

Influence of the probiotic *Streptococcus salivarius* strain M18 on indices of dental health in children: a randomized double-blind, placebo-controlled trial

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The prevalence of dental caries continues to increase, and novel strategies to reverse this trend appear necessary. The probiotic *Streptococcus salivarius* strain M18 offers the potential to confer oral health benefits as it produces bacteriocins targeting the important cariogenic species *Streptococcus mutans*, as well as the enzymes dextranase and urease, which could help reduce dental plaque accumulation and acidification, respectively. In a randomized double-blind, placebo-controlled study of 100 dental caries-active children, treatment with M18 was administered for 3 months and the participants were assessed for changes to their plaque score and gingival and soft-tissue health and to their salivary levels of *S. salivarius*, *S. mutans*, lactobacilli, β -haemolytic streptococci and *Candida* species. At treatment end, the plaque scores were significantly ($P=0.05$) lower for children in the M18-treated group, especially in subjects having high initial plaque scores. The absence of any significant adverse events supported the safety of the probiotic treatment. Cell-culture analyses of sequential saliva samples showed no differences between the probiotic and placebo groups in counts of the specifically enumerated oral microorganisms, with the exception of the subgroup of the M18-treated children who appeared to have been colonized most effectively with M18. This subgroup exhibited reduced *S. mutans* counts, indicating that the anti-caries activity of M18 probiotic treatments may be enhanced if the efficiency of colonization is increased. It was concluded that *S. salivarius* M18 can provide oral health benefits when taken regularly.

Received 22 December 2012

Accepted 26 February 2013

INTRODUCTION

Dental caries is the most common chronic disease of childhood and, despite major technological advances and the introduction of many new initiatives by the dental profession, its prevalence continues to increase in many populations worldwide (Bagramian *et al.*, 2009). Expression of the disease is characterized initially by dissolution of the mineral portion of the tooth (white spot lesions), progressing to localized destruction of the enamel and dentine, and followed ultimately by inflammation of the pulp and periapical tissues if left untreated. Whilst recent research indicates a multi-species aetiology

for dental caries, the mutans streptococci (MS) – a cluster of acidogenic, dental plaque-inhabiting streptococcal species – are still recognized as major constituents of most active dental caries lesions. Of the various MS species, it is *Streptococcus mutans* and *Streptococcus sobrinus* that have been principally implicated in dental caries development in humans. Dental caries imposes a major health and economic burden internationally, and a wide variety of approaches for its control has been developed and applied, with varying degrees of success. Treatments using conventional anti-streptococcal antimicrobials can be effective in the short-term to reduce dental plaque levels and to decrease counts of MS, but, as most therapeutic antibiotics have relatively broad-spectrum antimicrobial activity, they indiscriminately destroy both commensal and potentially

Abbreviations: BLIS, bacteriocin-like inhibitory substance; MS, mutans streptococci; OHI-S, simplified Oral Hygiene Index.

harmful bacteria and thereby create population imbalances within the microflora. In addition, conventional antimicrobial treatments are often unpalatable for young children, resulting in poor compliance and thereby compromising the likelihood of demonstrating beneficial outcomes.

The use of probiotics to effect an improvement in oral health without impacting negatively on the normal oral microbiota is a relatively new concept. Conventionally, probiotics – defined by the World Health Organization as ‘live organisms which, when administered in adequate amounts confer a health benefit on the host’ – have almost exclusively been bacteria of intestinal origin, and their application has largely been targeted at relieving maladies of the gastrointestinal tract. However, because it is now becoming clear that many human illnesses are related either directly (for example, dental caries and periodontal disease) or indirectly (for example, cardiovascular disease) to the development of oral microbiota disequilibria; new ways of reducing the disease burden imposed by these dysfunctional microbial populations are being investigated (Zarco *et al.*, 2012). A variety of putative commensal bacteria have been assessed for their potential to prevent dental caries. Studies based on use of the intestinal probiotics *Lactobacillus rhamnosus* GG (Näse *et al.*, 2001), *Lactobacillus reuteri* ATCC 55739 and *Bifidobacterium* DN-173 010 (Caglar *et al.*, 2005) have each reported achieving reduced levels of *S. mutans* and, moreover, the children taking *L. rhamnosus* GG developed fewer dental caries. Whilst these strains have shown some promise for the prevention of dental caries, a new generation of probiotic strains sourced from the human oral cavity and belonging to commensal species known to have extremely low pathogenic potential are now being developed. In this regard, a key species is *Streptococcus salivarius*, which has previously been investigated for its role in the prevention of pharyngitis caused by *Streptococcus pyogenes* (Dierksen & Tagg, 2000), dental caries (Tanzer *et al.*, 1985a, b), periodontal disease (Guglielmetti *et al.*, 2010; Teughels *et al.*, 2007) and halitosis (Burton *et al.*, 2006a).

Many *S. salivarius* strains produce bacteriocins, which are ribosomally synthesized antimicrobials that typically have a narrow inhibitory spectrum directed against relatively closely related bacteria (Wescombe *et al.*, 2009). In order to minimize widespread disruption within the oral microflora, bacteriocin-producing probiotics targeting MS are now under consideration as a replacement therapy approach to the control of dental caries (Hillman *et al.*, 1987). Key characteristics of an effector strain for use in replacement therapy include: (i) the absence of virulence determinants, (ii) colonization capability, and (iii) the ability to competitively displace the target bacterium (Burton *et al.*, 2011a). Perhaps not unexpectedly, the most common producers of anti-MS bacteriocins are other strains of MS. Indeed, a number of *S. mutans* producing strong anti-*S. mutans* activity have been identified as potential effectors of replacement therapy (Hillman *et al.*,

1987). However, unless stable non-cariogenic natural variants of these strains can be derived, their acceptance and application for dental caries control seems unlikely to gain either regulatory or public acceptance (Hillman *et al.*, 1990, 1998).

S. salivarius is a numerically predominant, exclusively human, oral streptococcal species that is not known to have any disease associations in healthy humans. Previously, *S. salivarius* TOVE-R (Tanzer *et al.*, 1985a, b) has been shown capable of effecting a reduction in dental caries in animal models, but this strain had only relatively weak bacteriocin activity *in vitro* (Wescombe *et al.*, 2011, 2006). Heightened interest in the probiotic potential of *S. salivarius* stems from the recognition that some strains produce a particularly diverse array of bacteriocins, together with the demonstration that the application of strain K12, the prototype of *S. salivarius* probiotics, can help control a variety of upper respiratory tract ailments including streptococcal sore throat, otitis media and halitosis (Burton *et al.*, 2006a, 2006b, 2011b; Wescombe *et al.*, 2009). In addition, some *S. salivarius* strains have been shown to produce the exoenzymes dextranase and urease, the activities of which could potentially help limit the progression of dental caries by reducing plaque accumulation and plaque acidification, respectively (Chen *et al.*, 1996; Li *et al.*, 2000; Nascimento *et al.*, 2009; Ohnishi *et al.*, 1995). *S. salivarius* M18 (also referred to as strain Mia or DSM 14685; Chilcott & Tagg, 2007), a strain originally isolated from a healthy adult subject during a specific search for *S. salivarius* capable of inhibiting MS, has subsequently been shown to have relatively broad-spectrum bacteriocin-like inhibitory substance (BLIS) activity against MS, and to produce both dextranase and urease enzymes (Heng *et al.*, 2011). The genome of M18 has recently been published, and its megaplasmid-encoded bacteriocin repertoire includes salivaricin A, salivaricin M, salivaricin MPS and salivaricin 9 (Chilcott & Tagg, 2007; Heng *et al.*, 2011; Wescombe *et al.*, 2006, 2011). These idiosyncratic characteristics of M18 make it an attractive candidate with potential application to the prevention and treatment of dental disease.

The present study provides a preliminary evaluation of *S. salivarius* M18 for its probiotic application to the prevention, or a reduction in the risk, of dental caries. The objective was to compare the influence on several readily measured indices of potential for dental caries activity in children who were given lozenges containing either M18 or placebo over a 3-month study period.

METHODS

Screening for antimicrobial activity. The spectrum of antimicrobial activity of M18 was detected using the deferred antagonism method, essentially as originally described by Tagg & Bannister (1979). Briefly, an agar plate, comprising tryptic soy broth (BBL) supplemented with 2% yeast extract (Difco), 1% CaCO₃ and 0.7% Bacto agar and adjusted to pH 6.5 before autoclaving (TSYCa), was

seeded diametrically with a 1 cm wide inoculum of the test strain from an 18 h culture of the producer strain grown on Columbia blood agar base (Difco) supplemented with 5% human blood using a cotton swab. Following incubation for 18 h at 37 °C in 5% CO₂ in air, the culture growth was removed using a glass slide and the agar surface sterilized by exposure to chloroform vapour for 30 min, followed by airing for a further 30 min. Indicator bacteria, from 18 h Todd–Hewitt broth (Difco) cultures, were then inoculated with a swab at right angles across the line of the original diametric streak culture of the test strain, and the plate was reincubated for 18 h at 37 °C in 5% CO₂ in air. Zones of inhibition were scored as ‘–’ for no inhibition or ‘+’ where definite interference with the growth of the indicator was evident.

Preparation of test material. Freeze-dried preparations of *S. salivarius* M18 cells were produced by the Microbial Fermentation Unit (Fonterra, Palmerston North, New Zealand), an ISO 9001 quality-accredited facility. The cell powder was blended with flavouring agents and the food-grade sugar substitutes trehalose and maltodextrin, prior to forming into lozenges by Good Manufacturing Practice-certified Alaron Products (Nelson, New Zealand). Cell counts were obtained just prior to commencement and at completion of the study, and each lozenge was determined to contain 3.6×10^9 c.f.u. *S. salivarius* M18, a level that was maintained throughout the 3 months of the active dosing period. The placebo differed only in containing additional sugar substitutes in place of the M18 cell powder. The active and placebo preparations were identical in appearance and taste.

Participants. The study was approved by the Otago Ethics Committee (approval no. 02/09/099). Six schools with dental clinics on site were randomly selected. One hundred Dunedin schoolchildren aged 5–10 years who had previously experienced dental caries (and had at least three dental restorations, including one placed within the previous 12 months) were recruited into the study through their school dental clinics. Two saliva specimens were procured from each child within a 2-week period and tested for their content of *S. mutans* and BLIS-producing *S. salivarius*. The inclusion criteria for the treatment phase of the study were: (i) no natural strong BLIS-producing *S. salivarius* detected, and (ii) $>10^4$ c.f.u. *S. mutans* ml⁻¹ in at least one of the two pre-screen saliva samples. During the recruitment phase, 18 children were excluded who were either lactose intolerant, allergic to dairy products, immunologically compromised or taking antibiotics. One hundred children meeting the selection criteria were assigned randomly into two groups of 50 and commenced the dosing programme; however, after exclusions due to non-compliance or incomplete data collection, 40 (80%) and 43 (86%) children, respectively, from each group completed the probiotic- and placebo-dosing courses. The probiotic group comprised 12 males and 28 females (mean age 8.5 years) and the placebo group had 21 males and 22 females (mean age 8.5 years). A combination of count back of the number of lozenges returned and a sticker chart administered by the parents was used for the monitoring of subject compliance. Parents/guardians were asked via telephone interview each month whether the children had experienced any ill effects and these were recorded.

Clinical examinations. At the first visit, a dental therapist (whose assessment regimen had previously been calibrated) carried out a clinical examination and recorded the numbers of decayed, missing or filled teeth and any new caries lesions that had occurred since the child's most recent school dental care visit. Also recorded at this visit (and at the 1-, 3- and 7-month visits) were data on soft-tissue health, gingival health and dental plaque.

Evaluation of soft-tissue health was based on the appearance of the oral mucosa and involved recording the presence of any tissue

abnormalities such as ulceration, redness/inflammation, abscesses or white patches. The surfaces examined were the lips, sulci, buccal mucosa, floor of the mouth, tongue, hard palate, soft palate and gingiva/alveolar processes. For analysis purposes, all abnormalities detected were treated as isolated events and the sum of abnormalities at each time point for each group was compared.

For the gingival health assessment, the Gingival Index of Loe & Silness (1963) was utilized. The appearance of the gingival tissues (colour, inflammation, swelling or signs of bleeding) was recorded for six teeth (the buccal aspects of the upper right second primary molar, upper left second primary molar, upper right central incisor and lower left central incisor, and the lingual aspects of the lower left second primary molar and lower right second primary molar). The categories and codes used were: normal gingivae, scoring 0; mild inflammation, slight difference in colour or slight oedema, 1; moderate inflammation, redness, oedema and glazing, 2; and severe inflammation, manifesting as redness and oedema with signs of bleeding, 3.

Plaque was scored using an adaptation of the simplified Oral Hygiene Index (OHI-S) of Greene & Vermillion (1964). The OHI-S has two components, the Debris Index and the Calculus Index. Each of these is based on numerical determinations representing the amount of debris or calculus on index tooth surfaces. The distribution of dental plaque was assessed following the use of a plaque-disclosing solution. The teeth scored were the same as those selected for the gingival health assessment.

Treatment protocol. On the first day of the study protocol, the children used a fluoride-containing toothpaste not containing any supplementary antibacterial agents and, under the supervision of a dental therapist, brushed their teeth until all of their disclosed plaque had been removed. The dental therapist then flossed the children's teeth. With a second brush, the teeth were then brushed for 1 min using a 2% chlorhexidine gel. Two hours later, the children were asked to suck two lozenges containing either *S. salivarius* M18 or the placebo. Two of the corresponding lozenges were also given to the children at the end of that school day. On days 2 and 3, the teeth were brushed for a timed 1 min with 2% chlorhexidine gel. Two hours later, the children were given two lozenges and then another two lozenges just before they went home. Beyond day 3, a supply of the appropriate lozenges (plus toothpaste and toothbrushes) was provided for home use, together with a sticker chart to encourage and record compliance. The protocol required the children to suck two lozenges each day for 3 months, one after brushing the teeth in the morning and one after teeth brushing at night. At 1, 3 and 7 months, the children's mouths were examined and scored for plaque distribution and clinical health as described above. Saliva samples for analyses of dental caries indicator microbes (MS, lactobacilli and yeast) and *S. salivarius* (total count and probiotic M18) were obtained pre-colonization and at 1, 2, 3 and 7 months. Approximately 1 ml saliva was collected at each assessment. If the child had difficulty salivating, he/she was asked to chew on a plastic film (Parafilm) to stimulate saliva flow.

Bacteriological analysis of saliva samples. *Candida* Chromogenic agar (for *Candida* spp.) (Fort Richard Laboratories), Rogosa SL agar (BD Difco) (for lactobacilli) and Mitis Salivarius agar (BD Difco) (for *S. salivarius*) were used. The MS selective medium was TYCSB, as described by Van Palenstein Helderma *et al.* (1983). CNA-P is a blood agar medium formulated to enhance the detection of haemolytic streptococci (Dierksen *et al.*, 2000). Incubation was in air for 48 h (for *Candida*), in 5% CO₂ in air for 24 h (for *S. salivarius*) or was anaerobic (85% N₂, 10% H₂, 5% CO₂) for 48 h (for MS or haemolytic streptococci). Saliva samples were serially diluted in sterile PBS in duplicate, and appropriate dilutions were plated in duplicate and cultivated under the appropriate conditions. CNA-P medium was used to determine the number of β -haemolytic

streptococci present in saliva samples; the numbers were graded from 0 (no haemolytic colonies) to 4 (all colonies apparently β -haemolytic) (Dierksen *et al.*, 2000). Streptex testing (Remel) established whether the haemolytic colonies on CNA-P were Lancefield group A (*S. pyogenes*). Data were \log_{10} transformed for analysis. To determine whether M18 had colonized in the child's oral cavity, 80 representative *S. salivarius*-like colonies from the Mitis Salivarius agar cultures were sampled using toothpicks and tested for simultaneous antagonism inhibitory activity against *S. mutans* OMZ 175 and *Micrococcus luteus* 11, as described previously (Tagg & Bannister, 1979). The characteristic activity of M18 against these two indicators provides a specific presumptive identification of M18. In addition, total streptococcal populations (taken from Mitis Salivarius agar) were tested for their deferred antagonism inhibitory profile (producer type) against a set of nine standard indicators, as described previously (Tagg & Bannister, 1979), to further confirm the prevalence of M18 as a significant proportion of the oral salivary population.

Statistical analysis. Following the computation of univariate descriptive statistics, bivariate associations were tested for statistical significance using analysis of variance or Kruskal–Wallis tests (as appropriate, depending upon the distribution of the dependent variable). Where the dependent variable was not normally distributed, it was log transformed prior to modelling. Linear regression modelling was used to examine the effect of the intervention while controlling for baseline status and putative confounding variables.

RESULTS

Antimicrobial spectrum of *S. salivarius* M18

To determine the potential applications of *S. salivarius* M18, an *in vitro* screen against a variety of bacterial species of significance for human health was carried out using the deferred antagonism test (Table 1). In particular, representative strains of a number of species identified as causative agents of either dental caries or periodontal disease in humans were tested and those inhibited were: *S. mutans* (11/11), *Actinomyces naeslundii* (1/1), *Actinomyces viscosus* (2/2), *Enterococcus faecalis* (1/1), *Lactobacillus* spp. (3/3) and *S. sobrinus* (1/1). Other important upper respiratory tract pathogens inhibited by M18 included all *S. pyogenes* (causative agent of streptococcal sore throat), all *Streptococcus pneumoniae* (associated with pneumonia, meningitis and otitis media), half of the *Moraxella catarrhalis* (otitis media), both *Streptococcus agalactiae* (major cause of infant septicaemia) and half of the *Staphylococcus aureus* (regularly carried in the nasopharynx and a common source of community- and hospital-acquired infections) isolates.

Compliance and adverse reactions

Dosing with probiotics twice daily required a significant commitment from the children, and therefore it was essential to determine the compliance rates in both the treatment and placebo arms of the trial. The chewable strawberry lozenges proved to be a suitable delivery format, with compliance found to be >80%. Compliance was monitored by the use of sticker charts and collecting and counting unused lozenges at the end of treatment months 1

Table 1. Spectrum of antibacterial activity of strain M18 when tested by the deferred antagonism method on TSYCa agar

Indicator species	No. strains inhibited/total tested
<i>Actinomyces naeslundii</i>	1/1
<i>Actinomyces viscosus</i>	2/2
<i>Bacillus cereus</i>	0/1
<i>Candida albicans</i>	0/3
<i>Clostridium perfringens</i>	1/1
<i>Clostridium sporogenes</i>	1/1
<i>Corynebacterium diphtheriae</i>	1/1
<i>Enterobacter aerogenes</i>	0/1
<i>Enterococcus faecalis</i>	1/3
<i>Enterococcus hirae</i>	1/2
<i>Escherichia coli</i>	0/1
<i>Haemophilus influenzae</i>	2/3
<i>Klebsiella pneumoniae</i>	0/1
<i>Lactobacillus acidophilus</i>	1/1
<i>Lactobacillus brevis</i>	1/1
<i>Lactobacillus casei</i>	1/1
<i>Lactococcus lactis</i>	1/1
<i>Listeria greyii</i>	3/5
<i>Listeria monocytogenes</i>	5/5
<i>Micrococcus luteus</i>	1/1
<i>Moraxella catarrhalis</i>	2/4
<i>Moraxella lacunata</i>	1/1
<i>Moraxella osloensis</i>	1/2
<i>Porphyromonas gingivalis</i>	0/2
<i>Prevotella intermedia</i>	0/2
<i>Proteus vulgaris</i>	0/1
<i>Rothia mucilagenosa</i>	1/1
<i>Staphylococcus aureus</i>	3/6
<i>Staphylococcus cohnii</i>	2/2
<i>Staphylococcus hominus</i>	1/1
<i>Staphylococcus saprophyticus</i>	2/2
<i>Staphylococcus simulans</i>	0/0
<i>Streptococcus agalactiae</i>	2/2
<i>Streptococcus mitis</i>	1/1
<i>Streptococcus mutans</i>	11/11
<i>Streptococcus pneumoniae</i>	8/8
<i>Streptococcus pyogenes</i>	8/8
<i>Streptococcus rattus</i>	0/1
<i>Streptococcus salivarius</i>	24/46
<i>Streptococcus sanguis</i>	0/1
<i>Streptococcus sobrinus</i>	1/1
<i>Streptococcus uberis</i>	2/2

and 2, and the subjects were assessed as compliant if they consumed $\geq 75\%$ of the prescribed lozenges each month. There were no significant differences in compliance rates between the treatment and placebo groups. Participants were excluded from the analysis if they failed to be assessed as compliant for both of the months monitored. Data for six participants were excluded from the treatment group and two from the placebo group based on this compliance criterion. Six participants dropped out of the study because they did not like the taste of the lozenges; one was eliminated because of protocol breaches, and four were lost

to follow-up due to moving away. There were four cases of adverse reactions, as monitored by self-reporting using the monthly questionnaires: three were for the M18 group and one was in the placebo group. None of the adverse events resulted in the participants leaving the trial, and none was of a serious nature.

Colonization with M18

One of the major aims of this trial was to determine whether dosing with M18 resulted in its persistent colonization of the oral cavity, with assessments being made at day 3 and after 1, 2 and 3 months. In the M18-treated group, nine subjects retained M18 populations comprising at least 5% of their total salivary *S. salivarius* population at the 3-month time point. Furthermore, P-typing of the 3-month samples of the total *S. salivarius* populations of these nine subjects showed that five gave inhibition profiles consistent with that given by pure cultures of M18. In addition to having their data evaluated together with that of the other members of the M18 treatment group, the plaque scores and *S. mutans* counts for these nine apparently more highly colonized individuals were also analysed separately to determine whether there might be additional oral health benefits associated with colonization efficacy and persistence.

Effect on plaque

Comparison of the total plaque scores of the two groups of subjects at the start of the study, after taking the lozenges for 1 and 3 months, and 4 months after dosing was terminated (i.e. at 7 months) demonstrated that, by the end of the treatment period, there was a significant difference in mean plaque scores between the M18 group and the placebo group (Table 2). As a consequence of the dental plaque treatment regimen given to all of the participants at the beginning of the study, there was also a decrease in plaque scores between the start of the study and after 1 month for both groups.

Although the plaque scores were lowest for the group taking the M18 lozenges at all sampling points, it should be noted that a disproportionate number of participants in

Table 3. Regression models for plaque score

	<i>B</i> (95% CI)*	<i>P</i> value
Plaque score at 1 month		
Intercept	4.26 (2.12, 6.40)	<0.001
Baseline plaque score	0.03 (-0.21, 0.27)	0.816
Treatment group†	-1.12 (-2.91, 0.67)	0.212
Plaque score at 3 months		
Intercept	5.86 (2.62, 9.10)	0.001
Baseline plaque score	0.27 (-0.09, 0.64)	0.137
Treatment group†	-2.71 (-5.4, 0.01)	0.050
Plaque score at 7 months		
Intercept	5.45 (2.64, 8.26)	0.001
Baseline plaque score	-0.03 (-0.36, 0.30)	0.847
Treatment group†	0.22 (-2.27, 2.70)	0.859

**B*, regression coefficient; CI, confidence interval.

†Reference category=control group.

this group also had relatively low preliminary plaque scores (Table 2). Closer analysis of the M18 group showed that it contained fewer children falling into the high plaque score category of ≥ 7 [$n=16$ (40%) for the M18 group versus $n=26$ (60%) for the placebo group]. However, controlling for baseline plaque scores using linear regression established that the plaque scores after 3 months were significantly lower in the M18 group (Table 3). Moreover, when the participants in the placebo and M18 groups who had high plaque scores (i.e. ≥ 7) at the beginning of the trial were followed separately, there was a strong difference between the two groups throughout the treatment phase of the study, with 87.5% of children in the M18 treatment group maintaining lower plaque scores than their pre-treatment scores (defined as a decrease in score of 3 or more), whilst only 44% of those in the placebo group had lower plaque scores at the same time point (Fig. 1).

While only small numbers of children were successfully colonized with M18 during the study period, the plaque scores of the nine who were well colonized showed a greater plaque reduction than both the entire M18- and placebo-treated groups. Indeed, the proportion of participants

Table 2. Summary data on plaque scores at each time point by group

Assessment time	Mean (SD)		<i>P</i> value
	Treatment group ($n=40$)	Placebo ($n=43$)	
Baseline	6.0 (3.3)	6.9 (3.2)	0.182
1 month	3.4 (2.1)	4.1 (2.6)	0.175
3 months	5.3 (3.2)	7.0 (4.1)	0.022
7 months	4.7 (2.7)	4.4 (2.9)	0.852

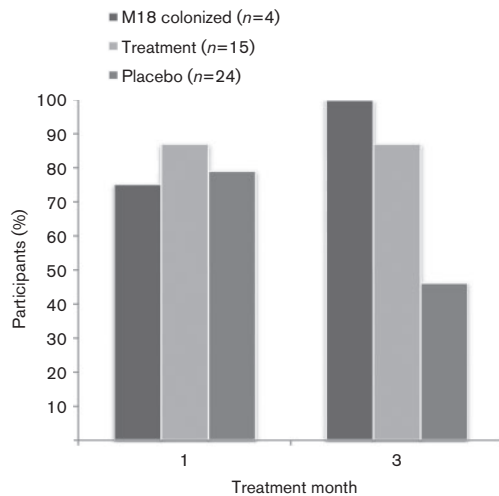


Fig. 1. Percentage of children for each group who had high pre-treatment plaque scores (≥ 7) and who demonstrated plaque score improvements of ≥ 3 at treatment months 1 and 3.

having reduced plaque scores was much greater in the colonized group than for the placebo group or for those who were not well colonized with M18 (Fig. 1).

Gingival and soft-tissue health

Analysis of the gingival health scores and soft-tissue scores showed no significant differences between the M18-treated children and the controls (Table 4). Whilst both scores were low at the beginning of the trial, the soft-tissue scores were observed to decrease further for both groups over the course of the monitored period.

Salivary microbial levels

No significant differences between the treatment and placebo groups were observed in the mean *S. mutans* counts at any of the time points assessed (Table 5). However, the five children who were identified as having been well colonized (by both the detection of M18-like colonies by the use of the simultaneous antagonism method and by having a total streptococcal P-type consistent with that of M18; i.e. P-type 677) tended to have lower *S. mutans* counts during the dosing phase when compared with their baseline salivary *S. mutans* levels (Fig. 2).

The presence of *S. pyogenes* was monitored at the preliminary, 1-, 2- and 3-month time points by plating the saliva onto CNA-P medium. Haemolytic colonies were identified as *S. pyogenes* if they were positive for Lancefield serogroup A using a Streptex agglutination test. The rate of *S. pyogenes* acquisition during the 3-month treatment phase for the M18 group was 17.9% (seven new acquisitions) and for the placebo group was 25.6% (ten new acquisitions).

Table 4. Summary data on gingival scores and soft-tissue frequency of abnormality at each time point by group

Assessment time	Gingival score		Soft-tissue frequency of abnormality		P value (between groups)*
	Group mean (SD)		Group mean (SD)		
	Treatment group (n=40)	Placebo (n=43)	Treatment group (n=40)	Placebo (n=43)	
Baseline	2.2 (1.9)	2.0 (1.4)	0.7 (0.7)	0.5 (0.5)	0.321
1 month	1.5 (1.7)	1.7 (1.9)	0.4 (0.6)	0.4 (0.7)	0.629
3 months	2.1 (2.6)	2.3 (3.1)	0.2 (0.4)	0.3 (0.5)	0.919
7 months	2.4 (2.2)	1.8 (1.9)	0.1 (0.4)	0.1 (0.3)	0.550

*Kruskal–Wallis test.

Table 5. Summary data on natural-logged *S. mutans* scores at each time point by group

Assessment time	Mean (sd)		P value
	Treatment group (n=40)	Placebo (n=43)	
Baseline 1	10.7 (2.1)	10.6 (2.6)	0.868
Baseline 2	10.3 (2.4)	11.3 (2.4)	0.129
Day 3	8.9 (2.2)	9.8 (2.1)	0.061
1 Month	11.3 (2.5)	11.6 (2.8)	0.593
2 Months	11.4 (2.2)	11.7 (2.5)	0.490
3 Months	10.6 (2.3)	10.9 (2.7)	0.755
7 Months	10.4 (2.6)	11.4 (2.3)	0.156

The salivary levels of *S. salivarius*, lactobacilli, haemolytic streptococci and *Candida* spp. did not differ substantially between the two groups (data not shown).

DISCUSSION

Previous studies of the applicability of probiotics to the prevention of dental caries have focused largely on the testing of gastrointestinal tract strains that had initially been developed as probiotics to help counter gut ailments. Here, we have provided evidence to support the use of *S. salivarius* strain M18 – a bacterium isolated from the human oral cavity and shown to have antibacterial activity against a number of clinically important human pathogens – to reduce dental plaque accumulation in schoolchildren. In addition, the findings of this preliminary double-blind, placebo-controlled trial, which included the longitudinal monitoring of the individuals' soft-tissue and periodontal clinical parameters, further attest to the safety of *S. salivarius* M18.

The treatment dosing regime of two lozenges per day, taken morning and night after tooth brushing, was well tolerated by the individuals (compliance >80 %) and this

indicates that this is a suitable format for use in primary-school-aged populations (5–12 years). Despite the high rate of compliance, only 22 % of the children in the M18 group had detectable probiotic in their saliva at the end of the treatment period. This observation indicates that any effects directly attributable to the presence of the probiotic are only likely to occur during the period of active dosing, which is in contrast to *S. salivarius* strain K12, a probiotic that has been shown to exhibit more persistent colonization of the human oral cavity (Horz *et al.*, 2007). It is, however, noteworthy that, in the time period since the present trial was conducted, significant improvements have been introduced to the production process for M18, resulting in considerably improved colonization efficacy (J. P. Burton, unpublished data).

In the present study, the primary clinical measure of clinical efficacy was the plaque score (as measured by the OHI-S), which has been ratified as an essentially valid method for measuring oral hygiene in large epidemiological studies (Broadbent *et al.*, 2011). The plaque score was monitored for each participant prior to the start of dosing and then at months 1, 3 and 7. The significant plaque score difference between the M18 group and the placebo group observed at the end of the treatment phase (month 3) indicated that the regimen implemented during this study was efficacious for a reduction in dental plaque. However, no significant differences were observed at any of the other time points between the two treatment groups, although both the placebo and M18 groups experienced a large reduction at the 1-month time point compared with their pre-treatment scores. The observed reduction in plaque score at 1 month appeared to be due to the efficient plaque removal carried out under the supervision of the dental hygienist as part of the initial pre-treatment regimen prior to beginning the trial. Both groups had similar plaque scores at the 7-month time point, indicating that the benefit of M18 treatment did not extend to 4 months past the termination of the probiotic treatment. However, for future studies, it would be beneficial to have examination time points closer to the end of active treatment to determine the extent of any persistent benefit occurring post-treatment. An interesting observation was that, for the subgroups with pre-treatment plaque scores of ≥ 7 , 87.5 %

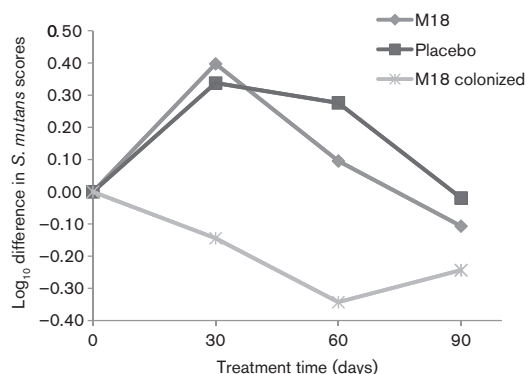


Fig. 2. Change in *S. mutans* scores (log₁₀) from the pre-treatment values for the total M18 group (n=40), placebo group (n=43) and subgroup (n=5) that had detectable levels of M18 following treatment (M18 colonized).

of the M18 group and 44% of the placebo group had plaque score reductions of ≥ 3 from their pre-treatment score at the end of treatment. This large difference between the two groups indicates that M18 treatment may provide greater benefit to individuals with existing high plaque levels and may be most efficacious when used in conjunction with a preliminary plaque removal procedure such as was implemented in the present study. Another group of individuals who may derive benefit from probiotic-mediated plaque reduction are adults experiencing gingival inflammation in whom the development of periodontitis is closely linked to the level of plaque accumulation (Broadbent *et al.*, 2011). Further evidence supporting a potential role for M18 in the control of gingivitis comes from recent experiments by Adam *et al.* (2011) who investigated whether M18 could potentially impact on pathogen-induced pro-inflammatory cytokine expression in gingival fibroblasts. A variety of pathogens have been implicated in the development of both gingivitis and periodontitis, and the aetiology of these diseases is now strongly linked to the inflammatory response of the host cells to the bacterial pathogens (Fisher *et al.*, 2010; Seymour & Gemmell, 2001). In earlier work, M18 was co-incubated with gingival fibroblasts both prior to and concomitantly with exposure to periodontal pathogens such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum*. Strain M18 significantly inhibited the expression of the pro-inflammatory cytokines IL-6 and IL-8 commonly associated with periodontitis, indicating that dosing with these probiotics may potentially be useful in the treatment of inflammatory periodontal diseases (Adam *et al.*, 2011).

The other clinical parameters examined in the present trial were the soft-tissue and periodontal tissue scores. These outcomes were monitored largely to establish whether there was evidence of any soft-tissue damage, ulcers or gum disease developing as a result of the twice-daily probiotic treatment regimen, thereby further monitoring and evaluating the safety of M18 and the efficacy of the delivery format for use in children. No significant difference was observed between the placebo or M18 treatment group for either of these clinical parameters; indeed, for the soft-tissue scores, both groups showed some improvement over the course of the trial, a finding providing some support for twice-daily oral dosing with a probiotic preparation having a positive rather than a detrimental general effect on oral health. This improvement may reflect either improved oral hygiene awareness and/or be related to seasonal changes over the period of the trial.

S. mutans has long been considered one of the principal aetiological agents of dental caries, and this has also been supported by more modern molecular analyses (Kanasi *et al.*, 2010). In the present study, the inter- and intra-individual salivary levels of cultivable *S. mutans* appeared quite variable, a finding in part due to the difficulty of selective propagation of these bacteria. A direct comparison

of the mean numbers of *S. mutans* in those receiving M18 probiotic treatment with those in the placebo group showed no significant difference. However, as the overall rates of colonization were quite low, the subgroup of individuals known to have been colonized with M18 were compared for their mean log reduction in *S. mutans* counts against both the placebo group and the entire M18 group. The M18 colonized subgroup showed a larger decrease in *S. mutans* counts than both the placebo group and the entire M18 group over the treatment period, indicating that the longer-term establishment of an M18 population may be required to impact significantly on the salivary levels of *S. mutans*. As the number of children in this colonized group was low ($n=5$), this observation must be considered preliminary and should now be supported by the conduct of further trials using the improved fermentation and manufacturing procedures that have been developed more recently for M18. Whilst it has been established that the presence of *S. mutans* is an important risk factor for the development of dental caries, recent molecular studies have highlighted the importance of microbial consortia in the aetiology and development of caries, and indeed *S. mutans* may simply act as a disease indicator organism, with its plaque predominance signifying that the oral conditions are changing to a more caries-active state (Marsh, 2003).

In previous studies, the impact of naturally occurring BLIS-producing *S. salivarius* on the acquisition of *S. pyogenes* has been determined to be of the order of a 47% reduction in new acquisitions (Dierksen & Tagg, 2000). In the present study, 18% of those in the M18 group experienced a new acquisition of *S. pyogenes* during the treatment phase and 26% of the children in the placebo group experienced new acquisitions. Unfortunately, this analysis suffers from type 2 error, as the numbers are too small. In order to demonstrate statistical significance at this level of difference, 100 individuals would have been required in each group. Nevertheless, the apparent difference in acquisition observed (in spite of poor colonization efficacy occurring) may provide encouragement for the implementation of a larger trial investigating the protective effect of M18 against *S. pyogenes* infections.

There were no significant differences in any of the other microbial indices that were measured [total *Lactobacillus*, *Candida* and *S. salivarius* c.f.u. (ml saliva)⁻¹]. This is noteworthy, as large quantities of exogenous bacteria were instilled into the oral cavity twice daily for an extended period of time and yet there were no detectable population shifts in these non-targeted microbes. Whilst the microorganisms quantified in the present study can constitute a large proportion of the cultivatable population, it is clear that the use of traditional microbiological culture technology does not suffice to detect relatively minor fluctuations in microbiota composition. In addition, the culture methodology employed did not support detection of the occurrence of more subtle intra-species (strain-specific) population changes. Further studies are now being undertaken using,

as a population analysis tool, higher-sensitivity, next-generation sequencing.

In this study, we demonstrated that twice-daily dosing with the probiotic strain *S. salivarius* M18 is a safe and efficacious way of effecting a significant reduction in plaque formation in primary-school-aged children. In addition, whilst there was no overall reduction in *S. mutans* carriage rates for the treatment group, the small subgroup of individuals who demonstrated persistent colonization by the probiotic strain showed lower *S. mutans* counts than their baseline scores, indicating that improved efficacy occurs when the probiotic strain is established within the oral microbiota of the host.

ACKNOWLEDGEMENTS

We would like to acknowledge excellent technical support from Hannah Clark, Vidya Kulkarni, Chris Moore, Erin Isdale, Nikolai Klesse and Megan Inglis. All funding for this trial was provided by BLIS Technologies Ltd. P. A. W. and J. D. F. H. are currently employed by BLIS Technologies Ltd. J. R. T. was the founding scientist of the company and currently is a consultant, whilst C. N. C. and J. P. B. are former employees. W. M. T. performed all statistical analysis of the data and has no affiliation (financial or otherwise) with BLIS Technologies Ltd.

REFERENCES

- Adam, E., Jindal, M., Seney, S., Summers, K., Hamilton, D. W., Hatibovic-Kofman, S. & Cadieux, P. A. (2011). *Streptococcus salivarius* K12 and M18 probiotics reduce periodontal pathogen-induced inflammation. In *IADR/AADR/CADR 89th General Session and Exhibition*, 16–19 March 2011, San Diego, CA, USA. Paper no. 150126.
- Bagramian, R. A., Garcia-Godoy, F. & Volpe, A. R. (2009). The global increase in dental caries. A pending public health crisis. *Am J Dent* **22**, 3–8.
- Broadbent, J. M., Thomson, W. M., Boyens, J. V. & Poulton, R. (2011). Dental plaque and oral health during the first 32 years of life. *J Am Dent Assoc* **142**, 415–426.
- Burton, J. P., Chilcott, C. N., Moore, C. J., Speiser, G. & Tagg, J. R. (2006a). A preliminary study of the effect of probiotic *Streptococcus salivarius* K12 on oral malodour parameters. *J Appl Microbiol* **100**, 754–764.
- Burton, J. P., Wescombe, P. A., Moore, C. J., Chilcott, C. N. & Tagg, J. R. (2006b). Safety assessment of the oral cavity probiotic *Streptococcus salivarius* K12. *Appl Environ Microbiol* **72**, 3050–3053.
- Burton, J. P., Wescombe, P. A., Cadieux, P. A. & Tagg, J. R. (2011a). Beneficial microbes for the oral cavity: time to harness the oral streptococci? *Benef Microbes* **2**, 93–101.
- Burton, J. P., Cowley, S., Simon, R. R., McKinney, J., Wescombe, P. A. & Tagg, J. R. (2011b). Evaluation of safety and human tolerance of the oral probiotic *Streptococcus salivarius* K12: a randomized, placebo-controlled, double-blind study. *Food Chem Toxicol* **49**, 2356–2364.
- Caglar, E., Sandalli, N., Twetman, S., Kavaloglu, S., Ergeneli, S. & Selvi, S. (2005). Effect of yogurt with Bifidobacterium DN-173 010 on salivary mutans streptococci and lactobacilli in young adults. *Acta Odontol Scand* **63**, 317–320.
- Chen, Y. Y., Clancy, K. A. & Burne, R. A. (1996). *Streptococcus salivarius* urease: genetic and biochemical characterization and expression in a dental plaque streptococcus. *Infect Immun* **64**, 585–592.
- Chilcott, C. N. & Tagg, J. R. (2007). Antimicrobial composition. US Patent 7226590.
- Dierksen, K. P. & Tagg, J. (2000). The influence of indigenous bacteriocin-producing *Streptococcus salivarius* on the acquisition of *Streptococcus pyogenes* by primary school children in Dunedin, New Zealand. In *Streptococci and Streptococcal Diseases Entering the New Millennium*, pp. 81–85. Edited by D. R. Martin & J. R. Tagg. Auckland: Securacopy.
- Dierksen, K. P., Ragland, N. L. & Tagg, J. R. (2000). A new alkaline pH-adjusted medium enhances detection of β -hemolytic streptococci by minimizing bacterial interference due to *Streptococcus salivarius*. *J Clin Microbiol* **38**, 643–650.
- Fisher, M. A., Borgnakke, W. S. & Taylor, G. W. (2010). Periodontal disease as a risk marker in coronary heart disease and chronic kidney disease. *Curr Opin Nephrol Hypertens* **19**, 519–526.
- Greene, J. C. & Vermillion, J. R. (1964). The simplified oral hygiene index. *J Am Dent Assoc* **68**, 7–13.
- Guglielmetti, S., Taverniti, V., Minuzzo, M., Arioli, S., Stuknyte, M., Karp, M. & Mora, D. (2010). Oral bacteria as potential probiotics for the pharyngeal mucosa. *Appl Environ Microbiol* **76**, 3948–3958.
- Heng, N. C., Haji-Ishak, N. S., Kalyan, A., Wong, A. Y., Lovric, M., Bridson, J. M., Artamonova, J., Stanton, J. A., Wescombe, P. A. & other authors (2011). Genome sequence of the bacteriocin-producing oral probiotic *Streptococcus salivarius* strain M18. *J Bacteriol* **193**, 6402–6403.
- Hillman, J. D., Dzuback, A. L. & Andrews, S. W. (1987). Colonization of the human oral cavity by a *Streptococcus mutans* mutant producing increased bacteriocin. *J Dent Res* **66**, 1092–1094.
- Hillman, J. D., Duncan, M. J. & Stashenko, K. P. (1990). Cloning and expression of the gene encoding the fructose-1,6-diphosphate-dependent L-(+)-lactate dehydrogenase of *Streptococcus mutans*. *Infect Immun* **58**, 1290–1295.
- Hillman, J. D., Novák, J., Sagura, E., Gutierrez, J. A., Brooks, T. A., Crowley, P. J., Hess, M., Azizi, A., Leung, K. & other authors (1998). Genetic and biochemical analysis of mutacin 1140, a lantibiotic from *Streptococcus mutans*. *Infect Immun* **66**, 2743–2749.
- Horz, H. P., Meinelt, A., Houben, B. & Conrads, G. (2007). Distribution and persistence of probiotic *Streptococcus salivarius* K12 in the human oral cavity as determined by real-time quantitative polymerase chain reaction. *Oral Microbiol Immunol* **22**, 126–130.
- Kanasi, E., Dewhirst, F. E., Chalmers, N. I., Kent, R., Jr, Moore, A., Hughes, C. V., Pradhan, N., Loo, C. Y. & Tanner, A. C. (2010). Clonal analysis of the microbiota of severe early childhood caries. *Caries Res* **44**, 485–497.
- Li, Y. H., Chen, Y. Y. & Burne, R. A. (2000). Regulation of urease gene expression by *Streptococcus salivarius* growing in biofilms. *Environ Microbiol* **2**, 169–177.
- Loe, H. & Silness, J. (1963). Periodontal disease in pregnancy. I. Prevalence and severity. *Acta Odontol Scand* **21**, 533–551.
- Marsh, P. D. (2003). Are dental diseases examples of ecological catastrophes? *Microbiology* **149**, 279–294.
- Nascimento, M. M., Gordan, V. V., Garvan, C. W., Browngardt, C. M. & Burne, R. A. (2009). Correlations of oral bacterial arginine and urea catabolism with caries experience. *Oral Microbiol Immunol* **24**, 89–95.
- Näse, L., Hatakka, K., Savilahti, E., Saxelin, M., Pönkä, A., Poussa, T., Korpela, R. & Meurman, J. H. (2001). Effect of long-term consumption of a probiotic bacterium, *Lactobacillus rhamnosus* GG, in milk on dental caries and caries risk in children. *Caries Res* **35**, 412–420.

Ohnishi, Y., Kubo, S., Ono, Y., Nozaki, M., Gonda, Y., Okano, H., Matsuya, T., Matsushiro, A. & Morita, T. (1995). Cloning and sequencing of the gene coding for dextranase from *Streptococcus salivarius*. *Gene* **156**, 93–96.

Seymour, G. J. & Gemmell, E. (2001). Cytokines in periodontal disease: where to from here? *Acta Odontol Scand* **59**, 167–173.

Tagg, J. R. & Bannister, L. V. (1979). “Fingerprinting” β -haemolytic streptococci by their production of and sensitivity to bacteriocine-like inhibitors. *J Med Microbiol* **12**, 397–411.

Tanzer, J. M., Kurasz, A. B. & Clive, J. (1985a). Inhibition of ecological emergence of mutans streptococci naturally transmitted between rats and consequent caries inhibition by *Streptococcus salivarius* TOVE-R infection. *Infect Immun* **49**, 76–83.

Tanzer, J. M., Kurasz, A. B. & Clive, J. (1985b). Competitive displacement of mutans streptococci and inhibition of tooth decay by *Streptococcus salivarius* TOVE-R. *Infect Immun* **48**, 44–50.

Teughels, W., Newman, M. G., Coucke, W., Haffajee, A. D., Van Der Mei, H. C., Haake, S. K., Schepers, E., Cassiman, J. J., Van Eldere, J. & other authors (2007). Guiding periodontal pocket recolonization: a proof of concept. *J Dent Res* **86**, 1078–1082.

Van Palenstein Helderma, W. H., Ijsseldijk, M. & Huis in 't Veld, J. H. (1983). A selective medium for the two major subgroups of the bacterium *Streptococcus mutans* isolated from human dental plaque and saliva. *Arch Oral Biol* **28**, 599–603.

Wescombe, P. A., Burton, J. P., Cadieux, P. A., Klesse, N. A., Hyink, O., Heng, N. C., Chilcott, C. N., Reid, G. & Tagg, J. R. (2006). Megaplasms encode differing combinations of lantibiotics in *Streptococcus salivarius*. *Antonie van Leeuwenhoek* **90**, 269–280.

Wescombe, P. A., Heng, N. C., Burton, J. P., Chilcott, C. N. & Tagg, J. R. (2009). Streptococcal bacteriocins and the case for *Streptococcus salivarius* as model oral probiotics. *Future Microbiol* **4**, 819–835.

Wescombe, P. A., Upton, M., Renault, P., Wirawan, R. E., Power, D., Burton, J. P., Chilcott, C. N. & Tagg, J. R. (2011). Salivaricin 9, a new lantibiotic produced by *Streptococcus salivarius*. *Microbiology* **157**, 1290–1299.

Zarco, M. F., Vess, T. J. & Ginsburg, G. S. (2012). The oral microbiome in health and disease and the potential impact on personalized dental medicine. *Oral Dis* **18**, 109–120.