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Preliminary assessment of safety and effectiveness in humans of ProBiora^{3} ™, a probiotic mouthwash

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Abstract

Aims: To conduct a pilot human clinical trial to assess the safety and to test the ability of a probiotic mouthwash, ProBiora^{3}, to affect the levels of *Streptococcus mutans* and certain known periodontal pathogens in the mouth when administered twice daily over a period of 4 weeks.
Methods and Results: The mouthwash contained three specific strains of naturally occurring oral bacteria and was tested at two dose levels: 10^{6} and 10^{8} colony forming units each of *Strep. oralis* strain KJ3sm, *Strep. uberis* strain KJ2sm, and the spontaneous lactic acid-deficient variant of *Strep. rattus*, strain JH145. Substantial decreases in the levels of the marker bacteria were observed. No safety issues were noted with the twice daily application of this mouthwash.
Conclusions: Despite the small number of subjects and the use of young, orally healthy adults, along with the inherent variability in the microbiological measurements, the probiotic mouthwash was able to substantially affect the levels of dental pathogens in saliva and periodontal pathogens in subgingival plaque.
Significance and Impact of the Study: The results of this pilot human study suggest that the probiotic mouthwash product may be safe for daily use as an aid in maintaining both dental and periodontal health.

Introduction

The use of beneficial micro-organisms has been proposed to treat a range of clinical conditions that have been linked to bacterial pathogens, including oral diseases like dental caries and periodontal diseases (Hillman and Socransky 1989; Taubman *et al.* 1989; Walker and Buckley 2006). The term 'probiotics' has been defined as live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host (Guarner *et al.* 2005). The selection of micro-organisms for use as probiotic treatments does, however, involve careful consideration. The selected organism(s) must be safe to the host and must be able to successfully compete with the pathogen(s) for space and/or nutrients at the site of action, or curtail the outgrowth of the pathogen(s) through negative interactions such as the production and excretion of inhibitory products. The potential for developing probiotics for oral health care has received increased attention during the past several years (Caglar *et al.* 2005a,b; Meurman 2005; Meurman and Stamatova 2007; Twetman and Stecksen-Blicks 2008), although most of the effort has been directed at merely adapting gastrointestinal probiotics for this purpose.

Some common problems of the human oral cavity are known to result from an increase in the populations of certain indigenous bacteria. In addition, it is known that a small number of species can help maintain a microflora balanced in favour of healthy teeth and periodontal tissues (Hillman and Socransky 1989; Taubman *et al.* 1989). In the case of periodontal diseases, a strong inverse relationship was demonstrated to exist between the presence of certain species of *Viridans streptococci* and the bacteria that are thought to cause most types of periodontal diseases (Socransky *et al.* 1988). In a healthy periodontal site, *Streptococcus oralis* (previously called *Strep. sanguis*

type II) and *Strep. uberis* are commonly found in significant amounts, while the levels of periodontal pathogens including *Tannerella forsythensis*, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Peptostreptococcus micros*, *Campylobacter rectus* and *Prevotella melaninogenica* are usually quite low. The opposite situation prevails in disease sites where, in fact, *Strep. oralis* and *Strep. uberis* are usually undetectable (Hillman and Socransky 1982; Socransky *et al.* 1988). Furthermore, the observed negative interactions between *Strep. oralis* and *Strep. uberis* and the various periodontal pathogens is dose dependent (Socransky *et al.* 1988). *Streptococcus oralis* and *Strep. uberis* have been reported to inhibit the growth of periodontal pathogens by producing hydrogen peroxide (Hillman and Socransky 1982; Hillman *et al.* 1985; Hillman and Shivers 1988). *Streptococcus oralis* and *Strep. uberis*, therefore, appear to have the necessary properties for use as probiotic aids in the maintenance of periodontal health.

With regard to the other major oral disease, dental caries, *Strep. mutans* has been generally accepted as the principle etiologic agent (Tanzer *et al.* 2001; Kuramitsu 2003; Caufield *et al.* 2005). *Streptococcus mutans* causes tooth decay by producing lactic acid from the metabolism of dietary sugar (Hillman 1978; Johnson *et al.* 1980; Hillman *et al.* 2000). The lactic acid, in turn, promotes loss of mineral from the tooth's enamel and dentin. It is interesting to note that *Strep. rattus* is a very close relative of *Strep. mutans*. In fact, until fairly recently, *Strep. rattus* was considered to be one of several subspecies of *Strep. mutans* (Coykendall 1989). A particular, completely natural strain of *Strep. rattus,* called JH145, makes less than three percent of the lactic acid from metabolism of sugar as does the parent clinical isolate (Hillman 1978; Johnson *et al.* 1980). The genetic defect in this strain has been identified as a spontaneous deletion mutation in the middle of the structural gene for the enzyme lactate dehydrogenase. Since the strain does not make lactic acid, it is virtually incapable of causing dental caries (Johnson *et al.* 1980). Daily administration of 10^6 or more colony forming units (CFU) of JH145 was shown to reduce the levels of indigenous *Strep. mutans* in Sprague Dawley rats and it showed no adverse side effects over a 26-week treatment period (J.D. Hillman, unpublished data). Based on these findings, JH145 represents a suitable candidate for use as an oral probiotic strain.

The purpose of the study was to determine if twice daily swishing with ProBiora^3, an oral probiotic mouthwash product, consisting of an equal mixture of *Strep. uberis* strain KJ2sm^{TM}, *Strep. oralis* strain KJ3sm^{TM}, and *Strep. rattus* strain JH145^{TM}, by human subjects is well tolerated and whether its use promotes periodontal and dental health by reducing the numbers of certain bacterial species known or believed to be absent or at very low levels in periodontally and dentally healthy individuals.

Materials and methods

Human subjects

A total of 20 healthy adult subjects were included in this first-in-human pilot study, with an equal number of each gender. Only subjects in the 21- to 35-year-old range were selected. Each subject was required to have no less than 20 natural, minimally restored teeth, and each subject needed to be in good general health according to a medical history, physical examination, and clinical judgment. Subjects with active carious lesions or periodontal pockets greater than 5 mm were excluded from the study. For inclusion in the effectiveness part of the study, each subject had to be colonized by measurable levels of *Strep. mutans* and one or more or the periodontal pathogens chosen as biomarkers for periodontal disease.

Probiotic mixture

ProBiora^3 (Oragenics Inc., Alachua, FL, USA), the probiotic mouthwash product, contains a proprietary blend of three select species of naturally occurring oral bacteria, each with a specific function for maintaining a healthy oral environment. These strains include *Strep. oralis* KJ3sm, *Strep. uberis* KJ2sm, and *Strep. rattus* JH145. The mouthwash product was supplied to study subjects as a dry powder in a 20 ml amber glass bottle with a rubber stopper and crimp. Each bottle contained either approx. 10^6 or 10^8 colony forming units (CFU) of each of the three strains. The lyophilized bacteria were dry blended with food grade maltodextrin as a bulking agent. The single-dose product bottles were stored sealed at room temperature until use. The subjects were instructed to mix the product with approx. 15 ml of bottled or tap water prior to application. The reconstituted mouthwash was then swished for 30 s before being expectorated.

Clinical protocol

The clinical trial design was an open-label study. The primary aim of the study was to assess product safety, while a secondary aim was to test for efficacy. The clinical testing of the safety of the probiotic mouthwash was conducted with a subset of the subjects ($n = 8$). Effectiveness of the probiotic mouthwash was evaluated in a second group of subjects ($n = 12$). The study was divided into three phases consisting of a Screening Phase, a Baseline Phase, and a Product Usage Phase. The Screening Phase provided the necessary medical and dental

information to assure that the subjects were in good health and therefore suitable for inclusion in the study. The Baseline Phase provided sufficient data to establish normal oral microbiological levels. The Product Usage Phase determined if twice daily rinsing with the product was well tolerated and/or resulted in a measurable change in the level(s) of one or more of the selected marker micro-organisms of oral health.

Each candidate underwent a routine set of tests to verify that he/she met the criteria for inclusion in the study. A medical history was obtained by questionnaire and interview, with special emphasis on chronic medical conditions, cardiovascular conditions, and conditions or circumstances that resulted in the implantation of a prosthetic device. A physical examination was performed to document vital signs, and a cardiovascular physical examination was performed on each subject. A complete oral examination, including bitewing radiographs, was also conducted to identify active carious lesions, periodontal pockets in excess of 5 mm, and signs of repeated interruption of the mucosa, such as by overly hard brushing or habitual cheek biting.

Group 1 Study: Saliva was collected at the Screening Visit 1 from potential candidates for the safety part of the study by instructing the subjects to chew a small (10 g) piece of paraffin to stimulate saliva production, and then to expectorate until 2 ml of saliva were collected in a sterile plastic container. The saliva samples were stored on ice until used. Decimal dilutions of the saliva were prepared in phosphate buffered saline (PBS) and triplicate samples of 10^{-4}–10^{-6} dilutions were spread on blood agar medium (Trypticase-soy agar supplemented with 5% sheep's blood) to determine the mean cultivable bacterial concentration and 10^{-1}–10^{-5} dilutions were spread on Mitis-Salivarius agar to determine mean total *Viridans streptococci* concentrations in saliva.

Only those subjects that met all of the inclusion conditions and demonstrated none of the exclusion conditions, as determined by the above outlined testing programme, were entered into the safety part of the study. Those subjects selected based on results obtained in the Visit 1 screening were scheduled for a Baseline Phase appointment on Visit 2. These subjects returned to the Clinic for two additional appointments at weekly intervals (Visits 3 and 4), during which saliva samples were obtained and tested as described above. The individual and group means and standard deviations for levels of *Viridans streptococci* [expressed as colony forming units (CFU) ml^{-1} of saliva] in the Baseline Phase were calculated. A total of four independent measures of *Viridans streptococci* and total cultivable bacteria levels were thus obtained. In addition to microbiological sampling, subjects also received a complete visual oral exam and their vital signs were taken at each of the baseline visits.

After saliva sampling and completion of the baseline examination at Visit 4, each subject was instructed in the use of the probiotic mouthwash, which entailed adding room temperature water (tap or bottled) to the indicated level on the vial, mixing gently, and swishing the contents in his/her mouth for 30 s before expectorating. The subjects were then given 14 applications of the product containing 10^{6} CFU each of KJ2sm, KJ3sm and JH145. This rinsing procedure was repeated each morning and evening, after brushing, for 1 week. The subjects were instructed to maintain their normal oral hygiene schedule with the exception of refraining from the use of any type of commercial mouthwash. A diary was provided to each subject in which to note the time of each usage and any unusual side effects or adverse events experienced during the Product Usage Phase of the study. At Visit 5, each subject returned to the clinic, and vital signs and an oral exam were performed. The subject's diary was reviewed and the examiner asked the subject directed questions to elicit any information regarding adherence to the protocol and unusual or adverse events that may not have been contained in the diary. Saliva samples were taken for microbiological assays as described previously. The subjects were given another 14 applications of the product for use during the following week leading up to Visit 6, again the following week leading up to Visit 7, and again the following week leading up to Visit 8. The individual and group means and standard deviations for levels of *Viridans streptococci* during the Product Usage Phase were calculated and compared to his/her Baseline Phase data to determine the occurrence of statistically significant differences.

Since no safety issues related to product use were reported for the low dose (10^{6} CFU) treatment, Group 1 subjects repeated the Product Use Phase protocol using product containing 10^{8} CFU for each of the three probiotic bacterial strains. Subject examinations and saliva sampling occurred weekly at Visits 9, 10, 11 and 12. Again the individual and group microbiological data derived from saliva samples taken at these four high-dose visits were compared to baseline data and analysed for statistically significant differences.

Group 2 Study: For a second group of potential candidates, saliva was collected at the Screening Visit in the same manner as for the Group 1 subjects. Decimal dilutions of the saliva were prepared in PBS and triplicate samples of 10^{-4}–10^{-6} dilutions were spread on blood agar medium (Trypticase-soy agar supplemented with 5% sheep's blood) to determine the cultivable bacterial concentration and 10^{-1}–10^{-5} dilutions were spread on S/S medium containing bacitracin and the pH indicator 2,3,5-triphenyl tetrazolium chloride (Bochner and Savageau 1977) to determine the mean mutans streptococci

concentration, where white colonies that arose following incubation were lactic acid producing, indigenous mutans streptococci and red colonies were JH145, the lactic acid-deficient probiotic strain. Only the white colonies were counted. The saliva dilutions were also spread on Mitis–Salivarius agar to determine total *Viridans streptococci* concentrations in saliva.

Buccal and interproximal subgingival plaque samples were obtained from the first and second bicuspids and first and second molars of the left and right maxillary quadrants of potential Group 2 candidates using a sterile curette. These two plaque samples were processed independently to help compensate for the expected variability in the results obtained. To help minimize contamination from supragingival plaque, the crowns of the teeth were scrubbed prior to plaque collection using dampened cotton gauze pads and air drying. The plaque recovered was placed in transport medium for microbiological evaluation for the presence and levels of five periodontal pathogens. Decimal dilutions (10^{-2}–10^{-7}) of the plaque samples were spread on TSBA-HK medium (Trypticase-soy agar supplemented with 5% sheep's blood, 0·005 mg ml^{-1} of hemin and 0·00005 mg ml^{-1} of menadione) and incubated under anaerobic conditions at 37°C for 5–7 days to determine the mean cultivable bacterial concentrations; and, 10^{-2}–10^{-5} dilutions were spread on selective media for the target periodontal micro-organisms, which included *Aggregatibacter actinomycetemcomitans, Tannerella forsythensis, Prevotella intermedia/nigrescens, Porphyromonas gingivalis* and *Campylobacter rectus*.

For *Aggregatibacter actinomycetemcomitans*, decimal dilutions of the plaque samples were spread on TSBV agar (Trypticase soy agar containing 10% horse serum, 0·1% yeast extract, 0·0075% bacitracin and 0·0005% of vancomycin) with aerobic plus 5% carbon dioxide incubation at 35°C for 2–3 days (Slots 1982). For *Campylobacter rectus*, all glistening black colonies (>1·5 mm in diam.) with entire edges arise when using Trypticase soy agar containing 0·03% sodium thiosulfate, 0·02% ferrous sulfate, 0·3% sodium fumerate, 0·2% sodium formate and 0·9% vancomycin, under anaerobic incubation at 37°C for 5–7 days (Hammond and Mallonee 1988). *Porphyromonas gingivalis* can be enumerated on the TSBA-HK medium, incubated under anaerobic conditions at 37°C for 5–7 days, as all dark pigmenting colonies that do not fluoresce red under long-wave UV light but fluoresce blue when sprayed with *N*-benzoyl-L-arginine-7-amido-4-methylcoumarin HCl (Chen *et al.* 2001). *Prevotella intermedia/nigrescens* shows up on TSBA-HK medium as all dark pigmenting colonies that fluoresce red under UV light when sprayed with *N*-benzoyl-L-arginine-7-amido-4-methylcoumarin HCl when incubated anaerobically at 37°C for 5–7 days, and are trypsin negative (Chen *et al.* 2001). For identification and enumeration, *Tannerella forsythensis* colonies appear small (*c.* 1 mm diam.) and round with either a white or pink speckled appearance on TSBA-HK medium supplemented with 0·01% *N*-acetyl muramic acid and incubated anaerobically at 37°C for 8–10 days, and give a positive trypsin reaction (Slots 1982).

Based on the results of the Visit 1 screening appointment, a total of 12 subjects were selected for participation in the efficacy part of the study. Only those subjects that met all of the inclusion conditions and demonstrated none of the exclusion conditions, as determined by the above outlined testing program, were enrolled into the study. These subjects were entered into the Baseline Phase of the study immediately following the conclusion of the Group 1 safety study.

Those subjects selected for the Group 2 efficacy testing were scheduled for a Baseline Phase appointment on Visit 2. These subjects returned to the Clinic for two additional Baseline Phase appointments at weekly intervals (Visits 3 and 4), during which, saliva and plaque samples were obtained and tested as described above. The individual and group means and standard deviations for baseline levels of *Strep. mutans*, expressed as colony forming units (CFU) ml^{-1} of saliva, and periodontal target organisms, expressed as percent of total anaerobes, were calculated. A total of four independent measures of *Strep. mutans* levels were obtained and eight independent measures of the periodontal pathogens were obtained. These data provided sufficient baseline information to determine the efficacy of the probiotic mouthwash product described in the following section. In addition to microbiological sampling, subjects also received a complete visual oral exam and their vital signs were taken at each of the baseline visits.

After saliva and plaque sampling and completion of the baseline examination at Visit 4, each subject in Group 2 was instructed in the use of the probiotic mouthwash, as described above. The subjects were then given 14 applications of the product containing 10^{8} CFU each of KJ2sm, KJ3sm and JH145. The rinsing procedure was repeated each morning and evening, after brushing, for 1 week. The subjects were instructed to maintain their normal oral hygiene schedule with the exception of refraining from the use of any type of commercial mouthwash. A diary was provided to each subject in which to note the time of each usage, and any unusual side effects or adverse events. At Visit 5 of the study, each subject returned to the clinic, and vital signs and an oral exam were performed. The subject's diary was reviewed and the examiner asked the subject directed questions to elicit any information regarding adherence to the protocol and unusual or adverse events that may not have been contained in the diary. Saliva and plaque samples were taken

for microbiological assays as described previously. The subjects were given another 14 applications of the product for use during the following week leading up to Visit 6, again the following week leading up to Visit 7, and again the following week leading up to Visit 8.

The individual and group means and standard deviations for levels of *Strep. mutans*, expressed as CFU ml^{-1} of saliva, and the periodontal target organisms, expressed as percent of total cultivable bacteria, during the Product Usage Phase were calculated and compared to Baseline Phase data to determine the occurrence of statistically significant differences in the levels of *Strep. mutans* and/or periodontal target organisms as the result of product usage.

Statistical analyses

Analyses were conducted separately for each of the six chosen dental and periodontal pathogens. Initial analyses used a repeated measures analysis of variance (ANOVA) on the four baseline samples to ensure that the samples were not significantly different from one another. Significant differences were not observed, and so the four samples were averaged to create one baseline value. Next, the baseline value was submitted to a repeated measures ANOVA along with four treatment samples (i.e. measured once per week for 4 weeks). The Wilks' Lambda statistic was used for analyses with three or more within-subject levels and significant differences were decomposed using paired-samples *t*-tests with Bonferroni correction procedure. The alpha level for all tests was set at $P = 0{\cdot}05$ prior to Bonferroni correction. If significant differences between the baseline and any of the four treatments were not observed, the procedure was conducted again using a second treatment.

Results

Group 1 safety study

The design of the first part of the study, in which the Product Usage Phase proceeded from low to high concentrations of the probiotic micro-organisms, was instituted to maximize the safety of the subjects in this first-in-humans pilot study. The mouthwash was well tolerated by all eight subjects at both the low dose (10^6 CFU per strain) and the high dose (10^8 CFU per strain). There were no complaints regarding taste or the administration of the product. With regard to safety, eight adverse events were reported during the 8 weeks of the product usage: slight tingle in the throat (two), sore throat (two), cold sore/ulcer (two), headache (one) and stomach ache (one). All of these adverse events were judged by the clinical staff as mild, all resolved on their own without medication or physician visit, and all were judged as unlikely to be related to the use of the product. Weekly soft tissue examinations by the principal investigator found no soft tissue changes during the 8 weeks of product usage, except for the two cases of ulcers/cold sores which appeared to be random, and resolved quickly on their own. An analysis of the adverse events data did not reveal any significant gender differences.

An analysis of the saliva microbiological data revealed that there were no significant changes or discernable trends in the mean total *Viridans streptococci* concentrations or total cultivable bacteria in saliva between the Baseline Phase and the Product Usage Phase of the study (data not shown). This result indicates that there were no obvious disruptions of the oral microbial flora caused by use of the probiotic mouthwash.

Group 2 efficacy study

In the second study, 11 of the 12 subjects completed a month of high-dose product use (10^8 CFU per strain per dose). One subject was dropped from the study during the Baseline Phase because that subject was placed on antibiotics for an unrelated infection. Since there were no clinically significant safety issues reported for the first group of subjects at the higher dose, this second group went directly from baseline to product use at the higher (10^8 CFU) dosage. With regard to safety in this group, a total of 10 adverse events were reported by the second set of subjects during the 4 weeks of product use: sore throat (2), mouth sore/fever blister (4), headache (1), cough (2) and congestion (1). None of these adverse events were judged to be severe by the principal investigator, all resolved quickly on their own without medication or physician visit, and all were judged as unlikely to be related to the use of the product. There were no significant gender differences with regard to reported adverse events.

With regard to the effects of the JH145 component of ProBiora^3 on *Strep. mutans* and dental health, the 11 subjects in Group 2 that completed the study showed a decrease in the salivary levels of this pathogen, with a mean drop of *c.* 60% from the baseline level (Table 1). The data were further analysed to take into account two reasonable concerns about the study design (i) the product was self-administered twice daily over an 8-week-period, which raises the question of compliance and (ii) a finite period is required in order to achieve optimum numbers of the probiotic strains at their site of action (J.D. Hillman, unpublished results). We anticipated that any effect would be seen first with *Strep. mutans* since this micro-organism is more accessible on the coronal surfaces than are the periodontal pathogens, which reside in the

Table 1 Total data summary for group 2

	Mean bacterial levels (±SD)	
	Baseline	High dose
Pathogen	Visits 1–4	Visits 5–8
Streptococcus mutans (10^5 CFU ml^{-1} saliva)	6·48 (±10·61)	2·54 (±5·20)
*Campylobacter rectus** (% of total bacteria)	0·0755 (±0·2084)	0·0053 (±0·0104)
Porphyromonas gingivalis† (% of total bacteria)	0·356 (±0·972)	0·056 (±0·114)

*Deleted four of 176 data points as obvious outliers (>3 SD from mean).
†Deleted one of 176 data points as outlier and Visit 5 data (excessive variability).

periodontal sulci. If we eliminate from the analysis one of the eleven subjects who did not appear to comply or respond to treatment, the mean salivary level of *Strep. mutans* (CFU per ml saliva) further decreased to about 15% of the baseline value (Table 2). Because of the small number of subjects, and the considerable sample-to-sample variability, the high dose decrease was not statistically significant at the $P < 0{\cdot}05$ level. It is also important to note here that no changes from baseline numbers of *Viridans streptococci* were evident at the treatment dosage level. This result agrees with the first study that there were no apparent disruptions of the oral microbial ecosystem caused by use of the probiotic mouthwash.

Of the five periodontal pathogens that were monitored in this study, *A. actinomycetemcomitans* and *T. forsythensis* occurred so infrequently that they were not considered in the analysis. Perhaps the absence, or very low level, of these two target periodontal pathogens was related to the generally good oral health of our subjects. At the conclusion of the study, data analysis showed a decrease by a factor of about 10 in the levels of *C. rectus* and *P. gingivalis* in subgingival plaque after ProBiora[3] mouthrinse use (Table 1), but these decreases were not statistically significant at the $P < 0{\cdot}05$ level because of the small subject number and the high variability of the test results. Because there were no statistically significant differences in the mean decrease in levels of *C rectus* and *P. gingivalis* for the males and females, the subject data were combined to obtain the mean bacterial levels reported in Tables 1 and 2. An analysis of trends in this pilot study suggests that the subject population for a second trial would need to be increased to 24 participants in order to assure statistical significance. However, taking into account the previously observed (J.D. Hillman, unpublished data) time dependence of a probiotic strain to exert its effect, a comparison of the baseline levels of *C. rectus* to the mean levels for just the last two treatment visits (Table 2) is informative. In this case, a decrease is observed from 0·0755% of total cultivable bacteria in the plaque samples at baseline to 0·0002% after treatment. It was remarkable that *C. rectus* was undetectable in six of 11 subjects collected at the last treatment visit (Visit 8), whereas all 11 subjects had detectable levels of this pathogen in at least three of the four baseline visits (Visits 1–4), and in both of the first two Product Use visits (Visits 5 and 6). This result further suggests that it may take several weeks of product use before a substantial, beneficial change in subgingival plaque composition can be observed.

Table 2 Adjusted data summary for group 2

	Mean bacterial levels (±SD)	
	Baseline	High dose
Pathogen	Visits 1–4	Visits 5–8
*Streptococcus mutans** ($\times 10^5$ CFU ml^{-1} saliva)	6·08 (±11·09)	0·99 (±0·97)
Campylobacter rectus† (% of total bacteria)	0·0755 (±0·2077)	0·0002 (±0·0007)
Porphyromonas gingivalis‡ (% of total bacteria)	0·487 (±0·994)	0·004 (±0·011)

*One of 11 subjects was dropped as non-complier/non-responder.
†Used only last two visits (allowed time for treatment effect).
‡Used only last two visits; dropped two of 11 non-compliers/non-responders and one of 11 with no *P. gingivalis* at baseline.

Similar to the *C. rectus* results, a decrease of more than two orders of magnitude in *P. gingivalis* plaque levels was found when only the data from the last two treatment visits were analysed, and if two subjects who did not comply/respond to treatment and one subject who did not harbour *P. gingivalis* at baseline were eliminated from the analysis (Table 2). The calculated decrease in the *P. gingivalis* level of subgingival plaque, though substantial (two orders of magnitude), was not significant at a $P < 0{\cdot}05$. This result, however, suggests a strong, time-dependent trend toward lower subgingival plaque levels of *P. gingivalis* with product use, despite the small number of participants, and the inherently large variation in plaque collection and analysis.

With *Prevotella intermedia/nigrescens*, there was no observable decrease in subgingival plaque levels with treatment, and there was substantial sample variation as well. It should be noted that *Prevotella intermedia* was the only periodontal pathogen whose levels in subgingival plaque samples, taken from healthy and diseased periodontal sites, was reported to not be inversely correlated with the presence of either *Strep. oralis* or *Strep. uberis* (Socransky *et al.* 1988).

Discussion

The application of probiotics to address oral health is a relatively new area of research; nevertheless, there is a growing body of scientific literature on this topic. The effect of oral administration of probiotics on the levels of *Strep. mutans* in saliva, and potentially on the incidence of dental caries, has been reported using micro-organisms revamped from their original application as gastrointestinal probiotics. For example, studies using *Lactobacillus rhamnosus* GG (Meurman *et al.* 1994; Nase *et al.* 2001; Ahola *et al.* 2002) and *Lactobacillus casei* (Busscher *et al.* 1999) reported a temporary reduction in *Strep. mutans* levels during the period of probiotic treatment. Similarly, Krasse *et al.* (2006) reported a decrease in gingival bleeding and reduced gingivitis with the repeated application of the traditional gastrointestinal probiotic, *Lactobacillus reuteri*.

Microbial interactions, including coaggregation, metabolic cross-feeding and cell signalling, are logically of enormous importance in determining the composition of the microflora in the mouth and may mediate the balance between oral health and disease (Demuth *et al.* 2001; Lamont and Yilmaz 2002; Kolenbrander *et al.* 2006). It seems likely that certain associations might favour the outgrowth of specific bacterial species that potentially could shift the oral population away from a healthy balance. Of equal interest is the likelihood that certain microbial species, such as probiotic strains, may interact with dental plaque and be antagonistic to the outgrowth of these potentially health-disruptive organisms. Beneficial oral micro-organisms, therefore, could be used to treat a range of clinical conditions that have been associated with pathogens, including dental caries and periodontal diseases.

It is clearly advantageous for a probiotic to use well tested strains of species that are indigenous to the human oral cavity and have proven, specific negative interactions with pathogenic species. ProBiora[3] satisfies these important prerequisites. The lactic acid-deficient *Strep. rattus* strain used in the present human trial is a very close relative of *Strep. mutans* and, at one time was classified as an *Strep. mutans* strain (Coykendall 1989). This *Strep. rattus* strain, therefore, should compete for the same binding sites on the tooth surface, nutrients and other resources as does the indigenous, decay-causing *Strep. mutans*. Thus, the specific *Strep. rattus* strain, JH145, selected for maintaining dental health most likely uses a competitive exclusion mechanism to reduce the numbers of *Strep. mutans* cells present on teeth. Based on the known correlation between salivary levels of *Strep. mutans* and the risk of developing new dental caries (Twetman *et al.* 1990; Bratthall 1991; Twetman and Frostner 1991), the change in mean *Strep. mutans* levels caused by ProBiora[3] in our pilot study would reduce the risk from moderate to low for this group of test subjects after using the product twice daily for as little as 4 weeks.

Streptococcus oralis and *Strep. uberis* are known to produce hydrogen peroxide, and their mechanism of probiotic action has been shown *in vitro* and in animal models to depend on this metabolic activity (Hillman and Socransky 1982; Hillman *et al.* 1985; Shivers *et al.* 1987; Hillman and Shivers 1988). Studies using periodontal plaque from healthy and diseased sites in human subjects indicate that *Strep. oralis* and *Strep. uberis* are the most effective of the *Viridans streptococci* in inhibiting the growth of periodontal pathogens (Socransky *et al.* 1988). This observation suggests that the habitat for *Strep. oralis* and *Strep. uberis* in the plaque biofilm may be close enough to the habitat of the pathogens for their peroxide production to have a negative interactive effect.

The unadjusted results (Table 1) from the efficacy study showed an obvious trend for a decrease in the proportions of *P. gingivalis* and *C. rectus* in subgingival plaque following 4 weeks of treatment with ProBiora[3]. This trend became much more pronounced when the microbiological data were analysed with the exclusion of those individuals who did not appear to comply with or benefit from product treatment. It routinely has been observed in other probiotic clinical studies (Jacobsen *et al.* 1999; Schrezenmeir and de Vrese 2001; Heczko *et al.* 2006) that certain individuals do not respond to treatment with a specific strain of a probiotic bacteria used in a clinical trial. This observation is potentially supported by the results of the present study and demonstrates the importance of employing only those specific strains of probiotic bacteria that have clinically demonstrated an ability to maintain a healthy flora in the majority of individuals tested. The consumer probiotic products industry is currently dominated by companies that effectively utilize the most scientifically supported strains of beneficial or probiotic bacteria in the formulation of their products.

The strains that comprise ProBiora[3] are expected to reach and saturate their target habitats within several weeks of regular product use. The rate at which the optimal beneficial effect of an oral probiotic strain can be achieved has been shown to be dependent on the dose (Johnson and Hillman 1982; J.D. Hillman, unpublished data). Once established, regular use of the oral probiotic product should help maintain effective levels of the helpful bacteria in the user's mouth over the long-term. The positive trend toward decreased levels of pathogenic bacteria achieved in just 4 weeks of treatment using 10^8 CFU per strain is encouraging, and suggests that use of the

product at a higher dose or for longer times will consistently provide a substantial positive oral health benefit for the user.

With regard to safety, the probiotic micro-organisms should not be pathogenic, should not have any growth-stimulating effects in bacteria-causing oral diseases, and should not have the ability to transfer antibiotic resistance genes (Grajek *et al.* 2005). The strains in ProBiora^3 appear to meet these safety guidelines. They were well tolerated by the subjects, and did not appear to alter the normal ecology of the oral microflora, as indicated by measurements of *Viridans streptococci* levels and total cultivable bacteria. All three oral probiotic strains are resistant to streptomycin, which is not commonly used as a therapeutic agent, but otherwise are sensitive to all antibiotics commonly used to treat Gram-positive infections (data not shown). The results of this pilot human study suggest that the probiotic mouthwash product may be safe for daily use as part of an effective oral hygiene regimen.

Finally, it is important to note that ProBiora^3 positively impacted on the levels of key bacterial markers of both dental and gingival disease in a group that was orally healthy, based on commonly accepted measures. This result provides preliminary but compelling evidence for the value of ProBiora^3 as an aid in helping to maintain a healthy oral bacterial composition for product users. The rapid and substantial decreases in observed levels of harmful bacteria in the oral cavity of the test subjects, most notably at the base of the periodontal sulcus following 4 weeks usage of the higher dose product, point to the potential for a substantially diminished risk of new cavities and gum disease in product users. As a first-in-human study, the protocol was designed primarily to establish safety using young, healthy adult volunteers, while effectiveness was a secondary study aim. Nevertheless, substantial changes were observed in the levels of key bacterial markers in both saliva and plaque. Further studies will be performed to determine the ameliorative effects of ProBiora^3 in subjects with ongoing dental caries and/or periodontal disease.

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ProBiora^3, KJ2sm, KJ3sm and JH145 are trademarks of Oragenics Inc., Alachua, FL.

Conflict of interest statement

The authors, R.T. Zahradnik, E. McDonnell and J.D. Hillman are employees of Oragenics, Inc., the distributors of ProBiora^3.

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