EVALUATION OF TWO ORAL PROBIOTIC PRODUCTS FOR MICROBIAL VIABILITY AND IN VITRO INHIBITION OF SELECTED PERIODONTAL BACTERIAL PATHOGENS.

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MASTER OF SCIENCE

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by
Joumana Salame, B.D.S., M.S.
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Thesis Approval(s):

Thomas E. Rams, Thesis Advisor, Department of Periodontology and Oral Implantology
Jon B. Suzuki, Department of Periodontology and Oral Implantology
Eugene J. Whitaker, Department of Restorative Dentistry

ABSTRACT

Objectives: One potential impact of oral probiotic products involves use of known bacterial antagonisms to alter the ecologic environment in periodontal pockets from one inhabited by pathogenic dental plaque microorganisms to one more favorable to colonization by non-pathogenic species (bacterial replacement). Until recently, the ability to introduce such beneficial effector bacteria into the oral cavity of periodontitis patients has been limited by the lack of specifically-formulated available commercial probiotic products. PerioBalance® (Sunstar GUM), with two strains of the gram-positive, aerobic species *Lactobacillus reuteri*, and EvoraPlus® (Oragenics), with freeze-dried strains of the gram-positive, aerobic species *Streptococcus oralis*, *Streptococcus uberis*, and *Streptococcus rattus*, are two recently-introduced commercial oral probiotic products proposed to have beneficial effects against periodontal disease. However, it is not known if the microbial species contained in these two oral probiotics are viable after the manufacturing process, and have the capability to exert inhibitory effects against putative periodontal bacterial pathogens when reconstituted in the oral cavity. Thus, the objective of the present study was to determine whether PerioBalance® lactobacilli and EvoraPlus® streptococci are viable upon product use, and possess in vitro inhibitory effects against fresh clinical strains of the putative periodontal bacterial pathogens, *Tannerella forsythia* and *Prevotella intermedia/nigrescens*, in the presence of anaerobic growth conditions.

Methods: Commercial lots of PerioBalance® and EvoraPlus® tablets were aseptically removed from the product packaging with sterile forceps, dissolved into Möller's VMG I anaerobic dispersion solution, plated onto pre-reduced, enriched

Brucella blood agar, and subjected to overnight anaerobic incubation at 35ºC in a culture cabinet containing 85% N_2-10% H_2-5% CO_2, and to overnight aerobic incubation in a 5% CO_2-95% air atmosphere. All culture plates were then visually examined under magnification for microbial colony growth.

In vitro solid media competition assays were used to assess the in vitro inhibition capability of the two oral probiotics against *T. forsythia* and *P. intermedia/nigrescens*. Pioneer PerioBalance® lactobacilli and EvoraPlus® streptococci colonies were first grown on enriched Brucella blood agar media, followed by secondary spotting of *T. forsythia* and *P. intermedia/nigrescens* isolates immediately next to the established pioneer EvoraPlus® and PerioBalance® bacterial colonies such that they almost touched each other. After an additional overnight anaerobic incubation period, growth inhibition of the putative periodontal bacterial pathogens by the pioneer PerioBalance® and EvoraPlus® colonies was noted as the visual presence without magnification of a proximal zone of inhibition at the intersection of the pioneer colonies and the *T. forsythia* and *P. intermedia*/*nigrescens* colonies.

Results: PerioBalance® lactobacilli grew readily and in abundance in vitro on anerobically and anaerobically-incubated EBBA, with no other colony types or contaminating organisms. In contrast, EvoraPlus® product samples purchased over-the-counter from drug stores in Maryland and Pennsylvania failed to exhibit any in vitro microbial growth under anaerobic and aerobic incubation conditions, with only EvoraPlus® tablets obtained directly from the manufacturer yielding in vitro streptococcal growth. No in vitro inhibition was noted under anaerobic conditions of established PerioBalance® lactobacilli and EvoraPlus® streptococci pioneer colonies against

subsequent growth of clinical isolates of *T. forsythia* and *P. intermedia*/*nigrescens*, with no zone of inhibition developing between their colonies and the immediately-adjacent established oral probiotic pioneer colonies.

Conclusions: The two commercial oral probiotics evaluated varied considerably in the viability of their microbial constituents, with abundant growth of PerioBalance® lactobacilli found in over-the-counter product material, and the lack of any EvoraPlus® streptococci growth in product tablets obtained from sources other than directly from the manufacturer. Both oral probiotic products failed in vitro, in solid media competition assays, to inhibit growth of fresh clinical isolates the putative periodontal bacterial pathogens *T. forsythia* and *P. intermedia*/*nigrescens* under anaerobic growth conditions. These findings question the potential effectiveness of the two oral probiotic products to alter the subgingival ecology in periodontal pockets when anaerobic environmental conditions are present. Additional research is needed to assess the inhibitory potential of PerioBalance® lactobacilli and EvoraPlus® streptococci against additional isolates of subgingival bacterial species, and in circumstances where microaerophilic or aerobic environmental conditions are found.

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CHAPTER 1

INTRODUCTION

During the latter half of the 20th century, the focus on controlling pathogenic microbial species centered on the discovery and use of various antibiotic compounds, following the initial observations and clinical applications by Fleming (1953) and co-workers. However, the development of antibiotic resistance among microorganisms at many different body sites, including the oral cavity, have led to concerns over the use of antibiotics in controlling pathogenic infections (Walker 1996).

In the early years of the 21st century, increasing attention is being given to the potential of probiotics to alter bacterial populations in the oral cavity as a way to possibly reduce antibiotic therapies for oral infections (Teughels et al. 2011). Probiotics are defined by the World Health Organization as "Live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (cited by Teughels et al. 2011). From a theoretical standpoint, probiotics in the oral cavity may 1.) provide nutrients of benefit to the host, 2.) compete with pathogenic microorganisms in occupying colonization sites (competitive exclusion), 3.) produce substances inhibitory to pathogenic species, or 4.) modulate host immune responses (Reddy & Babu 2010, Teughels et al. 2011).

Several oral health literature reviews of recent origin indicate that oral probiotics applied by dental patients may provide benefits in the treatment and prevention of periodontal diseases, primarily in regard to reducing gingivitis and dental plaque scores (Meurman & Stamatova 2007, Stamatova & Meurman 2009, Reddy & Babu 2010, Teughels et al. 2011).

Relatively few commercial products specifically promoted for their periodontal benefits have been introduced into the United States consumer market. Of those that can be identified, there is an urgent need to evaluate these probiotics independent of the manufacturer and distributor. An initial step in assessing the potential efficacy of an oral probiotic is to ascertain whether or not the commercial product provides viable microorganisms upon usage, since any potential probiotic effects would be dependant upon the availability of living bacterial species when the probiotic product is consumed. A second initial evaluation of an oral probiotic product is to examine in vitro interspecies interactions that occur between viable probiotic microorganisms and potential pathogenic species. Since human periodontal diseases represent the result of host-pathogenic interactions (Tatakis & Kumar 2005), generally in the absence of streptococcal bacterial species of low periodontopathic potential in subgingival sites (Socransky et al. 1998), it is of importance to determine if an oral probiotic intended to impact periodontal disease possesses any inhibitory properties against various putative periodontal bacterial pathogens.

PerioBalance® and EvoraPlus® are two commercially-available oral probiotic products in the United States that have been suggested to possess possible benefits against human periodontal diseases (Krasse et al. 2006, Zahradnik et al. 2009). However, no information is present available independent of the manufacturer on the actual microbial viability of microorganisms contained in the commercial product, and little is known about any inhibitory effects the probiotic species in the two products have against any putative periodontal pathogens.

Thus, the specific aims of the present study were to 1.) obtain commercially-available samples of PerioBalance® and EvoraPlus® probiotic product packages, 2.) evaluate the microbial viability of the bacteria contained in the two probiotic products with aerobic and anaerobic culture methodology, 3.) grow fresh clinical isolates of the putative periodontal bacterial pathogens, *Tannerella forsythia* and *Prevotella intermedia/ nigrescens*, isolated from the subgingival microbiota of human periodontitis patients, and 4.) perform in vitro solid media competition assays to assess the in vitro inhibition capability of the two oral probiotics against *T. forsythia* and *P. intermedia/nigrescens* under anaerobic environmental growth conditions.

CHAPTER 2

MATERIALS AND METHODS

Laboratory Facilities

All laboratory procedures were performed in the Oral Microbiology Testing Service (OMTS) Laboratory, located in Room 365-A of Building 600, which is part of the Temple University Maurice H. Kornberg School of Dentistry on the Temple University Health Sciences Center campus in north Philadelphia, PA. The OMTS Laboratory facilities are inspected and licensed by the Pennsylvania Department of Health for high-complexity bacteriological analysis - Clinical Laboratory Permit No. 021872 - in meeting the same proficiency and quality control standards required of hospital medical microbiology laboratories, and is CLIA-registered at a federal level - CLIA Certificate No. 39D0707385 - with the United States Department of Health and Human Services.

Oral Probiotic Products

Two oral probiotic products, commercially-available and sold over-the-counter in the United States, were evaluated in this study. The first oral probiotic product, PerioBalance®, manufactured and distributed by Sunstar GUM, contains two strains of the gram-positive, aerobic species *Lactobacillus reuteri*. The second oral probiotic product, EvoraPlus®, manufactured and distributed by Oragenics, contains freeze-dried strains of the gram-positive, aerobic species *Streptococcus oralis*, *Streptococcus uberis*, and *Streptococcus rattus* (Figures 1-2).

Figure 1. PerioBalance® commercial packaging (left) and tablet (right).

Figure 2. EvoraPlus® commercial packaging (left) and tablet (right)

Two separate lots of PerioBalance® were obtained from product packages purchased over-the-counter at drug stores in Maryland and Pennsylvania. Three EvoraPlus® lots were obtained from product packages purchased directly from the manufacturer through their on-line website, and from drug store shelves in Maryland and Pennsylvania. An additional fourth lot of EvoraPlus® was generously provided without charge directly from the manufacturer.

Microbial Viability of Oral Probiotic Products

To assess the viability of microorganisms contained in commercially-distributed packages of the two test oral probiotic products, tablets from each test product lot were aseptically removed from the product packaging with sterile forceps, placed into a sterile glass test tube, and dissolved into Möller's VMG I anaerobic dispersion solution, comprised of pre-reduced, anaerobically sterilized, 0.25% tryptose-0.25% thiotone E peptone-0.5% NaCl (Möller 1966) (Figure 3).

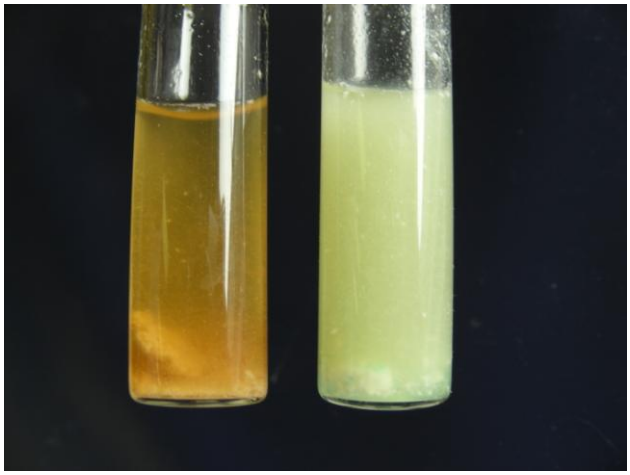

Figure 3. EvoraPlus® (left) and PerioBalance® (right) tablets being dissolved in VMG I.

Using a sterile bent glass rod, 0.1 ml aliquots of the undiluted solution from each dissolved product lot were plated onto sterile plastic petri plates with pre-reduced, enriched Brucella blood agar (EBBA), comprised of 4.3% Brucella agar (BBL Microbiology Systems, Cockeysville, MD) supplemented with 0.3% bacto-agar, 5% defibrinated sheep blood, 0.2% hemolyzed sheep red blood cells, 0.0005% hemin, and

0.00005% menadione. A set of the EBBA plates were incubated anaerobically overnight at 35ºC in a Whitley MG 500 anaerobic culture cabinet (Microbiology International, Frederick, MD) containing 85% N_2-10% H_2-5% CO_2, and another set incubated aerobically in an incubator with a 5% CO_2-95% air atmosphere. After the overnight incubation, all plates were visually examined with a 2.25x ring-light magnifying loupe (Luxo Taskmaster Magnifier, Lighting Specialists, Buffalo, Grove, IL, USA), as well as a Meijo Techno RZ 75x dissecting stereomicroscope with a Fostec Ace I fiberoptic light source, for microbial colony growth phenotypically-consistent with lactobacilli or streptococci, with other microbial colony types noted, if present. No additional phenotypic, biochemical or genetic characterization of the recovered organisms was performed.

Oral Probiotic In Vitro Inhibition of Selected Periodontal Bacterial Pathogens

Fresh clinical isolates of the putative periodontal bacterial pathogens, *Tannerella forsythia* and *Prevotella intermedia/nigrescens*, were recovered from the subgingival microbiota of human periodontitis patients, and maintained in the OMTS Laboratory stock library collection.

Since the *T. forsythia* and *P. intermedia/nigrescens* clinical isolates for the present study were obtained from the existing OMTS Laboratory stock library collection, and were not obtained through intervention or interaction with any living individuals, nor through any records with identifiable private information of patients, the present research activity thus does not involve human subjects, as defined by United States Department of Health and Human Services regulations at 45 CFR part 46.116(f). As a result, the present

research activity does not require a human subjects institutional review board approval (Department of Health and Human Services 2004).

T. forsythia was identified as a gram-negative, non-motile, anaerobic rod exhibiting grey-pink speckled, convex, pinpoint colonies seen with a stereomicroscope, lack of long-wave ultraviolet light autofluorescence (Slots & Reynolds 1982), and a positive CAAM test for trypsin-like activity (Slots 1987). *Prevotella intermedia/ nigrescens* was recognized as autofluorescent red-positive, black-pigmented colonies exhibiting lactose MUG-test negative (Alcoforado et al. 1987) and trypsin CAAM test-negative reactions. No attempt was made to further taxonomically differentiate the isolate between the two closely-related and phenotypically identical *Prevotella intermedia* and *Prevotella nigrescens* species.

Both *T. forsythia* and *P. intermedia/nigrescens* clinical strains were inoculated onto EBBA plate media incubated anaerobically for seven days at 35ºC in a Whitley MG 500 anaerobic culture cabinet containing 85% N_2-10% H_2-5% CO_2, with resulting colonies each dispersed and adjusted to a 1.0 McFarland turbidity standard into Möller's VMG I anaerobic dispersion solution (Möller 1966) in sterile glass test tubes.

PerioBalance® lactobacilli and EvoraPlus® streptococci colonies were similarly harvested from anaerobically-incubated EBBA plates, and each dispersed and adjusted to a 1.0 McFarland turbidity standard into Möller's VMG I anaerobic dispersion solution (Möller 1966) in sterile glass test tubes.

In vitro solid media competition assays to assess the in vitro inhibition capability of the two oral probiotics against *T. forsythia* and *P. intermedia/nigrescens* within anaerobic growth conditions were then performed using a modification of the protocol

described by Kreth et al. (2008). First, 8 µl of EvoraPlus® and PerioBalance® organisms in the 1.0 McFarland turbidity standard VMG I solution were each spotted as "pioneer colonizers" onto EBBA plates, and subjected to overnight anaerobic incubation at 35ºC in a Whitley MG 500 anaerobic culture cabinet (Microbiology International, Frederick, MD) containing 85% N_2-10% H_2-5% CO_2. Second, 8 µl of *T. forsythia* and *P. intermedia*/*nigrescens* human clinical isolates in the 1.0 McFarland turbidity standard VMG I solution were each spotted immediately next to the established pioneer EvoraPlus® and PerioBalance® bacterial colonies such that they almost touched each other, followed by an additional overnight anaerobic incubation period. Growth inhibition under anaerobic conditions by the pioneer EvoraPlus® and PerioBalance® colonies was noted as the visual presence without magnification of a proximal zone of inhibition at the intersection of the pioneer colonies and the *T. forsythia* and *P. intermedia*/*nigrescens* colonies. Positive controls for each bacterial group were spotted on the same culture plates separate and distant from the in vitro competition assay test areas. *T. forsythia* and *P. intermedia/nigrescens* in vitro growth immediately adjacent to pioneer EvoraPlus® and PerioBalance® colonies was scored as (-) = total inhibition/no species growth visually present; (+) = partial inhibition/species growth present, but less dense as compared to species positive control spot elsewhere on same culture plate; or (++) = no inhibition/species growth present, with same or greater density as compared to species positive control spot elsewhere on same culture plate.

All culture media preparation and specimen inoculation were carried out in a standardized fashion for all of the in vitro solid media competition assay runs.

Data Analysis

Only descriptive observations of oral probiotic in vitro microbial growth, and in vitro competitive inhibitory capability against *T. forsythia* and *P. intermedia/nigrescens* are noted, with no statistical hypothesis testing performed in the present study.

CHAPTER 3

RESULTS

Microbial Viability of Oral Probiotic Products

PerioBalance® lactobacilli grew readily and in abundance in vitro on anaerobically-incubated EBBA, with no other colony types or contaminating organisms present in the commercial, over-the-counter, store-bought product samples tested.

In contrast, EvoraPlus® product samples purchased over-the-counter from drug stores in Maryland and Pennsylvania failed to exhibit any in vitro microbial growth under both anaerobic and aerobic incubation conditions, even after additional days beyond the initial overnight incubation period. Only EvoraPlus® tablets either purchased or provided directly from the manufacturer yielded in vitro streptococcal growth (Figure 4).

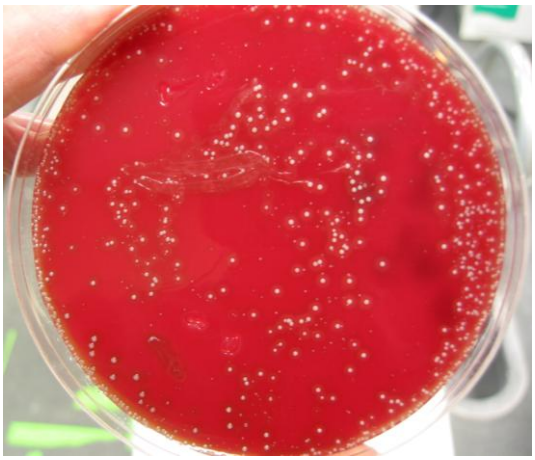

Figure 4. Typical EvoraPlus® in vitro streptococcal growth on EBBA media.

Recovered EvoraPlus® in vitro growth, noted only from tablets originating directly from the manufacturer, was comprised of three distinct streptococcal colony types characteristic of the test species *S. oralis*, *S. uberis* and *S. rattus* (Figure 5), with no other colony types or contaminating organisms.

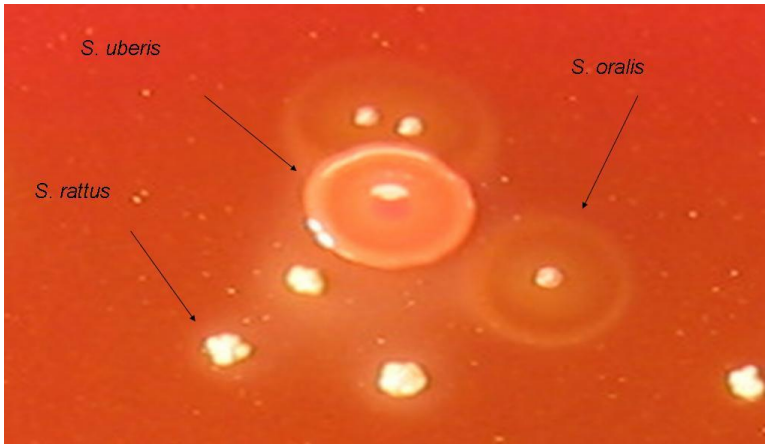

Figure 5. In vitro colony morphology of *S. oralis*, *S. uberis* and *S. rattus*.

Oral Probiotic In Vitro Inhibition of Selected Periodontal Bacterial Pathogens

The results of solid media competitive assays assessing the in vitro inhibition capability of the two oral probiotics, PerioBalance® and EvoraPlus® against *T. forsythia* and *P. intermedia/nigrescens* under anaerobic growth conditions is presented in Table 1.

No in vitro inhibition was noted under anaerobic conditions of established PerioBalance® lactobacilli pioneer colonies against subsequent growth of clinical isolates of *T. forsythia* and *P. intermedia*/*nigrescens*. Both organisms grew well on anaerobically-incubated EBBA without a zone of inhibition developing between their

Table 1. In Vitro Inhibition Capability of Two Oral Probiotics Against *T. forsythia* and *P. intermedia/nigrescens* in Anaerobic Growth Conditions

	Pioneer probiotic organisms	
Periodontal Pathogen	PerioBalance®	EvoraPlus®
T. forsythia	(++) [a]	(++)
P. intermedia/nigrescens	(++)	(++)

[a] *T. forsythia* and *P. intermedia/nigrescens* in vitro growth score adjacent to pioneer EvoraPlus® and PerioBalance® colonies; (-) = total inhibition/no species growth visually present; (+) = partial inhibition/species growth present, but less dense as compared to species positive control spot elsewhere on same culture plate; or (++) = no inhibition/species growth present, with same or greater density as compared to species positive control spot elsewhere on same culture plate.

colonies and the immediately-adjacent established PerioBalance® lactobacilli pioneer colonies (Figure 6).

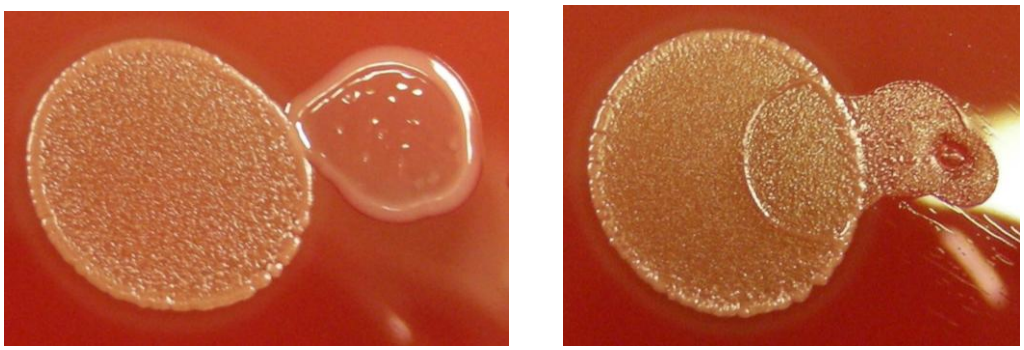

Figure 6. *T. forsythia* (left) and *P. intermedia/nigrescens* (right) colony growth (both on right side of each image) adjacent to PerioBalance® colony (located on left side of each image).

Similarly, no in vitro inhibition was seen under anaerobic conditions of established EvoraPlus® streptococcal pioneer colonies against subsequent growth of clinical isolates of *T. forsythia* and *P. intermedia*/*nigrescens*. Both organisms also grew well on anaerobically-incubated EBBA without a zone of inhibition developing between their colonies and the immediately-adjacent established EvoraPlus® streptococcal pioneer colonies (Figure 7).

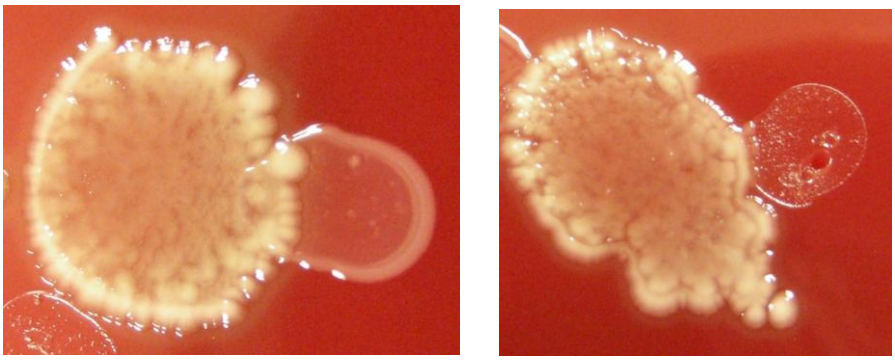

Figure 7. *T. forsythia* (left) and *P. intermedia/nigrescens* (right) colony growth (both on right side of each image) adjacent to EvoraPlus® colony (located on left side of each image).

CHAPTER 4

DISCUSSION

The present study uncovered two major findings relative to the two evaluated oral probiotic products. First, it was noted that microbial viability of the contained probiotic microorganisms varied considerably between the two products, and between that obtained directly from the manufacturer and from drug stores for one of the probiotics. Store-purchased samples of the PerioBalance® probiotic product yielded abundant lawns of viable lactobacilli following overnight anaerobic incubation. In contrast, store-purchased boxes from drug stores in Maryland and Pennsylvania of the EvoraPlus® probiotic product were totally devoid of all microbial growth following overnight and additional days of both aerobic and anaerobic incubation on EBBA media. Only EvoraPlus® tablets obtained directly from the manufacturer, and not from an over-the-counter source, were found to contain viable microorganisms of *S. oralis*, *S. uberis* and *S. rattus*.

The exact reasons for this variance in microbial viability of the EvoraPlus® probiotic between product sources remains to be determined. However, in communication of these findings to EvoraPlus® manufacturer executives, it was learned that similar problems in maintaining microbial viability of *S. oralis*, *S. uberis* and *S. rattus* within over-the-counter product materials has also been recently discovered by the manufacturer. In this regard, the manufacturer believes that improper storage of EvoraPlus® at excessive temperatures, as well as product exposure to excessive humidity, at drug store locations, is responsible for the loss of streptococci microbial viability in the

drug-store obtained EvoraPlus® tablets used in the present study. As a result of the manufacturer's own observations, and the findings of the present study shared with the manufacturer, it was learned that the EvoraPlus® product will be subjected to a revised re-packaging process in the near future, with the present cardboard product box and bubble sheet delivery system replaced with a temperature-resistant dark glass/plastic bottle containing a moisture-absorbing desiccant. However, in the meantime, the results of the present study raise significant doubt about the potential effectiveness of EvoraPlus® probiotic tablets obtained from over-the-counter drug store sources, since the contained probiotic streptococci are likely to be non-viable and unable to exert any beneficial probiotic impact upon consumer use. It is important to point out that EvoraPlus® probiotic tablets are approved by the United States Food & Drug Administration for distribution in the United States as a "food additive" for cosmetic purposes (i.e., prevention of halitosis, tooth whitening), without any medical or dental therapeutic claims related to any human disease process. As a result, there is no regulatory requirement that the *S. oralis*, *S. uberis* and *S. rattus* microbial constituents within EvoraPlus® be actually viable upon usage for the product to be available in the consumer marketplace. However, until such time as the manufacturer changes the product packaging to one which insures the viability of EvoraPlus® streptococci against temperature and humidity changes associated with various retail distribution environments, the findings of the present study strongly support a recommendation against consumer use of the EvoraPlus® probiotic product, if there is any expectation of a beneficial, non-placebo effect from its consumption. It is difficult to envision any non-placebo activity of non-viable *S. oralis*, *S. uberis* and *S. rattus* cells in present over-the-

counter EvoraPlus® tablets working against any type of oral or periodontal cosmetic or disease condition in humans.

The second major finding of the present study was that when viable microbial species of the PerioBalance® and EvoraPlus® probiotic products were each tested in an in vitro anaerobic environment with two putative periodontal bacterial pathogens, both failed to exert detectable in vitro inhibitory effects against fresh clinical isolates of *T. forsythia* and *P. intermedia/nigrescens* recovered from the subgingival microbiota of chronic periodontitis patients. In solid media competitive assays where viable PerioBalance® lactobacilli and EvoraPlus® streptococci were initially grown as pioneer colonies on EBBA media, subsequent plating of *T. forsythia* and *P. intermedia/ nigrescens* isolates immediately adjacent to the established probiotic bacterial colonies, followed by overnight anaerobic incubation, resulted in unimpaired *T. forsythia* and *P. intermedia/nigrescens* growth that was equal in density to control inoculations of the putative periodontal pathogens that were plated distant from the probiotic organisms. Thus, under anaerobic environmental growth conditions, neither established, viable colonies of PerioBalance® lactobacilli nor EvoraPlus® streptococci were able to inhibit in vitro subsequent growth of *T. forsythia* and *P. intermedia/nigrescens*.

The choice of testing clinical subgingival strains of *T. forsythia* and *P. intermedia/nigrescens* was based upon their potential role in the pathogenesis of human periodontitis. *T. forsythia* is classified as one of the three "red complex" microbial species associated with the most severe forms of human periodontitis, and *P. intermedia/nigrescens* is classified as one of the "orange complex" microorganisms associated with moderate periodontitis (Socransky et al. 1998). Thus, representatives

from each of the two most pathogenic microbial groups in periodontitis (i.e., the red and orange complex groups) were employed in the in vitro solid media competitive assays. Since both of these organisms are strictly anaerobic, gram-negative, non-spore forming, non-motile rods, it was necessary to perform the in vitro testing of the oral probiotic product microorganisms against them in anaerobic growth conditions.

Previous research studies have indicated the presence of antagonistic relationships between bacterial species present in the EvoraPlus® probiotic tablets. Socransky et al (1988) reported that among 35 subjects with periodontitis, *S. uberis* exhibited a markedly negative subgingival colonization pattern with *T. forsythia* (previously *Bacteroides forsythus*) (mean odds ratio for joint subgingival colonization = 0.18), as well as a negative subgingival association with *P. intermedia/nigrescens* (previously *Bacteroides intermedius*) (mean odds ratio for joint subgingival colonization = 0.68). In contrast, *S. oralis* (previously *Streptococcus sanguis* II) demonstrated neutral to weakly positive subgingival colonization patterns with both *T. forsythia* (mean odds ratio for joint subgingival colonization = 1.01) and *P. intermedia/nigrescens* (mean odds ratio for joint subgingival colonization = 1.21), while the subgingival microbial associations with *S. rattus* were not evaluated (Socransky et al. 1988). In a previous in vitro study where a microaerophilic growth atmosphere (0.1% oxygen) was used for incubation, as compared to the strict anaerobic incubation growth conditions employed in the present study, it was found that *S. uberus* and *S. oralis* both exerted antagonism against both *T. forsythia* and *P. intermedia/nigrescens* (Hillman et al. 1985). This positive in vitro inhibitory effect of *S. uberis* and *S. oralis* was ascribed to their considerable extracellular production of hydrogen peroxide during the microaerophilic growth conditions, since the incorporation

of catalase (which mitigates the antmicrobial effects of hydrogen peroxide) into the test media generally eliminated the streptococcal inhibitory effects (Hillman et al. 1985). Since oral streptococcal hydrogen peroxide production is markedly greater in aerobic as compared to anaerobic growth conditions (Kreth et al. 2008), the lack of in vitro inhibition by EvoraPlus® streptococci of *T. forsythia* and *P. intermedia/nigrescens* in the present study appears related to the diminished or absent hydrogen peroxide production by *S. uberis* and *S. oralis* in anaerobic growth conditions.

These observations suggest that in the presence of established periodontal pockets, where low oxygen-reduction conditions exist providing a highly anaerobic environment (Kenney & Ash 1969), administration of EvoraPlus® streptococci would likely be of little beneficial effect in the inhibition of periodontal bacterial pathogens, since their capacity to produce antimicrobial levels of hydrogen peroxide would be limited or absent. Instead, the use of EvoraPlus® streptococci appears more therapeutically promising in clinical periodontal situations where existing periodontitis lesions are first treated to a point where the pocket environment is sufficiently altered away from strict anaerobic to more microaerophilic conditions, which would then provide a more suitable ecologic niche to introduce EvoraPlus® streptococci, where they could elaborate inhibitory hydrogen peroxide against periodontal bacterial pathogens.

In this regard, EvoraPlus® streptococci in a mouth rinse used over a 4-week period in adults with periodontal health and/or gingivitis, and positive for one or more subgingival periodontal pathogens, demonstrated decreases in subgingival *Campylobacter rectus* and *Porphyromonas gingivalis*, but not *P. intermedia/nigrescens* (Zahradnik et al. 2009).

This study also found PerioBalance® lactobacilli to be unable to inhibit in vitro subsequent growth of *T. forsythia* and *P. intermedia/nigrescens* in anaerobic growth conditions. Lactobacilli produce lactic acids that can inhibit bacterial growth (Kõll-Klais et al. 2005), but that antimicrobial properties are lactobacilli strain, species and origin specific in nature (Kõll-Klais et al. 2005). It appears that the two strains of *L. reuteri* in PerioBalance® lack inhibitory activity against *T. forsythia* and *P. intermedia/nigrescens*, unlike other lactobacilli strains, such as *Lactobacillus paracasei* species active in inhibition of *P. intermedia* (Kõll et al. 2008).

Interestingly, a randomised, placebo-controlled, double blind clinical trial over a 2-week time period with 59 persons with moderate to severe gingivitis found significantly reduced gingivitis and plaque scores with use of a *L. reuteri* probiotic as a supplement to toothbrushing and dental flossing, as compared to a placebo, with the favorable clinical effects associated with positive subject colonization by *L. reuteri* organisms (Krasse et al. 2006). It could be that the positive clinical outcomes associated with the *L. reuteri* probiotic used in the Krasse et al. (2006) study were due to antimicrobial activity against periodontal organisms other than *T. forsythia* and *P. intermedia/nigrescens*, particularly since the subject population exhibited gingivitis instead of periodontitis, or due to other non-antimicrobial mechanisms.

Similar to the present study conclusions on EvoraPlus®, administration of PerioBalance® lactobacilli into existing periodontal pockets colonized by *T. forsythia* and/or *P. intermedia/nigrescens* would likely be of little beneficial effect, based on the present in vitro study findings. Additional research is needed to assess the inhibitory potential of both of these oral probiotic products against additional isolates of subgingival

bacterial species, and in circumstances where microaerophilic or aerobic environmental growth conditions are found.

CHAPTER 5
CONCLUSIONS

The present study aimed to determine whether PerioBalance® and EvoraPlus® oral probiotic product microorganisms are viable upon product use, and possess in vitro inhibitory effects against fresh clinical strains of the putative periodontal bacterial pathogens, *Tannerella forsythia* and *Prevotella intermedia/nigrescens*, in the presence of anaerobic growth conditions.

PerioBalance® lactobacilli exhibited abundant in vitro growth from over-the-counter product material, whereas EvoraPlus® streptococci growth was negative from product tablets obtained from sources other than directly from the manufacturer. In solid media competition assays, both oral probiotic products failed in vitro to inhibit growth of fresh clinical isolates the putative periodontal bacterial pathogens *T. forsythia* and *P. intermedia*/*nigrescens* under anaerobic growth conditions.

These findings question the potential effectiveness of the two oral probiotic products to alter the subgingival ecology in periodontal pockets when anaerobic environmental conditions are present. Additional research is needed to assess the inhibitory potential of PerioBalance® lactobacilli and EvoraPlus® streptococci against additional isolates of subgingival bacterial species, and in circumstances where microaerophilic or aerobic environmental growth conditions are found.

REFERENCES CITED

Alcoforado, G. A. P., McKay, T. L., & Slots, J. (1987). Rapid method for detection of lactose fermenting oral microorganisms. *Oral Microbiology and Immunology, 2,* 35-38.

Department of Health and Human Services (2004). Guidance on research involving coded private information on biological specimens. Accessed at http://www.hhs.gov/ohrp/humansubjects/guidance/cdebiol.pdf

Fleming, A. (1953). Review of the development of the antibiotics, principles underlying choice of a particular antibiotic for a particular patient; the combination of different antibiotics. *Acta medica Scandinavica 146,* 65-66.

Hillman, J. D., Socransky, S. S., & Shivers, M. (1985). The relationships between streptococcal species and periodontopathic bacteria in human dental plaque. *Archives of Oral Biology, 30,* 791-795.

Kenney, E. B., & Ash, M. M. Jr. (1969). Oxidation reduction potential of developing plaque, periodontal pockets and gingival sulci. *Journal of Periodontology 40,* 630-633.

Kõll, P., Mändar, R., Marcotte, H., Leibur, E., Mikelsaar, M., & Hammarström, L. (2008). Characterization of oral lactobacilli as potential probiotics for oral health. *Oral Microbiology and Immunology, 23,* 139-147.

Kõll-Klais, P., Mändar, R., Leibur, E., Marcotte, H., Hammarström, L., & Mikelsaar, M. (2005). Oral lactobacilli in chronic periodontitis and periodontal health: species composition and antimicrobial activity. *Oral Microbiology and Immunology, 20,* 354-361.

Krasse, P., Carlsson, B., Dahl, C., Paulsson, A., Nilsson, A., & Sinkiewicz, G. (2006). Decreased gum bleeding and reduced gingivitis by the probiotic *Lactobacillus reuteri*. *Swedish Dental Journal 30,* 55-60.

Kreth, J., Zhang, Y., & Herzberg, M. C. (2008). Streptococcal antagonism in oral biofilms: *Streptococcus sanguinis* and *Streptococcus gordonii* interference with *Streptococcus mutans*. *Journal of Bacteriology,* 190, 4632-4640.

Meurman, J. H., & Stamatova, I. (2007). Probiotics: contributions to oral health. *Oral Diseases 13,* 443-451.

Möller, A. J. R. (1966). Microbiological examination of root canals and periapical tissues of human teeth. *Odontologisk Tidskrift, 74,* 1-380.

Reddy, M. S. & Babu, M. N. (2010). How beneficial is bacterial prophylaxis to periodontal health? *Journal of Investigative and Clinical Dentistry 1,* 1-7.

Slots, J. (1987). Detection of colonies of *Bacteroides gingivalis* by a rapid fluorescence assay for trypsin-like activity. *Oral Microbiology and Immunology, 2,* 139-141.

Slots, J., & Reynolds, H. S. (1982). Long-wave UV light fluorescence for identification of black-pigmented *Bacteroides* spp. *Journal of Clinical Microbiology, 16,* 148-151.

Socransky, S. S., Haffajee, A. D., Cugini, M. A., Smith, C., & Kent, R. L. Jr. (1998). Microbial complexes in subgingival plaque. *Journal of Clinical Periodontology, 25,* 134-144.

Socransky, S. S., Haffajee, A. D., Dzink, J. L., & Hillman, J. D. (1988). Associations between microbial species in subgingival plaque samples. *Oral Microbiology and Immunology, 3,* 1-7.

Stamatova, I., & Meurman, J. H. (2009). Probiotics and periodontal disease. *Periodontology 2000 51,* 141-151.

Tatakis, D.N., & Kumar, P. S. (2005). Etiology and pathogenesis of periodontal diseases. *Dental Clinics of North America, 49,* 491-516.

Teughels, W., Loozen, G., & Quirynen, M. (2011). Do probiotics offer opportunities to manipulate the periodontal oral microbiota? *Journal of Clinical Periodontology 38 (Supplement 11),* 159-177.

Walker, C. B. (1996). The acquisition of antibiotic resistance in the periodontal microflora. *Periodontology 2000 10,* 79-88.

Zahradnik, R. T., Magnusson, I., Walker, C., McDonell, E., Hillman, C. H., & Hillman, J. D. (2009). Preliminary assessment of safety and effectiveness in humans of ProBiora3, a probiotic mouthwash. *Journal of Applied Microbiology 107,* 682-690.