Multi-Omics Analysis of Oral Bacterial Biofilm on Titanium Oxide Nanostructure Modified Implant Surface: in Vivo Sequencing-Based Pilot Study in Beagle Dogs

Hanyu Sun

Sun Yat-Sen University

Yuki Chan

The University of Hong Kong Faculty of Dentistry

Xuan Li

The University of Hong Kong Faculty of Dentistry

Ruogu Xu

Sun Yat-Sen University

Zhengchuan Zhang

Sun Yat-Sen University

Feilong Deng

Sun Yat-Sen University

Xiaolin Yu (✉ yuxlin3@mail.sysu.edu.cn)

SYSU: Sun Yat-Sen University <https://orcid.org/0000-0001-9532-3598>

Research Article

Keywords: TiO₂ nanotubes, Oral biofilm, 16S, Metatranscriptomics, Dental implants, Calcium phosphate, Surface modification

Posted Date: December 29th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1164895/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background and aims

Surface modifications of titanium implants play essential role in facilitating osteointegration and enhancing their antimicrobial properties, while the latter is critical for preventing infectious diseases caused by the biofilm. However, it remains unknown about how the surface modifications could affect the composition and functional gene expression of oral microbiota deposited on the titanium implants. In this study, we aimed to investigate the impact of different nanostructured surfaces on the biofilm *in vivo*.

Results

Nanophase calcium phosphate were successfully deposited into or between the TiO₂ nanotubes with a diameter of 70–90 nm. NT and NTN surfaces showed increased roughness than the MP surface. XPS spectra showed that the O 1s was mainly divided into two bands in MP and NT samples, including Ti-O and -OH, while the surface modification of TiO₂ nanotube in NT accounted for the increased intensity of Ti-O with the reference to that in MP samples. After the deposition of calcium phosphate, two new elemental peaks of Ca and P can be identified from the XPS survey spectrum of NTN. Moreover, the O 1s of NTN sample could be differentiated into three peaks, while the new one represented the -PO band. The 16S rDNA sequencing results showed that NT and NTN had minimal impact on the diversity and community structure of oral microbiota. Metatranscriptomic sequencing revealed that differentially expressed genes (DEGs) mostly differed in the terms of the biological process and cellular component on different surfaces. Gene Ontology (GO) terms enrichment indicated that NTN down-regulate the genes associated in localization and locomotion. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis revealed that the DEGs were associated with microbial metabolism, protein synthesis and bacterial invasion of epithelial cells.

Conclusion

TiO₂ nanotube and calcium phosphate-coated TiO₂ nanotube despite improving the antimicrobial properties of implant surfaces, had unexpectedly minimal impact on the microbiome composition and diversity. Notably, nanostructured titanium surfaces could inhibit the bacterial migration and colonization, down-regulate the pathogen invasion pathways, and further destruct bacterial cellular membrane, all in all, conferred the bactericidal properties.

Background

Titanium and its alloys have been widely used as medical materials due to their ideal chemical and mechanical properties, such as high strength to weight ratio, high yield and fatigue strength, a relatively low Young's modulus counteracting the effects of stress shielding [1], an excellent corrosion resistance, as well as biocompatibility provided by the oxide layer [2-4]. Recently, surface modifications of titanium implants have received increasing interest to facilitate the physiological healing process and obtain

biologically active materials for promoting the tissue regeneration [5, 6], among which titanium oxide (TiO₂) nanotubes has been a biomaterial surface modification of more recent interest for orthopedic applications and medical implants [6-8]. It has been found to increase the osseointegration by promoting adhesion and proliferation of osteoblasts [9, 10], and stem cell differentiation [11], and increasing strength of adhesion [12] and mineralization rate [13]. Notably, TiO₂ nanotubes also improved the anti-microbial properties [14-18]. Antibacterial coatings are important to prevent biofilm formation and infectious diseases [19, 20]. Since up to 65% of all the infectious diseases, including peri-implant and periodontal diseases, were associated with the formation of bacterial biofilms on the medical implant surfaces. Since they are usually problematic to treat or completely eradicate these biofilms, this eventually led to the implant removal and repeated surgeries [21]. Peng *et al.* found that TiO₂ nanotubes coating significantly decreased the initial adhesion and colonization of *Staphylococcus epidermidis* compared with the acid-etched or polished Ti surfaces *in vitro* [14]. Moreover, additively manufactured porous titanium biomaterials covered with nanotubular surfaces had less adherent *S. aureus* compared with the porous titanium surface on day one, suggesting that nanotubes alone could be effective in preventing biofilm formation within the first few hours to the first day after the implant surgery [15]. This property keeps in line with the reported bactericidal capacity caused by the high aspect ratio nanotopographies [22, 23].

Selective laser melting (SLM) offers an ideal platform to fabricate customized substitutes [24]. Due to its great flexibility, it provides a potential for producing complexly shaped titanium and its alloy substitutes [25]. However, SLM-processed Ti implant demonstrated less osseointegration has limited its applications [26], while the rough SLM surface also increases the bacterial adhesion [27]. Therefore, surface modifications are essential for SLM-processed Ti implants to enhance the osseointegration and antibacterial efficacy.

Our previous study indicated that the nanotubes with nanophase calcium phosphate on titanium surface showed fewer *S. mutans* and *S. sanguinis* bacteria colonies after 8h and 24h cultivation compared with TiO₂ nanotubes [27]. Moreover, a recent study reported that the significant decrease of *S. aureus* adhesion after 3h and 22h was detected on calcium phosphate-coated TiO₂ nanotubes compared with those on the TiO₂ nanotubes, Ag coated TiO₂ nanotubes, and Ag/calcium phosphate-coated TiO₂ nanotubes [7]. However, the antibacterial activity of calcium phosphate deposited on TiO₂ nanotubes is not fully clear, while the investigations of the composition and functional gene expression of oral microbiota presenting on the calcium phosphate-coated TiO₂ nanotubes versus TiO₂ nanotubes *in vivo* may facilitate the understanding the antibacterial surface coating and benefit the clinical outcome.

Development of next-generation sequencing (NGS) technology makes it possible to rapidly generate sequences and provide extensive information of the inhabitant microorganisms in the oral cavity [28]. Metagenomics reveals a taxonomical profile of a microbial community and metatranscriptomics inform us the functional profile of a community [29]. The retrieved information can be used for detecting microbiome-based biomarkers for early diagnosis of oral and associated diseases [28]. However, either

metagenomic or metatranscriptomic sequencing technology has been utilized for *in vivo* evaluation of the effect of titanium materials on the antibacterial properties.

Herein, we combined metagenomic and metatranscriptomic sequencing to characterize the diversity and community structure of the oral microbiota, and then identify the differentially expressed microbial genes on calcium phosphate-coated TiO₂ nanotubes and TiO₂ nanotubes in a beagle mandible model.

Materials And Methods

Specimen preparation and treatment

Specimen preparation and treatment of the mechanical polishing group (MP) and TiO₂ nanotube group (NT) were reported in our previous studies [27]. Briefly, screw-type titanium implants were designed using SolidWorks® 12.0 (SolidWorks Corp, Concord, MA, USA) and manufactured using an SLM machine (SLM125HL, SLM solutions GmbH, SLM125HL, Lubeck, Germany). A total of two types of specimen shapes were prepared. Titanium round discs with a thickness of 1 mm and a diameter of 10 mm were used for titanium surface characterization. Titanium implant screws were fabricated for the implant surgery *in vivo*. Each implant body was 13 mm in length and 4.5 mm in diameter as shown in Figure S1 [see Additional file 1]. Then SLM-processed titanium specimens were immersed in a cleaning solution (0.05mol/L Na₂SiO₃, 0.1mol/L Na₃PO₄ and 0.2mol/L Na₂CO₃) for 15 min followed by sonication in deionized water for 15 min. A total of three groups of specimens were fabricated in this study: 1) Mechanical polishing group (MP, n=5): specimens were mechanically polished successively by P80, P220, P400, P600, P800, P1000 sandpapers; 2) TiO₂ nanotube group (NT): SLM-processed titanium specimens were first sandblasted with 250 µm ZrO₂ particles and then chemically polished in a solution of 5% hydrofluoric acid (HF) for 1 min. TiO₂ nanotube arrays was fabricated on the SLM-processed titanium substrate using anodic oxidation methods (voltage of 20 V in a 0.5 wt % HF electrolyte for 5 min) followed by annealing at 500 °C for 120 min; 3) Nanophase calcium phosphate embedded to TiO₂ nanotubes groups (NTN): calcium phosphate nanoparticles were in or between the TiO₂ nanotubes by electrochemical deposition. The details were described as below. Nanotube samples were anchored onto a platinum cathode and subjected to a constant voltage of -3V for 5min, with the counter-electrode platinum using an electrochemical workstation (CHI-660D, USA). The solution for electrochemical deposition contained 0.20mmol/L of Ca(NO₃)₂, 0.12mmol/L of (NH₄)₂HPO₄ and 0.05mol/L NaNO₃ in an 80°C water bath environment. The duration of electrochemical deposition in this study was shortened compared with our previous protocol [27] in order to maintain the topography of nanotubes.

Surface characterization

The surface topography and elemental compositions were analyzed using field-emission scanning electron microscopy (FE-SEM, Hitachi, S-4800, Tokyo, Japan) equipped with an energy-dispersive X-ray spectrometer (EDS) and X-ray photoelectron spectroscopy (XPS) (Thermo Scientific ESCALAB 250Xi,

USA). Surface roughness parameters, including the roughness average (Ra) and root mean square roughness (Rq), were inspected using a three-dimensional surface topography analyzer (BMT EXPERT, Lorsch, Germany).

Animal experiment

This study was ethically approved by the Ethics Committee for Animal Experiments of Sun Yat-Sen University (No. SYSU-IACUC-2018-000207). A total of 3 adult beagle dogs weighing 11.5-13.5 kg were used. All surgical procedures were performed under general anaesthesia using pentobarbital sodium (30 mg/kg/i.m., Pelltobarbitalum, Sigma, USA) and xylazine hydrochloride (0.10 mg/kg/i.m., Sumianxin, Guilin Huamei, China). The premolar and molar teeth in both mandibular quadrants were extracted gently using a rotational movement after hemi-sectioning. The sockets were curetted and then interrupted suturing was performed. After a 3-month healing period, implant surgery was performed; a carbon blade no. 15 was used to make a full-thickness crestal incision along the edentulous ridge, and the mucoperiosteal flap was elevated. Profuse irrigation with saline water was used for cooling during drilling. After implant site preparation, titanium implants were inserted and then interrupted suturing was performed. The animals received prophylaxis penicillin administration (80,000 μ /kg/i.m., Reyoung, China) during the first three days after surgery. After approximately 8 weeks, clinical parameters were recorded and supra-mucosal and sub-mucosal plaque were collected. All of the animals were euthanized by the administration of a sodium pentobarbital overdose.

Clinical evaluation and sampling methods

The following clinical parameters were examined to characterize the status of peri-implant lesions: modified Plaque Index (mPI) [30], modified Bleeding Index (mBI) [30], bleeding on probing (BOP) scores [31] and probing pocket depth (PPD) [32].

Prior to sampling, the clinical sites were firstly isolated and dried with sterile cotton rolls. Supra-mucosal plaque samples were collected using sterile Gracey or universal curettes and placed into labeled sterile Eppendorf tubes with 1 ml phosphate buffered saline (PBS) (0.01 M phosphate, 137 mM NaCl, 2.7 mM KCl; pH 7.4). After complete removal of supra-mucosal plaque, sub-mucosal plaque was collected using the same method as the supra-mucosal plaque collection. Supra-mucosal samples which were collected from MP, NT and NTN groups were labeled as MPP, NTP and NTNP, respectively. Sub-mucosal samples which were collected from MP, NT and NTN groups were labeled as MPB, NTB and NTNB, respectively. In the laboratory, Eppendorf tubes were briefly mixed by vortexing, before being centrifuged (5,000 g, 10 min), the PBS buffer was carefully discarded, to obtain washed plaque samples. Then all the samples were stored at -80°C until further analysis

16S rRNA gene sequencing and data analysis

Total bacterial genomic DNA was extracted using QIAamp DNA Mini kits (Qiagen, CA, USA) according to the manufacturer's instructions. PCR amplification of the hypervariable V3-V4 region of the 16S ribosomal RNA (rRNA) genes was performed using the universal bacterial primer pairs, 341F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). PCR amplification and library construction were performed according to Illumina's standard protocol for 16S Metagenomic Sequencing Library Preparation (Part # 15044223 Rev. B). Paired-end MiSeq sequencing was performed on the amplicon libraries using an Illumina MiSeq system (300PE) at the Beijing Genomics Institute (BGI Institute, Wuhan, China). The paired-end MiSeq sequences were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME1) package [33] following the MiSeq SOP pipeline. Mothur [34] was also used in the downstream sequence analysis. Briefly, the paired ca. 300-bp forward and reverse MiSeq sequence reads were assembled, followed by the filtering of noisy sequences, chimera checking, and Operational Taxonomic Units (OTU) picking based on 97% identity using the usearch quality filtering script. Taxonomy assignments were made against the RDP databases. The Venn plots in OTUs were plotted with R package. The structure of different peri-implant microbial communities, the relative abundance, alpha diversity and beta-diversity were estimated using Mothur and Qiime. Beta-diversity comparisons were performed by both the unweighted and weighted UniFrac analyses, which were visualized in principal coordinate analysis (PCoA) plots. The comparisons of the relative abundance and alpha diversity among different groups were determined by R package based on Kruskal-Test. The p value threshold was set to 0.05.

RNA sequencing and data analysis

Total RNA was extracted using Promega SV Total RNA Isolation System kit (Promega, USA) following the manufacturer's protocol. After rRNA was removed, mRNA was obtained by oligo dT. Then fragmentation buffer was added to interrupt mRNA to short reads. Taking these short reads as templates, Random hexamer-primer were used to synthesize the first-strand cDNA. The second-strand cDNA was synthesized using buffer, dATPs, dGTPs, dCTPs, dUTPs, RNase H and DNA polymerase I respectively after removing dNTPs. Short reads were purified with QiaQuick PCR extraction kit and resolved with EB buffer for end reparation and adding poly(A). Then the short reads were connected with sequencing adapters. After that, the UNG enzyme was used to degrade the second-strand cDNA, and the product was purified by MiniElute PCR Purification Kit before PCR amplification. Sequencing of mRNA was carried out at the Beijing Genomics Institute (BGI Institute, Wuhan, China) using an Illumina HiSeq2000 platform.

Clean data was obtained using SOAP2 software [35]. The detailed method was shown below: 1) Remove reads with 10% N; 2) Remove reads contaminated by adapter (default:15 bases overlapped by reads and adapter); 3) Remove reads with 20% low quality (20) bases; 4) Remove 16S rRNA, 18S rRNA contamination reads (consistency of 90%). Short reads were assembled using Trinity software [36]. Based on NOIseq package method [37], differentially expressed genes (DEGs) were screened among groups. Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the GOrse R package [38]. KOBAS software [39] was used to test the statistical enrichment of differential

expression genes in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The *p* values were adjusted using the Benjamini & Hochberg method. GO terms and KEGG terms with corrected *p* value less than 0.05 were considered significantly enriched by differential expressed genes.

Statistical analysis

Statistical analysis was conducted using SPSS software 25 (SPSS Inc., Chicago, IL, USA) and RStudio interface (Team, R., RStudio: Integrated Development Environment for R. RStudio Inc., Boston, MA. In 2015) [40]. One-way ANOVA test followed by a Bonferroni's test was used to identify any clinical parameter between multiple groups.

Data depository

The sequences generated in this study were deposited in NCBI BioProject: PRJNA776400 (Reviewer link: <https://www.ncbi.nlm.nih.gov/sra/PRJNA776400>) for 16S rRNA gene sequencing data; and PRJNA776641 (<https://www.ncbi.nlm.nih.gov/sra/PRJNA776641>) for RNA sequencing data.

Results

Surface characterization

The surface topographies of specimens were shown in Figure 1. The surface of the MP specimen was smooth with regular scratches resulting from the polishing process (Figure 1A & 1B). TiO₂ nanotube structures of approximately 70-90 nm in diameter were observed on the surface of NT specimen (Figure 1D & 1E). As shown in Figure 1G & 1H, the nanophase calcium phosphate was successfully deposited into or between the TiO₂ nanotubes on the surface of NTN specimen.

Table 1

Element compositions of specimen surfaces (wt %).

Specimen	Titanium	Oxygen	Calcium	Phosphorus
MP	93.03	6.97	–	–
NT	80.82	19.18	–	–
NTN	71.36	28.32	0.19	0.13

The elemental compositions of specimen surfaces were measured by EDS (Table 1). It was noted that titanium and oxygen were the major elements in all specimens, while the oxygen proportion increased dramatically in NT and NTN samples, indicating a significant surface elemental change. For NTN specimen, calcium and phosphorus were newly found, suggesting the successful deposition of calcium

phosphate nanoparticles. The surface roughness parameters of specimen surfaces were shown in Table 2, and the NT and NTN surfaces showed higher average roughness values (Ra) (2.78 μm and 2.94 μm) than the MP surfaces (0.42 μm).

Table 2

Surface roughness parameters of specimen surfaces.

Specimen	Ra	Rq
MP	0.42±0.01	0.55±0.05
NT	2.78±0.02	3.49±0.02
NTN	2.94±0.07	3.76±0.17

The XPS spectra in Figure 2 provide more information about the elemental changes resulting from the surface modification. As Ti and O were the major elements found in all the samples, we also analyzed these two elements using XPS. It was found that the O 1s was mainly divided into two bands in MP and NT samples, including Ti-O and -OH, while the surface modification of TiO₂ nanotube in NT accounted for the increased intensity of Ti-O with the reference to that in MP samples. After the deposition of calcium phosphate, two new elemental peaks of Ca and P can be identified from the XPS survey spectrum of NTN. Moreover, the O 1s of NTN sample could be differentiated into three peaks, while the new one represented the -PO band.

Clinical evaluation

Because MP and NTN group lost 2 and 1 implants respectively during the observation period, a total of 12 implants (MP=3, NT =5, NTN =4) were recruited in this study. The clinical profiles of titanium implants were summarized in Table 3. No significant differences of clinical parameters were detected between groups.

Table 3

Clinical characteristics of titanium implants in 3 groups.

Clinical parameters	MP	NT	NTN	<i>p</i> value
mPLI	1.67±0.58	1.60±0.89	1.75±0.50	0.953
PPD (mm)	2.33±0.58	2.20±0.57	1.88±0.48	0.525
mBI	2.33±0.58	2.00±1.41	1.00±1.16	0.326
BOP (% (frequency of detection))	100	80	50	0.366

16S rRNA gene sequencing results

A total of 648,953 assembled quality-filtered sequencing reads were obtained, which were assigned to 572 operational taxonomic units (OTUs) with the identity threshold set at 97%. For sub-mucosal plaque, the three categories shared 290 OTUs in common (i.e. OTUs that were detected on all three categories of titanium surfaces: MP, NT and NTN). This accounted for nearly half of the total number of OTUs detected, as shown in Venn diagram (Figure 3A). Within the supra-mucosal plaque niches, MP, NT and NTN shared 226 OTUs in common (Figure 3B). As Figure S2 showed, the rarefaction curves of observed OTUs appeared to approach asymptotes, which indicated high levels of coverage and thus sufficient sampling effort in each biofilm sample [see Additional file 1].

Bacterial community composition at different taxonomic levels

Diverse bacterial taxa were assigned to 14 phyla, 189 genera and 229 species from the clinical samples. As Figure 4 showed, no significant differences in terms of the relative abundance were detected in the sub-mucosal or supra-mucosal samples at the phylum level. As Table S1 [see Additional file 2] showed, a total of 6 genera and 9 species showed significant differences in the sub-mucosal samples among groups. There were no significant differences at the genus level but 3 species showed statistical differences in supra-mucosal samples among groups.

The X-axis indicates different groups; the Y-axis indicates the relative abundance of the phylum annotated. The phyla with the relative abundance of less than 0.5 % in the samples are merged into "Others" item.

Alpha-diversity comparisons

Based on results from alpha-diversity analysis (Figure 5 & 6), which included the Shannon index, Simpson index, ACE index, Chao1 index, sobs and the coverage index in the supra-mucosal and sub-mucosal samples; no statistically significant difference was found across the three implant surface categories.

Beta-diversity comparisons

To compare the respective community structures within each titanium surface, the overall bacterial community compositions in each group were compared based on their unweighted and weighted UniFrac distances, and visualized in a PCoA plot. As shown in Figure 7, a PCoA plot was generated with two principal axes (PCoA1 and PCoA2), which respectively explained 26.85% and 14.71% of the variance based on the unweighted UniFrac distances as well as 32.25% and 15.95% based on the weighted

UniFrac distances amongst the bacterial communities on each kind of titanium surface. No clear patterns (clustering arrangements of different samples) were visually apparent in the PCoA plots coloured according to the three titanium surface categories (Permutational multivariate analysis of variance (PERMANOVA): $p = 0.31$ for weighted UniFrac distances and $p = 0.06$ for unweighted UniFrac distances).

Differential gene expression

By applying comparative metatranscriptome analysis, we identified major shifts in the overall community activities on different titanium surfaces. Compared with the NTN group, 10,401 genes were significantly down-expressed and 9,497 genes up-expressed in the NTB group (Table S2). Compared with the MPB group, the NTB group showed 9,640 up-regulated genes and 9,797 down-regulated genes (Table S3). A total of 28,674 genes (14,121 down-regulated genes and 14,553 up-regulated genes) significantly changed their expression levels in NTN group than in MPB group (Table S4) [see Additional file 2].

GO functional enrichment analysis

As shown in Figure 8 and Table S5-S10 [see Additional file 2], a total of 23 and 35 GO terms significantly up-regulated in the NTB group and the NTN group, respectively when compared with MPB groups. On the other hand, the NTB group and the NTN group presented a total of 3 and 16 significantly down-regulated GO terms. The NTB group showed 4 down-regulated GO terms but no up-regulated GO terms when compared to the NTN group.

KEGG pathway enrichment analysis

Table S11-16 [see Additional file 2] and Figure 9 showed the results of the KEGG pathway enrichment analysis. When compared with the MPB groups, the differentially expressed genes of the NTN group were enriched in the pathways associated with ribosome, two-component system, *Salmonella* infection, Shigellosis, bacterial invasion of epithelial cells, pathogenic *Escherichia coli* infection, oxytocin signaling pathway and mRNA surveillance pathway. The differentially expressed genes of the NTB group compared with the MPB group were mostly enriched in the pathways of glyoxylate and dicarboxylate metabolism, *Salmonella* infection, and protein processing in endoplasmic reticulum. The differentially expressed genes between the NTB group and the NTN group were significantly enriched in protein processing in endoplasmic reticulum pathway.

Discussion

With the development of oral implantology, the dental implants have been a common procedure to rehabilitate partial and full edentulism [41]. However, there is an increasing number of patients with peri-implant diseases recent years. Peri-implant diseases are biological complications of dental implantation

which are mainly caused by microbial biofilm [42, 43]. To handle the challenge of peri-implant diseases, it is essential to improve the antimicrobial properties of dental implants [44]. Recent studies demonstrated that the antibacterial properties of the materials can be improved by the construction of nanostructured surfaces [45, 46]. An anti-bacterial composite nanostructure of nanophase calcium phosphate embedded to TiO₂ nanotubes on microrough SLM titanium substrates was constructed in previous studies [27]. Although it showed satisfying results in *in-vitro* study, little is known regarding the *in-vivo* antibacterial activities of the nanostructured titanium and how nanostructures affect the profiles and functions of the microbial biofilms. In this study, peri-implant microbiota on the different titanium surfaces were systematically compared in the oral cavity of beagle dogs by using the next-generation sequencing (NGS) technique. We not only described the taxonomy of the oral bacteria sampled, but also profiled and compared the expressed genes, and thus explored the bacterial functions on the different nanostructured surfaces. To the best of our knowledge, it is the first study that high-throughput DNA and RNA sequencing to evaluate the antibacterial activity of the microbiome established on the titanium nanostructured surfaces *in vivo*.

Base on the results from the 16SrRNA gene sequencing, we found no significant differences in the microbial diversity of the biofilms among different titanium surfaces. The α -diversity parameters, indicating both species richness and evenness, in the sub-gingival and supra-gingival microbiota presented on the three kinds of titanium surface were tested ($p > 0.05$). In addition, the overall composition of the sub-gingival and supra-gingiva microbiota among the 3 groups were detected by the weighted and unweighted UniFrac metrics. The resultant PCoA plot showed no significant clustering. Together, these results illustrated that the nanostructured titanium had little effect on the community composition of the sub-gingival and supra-gingiva peri-implant microbiota established on the surfaces.

The dominant bacterial taxa identified in the sub-gingival and supra-gingival niches in this study were in the phyla *Bacteroidetes*, *Proteobacteria* and *Firmicutes*, which were constant with previous studies in beagles [47, 48]. However, when compared to a study in human, peri-implantitis communities were enriched with the phyla *Bacteroidetes*, *Spirochetes*, and *Synergistetes*, whereas *Actinobacteria* prevailed at healthy sites [49]. This might imply different microbial composition in the sub-mucosal microbiota in different hosts or heterogeneity in the methods such as different sampling techniques, sequencing technique and analysis [50, 51]. No significant difference of diversity and species richness were found among all groups. These results showed that although antibacterial materials have significant results *in vitro*, they may not present obvious advantages in the bacterial environment of the oral cavity when implanted *in vivo*. The diversity and richness of the peri-implant microbiota community may be more related to the condition of the surrounding tissue of hosts.

Despite the fact that the bacterial community composition of peri-implant niches did not show significant differences, comparative metatranscriptomic analysis might reveal the underlying mechanisms. Several investigations showed that nanostructured titanium surfaces were considered to have considerable impact on inhibiting bacteria adhesion [52, 53]. GO functional analysis showed that the differentially expressed genes between the 3 groups were mainly enriched in the GO terms of biological process and

cellular component. Compared to MPB group, the differentially expressed genes were significantly enriched in locomotion and localization, especially there were 231 genes significantly down regulated in the GO terms of localization in the NTN group. It can be inferred that calcium phosphate coated TiO₂ nanotube could inhibit bacterial migration and colonization. Compared to NTB groups, GO terms of integral to membrane, cytosolic ribosome, intrinsic to membrane and cytosolic large ribosomal subunit were up-regulated significantly in the NTN groups. In our previous study, dead bacteria with deformed and damaged membranes had been observed on the surfaces of NTN samples by SEM suggested that the sharp nanophase calcium phosphate can affect the integrity of bacterial cell membrane [27]. Combining this with the RNA sequencing results presented in this study, we propose that the destruction of bacterial cellular membrane on the NTN surface could be one of the vital mechanisms of antibacterial activities of nanostructured titanium surfaces. KEGG pathway enrichment analysis revealed that over 300 differently expressed genes (DEGs) enriched in the term of ribosome between the NTN group and MPB group, which means that the titanium surface nanostructures affect the gene expression of the microbiota by down-regulating the ribosome-associated pathway. It is noteworthy that several DEGs enriched in the pathway of bacterial invasion of epithelial cells, which implies that the titanium nanostructures regulated invasiveness of pathogens. Bacteria invasion into soft tissue is a vital event in the progress of periodontal disease [54, 55]. The oral mucosal barrier is exposed to oral commensal microbiome and a subtle balance is kept through host specific and unspecific immunity. Homeostasis disruption would lead to the periodontal disease [56]. Healthy peri-implant soft tissue plays a crucial role in maintaining peri-implant tissue health. Collagen fibers in the connective tissue around the implant array in a parallel orientation to the implants while fibers around teeth perpendicularly and tightly attached to the cement. Besides, lower density of vascular around implant may make implants more susceptible to get infection and progression than teeth [57]. It can be inferred that titanium nanostructured surface affect the progression of peri-implant diseases through the pathway of bacterial invasion of epithelial cells, whilst the exact mechanism entails further exploration.

The antibacterial properties of materials were usually estimated by traditional methods such as *in vitro* antibacterial experiments, although it only verified the influence of materials on specific tested pathogens. Currently, it is the standard assay in most studies. However, the complex host-biofilm interactions make it more unpredictable to evaluate the in-vivo antimicrobial activities. The results of this *in-vivo* study and previous *in-vitro* experiments [27, 53] show that the biological behavior of specific pathogens in the *in-vitro* experiment cannot represent the response of the whole microbiome established as biofilm on the material surface. This also suggests that the evaluation and estimation of antibacterial properties of materials should be taken with more comprehensive examination.

The results of antibacterial activity *in vivo* were affected by not only the material characteristics but also the condition of the surrounding tissue. Our previous study indicated that calcium phosphate nanoparticles deposited in TiO₂ nanotube could enhanced the adhesion and proliferation of human gingival epithelial cells (HGEs) and human gingival fibroblasts (HGFs) which means nanostructured titanium surfaces were beneficial to the mucosal barrier [58]. Besides, TiO₂ nanotubes can modulate

macrophage immune response [59]. Host immune responses and inflammatory state may also influence the complex host-biofilm interaction and therefore influence the composition of the colonizing microbiota and their expressed functions [48, 60].

This study estimated the antibacterial abilities by exploring the differences in the bacterial community composition and their biological function within the oral microbiome associated to 3 different surfaces using high-throughput sequencing. Insignificant differences were observed in the microbiome composition and diversity between the 3 groups, nevertheless differentially expressed genes were detected. This work provides a new perspective to evaluating the *in-vivo* antibacterial abilities of biomaterials and helps us understand the underlying mechanisms. However, the empirical results of this study have to be seen in light of some limitations. The present investigation is a pilot study based on a relatively limited sample size. Future study in a larger population is needed to validate and generalize the findings. There is also a known heterogeneity between different hosts (beagles and humans) of the oral microbiomes, thus the results must be interpreted with caution, and should not be directly transferred onto different hosts. Further study in the relevant hosts are required to establish the antibacterial abilities of biomaterials. Nonetheless, this study has important implications for future studies exploring the antibacterial abilities of biomaterials, especially highlighting the differences between the traditional *in vitro* antibacterial experiments focusing on limited number of pathogens and the full sequencing-based examination of the biofilm microbiome established on the biomaterial surfaces *in vivo*.

Conclusion

In the current study, high-throughput sequencing analysis showed that TiO₂ nanotube and calcium phosphate coated TiO₂ nanotube did not change the diversity and community structure of oral microbiota, but affected the differentially expressed genes associated with microbial metabolism, protein synthesis, bacterial locomotion and bacterial localization.

This study provided a new strategy for evaluating the antibacterial properties of materials *in vivo* by comparing the composition differences and differential genes expression of the supramucosal and submucosal biofilm on different titanium surfaces with high-throughput sequencing.

Abbreviations

TiO₂: Titanium oxide; SLM: Selective laser melting; NGS: Next-generation sequencing; MP: Mechanical polishing; NT: TiO₂ nanotube; HF: Hydrofluoric acid; NTN: Nanophase calcium phosphate embedded to TiO₂ nanotubes; FE-SEM: Field-emission scanning electron microscopy; SEM: scanning electron microscopy; EDS: Energy-dispersive X-ray spectrometer; XPS: X-ray photoelectron spectroscopy; Ra: Roughness average; Rq: Root mean square roughness; mPI: Modified Plaque Index; mBI: Modified Bleeding Index; BOP: Bleeding on probing; PPD: Probing pocket depth; PBS: Phosphate buffered saline; rRNA: ribosomal RNA; OTU: Operational Taxonomic Unit; PCoA: Principal coordinate analysis; DEG:

Differentially expressed gene; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; HGECS: Human gingival epithelial cells; HGFs: Human gingival fibroblasts

Declarations

Ethics approval and consent to participate

This study was ethically approved by the Ethics Committee for Animal Experiments of Sun Yat-Sen University (No. SYSU-IACUC-2018-000207) and performed complying to the national and institutional guidelines.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare no conflict of interest.

Funding

This work was funded by National Natural Science Foundation of China (No. 81801012).

Authors' contributions

HS, RX, ZZ and XY performed the animal experiments. HS did the clinical examination and sampling. YC and XY performed the DNA library preparation and bioinformatic analysis. XL and XY performed the material characteristic analysis. XY and FD conceived and designed this study, analyzed the data, and interpreted the results. XY, YC, XL, HS and FD wrote the manuscript. All authors have read and approved the final version of the manuscript.

Acknowledgements

The authors sincerely acknowledge Mr. Fan Wu and Mr. Xiucheng Hu for help with animal experiments.

References

1. Damiani L, Eales MG, Nobbs AH, Su B, Tsimbouri PM, Salmeron-Sanchez M, Dalby MJ: **Impact of surface topography and coating on osteogenesis and bacterial attachment on titanium implants.** *J Tissue Eng* 2018, **9**:2041731418790694.
2. Jiang X, Yao Y, Tang W, Han D, Zhang L, Zhao K, Wang S, Meng Y: **Design of dental implants at materials level: An overview.** *J Biomed Mater Res A* 2020, **108**:1634–1661.
3. Zhang Y, Gulati K, Li Z, Di P, Liu Y: **Dental Implant Nano-Engineering: Advances, Limitations and Future Directions.** *Nanomaterials (Basel)* 2021, **11**.
4. Bonilla-Represa V, Abalos-Labruzzo C, Herrera-Martinez M, Guerrero-Pérez MO: **Nanomaterials in Dentistry: State of the Art and Future Challenges.** *Nanomaterials (Basel)* 2020, **10**.
5. Guo T, Gulati K, Arora H, Han P, Fournier B, Ivanovski S: **Orchestrating soft tissue integration at the transmucosal region of titanium implants.** *Acta Biomater* 2021, **124**:33–49.
6. Su EP, Justin DF, Pratt CR, Sarin VK, Nguyen VS, Oh S, Jin S: **Effects of titanium nanotubes on the osseointegration, cell differentiation, mineralisation and antibacterial properties of orthopaedic implant surfaces.** *Bone Joint J* 2018, **100-b**:9-16.
7. Chernozem RV, Surmeneva MA, Krause B, Baumbach T, Ignatov VP, Prymak O, Loza K, Epple M, Ennen-Roth F, Wittmar A, et al: **Functionalization of titania nanotubes with electrophoretically deposited silver and calcium phosphate nanoparticles: Structure, composition and antibacterial assay.** *Mater Sci Eng C Mater Biol Appl* 2019, **97**:420–430.
8. Yu S, Guo D, Han J, Sun L, Zhu H, Yu Z, Dargusch M, Wang G: **Enhancing Antibacterial Performance and Biocompatibility of Pure Titanium by a Two-Step Electrochemical Surface Coating.** *ACS Appl Mater Interfaces* 2020, **12**:44433–44446.
9. Oh S, Daraio C, Chen LH, Pisanic TR, Finones RR, Jin S: **Significantly accelerated osteoblast cell growth on aligned TiO₂ nanotubes.** *J Biomed Mater Res A* 2006, **78**:97–103.
10. Zhang Y, Luo R, Tan J, Wang J, Lu X, Qu S, Weng J, Feng B: **Osteoblast behaviors on titania nanotube and mesopore layers.** *Regen Biomater* 2017, **4**:81–87.
11. Oh S, Brammer KS, Li YS, Teng D, Engler AJ, Chien S, Jin S: **Stem cell fate dictated solely by altered nanotube dimension.** *Proc Natl Acad Sci U S A* 2009, **106**:2130–2135.
12. Bjursten LM, Rasmusson L, Oh S, Smith GC, Brammer KS, Jin S: **Titanium dioxide nanotubes enhance bone bonding in vivo.** *J Biomed Mater Res A* 2010, **92**:1218–1224.
13. Frandsen CJ, Brammer KS, Noh K, Johnston G, Jin S: **Tantalum coating on TiO₂ nanotubes induces superior rate of matrix mineralization and osteofunctionality in human osteoblasts.** *Mater Sci Eng C Mater Biol Appl* 2014, **37**:332–341.

14. Peng Z, Ni J, Zheng K, Shen Y, Wang X, He G, Jin S, Tang T: **Dual effects and mechanism of TiO₂ nanotube arrays in reducing bacterial colonization and enhancing C3H10T1/2 cell adhesion.** *Int J Nanomedicine* 2013, **8**:3093–3105.
15. Amin Yavari S, Loozen L, Paganelli FL, Bakhshandeh S, Lietaert K, Groot JA, Fluit AC, Boel CH, Alblas J, Vogely HC, et al: **Antibacterial Behavior of Additively Manufactured Porous Titanium with Nanotubular Surfaces Releasing Silver Ions.** *ACS Appl Mater Interfaces* 2016, **8**:17080–17089.
16. Shivaram A, Bose S, Bandyopadhyay A: **Mechanical degradation of TiO₂ nanotubes with and without nanoparticulate silver coating.** *J Mech Behav Biomed Mater* 2016, **59**:508–518.
17. Stolzoff M, Burns JE, Aslani A, Tobin EJ, Nguyen C, De La Torre N, Golshan NH, Ziemer KS, Webster TJ: **Decreased bacterial growth on titanium nanoscale topographies created by ion beam assisted evaporation.** *Int J Nanomedicine* 2017, **12**:1161–1169.
18. Li Y, Yang Y, Li R, Tang X, Guo D, Qing Y, Qin Y: **Enhanced antibacterial properties of orthopedic implants by titanium nanotube surface modification: a review of current techniques.** *Int J Nanomedicine* 2019, **14**:7217–7236.
19. Liu Z, Liu X, Ramakrishna S: **Surface engineering of biomaterials in orthopedic and dental implants: Strategies to improve osteointegration, bacteriostatic and bactericidal activities.** *Biotechnol J* 2021, **16**:e2000116.
20. Djošić M, Janković A, Mišković-Stanković V: **Electrophoretic Deposition of Biocompatible and Bioactive Hydroxyapatite-Based Coatings on Titanium.** *Materials (Basel)* 2021, **14**.
21. Khoury F, Keeve PL, Ramanauskaite A, Schwarz F, Koo KT, Sculean A, Romanos G: **Surgical treatment of peri-implantitis - Consensus report of working group 4.** *Int Dent J* 2019, **69** Suppl 2:18–22.
22. Hasan J, Crawford RJ, Ivanova EP: **Antibacterial surfaces: the quest for a new generation of biomaterials.** *Trends Biotechnol* 2013, **31**:295–304.
23. Mahanta U, Khandelwal M, Deshpande AS: **Antimicrobial surfaces: a review of synthetic approaches, applicability and outlook.** *J Mater Sci* 2021:1–27.
24. Yuan L, Ding S, Wen C: **Additive manufacturing technology for porous metal implant applications and triple minimal surface structures: A review.** *Bioact Mater* 2019, **4**:56–70.
25. Wauthle R, Ahmadi SM, Amin Yavari S, Mulier M, Zadpoor AA, Weinans H, Van Humbeeck J, Kruth JP, Schrooten J: **Revival of pure titanium for dynamically loaded porous implants using additive manufacturing.** *Mater Sci Eng C Mater Biol Appl* 2015, **54**:94–100.
26. Han C, Wang Q, Song B, Li W, Wei Q, Wen S, Liu J, Shi Y: **Microstructure and property evolutions of titanium/nano-hydroxyapatite composites in-situ prepared by selective laser melting.** *J Mech Behav Biomed Mater* 2017, **71**:85–94.
27. Hu X, Xu R, Yu X, Chen J, Wan S, Ouyang J, Deng F: **Enhanced antibacterial efficacy of selective laser melting titanium surface with nanophase calcium phosphate embedded to TiO₂ nanotubes.** *Biomed Mater* 2018, **13**:045015.
28. Verma D, Garg PK, Dubey AK: **Insights into the human oral microbiome.** *Arch Microbiol* 2018, **200**:525–540.

29. Aguiar-Pulido V, Huang W, Suarez-Ulloa V, Cickovski T, Mathee K, Narasimhan G: **Metagenomics, Metatranscriptomics, and Metabolomics Approaches for Microbiome Analysis.** *Evol Bioinform Online* 2016, **12**:5–16.
30. Mombelli A, Lang NP: **Clinical parameters for the evaluation of dental implants.** *Periodontol 2000* 1994, **4**:81–86.
31. Lang NP, Joss A, Orsanic T, Gusberti FA, Siegrist BE: **Bleeding on probing. A predictor for the progression of periodontal disease?** *J Clin Periodontol* 1986, **13**:590–596.
32. Glavind L, Löe H: **Errors in the clinical assessment of periodontal destruction.** *J Periodontal Res* 1967, **2**:180–184.
33. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI: **QIIME allows analysis of high-throughput community sequencing data.** *Nature methods* 2010, **7**:335–336.
34. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ: **Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities.** *Applied and environmental microbiology* 2009, **75**:7537–7541.
35. Li R, Yu C, Li Y, Lam TW, Yiu SM, Kristiansen K, Wang J: **SOAP2: an improved ultrafast tool for short read alignment.** *Bioinformatics* 2009, **25**:1966–1967.
36. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q, et al: **Full-length transcriptome assembly from RNA-Seq data without a reference genome.** *Nat Biotechnol* 2011, **29**:644–652.
37. Tarazona S, García-Alcalde F, Dopazo J, Ferrer A, Conesa A: **Differential expression in RNA-seq: a matter of depth.** *Genome Res* 2011, **21**:2213–2223.
38. Young MD, Wakefield MJ, Smyth GK, Oshlack A: **Gene ontology analysis for RNA-seq: accounting for selection bias.** *Genome Biol* 2010, **11**:R14.
39. Mao X, Cai T, Olyarchuk JG, Wei L: **Automated genome annotation and pathway identification using the KEGG Orthology (KO) as a controlled vocabulary.** *Bioinformatics* 2005, **21**:3787–3793.
40. Team R: **RStudio: Integrated Development Environment for R.** Boston, MA.: RStudio Inc.,; 2015.
41. Fu JH, Wang HL: **Breaking the wave of peri-implantitis.** *Periodontol 2000* 2020, **84**:145–160.
42. Renvert S, Quirynen M: **Risk indicators for peri-implantitis. A narrative review.** *Clin Oral Implants Res* 2015, **26 Suppl 11**:15–44.
43. Daubert DM, Weinstein BF: **Biofilm as a risk factor in implant treatment.** *Periodontol 2000* 2019, **81**:29–40.
44. Chen Z, Wang Z, Qiu W, Fang F: **Overview of Antibacterial Strategies of Dental Implant Materials for the Prevention of Peri-Implantitis.** *Bioconj Chem* 2021, **32**:627–638.
45. Ganjian M, Modaresifar K, Zhang H, Hagedoorn PL, Fratila-Apachitei LE, Zadpoor AA: **Reactive ion etching for fabrication of biofunctional titanium nanostructures.** *Sci Rep* 2019, **9**:18815.

46. Vassallo E, Pedroni M, Silvetti T, Morandi S, Toffolatti S, Angella G, Brasca M: **Bactericidal performance of nanostructured surfaces by fluorocarbon plasma.** *Mater Sci Eng C Mater Biol Appl* 2017, **80**:117–121.
47. Jiang Q, Yu Y, Xu R, Zhang Z, Liang C, Sun H, Deng F, Yu X: **The temporal shift of peri-implant microbiota during the biofilm formation and maturation in a canine model.** *Microb Pathog* 2021, **158**:105100.
48. Qiao S, Wu D, Wang M, Qian S, Zhu Y, Shi J, Wei Y, Lai H: **Oral microbial profile variation during canine ligature-induced peri-implantitis development.** *BMC Microbiol* 2020, **20**:293.
49. Sanz-Martin I, Doolittle-Hall J, Teles RP, Patel M, Belibasakis GN, Hämmerle CHF, Jung RE, Teles FRF: **Exploring the microbiome of healthy and diseased peri-implant sites using Illumina sequencing.** *J Clin Periodontol* 2017, **44**:1274–1284.
50. Sahrmann P, Gilli F, Wiedemeier DB, Attin T, Schmidlin PR, Karygianni L: **The Microbiome of Peri-Implantitis: A Systematic Review and Meta-Analysis.** *Microorganisms* 2020, **8**.
51. Kensara A, Hefni E, Williams MA, Saito H, Mongodin E, Masri R: **Microbiological Profile and Human Immune Response Associated with Peri-Implantitis: A Systematic Review.** *J Prosthodont* 2021, **30**:210–234.
52. Resnik M, Benčina M, Levičnik E, Rawat N, Iglič A, Junkar I: **Strategies for improving antimicrobial properties of stainless steel.** *Materials (Basel)* 2020, **13**.
53. Camargo SEA, Xia X, Fares C, Ren F, Hsu SM, Budei D, Aravindraja C, Kesavalu L, Esquivel-Upshaw JF: **Nanostructured Surfaces to Promote Osteoblast Proliferation and Minimize Bacterial Adhesion on Titanium.** *Materials (Basel)* 2021, **14**.
54. Ji S, Choi YS, Choi Y: **Bacterial invasion and persistence: critical events in the pathogenesis of periodontitis?** *J Periodontol Res* 2015, **50**:570–585.
55. Bartold PM, Van Dyke TE: **An appraisal of the role of specific bacteria in the initial pathogenesis of periodontitis.** *J Clin Periodontol* 2019, **46**:6–11.
56. Moutsopoulos NM, Konkel JE: **Tissue-Specific Immunity at the Oral Mucosal Barrier.** *Trends Immunol* 2018, **39**:276–287.
57. Ivanovski S, Lee R: **Comparison of peri-implant and periodontal marginal soft tissues in health and disease.** *Periodontol 2000* 2018, **76**:116–130.
58. Xu R, Hu X, Yu X, Wan S, Wu F, Ouyang J, Deng F: **Micro-/nano-topography of selective laser melting titanium enhances adhesion and proliferation and regulates adhesion-related gene expressions of human gingival fibroblasts and human gingival epithelial cells.** *Int J Nanomedicine* 2018, **13**:5045–5057.
59. Qiao X, Yang J, Shang Y, Deng S, Yao S, Wang Z, Guo Y, Peng C: **Magnesium-doped Nanostructured Titanium Surface Modulates Macrophage-mediated Inflammatory Response for Ameliorative Osseointegration.** *Int J Nanomedicine* 2020, **15**:7185–7198.
60. Wang Q, Lu H, Zhang L, Yan X, Zhu B, Meng H: **Peri-implant mucositis sites with suppuration have higher microbial risk than sites without suppuration.** *J Periodontol* 2020, **91**:1284–1294.

Figures

Figure 1

SEM and EDS results of 3 groups.

SEM (scanning electron microscopy) images of MP (A & B), NT (D & E), and NTN samples (G & H) at different magnifications. EDS results showed the elemental compositions of MP (C), NT (F), and NTN (I) samples.

Figure 2

XPS spectra of 3 groups.

XPS spectra (Ti 2p, O 1s, Ca 2p and P 2p) of MP (A), NT (B) and NTN (C) samples.

Figure 3

Venn diagram of OTUs on the titanium surfaces of 3 groups.

Venn diagram showing the distributions of all 572 OTUs on the titanium surfaces within the sub-mucosal plaque niches (A) and the supra-mucosal plaque niches (B).

Figure 4

The relative abundance of sub-mucosal microbiota (A) and supra-mucosal microbiota (B) at the phylum level.

The X-axis indicates different groups; the Y-axis indicates the relative abundance of the phylum annotated. The phyla with the relative abundance of less than 0.5 % in the samples are merged into "Others" item.

Figure 5

Boxplots showed the Alpha diversity metrics in the sub-mucosal samples among groups.

The comparisons of the Alpha diversity metrics including Shannon (A), Simpson (B), ACE (C), Chao1 (D), sobs (E) and coverage (F). The error bars show the respective minimum and maximum values; the thick line represents the median value; the boxes demarcate the 1st to 3rd quartiles (Q1-Q3), and the abnormal value is shown as 'o'.

Figure 6

Boxplots showed the Alpha diversity metrics in the supra-mucosal samples among groups.

The comparisons of the Alpha diversity metrics including Shannon (A), Simpson (B), ACE (C), Chao1 (D), sobs (E) and coverage (F). The error bars show the respective minimum and maximum values; the thick

line represents the median value; the boxes demarcate the 1st to 3rd quartiles (Q1-Q3), and the abnormal value is shown as 'o'.

Figure 7

PCoA for each single site samples.

Principal coordinates analysis (PCoA) of the unweighted (A) and weighted (B) UniFrac distances for each of the single site samples. PCoA plot is coloured according to different titanium surface categories.

Figure 8

The top 30 mostly enriched GO terms between different groups.

The top 30 enriched GO terms of the up-regulated (A) and down-regulated (B) DEGs between NTB and MPB; the top 30 enriched GO terms of the up-regulated (C) and down-regulated (D) DEGs between NTNB and MPB; the top 30 enriched GO terms of the up-regulated (E) and down-regulated (F) DEGs between NTB and NTNB. The Y-axis represents GO terms. The X-axis represents the number of differential genes. The color of the bar represents the type of GO terms. "*" indicates corrected p value < 0.05.

Figure 9

The top 20 mostly enriched KEGG terms between different groups.

The top 20 enriched KEGG terms of the up-regulated (A) and down-regulated (B) DEGs between NTB and MPB; the top 20 enriched KEGG terms of the up-regulated (C) and down-regulated (D) DEGs between NTNB and MPB; the top 20 enriched KEGG terms of the up-regulated (E) and down-regulated (F) DEGs

between NTB and NTNB. The Y-axis represents KEGG terms. The X-axis represents the number of differential genes. The color of the bar represents the type of KEGG terms. "*" indicates corrected p value < 0.05 .

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [GraphicalAbstract.jpg](#)
- [Additionalfile1.docx](#)
- [Additionalfile2.xlsx](#)