2020

Oral Probiotic Supplementation as an Adjunct to Non-surgical Therapy in Peri-implantitis Lesions: A Pilot Study

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ORAL PROBIOTIC SUPPLEMENTATION AS AN ADJUNCT TO NON-SURGICAL THERAPY IN PERI-IMPLANTITIS LESIONS: A PILOT STUDY

PO-NING POLLY HUANG, D.M.D.

A Thesis Presented to the Faculty of the College of Dental Medicine of

Nova Southeastern University in Partial Fulfillment of the Requirements for the

Degree of

MASTER OF SCIENCE

September 2020

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Department of Periodontics

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September 2020

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COURSE DESCRIPTION: Master of Science

TITLE OF SUBMISSION: Oral probiotic supplementation as an adjunct to non-surgical therapy in peri-implantitis lesions: a pilot study

DATE SUBMITED: September 2020

I certify that I am the sole author of this thesis, and that any assistance I received in its preparation has been fully acknowledged and disclosed in the thesis. I have cited any sources from which I used ideas, data, or words, and labeled as quotations any directly quoted phrases or passages, as well as providing proper documentation and citations. This thesis was prepared by me, specifically for the MSc degree and for this assignment.

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DEDICATION

To my husband, Jason, for the unwavering support throughout the years.

To Mom, Angela and my dog, Jack, for always standing by me no matter what.

To my grandparents who I know are watching over me.

Thank you all for your unconditional love.

Acknowledgement

I would like to acknowledge the following individuals:

Dr. Saynur Vardar as not just the chair of my thesis committee but also a mentor, colleague and a dear friend. Thank you for your insight and guidance throughout my residency journey. I am grateful to have your support even after residency. You are always there, rain or shine and I truly appreciate it.

Dr. Toshihisa Kawai for helping me throughout this entire journey. I would not have accomplished this thesis if I did not have you by my side. You have always encouraged me and are with me every step of the way. I truly appreciate your presence and all the time you dedicated to help me succeed.

Dr. Maria Hernandez for always being a great listener even out of your very busy schedule. Your door is always open and I am truly thankful for all the times you've been there for me.

Alireza Heidari for your constant support and help throughout the research time. You are always there for me and I could not have finished this without your help. You are not only one of the most dependable helpers but also a friend.

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DEGREE DATE: September 2020

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Abstract:

Introduction: During the last decade, the results of several clinical studies suggested that oral probiotics may potentially improve oral health. The first study examining the effects of probiotics on oral health demonstrated that almost every patient with gingivitis, periodontitis or pregnancy gingivitis, had significant improvements in measurable periodontal indices when they were treated with a locally administered culture supernatant of a *Lactobacillus acidophilus* strain. This finding sparked several other studies to further examine the potential for treating oral diseases, such as, combating halitosis, oral candidiasis, and dental caries with probiotics. Of our interest, Fernandez et al. and Hollstrom et al. published their clinical studies investigating the effects of probiotics on peri-implant mucositis lesions and had conflicting results. In a recent study investigating the effect of *Lactobacillus reuteri* on peri-implantitis lesions, it was concluded that the oral probiotics together with mechanical debridement gave additional improvement for all clinical parameters. However, more studies are needed to confirm this finding as the majority of published studies explored the effects of probiotics on

periodontitis and peri-implant mucositis lesions, very few focused specifically on peri-implantitis lesions. Therefore, the goals of this study were to examine the clinical, inflammatory and microbiological effects of oral probiotics when used as an adjunct in addition to non-surgical debridement in treating peri-implantitis lesions. **Methods:** A double blind pilot study was conducted between the test group (probiotics) versus the control group (placebo) as an adjunct to non-surgical treatment of peri-implantitis sites. Peri-implantitis is defined when there is a probing depth of 6mm or more with bleeding on probing and 3mm or more radiographic bone loss compared to radiographs when implants were placed. A full mouth non-surgical treatment was performed and peri-implantitis sites were debrided using titanium curettes. Subsequently, oral probiotics containing strains of *S. salivarius K12*, *S. salivarius M18*, *L. reuteri* and *L. paracasei* were given to the test group while the control group received placebo tablets. Subgingival plaque, gingival crevicular fluid/peri-implant crevicular fluid volume and clinical parameters such as probing depth,clinical attachment level, bleeding on probing plaque index and gingival index were recorded for analysis. Moreover, these data were collected from not only peri-implantitis sites of each subject but also from healthy and periodontitis sites of all patients. Microbiological testing was done by 16sRNA analysis and GCF/PICF analysis were done by ELISA. Statistical analyses were done by using One-way Anova. **Results:** There were no statistically significant differences for all clinical parameters comparing baseline to 90 days for the test and control groups at all sites. However, biologically, only probiotic group in peri-implantitis sites demonstrated statistically significant reduction after 90 days. Moreover, there were no statistical differences for MMP-9 or interleukin-1β. There was a statistically significance increase for *P. gingivalis* for peri-implantitis sites ($p=0.04$) for the probiotic group. There was

statistically significant increase in commensal bacteria of the green, purple and blue complexes specifically *C.concisus* (p=0.028), *A. graevenitzii* (p=0.023) and Actinomyces species (p=0.015) in probiotic group in peri-implantitis sites compared to placebo. .

Conclusions: Our findings suggest that adjunctive use of oral probiotics appeared to have limited effects on clinical parameters, however, biologically, they significantly decreased PICF TNF-α levels, a pro-inflammatory cytokine which is associated with periodontal and peri-implant breakdown. Furthermore, oral probiotics may help to shift the microbial flora towards commensal bacteria as it was shown to be statistically increased. in peri-implantitis sites. This suggests that oral probiotics may play a role in affecting the overall microbiota of the oral cavity shift towards a healthier and more symbiotic environment.

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Chapter 1: Introduction

1.1. Background

1.1.1. History of Probiotics

Probiotics, derived from the Greek word *bio-tikos* "for life", has been a subject of interest in modern health care. In 1907, Metchnikoff, a Nobel Prize winner, was the first to discover the positive role of selected bacteria.[1] He advocated that "the dependence of intestinal microbes on food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes".[1] Around the same time, Tissier, a French pediatrician, recognized that children with diarrhea had minimal numbers of "bifid" bacteria compared to healthy children.[2] He proposed that these bacteria could restore a healthy gut flora in patients with diarrhea.[2] Even though Metchnikoff & Tissier were amongst the first to explore the probiotic usage of bacteria, the term "probiotic" was not coined until 1960 to describe the substance produced by microorganisms that promote growth of other microorganisms.[3] In 2001, the Food & Agriculture Organization of the United Nations-World Health Organization (FAO-WHO) officially defined probiotics as "live microorganisms that when administered in adequate amounts confer a significant health benefit on the host".[4] This definition was later adopted by the International Scientific Association for Probiotics and Prebiotics (ISAPP) and remained to date to be the most accepted definition of probiotics worldwide.

1.1.2 Probiotics and Oral Health:

During the last decade, several authors speculated that probiotic bacteria, used traditionally for improving gut health, could also improve oral health. Some

suggested that benefits include combating halitosis, oral candidiasis, and dental caries.[6] In addition, the first studies to use probiotics for oral health were targeted to treat periodontal inflammation.[7] Significant improvement in measurable periodontal indices was observed for almost every patient when patients with various periodontal diseases such as gingivitis, periodontitis and pregnancy gingivitis were locally treated with a culture supernatant of a *Lactobacillus acidophilus* strain.[7] This sparked several other studies that further examined the potential of probiotics for treating periodontal diseases. The strains used in these studies include *Lactobacilus reuteri, Lactobacillus brevis (CD2), Lactobacillus casei Shirota, Lactobacillus salivarius WB21* and *Bacillus subtilis spp*. There was a decrease in gingival bleeding with the use of *L. reuterui* and *L brevis*.[8-10] Specifically, *L. reuteri* in chewing gum was shown to decrease the levels of pro-inflammatory cytokines in gingival crevicular fluid (GCF) while *L. brevis* decreased inflammatory markers in saliva, including MMP (collagenase) activity.[9,10] *L. casei Shirota* decreased gingival inflammation, PMN elastase, and MMP-3 activities in GCF after 4-days and *B. subtilis* reduced the number of periodontal pathogens.[11,12] Gingival pocket depths and sum of periodontal pathogens in plaque decreased with *L. salivarius WB21*, especially in smokers.[13,14]

With the growing interest in using probiotics in periodontal therapy, newly emerging research assessed the effects of probiotics on oral health. Kuru et al. (2017) assessed the effect of yogurt supplemented with *Bifidobacterium animalis* for 4 weeks versus a placebo yogurt followed by a non-brushing period of 5 days.

They concluded in this single-blinded randomized controlled study that the use of a probiotic yogurt supplemented with *B. animalis* appeared to have a positive effect on plaque accumulation and gingival parameters after the oral hygiene regimen was stopped.[15] Moreover, Shah et al. (2017) recognized the concerns regarding the overuse and broad use of antibiotics. As a result, the authors conducted a randomized controlled trial using *L. brevis CD2* lozenges, L. *brevis CD2* with oral doxycycline, or doxycycline alone to assess their effects on patients with aggressive periodontitis. After 14 days of treatment, they found that lozenges containing *L. brevis CD2* had a lasting and positive effect on clinical measures of aggressive periodontitis, especially the gingival index. In addition, they found the effects of *L. brevis CD2* to be equivalent to those of doxycycline.[16]

1.1.3. Why Probiotics?

Globally, we have seen that increasing and often inappropriately widespread use of antibiotics has, correspondingly, increased the antibiotic-resistance of bacteria in all major human organ systems, including the subgingival microbiota of adult periodontitis patients.[15] According to the Centers for Disease Control and Prevention, "at least 2 million people in the US become infected with bacteria that are resistant to antibiotics and at least 23,000 people die each year as a direct result of these infections.[16] Thus, it is important to seek alternative anti-microbial approaches such as the exploring the potential of health-promoting bacteria for therapeutic purposes. The concept itself is straightforward. Antibiotics not only destroy the harmful bacteria but can also suppress the beneficial bacteria that help fight infection. On the contrary, probiotics

repopulate the beneficial bacteria to strengthen the fight against infection. Probiotics are already often recommended to patients with a history of diarrhea or overall gastrointestinal irritation, while, at the same time, taking antibiotics to minimize symptoms.

1.1.4. Probiotics: Proposed Mechanisms

Numerous major mechanisms have been proposed to explain the bio-protective role of probiotics. For instance, oral health may benefit from probiotics by preventing the growth of harmful microbiota or by modifying immunity in the oral cavity.[15] Haukiojia et al. (2006) demonstrated that certain strains of probiotics remove a crucial adhesion protein, salivary agglutinin gp340, which is necessary for the adhesion of *S. mutans ,* thus decreasing the colonizing efficiency of *S. mutans*. In addition, the competitive exclusion mechanism of probiotics may hinder adhesion of pathogenic bacteria or compete for the same nutrients and thus, shift the species to another niche or lead to extinction of the weaker competitor.[17] Probiotics may possibly compete for certain essential nutrients or chemicals necessary for the growth of pathogens and block them out, thus, improving oral health. Moreover, probiotics can produce a diverse range of antimicrobial substances such as lactic acid, hydrogen peroxide, bacteriocins and bacteriocin-like inhibitory substances.[18] Sookkhee et al. (2001) isolated lactic acid bacteria from healthy oral cavities and demonstrated their antimicrobial effect against *Porphyromonas gingivalis* and *Streptococcus mutans*.[19] Koll-Klais et al. (2005) then supported this finding when he found higher prevalence of obligatory

homofermentative *Lactobacilli*, especially *Lactobacillus gasseri* in healthy individuals versus individuals with periodontitis.[20] Since higher concentrations of lactic acid was produced by homofermentative *Lactobacilli* in comparison with heterofermentative lactobacilli, *P. gingivalis* and *Prevotella intermedia* were significantly inhibited.[20]

Probiotics can also indirectly exert their actions in the oral cavity by modulating the body's immune function.[21] Host cells such as epithelial cells and immune cells can recognize probiotic bacteria and their products, including, metabolites, DNA and cell wall components.[21] When *Lactobacillus acidophilus* and *Lactobacillus casei* were administered, the macrophages exhibited increased phagocytic abilities.[22] In healthy individuals, probiotics are shown to upregulate the expression of phagocytosis receptors in neutrophils and strengthen natural killer (NK) cell activity.[23,24] Most importantly, they are able to modulate immune response through adaptive immunity.[25] Cosseau et al. (2008) proposed that *S. salivarius K12* is not only tolerated by the host but also promotes homeostasis and cellular health, leading to the protection of host tissues from damage caused by other immunostimulatory cells and products.[26] Based on their *in vivo* study, Della et al.(2007) found that *Lactobacillus brevis* significantly decreased inflammatory markers in saliva, such as metallo-proteinase, nitric oxide synthase activity, PGE_2 and interferon γ levels.[27]

1.2 Probiotics and Peri-Implant Conditions

1.2.1. Peri-Implant diseases: Background and Prevalence

These encouraging results have kindled growing interest in probiotics and very recently, authors have begun to expand the scope of probiotic research to look into peri-implant diseases. Peri-implant diseases exist as either peri-implant mucositis or peri-implantitis. Though both are inflammatory reactions in the tissues that surround an implant, peri-implant mucositis is defined as the result of inflammation confined to the tissues that surround the implant with no loss of supporting bone after initial bone remodeling, while peri-implantitis is an inflammatory process characterized by both soft tissue inflammation and progressive loss of supporting bone in relation to radiographic bone level one year after implant supported prosthetic delivery.[28] While measuring the true prevalence for peri-implant diseases remains controversial in today's literature, it is still broadly regarded as highly prevalent. Zitzmann and Berglundh's review concluded that up to 80% of all dental implant patients and 50% of all implants have peri-implant mucositis while 28-56% of all dental implant patients and 12-43% of all implants have peri-implantitis.[29] The literature has demonstrated that the microbiota associated with peri-implantitis is more complex, consisting of mainly anaerobic gram-negative bacteria versus microbiota associated with healthy peri-implant tissues.[30] Particularly, *Staphylococcus aureus* may be a critical pathogen in initiating peri-implantitis.[31,32] Studies found that tissue destruction both clinically and radiographically, was significantly more destructive for peri-implantitis lesions versus periodontitis.[33,34] The size and extent of the inflammatory cell infiltrates in the connective tissue were greater in peri-implantitis lesions. The self-limiting process that normally results in a

protective tissue capsule that separates lesion from alveolar bone did not occur for peri-implantitis lesions.[35] In addition, animal studies demonstrated that the disease continued to progress insidiously even after post-ligature removal in experimental peri-implantitis.[36-38]

1.2.2. Treatment for Peri-Implant Diseases

Peri-implant disease is an important pathology worth understanding owing to its prevalence and incidence. A full 80% of subjects and 50% of implants were reported to have peri-implant mucositis while peri-implantitis affects a range between 28 and 56% of subjects and between 12 and 43% of implant sites.[39] Since the main etiology for peri-implant disease is the development of bacterial biofilm on the implant surface, the goal of treatment of peri-implant disease is to remove the bacterial biofilm and disinfect the implant surface.[40] Unfortunately, the screw threads and surface roughness of implants make decontamination of the implant especially difficult. Several studies using the non-surgical approach have demonstrated promising results in controlling inflammation with decreased bleeding on probing and may heal peri-implant mucositis lesions. Nonetheless, using this modality still gives unpredictable results in treating peri-implantitis lesions.[41] A systemic review by Giacomo et al. concluded that non-surgical therapy of peri-implantitis had limited efficacy for treating peri-implantitis.[42] However, among various non-surgical options investigated, mechanical debridement and adjunctive measure appeared to provide slightly greater benefits. Specifically, Gomi et al. found that using systemic azithromycin, along with mechanical debridement could give better outcomes clinically reporting more

reduction of 0.96 +/- 0.4 mm in the test group versus control after the 1 year follow up.[43] Nevertheless, it is concerning that the incidence of peri-implantitis seems to further increase as more and more implants are placed by a number of clinicians with various degree of expertise. The11th European Workshop of Periodontology raised awareness for research to recognize effective protocols in treating peri-implantitis.[44] Current research has, however, still failed to identify a gold standard in treating peri-implantitis. Though several protocols were documented including nonsurgical, surgical, resective, regenerative and combined approaches, the best management in treating peri-implantitis remained a mystery. Perhaps these results from the heterogeneity of the study designs, patient profiles, defect characteristics, implant and prosthesis design, clinician experience and skills, or disease definitions. The systematic review and meta-analysis of Chan et al. attempted to assess the surgical management of peri-implantitis.[45] They concluded that the use of grafting material and barrier membranes gave greater pocket depth reduction and radiographic bone fill, leading to a PD reduction of 33.4% to 48.2%.[45] However, the authors recognized that more high quality comparative studies need to be conducted before this conclusion can be supported. However, the newest systematic review published by Roccuzzo et al. analyzed clinical outcomes of peri-implantitis treatment and supportive care, which seemed to give hope in treating this tricky lesion.[46] Studies with $\geq$10 patients with at least 3 years follow up were searched and analyzed looking at cumulative survival at both implant and patient level. This systematic review found that peri-implantitis can be treated successfully in patients who adhered to a

supportive care program that involves professional biofilm removal demonstrating that over 90% of implants and over 85% of patients who received the supportive care treatment could retain their implants successfully after 5 years.[46] It is important to note that 20% of the included studies were found using grey literature and that the definition of survival did not include tissue health, tissue appearance or patient satisfaction.[46] Thus, a surviving implant in one patient compared to another surviving implant in a different patient may be extremely different, serving as yet another limitation of this review. A time passes, we incrementally learn more and more about peri-implantitis lesions. Notwithstanding this growth in relevant studies, we still suffer from lack of comprehensive evidence to support recommending one treatment over another in the management of peri-implantitis lesions.

1.2.3. Probiotics and their Effects on Peri-implant Mucositis and Peri-implantitis:

Only a few studies have investigated the effects of oral probiotics for peri-implant mucositis and peri-implantitis. In a very recent study, Tada et al. showed that probiotics prevent inflammation by affecting host responses rather than improving microbial flora in peri-implant sulci in peri-implantitis patients.[47] In 2015, Flichy-Fernandez et al. carried out a double blind, placebo-controlled cross-over study evaluating the effects of taking *Lactobacillus reuteri* on edentulous implant patients by comparing the peri-implant health of those without peri-implant disease versus those with peri-implant mucositis.[48] They concluded

that both groups demonstrated improvements in plaque, diminished probing depth, gingival index and gingival crevicular fluid levels with decreased cytokine levels.[48] Hollstrom et al. evaluated the effects of probiotics as an adjunct to mechanical debridement for peri-implant mucositis lesions.[49] They administered topical oil application followed by *Lactobacillus reuteri* to patients with peri-implant mucositis for 3 months and clinically evaluated the patients' probing depths, plaque index, bleeding on probing and subgingival microbiota using checkerboard DNA-DNA hybridization.[49] While all patients improved clinically, they found no significant differences between the two groups in their subgingival microflora and levels of inflammatory mediators in GCF.[49] In contrast to the study by Flinchy-Fernandez et al., Hollstrom et al. concluded that probiotic supplements did not improve clinical, microbial or inflammatory variables of peri-implant mucositis as opposed to a placebo.[48-49] The newest study investigating peri-implant mucositis and the effects of probiotics was conducted by Pena et al. in 2017.[50] They investigated, both clinically and microbiologically, the effects of oral probiotics in 50 patients with peri-implant mucositis lesions. The test group involved mechanical debridement with 0.12% chlorhexidine mouthwash plus the administration of a *Lactobacillus reuteri* tablet, while the control group substituted the oral probiotic tablet for a placebo.[50] After following the patients for 135 days, they concluded that the probiotics did not appear to give any additional clinical or microbiological benefit but that the treatment with mechanical debridement plus chlorhexidine rinse was effective in decreasing mucositis though the lesion may not resolve completely.[50] It is obvious that the

effects of oral probiotics for peri-implant mucositis lesions are still controversial and that studies assessing the effects of oral probiotics on peri-implantitis lesion are even more scarce with limited evidence. To the best of our knowledge, only one study has, so far, published on this topic. A triple blind randomized clinical trial published by Galofre et al. assessed the effects of probiotic administration of *Lactobacillus reuteri* on both mucosisits and peri-implantitis.[51] A total of 44 patients, 22 of whom had mucositis and 22 of whom had peri-implantitis, were recruited in this study. For both mucositis and peri-implantitis lesions, it was found that *L. reuteri,* together with mechanical therapy, gave additional improvement compared to treating with only mechanical therapy for all clinical parameters, such as bleeding on probing and probing pocket depth.[51] However, interestingly, very limited effect microbiologically was observed, as the only significant decrease in bacterial load was for *P. gingivalis* in mucositis lesions.[5]

1.3. Oral Microbiome and Peri-Implant Tissues

1.3.1. Background: Oral Microbiome

The oral cavity is continuously colonized by various microorganisms, collectively called the oral microbiome. This microbiome resides in the oral cavity and consists of different anatomic structures in the form of biofilm which produces an equilibrium that maintains health. However, ecological shifts in the microbiome lead to development of pathogenic species that may cause destruction. Biofilm not only occurs on teeth and mucosa, but also it also develops in artificial structures, such as, prosthesis and implants. Thus, oral biofilm creates a pathogenic environment wherein microorganisms become less

sensitive to immune cells and other anti-microbial agents.[52] Currently, the ecological hypothesis of plaque can explain how oral biofilm causes diseases, such as caries, periodontitis or peri-implantitis by the interactions between microorganisms and the host which then establish the state of either health or diseases.[53] Recent studies have suggested a new model of periodontal disease pathogenesis whereby individual pathogens do not cause chronic periodontitis, but rather, a polymicrobial synergy and dysbiosis combined with an unbalanced immune response is responsible for inflammation-mediated tissue damage.[54] For example, changes in microflora environment as influenced, for example, by diet, pH, oral hygiene regimen and use of antibiotics, immune system integrity may also alter biofilm composition. As a result of these changes, certain species of bacteria may exhibit greater virulence thereby allowing opportunist microorganisms to cause disease.

1.3.2. Differences between peri-implant tissues versus periodontal tissues:

Though similarities exist in microbial colonization during biofilm formation between peri-implant tissues and periodontal tissues, fundamental differences exist. Peri-implant tissues lack Sharpey fibers, i.e., collagen fibers of the submucous connective tissue situated perpendicular in periodontal tissues. More specifically, collagen fibers surrounding implant body are arranged parallel to the implant surfaces. This allows a greater gap compared to the gingival sulcus leading to easier penetration of microorganisms. Moreover, peri-implant tissues lack periodontal ligament, leading to weaker physical barriers, in turn allowing

submucosal tissues to be more susceptible to microorganisms. In addition, blood supply is decreased, leading to fewer nutrients and host anti-bacterial system represented by immune cell infiltrates that are crucial especially in the early stages of infection .[55]

1.3.3 Peri-Implant Microbiome Development:

So far, researchers have reached no consensus on the exact time that the peri-implant microbiome becomes established, but Persson net al. believed that implant contamination happens during implant surgery and subsequent prosthetic component delivery.[55.] This agrees with van Winkelhoff and Winkel's who reported the presence of *Peptostreptococcus micros*, Fusobacterium sp. and *Prevotella intermedia* after implant installation.[56] Salvi et al. discovered that bacteria associated with periodontitis such as *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* were found in the same prevalence 30 minutes or 1 year after implant installation, with *P. gingivalis* being the most prevalent species in both teeth and implants.[57] After just after a few weeks following implant installation, other studies found the presence of microorganisms in peri-implant groove or from the surface of implants.[58-60] Many believed that peri-implant microbiome establishment occurs via transmission of microbiome from teeth to implants. Gerber et al. believed that the oral microbiome present before implant insertion can determine the microbiome composition in peri-implant sites. They proposed that patients with a history of periodontal disease may transfer pathogens from teeth to implants.[61-62] This

theory is supported by Botero et al. who found that in partially edentulous patients with periodontitis who had dental implant rehabilitation exhibited pocket and bone loss around implants with high levels of periodontal pathogens. In fact, facultative anaerobes were found after 6 months of implant placement in the subgingival flora which support the theory that teeth may serve as a reservoir in transmitting pathogens to implants. On the other hand, patients with history of periodontitis that is aggressively monitored and controlled did not have changes or inflammation after osseointegration of their implants.[63]

1.3.4 Oral microbiome associated with dental implants

In healthy peri-implant sites, a high proportion of Gram positive coccus if found, but for peri-implantitis cases, high amounts of *Aggregatibacter actinomycetemcomitans* and *P. gingivalis* were discovered, supporting the argument that these are the predominant microorganisms responsible for the destructive nature of peri-implantitis lesions.[64-65] Other species, such as *Fusobacterium*, *Pseudomonas aeruginosa* and *T. forsythia* in symbiosis, were also discovered in peri-implantitis sites.[66] Kohavi et al. suggested that the subgingival microbiome is similar in teeth and implants as similar amounts of *A. actinomycetemcomitans* and *A. viscosus* were found in supragingival biofilm of teeth (92% *vs*. 57%) and implants (90% *vs*. 73%).[67] Quirynen et al. supported this suggestion in 2006 by using molecular biology techniques and found small differences in microflora between teeth and implants.[57] Moreover, Kohavi et al. found that healthy teeth of partially and totally edentulous patients had similar

microbiome composed of mainly Gram positive coccus with little amounts of spirochetes and mobile bacilli.[67] Then Quirynen et al., along with similar studies, discovered high levels of bacteria associated with periodontitis and peri-implantitis in totally edentulous patients.[59,68,69.] Other studies reported high levels of the red and orange complexes, such as, *P gingivalis*, *T. forsythia*, *T. denticola* and *F. nucleatum*, were in the subgingival biofilm of peri-implantitis sites.[70-71] However, Zhuang et al. reported differences between periodontitis and peri-implantitis for pathogens in terms of prevalence.[72] For example, levels of *P. gingivalis* and *F. nucleatum* were significantly associated with periodontitis but not associated with peri-implantitis. On the other hand, *A. actinomycetemcomitans* was associated with both periodontitis and peri-implantitis.[72] These differences among studies may be due to the heterogeneity of the studies and differences in methodologies for pathogen identification.[73] However, one can still conclude that there appear to be differences between healthy implants versus implants with peri-implantitis both in supra and subgingival biofilm. Overall, observational studies revealed that peri-implantitis was frequently associated with opportunistic pathogens such as pseudomonas aeruginosa and Staphylococcus aureus, fungal organisms such as candida albicans and viruses like human cytomegalovirus and Epstein Barr virus.[74] Although the submucosal microbiota of peri-implantitis lesions has not been extensively investigated with culture independent techniques, it is clear that peri-implantitis lesion is rather complex and heterogeneous. In fact, Schwarz et al. emphasized in

his 2017 world workshop narrative review on peri-implantitis that the “microbial picture associated with peri-implantitis should be regarded as incomplete”.[74]

1.4. Current Study

1.4.1. Purpose

Currently, no gold standard guides the treatment of peri-implantitis lesions. Since bacteria were believed to be the main causative factor for periodontal and peri-implant diseases, it is crucial to examine the role of microorganisms in the development and progression of peri-implantitis. Therefore, the present study aimed to examine and compare the range of microorganisms harvested from healthy, periodontitis and peri-implantitis sites and to examine if probiotics, taken orally, as an adjunct to non-surgical treatment would affect the microbiome within the oral cavity compared to placebo. In addition, to examine the effects of adjunctive probiotic intake on GCF/PICF inflammatory markers. To accomplish this, clinical, inflammatory and microbiological effects of oral probiotics were tested as an adjunct to non-surgical therapy in peri-implantitis lesions in a 90-day period while samples from healthy, periodontitis and peri-implantitis sites were compared.

1.4.2. Specific Aim

Specific Aim:

1. To evaluate the clinical, microbiological and biological effects of oral probiotics used as an adjunct to non-surgical therapy in patients with peri-implantitis lesions.
2. To evaluate these effects in probiotic group compared with placebo group.
3. To compare the effects in peri-implantitis sites with healthy and periodontitis sites.

Hypothesis: Patients receiving oral probiotics as an adjunct to non-surgical periodontal therapy for 90 days will demonstrate better clinical, microbiological and inflammatory outcome at 90 days compared to placebo group.

Chapter 2: Materials and Methods

2.1: Regulatory approvals:

This study was designed and performed as a double-blind randomized clinical trial of 90 days in duration. The study protocol was submitted to and approved by the Institutional Review Board for Research with Human Subjects (IRB) of Nova Southeastern University, Florida, USA.

2.2. Study Design

This is a pilot study to investigate the clinical effects of orally administered probiotics as an adjunct to non-surgical treatment on peri-implantitis lesions. It also aims to assess the microbiological composition of healthy, periodontitis and peri-implantitis sites of the samples collected and determine if adjunctive probiotics had any anti-inflammatory effects on GCF/PICF levels.

2.3: Patient Selection: Inclusion and Exclusion Criteria:

Patients with peri-implantitis were enrolled in the post graduate periodontics clinic at the College of Dental Medicine of Nova Southeastern University.

Inclusion criteria:

1. Patients that have one or more implants diagnosed with peri-implantitis

a) Peri-implantitis is defined as inflammation in peri-implant mucosa and subsequent progressive loss in relation to radiographic bone level at 1 year after implant supported prosthetic delivery. This definition is based on the 2017 World Workshop on the classification of Periodontal and Peri-implant diseases and conditions (Swartz 2017 classification).

2. Age ≥ 18 years old

3. Willing and able to give informed consent

Exclusion criteria:

1. Uncontrolled medical conditions
2. Pregnant or lactating females
3. Use of antibiotics for the last 3 months.
4. Subjects treated for ≥ 2 weeks with any medication known to affect soft tissue inflammation, such as, cyclosporine, phenytoin, or Coumadin.
5. Patients taking bisphosphonate medication
6. Patients diagnosed with aggressive periodontitis

2.4 Patient sampling

The samples were taken from patients affected by peri-implantitis who attended the post graduate periodontics clinic of the College of Dental Medicine at Nova Southeastern University. A total of 13 systemically healthy patients were initially recruited but 4 subjects dropped out of the study due to reasons such as loss of implants, recent surgery requiring antibiotics and scheduling conflicts not being able to keep up with follow-ups. Thus, the study was ultimately made up of a total of 9 patients. They were randomly assigned to two different treatment groups: the test group (probiotics) and controlled group (placebo). The study was conducted over a 90-day period with each patient coming in for four appointments total. Subjects were advised to maintain their usual diet during the study period but to avoid intake of fermented milk products and foods that may contain high quantities of fermentable carbohydrates. Moreover, at the end of each subject completion of 90 days, each subject was given the opportunity to re-enroll in the study after a 4-week wash-out period. Patient has the right to either continue or decline the offer. If they decide to re-enroll themselves for another 90-day period of the experiment as a new subject of the study, they were then randomly assigned to either the test or controlled group. Ultimately, four out of the nine participants decided to re-enroll in the study after wash-out period making up a total of 13 subjects for this pilot study.

2.5: Evaluation of Parameters:

Two designated examiners (PH and JW), both appropriately trained and calibrated clinicians, collected clinical, radiographic and microbiological data. Six sites (disto-buccal, buccal, mesio-buccal, disto-lingual, lingual and mesio-lingual) were recorded for periodontally healthy (n=13), periodontitis (n=13) and peri-implantitis sites (n=18) for each subject. Total samples for sites were (n=44) per subject. The subjects were clinically assessed at baseline and 90-days using UNC probe (UNC, Hu-Friedy, Chicago, Il, USA), which has marked millimeters from 1 to 15 was used to conduct these measurements. The implant shoulder was used as a landmark for clinical attachment level and mucosal recession.

The clinical measurements taken were as follows:

- Probing depth (PD): in millimeters
- Clinical Attachment Level (CAL): The implant shoulder was used as a landmark for the clinical attachment level and for the mucosal recession.
- Bleeding on Probing (BOP)
- Modified Plaque Index
- Gingival Index

The microbiological samples taken were as follows:

- **Subgingival plaque sampling:** Subgingival plaque samples were collected from 3 sites (periodontally healthy site, periodontitis site, peri-implantitis site) using a sterile Gracey curette at baseline and at 90 days and transported into the sampling tube containing TE buffer (150 ul/tube, 10 mM Tris-HCl, 1.0

mM EDTA, pH 7.6). Before collecting subgingival plaque, supragingival plaque was removed by sterile gauze and scalers. Sites of collection for subgingival plaque were isolated with sterile cotton roll.

- **Peri-implant crevicular fluid (PICF) and gingival crevicular fluid (GCF) sampling:**

PICF and GCF samples were collected from the implant site with peri-implantitis, tooth with periodontitis (if existed in patients' mouths) and periodontally healthy tooth using a PerioPaper® (Oraflow, Inc) placing in the crevice for 30 seconds. Immediately after the collection, the volume of collected fluid in the paper points was measured by using a Periotron 6000 (Oraflow). The PerioPaper® were collected in a fresh tube (1.5 ml Eppendorf tube) and saved at -80°C to determine measurement of biomarkers related to the inflammation and bone tissue destruction using ELISA.

2.6: Tablets: Test Group (Probiotics) versus Placebo Group

Probiotics used for the test group:

- Probiotic tablets (Hyperbiotics PRO Dental tablets) were made in Washington State then tableted in a Good Manufacturing Practice (GMP) certified facility in Colorado. The content of one tablet is composed of proprietary probiotic blend of 3 billion CFU (*S. salivarius K 12, S. salivarius M18, L. reuteri, L. paracasei*) and zinc amino acid chelate of 2mg. This probiotic is also lactose free, vegetarian, yeast free, soy free,

iron free, and gluten-free. It has no wheat, no nuts, no preservatives, no sugar and no artificial colors, flavors and sweeteners.

Placebo used for the control group:

- Hyperbiotics Inc, the company that manufactured the test tablets, also manufactured the placebo tablets that contained the same ingredients, except the active ingredient of probiotic bacteria.

2.7 Timeline for the study:

Visit 1: Data collection + Non-surgical treatment and start of tablet consumption

Subjects' medical and dental history was obtained, reviewed and kept in each study file stored in a secured cabinet. They received a complete periodontal exam where the designated examiner measured clinical parameters listed above in section 2.5. Subgingival plaque was collected with a sterile Gracey curette in 3 sites: healthy site (≤ 3mm PD), periodontitis site (≥5mm with BOP), and the peri-implantitis site at baseline and 90 days. In addition, PerioPapers was used to collect PICF & GCF samples at baseline and 90 days. Last, standardized digital radiographs (bitewings and PAs) of the peri-implantitis lesions were taken.

Full mouth non-surgical debridement (where necessary scaling and root planing – SRP) were performed and subjects were randomly assigned to a treatment group (placebo or test group). The randomization was performed by means of computer-generated sequencing. Neither investigators nor patients were told how the group

allocations were assigned. The record book remained in the hands of the PG periodontics clinic coordinator, who did not share this information with patients or clinicians. One bottle of 30 tablets containing either placebo or probiotics, was given to the patients. Proper administration instructions were given to the patients. – Both groups were instructed to take 2 tablets once per day. The subjects were asked to bring the tablets left with them in the bottle at each follow-up appointment.

Visit 2: 30 days after visit 1:

Subjects reported back to PG periodontics clinic and their medical histories were updated. Oral hygiene was checked, and oral hygiene instructions were given and reinforced. Patients were reminded to continue taking the tablets.

Visit 3: 60 days after visit 2:

Subjects reported back to PG periodontics clinic and their medical histories were updated. Oral hygiene was checked, and oral hygiene instructions were given and reinforced. Patients were reminded to continue taking the tablets.

Visit 4: 90 days after baseline (final appointment):

Subjects reported back to PG periodontics clinic and medical history was updated. They returned the empty bottles to ascertain the compliance of the patients. They received a complete periodontal exam where the examiner would measure clinical parameters listed above in section 2.5. Subgingival plaque was collected with a sterile Gracey curette at three sites: periodontally healthy site, periodontitis site and

peri-implantitis sites. In addition, Perio papers were used to collect PICF and GCF. Last, standardized digital radiographs (bitewings and PAs) of the peri-implantitis lesions were taken.

2.8. Statistical Analysis and Data Interpretation:

Clinical data was analyzed with one-way ANOVA with p-value <0.05 as being statistically significant to compare statistical differences between treatment groups. In addition, plaque samples were shipped to the Laboratories of Molecular Anthropology and Microbiome Research (LMAMR, Director, Dr. Cecile Lewis) at the University of Oklahoma for 16S-ribosomal RNA (rRNA) based microbiome sequencing service. The PICF/GCF samples were analyzed to measure TNF-α, IL-β, MMP-9 and RANKL in the collected samples using ELISA in the Department of Periodontology at NSU College of Dental Medicine.

Chapter 3: Results

3.1 Clinical outcomes:

A) Pocket depth:

PD measured at baseline showed significantly higher levels in periodontitis and peri-implantitis group compared to that in periodontally healthy group. The trend of higher PD levels in periodontitis and peri-implantitis were retained at 90 days after the non-surgical treatment followed by oral administration with probiotic or placebo tablets. We did not find any statistically significant difference on PD between probiotic and placebo administrations in

periodontitis and peri-implantitis groups in Day-90 compared to baseline (Figure 1, Table 1 and 2)

B) GCF/PICF:

It is well established that flow rate of GCF/PICF and severity of periodontitis/peri-implantitis at the disease affected site are positively correlated. In the present study, GCF and PICF were collected using PerioPaper by inserting it into the periodontal and peri-implant sulcus for 30 seconds. Subsequently, the volume of fluid collected in PerioPaper was measured using Periotron 4000. We could not find any significant difference in the levels of GCF/PICF measured at baseline. However, GCF volume as well as PICF volume measured in periodontitis and periimplantitis at Day-90 were both significantly higher than GCF measured in periodontally healthy sites at Day-90. There was no statistically significant difference in the GCF and PICF volume between the probiotic and placebo groups at Day-90 (Figure 2, Table 1 and 2)

C) Clinical Attachment Level (CAL):

CAL was also measured at Baseline and Day-90 on all study participants. The levels of CAL were significantly higher in the sites of periodontitis as well as peri-implantitis than periodontally healthy sites at both baseline and Day-90. Between two different diseased sites, the CAL values were higher in peri-implantitis than periodontitis (no significant difference, Baseline: P=0.62, Day-90: P=0.052). Furthermore, there was no significant difference in the CAL

measured for all three sites, i.e., healthy, periodontitis and peri-implantitis sites between baseline and Day-90 (healthy: P=0.81, Periodontitis: P=0.43, Peri-implantitis: P=0.98), indicating non-surgical treatment was not effective in CAL changes. Finally, adjunctive oral administration of probiotic tablets did not show any statistically significant difference on CALs compared to placebo group. (Figure 3 and Table 1 and 2)

3.2 Pro-inflammatory biomarkers in GCF/PICF

Using the GCF/PICF collected at baseline and day-90, pro-inflammatory cytokines and bone/tissue destructive factors produced in each site were measured using Luminex Multianalyte System. More specifically, TNF-α, IL-β, RANKL and MMP-9, were measured in GCF/PICF. At baseline, there was an increasing trend of TNF-α IL-1β and RANKL productions in both periodontitis and periimplantitis sites compared to healthy sites, indicating that inflammatory responses are induced in those diseased sites. Such a trend of higher production in periodontitis and peri-implantitis sites than periodontally healthy sites was not detected for MMP-9 measured at baseline. (Figure 4,5,6, and 7) Among those four factors detected in GCF/PICF, only TNF-α showed some responses to treatment. In the GCF collected from periodontitis sites at Day-90, non-surgical treatment followed by either oral administration with probiotic or placebo tablets suppressed significantly the production of TNF-αin periodontal pocket, suggesting that non-surgical treatment was effective in down-regulating the production of proinflammatory TNF-α, and that probiotics was not effective in periodontitis

sites. However, in the PICF collected from peri-implantitis sites at Day-90, non-surgical treatment followed by oral administration with probiotics, but not placebo, showed significant suppression of TNF-α production, indicating that adjunctive oral probiotic administration may be effective in down-regulating the production of TNF-α levels in PICF in peri-implantitis lesions. (Figure 4, 5, 6, and 7)

3.3 Microbiological analysis of subgingival plaque samples in periodontally healthy, periodontitis and peri-implantitis sites

According to the 16S rRNA sequence performed for dental/peri-implant plaque, which is commonly used for identification, classification and quantitation of microbes within the plaque, we identified a total of 838 bacterial species. The 16S rRNA gene is the DNA sequence corresponding to rRNA encoding bacteria, which exists in the genome of all bacteria and highly conserved, specific to each bacterial species, and the data base include the all species of bacteria of which 16S rRNA was already clarified. Nonetheless, we could not detect any of the four probiotics administered orally via probiotic tables at Day- 90 samples, including, *S. salivarius* K 12, *S. salivarius* M18, *L. paracasei*, and *L. reuteri,* suggesting that probiotic bacteria did not colonize in any of sites tested.

The formation of oral bacterial biofilm, which is responsible for onset and progression of periodontitis, was thoroughly studied by Socransky's group at the Forsyth Institute (16930311, 11350499, 15790740). They, then, established a

theory that the tooth surface is consecutively colonized by distinct complexes of bacteria which were color coded in following order: 10 yellow/purple, 2) orange and 3) red. For instance, in the red complex, three periodontal pathogens, *Porpyhromonas gingivalis, Treponema denticola,* and *Tannerella forsythia*, are included. According to their theory, the clinical symptoms of periodontal infection usually reflect the amount and composition of dental plaque harbored around the tooth. This theory of bacterial settlement was also translated into dental implant infections (29238198). The elevated species in microbial flora in the peri-implant lesions compared to healthy peri-implant tissue were found to be similar to those found elevated in periodontitis compared to healthy periodontal tissue, including the red complex bacteria and orange complex species (*Fusobacterium* and *Prevotella intermedia*) defined by Socransky's color complex theory (PMID: 27833735, 25622536, 26424287, 26252036). Therefore, we have evaluated the effects of orally administered mixed probiotics on the possible alteration of different color complex bacteria.

According to 16S rRNA sequence results, at the baseline, significantly elevated relative sequence counts of red complex bacteria, including, *Porpyhromonas gingivalis,* and *Tannerella forsythia* were found in peri-implantitis (both *Pg* and *Tf*) and periodontitis sites (*Tf*) compared to healthy sites (Figure 9). Among 6 bacteria in the orange complex, only *Parvimonas micra* showed the significantly higher relative sequence counts in peri-implantitis lesions compared to healthy sites (Figure 10). In the green complexes, *Eikenella*

corrodens and *Campylobacter concisus*, showed the decreased incidence in periodontitis and peri-implantitis sites compared to healthy sites (Figure 11). In the yellow complex, the relative sequence counts of Streptococcus sanguinis was significantly lower than that of healthy site (Figure 12). In sum, the color complex distribution in the microbiome of peri-implantitis lesion was similar to the Socransky's theory established for periodontitis.

The effects of oral administration of probiotics on the relative sequence count of each bacteria in different color complexes were determined in the plaque samples collected from periodontally healthy, periodontitis and peri-implantitis sites at Day 90, in comparison to the placebo control. There was no significant effect of probiotics used in this study on the relative sequence count of any bacteria in red and orange complex compared to placebo or baseline level. Instead, adjunctive probiotics significantly increased the relative sequence count of *Prevotella intermedia* in periodontitis site compared to placebo control or baseline in the orange complex. On the other hand, relative sequence count of *Campylobacter concisus* in green complex, *Actinomyces* species in yellow complex and *Actinomyces graevenitzii* in purple complex were significantly increased in the peri-implantitis sites by the oral administration of probiotics at Day-90, but not in control group. These results indicated that probiotics appeared to elicit the host beneficial influence to restore the healthy, more symbiotic microbiome in the peri-implantitis sites by promoting the population sizes of commensal bacteria (green, yellow and purple complex), while probiotics used in

this study did not affect the population size of pathogenic complexes (red and orange complexes).

Chapter 4: Discussion

As infectious diseases present a problem in the current healthcare society, bacterial resistance to antibiotics became a realistic concern. It is true that, after bacterial resistance was reported to Daptomycin, an alternative to Vancomycin that had been used as the last resort antibiotics for MRSA treatment, pharmaceutical industry has never developed any novel class of antibiotics that can kill Daptomycin-resistant MRSA. Thus, alternative therapies such as probiotics became more of interest in recent research. Current research investigated the use of *L.reuteri* as an adjunct to non-surgical therapy in treating gingivitis and periodontitis and found clinical and microbiological improvement in addition to the benefit of implementing mechanical treatment alone.[75,76] A recent systematic review and meta-analysis published in March 2020 evaluated specifically, the effect of probiotic *Lactobacillu,* in the nonsurgical management of peri-implant diseases found that there was a slight reduction of probing depth after treatment termination but concluded that overall, *Lactobacillus* gave limited benefits in peri-implant mucositis.[77] This systematic review included 7 studies with only one of them looking at the effect of *Lactobacillus* on peri-implantitis lesions while the other studies focused only on peri-implant mucositis lesions.[77] However, it is noteworthy that the evidence withdrawn from those 7 published studies is weak, due to the small sample sizes which did not provide sufficient statistical power.[77] Though this review concluded that there were limited benefits

in managing peri-implant mucositis and peri-implantitis with *lactobacillus* in conjunction with non-surgical treatment, this result can only be considered preliminary due to its small sample size.[77] Thus, more studies are needed especially for investigating the potential benefit of oral probiotics in treating peri-implantitis. To the best of our knowledge, there was only one published study by Galofre et al. to date that examined on the oral intake of probiotic as an adjunct treatment to non-surgical mechanical therapy.[51] They concluded that the probiotic, *L. reuteri* led to a statistically significant improvement for clinical parameters such as bleeding on probing, probing pocket depth while microbiologically, there were no effect on the bacterial load for peri-implantitis sites.[51] However, the subjects only took the oral probiotic for 30 days and real-time PCR was used to quantify bacterial loads of only 9 bacteria in the red and orange complexes.[51] Contrast to the above noted published studies that evaluated the possible therapeutic effects of administrating single strain of *L. reuteri*, the present study, for the first time, utilizes a mixture of oral probiotics that contains three additional strains of bacteria including *S. salivarius* K 12, *S. salivarius* M18 and *L. paracasei*, in addition to the conventionally used *L. reuteri*. In Chapman et al.'s review in comparing mixture of probiotic strains versus single strain in efficacy, it was concluded that probiotic mixtures were more effective against a wide variety of end points including irritable bowel syndrome, atopic diseases, immune function, respiratory infections and more.[78.] It is, then, conceivable that the superior efficacy of mixed probiotic strains may be attributed to synergistic interactions among the administered mixed probiotic strains. Otherwise, it is also

plausible that, because of mixture of different strains, the increase total number of probiotic bacteria used in those clinical studies are responsible for the pronounced clinical efficacy compared to that used single probiotic strain. Thus, with the purpose to augment the efficacy of the oral probiotics as much as possible, the present study used a higher dosage of mixed probiotic strains compared to other published studies; more specifically, the patients were instructed to take 2 tablets of 3 billion colony forming units (CFU) of mixture of four probiotic strains (a total of 6 billion CFU), contrast to one tablet containing a single strain of *L. reuteri* (2 hundred million CFU) as implemented by the majority of the studies.[48,49] Furthermore, this study explored microbiome changes on not only peri-implantitis lesions but also, periodontally healthy and periodontitis sites and used 16S ribosomanl RNA (rRNA) sequencing to conduct the microbiological analysis. This metagenomic bacterial sequencing method can better recognize poorly described, rarely isolated or phenotypically aberrant strains than classical PCR method which may, in turn, lead to identifying novel pathogens and/or uncultured bacteria.[79] This pilot study concluded that this multiple strain blend of probiotics had no statistical significant effects on any of the clinical parameters tested including probing depth, GCF/PICF volume and clinical attachment levels in the probiotic group compared to the placebo group for peri-implantitis and periodontitis sites. This could be due to no effect of adjunctive use of probiotics in peri-implantitis lesion and/or small sample size. Such findings did not correspond to the studies by Galofre et al. and Flichy-Fernandez et al.[48,51] Galofre et al. demonstrated that probing depth at peri-implantitis lesions improved by the

probiotic administration whereas Flichy-Fernandez et al. also reported the findings similar to Galofre et al. in peri-implant mucositis sites.[48,51] Nonetheless, the present results were in accordance with Hallstrom et al. who reported little or no effects of probiotics on the clinical parameters in the peri-implant mucositis sites.[49] Since it is well-accepted that multiple different factors, such as ethnic background, gender and age, are also associated with pathogenesis of peri-implantitis, large scale comprehensive clinical studies are required in future to gain insight into the effects of probiotics on the peri-implantitis lesions.

The key finding of this study is the impact of probiotics on the microbiological composition as well as on the production patterns of pro-inflammatory cytokine TNFα produced in GCF/PICF. There was no difference in the levels of MMP-9 and sRANKL detected in GCF/PICF between the baseline (Day-0) and two treatment groups (placebo and probiotics) at Day-90. Both placebo and probiotic groups at Day-90 compared to baseline (Day-0) showed a statistically significant decrease in TNF-α levels at periodontitis sites in the present study. However, the probiotic group only, but not placebo group, demonstrated a statistically significant reduction in PICF TNF-α levels at peri-implantitis sites at Day-90 compared to baseline. A recently published systematic review study showed that peri-implantitis sites were associated with a significant increase in TNF-α levels compared to healthy implant sites.[80-81] Furthermore, TNF-α levels have been proposed to be used as one of early biomarkers to detect the onset of peri-implant diseases that may not be clinically apparent.[80-81.] This pro-inflammatory cytokine induces fibroblast apoptosis and

impairs the regenerative ability of the peri-implant tissue. The review also illustrated that TNF-αlevels increase with the progression of attachment loss around implants suggesting that the levels of TNF-α could be proportional to the loss of attachment.[80] Thus, our findings showing statistically significant reduction in PICF TNF-α levels at peri-implantitis lesions of probiotic group indicated that administration of oral probiotics may reduce inflammation around peri-implantitis sites by shifting the subgingival microbiome towards more symbiotic bacteria. Therefore, we speculate that oral probiotic administration as an adjunct to regular home care could prevent the progression of peri-implantitis. To test it, large scale longitudinal studies are needed to evaluate the effect of this probiotic blend on suppression of pathological tissue destruction in peri-implantitis lesions by monitoring the level of TNF-α as a pathologic biomarker.

This study investigated the impact of mixed probiotics on the incidence of specific bacteria in the Socransky's color complexes (red, orange, purple and yellow), whereas effects of mixed probiotics on the shift of bacterial phyla in microbiome were also monitored.[82] Although there were no reduction in the incidences of the red and orange complexes, an intriguing trend of elevated incidence the commensal bacterial species was detected in peri-implantitis sites, but not periodontitis sites, in a manner dependent on probiotics treatment. There was a statistically significant increase in the bacteria from the green, yellow and purple complexes namely the *Actinomyces species, Actinomyces graevenitzii and Campylobacter concius*. Based on Socransky et al., the blue, yellow, green and purple complexes are known to be compatible with periodontal health.[82] These

bacteria are known to be the first bacteria to colonize the supragingival and subgingival biofilms and are believed to be non-harmful or host-beneficial commensal bacteria that are detected as high incidence groups of bacteria in periodontally healthy individuals.[84] In sum, our findings suggested that, although this mixture of probiotics did not suppress the incidence of red and orange complexes, it was able to increase the incidence of commensal bacteria that represent periodontal health and may therefore help restore the more symbiotic oral microbiota from the imbalanced pathogenic microbiota that is in favor of upregulating the progression of peri-implantitis. Therefore, we speculate that adjunctive and preventive use of oral probiotics can be considered as a treatment modality in the future for the treatment of peri-implant diseases.

Moreover, this study was able to compare the microbiome at baseline among periodontal health versus peri-implantitis sites and its findings are in agreement with several studies that *P. micra, T. denticola* were associated with peri-implantitis lesions while Streptococcus sanguinis was associated with health.[84.]

Furthermore, out of more than the 800 bacterial species detected from the 16S rRNA gene sequence analysis, there were no specific strains from the oral probiotic tablet consumed that were detected. This could be attributed to the colonization effect of oral probiotics may vary among different individuals, products and strains as demonstrated by both in vitro and in vivo studies.[85,86,87.]

For example, two different *L.reuteri* strains were reported to colonize the oral cavity of 48-100% of the subjects who consumed the products containing them.[86,87.] Not only was this a wide range but a mixture of different oral probiotic strains could lead to changes in the microbiota overall. For example, by adding a mixture of 7 different *Lactobacillus* strains, the number of salivary *Lactobacillus* counts increase significantly.[41] Maukonen et al. suspected that probiotic bacteria may only colonize the oral cavity when they were used in products that are contacting the mouth as they did not find any probiotic bacteria that were administered through capsules in saliva samples.[88.] Our finding agreed with this phenomenon. However, Haukioja et al. found in his study that *Lactobacillus rhamnous* survived well in saliva and was even found 3 weeks after discontinuing the oral probiotics.[89] What was agreed among all the studies investigating colonization though was that there was a large variation in binding to saliva-coated surfaces and buccal epithelial cells and different probiotic strains and their interactions could affect the binding tremendously along with high changeability among different hosts.[89,17, 90.]

This brought the point of one of the limitations of this study being the colonization of the multiple blend of bacteria strands over time was not evaluated. Though previous literature had suggested that the colonization of *L. reuteri* in subgingival sites start between 2-3 weeks after the start of probiotic treatment and remain until 70-75 days post-therapy, it is unknown regarding the duration of effect for this particular blend of probiotic strains that this study had implemented.[48,49,50] Thus, future studies could explore the temporal change of

colonization by each bacterium in the probiotic mixture and evaluate the microbiome changes through the time after stopping of the probiotic administration. Such would help to establish the optimal time required for treatment and the dosage required for probiotics to take effect. It is noteworthy that, although probiotics were hypothesized to only colonize the oral cavity temporarily, some studies revealed clinical and microbiological improvements even when *L. reuteri* were no longer detected in subgingival samples.[91,92] Another limitation of this study was the relatively low number of total subjects (n=15; n=9/group for probiotics and n=6/group for placebo).[91,92.] According to our power calculation, at least n=12/group is required to get the statistically significant difference by the intervention provided to the patients with peri-implantitis. Furthermore, the overhanging prosthesis of the implant crown may interfere with the examiner trying to introduce the sampling paper strips (PerioPaper) in order to collect the microbiological sample, especially when the sulcus was tightly confined.

Overall, this study demonstrated that the oral administration of probiotic mixture for 90 days may help increase the commensal bacteria associated with health Nonetheless, longer-term longitudinal studies are needed 1) to determine stability of increased commensal bacteria associated with health 2) to ascertain the optimal dosage that is needed for the stable change of microbiome towards health and 3) to define the duration that the oral probiotics would be able to exert their influence after stopping the oral administration of probiotic mixture.

Chapter 5: Conclusions

The daily oral intake of the probiotic blend consisting of *S. salivarius K 12, S. salivarius M 18, L. reuteri, L. paracasei*, as an adjunct to non-surgical periodontal therapy did not improve the clinical parameters for peri-implantitis sites. However, biologically, such an administration of probiotic mixture, but not placebo, were able to significantly reduce the PICF TNF-α levels in peri-implantitis lesions at Day-90 compared to baseline. This suggested that oral probiotics may be indirectly suppressing PICF TNF-α levels possibly decreasing the magnitude of inflammation in peri-implantitis lesions. Moreover, though the population sizes of red and orange complex bacteria were not reduced by oral administration of probiotic mixture, there was a shift in the population size of yellow, blue and purple complex bacteria which may imply that oral probiotic mixture may alter the microbiome composition in peri-implant sites towards healthy symbiotic microbiome. According to our results, the oral probiotics may initiate greater positive microbiological and biological effects in the early stages of treatment. However, clinical effects were not shown in the present study as the early effects of probiotics. Clinical effects of them may follow in the later stages of treatment which can be studied in a long-term clinical study. Therefore, long-term longitudinal clinical studies are needed to support these findings as the research of oral probiotics' effect on peri-implant diseases has just emerged in the oral science research field. In addition, there is an overall high heterogenicity within the studies published in terms of the dosages and duration used for oral probiotics-based therapy. It is also intriguing to explore if the change of bacterial

strains in probiotic mixture would affect the clinical outcomes as well as population size of pathogenic red and orange complexes.

It is still immature to conclude that oral probiotics are able to effectively improve peri-implantitis from the present study. We are confident in stating that oral probiotics were able to exert impact on the microbiota within the oral cavity, especially in the peri-implantitis lesions within the limitations of the present study. One of the strengths of probiotic approaches is that, unlike notorious antibiotics, probiotics do not induce bacterial resistance in the subgingival flora even if they were ingested frequently. Only future research will unveil the possible host beneficial property of probiotics in response to peri-implantitis with the ultimate goal of developing a side-effect free sufficiently efficient clinical approach for periimplantitis using the probiotics.

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Appendix A

Table 1

Table 1: Clinical Data Collected at Baseline for Test and Control Groups.

Subject #	Test (A)/Control (B)	Tooth/Implant	GCF1b	GCF2	PD MB	PD MidB	PD DB	PD ML	PD MidL	PD DL	CAL MB	CAL MidB	CAL DB	CAL ML	CAL MidL	CAL DL
1	B	8H	181	195	3	2	2	2	3	2	3	3	3	2	3	2
		3P	199	185	5	3	3	5	3	2	9	6	6	5	3	2
		19PI	170	164	3	6	3	3	4	5	3	6	3	5	4	3
2	A	27H	60	74	3	2	3	3	2	3	2	1	2	2	1	2
		5P	84	60	4	2	4	4	2	4	3	1	3	3	4	4
		12PI	150	158	6	4	9	8	5	4	9	9	9	11	10	11
		13PI	190	90	7	4	6	4	3	4	9	10	10	11	7	7
3	A	25H	142	140	2	2	2	2	2	2	1	1	2	1	3	1
		2P	156	125	9	2	3	7	4	4	11	5	6	8	5	4
		19PI	198	174	6	4	4	8	3	4	6	4	4	8	4	4
4	A	22H	125	125	3	2	3	3	2	3	2	1	2	1	1	2
		13P	83	138	3	3	4	4	3	3	2	2	3	2	2	2
		9PI	65	40	4	10	6	3	3	3	4	9	7	5	5	10
		10PI	95	50	4	3	3	4	2	3	7	6	6	5	3	4

5	A	28H	55	62	2	2	3	3	2	3	1	2	2	2	3	3
		22P	73	50	2	1	3	3	2	3	8	6	3	6	5	5
		7PI	16	17	3	2	3	5	3	5	6	7	6	8	6	8
		10PI	29	99	3	2	3	5	4	4	6	6	6	8	8	8
6	A	28H	70	NA	2	2	3	2	2	3	3	5	4	1	3	2
		22P	69	NA	4	1	3	3	2	3	3	0	2	6	5	5
		7PI	67	NA	2	1	3	7	3	4	5	5	6	9	5	6
		10PI	68	NA	2	2	2	6	3	3	6	7	6	8	6	6
7	B	24H	120	130	3	2	3	3	2	3	2	2	3	3	3	2
		30P	123	104	3	5	5	5	3	5	4	6	5	6	4	6
		3PI	145	140	8	7	7	6	4	8	8	7	7	7	6	11
8	B	9H	50	67	2	2	2	2	2	2	4	3	4	1	1	1
		20P	158	128	5	3	4	4	3	5	4	4	5	3	4	6
		12PI	76	58	6	3	7	6	4	9	6	3	7	6	3	9
9	B	9H	55	148	3	2	3	3	2	3	3	2	3	5	4	4
		25P	87	149	3	3	4	3	3	3	4	4	4	6	5	7
		11PI	98	56	4	4	4	4	3	5	4	3	3	4	4	3
		12PI	170	149	4	4	6	4	3	6	2	1	2	3	3	3
10	B	7H	90	60	3	2	3	3	3	3	2	2	2	3	2	3

		29P	170	153	6	4	4	3	3	2	3	3	4	3	3	5
		3PI	145	190	5	5	5	7	5	6	5	5	5	7	6	9
		14PI	110	122	6	3	5	4	5	4	6	3	8	9	8	5
11	B	24H	84	92	3	2	3	3	2	3	2	1	2	2	1	2
		30P	116	100	5	3	3	4	2	3	2	4	3	2	4	4
		3PI	193	172	8	9	10	9	9	9	10	9	8	9	9	9
12	B	8H	88	105	3	2	2	3	2	3	1	3	2	2	1	2
		3P	147	75	3	3	4	4	3	4	5	5	5	4	4	6
		19PI	175	185	4	4	6	5	6	4	5	6	4	4	6	6
13	B	27H	77	40	3	3	3	2	2	3	2	1	1	2	2	3
		5P	169	70	3	2	3	3	2	3	2	1	2	4	3	2
		12PI	175	125	9	9	9	6	6	7	9	9	10	8	9	9
		13PI	190	190	9	9	7	7	7	6	10	10	9	9	10	9

Note: GCF1b: gingival crevicular fluid volume at baseline**;** GCF2: gingival crevicular fluid volume at final appointment (90 days); PD: probing depth (mm); MB: mesiobuccal; MidB: mid-buccal; DB: disto-buccal; ML: mesiolingual; MidL: mid-lingual; DL: disto-lingual; CAL: clinical attachment loss; H: healthy; P: periodontitis; PI: peri-implantitis.

Appendix B

Table 2

Table 2: Clinical Data Collected at Final Appointment (90 days).

Subject #	Test (A)/Control (B)	Tooth/ Implant	GCF1	GCF2	PDMB	PDMidB	PDDB	PDML	PDMidL	PDDL	CALMB	CALMidB	CALDB	CALML	CALMidL	CALDL
1	B	8H	62	74	2	2	2	3	2	3	1	2	2	1	1	1
		3P	72	77	3	2	4	3	3	5	2	4	4	2	5	7
		19PI	83	133	5	7	7	4	5	7	3	5	3	2	3	3
2	A	27H	90	105	3	2	3	3	2	3	2	1	2	2	1	2
		5P	170	125	4	3	3	4	3	3	3	2	2	3	4	2
		12PI	125	181	8	8	9	7	7	8	7	7	8	9	10	12
		13PI	128	119	8	7	6	8	5	4	7	8	9	12	8	7
3	A	25H	82	105	2	2	3	2	2	3	1	3	2	1	1	2
		2P	150	95	3	3	3	3	2	5	6	6	5	2	3	6
		29PI	180	103	3	4	5	5	3	4	2	5	4	4	6	3
4	A	22H	65	40	3	3	3	3	2	3	2	2	2	2	1	2
		13P	117	72	4	3	4	4	3	3	3	2	3	3	2	2
		9PI	152	172	4	9	10	10	5	5	3	9	10	10	5	5
		10PI	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
5	A	28H														
		22P														
		7PI														
		10PI														
6	A	28H	59	66	3	2	3	3	2	3	2	3	2	4	5	4
		22P	189	89	3	2	4	3	2	3	6	5	6	2	1	2
		7PI	69	106	3	1	2	4	3	4	6	5	5	6	5	6

		10PI	62	77	2	2	2	5	3	4	6	7	6	7	6	7
7	B	24H	170	160	3	2	3	2	2	3	2	1	2	1	1	2
		30P	180	104	4	2	3	5	2	3	3	1	2	4	5	2
		3PI	195	180	9	7	6	7	9	5	8	6	15	6	8	4
8	B	9H	35	45	2	2	2	3	3	3	1	1	1	2	1	2
		20P	119	53	4	3	3	3	2	3	3	2	2	2	2	2
		12PI	148	95	5	4	4	3	4	6	5	4	4	3	4	6
9	B	9H	25	25	4	1	3	3	4	4	4	2	4	5	5	5
		25P	102	95	3	3	3	2	2	2	5	5	5	5	4	5
		11PI	105	110	7	3	5	3	3	4	3	3	5	3	3	4
		12PI	90	60	3	5	6	5	2	4	4	5	5	3	2	4
10	B	7H	60	58	3	2	3	4	3	4	2	2	2	3	2	3
		29PI	190	160	5	3	3	4	3	3	5	3	3	4	3	3
		3PI	92	100	5	5	5	9	6	7	5	5	5	9	6	7
		14PI	188	185	6	3	5	6	6	5	6	3	8	9	8	5
11	B	24H	162	64	3	2	3	3	2	3	2	1	2	2	3	2
		30P	186	132	4	2	6	4	2	5	3	3	7	3	1	4
		3PI	160	160	6	10	10	6	5	10	6	10	10	6	5	10
12	B	8H	48	72	2	2	2	2	2	2	1	1	1	1	1	1
		3P	43	68	3	2	3	3	3	5	5	4	2	7	5	2
		19PI	195	135	3	4	4	4	6	4	3	4	4	4	6	4

13	B	27H	39	30	3	2	3	3	3	2	2	1	2	2	2	1
		5P	114	80	3	2	3	3	3	4	2	1	2	2	2	3
		12PI	199	180	8	7	9	7	5	7	8	7	10	9	8	9
		13PI	86	178	8	7	6	7	6	5	9	8	8	9	9	8

Note: GCF1b: gingival crevicular fluid volume at baseline**;** GCF2: gingival crevicular fluid volume at final appointment (90 days); PD: probing depth (mm); MB: mesiobuccal; MidB: mid-buccal; DB: disto-buccal; ML: mesiolingual; MidL: mid-lingual; DL: disto-lingual; CAL: clinical attachment loss; H: healthy; P: periodontitis; PI: peri-implantitis.

Appendix C

Table 3

Table 3: Subject Data (Test Group n= 5).

	Age	Gender	Smoking status	Periodontal status* (Stage = S & Grade =G)	Peri-Implantitis Status**
1	33	M	Non-smoker	S III, G C	advanced
2	70	M	Non-smoker	S III, GB	moderate
3	60	F	Non-smoker	S III, GB	moderate
4	72	M	Non-smoker	S III, GB	advanced
5	76	M	Past smoker (quit in 2007)	S III, GB	moderate

Note: Periodontal status is based on 2018 AAP Periodontal Classification. All peri-implantitis lesions qualified as "true peri-implantitis lesions" based on the 2018 AAP peri-implant classification definition: 6mm or more with BOP or 3mm radiographic bone loss. Peri-Implantitis status is further defined based on Froum & Rosen's classification to further categorize the disease status.[93]

Appendix D

Table 4

Table 4: Subject Data (Placebo Group n= 8).

	Age	Gender	Smoking status	Periodontal status* (Stage = S & Grade =G)	Peri-Implantitis Status**
1	70	M	Non-smoker	S II, GB	moderate
2	63	F	Non-smoker	S III, GB	moderate
3	81	M	Non-smoker	S III, GB	moderate
4	65	M	Non-smoker	S III, GB	advanced
5	45	M	Past smoker (quit in 2000)	S III, GB	moderate
6	65	M	Non-smoker	S III, GB	advanced
7	63	F	Non-smoker	S III, GB	moderate
8	70	M	Non-smoker	S II, GB	moderate

Note: Periodontal status is based on 2018 AAP Periodontal Classification. All peri-implantitis lesions qualified as "true peri-implantitis lesions" based on the 2018 AAP peri-implant classification definition: 6mm or more with BOP or 3mm radiographic bone loss. Peri-Implantitis status is further defined based on Froum & Rosen's classification to further categorize the disease status.[93]

Appendix E

Figure 1

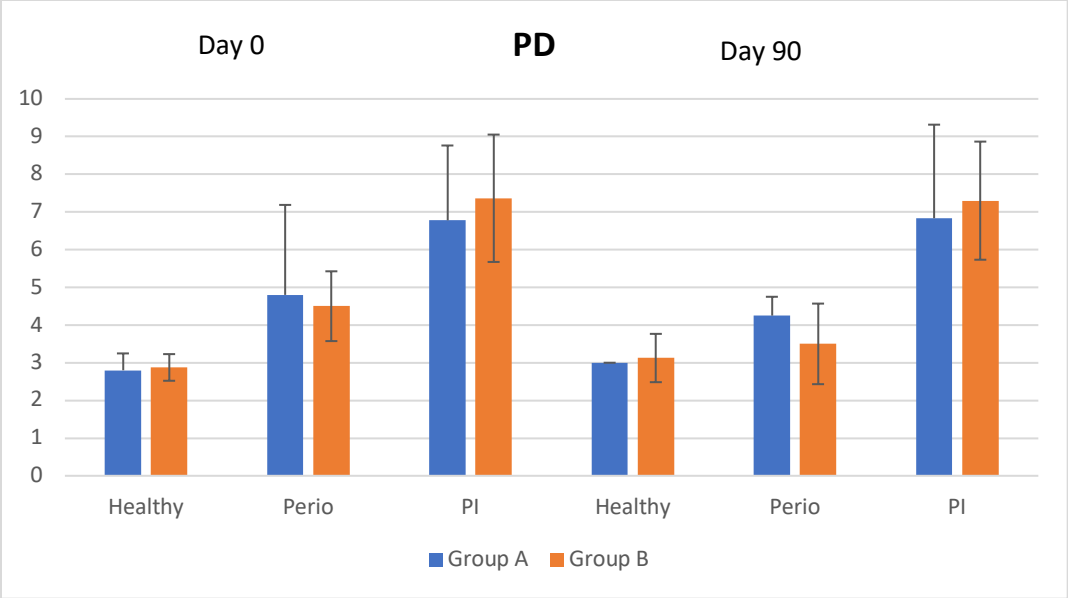


Figure 1: Pocket depths (PD) comparing Group A (probiotic-test) versus Group B (placebo-control). No statistically significant difference between Day-0 and Day-90 in healthy, periodontitis and peri-implantitis sites and between probiotic and placebo groups.
Periodontally healthy sites (Healthy); Periodontitis sites (Perio); Peri-Implantitis sites (PI).

Appendix E

Figure 2

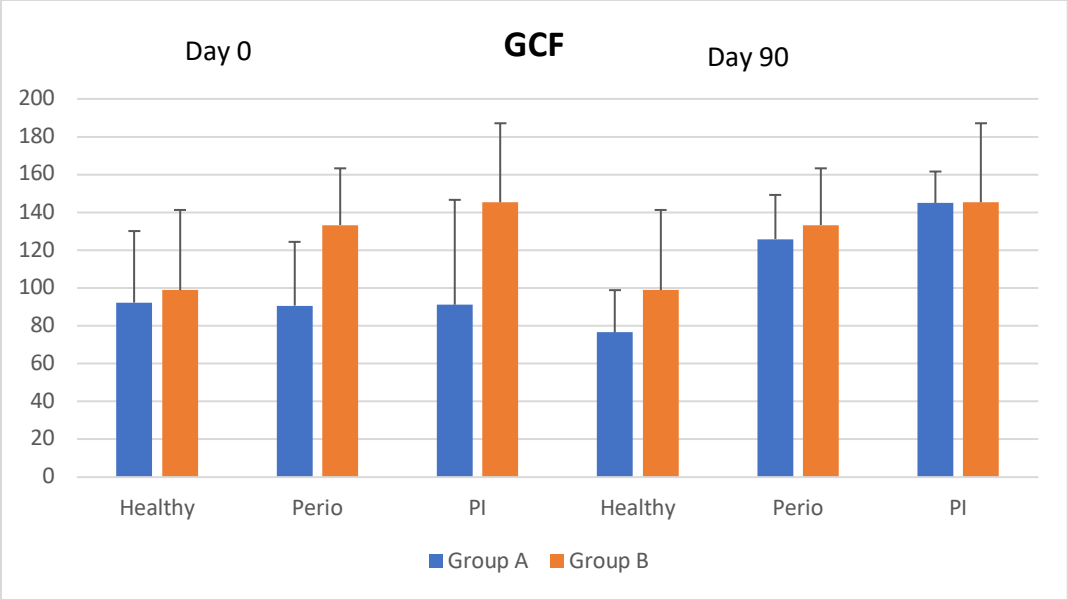


Figure 2: Gingival crevicular fluid (GCF/PICF) volume comparing Group A (probiotic-test) versus Group B (placebo-control). No statistically significant difference between Day-0 and Day-90 in healthy, periodontitis and peri-implantitis sites and between probiotic and placebo groups. Periodontally healthy sites (Healthy); Periodontitis sites (Perio); Peri-Implantitis sites (PI).

Appendix E

Figure 3

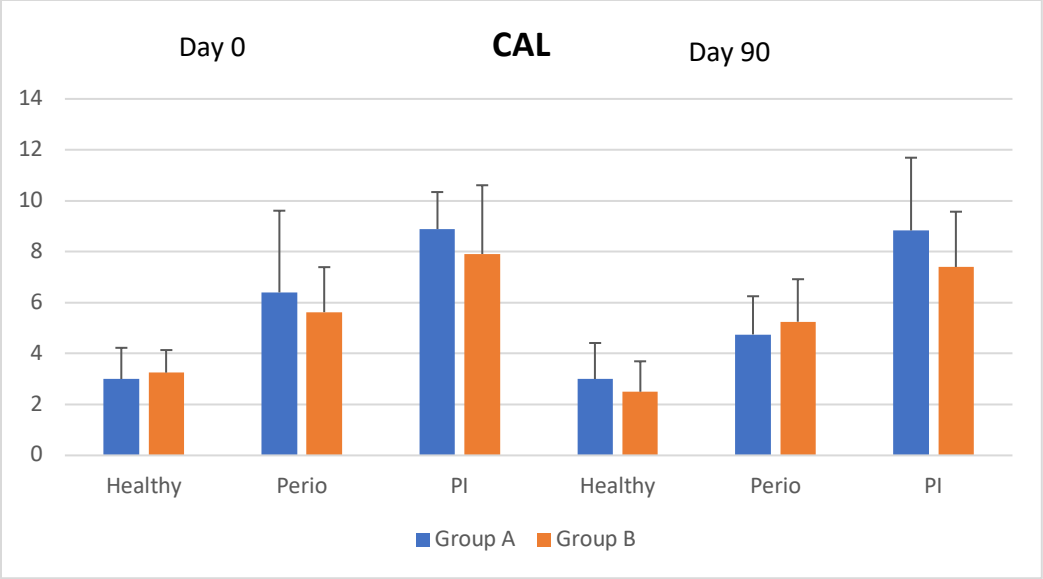


Figure 3: Clinical attachment level (CAL) comparing group A (probiotic-test) versus Group B (Placebo-control). No statistically significant difference between Day-0 and Day-90 in healthy, periodontitis and peri-implantitis sites and between probiotic and placebo groups. Periodontally healthy sites (Healthy); Periodontitis sites (Perio); Peri-Implantitis sites (PI).

Appendix E

Figure 4

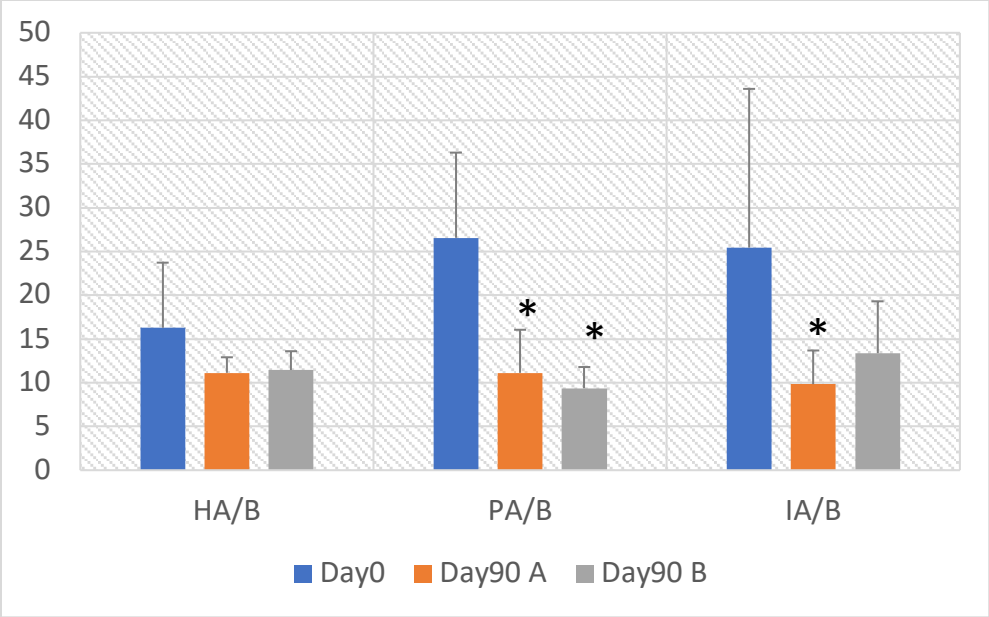


Figure 4: GCF/PICF TNF-α levels comparing group A (Probiotic-test) versus Group B (Placebo-control). Statistically significant difference in Day-90 compared to Day-0 in periodontitis and peri-implantitis sites in probiotic group. Periodontally healthy sites (H); Periodontitis sites (P), Peri-implantitis sites (I); Probiotic group (A), Placebo group (B).

Appendix E

Figure 5

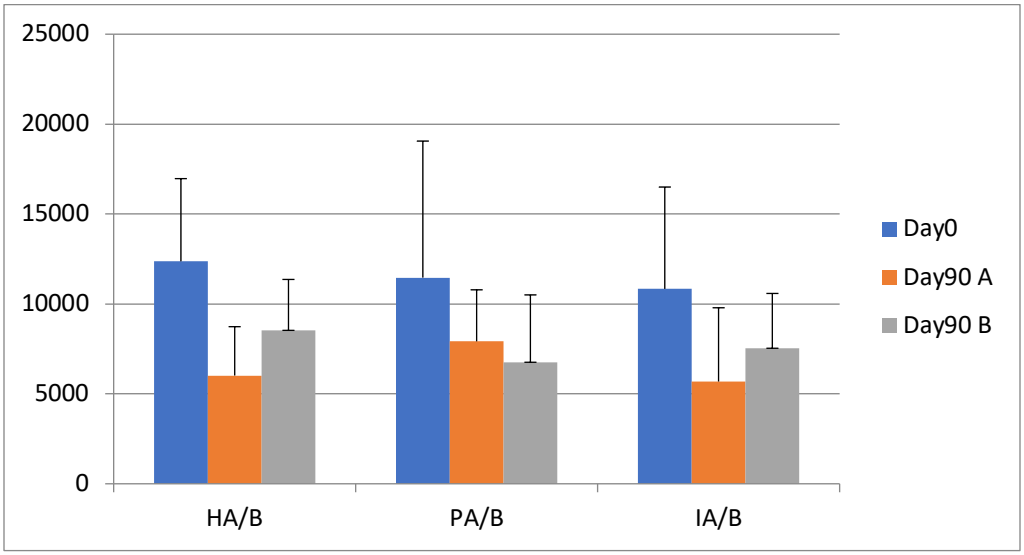


Figure 5: GCF/PICF MMP-9 levels between group A (probiotic-test) versus group B (placebo-control) among H (healthy), P (Periodontitis) and I (peri-implantitis) sites. No statistically significant differences were detected among the groups and sites.

Appendix E

Figure 6

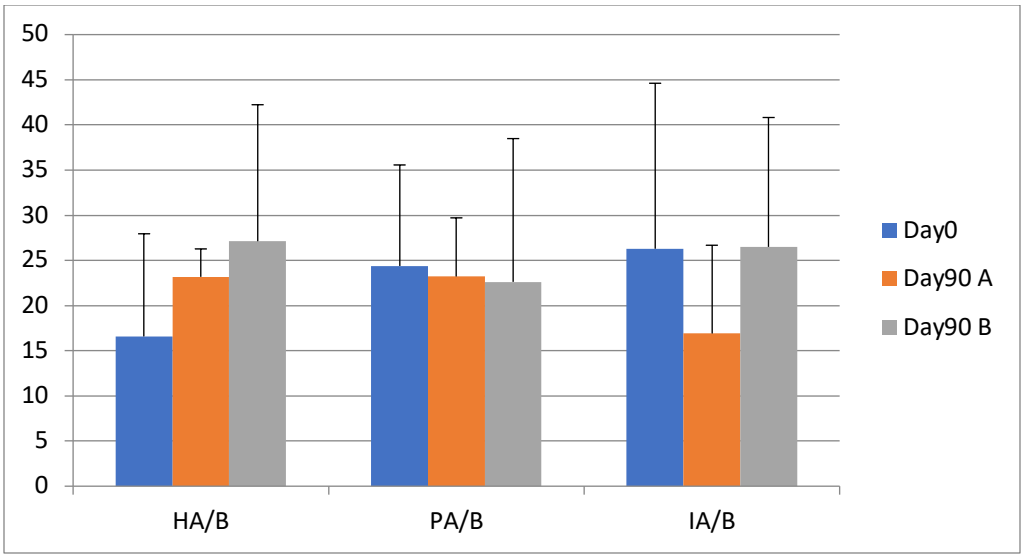


Figure 6: GCF/PICF RANKL levels between group A (probiotic-test) versus group B (placebo-control) among H (healthy), P (Periodontitis) and I (peri-implantitis) sites. No statistically significant differences detected among the groups and sites.

Appendix E

Figure 7

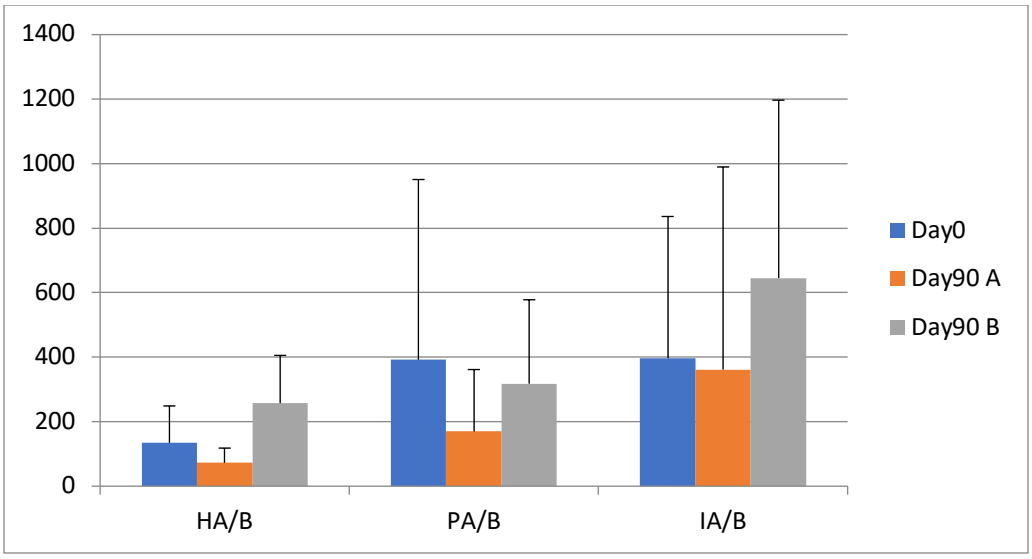


Figure 7: GCF-PICF IL-1β levels between group A (probiotic-test) versus group B (placebo-control) among H (healthy), P (Periodontitis) and I (peri-implantitis) sites. No statistically significant differences were detected among the groups and sites.

Appendix E

Figure 8

		Healthy				Periodontits				Peri-implantitis		
		Baseline	90d Placebo	90d Probio		Baseline	90d Placebo	90d Probio		Baseline	90d Placebo	90d Probio
Red	Porphyromonas gingivalis	11.9	0.7	47.8		261.3	0.8	176.6		282.0625	6	445.1
	Tannerella_forsythia	33.6	45.1	16.3		113.5	161.0	71.0		84.90625	83.5	46.0
	Treponema_denticola	24.6	105.1	17.8		186.1	63.5	327.0		257.2813	155.7	150.6
Orange	Prevotella_intermedia	111.8	691.4	4.6		22.33	1.8	789.1		316.469	165.4	690.7
	Fusobacterium_nucleatum	690.7	20.1	58.1		123.4	17.0	97.8		70.8125	626.4	78.5
	Parvimonas_micra	51.2	58.5	28.1		88.2	71.2	62.0		189.1563	79.2	64.0
	Prevotella_nigrescens	226.7	425.1	124.5		167.5	450.8	114.3		248.6875	304.7	392.5
	Campylobacter_gracilis	250.6	247.4	169.0		330.1	284.1	132.8		140.6563	147.4	252.5
	Eubacterium_nodatum	0.1	0	0.6		0.8	0	1.5		23.6875	8.0	40.5
Green	Eikenella_corrodens	150.9	46.4	61.6		41.4	64.7	34.3		37.9375	49.1	15.5
	Capnocytophaga granulosa	253.5	208.8	545.0		219.8	202.2	214.6		225.625	149.6	355.0
	Campylobacter concisus	11.6	36.7	10.5		3.8	3.2	45.5		3.59375	6.0	***54.5**
Yellow	Streptococcus_sanguinis	1133.0	362.1	556.3		763.0	885.7	170.8		380.3125	279.5	212.9
	Streptococcus_intermedius	167.8	99.7	220.0		147.7	96.0	12.8		103.0938	10.8	9.42
	Actinomyces species	3	0.7	2.830		4.8	8.7	9.3		13.71875	9.8	***186.2**
Purple	Actinomyces graevenitzii	0.3	0.1	0		0.1	0.2	0		0.21875	0.1	***45.4**
	Veillonella parvula	938.8	1904.1	516.0		821.1	1055.2	1006.3		1303	1974.7	999.6

Figure 8: Bacteria from the Socransky's Complex Detected in Healthy (H), Periodontitis and Peri-implantitis sites in terms of colony-forming units (CFU). Statistically significant difference compared to baseline with *p*-value <0.05 are seen in bold with asterisks.

Appendix E

Figure 9

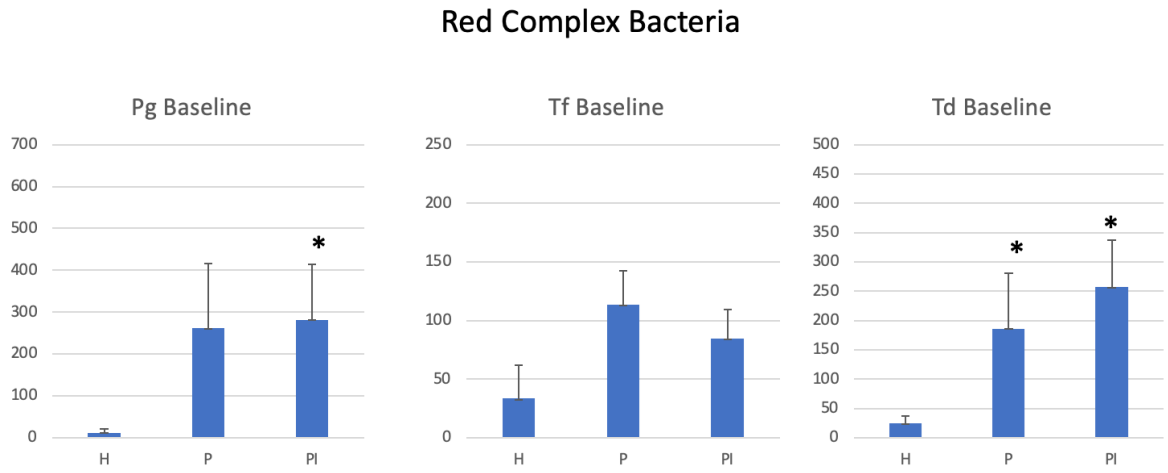


Figure 9: Red complex bacteria units at baseline. Statistically significant difference compared to healthy sites were marked in asterisks. Periodontally healthy sites (H); Periodontitis sites (P); Peri-implantitis sites (PI); *Porphyromonas Gingivalis* species (Pg); *Tannerella Forsythia* species (Tf); *Treponema Denticola* species (Td).

Appendix E

Figure 10

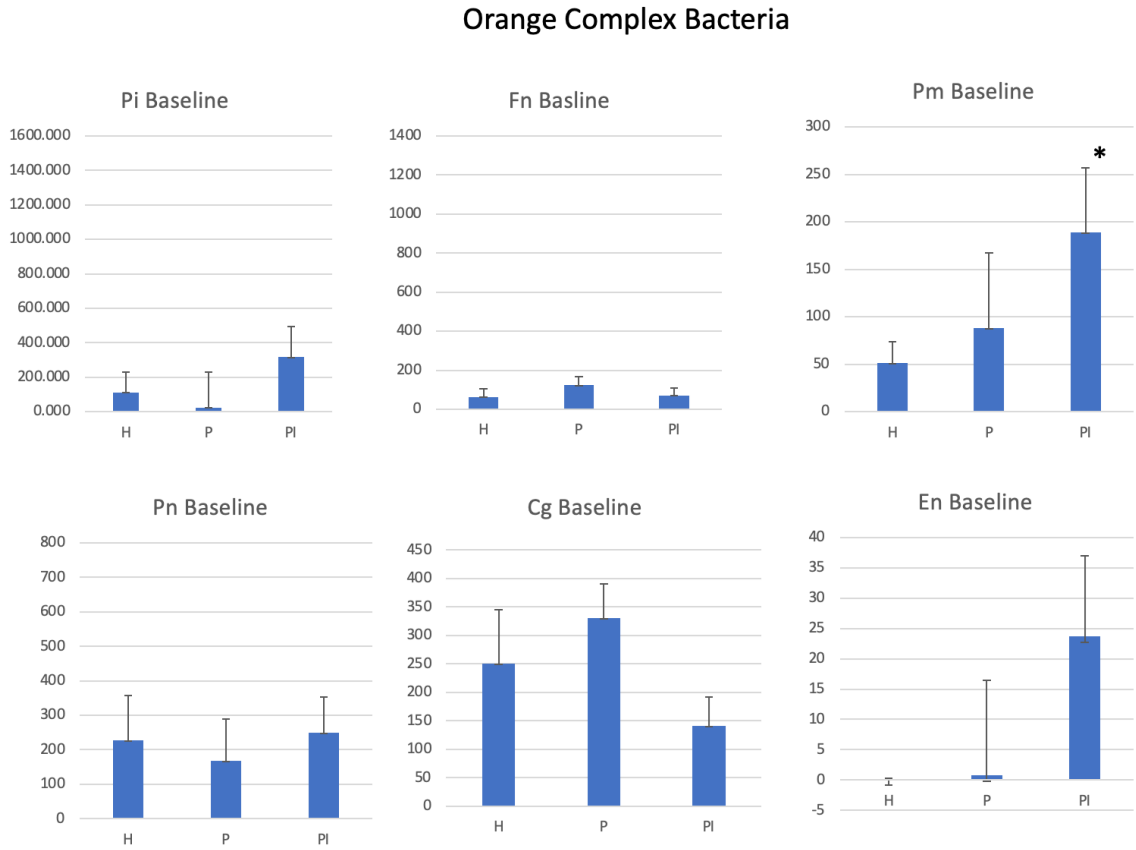


Figure 10: Orange Complex Bacteria Units at Baseline Statistically significant difference compared to healthy sites marked with asterisks. Peridontally healthy sites (H); Periodontitis sites (P); Peri-implantitis sites (PI); *Prevotella intermedia* species (Pi); *Fusobacterium nucleatum* (Fn); *Parvimonas micra* (Pm); *Prevotella nigrescens* (Pn); *Campylobacter gracilis* species (Cg); *Eubacterium nodatum* (En).

Appendix E

Figure 11

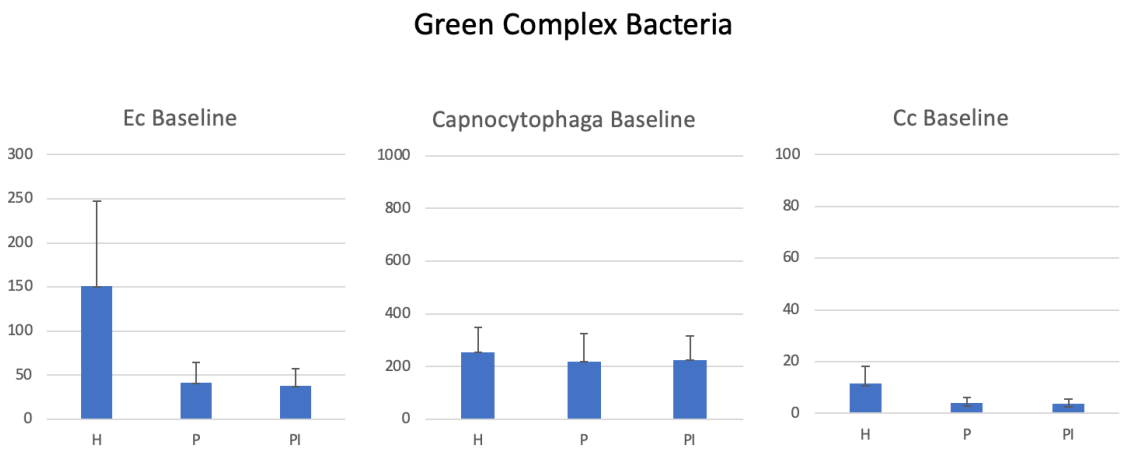


Figure 11: Green Complex Bacteria Units at Baseline. Periodontally healthy sites (H); Periodontitis sites (P); Peri-implantitis sites (PI); *Eikenella corrodens* species (Ec); *Campylobacter concisus* species (CC).

Appendix E

Figure 12

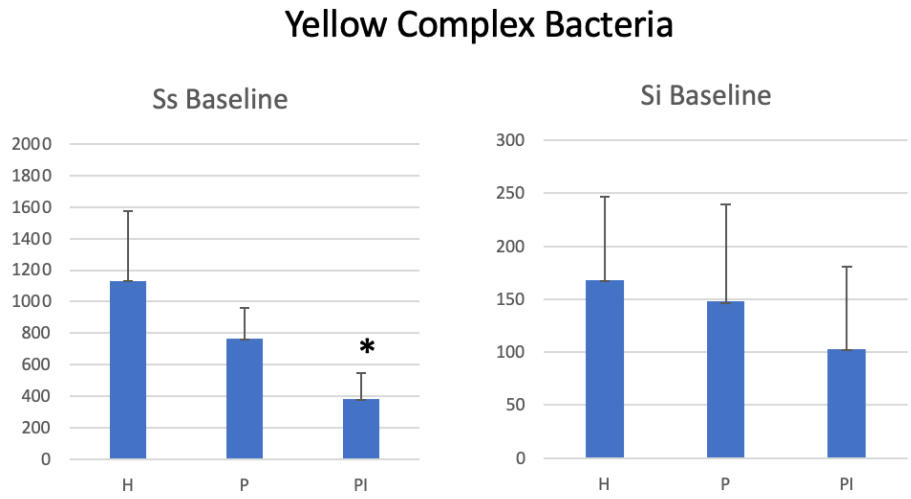


Figure 12: Yellow Complex Bacteria Units at Baseline. Statistically significant difference compared to healthy sites marked with asterisk. Periodontally healthy sites (H); Periodontitis sites (P); Peri-implantitis sites (PI); *Streptococcus sanquinis* species (Ss); *Streptococcus intermedius* species (Si).

Appendix E

Figure 13

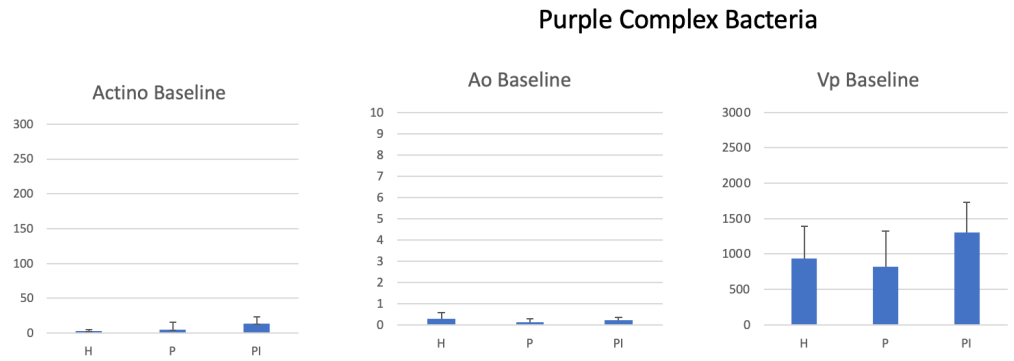


Figure 13: Purple Complex Bacteria Units at Baseline. Periodontally healthy sites (H); Periodontitis sites (P); Peri-implantitis sites (PI); *Actinomyces speciesv* (Ac); *Actinomyces odontolyticus* species (Ao); *Veillonella parvula* species (Vp).

Appendix E

Figure 14

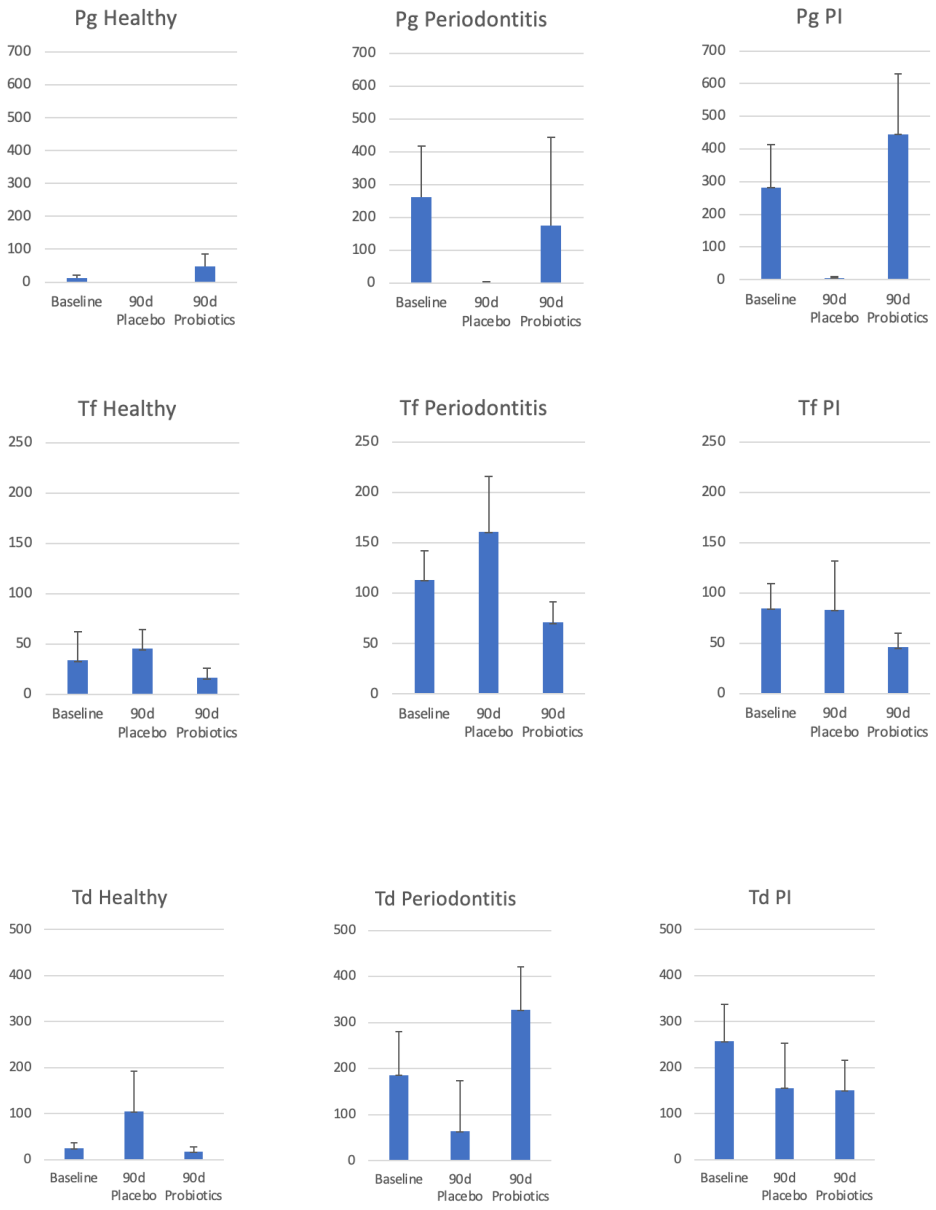
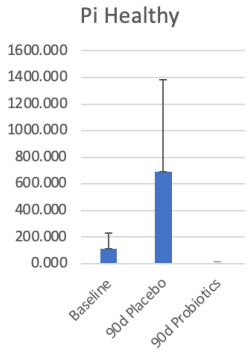


Figure 14: Red Complex Bacteria (Compared Baseline with Placebo and Probiotic groups at 90 days) for healthy, periodontitis and peri-implantitis sites.
Porphyromonas gingivalis species (Pg); *Tannerella forsythia* species (Tf); *Treponema denticola* (Td).

Appendix E

Figure 15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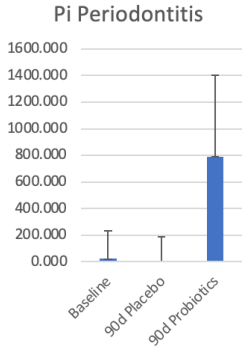
600.000
400.000
200.000
0.000
Baseline
90d Placebo
90d Probiotics


Pi Periodontitis
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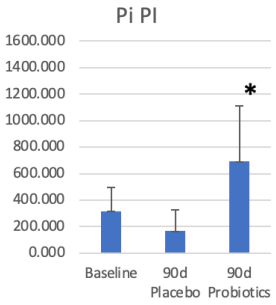
200.000
0.000
Baseline
90d Placebo
90d Probiotics


Pi PI
1600.000
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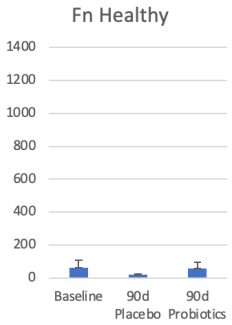
*
Baseline
90d Placebo
90d Probiotics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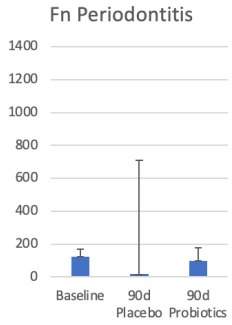
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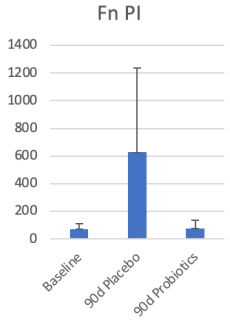
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800
600
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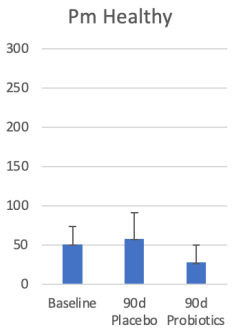
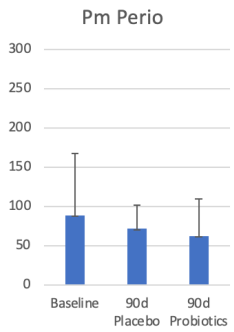
Baseline
90d Placebo
90d Probiotics


Pm Healthy
300
250
200
150
100
50
0
Baseline
90d Placebo
90d Probiotics


Pm Perio
300
250
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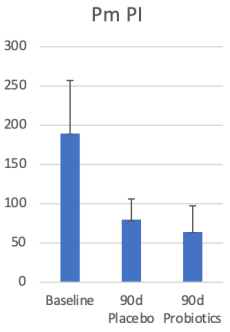
Baseline
90d Placebo
90d Probiotics


Pm PI
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Baseline
90d Placebo
90d Probiotics

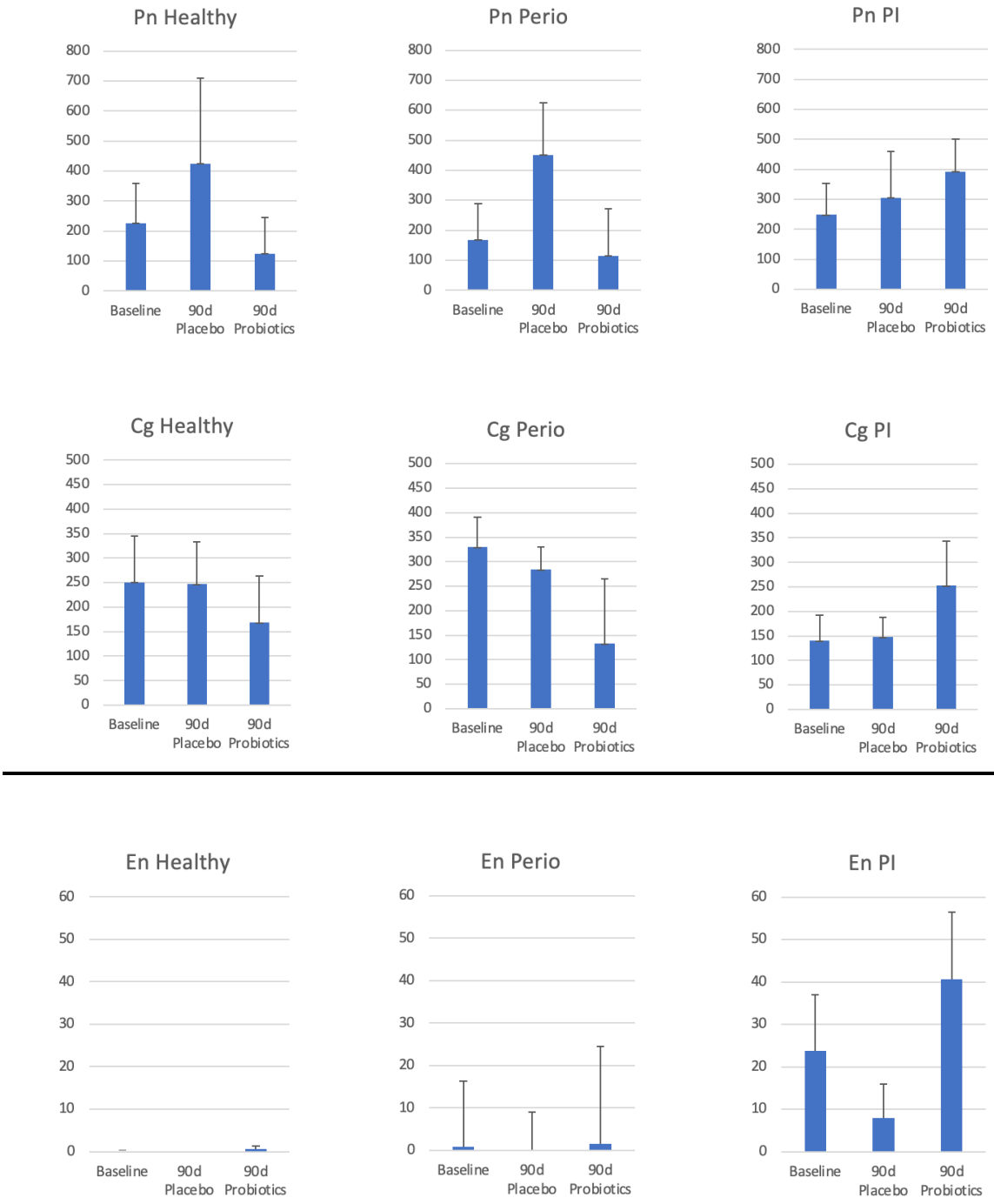

Figure 15: Orange Complex Bacteria (Compared Baseline with Placebo and Probiotic groups at 90 days) for healthy, periodontitis and peri-implantitis sites. Statistically significant difference compared to baseline marked with an asterisk. *Prevotella intermedia* species (Pi); *Fusobacterium nucleatum* species (Fn); *Parvimonas micra* species (Pm); *Prevotella nigrescens* (Pn);
Cg: *Campylobacter gracilis* En: *Eubacterium nodatum*

Appendix E

Figure 16

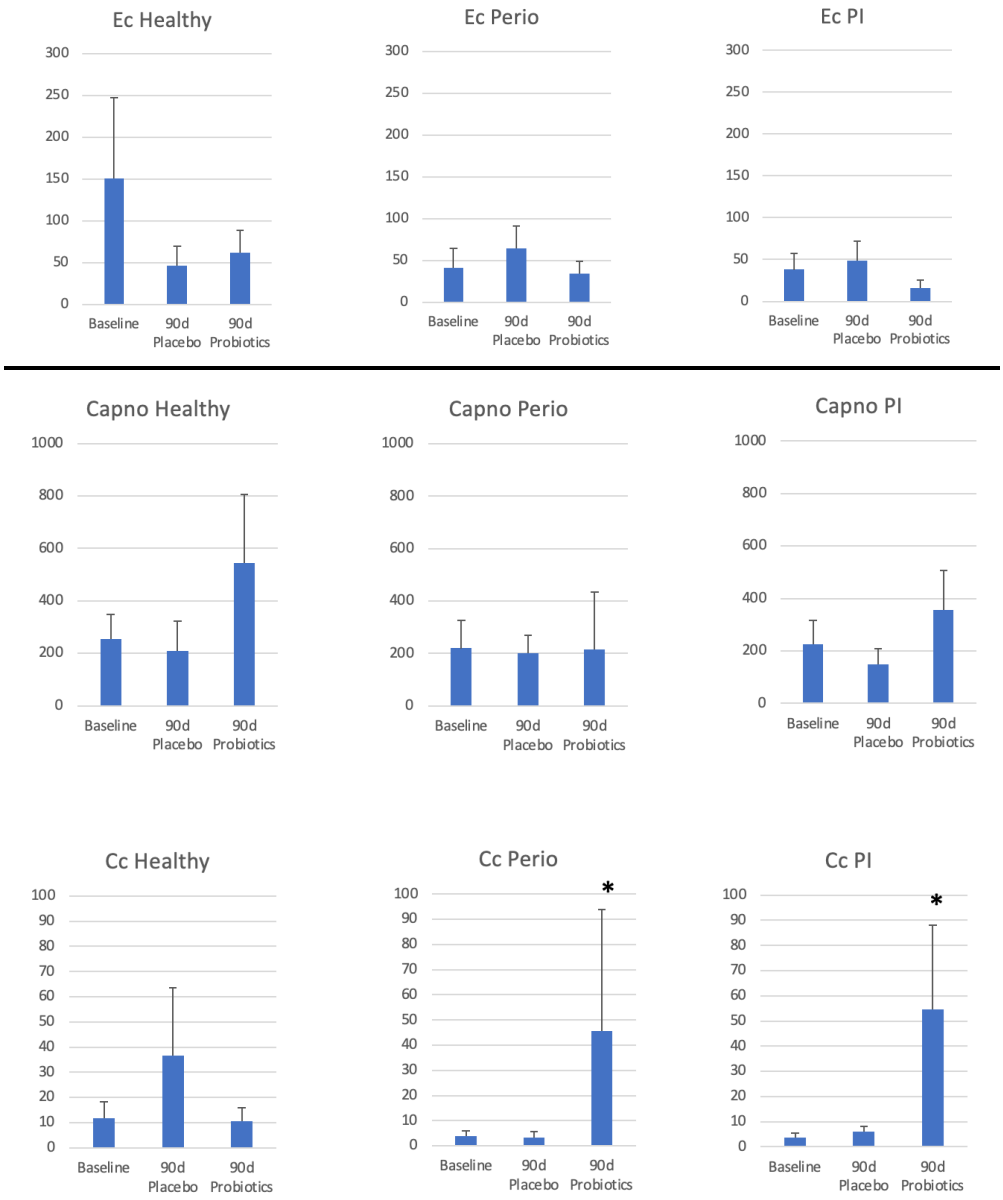

Figure 16: Green Complex Bacteria (Compared Baseline with Placebo and Probiotic groups at 90 days) for healthy, periodontitis and peri-implantitis sites. Statistically significant differences compared to baseline are marked with asterisks. *Eikenella corrodens* species (Ec); *Capnophiles* species (Capno), *Campylobacter concisus* species (Cc)

Appendix E

Figure 17

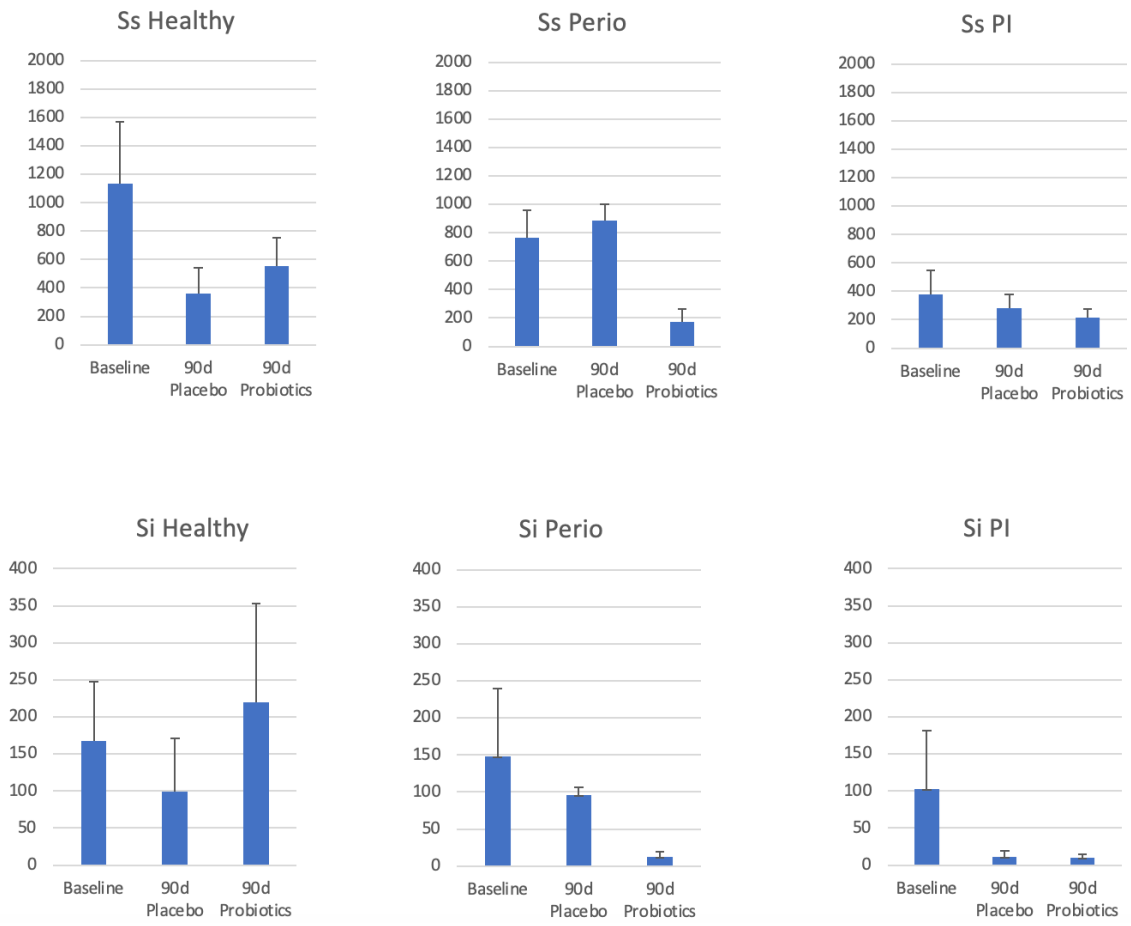

Figure 17: Yellow Complex Bacteria (Compared Baseline with Placebo and Probiotic groups at 90 days) for healthy, periodontitis and peri-implantitis sites. No statistically significant differences were found among the placebo and probiotic groups. *Streptococcus sanquinis* species (Ss); *Streptococcus intermedius* (Si).

Appendix E

Figure 18

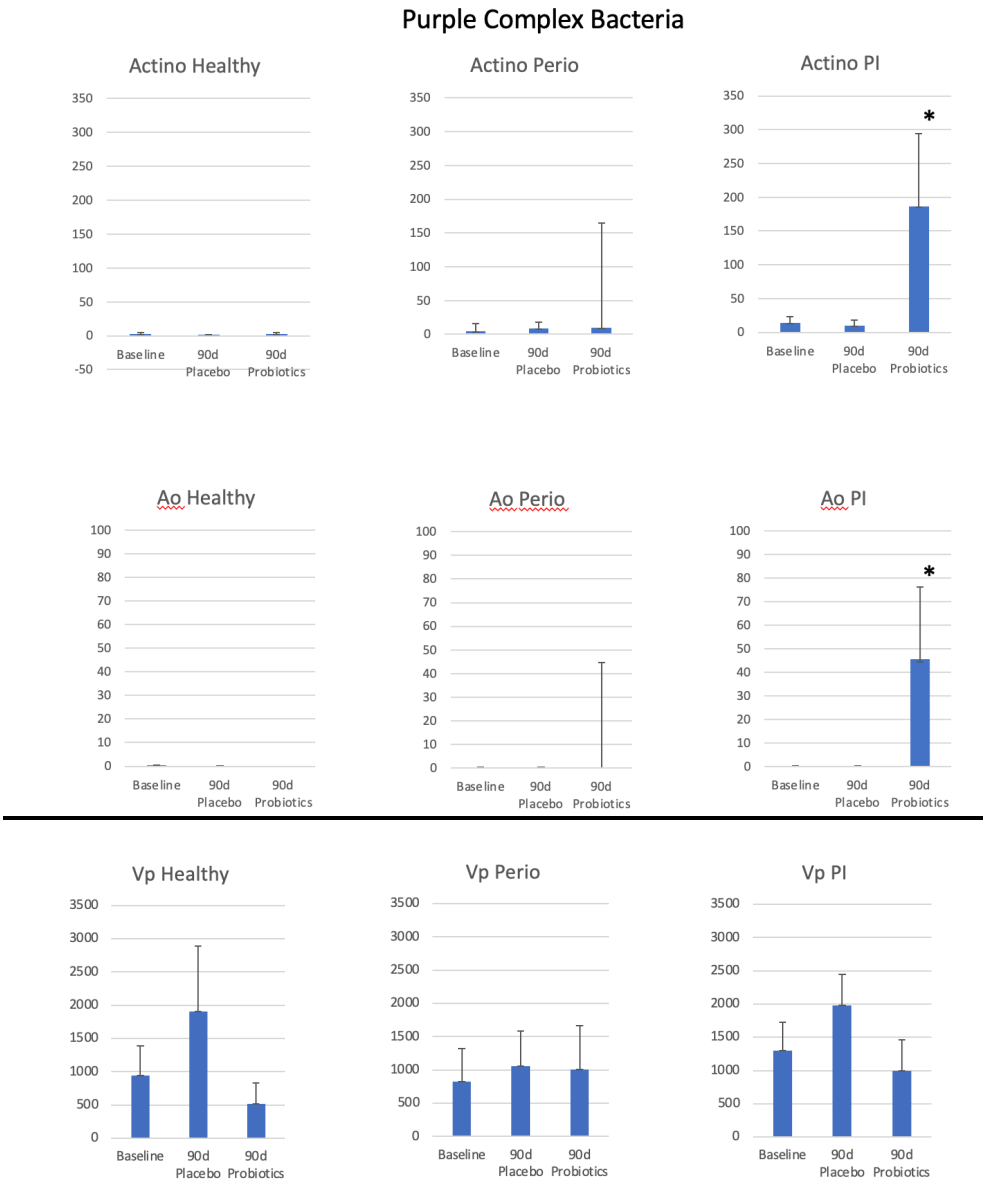

Figure 18: Purple Complex Bacteria (Compared Baseline with Placebo and Probitic groups at 90 days). Statistically significant difference compared to baseline are marked with asterisks. *Actinomyces species*(Actino); *Actinomyces Odontolyticus* (Ao); *Veillonella parvula* (Vp).

Appendix F

Informed Consent

NOVA SOUTHEASTERN UNIVERSITY

Health Professions Division – College of Dental Medicine

General Informed Consent Form
NSU Consent to be in a Research Study Entitled

NSU IRB APPROVED: Approved: December 14, 2017 Expired: December 13, 2018 IRB#: 2018-26-NSU

Probiotic Supplements as Adjunct Therapy to Peri-Implantitis Lesions: A Double-Blind Randomized Controlled Trial

Who is doing this research study?

College: Nova Southeastern University Department of Periodontics Principal Investigator: Po Ning Polly Huang D.M.D.

Faculty Advisor/Dissertation Chair: Saynur Vardar-Sengul D.D.S. Ph.D Co-Investigator(s): Toshihisa Kawai
Site Information:
Funding: This study is funded by NSU grant

What is this study about?

You are invited to be in this research study. This study will evaluate the effectiveness of a probiotic on bone loss (peri-implantitis) after receiving a dental deep cleaning. The probiotics pill (Hyperbiotics-PRODENTAL) has been shown to bring good bacteria to the gums and decrease inflammation of the gums. This deep cleaning around the implants have been used for many years as part of the non-surgical procedure to treat peri-implantitis and is not considered new or experimental.

Why are you asking me to be in this research study?

We are inviting you to participate because you are a patient at the Nova Southeastern University College of Dental Medicine, and currently, you have at least one implant with bone loss around it that we called “peri-implantitis”. It is an inflammatory process around an implant where both the gums and the bone are affected and if not treated, you can eventually lose the implant(s).

To qualify for this study, you need to be:

- ¬ At least 18 or older
- ¬ Willing and able to give informed consent.

In addition, you cannot have any of the following criteria listed below:

- ¬ Uncontrolled medical conditions
- ¬ Pregnant or lactating females
- ¬ Use of antibiotics for the last 3 months.
- ¬ Subjects treated for = 2 weeks with any medication known to affect soft tissue conditions (cyclosporine, phenytoin, Coumadin, etc.)
- ¬ Patients taking bisphosphonate medication
- ¬ Patients diagnosed with aggressive periodontitis
- ¬ Patients taking immunosuppressive medications (steroids, biologics (like Humira, Enbrel) etc) or with a diagnosis of immunodeficiency.
- Patients who usually require antibiotic prophylaxis for a dental procedure (prosthetic heart valves, prior history of infectious endocarditis, congenital heart defects etc)

What will I be doing if I agree to be in this research study?

You will be required to attend a total of 5 appointments at different time periods at NSU Periodontics Dental Clinic over the course of 90 days. You will be asked to come back for several appointments after your cleaning for us to take some x-rays of your implants and do some clinical measurements around the implants. The measurements done are the same kind of measurements that your hygienist regularly do to check the status of gums. We will use a paper point to collect bacteria sample around your affected implants. This procedure is non- invasive and is something that hygienists or dentists can do regularly to see what kind of bacteria are in your gums. This study will be completed in 90 days.

Research Study Procedures - as a participant, this is what you will be doing:

The following is a summary of what you can expect:

Visit 1: Data collection (will take approximately 60 minutes)
You will be asked to read and sign an Informed Consent form and will be given a copy. Your medical and dental history will be obtained, reviewed and kept in each study file stored in a secured cabinet. You will receive an oral exam to evaluate the general condition of your gums and especially the ones around your affected implant. Dr. Huang would remove some plaque from certain sites of the mouth with an instrument that is used to clean teeth (sterile curette). The collected plaque will be used as microbiological sample in this study. Moreover, we will use paper points to collect the fluid around the gums of your implant. It is a non-invasive procedure and is pain-free to measure the amount of inflammation of your gums. In addition, x-rays will be taken to evaluate the peri-implantitis lesion(s).

Visit 2: Non-Surgical Therapy (Deep Cleaning) & Start taking tablets: (will take approximately 60 minutes)
You will be asked to come back to the NSU PG Periodontics clinic for non-surgical therapy (deep cleaning) on teeth with deep pockets and also around the implant(s). Dr. Huang or Dr. Vardar will show you appropriate home care techniques for optimal home care before you leave. Before leaving the appointment, you will be randomly assigned to a group. One group will take the probiotics pill and another group will take the placebo pill. The pills will be given to you in a bottle and you will be instructed to take 2 pills per day (one in morning and one at night). You will be asked to take the pills for 90 days.

Visit 3: 7 days after last visit (will take approximately 60 minutes)
You will report back to NSU PG Periodontics clinic. You will have a exam to evaluate the general condition of your gums and especially the ones around your affected implant. Some measurements will be taken with an instrument (probe) to assess the lesion(s) clinically. You will be reminded to continue to take the tablets given to you at your first (baseline) appointment as directed (two tablets per day) and home care techniques will be reviewed by Dr. Huang or Dr. Vardar.

Visit 3: 30 days after visit 2 (will take approximately 60 minutes)
You will report back to NSU PG Periodontics clinic. You will have a periodontal exam to evaluate the general condition of your gums and especially the ones around your affected implant. Some measurements will be taken with an instrument (probe) to assess the lesion(s) clinically. You will be reminded to continue to take the tablets given to you at your first (baseline) appointment as directed (two tablets per day) and home care techniques will be reviewed by Dr. Huang or Dr. Vardar.

Visit 4: 60 days after visit 2 (will take approximately 60 minutes)
You will report back to NSU PG Periodontics clinic. You will have a periodontal exam to evaluate the general condition of your gums and especially the ones around your affected implant. Some measurements will be taken with an instrument (probe) to assess the lesion(s) clinically. You will be reminded to continue to take the tablets given to you at your first (baseline) appointment as directed (two tablets per day) and home care techniques will be reviewed by Dr. Huang or Dr. Vardar.

Visit 5: 90 days after baseline (final appointment) (will take approximately 60 minutes)
You will report back to NSU PG Periodontics clinic. You will have a periodontal exam to evaluate the general condition of your gums and especially the ones around your affected implant. Some measurements will be taken with an instrument (probe) to assess the lesion(s) clinically. Microbiological sampling will be collected. Moreover, the fluid around your implants will be collected with paper points. In addition, digital radiograph (xrays) will be taken to evaluate the peri-implantitis lesion(s).

Note: *Throughout the appointments, we may take some pictures using the Nikon SLR camera. Your face will NOT be photographed.

*The probiotics tablets are made in the USA in a facility that is Good Manufacturing Practice (GMP) certified. Contents of 1 tablet include: Zinc 2mg and Proprietary Probiotics Blend of 3 billion CFU (*S. salivarius K 12, S .Salivarius M 18, L. reuteri, L. paracasei*). It is also lactose free, vegetarian, non-GMO, yeast free, no lactose, no soy, no iron, no gluten, no wheat, no nuts, no preservatives.

Could I be removed from the study early by the research team?

Yes, you may be removed early from the research team. These reasons may be (but not limited to):

- - If you no longer meet the criteria to participate
- - If you fail to show up to scheduled appointments
- - If you fail to follow the study directions

Are there possible risks and discomforts to me?

This research study involves minimal risk to you. To the best of our knowledge, the things you will be doing have no more risk of harm than you would have in everyday life.
This study is completely NON SURGICAL. This means no surgeries are involved. Thus, the risks are minimal. Some mild discomfort may be felt during the deep cleaning but the discomfort is the same as a regular cleaning with the hygienist. If you have more sensitive gums, we may numb you so you feel more comfortable. Minimal to none discomfort may be felt after the procedure for most people. In fact, the discomfort, if any, will not require any pain medications. If you have any pain post-procedure, please follow your dentist's instructions for the post-procedure management of pain.
.
The National Center for Complementary and Integrative Health stated "in healthy people, probiotics usually have only minor side effects, if any." and " in people who are generally healthy, probiotics have a good safety record. Side effects, if they occur at all, usually consist only of mild digestive symptoms such as gas".

If you feel slightly "gassy" in the beginning, that is completely normal as probiotics may increase some gas but most people will not feel any different. The gassy feeling should subside in less than a week if it is felt.

What other treatment options are there to being in this research study?

There are other options available to you. Your other choices may include: 1) get treatment or care without being in a study
2) getting no treatment

What if a research-related injury occurs?

The researchers have taken steps to minimize the known or expected risks. However, you may still have problems or get side effects, even though the

researchers are careful to avoid them. In the event of a research-related injury or if you have a bad reaction, please contact Principal Investigator right away. See the contact section at the end of this form for phone numbers and more information.

Nova Southeastern University does not have a program to pay you if you are hurt or have other bad results from being in this study. However, medical care at Nova Southeastern University is open to you as it is to all sick or injured people. If you have health insurance, the costs for any treatment or hospital care you receive as result of a study-related injury will be billed to your health insurer. Any costs that are not paid for by your health insurer will be billed to you. If you do not have health insurance, you will be billed for the costs of any treatment or hospital care you receive because of a study-related injury. If you sign this form, you do not give up your right to seek additional compensation if you are harmed because of participation in this study.

What happens if I do not want to be in this research study?

You have the right to leave this research study at any time or refuse to be in it. If you decide to leave or you do not want to be in the study anymore, you will not get any penalty or lose any services you have a right to get. If you choose to stop being in the study before it is over, any information about you that was collected **before** the date you leave the study will be kept in the research records for 36 months from the end of the study and may be used as a part of the research.

Are there risks related to withdrawing from the study early?

If you decide to stop being in the study before it is over, please talk to the principal investigator about why you don't want to be in the study any more.

There is no risk to you if you do not complete the final withdrawal procedures and you can choose not to participate in them. However, note that not seeking any treatment for peri- implantitis lesions, you may be at risk for the lesion to progress and if lesion becomes more advanced, you may be at risk for losing the dental implant.

What if there is new information learned during the study that may affect my decision to remain in the study?
If significant new information relating to the study becomes available, which may relate to whether you want to remain in this study, this information will be given to you by the investigators. You may be asked to sign a new Informed Consent Form, if the information is given to you after you have joined the study.

Are there any benefits for taking part in this research study?

There are no direct benefits for participating in this study.

Will I be paid or be given compensation for being in the study?

You will not be given any payments or compensation for being in this research study.
The benefit of you being in this research study is that your treatment such as deep cleaning around the peri-implantitis lesions, microbiological sampling, digital radiographs and tablets given to you will be free of charge.

Will it cost me anything?

There are no costs to you for being in this research study.

Will clinically relevant research results be shared with me?

The study investigators do not plan to share research results with people in the study.

How will you keep my information private?

Information we learn about you in this research study will be handled in a confidential manner, within the limits of the law and will be limited to people who have a need to review this information. Organizations that may review and copy your information include the Institutional Review Board and other representatives of this institution. If we publish the results of the study in a scientific journal or book, we will not identify you. All confidential data will be kept securely All data will be kept for 36 months and destroyed after that time by deleting all encrypted information on the hard drive and by burning all the paper records.

Will my biological specimens be used in future research studies?

There is a possibility that the data collected from you may be shared with other investigators in the future. If that is the case, the data will not contain information that can identify you. You will not be contacted or asked to provide consent for the use of this data and/or specimens.

Whom can I contact if I have questions, concerns, comments, or complaints?

If you have questions now, feel free to ask us. If you have more questions about the research, your research rights, or have a research-related injury, please contact:

Primary contact:
Po- Ning Polly Huang D.M.D. can be reached at 412-387-8585

If primary is not available, contact:
Saynur Vardar-Sengul D.D.S., PhD can be reached at 954-262-1909

Research Participants Rights

For questions/concerns regarding your research rights, please contact:

Institutional Review Board
Nova Southeastern University
(954) 262-5369 / Toll Free: 1-866-499-0790 **IRB@nova.edu**

You may also visit the NSU IRB website at **www.nova.edu/irb/information-for-research- participants** for further information regarding your rights as a research participant.

All space below was intentionally left blank.

Voluntary Participation - You are not required to participate in this study. In the event you do participate, you may leave this research study at any time. If you leave this research study before it is completed, there will be no penalty to you, and you will not lose any benefits to which you are entitled.

If you agree to participate in this research study, sign this section. You will be given a signed copy of this form to keep. You do not waive any of your legal rights by signing this form.

SIGN THIS FORM ONLY IF THE STATEMENTS LISTED BELOW ARE TRUE:

- You have read the above information.
- Your questions have been answered to your satisfaction about the research.

Adult Signature Section

I have voluntarily decided to take part in this research study.

Printed Name of Participant

Printed Name of Person Obtaining Consent and Authorization

Signature of Participant Date

Signature of Person Obtaining Consent & Date Authorization

Initials: _____ **Date**: _____

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