systematic coating techniques have been created to effectively address pathogenic activities, providing outstanding outcomes in terms of infection control and promoting bone integration. [46]

The utilization of inorganic ions (such as $Ag^{+}$, $Cu^{2+}$, and $Zn^{2+}$) as coatings for various materials continues to be highly effective. However, due to their non-specific action and ability to target cell membranes, ribosomes, and genetic material, their impact can be unpredictable and may result in unintended consequences. This broad-spectrum activity can disrupt the natural balance of oral microorganisms, leading to oral dysbiosis and creating favorable conditions for the growth of pathogenic bacteria. [47-48]

To address these challenges, gallium ($Ga^{3+}$) has emerged as a promising alternative. it has gained attention due to its ability to mimic the behavior of iron ions ($Fe^{3+}$) and interfere with bacterial metabolism. Interestingly, $Ga^{3+}$ shares many similarities with $Fe^{3+}$ in terms of charge, ionic radius, coordination number, and electronic configuration. These similarities enable Ga3+ to effectively interact with bacterial cells, disrupting their normal functions. [49-50]

However, it is important to note that $Ga^{3+}$ cannot be reduced to a lower oxidation state like $Fe^{3+}$. This characteristic has a significant impact on bacterial biology as it hinders the function of siderophores, which are small, high-affinity iron-chelating compounds that secreted by

microorganisms and they are essential for the acquisition of iron by bacteria. By blocking the activity of siderophores, $Ga^{3+}$ impairs several crucial bacterial functions, further contributing to its antibacterial effects. [51]

In the context of implant dentistry, surface modification of implants through the application of coatings represents an optimal approach. This strategy allows for the enhancement of essential features such as biocompatibility and osseointegration of the implant materials. Moreover, the incorporation of antibacterial properties into the coatings provides an additional layer of protection against implant failure. [52-53-54-55-56]

By introducing gallium-doped coatings, implants can exhibit improved biocompatibility, successful integration with surrounding bone tissue, and the ability to combat bacterial colonization, thereby reducing the risk of complications.

The effectiveness of doped $ZrO_2$ coatings with calcium ions ($Ca^{2+}$) or gallium ions ($Ga^{3+}$) in promoting the proliferation of Saos-2 human osteoblastic cells and exhibiting antibacterial properties against oral pathogens such as *Porphyromonas gingivalis* and *A. actinomycetemcomitans* has been scientifically validated. [57]

Despite the increasing popularity of zirconia in implant dentistry there are limited studies on surface modifications of this material using the sol-gel approach which is a cheap and low-temperature technique that allows the fine control of the product's chemical composition this allow to mitigate bacterial colonization and reduce the risk of peri-implantitis. [58-59]

# THE AIM OF THE STUDY

The study aims to demonstrate that gallium-doped zirconia coatings serve as a promising foundation for enhancing zirconia dental implant performance. This is achieved through several key evaluations, including testing the cytocompatibility of gallium towards human gingival fibroblasts (HGF), assessing its antimicrobial activity against the *A. actinomycetemcomitans* pathogen and examining its capacity to preserve the commensal *S. salivarius* and maintain the normal oral microbiota.

# MATERIALS AND METHODS

## 2.1 MATERIALS

In our study, the materials have been provided by the department of materials chemistry and chemical engineering of Politecnico of Milano. The chemicals used, including Zirconium chloride (ZrCl4), ethanol (EtOH), Pluronic F-127, and Gallium (Ga3+) nitrate hydrate (Ga(NO3)3•H2O), were purchased from Merck (Germany) without purification. Millipore water was used for preparing aqueous solutions. Polycrystalline zirconia discs, obtained from Zirkonzahn GmbH (Gais, Italy), with dimensions of 1 mm thickness and 15 mm diameter, were employed as substrates for the coatings. Prior to functionalization, the disc surfaces were sterilized using ethanol. Tested materials included: Zirconia discs (Z) and Zirconia discs doped with Gallium (ZGa).

## 2.2 IN VITRO CYTOCOMPATIBILITY EVALUATION

Prior to conducting biological assays, the zirconia coated discs (Z and ZGa samples) underwent sterilization under UV light. Each side of the discs was exposed to UV-C light for a duration of 30 minutes.

For the assessment of cytocompatibility, primary human gingival fibroblasts (HGF) obtained from the American Type Culture Collection (ATCC CRL2014) were used. The cells were cultured in alpha-modified Minimal Essential Medium (α-MEM) from Merck, supplemented with 10% fetal bovine serum (FBS) from Lonza (Basel, Switzerland) and 1% Penicillin/Streptomycin (PS, Lonza). The cells were maintained at 37 °C in a 5% $CO_2$ atmosphere with 95% humidity. Once the cell confluence reached 80-85%, the cells were detached using a trypsin-EDTA solution (0.25%), harvested, and utilized for the *in vitro* experiments.

To assess cytocompatibility, the following procedures were carried out according to the manufacturer's instructions. Cell viability was determined using the Alamar blue assay (Thermo-Scientific, AlamarBlue) based on metabolic activity. The assay involves the conversion of the blue, non-fluorescent molecule Resazurin in the violet and fluorescent molecule Resorufin.

The conversion of Resazurin to Resorufin is based on a redox reaction. Metabolically active cells possess enzymes, such as mitochondrial reductases, which can reduce Resazurin. These enzymes

transfer electrons from cellular metabolic processes to Resazurin, resulting in its reduction and the formation of Resorufin.

The fluorescence can be detected and quantified using appropriate fluorescence measurement techniques.

Sterile specimens (Z and ZGa) were placed individually into a sterile 12-multiwell plate. A specific number of human gingival fibroblasts (HGFs) amounting to $3.5 \times 10^4$ cells per specimen were dropwise spotted directly onto the surface of each specimen. The plate was then incubated for 3-4 hours to allow for cell adhesion and spreading. Subsequently, each well was rinsed with 1 ml of complete medium, and the cells were cultivated for 72 hours at 37 °C in a 5% $CO^2$ environment.

Following the incubation period, the Alamar blue solution (0.015% in complete culture medium) was added to the wells, and the plate was kept in the dark at 37 °C for 4 hours. Afterward, 100 µl of the solution from each specimen was collected and transferred to a dark 96-well plate. Fluorescence signals were measured using a spectrophotometer (Tecan Trading AG, Mannedorf, Switzerland) with a fluorescence excitation wavelength of 530 nm and a fluorescence emission reading of 590 nm.

Furthermore, the adhesion, spread, and morphology of the cells cultivated on the specimen surfaces were assessed using immunofluorescence (IF) and scanning electron microscopy (SEM). For immunofluorescence, the cells were fixed in 4% paraformaldehyde in PBS for 1 hour at room temperature, washed twice with PBS, and permeabilized with 0.2% Triton X-100 on ice for 5 minutes. The samples were then incubated with Phalloidin-TRITC (1:500) and DAPI (1:1000) diluted in PBS containing 0.1% BSA for 45 minutes. After washing twice with PBS, the samples were observed under a fluorescent microscope (ThermoFisher, EvosFloid).

For scanning electron microscopy, the specimens were fixed in 2.5% glutaraldehyde for 1 hour at room temperature to preserve the structural integrity and morphology of biological specimens. It forms cross-links between proteins, essentially "freezing" the cellular structures in place and preventing degradation or distortion during subsequent processing and imaging, then the specimens were washed with PBS, and dehydrated using an increasing ethanol concentration series (50%, 70%, 90%, 100% for 1 hour each). Subsequently, the specimens were submerged in hexamethyldisilazane (HMDS) for 20 minutes. To visualize the specimens under the scanning electron microscope, they were coated with a 10 nm gold layer (SmartCoater, Jeol) and examined using a scanning electron microscope (Jeol, JSM-IT500 InTouchScope™).

## 2.3 ANTIBACTERIAL ACTIVITY EVALUATION

To evaluate the antibacterial activity of the materials, two bacterial strains were used: *A. actinomycetemcomitans* (ATCC 33384), a Gram-negative strain commonly associated with periodontal and peri-implant diseases, and *S. salivarius* (DSM 20067), a Gram-positive bacterium considered a commensal.

The bacteria were cultured on Tryptic soy agar plates (TSA, Merck, Darmstadt, Germany) and incubated at 37 °C until single colonies were formed. A few colonies were then collected and transferred into 15 ml of Tryptic soy broth (TSB, Sigma-Aldrich). The broth culture was incubated overnight at 37 °C with agitation (120 rpm). The following day, a fresh broth culture was prepared by diluting the bacteria in a fresh medium to achieve a final concentration of $1 \times 10^5$ bacteria/ml, corresponding to an optical density of 0.001 at a wavelength of 600 nm, as measured by spectrophotometry.

For the *in vitro* antibacterial test, the metabolic activity of the bacteria in contact with the functionalized materials was assessed using the colorimetric-metabolic assay Alamar blue, as described previously (0.0015% in PBS). Sterile specimens were placed individually into a sterile 12-multiwell plate. A volume of 300 µl of the bacterial suspension, containing $1 \times 10^5$ bacteria per specimen, prepared as described earlier, was brought into contact with the surface of each material specimen.

## 2.4 THE EFFECT OF THE MATERIAL ON ORAL PLAQUE

Once the antibacterial properties of the material have been assessed, the specimens were exposed to oral plaque to assess the selective ability of gallium in killing pathogenic bacteria while preserving commensal ones.

Samples of oral plaque were collected from three healthy volunteers using non-invasive procedures with their informed consent obtained under the Declaration of Helsinki. Oral plaque samples were obtained from the supragingival areas of premolars or molars using individual sterile Gracey curettes and gentle scraping. After collection, the samples were pooled and placed in sterile cooked meat culture broth (Merck, Darmstadt, Germany). The microorganisms present in the plaque were dispersed by vortexing and transferred to 30 ml of fresh media. The samples were then maintained under anaerobic conditions using a Baker Ruskinn Concept 400 Workstation to allow the growth of the oral microbial species. After 24 hours, the bacterial

community within the oral plaque was frozen and stored at -80 °C to preserve the initial population.

To assess the effect of gallium on oral plaque, the previously described oral plaque samples were submerged in 500 µl of a bacterial suspension containing $1 \times 10^5$ bacterial cells, adjusted based on the optical density at 600 nm. The samples were then incubated under anaerobic conditions for 24 hours. Following incubation, the bacterial biofilm derived from both samples (with and without gallium) was washed once with a PBS solution to remove unattached bacterial cells. Samples were transferred in an appropriate tube, submerged with an appropriate volume of sterile PBS and then sonicated three times, with each sonication lasting 5 minutes followed by 30 seconds of vortexing. The solution containing the detached biofilm bacteria was transferred to a new sterile test tube and centrifuged at 10,000 rpm for 10 minutes.

To extract proteins from the bacterial biofilm, 200 µl of lysis buffer was added to all the samples obtained in the previous section. The lysis buffer consisted of an 8 M urea buffer (pH 8.5) and Tris-HCl. To ensure complete release of bacterial cell proteins, the samples were sonicated six times, with each sonication step at 27% amplitude for 10 seconds followed by 10 seconds on ice.

After protein extraction, the protein concentration was determined using the Bradford reagent (Sigma-Aldrich). A volume of the protein samples corresponding to 80 µg (a selected threshold concentration) was mixed with 25 µl of 100 mM ammonium bicarbonate (NH4HCO3).

To initiate protein reduction, 15 µl of trifluoroethanol (TFE, 99%) and 2.5 µl of dithiothreitol (200 mM DTT stock solution) (Sigma-Aldrich) were added to the protein samples, and the mixture was incubated at 60 °C for 30 minutes.

Next, the proteins were alkylated by adding 10 µl of cysteine blocking reagent (Iodoacetamide, IAM, 200 mM; Sigma-Aldrich) and incubating the mixture at room temperature in the dark for 30 minutes. The proteins were then digested with trypsin (Promega, Sequence Grade) overnight at 37 °C. Trypsin activity was stopped by adding 2 µl of neat formic acid, and the digests were dried using a speed vacuum.

Additionally, the surfaces of the materials contaminated with bacteria and the features and development of the bacterial biofilm were examined using scanning electron microscopy (SEM), as described earlier.

## 2.5 PROTEOMICS ANALYSIS

The proteomics analysis has been done by biological mass spectrometry laboratory headed by professor Marcello Manfredi.

In order to investigate the impact of gallium on the oral biofilm collected from healthy volunteers, a proteomics analysis was conducted using a well-established methodology. [60] The protein samples prepared in the previous section were used for this analysis.

The digested peptides were analyzed using an Ultra-High-Performance Liquid Chromatography (UHPLC) system (Vanquish, Thermo Scientific, Rodano, Italy) coupled with an Orbitrap QExactive Plus mass spectrometer (Thermo Scientific, Rodano, Italy). A reverse phase column (Accucore™ RP-MS, 100 x 2.1 mm, particle size 2.6 µm) was used to separate the peptides. The column was maintained at a constant temperature of 40 °C, and the peptides were eluted at a flow rate of 0.2 mL/min.

For mass spectrometry analysis, Mascot software version 2.4 (Matrix Science Inc., Boston, USA) was used. Trypsin was selected as the digestion enzyme with allowance for 2 missed cleavages. A peptide mass tolerance of 10 ppm and an MS/MS tolerance of 0.1 Da were specified. The charges of the peptides to search for were set at 2+, 3+, and 4+, and the search was conducted using monoisotopic mass. The search included carbamidomethyl cysteines as fixed modifications and oxidized methionine as a variable modification.

The Human Oral Microbiome Database V3 was utilized, and a target-decoy database search was performed. The false discovery rate (FDR) was set at 1%. To map the peptides to their respective taxa of origin, Unipept software was employed. [61-62]

## 2.6 STATISTICAL ANAYLSIS OF THE DATA

The statistical analysis was conducted using GraphPad version 6 software (GraphPad Software, CA, USA). The in vitro cytocompatibility and antibacterial activity evaluations were performed in triplicate, while the assessment of contact with oral plaque was conducted using six samples.

To compare the groups, a one-way analysis of variance (ANOVA) was performed, followed by Tukey's test as a post-hoc analysis. Significant differences were considered when $p < 0.05$.

# RESULT AND DISSCUSSION

The physiochemical and morphological characterizations of the zirconia coatings were tested by department of materials chemistry and chemical engineering of Politecnico of Milano, including coating the zirconia discs with gallium using sol-gel technique that enables the equal distribution of gallium on zirconia discs.

Also the Mechanical stability and wettability properties have bene tested also by department of materials chemistry and chemical engineering of Politecnico of Milano, indicating optimal coating stability and decrease of contact angle values and hydrophobic behavior.

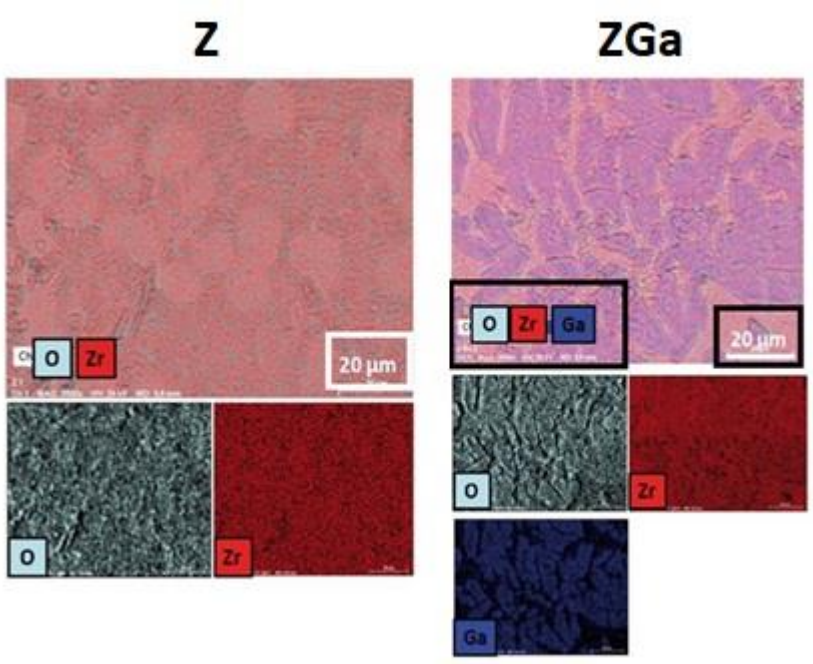


**Figure 7.** The pictures show SEM-EDS observation of Z and ZGa samples, including the elements mapping of oxygen and zirconium and gallium

In figure 7, the coated zirconia disc (Z) and Ga- doped zirconia coatings (ZGa sample) was subjected to micro-morphological characterization using scanning electron microscopy (SEM). The SEM analysis involved the use of secondary electrons (SE) and backscattered electrons (BSE) to capture micrographs of the sol-gel coating morphology on the Z and ZGa specimens.

The micrographs obtained from the SE and BSE imaging techniques revealed a uniform and consistent sol-gel coating morphology on the Z specimens. This also support that the sol-gel spin-coating method employed in the study resulted in a homogeneous and well-adhered coating on the surface of the zirconia disc.

In ZGa specimens, the observed compositional contrast in the BSE image is attributed to the presence of an element with a lower atomic number in the coating. This element generates fewer backscattering electrons. The EDS spectrum analysis confirmed that this element was gallium. The presence of gallium in the coating was verified through the mapping of elements This mapping technique provided visual confirmation of the inclusion of the doping agent (gallium) within the zirconia coating.

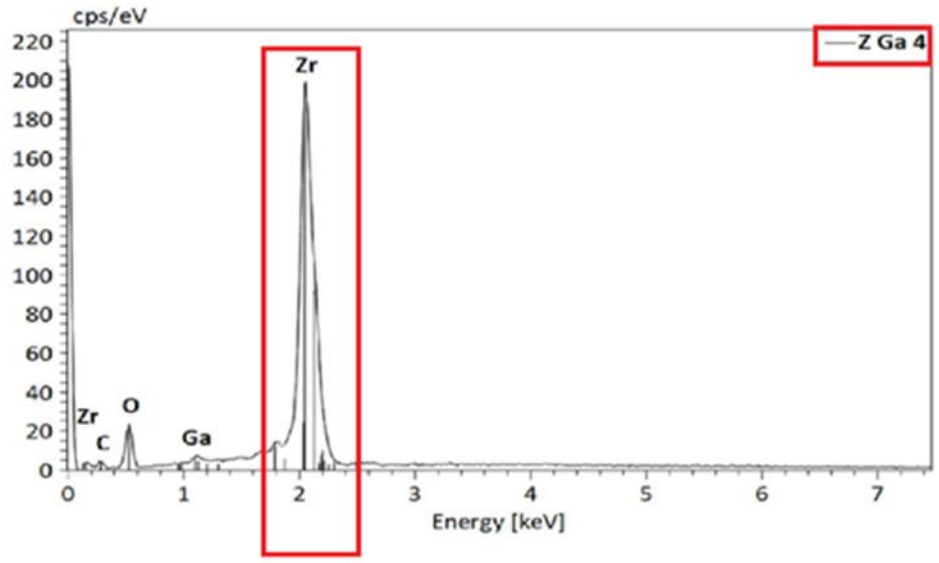


**Figure 8,** The figure shows the presence of the chemical elements on the coating discs**.**

The Biological investigations included cytocompatibility assessment towards human gingival fibroblasts (HGF). The human gingival fibroblasts were put in contact with the zirconia discs, and the metabolic activity of cells cultivated on the gallium-containing disc was found to be comparable to that of the bare materials at all examined time points (24 h, 48 h, 72 h) with no significant differences ($p>0.05$). This indicates that the safety of the gallium-containing disc is not influenced by gallium and the duration of gallium exposure as already was demonstrated previously. [51-56-63]

Furthermore, the cell morphology and spread were well-preserved, as confirmed by immunofluorescence and SEM analyses.

This finding is promising for the potential clinical application of these coatings, as it suggests their compatibility with the surrounding oral tissues and their ability to facilitate proper healing processes.

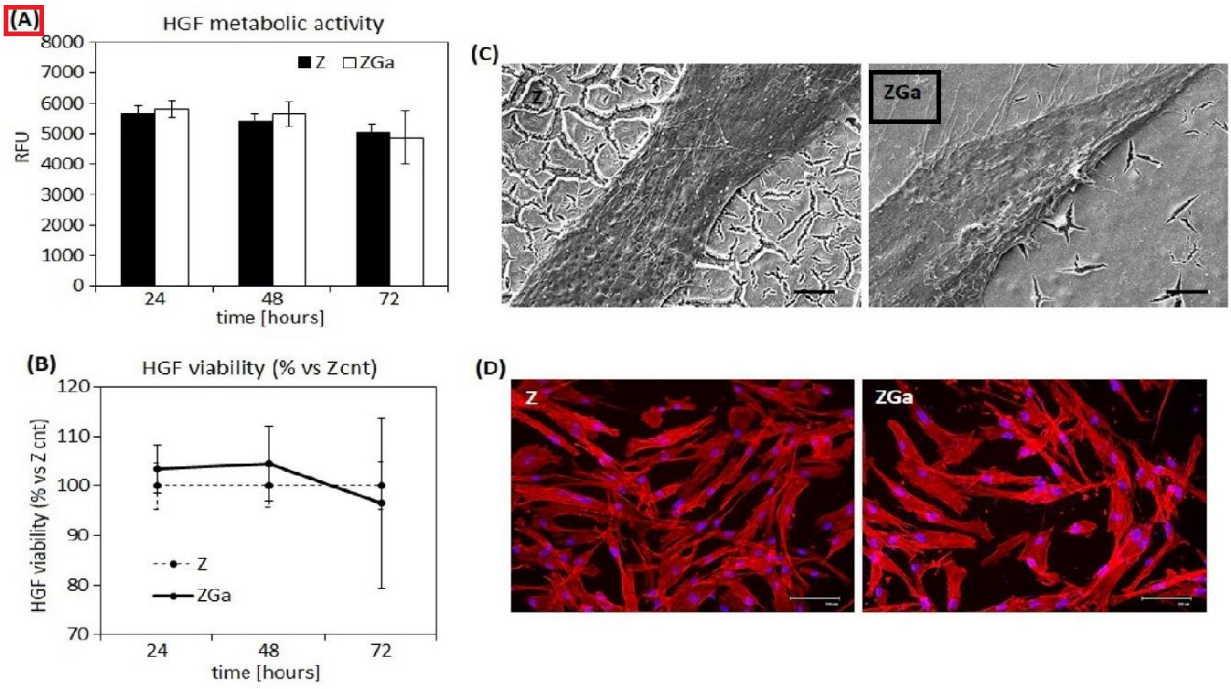


**Figure 9.** The picture displays the specimens' cytocompatibility results. Metabolic activity and viability of cells cultivated on no doped zirconia samples (Z) and Ga-doped zirconia samples (ZGa) are represented in **(A)** and **(B)** respectively. No significant differences have been noticed ($p > 0.05$). Cells' morphology, spread and distribution on both samples are represented by **(C)** Scanning Electron Microscopy (scale bar 5 µm) and **(D)** immunofluorescence (scale bar 100 µm).

In contrast, gallium ($Ga^{3+}$), which mimics the behavior of iron ($Fe^{3+}$), efficiently inhibits bacterial metabolism while remaining safe for eukaryotic cells, as previously demonstrated in other study. [63]

The use of both pathogenic and commensal bacterial strains allows for a comparison that can help confirm the selectivity of gallium against pathogenic bacteria. By examining the differential response of these bacteria to the gallium-doped material, it becomes possible to determine if gallium exhibits preferential antimicrobial activity against pathogenic strains while sparing commensal bacteria. [64]

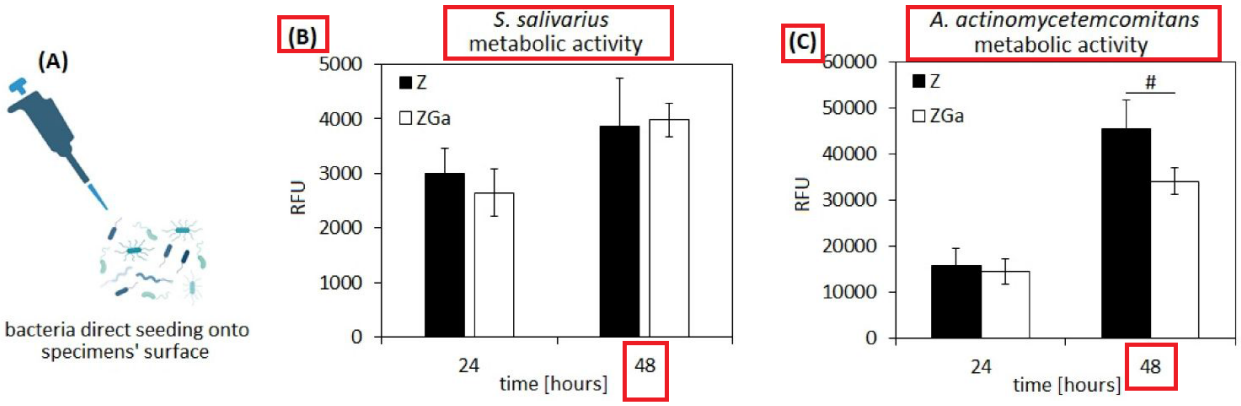


**Figure 10.** Single strains antibacterial effect. **(A)** Cartoon representing the infection methodology. The metabolic activity of the commensal and pathogenic bacteria is represented in **(B)** and **(C)** respectively. In the commensal there are no significant differences ($p > 0.05$) between the materials after 24 and 48 h from the contact. In the pathogen, after 24 h from the contact we can't notice a significant difference between the no doped and the doped scaffold ($p > 0.05$) but after 48 h the gallium-doped specimens reduce significantly ($p < 0.05$) the AA metabolic activity.

The results of the antibacterial activity are presented in Figure 10(B-C). It indicates the effect of the coating on the individual bacterial strains is found to be diametrically opposite. Specifically, in the case of *A. actinomycetemcomitans* (pathogenic), there is initially a similarity in metabolic activity between the samples during the first 24 hours. However, a significant reduction ($p<0.05$) in metabolic activity is observed at the second time point. On the other hand, this trend is absent in the commensal bacteria, where the metabolic activity remains unchanged for both materials at both time points.

These results are consistent with previous findings that demonstrated the ability of the gallium coating to significantly reduce the viability of the pathogenic strain while preserving the commensal bacteria, which plays a beneficial role in maintaining oral health. As it was

demonstrated previously [64-65]. This selective effect of gallium is likely related to the higher capability of pathogens to quickly uptake nutrients from the environment compared to commensal strains. This selective antibacterial action of the coatings is highly desirable as it targets harmful pathogens while preserving the beneficial microflora, promoting a healthy oral environment.

Having confirmed the selective effect of gallium on single strains, we shift our focus to discover the effect of Ga-coated material on the normal human microbiota by testing the coated materials in contact with oral plaque collected from three healthy volunteers.

The presence of gallium in the oral environment did not negatively affect the normal oral microenvironment. However, it was found to decrease the presence of *Fusobacterium nucleatum*, a Gram-negative strictly anaerobic bacterium that is naturally present in the gastrointestinal tract (GI) and can be found from the oral cavity to the colon [66]. *F. nucleatum* is known to play a role in oral dysbiosis, which is often caused by factors like chronic alcohol intake and poor dental hygiene. Oral dysbiosis can lead to an increase in the number of pathogenic bacteria, including *F. nucleatum*.

*F. nucleatum* acts in synergy with other bacteria such as *S. mutans*, *A. actinomycetemcomitans*, and *P. gingivalis*, and together they can contribute to the development of periodontal and endodontic diseases. [67]

Additionally, the rod-shaped structure of *F. nucleatum* provides structural support for the adhesion of other bacteria, leading to the formation of thicker polymicrobial biofilms. The reduction in the population of *F. nucleatum* observed in the Ga-doped samples resulted in a significant decrease in the distribution of the biofilm, as demonstrated by scanning electron microscope (SEM) analysis.

These findings suggest that the presence of gallium in the oral environment can have important implications for preventing and treating periodontal and endodontic diseases and contribute to the maintenance of oral health. [67-68-69]

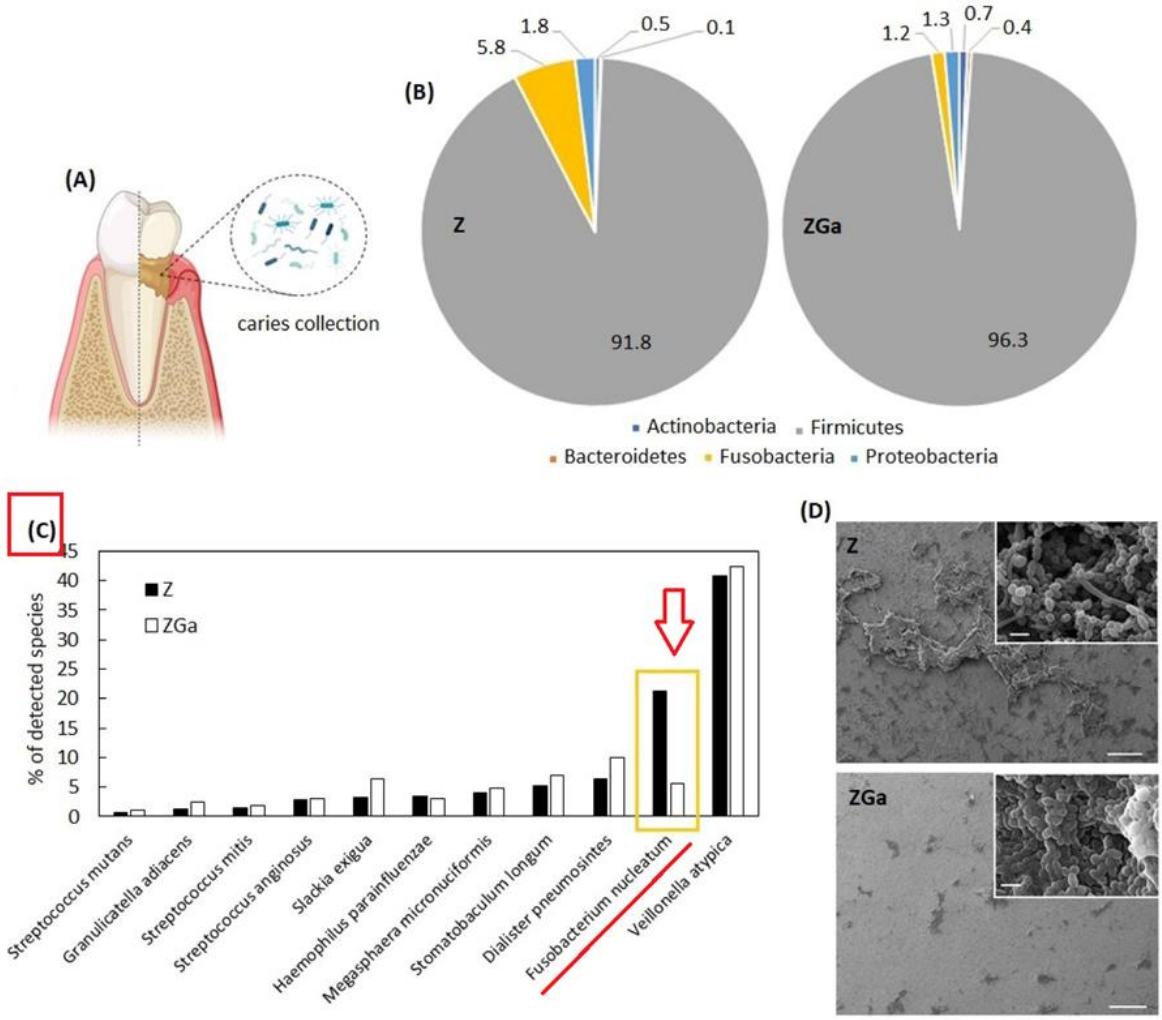


**Figure. 11.** The figure shows Pathogens targeted activity. In **(A)** it is represented the oral plaque collection site. **(B)** and **(C)** represents the phylum and the species respectively. The analysis of **(B)** evidence a decrease in the *fusobacteria* whilst the analysis of **(C)** clearly point out a reduction in *F. nucleatum* (in the yellow box); **(D)** show the biofilm distribution on both materials (scale bar 200 µm). The zoomed box (14 Kx, scale bar 1 µm) shows the differences in terms bacterial species. The no doped zirconia coating (Z sample) is rich in fusiform rods bacteria, typical form of *F. nucleatum*, absent in gallium doped zirconia. This absence may have an impact on biofilm structure stability.

(Figure 11C). Protein analysis of the oral plaque in contact with both the non-doped zirconia coatings and the Ga-doped materials identified a total of 40 bacterial species. However, only 11 species, representing more than 1% of the population, were considered for comparison. The remaining 29 species are listed in the following Table:

| Species | Population % | | Species | Population % | |
|---|---|---|---|---|---|
| | *Z* | *ZGa* | | *Z* | *ZrGa* |
| *Anaeroglobus geminatus* | 0.18 | 0.23 | *Prevotella oris* | 0.37 | 0.92 |
| *Archangium gephyra* | #N/D | 0.23 | *Shuttleworthia satelles* | 0.18 | 0.46 |
| *Brevundimonas diminuta* | 0.18 | 0.23 | *Streptococcus cristatus* | 0.37 | 0.23 |
| *Brochothrix thermosphacta* | 0.55 | #N/D | *Streptococcus gordonii* | 0.18 | 0.46 |
| *Candidatus Pseudoramibacter fermentans* | #N/D | 0.23 | *Streptococcus intermedius* | #N/D | 0.69 |
| *Catonella morbi* | 0.18 | 0.23 | *Streptococcus mutans* | 0.74 | 1.15 |
| *Eggerthella lenta* | 0.18 | 0.23 | *Streptococcus parasanguinis* | 0.18 | 0.46 |
| *Escherichia coli* | 0.18 | #N/D | *Streptococcus sanguinis* | 0.18 | #N/D |
| *Granulicatella elegans* | #N/D | 0.23 | *Streptococcus sobrinus* | #N/D | 0.23 |
| *Lachnoanaerobaculum orale* | 0.18 | 0.23 | *Treponema denticola* | #N/D | 0.23 |
| *Melioribacter roseus* | #N/D | 0.23 | *Variovorax paradoxus* | 0.18 | 0.23 |
| *Peptoniphilus harei* | #N/D | 0.23 | *Veillonella denticariosi* | 0.18 | #N/D |
| *Polyangium fumosum* | 0.18 | 0.23 | *Veillonella dispar* | 0.92 | 0.46 |
| *Prevotella loescheii* | 0.18 | #N/D | *Vibrio algivorus* | 0.18 | 0.23 |
| *Prevotella melaninogenica* | #N/D | 0.23 | | | |

**Figure 12.** The schedule shows the effect of Z and ZGa on the different population of oral microbiota.

Overall, the comprehensive characterization of the gallium-doped zirconia coatings in this study has shed light on their structural, mechanical, and biological properties, our results have demonstrated their promising potential in the field of implant dentistry, as they exhibit excellent stability, favorable surface properties, biocompatibility, and targeted antibacterial activity. Further research and clinical studies are warranted to validate these findings and explore the full extent of the benefits offered by gallium-doped zirconia coatings in preventing implant failure and promoting long-term oral health.

# CONCLUSION

In addition to the successful application of tetragonal zirconia coatings onto bulk zirconia discs using the sol-gel spin coating method, the incorporation of gallium has shown promising anti-plaque properties in preserving the normal human oral microbiota based on in vitro studies. However, the translation of these findings into clinical practice necessitates further comprehensive investigations. Specifically, there is a crucial need to thoroughly assess the biological properties of the zirconia coatings, including their long-term stability in promoting osseointegration and their ability to mitigate inflammation of soft tissue surrounding dental implants.

The complexity of the oral environment and the dynamic interactions between the implant surface, surrounding tissues, and oral microbiota underscore the importance of conducting extensive in vivo studies to validate the efficacy and safety of these novel coatings. Additionally, long-term clinical trials involving human subjects are essential to evaluate the durability and performance of zirconia dental implants coated with gallium-doped sol-gel-derived coatings.

Despite these necessary avenues for further research, the current findings provide a compelling rationale for embracing the sol-gel approach as a means to develop a novel class of inorganic functional coatings with tailored features. These coatings hold immense potential for revolutionizing the field of implant dentistry by improving the biocompatibility, antibacterial properties, and overall performance of zirconia dental implants, ultimately enhancing patient outcomes and satisfaction.

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