Effectiveness of CRT at Measuring the Salivary Level of Bacteria in Caries Prone Children with Probiotic Therapy

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Aim: *This IRB approved clinical trial was to determine the effect of "over the counter" probiotic supplements on the Caries Risk Test- CRT- (Ivoclar) results of the oral microflora in high caries risk children.* ***Study design****: Sixty subjects 6 to 12 years old with a caries risk assessment (CAMBRA) of moderate to high (caries prone) were evaluated by an analysis of the difference in the salivary levels of pathogenic bacteria (mutans streptococci and Lactobacilli). The subjects were randomly selected by randomizing software and assigned to two different Groups. Group A used PerioBalance (Lactobacilli reuteri-CFU of 200 million) lozenges for 28 days. Group B used the EvoraKids (Streptococcus uberis KJ2, Streptococcus oralis KJ3, Streptococcus rattus JH145, ≥ 100 million) probiotics chewable tablets for 30 days. Salivary samples were collected then incubated for 48 hours for colony counting and ranking. Follow up testing with the CRT was performed after 60 days at a follow up visit.* ***Results****: There was a statistically significant difference in the CRT results between the pre and post use of the probiotics. PerioBalance; SM results t= -6.78 p< .0001 Lactobacilli results t= -5.762, p< .0001, EvoraKids SM results t= -7.33, p< .0001, Lactobacilli results t= -2.952, p= .0068.* ***Conclusions****: The CRT values obtained with caries prone children may be significantly affected by probiotic use. Based on this study's results the following conclusions can be made: Both EvoraKids and PerioBalance affected the CRT results by significantly decreasing the number of S. mutans and lactobacilli present in the salivary samples.*

Keywords: *probiotics, caries prevention, CRT, mutans streptococci, children.*

INTRODUCTION

Dental caries is the most common chronic disease of childhood, with its prevalence largely surpassing asthma.[1-2] It is a transmissible disease typically passed on vertically by caregiver to child through salivary contact. Horizontal transmission can also occur between siblings or children in the same daycare.[3] The main microorganisms involved have been shown to be a group of phenotypically similar, but genetically different streptococcal species known as mutans streptococci. Based on DNA homology, mutans streptococci are divided into seven species (*Streptococcus mutans, S. sobrinus, S. rattus, S. riceti, S. downei, S. ferus, and S. macacae)*. Mutans streptococci may be further subdivided into eight serotypes: a, b, c, d, e, f, g and h. Of these species, *S. mutans and S. sobrinus* have been implicated as the main causative agents of dental caries in humans.[4-7] Indeed changes in the balance of the oral cavity with an overgrowth of *Streptococcus mutans* create an ideal condition for the development of dental decay.[8] In fact, *Streptococcus mutans* usually comprises less than 1% of the flora of children with negligible caries activity.[9] Since caries is a complex disease with a multifactorial origin, it requires more than mutans streptococci to produce dental decay. Four principal factors are involved: the host, microflora, diet, and time. There are modifying factors such as race/ethnicity, special needs, socioeconomic status, saliva composition and flow rate, and genetic factors which can also contribute to the dynamic caries process.[10] Initially, the caries process begins by *Streptococcus mutans* strongly adhering to tooth surface and subsequently releasing acids by fermentation of carbohydrates, which leads to demineralization. This attachment is mediated mostly by the interaction of surface proteins and bacterial polysaccharides within the biofilm.[11] Other contributing risk or protective factors can mediate the caries process, altering it over time.[12]

The incidence of tooth decay in children has been well established as highlighted in the Surgeon General's Report on Oral Health. The Surgeon General's Report stated that there are profound disparities in both access to care and the epidemiology of oral disease, especially, but not limited to, dental caries.[13] The report went so far as to call dental and oral disease a "silent epidemic" that most severely affects the poor, children, and those with disabilities and complex health problems. In an effort to combat this epidemic (pandemic) there are numerous preventative measures that have been implemented in various countries with marked success.[14] Certain preventive approaches, such as the use of probiotic therapy to modify the oral microflora in young children, require further research. Thus,

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examining salivary level of mutans streptococci and lactobacilli before and after probiotic therapy will be a focus of this study.

Protective factors have been of increasing interest over the last several decades as dentistry has shifted from treating the existing disease to preventing future disease.[15] The application of health-promoting bacteria for therapeutic purposes is one of the strongest emerging topics, not only in medical, but dental science. The expanding research in herbal treatments has led to the discovery of various phytochemicals to limit the virulence of *S. mutans*.[16] Most treatments are now directed at either elimination of this bacterium or suppression of its virulence. The term 'probiotic' as officially adopted by the International Scientific Association for Probiotics and Prebiotics can be defined as 'Live microorganisms, which when administered in adequate amounts, confer a health benefit on the host'.[17] There is considerable scientific support of their potential and real benefits in vitro and animal experiments and to a lesser extent in humans. The best known and studied probiotics are the lactic acid bacteria and bifidobacteria, which are widely used in dairy products. They retain viability during storage and endure passage though the stomach and small bowel. They are also generally regarded as safe (GRAS), that is, nonpathogenic and non-toxic.[18] Importantly, bacteria residing in the oral microflora have been implicated in cardiac disease, atherosclerosis, diabetes, pneumonia, pre-eclampsia, arthritis, Alzheimer's and many other systemic disorders.[19] Being able to exactly determine the pathogenicity of the oral microflora and also creating the ability to successfully modify the oral microflora has become essential in the maintenance of patient's overall health. The oral rinse and spit diagnostic test may soon be the best and most effective method to screen for many pathologic states.[20] Thus testing may be considered one of the most significant advances in oral health diagnosis. The effect of OTC probiotics on this test may also be very significant, therefore necessitating immediate investigation.

Probiotic organisms are thought to act through a variety of mechanisms including antagonism with potential pathogens for nutrients or enterocyte adhesion sites, the degradation of toxins, production of antimicrobial substances, and through local and systemic immunomodulation.[21-23] *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus lactis, Lactobacillus helviticus, Lactobacillus salivarius, Lactobacillus plantarum, Lactobacillus bulgaricus, Lactobacillus rhamnosus, Lactobacillus johnsonii, Lactobacillus reuteri, Lactobacillus fermentum, Lactobacillus delbrueckii, Streptococcus thermophilus, Enterococcus faecium, Enterococcus faecalis, Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium longum and Saccharomyces boulardii* are some of the more commonly used bacterial probiotics.[24] In dentistry, published studies with *Lactobacillus rhamnosus* GG,[25-27] *Lactobacillus reuteri,*[28] and *Lactobacillus casei*[29] have demonstrated their possible role in preventive care by reducing the number of the caries initiating pathogen *S. mutans*, thereby suggesting probiotics for caries prevention.[30] *Lactobacillus rhamnosus* belongs to the heterofermentative lactobacilli group, which ferments neither sucrose nor lactose and has also shown to increase humoral immunity.[21, 26] Yet, there is overall little information regarding the contributions of probiotics to oral health.

While a healthy mouth generally includes a well-balanced range of diverse oral bacteria, risk of caries has historically been reported to increase when the numbers of mutans streptococci and lactobacilli significantly rise, and the mouth's natural protective factors cease to function optimally. The Caries Risk Test, made by Ivoclar Vivadent, works by rendering bacteria that cause caries more visible. The CRT (Caries Risk Test) Bacteria Kit allows simultaneous determination of mutans streptococci and lactobacilli counts in saliva by means of selective agars.[31] The kit's blue mitis-salivarius-agar with bacitracin has historically been used to detect mutans streptococci, while the light culture medium Rogosa agar is used to evaluate lactobacilli. The kit includes foils that protect the agars from contamination and drying out, while the deep indentation in the carriers prevents the culture media from slipping out. Test results are available within 48 hours wherein the colonies may be discerned and the number relatively categorized.

The difference between a diseased and a healthy oral condition appears not only to be related to the presence of oral pathogens but also to the level of "healthy" bacteria.[32] The oral flora of young children that do not develop pathology contains a number of inhibitory bacteria.[33] Based on this concept Oragenics, Inc. has introduced EvoraKids™, EvoraPro™ and Evora Plus™ with a proprietary mixture (ProBiora3 ™) containing three GRAS (generally regarded as safe) probiotics; *Streptococcus rattus* JH145, *Streptococcus oralis* KJ3 and *Streptococcus uberis* KJ2. These low acid producing oral inhabitants quickly colonize the oral cavity inhibiting growth of the pathogenic Streptococci strains.[34] *Streptococcus oralis* KJ3 also reportedly produces hydrogen peroxide to inhibit adjacent pathogenic bacteria by oxygenating the plaque. A probiotic mouthwash with ProBiora3, a mixture of the three previously mentioned bacteria strain, has been demonstrated as safe in the laboratory model.[35,36]

Threshold levels of certain pathogenic bacteria have been reported by Simark-Mattsson *et al* in young adults and research on the presence of the pathogens in children (6-12 years of age) has also been published.[37,38] In addition, studies by Simark-Mattsson suggest that *Streptococci mutans* strains distribution may be influenced through the oral administration of Lactobacilli (such as *Lactobacilli reuteri*). The *Lactobacilli reuteri* probiotics may be administered orally either in liquid drops, chewable lozenge or gum formulation. For this study, *Lactobacilli reuteri* lozenges (PerioBalance) were administered to one of the groups. Previous studies have demonstrated significant lowering of pathogenic bacteria in the saliva by using probiotic therapy.[39-41] No studies have been done to date in the young child population. No long-term studies have been reported demonstrating any long-term benefit of *Lactobacilli reuteri* lozenges (PerioBalance).

MATERIALS AND METHOD

This clinical study included 60 total patients-30 children taking the EvoraKids probiotic and 30 children taking the PerioBalance oral probiotic. The study was approved by the Institutional Review Board of Children's Memorial Hospital in Chicago, Illinois and consent forms were obtained for each patient. The study population included both healthy well and medically compromised children, ages 6 to 12 years who are patients of the principal investigator at his private office. The study population had a caries risk of moderate to high (caries prone) using the standard risk assessment (CAMBRA). There are no published studies demonstrating a difference in the levels of pathogenic bacteria between the two groups. Indeed, the two groups may be considered as one (the caries prone group) for the purpose of microbiological studies. Another inclusion criterion was the ability to rinse and spit into the collection device. Depending on the assigned group, subjects agreed to use one PerioBalance lozenge

daily or one EvoraKids chewable tablet two times a day, and keep a diary of usage. Subjects also agreed to return for all required recall or follow up appointments and to return PerioBalance or EvoraKids packages with all lozenges or chewable tablets accounted for.

Exclusion criteria included failure to meet age requirements, failure to return for required recall or follow up appointments, failure to use home care products as recommended, home diary incomplete, failure to use all 28 lozenges or 60 chewable tablets as instructed, and/or un-used lozenges or chewable tablets present in package.

Each subject was evaluated at a screening visit where parents (and patient, depending on his/her age) gave written informed consent to participation in the study. Parents completed a comprehensive health history, hygiene, and nutrition survey for each child. Parents also completed a dental history pertaining to their own caries experience. Each child received a thorough dental examination and modified Oral Hygiene Index (simplified) with Lorvic Plaque Indicator. All subjects continued with their previous preventive regimen.

After the initial exam, each subject was placed into the caries prone group (the moderate to high risk group) based on the standard risk assessment (CAMBRA). Examples of risk factors include:

1.) The positive presence of white spot lesions or cavitated lesions.

2.) High susceptibility due to health, hygiene, or nutrition history.

3.) The presence of tight interproximal contacts.

4.) The presence of plaque.

The sixty caries prone subjects (at moderate to high risk) were randomly assigned by Research Randomizer software to either Group A: the PerioBalance *Lactobacilli reuteri* lozenges (28 lozenges, one per day for 28 days), or Group B: the EvoraKids chewables (60 tablets, two per day, following tooth brushing) for 30 days. (This complied with the manufacturer's instruction for the use of each OTC product). Follow up testing with the CRT was completed for all the subjects at the eight week visit (roughly 60 days). The 8 week period had been suggested as the most appropriate timing for follow-up, as the effects of the Probiotic supplementation continue after the product has been discontinued. Previously reported pilot studies have determined that 4 weeks would be sufficient to measure the effect, if any, of the probiotics on the test results.[42] This study also evaluated the subject's SM determination of either positive or negative changes from pre- to post- probiotic supplementation. The primary evaluation of the salivary status (+/-) of SM was tested using the McNemar test, comparing the post PerioBalance and EvoraKids result to the baseline status for each subject.

Both PerioBalance and EvoraKids were included in the study because they are the only two Probiotic products available for oral health that claim documented benefits. Previously reported studies have indicated that currently utilized preventive dentistry measures are influencing the strain distribution of SM creating a more pathogenic oral microflora.[43] Due to that research result, this protocol did not utilize any anti-microbial agents which have been routinely utilized in some other dental preventive programs.

For initial data collection, the CRT analysis from Vivadent/Ivoclar was used for direct measurement of the Streptococcus mutans colonies formed. The subject chewed on paraffin wax to stimulate salivary flow and expectorated into a collection device. The collected saliva was carefully poured over the selective agar test strips after removing the protective foil. The CO2 producing tablet was then placed in the bottom of the collection tube and the top carefully sealed. The collection tubes were numbered as to prevent investigator bias and placed in the supplied incubator for 48 hours. After 48 hours, the colonies formed were visually compared to the supplied manufacturer's chart. The chart had been adapted to a numerical scale (0-4.0) allowing for ranking of the colony formation density. The colonies were harvested under a sterile hood with the "stab" technique and the bacteria frozen for future DNA-PCR determination as per the IRB approved protocol.

At 6-8 weeks following the initial screening visit, subjects were again evaluated and presented with consultation on the results of the initial CRT, followed by an post probiotic sampling of the saliva. The same procedure for analysis of the saliva was performed as before, with post probiotic numerical ranking of the mutans streptococci and Lactobacilli colonies. More stabs were collected for future analysis of strain shifting, as per the IRB approved protocol. The colonies were not directly examined by a microbiologist for phenotypical evaluation as the frozen cultures will later by processed with DNA-PCR analysis. An update of all health, nutrition, and hygiene information was performed. Each subject was given a dental prophylaxis, along with new oral hygiene and diet instructions. Parents and subjects received education on the importance of good oral hygiene and proper nutrition. The oral healthcare professional demonstrated proper oral hygiene for the parent/subject, after which, each parent/subject demonstrated for the oral healthcare professional. Finally, fluoride foam 1.23 % APF was applied to the dentition of all subjects for 1minute.

RESULTS

The final sample size was 60 patients 6-12 years of age who were diagnosed with 4 or more dental restorations or dental lesions. All patients completed the probiotic regimen except two brothers who were involved in an automobile accident and another two patients who became ill (not related to the study). The four patients were immediately replaced with other subjects as subjects were still being screened. The empty containers of the probiotics were returned for confirmation of compliance with the study protocols and parent diaries of appropriate consumption were also obtained, as per study protocol.

The Results were statistically significant. Using ANOVA stat advisor there is a significant result with a decrease of colony forming units for both the EvoraKids and the PerioBalance groups. The results were statiscally significant, between groups, sum of squares-84.3711 Df- 7, mean square 12.053, F Ratio-10.36 and P Value-0.000. Both probiotics suppressed the level of mutans streptococci and Lactobacilli. The difference between the two probiotics was not statistically significant; however, there may still be a clinically relevant difference. The importance of colony suppression should not be under emphasized, but this does not include any beneficial changes from effects on inflammatory mediators or the rest of the oral microflora. There was no significant difference within the groups (Sum of Squares- 242.087, Df-208, Mean Square-1.16388), meaning that both probiotics had a smiliar influence on the CRT results. Further analysis with the Wilcoson Two-Sample Test revealed no statistically significant differences between the two probiotics on the CRT results for either Lactobacilli (Z- 0.1846) or mutans streptococci (Z- 0.8244). The Kruskal Wallis test for the effect of either probiotic on lactobacilli (Chi Square -0.0374, Df -1, Pr>Chi Square - 0.8467) or mutans streptococci (Chi Square -0.6972, Df -1,Pr > Chi Square

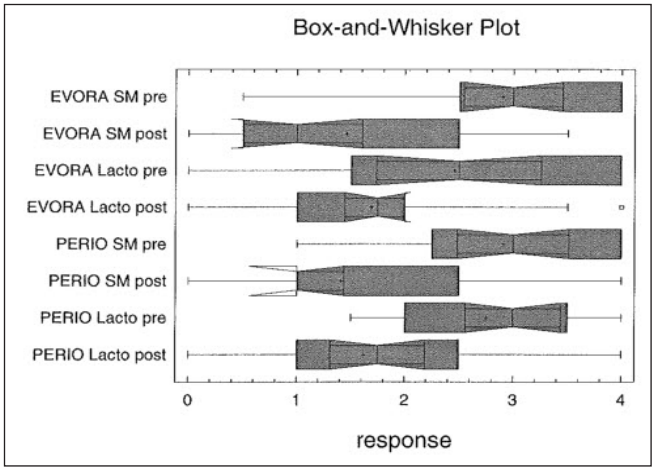

Figure 1. Box and Whisker Plot demonstrating the CRT Rankings in Children before and after probiotic use (EvoraKids and PerioBalance). The response is the Rank values determined by comparing colony formation to the supplied chart.

- 0.4047), also demonstrated no significant difference. Although the graphs would indicate that the specifics of Lactobacilli and mutans streptococci inhibition by the two different probiotics are distinct, as demonstrated in Figures 2 and 3, the overall effect of the probiotics does not significantly differ. Both apparently inhibit growth of oral cariogenic bacteria as determined by the CRT results.

In Figure 1 the visual Box and Whisker Plot demonstrates the CRT results in children before and after probiotic use (EvoraKids and PerioBalance) and clearly shows the significant suppression of MS and Lactobacilli colony formation. The drop in colonies would change the status of the subjects from being caries prone, to what is reported in the average patient population. The Rank of 2 would be considered the "cut off" between caries prone and caries inhibited patients, with a Rank above 2 correlating with the CAMBRA determination of caries prone for this experimental subject group (Findings higher than 10^5 CFU of mutans streptococci and/or lactobacilli per milliliter of saliva indicate a high risk (Krasse 1988; Andersson *et al* 1993). The very apparent shift below the mid-point Rank of 2 should signify a clinically significant result.

The changes in the CFU level for mutans streptococci before and after EvoraKids and PerioBalance probiotic treatment is demonstrated in Figure 2. The vertical columns are the frequency of the changes in the mid-point whereas the horizontal values are the changes in the Ranking, signifying the suppression of colony formation. The subjects obviously responded differently to probiotic supplementation, with the majority of the patients having a moderate decrease while only a few did not (9), while some had a more pronounced response (13 subjects).

The changes in Lactobacilli before and after EvoraKids and PerioBalance probiotic treatment is demonstrated in Figure 3. Again it is demonstrated that patients respond differently to probiotic supplementation. With both probiotics there was an increase in a few patients (12) in Lactobacilli colony formation although with the exception of only one subject, the increase was mild. The variance in response would prompt the conclusion that the probiotics would also demonstrate significant differences. However, the Wilcoxon two sample test demonstrated that there is no significant difference between the EvoraKids and the PerioBalance probiotic treatment result. The chi square also showed no significant difference between EvoraKids and PerioBalance (P=0.84676) probiotics results. But as mentioned before, the exact specifics of the oral cariogenic inhibition by the two probiotics, may indeed differ.

DISCUSSION

Dental caries is a debilitating oral disease and current research has been focused on finding preventive options to help decrease pathogenic bacteria and thus the caries process in high risk patients. There has been an emphasis in pediatric dentistry towards identifying early factors to decrease the progression of the disease process or eliminate it. The etiology of caries is multifactorial but with a direct correlation to mutans streptococci.

Few studies have analyzed preventive approaches such as the use of probiotic therapy to modify the oral microflora in young children. Thus, this study was designed to examine the salivary level of mutans streptococci and lactobacilli before and after probiotic therapy in children. "Mutans streptococci" are identified by means of standard test procedures involving mitis-salivarius agar, which

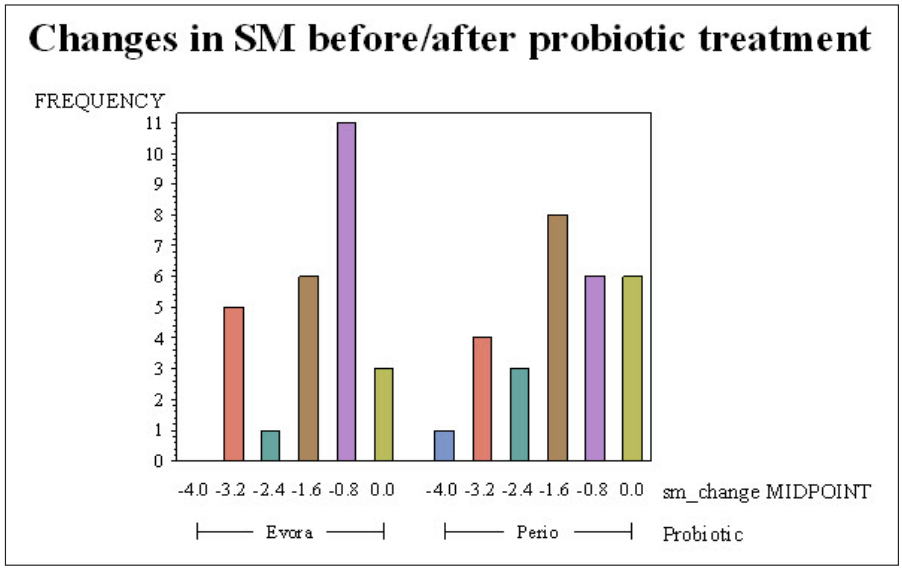

Figure 2. Changes in the *mutans streptococci* CFU rankings before and after probiotic use.

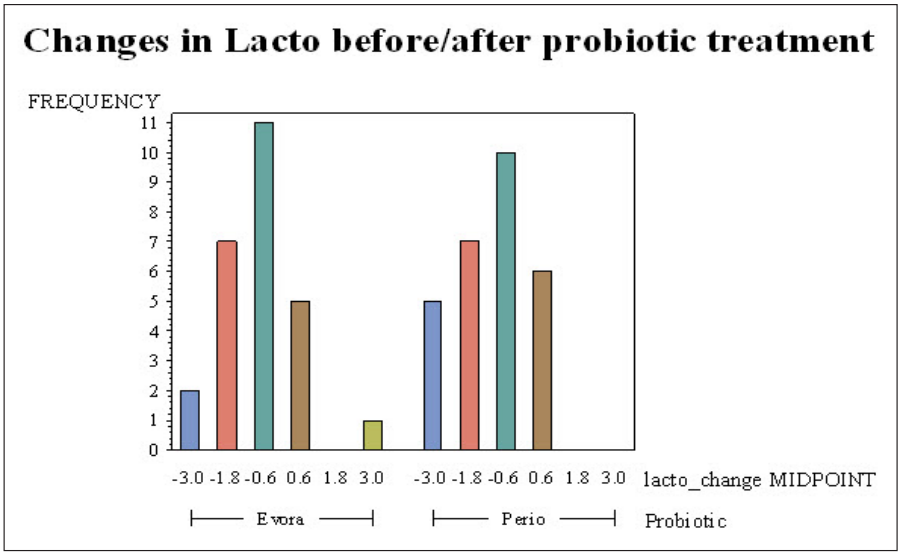

Figure 3. Changes in the *Lactobacilli* CFU rankings before and after probiotic use.

contain bacitracin (Gold *et al*). Several substances ensure the high selectivity of this procedure, such as sucrose and bacitracin, a polypeptide antibiotic inhibiting growth of bacteria other than mutans streptococci, as well as various salts, which are responsible for the blue coloring of the agar. Mutans streptococci demonstrate a high resistance to this combination whereas other microorganisms are inhibited. Rogosa agar permits the selective detection of lactobacilli and has remained the laboratory standard to this day.

The Results demonstrated that probiotics will definitely affect the results of CRT analysis. Both probiotics resulted in suppression of the colony formation of mutans streptococci and Lactobacilli. Although the differences in the probiotics were not statistically significant, they could indeed be clinically different in effectiveness. The study did not measure new caries formation or the effect on the oral microbiome as a whole. More research will be necessary to determine under which circumstance the clinician should choose what probiotic is best for the individual patient. The changes in the Rankings were not uniform at all, demonstrating an individual response to the probiotics given. Oddly enough, the level of Lactobacilli actually increased in a few patients. This would not be so surprising with PerioBalance, which is after all, Lactobacilli itself. But this was also observed with the EvoraKids probiotic. The shifting of the oral microbiome is therefor hard to predict. The interplay between the many different species of bacteria is not that well documented.

Due to this strain shifting, future plans include further analysis of the frozen salivary samples with DNA-PCR. One of the strengths of DNA-PCR is the capability to detect non-cultivatable and slow-growing microorganisms, which the CRT is unable to show. The ability to identify infectious agents and to discriminate non-pathogenic from pathogenic strains by virtue of particular genes is what makes DNA-PCR so critical to advanced medical care. For our purposes, DNA-PCR will allow selective isolation of specific bacteria and create a pathway for genetic fingerprinting. These keys may unlock the door of uncertainty and allow clinicians to prescribe certain probiotics based on an individual patient's genetic background. Tailoring every probiotic regime to the individual patient will undoubtedly improve effectiveness and ultimately lead to more caries prevention. The protocol submitted to and approved by the Institutional Review Board included DNA-PCR analysis of the saliva. Extra saliva was obtained and subjected to DNA-PCR analysis with an Applied Biosystems 7900HT utilizing a *Streptococcus mutans* primer set from Primer Designs (glucosyltransferase-I (gtfb) gene) genesig LTD. The DNA-PCR analysis of the *Streptococcus mutans* levels, however, did not demonstrate any statistically significant changes. This may have been due to the selective agar complicating DNA extraction, and possible contamination of some samples. Furthermore, *Streptococcus rattus* (included in EvoraKids) was mis-identified as SM but is a mutans streptococci. This technical difficulty occurred because the glucosyltransferase-I gene has previously been identified as a highly specific marker for *Streptococcus mutans* (Lett Appl Microbiol. 2006 Feb; 42(2):127-31). The primers and probe have 100% homology with all reference sequences for *Streptococcus mutans* in the NCBI database but unfortunately also with other mutans streptococci

The Results clearly indicate that although there is so much to be resolved, the two probiotics in the present study did significantly suppress the colony formation of bacteria associated with the development of dental caries. The decrease in the colony formation correlated well with what would clinically change the caries prone patient into a patient not at significant risk. Translating the CFU chart into numerical Ranking allowed for extrapolation of the results into clinical application. The study group that was caries prone according to CAMBRA standards would be much less at risk according to previously published and long accepted research.[44-50]

But from a diagnostic standpoint, clinicians, when performing caries risk assessment, should ask parents about the use of probiotic therapy which may affect the CRT results.

CONCLUSION

Both EvoraKids and PerioBalance probiotics affected the CRT results by significantly decreasing the number of bacteria associated with dental caries. The present research study would indicate that by introducing specific "inhibitory" bacteria through the use of "probiotics", that it may be possible to alter the oral microflora and create a less pathogenic environment, especially for the development of dental disease.

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