Title
Nisin and its probiotic modulate oral pathogens on Titanium discs in peri-implantitis in vitro settings

Author
Brody, Hanna

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Nisin and its probiotic modulate Oral Pathogens on Titanium discs in peri-implantitis in vitro settings

by
Hanna Brody

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Approved:

DocuSigned by:
Yvonne Kapila
CE032F3A4E984CF...

Yvonne Kapila
Chair

DocuSigned by:
Guo-Hao Lin
DocuSigned by:

Guo-Hao Lin

DocuSigned by:
Yogalakshmi Rajendran
7478C3EBE4A74BB...

Yogalakshmi Rajendran

Committee Members

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Abstract: Nisin and its probiotic modulate Oral Pathogens on Titanium discs in peri-implantitis in vitro settings

Hanna Brody

Peri-implantitis is characterized by chronic inflammation of the peri-implant supporting tissues that progressively and irreversibly leads to bone loss and consequently, implant loss. Similar to periodontal disease, oral dysbiosis is thought to be a driver of peri-implantitis. However, man-aging peri-implantitis with traditional treatment methods, such as non-surgical debridement or surgery, is not always successful. Thus, novel strategies have been proposed to address these shortcomings. One strategy is the use of probiotics as antimicrobial agents since they are considered safe for humans and the environment. Specifically, the probiotic Lactococcus lactis, produces nisin, which has been used worldwide for food preservation. The objective of this study was to determine whether nisin and a nisin-producing L. lactis probiotic can disrupt oral pathogenic biofilms and promote a healthier oral microbiome within these oral biofilms on titanium discs. Using confocal imaging and 16S sequencing, this study revealed that nisin and the L. lactis probiotic disrupt oral pathogenic biofilms in a peri-implantitis setting In vitro. Further, both treatments shift the composition, relative abundance, and diversity of the microbiome within these biofilms towards healthy control levels. Thus, nisin and its nisin-producing L. lactis probiotic may be useful in treating peri-implantitis by promoting healthier oral biofilms, which may be useful for im-proving patient oral health.

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Introduction

Since the beginning of the mankind, humans have tried to replace their missing teeth. It is well documented that the ancient Egyptians, in 2500BCE, stabilized periodontally missing tooth using ligatures made of golden wires[1]. Yet, the introduction of the two-stage threaded titanium root-form implant in the late 1960's revolutionized both dental implant research and industry by facilitating long-term implants through osseointegration of the patient bone to the titanium surface of the implant[1,2].

Modern dental implants consist of four main parts (Figure 1) – the crown, abutment, abutment screw, and implant post, which can be further divided into the collar and the screw[3]. The crown is usually fabricated from metal and ceramic[3]. The abutment and the implant post are made of titanium alloy, typically Titanium alloy Grade 5, which is a titanium alloy strengthened by the inclusion of 6% Aluminum and 4% Vanadium (i.e., Ti-6Al-4V). This alloy has optimal osseointegration, but it can be texturized to improved properties. Among these textures, the Laser-Lok® surface, in particular, is made of optimally sized microchannels on the implant post collar to attach and organize both osteoblasts and fibroblasts, eliciting inhibition of epithelial downgrowth and increased attachment of bone and connective tissues to the implant[4–7].

This structure made modern dental implants to become so successful that recent studies indicate an increase of implant prevalence by 8-fold in the partially edentulous US population in 2015-2016 compared to 1999-2000, with an average covariate-adjusted increase in dental implant prevalence of 14% per year, achieving a market share of USD 4.3 billion dollars in 2021[8,9]. Within the US population, implant prevalence is estimated to reach 23% by 2026, and a revenue forecast of USD 8 billion by 2028[8,9]. Drivers of this trend include, but are not limited to, the increased size of the geriatric population and the prevalence of tooth-related diseases, such as dental caries and periodontitis. Among these tooth-related diseases, peri-implant diseases are an emerging area of interest, as partial edentulism is not limited

to missing natural teeth, but also loss of dental implants. Peri-implant mucositis and peri-implantitis remain an issue for dental implant morbidity and loss.

Mirroring the periodontal disease progression[10], peri-implant mucositis is the first stage of peri-implant disease, characterized by the inflammation restricted to the peri-implant soft tissues without bone loss; while peri-implantitis is the later, more chronic phase, characterized by the chronic inflammation of the peri-implant mucosa that progressively and irreversibly damages the implant supporting tissues, to the point of bone resorption, decreased osseointegration, increased pocket formation, purulence, bone loss and, consequently, implant loss[2,11–13].

Frequently, a spectrum of bacteria is detected in peri-implant lesions and recent data have indicated that peri-implantitis correlates to an unbalanced oral microbiome state similar to periodontitis, known as dysbiosis[13,14]. The oral microbiome is complex ecosystem holding up to a thousand microbial species that thrives in a very dynamic environment – the oral cavity – stablishing several host-microbiome interactions with us, humans, known as oralome[10,15]. These microorganisms live in a symbiotic/commensal relationship with the human host, assisting with several benefits to the host, including the development and priming of the host defense system, regulation of the gastrointestinal and cardiovascular system, nutrient absorption and energy regulation[10,15–19].

Despite its resilience, certain perturbations seems to shift the oralome into a unbalanced state, known as dysbiosis. This dysbiosis seems to be increasingly related to severe human diseases, including atherosclerosis, Alzheimer's disease and cancer, as well as, caries, periodontal disease and peri-implantitis[10,14,15]. On peri-implantitis, specifically, a systematic review including 29 reports have found similar pathogens in peri-implantitis and periodontitis lesions, such as *Tannarella forsythia, Porphyromonas gingivalis,* several *Fusobacterium species, including Fusobacterium nucleatum, Prevotella intermedia, Prevotella nigrescens* and *Aggregatibacter actinomycetemcomitans*[20]. Others, on

the other hand, argue that peri-implantitis is more complex and do have more distinct microbiological profile from periodontitis, such as higher *Treponema denticola*, Staphylococci, *Peptostreptococci*, *Streptococci,* enterics, and several yeasts species on peri-implantitis compared to periodontitis[20–23]. Some studies, also suggest that the lack of periodontal ligament and cementum makes the peri-implant tissues more susceptible to infection and trauma, which leads to a faster progression of the disease, compared to periodontitis[21].

In this context, the aim for the treatment of peri-implant disease is disease resolution, with no further loss of support[24]. There are currently three main treatment modalities for peri-implantitis – nonsurgical debridement, resective treatment with or without implantoplasty, and reconstructive treatment[25]. Nonsurgical therapy can include curettage, ultrasonic use, locally delivered agents, such as chlorhexidine or antibiotics, or systemic antibiotics. While nonsurgical therapy can lead to minimal soft tissue recession or discomfort, it is often inefficient in surface detoxification, and mainly used to treat more mild cases of peri-implant mucositis[25]. While predictably reducing pocket probing depths around implants, resective treatment such as surgical debridement can lead to further loss of hard and soft tissues[26]. Reconstructive treatment is extremely sensitive to defect containment and often requires submerged healing[27]. Use of local or systemic antibiotics on the other hand, may induce further dysbiosis in oral and gut microbiomes, which both microbiotas may not able to recover from[28,29]. For all treatment options, great variability has been reported in outcomes mainly attributed to patient factors, defect morphology, and the reconstructive methods used, there is currently no major consensus on the treatment of peri-implant disease[30]. Thus, addressing the etiology of peri-implant disease is paramount to successful resolution and also highlights the urgent need for new strategies to treat it.

One of these new strategies is the use of bacteriocins and probiotics to assist in mitigating this dysbiosis by suppressing oral pathogens within these communities [10,31–33]. Recently, the potential for using a nisin bacteriocin and nisin probiotic in biomedical applications has been highlighted[10,15,32–34]. Nisin is a class I

lantibiotic bacteriocin produced by the Gram-positive bacteria *Lactococcus lactis*, which contains 34 amino acids in a penta-cyclic structure[10,33]. Nisin is active against both gram-positive and gram-negative bacteria, including *Streptococcus aureus*, *Listeria monocytogenes*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and *Treponema denticola*[33,35]. Nisin itself and nisin-expressing *Lactococcus lactis spp.* have been used successfully to abrogate infections associated with drug-resistant pathogens, gastrointestinal infections, respiratory tract infections, skin and soft tissue infections, mastitis, head and neck cancer (HNC), and other oral diseases using *in vitro* and *in vivo* models[10,15,31,32,34,36]. In addition, studies led by our group demonstrate nisin and its probiotic (Nisin-producer *Lactococcus lactis*) dose-dependently abrogate the growth of pathogens associated with periodontal disease in their planktonic state, and dose-dependently modulate pathogenic human-derived *in vitro* oral biofilms towards health, without inducing cytotoxicity to human oral cells[31,35]. Despite these advances, we were unable to find any other studies in literature testing nisin and its probiotic on peri-implantitis settings, indicating this may be the first-time testing nisin and the probiotic on peri-implantitis settings.

Therefore, the objective of this study was to test whether the nisin and its probiotic form (nisin-producing *Lactococcus lactis*) can disrupt peri-implantitis pathogenic oral biofilms and drive implant biofilms to health.

Materials and Methods

Nisin solution preparation

NisinZ®P powder (Handary, Belgium), which has >95% purity, were gently mixed in Mili-Q water at 5mg/mL in a 15mL tube covered in aluminum foil. The tube was, then, placed on a rotator and mixed for 4h to completely solubilize Nisin. Finally, the solution was filtered using a 0.22µm syringe filter before use to remove any potential nisin crystals in the solution.

Human Saliva Collection and Informed Consent

Human saliva collection was approved by the University of California San Francisco Institutional Review Board (IRB #17-21912, Reference #186994, approved on April 25th, 2017). The collection protocol was previously published by our group[31,35]. Briefly, ten healthy volunteers with no known health issues verbally consented to donate saliva for this study. No information from the volunteers was collected at any time prior to or at the time of saliva donation. Prior to the collection, the volunteers were informed not to eat, drink and/or smoke for, at least, 30 minutes before the donation. They were comfortably seated and given a sterile tube for saliva collection. About 10-15mL of saliva was obtained from each volunteer. All the collected saliva was pooled, centrifugated (10,000 x RPM for 30 minutes) and separated into a Cell-Containing Saliva (CCS) and Cell-Free Saliva (CFS). CCS was used as the biofilm inoculum, and it was obtained by adding glycerol (50% v/v) to the precipitate of the centrifuged pooled saliva and stored at -80ºC. CFS was used as biofilm media and it was obtained by collecting supernatant of the centrifuged pooled saliva, diluted with sterile Phosphate Buffer Saline (PBS) (1:4 v/v) and stored at -80ºC.

Bacteria and Biofilm Growth

Treponema denticola (ATCC 35405), *Porphyromonas gingivalis* (ATCC 33277), and *Fusobacterium nucleatum* (ATCC 25586) were grown as described previously [37–39]. *T. denticola* was cultured in Oral Treponeme Enrichment Broth (OTEB; Anaerobe Systems, USA), while *P. gingivalis* and *F. nucleatum* were cultured in Brain-Heart Infusion broth (BHI; Sigma-Aldrich, USA) supplemented with hemin (5 µg ml^{-1}) and vitamin K (1 µg ml^{-1}) under anaerobic conditions. Anaerobic conditions were obtained by placing bacterial samples into sealed anaerobic jars that underwent five cycles of depressurization (vacuum formation) and Nitrogen (N_2) pressurization (1 ATM) and kept in a 37ºC in a Fisher-Scientific Isotemp Incubator. The bacteria were split every 3-7 days. Strains were passaged at least once before using in the experiments to ensure log-phase growth. Previous literature determined that a 0.1 absorbance at 600nm of *Treponema denticola*[37], *Porphyromonas gingivalis*[40,41], or *Fusobacterium nucleatum*[42] suspention contains $2.4x10^8$ CFU/mL, $2.4x10^8$ CFU/mL, and $1x10^8$ CFU/mL of each the bacteria,

respectively. Purity of the cultures were confirmed by 16S sequencing prior to their use in the experiments.

Nisin-producer *Lactococcus lactis* (ATCC 11454) and non-nisin-producer *L. lactis* (gift from Cork Institute of Technology, Ireland) strains were grown in BHI at 37°C with shaking and under aerobic conditions in an Eppendorf G24 Environmental Incubator Shaker and passaged every 2-3 days. To ensure nisin production, strains were passaged at least once before using in the experiments. Previous literature determined that a 0.1 absorbance at 600nm of the *L. lactis* suspension contains $6x10^7$ CFU/mL of the bacterium[43].

The human saliva-derived oral biofilms were grown by adding 20µL of CCS to 600µL of CSF per well in 24-well plates containing one 13mm diameter by 1mm thickness grade V (Ti-6Al-4V) titanium disc with either RBT or the Laser-Lok® surface textures (Biohorizons, USA). The plates were, then incubated under aerobic conditions for 48h at 37°C in a humidified Thermo-Fisher Forma Series II Incubator. CFS media was changed every 24h. For the pathogenic-spiked biofilms, 24h preformed biofilms were spiked with $6x10^5$ CFU/mL of each periodontal pathogen (i.e., *Treponema denticola*, *Fusobacterium nucleatum,* and *Porphyromonas gingivalis)* and incubated under aerobic conditions for another 24h.

Oral Biofilm Disruption

The pre-formed oral biofilms were challenged with either *L. lactis* strains ($6x10^3$ and 10^5 CFU/mL) or nisin (1, 10 or 50µg/mL) for 24h. Biofilms were then, fixed for 5 min using paraformaldehyde (4% in PBS). Next, the samples were stained for 15min using LIVE/DEAD BacLight Bacterial Viability Kit (ThermoFisher Scientific, USA). This kit uses two dies – Syto9, which is membrane permeable, thus staining all bacterial cells, and Propidium Iodide, which is membrane impermeable, thus staining only cells with damaged cell membranes. Propidium Iodide have a higher DNA affinity factor, thus displacing Syto9, in case of both dyes are competing for the site[31]. Then, the samples were washed three-times with

PBS to remove the stain excess and mounted on slides, by adding a small drop of mounting media (Agilent, USA) on top of the Titanium discs and a 13mm coverslip was added on top of the disc to seal the biofilms. Finally, the mounted biofilms fluorescence was observed using a TCS SP8 X Confocal Microscope (Leica, Germany).

16S RNA Sequencing

The pre-formed oral biofilms were challenged with either *L. lactis* strains ($6x10^3$ and 10^5 CFU/mL) or nisin (1 or 10μg/mL) for 24h. Then, the samples DNA were extracted using a QIAmp DNA Mini Kit (Qiagen, USA), according to the manufacturer protocol for bacterial samples. Next, the samples extracted were submitted to Novogene (USA) for 16S Sequencing.

Statistical Analysis

Oral biofilm disruption, biofilm richness, diversity and unweighted UniFrac indexes data were analyzed by one-way ANOVA and intergroup differences were analyzed by Tukey's post hoc test (p significant values are displayed in figure legends). Phyla and Genus OTU data were analyzed by two-way ANOVA and intergroup differences were analyzed by Tukey's post hoc test (p significant values are displayed in figure legends). Oral biofilm disruption experiment was performed two times in triplicates and results are presented as mean ± SD. 16S sequencing experiment was performed two times in duplicates. Phyla and genus OTU results are presented as means ± SD, while richness, diversity and unweighted UniFrac indexes are presented as box-plots (medians, minimums and maximums).

Results

Nisin-Producing Probiotic Disrupts Oral Biofilm on Titanium Discs

We started by growing the biofilms on top of titanium discs (no pathogens) for 48h, we tested the ability of nisin (Figure 1A), wild-type nisin-producing *Lactococcus lactis* (WT *L. lactis*, Figure 1B) and non-

nisin-producing *Lactococcus lactis* (NN *L. lactis*, Figure 1C) to abrogate the grown biofilms. Then, the obtained images were semi-quantified and biofilm viability were obtained (Figure 1D). The control biofilm reported 84.15±3.35 % of viability. Nisin significantly decreases the biofilm viability dose-dependently to 74.72±6.10% and 58.80±5.31%, respectively. Similarly, WT *L. lactis* significantly decreases the biofilm viability dose-dependently to 71.37±4.32 % and 65.55±6.18%, respectively. Interestingly, both concentrations of NN *L. lactis* significantly decreases the biofilm viability to 64.64±5.25% and 69.29±3.77%, compared to control, although not dose-dependently.

Then, we spiked the biofilms with known peri-implantitis pathogens, namely T. denticola, F. nucleatum and P. gingivalis, and tested the ability of nisin (Figure 2A), WT *L. lactis* (Figure 2B) and NN *L. lactis* (Figure 2C) to abrogate this pathogenic-spiked biofilms. The obtained images were semi-quantified and biofilm viability were obtained (Figure 2D).

Similar to previous test, the control biofilm reported a viability of 83.04±3.00%. Interestingly, spiking the pathogen to the control biofilm significantly decreases its viability to 62.53±3.69%. Nisin is able to decrease the viability of the pathogenic-spiked biofilm dose-dependently to 54.26±3.35% and 44.88±2.98%. Similarly, 10^5 CFU/mL of WT *L. lactis* were able significantly decrease it to 52.45±3.41%. On the other hand, 10^3 CFU/mL of WT *L. lactis* and both concentrations of NN *L. lactis* did not decreased the pathogen-spiked biofilm viability significantly.

Microbiome Sequencing of Titanium-Derived Oral Biofilms Reveals Unique and Divergent Species Upon Spiking with Pathogens

Next, we 16S sequenced the biofilms (both control and pathogen-spiked), as well as the biofilms after treatments. Initially, we compared the healthy control biofilm to the pathogen-spiked one (Figure 3). Fascinatingly, we found that both biofilms shared 159 genera and 71 species and diverged in 31 genera

and 16 identified species – 11 genera and 8 identified species were unique to the healthy control biofilm and 20 genera and 8 identified species were unique to the pathogen-spiked biofilm. A list containing these divergent genera and species can be found on Table 1.

Nisin and L. lactis Probiotic Shift Specific Phyla and Genera in Pathogen-Spiked Oral Biofilms Back Towards Control Levels

Next, we evaluated Phyla relative abundance in the samples (Figure 4). The healthy control biofilm reported a high dominance of the Proteobacteria phylum (77.5±3.4%), followed by Firmicutes (14.4±1.5%), Actinobacteriota (4.1±0.7) and Bacteroidota (3.9±1.6%) phyla. Compared to healthy control biofilm, the pathogen-spiked biofilm showed a significant increase on the Proteobacteria (to 80.5±2.9%) and Fusobacteriota phyla levels (from 0.7±0.6% to 2.75±0.9%), followed by a significant decrease on the Firmicutes phylum levels (to 11.6±1.6%). Although the Bacteroidota phylum seems to be decreased on the pathogen-spiked biofilm, compared to the health control, the decrease is not statistically significant. Remarkably, 1µg/mL of Nisin, 103CFU/mL of WT and NN *L. lactis* were able to revert the pathogens changes to the Proteobacteria (to 75.6±2.0%, 78.0±2.8%, and 75.1±5.3% respectively) and Firmicutes (to 15.4±1.3%, 13.8±1.8%, and 13.7±2.6%, respectively) phyla, similar to control levels. Additionally, Nisin 1µg/mL and NN *L. lactis* 10^3 CFU/mL were able to decrease the p-value on the Fusobacteria phylum added by the pathogen-spiked biofilm (from p=0.0062 (pathogen-spiked) to p=0.0130 and p=0.0296, respectively), although it is still significantly different than the healthy control.

Interestingly, 10µg/mL of Nisin seems to significantly over-suppress the Proteobacteria phylum (to 73.5±1.3%), compared to control, while significantly over-expanding the Firmicutes relative abundance (to 17.8±0.7%), compared to control. A similar significant effect seems to be caused by 10^5 CFU/mL of NN L. lactis to the Proteobacteria phylum (further reduced to 74.1±2.8%, compared to control).

Intriguingly, 10^5 CFU/mL of WT *L. lactis* does not affect the pathogen-spiked Proteobacteria/Firmicute pattern. Other changes in the rest of Top 10 phyla are not statistically significant, compared to the healthy and pathogen-spiked controls.

Next, we evaluated Genera relative abundance in the samples (Figure 5). The healthy control seems to be co-dominated by Serratia (36.3±5.1%) and Stenotrophomonas (26.2±3.1%) genera, followed by Streptococcus (11.1±0.8%), Neisseria (6.0±1.3%), Rothia (3.9±0.7%), and Porphyromonas (3.0±1.5%). Compared to the healthy biofilm, the pathogen-spiked biofilm showed a significant increase in the Serratia levels (to 41.9±7.2), overly dominating the biofilm. Except for WT *L. lactis* 10^5CFU/mL, all treatments were able to singificantly decrease Serratia levels back to healthy control levels. Similarly to Phyla relative abundance, WT *L. lactis* 10^5CFU/mL seems to further increase Serratia levels (to 50.7±9.1%). Other changes in the rest of Top 10 genera are not statistically significant, compared to the healthy and pathogen-spiked controls.

Nisin and L. lactis Probiotic Shift alpha and beta diversity indices, as well as the Principal Coordinate Analysis (PCoA) distances in Pathogen-Spiked Oral Biofilms Back Towards Control Levels

Next, we analyzed the alpha diversity of the biofilms. For this analysis, we evaluated species richness (Figure 6A), Shannon Diversity (Figure 6B), Phylogenic Diversity (Figure 6C) and UniFrac Index (Figure 6D).

Species richness is, basically, the number of species within a defined area or sample[44]. In our case, healthy control biofilm reported a median of 155.5 (155.0-160.0) species, while the pathogen-spiked biofilm reported a slight increase to 157.0 (155.0-169.0) species; although the difference is not statistically significant. Except for WT *L. lactis* 10^3CFU/mL, all treatments were able to slightly decrease the species richness medians, with a tendency of turning back to control levels. Interestingly, WT *L. lactis*

10^3 CFU/mL reported a median of 168.5 (148.0-171.0) species. Nonetheless, these changes were not statistically significant.

Shannon's diversity index takes into account, not only species richness, but also the proportion of each species in an ecosystem studied, thus giving a better description of the ecosystem's diversity compare to species richness only[45]. In our case, healthy control biofilm reported a diversity index median of 3.487 (3.354-3.622), while the pathogen-spiked biofilm reported a slight index decrease to 3.212 (2.977-3.603). Except for WT *L. lactis* 10^5 CFU/mL, all treatments showed a slight tendency to increase Shannon's diversity index, back to control levels. Interestingly, WT *L. lactis* 10^5 CFU/mL reported an index median of 3.055 (2.497-3.353). Again, none of these changes were statistically significant.

Phylogenetic diversity (PD) index takes a different approach towards diversity, as it describes the total amount of phylogenetic distance among species within a community[46]. Phylogenetic distance measures provide far more power than abundance measures, as they exploit the degree of divergence between different sequences[47]. In our case, healthy control biofilm reported a PD Index of 11.87 (11.30-12.08), while the pathogen-spiked biofilms reported a slight increase to 13.35 (11.94-14.48) in the index. All the treatments show a decrease trend in the median index compared to the pathogen-spiked biofilm, although, none of these changes were statistically significant.

The unique fraction metric (UniFrac) measures the phylogenetic distance in the phylogenetic tree as the fraction of the branch length of the tree between two samples or regions. In this sense, UniFrac is a Beta-diversity index. There are two versions of UniFrac - Unweighted UniFrac metric considers only species presence and absence information and counts the fraction of branch length unique to either community, while weighted UniFrac metric weights the branch length with species abundance information. Unweighted UniFrac index is most efficient in detecting abundance change in rare lineages[47]. In our case, the healthy control biofilm reported an UniFrac metric of 0.22 (0.21-0.24), while the pathogen-spiked

biofilm reported a slight increase to 0.30 (0.26-0.32) compared to healthy control biofilm. All treatments were able to slightly decrease this index back to control levels, although, none of these changes were statistically significant.

Finally, we analyzed the Unweighted Unifrac distances via Principal Coordinate Analysis (PCoA) for nisin (Figure 7A), WT *L. lactis* (Figure 7B) and NN *L.lactis* (Figure 7C). Basically, PCoA transforms the original UniFrac metrics into a new set of orthogonal axes[48]. In the resulting plot, samples coordinated closer to one another are more similar than those ordinated further away. Even though the pathogen-spiked biofilm has some similarities with the healthy control biofilm, its PCoA area seems to be spread out of the healthy control levels. Nisin 1µg/mL, WT *L. lactis* 10^5 CFU/mL an both NN *L. lactis* concentrations were able to significantly reduce the pathogen-spiked area back to the healthy control area. Interestingly, Nisin 10µg/mL and WT *L. lactis* 10^3 CFU/mL are able to partially reduce the sample spread area, but not completely.

Discussion

Oral biofilms play an essential role both in the development of natural oral physiology and defense of the host[10,49]. Because of these important roles, an imbalance in the oral microbiome or dysbiosis is associated with a variety of oral and systemic diseases, including Alzheimer's disease, head and neck cancer, periodontal disease, caries, and peri-implantitis[10,14,15]. Although antibiotics can be used to address this, recent data indicate that antibiotics may even induce dysbiosis in such a way that the oral mcirobiota cannot recover from [28,29]. Peri-implant diseases are biofilm mediated, and exacerbation of disease can lead to loss of implants over time. Thus, new strategies to modulate oral biofilm dysbiosis, especially those associated with peri-implantitis are needed.

Lactobacillus reuteri is one of the most studied probiotic as peri-implantitis treatment in the literature. The probiotic has been shown to have antagonistic effects towards peri-implantitis pathogens[23], yet, a

recent metadata analyses demonstrated that the bacterium still provide limited benefits to patients with peri-implant mucositis or peri-implantitis[50,51].

Interestingly, our group have demonstrated that nisin-producer WT *L. lactis* inhibit and disrupt *in vitro* human-derived oral biofilms and shift the bacterial population towards health[31,35]. Additionally, the probiotic has been considered was safe and well tolerated by head and neck patients receiving chemotherapy[52]. However, we were unable to find any studies testing the probiotic or nisin in peri-implantitis settings. Therefore, the objective of the current study was to test whether nisin and its probiotic (WT *L. lactis*) can disrupt pathogenic peri-implantitis biofilms and shift it back to a healthier state *in vitro*.

Overall, our results point out that both nisin and WT *L. lactis* are able not only to reduce the viability of pathogen-spiked biofilms, but also revert the pathogen-spiked biofilms changes, including significantly restoring Proteobacteria and Firmicutes phyla relative abundance to healthy control levels; significantly restores Serratia genus back to healthy control levels, and driving richness and diversity indexes and metrics back to healthy control levels.

As WT *L. lactis* probiotic have a double action on biofilms - it produces nisin, which by itself can abrogate oral biofilms[31,35], and the bacterium may compete for nutrients and space inside the biofilms[31] –, we added a control *L. lactis* which does not produces nisin (Non-nisin-producer *L. lactis*; NN *L. lactis*). This probiotic control would affect the biofilms only through nutritional and space competition, removing the overall effects of nisin with the treatment. Even though NN *L. lactis* was not able to significantly decrease the pathogen-spiked biofilm, as their wild-type probiotic, it was still able to drive significant changes to the pathogen-spiked biofilm relative abundance. These data point out that the viability effect may be solely or in great part due to nisin, while probiotic competition may play a greater part in driving relative abundance changes to the pathogenic-spiked biofilm. Interestingly, a similar effect was found in

our previous study on periodontitis, where, again, NN *L. lactis* were not able to significantly decrease the viability of the biofilms, while still driving changes in the relative abundance and diversity index[31]. All this indicate how important it is to evaluate bacterial competition, especially when working with bacteriocin-producer probiotics.

Conclusion

We conclude that the nisin-producing probiotic *L. lactis* and its purified bacteriocin, namely, nisin, can disrupt oral biofilms, return the peri-implantitis dysbiotic biofilm relative abundance and diversity indexes back to healthy control levels. Therefore, both nisin and its probiotic may be useful to promote healthier oral biofilms to peri-implantitis patients, improving patient's oral health.

Table 1 – Distinct genera and species found between control and pathogen-spiked biofilms

Control Biofilms		Pathogen-Spiked Biofilms	
Genera	**Species**	**Genera**	**Species**
Selenomonas	*Veillonellaceae bacterium*	Bifidobacterium	*Bifidobacterium dentium*
Oribacterium	*Oribacterium sp.*	Selenomonas	*Schwartzia sp.*
Filifactor	*Filifactor alocis*	Veillonella	*Veillonella sp.*
Rikenellaceae RC9 gut group	*Bacteroidales oral*	Shuttleworthia	*Shuttleworthia satelles*
Chryseobacterium	*Chryseobacterium soldanellicola*	Massilia	*Massilia timonae*
Slackia	*Slackia exigua*	Gracilibacteria	*Gracilibacteria bacterium*
Prevotella	*Prevotella loescheii*	Prevotella	*Prevotella saccharolytica*
Treponema	*Treponema socranskii*	Treponema	*Treponema lecithinolyticum*
Pelomonas	-	Fusobacterium	-
Gemella	-	Phenylobacterium	-
Comamonas	-	Olsenella	-
		Reyranella	-
		Pseudarcicella	-
		Mycoplasma	-
		Family XIII UCG-001	-
		Bacteroides	-
		Fretibacterium	-
		Rheinheimera	-
		Chloroplast	-
		Campylobacter	-

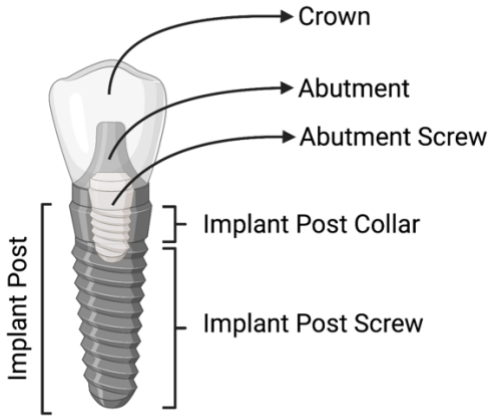

Figure 1 – Dental Implant Structure.

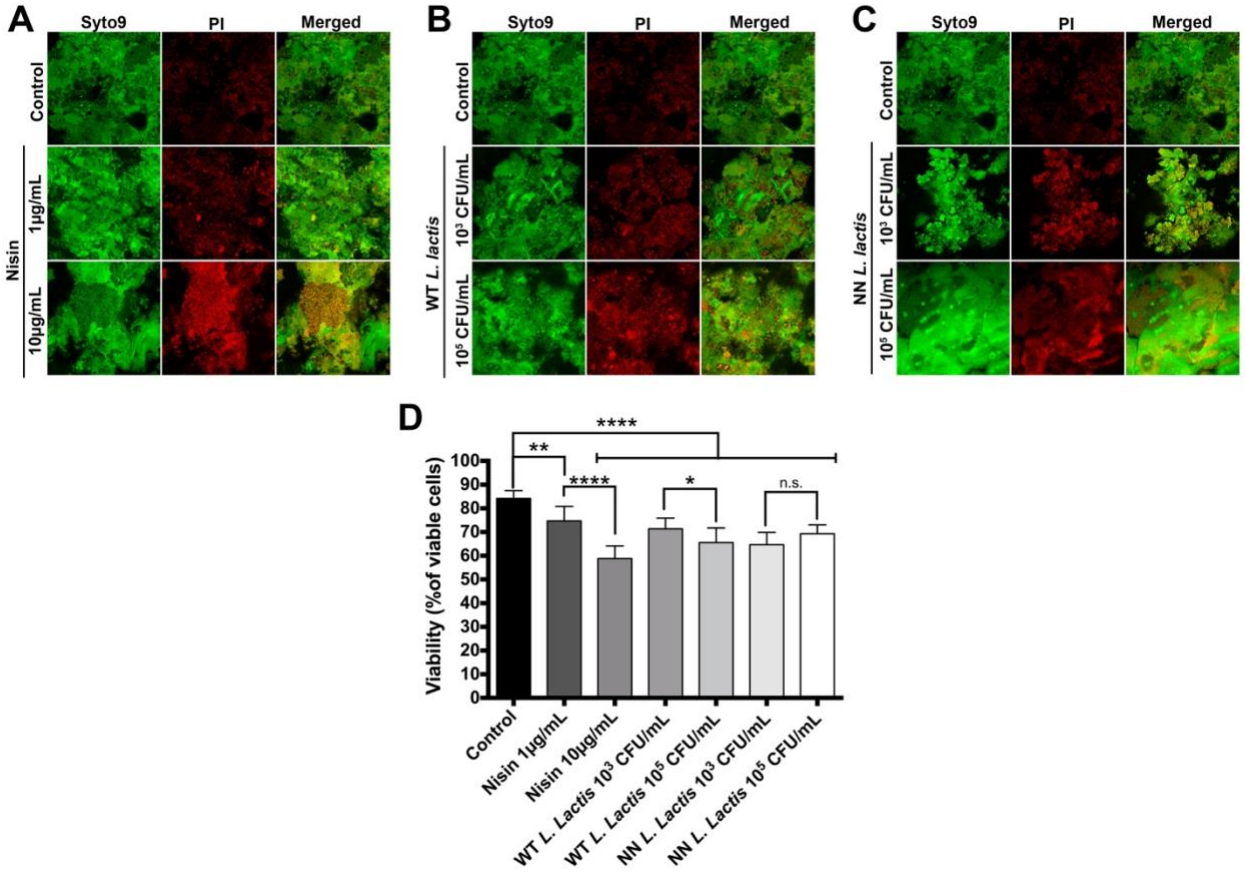

Figure 2 – Nisin effectively disrupts oral biofilms on Laser-Lok® grade V titanium discs. Biofilms were treated with either nisin (A), WT *L. lactis* (B) or NN *L. lactis* (C) for 24 h. (A-C) Representative images of fluorescently labeled biofilms are shown. The columns, from left to right, represent the different staining dyes/protocols; SYTO9 is a live cell stain, whereas propidium iodide (PI) is a dead-cell stain, and the merged image shows the overlap of both stains. The rows represent the different treatments. (D) Quantification of the bacterial viability assessed from the confocal images is shown. n.s. means not significant; *means $p \leq 0.05$; ** means $p \leq 0.01$; and **** means $p \leq 0.0001$ between the marked samples.

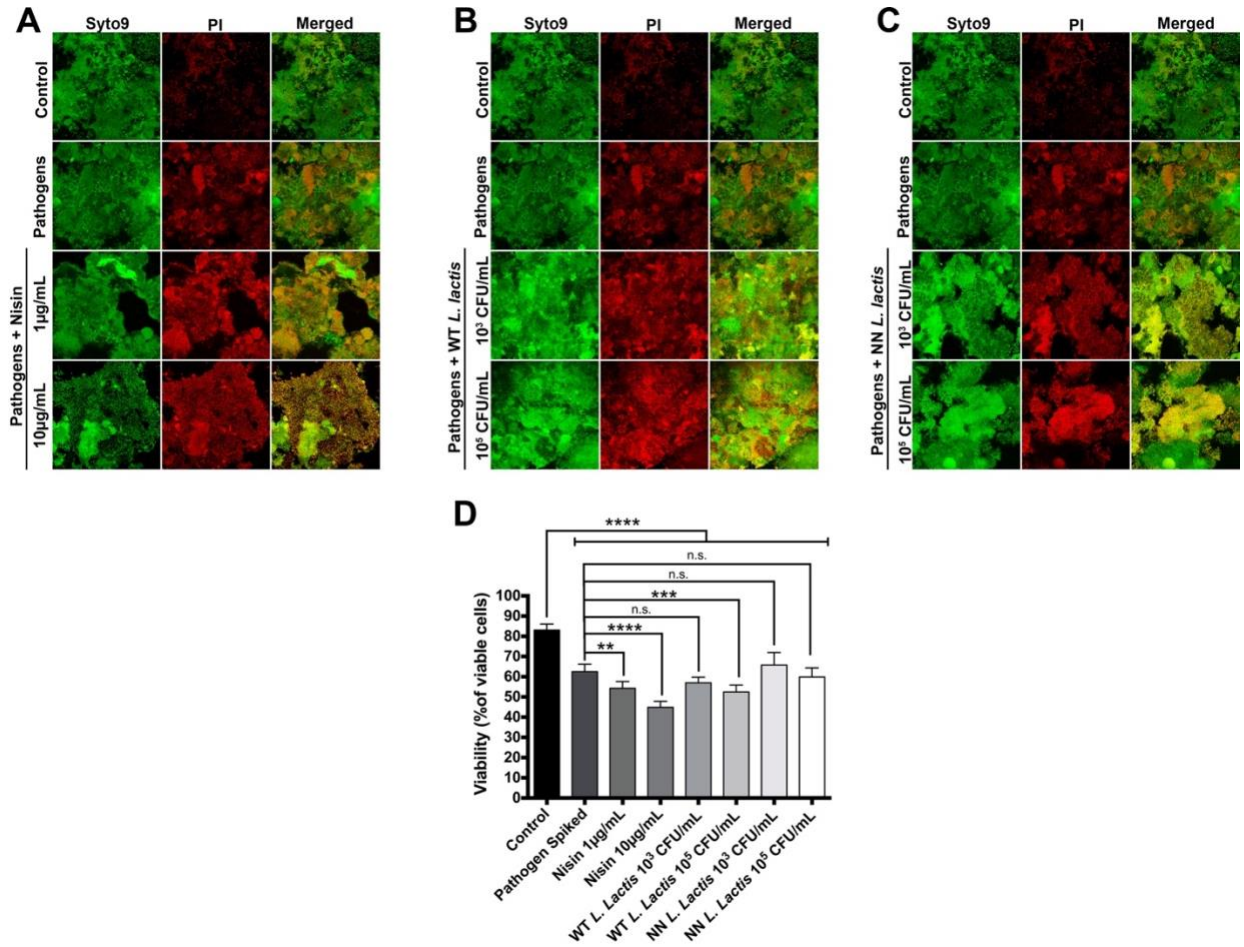

Figure 3 – **Nisin effectively disrupts pathogen-spiked oral biofilms on Laser-Lok® grade V titanium discs.** Pathogen-spiked biofilms were treated with either nisin (A), WT *L. lactis* (B) or NN *L. lactis* (C) for 24 h. (A-C) Representative images of fluorescently labeled biofilms are shown. The columns, from left to right, represent the different staining dyes/protocols; SYTO9 is a live cell stain, propidium iodide (PI) is a dead-cell stain, and the merged images shows the overlap of both stains. The rows represent the different treatments. (D) Quantification of bacterial viability assessed from the confocal images is shown. n.s. means not significant; *means $p \leq 0.05$; *** means $p \leq 0.001$; and **** means $p \leq 0.0001$ between the marked samples.

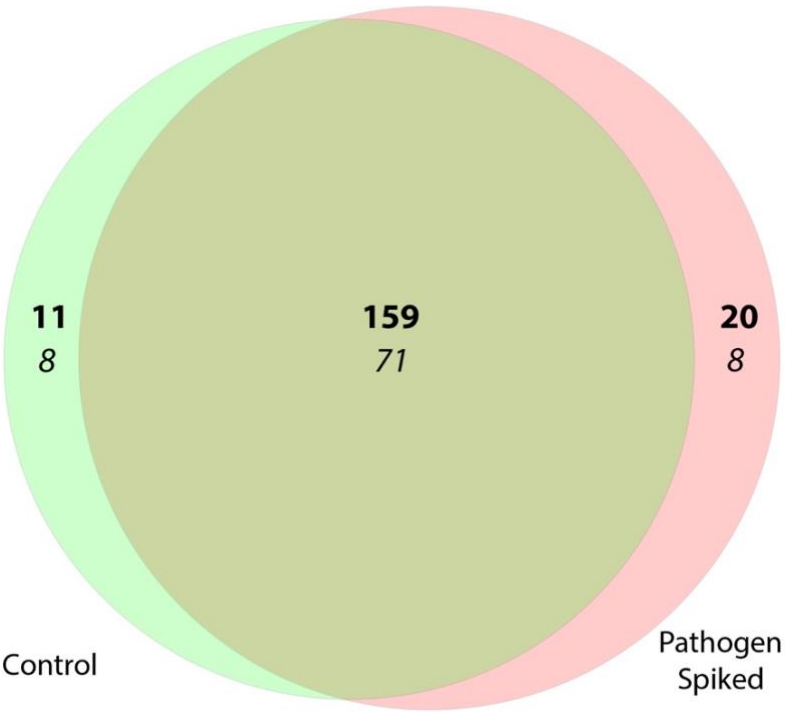

Figure 4 – **Distinct species and genera found in the pathogen-spiked biofilms com-pared to control biofilms.** Venn diagram showing the comparisons of the microbiome of the different biofilms with the number of genera (bold) and species (italic) found in the control biofilms (green) and in the pathogen-spiked biofilms (red).

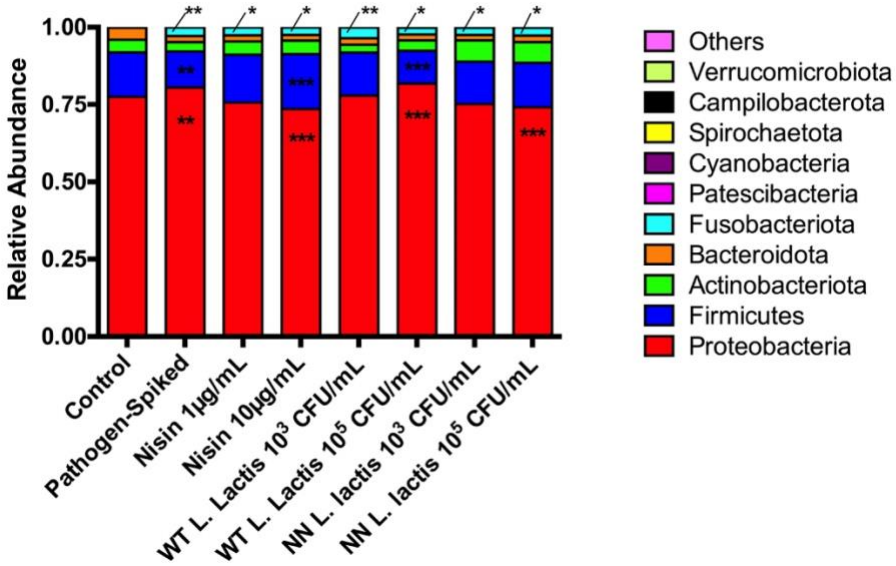

Figure 5 – Lower doses of nisin and probiotic revert Proteobacteria and Firmicutes phyla back to control levels. Top 10 phyla relative abundance are displayed; *means $p \leq 0.05$; ** means $p \leq 0.01$, *** means $p \leq 0.001$ between the marked sample and control.

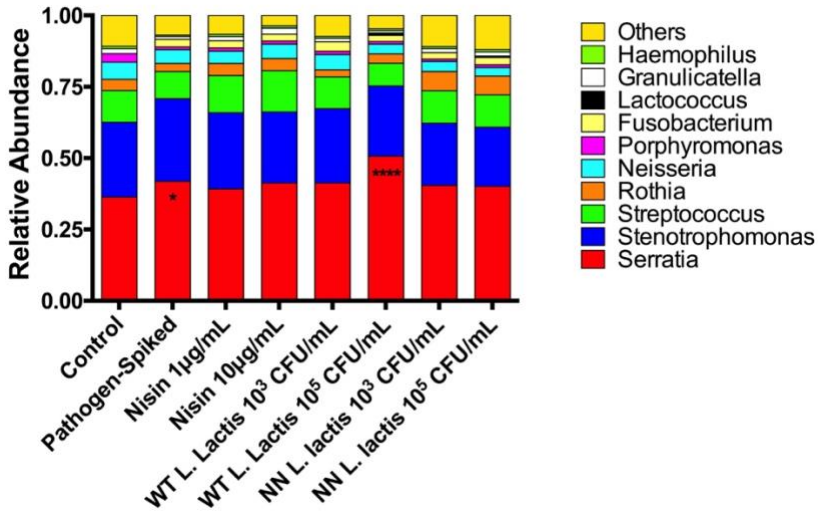

Figure 6 – Lower doses of nisin and probiotic revert the *Serratia* genus back to control levels. Top 10 genera relative abundance are displayed; *means $p \leq 0.05$; while **** means $p \leq 0.0001$ between the marked sample and control.

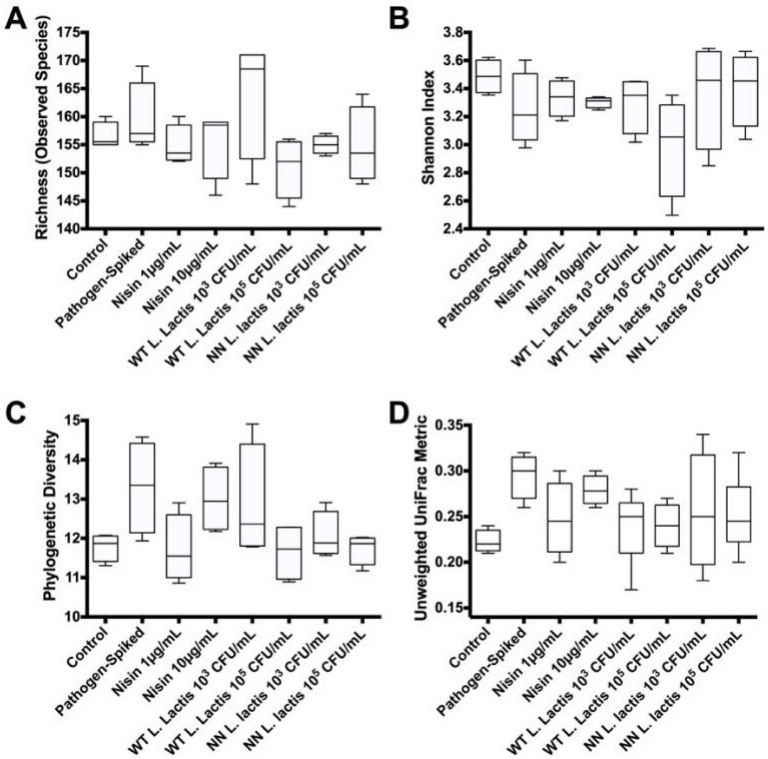

Figure 7 – Nisin and probiotic show tendency to revert pathogen-spiked biofilm richness and diversity indices back to control levels. (A) Species richness (number of observed species); (B) Shannon Diversity Index; (C) Phylogenic Diversity Index; (D) Unweighted UniFrac Metric.

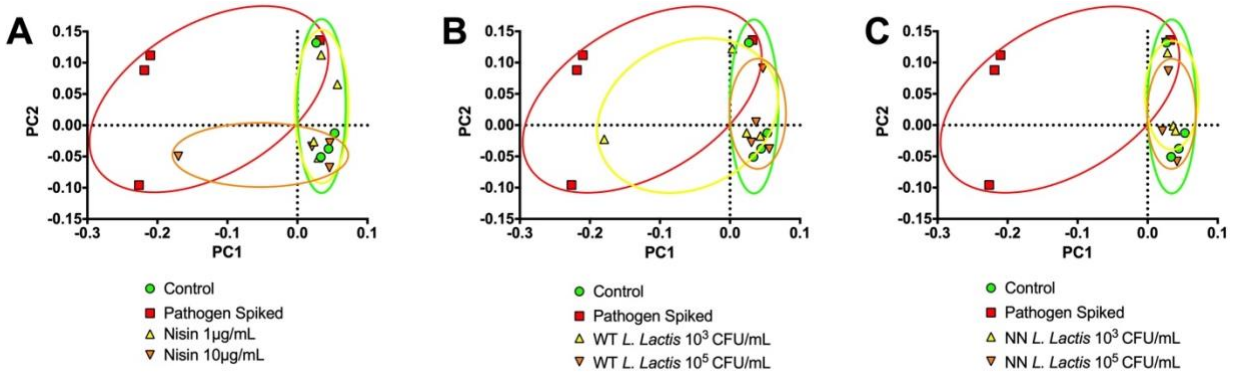

Figure 8 – Nisin and the probiotic effectively bring PCoA variables/distance of the pathogen-spiked oral biofilms back toward control levels. Images show the Principal Coordinate Analysis (PCoA) of the Unweighted UniFrac distances for control biofilms and pathogen-spiked biofilms treated with (A) Nisin 1 and 10 µg/mL, (B) WT *L. lactis* 10^3 and 10^5 CFU/mL and (C) NN *L. lactis* 10^3 and 10^5 CFU/mL. Colored ellipses highlight sample group distance.

References

1. Abraham, C. M. A brief historical perspective on dental implants, their surface coatings and treatments. *Open Dent. J.* **8**, 50–55 (2014).

2. Monje, A., Insua, A. & Wang, H.-L. Understanding Peri-Implantitis as a Plaque-Associated and Site-Specific Entity: On the Local Predisposing Factors. *J. Clin. Med.* **8**, (2019).

3. Lin, P.-J. & Su, K.-C. Biomechanical Design Application on the Effect of Different Occlusion Conditions on Dental Implants with Different Positions—A Finite Element Analysis. *Appl. Sci.* **10**, 5826 (2020).

4. Ricci, J. L., Grew, J. C. & Alexander, H. Connective-tissue responses to defined biomaterial surfaces. I. Growth of rat fibroblast and bone marrow cell colonies on microgrooved substrates. *J. Biomed. Mater. Res. A* **85**, 313–325 (2008).

5. Grew, J. C., Ricci, J. L. & Alexander, H. Connective-tissue responses to defined biomaterial surfaces. II. Behavior of rat and mouse fibroblasts cultured on microgrooved substrates. *J. Biomed. Mater. Res. A* **85**, 326–335 (2008).

6. Nevins, M. *et al.* Maintaining interimplant crestal bone height via a combined platform-switched, Laser-Lok implant/abutment system: a proof-of-principle canine study. *Int. J. Periodontics Restorative Dent.* **33**, 261–267 (2013).

7. Nevins, M., Nevins, M. L., Camelo, M., Boyesen, J. L. & Kim, D. M. Human histologic evidence of a connective tissue attachment to a dental implant. *Int. J. Periodontics Restorative Dent.* **28**, 111–121 (2008).

8. Elani, H. W., Starr, J. R., Da Silva, J. D. & Gallucci, G. O. Trends in Dental Implant Use in the U.S., 1999-2016, and Projections to 2026. *J. Dent. Res.* **97**, 1424–1430 (2018).

9. Grand View Research. Dental Implant Market Size & Share Report, 2021-2028. *Dental Implant Market Size, Share & Trends Analysis Report By Implants Type (Titanium, Zirconium), By Region (North America, Europe, Asia Pacific, Latin America, MEA), And Segment Forecasts, 2021 - 2028* https://www.grandviewresearch.com/industry-analysis/dental-implants-market (2021).

10. Radaic, A. & Kapila, Y. L. The oralome and its dysbiosis: New insights into oral microbiome-host interactions. *Comput. Struct. Biotechnol. J.* **19**, 1335–1360 (2021).

11. Schwarz, F., Derks, J., Monje, A. & Wang, H.-L. Peri-implantitis. *J. Periodontol.* **89 Suppl 1**, S267–S290 (2018).

12. Rokaya, D., Srimaneepong, V., Wisitrasameewon, W., Humagain, M. & Thunyakitpisal, P. Peri-implantitis Update: Risk Indicators, Diagnosis, and Treatment. *Eur. J. Dent.* **14**, 672–682 (2020).

13. Smeets, R. *et al.* Definition, etiology, prevention and treatment of peri-implantitis--a review. *Head Face Med.* **10**, 34 (2014).

14. Kröger, A. *et al.* The severity of human peri-implantitis lesions correlates with the level of submucosal microbial dysbiosis. *J. Clin. Periodontol.* **45**, 1498–1509 (2018).

15. Radaic, A. *et al.* Paradigm shift in the pathogenesis and treatment of oral cancer and other cancers focused on the oralome and antimicrobial-based therapeutics. *Periodontol. 2000* **87**, 76–93 (2021).

16. Chow, J., Lee, S. M., Shen, Y., Khosravi, A. & Mazmanian, S. K. Host-bacterial symbiosis in health and disease. *Adv. Immunol.* **107**, 243–274 (2010).

17. Dagli, N., Dagli, R., Darwish, S. & Baroudi, K. Oral Microbial Shift: Factors affecting the Microbiome and Prevention of Oral Disease. *J. Contemp. Dent. Pract.* **17**, 90–96 (2016).

18. Relman, D. A. The human microbiome: ecosystem resilience and health. *Nutr. Rev.* **70 Suppl 1**, S2-9 (2012).

19. Marsh, P. D. In Sickness and in Health-What Does the Oral Microbiome Mean to Us? An Ecological Perspective. *Adv. Dent. Res.* **29**, 60–65 (2018).

20. Mombelli, A. & Décaillet, F. The characteristics of biofilms in peri-implant disease. *J. Clin. Periodontol.* **38 Suppl 11**, 203–213 (2011).

21. Rakic, M., Grusovin, M. G. & Canullo, L. The Microbiologic Profile Associated with Peri-Implantitis in Humans: A Systematic Review. *Int. J. Oral Maxillofac. Implants* **31**, 359–368 (2016).

22. Persson, G. R. & Renvert, S. Cluster of bacteria associated with peri-implantitis. *Clin. Implant Dent. Relat. Res.* **16**, 783–793 (2014).

23. Mulla, M., Hegde, S., Koshy, A. & Mulla, M. Effect of Probiotic Lactobacillus salivarius on Peri-Implantitis Pathogenic Bacteria: An In Vitro Study. *Cureus* **13**, e20808 (2021).

24. Berglundh, T. *et al.* Peri-implant diseases and conditions: Consensus report of workgroup 4 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *J. Periodontol.* **89 Suppl 1**, S313–S318 (2018).

25. Schwarz, F., Schmucker, A. & Becker, J. Efficacy of alternative or adjunctive measures to conventional treatment of peri-implant mucositis and peri-implantitis: a systematic review and meta-analysis. *Int. J. Implant Dent.* **1**, 22 (2015).

26. Claffey, N., Clarke, E., Polyzois, I. & Renvert, S. Surgical treatment of peri-implantitis. *J. Clin. Periodontol.* **35**, 316–332 (2008).

27. Chan, H.-L., Lin, G.-H., Suarez, F., MacEachern, M. & Wang, H.-L. Surgical management of peri-implantitis: a systematic review and meta-analysis of treatment outcomes. *J. Periodontol.* **85**, 1027–1041 (2014).

28. Wu, H. *et al.* Antibiotic-induced dysbiosis of the rat oral and gut microbiota and resistance to Salmonella. *Arch. Oral Biol.* **114**, 104730 (2020).

29. McDonnell, L. *et al.* Association between antibiotics and gut microbiome dysbiosis in children: systematic review and meta-analysis. *Gut Microbes* **13**, 1–18 (2021).

30. Khoshkam, V. *et al.* Reconstructive procedures for treating peri-implantitis: a systematic review. *J. Dent. Res.* **92**, 131S–8S (2013).

31. Radaic, A. *et al.* Modulation of pathogenic oral biofilms towards health with nisin probiotic. *J. Oral Microbiol.* **12**, 1809302 (2020).

32. Nguyen, T., Brody, H., Radaic, A. & Kapila, Y. Probiotics for periodontal health-Current molecular findings. *Periodontol. 2000* **87**, 254–267 (2021).

33. Radaic, A., de Jesus, M. B. & Kapila, Y. L. Bacterial anti-microbial peptides and nano-sized drug delivery systems: The state of the art toward improved bacteriocins. *J. Control. Release* **321**, 100–118 (2020).

34. Shin, J. M. *et al.* Biomedical applications of nisin. *J. Appl. Microbiol.* **120**, 1449–1465 (2016).

35. Shin, J. M. *et al.* Antimicrobial nisin acts against saliva derived multi-species biofilms without cytotoxicity to human oral cells. *Front. Microbiol.* **6**, 617 (2015).

36. Shin, J. M. *et al.* Microbial Communities Associated with Primary and Metastatic Head and Neck Squamous Cell Carcinoma - A High Fusobacterial and Low Streptococcal Signature. *Sci. Rep.* **7**, 9934 (2017).

37. Fenno, J. C. Laboratory maintenance of Treponema denticola. *Curr. Protoc. Microbiol.* **Chapter 12**, Unit 12B.1 (2006).

38. Mishra, A. *et al.* Role of Acetyltransferase PG1842 in Gingipain Biogenesis in Porphyromonas gingivalis. *J. Bacteriol.* **200**, (2018).

39. Guo, M., Reynolds, H., Stinson, M. & De Nardin, E. Isolation and characterization of a human neutrophil aggregation defective mutant of Fusobacterium nucleatum. *FEMS Immunol. Med. Microbiol.* **27**, 241–246 (2000).

40. Joe, A., Murray, C. S. & McBride, B. C. Nucleotide sequence of a Porphyromonas gingivalis gene encoding a surface-associated glutamate dehydrogenase and construction of a glutamate dehydrogenase-deficient isogenic mutant. *Infect. Immun.* **62**, 1358–1368 (1994).

41. Martin, B. *et al.* A new mathematical model of bacterial interactions in two-species oral biofilms. *PLoS ONE* **12**, e0173153 (2017).

42. Gaetti-Jardim Júnior, E., Luvizotto, M. C. R. & Avila-Campos, M. J. Virulence of oral Fusobacterium nucleatum from humans and non-human primates in mice. *Braz. J. Microbiol.* **31**, (2000).

43. Pedersen, M. B., Koebmann, B. J., Jensen, P. R. & Nilsson, D. Increasing acidification of nonreplicating Lactococcus lactis deltathyA mutants by incorporating ATPase activity. *Appl. Environ. Microbiol.* **68**, 5249–5257 (2002).

44. Moore, J. C. Diversity, Taxonomic versus Functional. in *Encyclopedia of Biodiversity* 648–656 (Elsevier, 2013). doi:10.1016/B978-0-12-384719-5.00036-8.

45. Morris, E. K. *et al.* Choosing and using diversity indices: insights for ecological applications from the German Biodiversity Exploratories. *Ecol. Evol.* **4**, 3514–3524 (2014).

46. Srivastava, D. S., Cadotte, M. W., MacDonald, A. A. M., Marushia, R. G. & Mirotchnick, N. Phylogenetic diversity and the functioning of ecosystems. *Ecol. Lett.* **15**, 637–648 (2012).

47. Chen, J. *et al.* Associating microbiome composition with environmental covariates using generalized UniFrac distances. *Bioinformatics* **28**, 2106–2113 (2012).

48. Navas-Molina, J. A. *et al.* Advancing our understanding of the human microbiome using QIIME. *Meth. Enzymol.* **531**, 371–444 (2013).

49. Do, T., Devine, D. & Marsh, P. D. Oral biofilms: molecular analysis, challenges, and future prospects in dental diagnostics. *Clin. Cosmet. Investig. Dent.* **5**, 11–19 (2013).

50. Gao, J. *et al.* Does Probiotic Lactobacillus Have an Adjunctive Effect in the Nonsurgical Treatment of Peri-Implant Diseases? A Systematic Review and Meta-analysis. *J. Evid. Based Dent. Pract.* **20**, 101398 (2020).

51. Laleman, I., Pauwels, M., Quirynen, M. & Teughels, W. The usage of a lactobacilli probiotic in the non-surgical therapy of peri-implantitis: A randomized pilot study. *Clin. Oral Implants Res.* **31**, 84–92 (2020).

52. Limaye, S. A. *et al.* Phase 1b, multicenter, single blinded, placebo-controlled, sequential dose escalation study to assess the safety and tolerability of topically applied AG013 in subjects with locally advanced head and neck cancer receiving induction chemotherapy. *Cancer* **119**, 4268–4276 (2013).

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