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ORIGINAL ARTICLE

A preliminary study of the effect of probiotic *Streptococcus salivarius* K12 on oral malodour parameters

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Abstract

Aims: To determine whether dosing with bacteriocin-producing *Streptococcus salivarius* following an antimicrobial mouthwash effects a change in oral malodour parameters and in the composition of the oral microbiota of subjects with halitosis.

Materials and Results: Twenty-three subjects with halitosis undertook a 3-day regimen of chlorhexidine (CHX) mouth rinsing, followed at intervals by the use of lozenges containing either *S. salivarius* K12 or placebo. Assessment of the subjects' volatile sulphur compound (VSC) levels 1 week after treatment initiation showed that 85% of the K12-treated group and 30% of the placebo group had substantial (>100 ppb) reductions. The bacterial composition of the saliva was monitored by culture and PCR-denaturing gradient gel electrophoresis (PCR-DGGE). Changes in the PCR-DGGE profiles occurred in most subjects following K12 treatment. *In vitro* testing showed that *S. salivarius* K12 suppressed the growth of black-pigmented bacteria in saliva samples and also in various reference strains of bacteria implicated in halitosis.

Conclusions: Administration of bacteriocin-producing *S. salivarius* after an oral antimicrobial mouthwash reduces oral VSC levels.

Significance and Impact of the Study: The outcome of this preliminary study indicates that the replacement of bacteria implicated in halitosis by colonization with competitive bacteria such as *S. salivarius* K12 may provide an effective strategy to reduce the severity of halitosis.

Introduction

Halitosis, more commonly known as oral malodour, afflicts up to half the adult human population to various degrees (Rosenberg 1996; Yaegaki and Coil 2000). Although generally not considered to be a medical concern, it certainly can confer a significant social stigma (Rosenberg 2002). The most common oral malodour compounds are by-products of the metabolism of oral bacteria, especially anaerobes, located on the dorsum of the tongue (Scully *et al.* 1997; Loesche and Kazor 2002). Volatile sulphur compounds (VSC), valeric acid, butyric acid and putrescine are thought to contribute to the odour (Loesche and Kazor 2002). Previous culture-based studies have indicated that *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Micromonas micros*, *Campylobacter rectus*, *Eikenella corrodens* and *Treponema denticola*, as well as various species of *Bacteroides*, *Desulfovibrio* and *Eubacterium* are largely responsible for the production of the VSC that are the principal contributors to halitosis (De Boever and Loesche 1995; Khaira *et al.* 2000; Loesche and Kazor 2002). One recent nonculture based study has shown that certain oral bacterial species are relatively prevalent in subjects who are healthy, whereas other species predominate in individuals who are afflicted with halitosis. *Atopobium pavulum*, *Eubacterium sulci*, *Fusobacterium periodonticum*, *Dialister* species, *Solobacterium moorei* and

certain uncharacterized *Streptococcus* species were relatively common in subjects with halitosis (Kazor *et al.* 2003). By contrast, *Streptococcus salivarius*, *Rothia mucilaginosa* and an uncharacterized species of *Eubacterium*, were more commonly detected in healthy individuals (Kazor *et al.* 2003).

Various strategies have been developed for either the prevention or at least the alleviation of halitosis. Current treatments focus on either nonselective anti-bacterial treatment to reduce the total numbers of oral bacteria or the use of agents that mask or neutralize the odour. These protocols typically require complex and expensive physical or chemical therapy to be carried out daily and generally only provide short-term benefit, as the malodour-causing oral bacteria quickly recover to their former numbers as soon as the treatment is stopped. None of the protocols described to date incorporate a step in which a beneficial bacterial population is introduced to help counter the proliferation of odiferous species. It is our view that probiotics, defined by the WHO as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' may have beneficial application to the reduction of halitosis (Reid 2005). Probiotics have had some success when applied to the intestinal tract and vagina after antibiotic therapy and for the management of infections in which it is perceived that there is an 'imbalance' of the normal microbiota allowing unregulated growth of 'problematic' micro-organisms (Cadieux *et al.* 2002; Reid *et al.* 2003). Additionally, there is a growing body of evidence that probiotics can stimulate host immunity, occupy potential pathogen colonization sites and interrupt host–pathogen 'cross-talking' (Connolly *et al.* 2003; Freitas *et al.* 2003; Gill 2003; Valeur *et al.* 2004).

The aim of the present study was to alleviate halitosis by pre-emptively colonizing the oral cavity with a competitive commensal bacterium following a short course of mechanical and chemical treatment to reduce the numbers of odour-causing organisms and possibly provide additional attachment sites for the colonizing strain. *Streptococccus salivarius* appears to have excellent credentials as an oral probiotic. It is known to be a pioneer colonizer of oral surfaces and is a numerically predominant nondisease-associated member of the oral microbiota of 'healthy' humans (Carlsson *et al.* 1970; Kazor *et al.* 2003). This species has only a very limited capability for producing VSC (Yoshida *et al.* 2003) and thus is unlikely to contribute significantly to oral malodour. *Streptococcus salivarius* K12 is known to produce at least two lantibiotic bacteriocins, salivaricin A (Ross *et al.* 1993; Upton *et al.* 2001) and salivaricin B (Tagg and Dierksen 2003). The present pilot study had two specific objectives: (i) assessment of the impact on halitosis parameters and on the composition of the oral microbiota after the administration of a short oral antimicrobial treatment followed by daily dosing with *S. salivarius* K12 for a total of 2 weeks and (ii) evaluation of the inhibitory activity *in vitro* of bacteriocin-producing *S. salivarius* strains against bacterial species implicated in halitosis.

Materials and methods

Subjects, pretreatment, probiotic instillation and sample collection

Subjects who considered that they may have halitosis were initially screened for the levels of VSC in their oral cavity air samples (breath scores) using a halimeter (Interscan Corp., Chatsworth, CA, USA) based on the average of three readings. The average subject age was 43·3 years with a range of 18–69 years. Ten females and 13 males participated. All measurements were taken in the morning prior to the subjects eating, drinking, smoking or using any oral care. Subjects having VSC scores higher than 200 ppb on two separate visits were recruited into the study. Each gave informed consent according to an approved protocol by the Otago Ethics Committee. Thirteen subjects received the active treatment. Ten subjects (treated the same as the test group but having no bacteria in their lozenges) were included to determine the effect of the pretreatment protocol on VSC readings. All subjects undertook a mechanical and chemical oral cleansing treatment which consisted of brushing their teeth and tongue for 2 min using toothpaste (Colgate Total, Colgate, Sydney, Australia), then using a plastic tongue scraper for 30 s, followed by 2 min brushing of their teeth and tongue with 2·0% chlorhexidine (CHX) mouth gel (Colgate Perioguard) and finally a 30 s CHX rinse (0·2% CHX in 10% ethanol). At 2-h intervals, over the next 8 h, the subjects sucked a lozenge containing either $>1 \times 10^9$ colony forming units (CFU) of *S. salivarius* $K12^{Str}$ (a variant of strain K12 resistant to 100 μg ml^{-1} streptomycin; BLIS Technologies Ltd, Dunedin, New Zealand) or a placebo lozenge containing no bacteria. The lozenges took *c.* 5 min to dissolve by sucking. On days 2 and 3 the subjects brushed their teeth and tongue in the morning with Colgate (Total) toothpaste and then rinsed with CHX, followed by the taking of K12 lozenges as on day 1. Subsequently, the subjects refrained from any use of CHX, but took a lozenge morning and night after normal oral care for 2 weeks in the case of the active group.

At each pretreatment visit and 1 and 2 weeks (for the test group) after treatment initiation the subjects were tested for VSC levels and saliva samples were taken (prior to taking the morning lozenge). Two subjects (S1 and S3) in the active group were sampled more frequently and

continued to take two lozenges a day for 28 days. These subjects provided additional samples at days 4, 11, 21, 28 and also 2 weeks after completing their courses of K12 administration. Three subjects (S4, S8 and S12) in the test group who had responded positively to the treatment were recalled 6 weeks later to determine the effect of CHX treatment alone on their breath scores. These subjects were asked to repeat the 3-day CHX regimen (but without taking lozenges) and 7 days after commencing this protocol their VSC levels were assessed. Repeated measures ANOVA with a Tukey–Kramer post-test of VSC readings on the active treatment was used to determine differences before and after treatment. To determine the significance of the variation of individual time points between the active and placebo groups a Mann–Whitney unpaired *t*-test was used.

Culture analysis

Saliva samples were immediately diluted 10-fold in sterile phosphate-buffered saline (PBS, pH 7·5) and then vortex mixed for 5 s. Fifty-microlitre aliquots were spiral plated onto Mitis-salivarius agar (Difco) for *S. salivarius* enumeration and on Mitis-salivarius agar containing streptomycin (100 μg ml^{-1}, Sigma, MO, USA) to specifically enumerate *S. salivarius* K12. Incubation was in a 5% CO_2 in air atmosphere for 24 h at 37°C.

Extraction of bacterial DNA from saliva samples, PCR-DGGE

The cells from 400 μl samples of saliva (diluted to 1 ml by mixing with phosphate-buffered saline, PBS, pH 7·5) were harvested by centrifugation (10 000 × *g*, 5 min) and total DNA was extracted using Instagene Matrix (Bio-Rad Laboratories, Hercules, Ca) according to the manufacturer's instructions. PCR reactions were carried out in 0·2 ml tubes in a thermocycler (Eppendorf Mastercycler, Hamburg, Germany). The bacterial DGGE PCR primers and amplification conditions have been previously described and tested on clinical samples (Walter *et al.* 2000; Burton and Reid 2002; Rudney *et al.* 2003).

Preparation of DGGE gel gradients and electrophoresis procedures were according to the manufacturer's guidelines for the CBS Scientific DGGE apparatus (Del Mar, CA, USA). A 100% denaturing solution was taken as a mixture of 7 mol l^{-1} urea and 40% formamide. The concentrations of polyacrylamide and denaturant were 8% and 40–55% respectively. Gels were run at 70 V for 15·5 h. Fragments of interest were excised from the gels using sterile scalpel blades, washed once in 1x PCR buffer and incubated in 20 μl of the same buffer overnight at 4°C. Five microlitres of the buffer solution was used as template for PCR. Re-amplification was conducted using the same primers, but without the 'GC-clamps' (Walter *et al.* 2000). Sequences of the re-amplified fragments were determined by the dideoxy chain termination method (Sequencing Facility, Center for Gene Research, University of Otago). Analysis of the partial 16S rRNA sequences was conducted using Genbank and the BLAST algorithm. Identities of sequences were determined on the basis of the highest score.

Inhibitory activities of *S. salivarius* strains against known odiferous bacteria

The inhibitory spectra of *S. salivarius* strains were established by the use of deferred antagonism test, essentially as described previously (Tagg and Bannister 1979). In brief, a 1-cm wide diametric streak culture of *S. salivarius* (test strain) was inoculated onto blood agar–calcium carbonate medium. Following incubation in a 5% CO_2 in air atmosphere, for 24 h at 37°C, the macroscopic cell growth was removed with a glass slide and any residual cells on the agar surface were killed by exposure to chloroform vapours for 30 min. The agar surface was then aired for 30 min. Bacterial strains implicated in halitosis (referred to as indicator strains) were pregrown for 48 h on blood agar–calcium plates under anaerobic conditions. Several colonies of the indicator strain were suspended in 3 ml of Todd Hewitt broth and this was used to inoculate at right angles across the line of the original streak culture with the use of a swab. After incubation for 48 h in an anaerobic environment at 37°C, the extent of inhibition of each indicator strain was recorded. The scoring system for the recording of inhibition was as follows: a negative sign (−) denotes no inhibition of the indicator strain; a positive symbol (+) indicated that there was partial inhibition of the indicator growth on the plate where the test bacterium had previously been grown; two positive symbols (++) denoted complete inhibition of the indicator strain growth in the vicinity of where the test strain had grown; three positive symbols (+++) indicated inhibition of the indicator strains beyond the producer growth zone.

Inhibition of salivary black-pigmented bacteria by *S. salivarius* strains

To test the inhibitory activity of known *S. salivarius* K12 bacteriocins against black-pigmented bacteria implicated in halitosis, strains K12 (salivaricin A and salivaricin B producer) and MU (salivaricin nonproducer) were swabbed from master plates to cover one half (semicircle) of blood agar–calcium carbonate plates. Following incubation for 18 h at 37°C under 5% CO_2 in air conditions, the bacterial growth was removed using cotton-tipped

swabs and chloroform vapours were used to kill any remaining bacteria. An agar overlay (15 ml per plate) comprising (per l), 30 g trypticase soy broth (BBL), 15 g agar (BBL) and (added just prior to pouring) 50 ml of defibrinated human blood and 10 ml of vancomycin stock solution (14 mg ml^{-1}) were then added to the plates. Serial 10-fold dilutions (to 10^{-4}) of saliva samples from two subjects were spiral plated in duplicate onto the double-layer agar plates. Following anaerobic incubation at 37°C for 72 h the counts of black-pigmented bacteria were recorded for the respective test and control halves of the plates.

Results

Effect of treatment protocol on VSC readings

The VSC readings of 11 of the 13 subjects in the test group had decreased by more than 100 ppb when measured at 7 days, and eight of these subjects continued to exhibit reduced levels at day 14 (Fig. 1 and Table 1). The mean day 7 reading was significantly lower than the baseline readings at days 0 and 1. The VSC readings of the 10 subjects in the control group were similar to that of the subjects in the active group before treatment (Fig. 1). Although the VSC readings of the control group were slightly higher for the second pretreatment samples, the difference was not significant. One week after commencing treatment with placebo lozenges (bacteria free) the mean VSC reading of these subjects had decreased, but not significantly when compared with the pretreatment readings (data not shown). The VSC readings of the test group were significantly lower than those of the placebo group when tested at 7 days (Table 2).

Two subjects (S1 and S3) remained on active treatment for 28 days and generally yielded reduced VSC levels (Fig. 2). Upon ceasing K12 treatment, one of these subjects (S1) returned to pretreatment halitosis levels, while the VSC levels of subject 3 remained relatively low. Three other test subjects (S4, S8 and S12) who had experienced

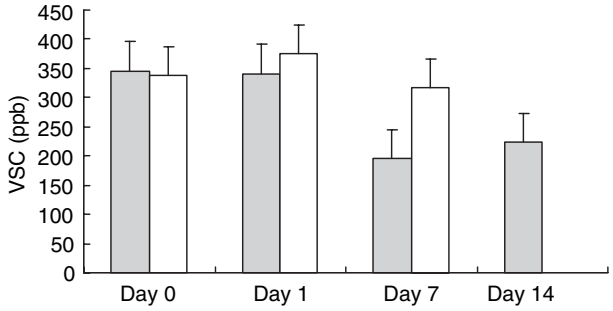

Figure 1 Mean volatile sulphur compound (VSC) levels of mouth air from subjects and before and after treatment with either *Streptococcus salivarius* K12 or placebo. ■, Active; □, the Placebo

Table 1 Significance of halimeter readings of the active treatment group between time points

Comparison time points	*P*-value
Day 0 *vs* day 1	>0·05
Day 0 *vs* day 7	**<0·05***
Day 0 *vs* day 14	>0·05
Day 1 *vs* day 7	**<0·05***
Day 1 *vs* day 14	>0·05
Day 7 *vs* day 14	>0·05

Underlining denotes sample times when subjects are on active treatment. Numbers in bold denote significance.

Table 2 Difference between active treatment *vs* placebo group halimeter readings at corresponding time points

Sample time	*P*-value
Day 0 (baseline reading 1)	0·2917
Day 1 (baseline reading 2)	0·7844
Day 7	**0·0080****
Day 14	ND

ND, not determined.
Number given in bold denotes significance.

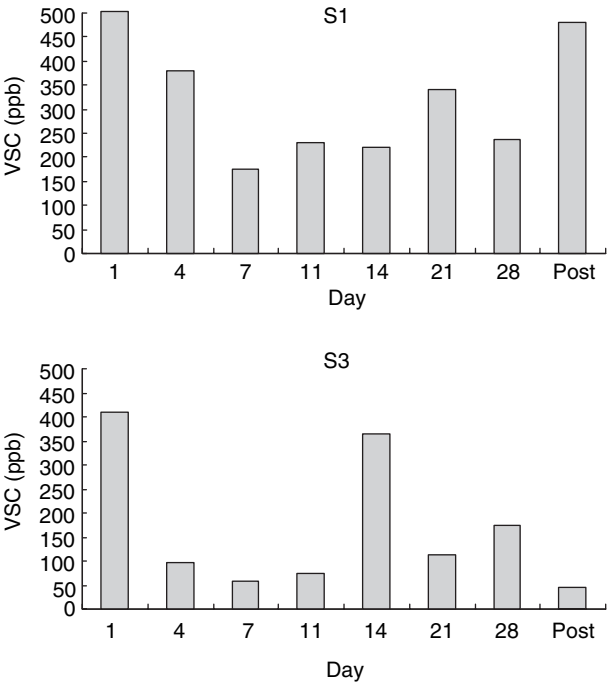

Figure 2 Volatile sulphur compound (VSC) levels of mouth air from subjects (S1 and S3) over 28 days and after treatment.

substantial drops in their VSC readings while on the active treatment were re-evaluated 6 weeks later. The VSC levels of two of these subjects (S4 and S8) had increased significantly (532 and 298 ppb respectively) when compared with treatment levels (as low as 128 and 126 ppb respectively). By contrast, the third subject (S12) appeared to have maintained relatively reduced VSC levels (216 ppb). All three subjects were then treated with CHX (but not with the lozenges containing the *S. salivarius*) for 3 days to determine whether the use of CHX alone had any impact on their VSC levels. No significant reduction could be detected at day 7.

Culture data

Streptococcus salivarius K12 was detected in the saliva of each subject in the active group when tested at days 7 and 14. There was a slight though not significant increase in total *S. salivarius* CFU ml^{-1} of saliva. The mean (and standard deviation) salivary counts for *S. salivarius* were: $4{\cdot}4 \times 10^7$ ($6{\cdot}8 \times 10^7$) (for initial screen); $4{\cdot}5 \times 10^7$ ($6{\cdot}2 \times 10^7$) (just prior to commencing treatment); $6{\cdot}1 \times 10^7$ ($7{\cdot}8 \times 10^7$) (after 7 days of treatment); and $6{\cdot}1 \times 10^7$ ($6{\cdot}6 \times 10^7$) (after 14 days). Mean cell counts of strain K12 were $1{\cdot}8 \times 10^7$ ($4{\cdot}9 \times 10^7$), $5{\cdot}8 \times 10^7$ ($1{\cdot}4 \times 10^7$) CFU ml^{-1}, respectively, at days 7 and 14 after commencing treatment.

DGGE analysis

The saliva samples prior to treatment generally had similar DNA banding profiles, especially with respect to the predominant fragments (Fig. 3). Sequence similarities for some of the major fragments are listed in Table 3. Most of the DGGE profiles changed after treatment. The basic profiles of subjects who either did or did not maintain low VSC readings at day 14 were in general quite similar. However, one feature, was that the subjects who main-

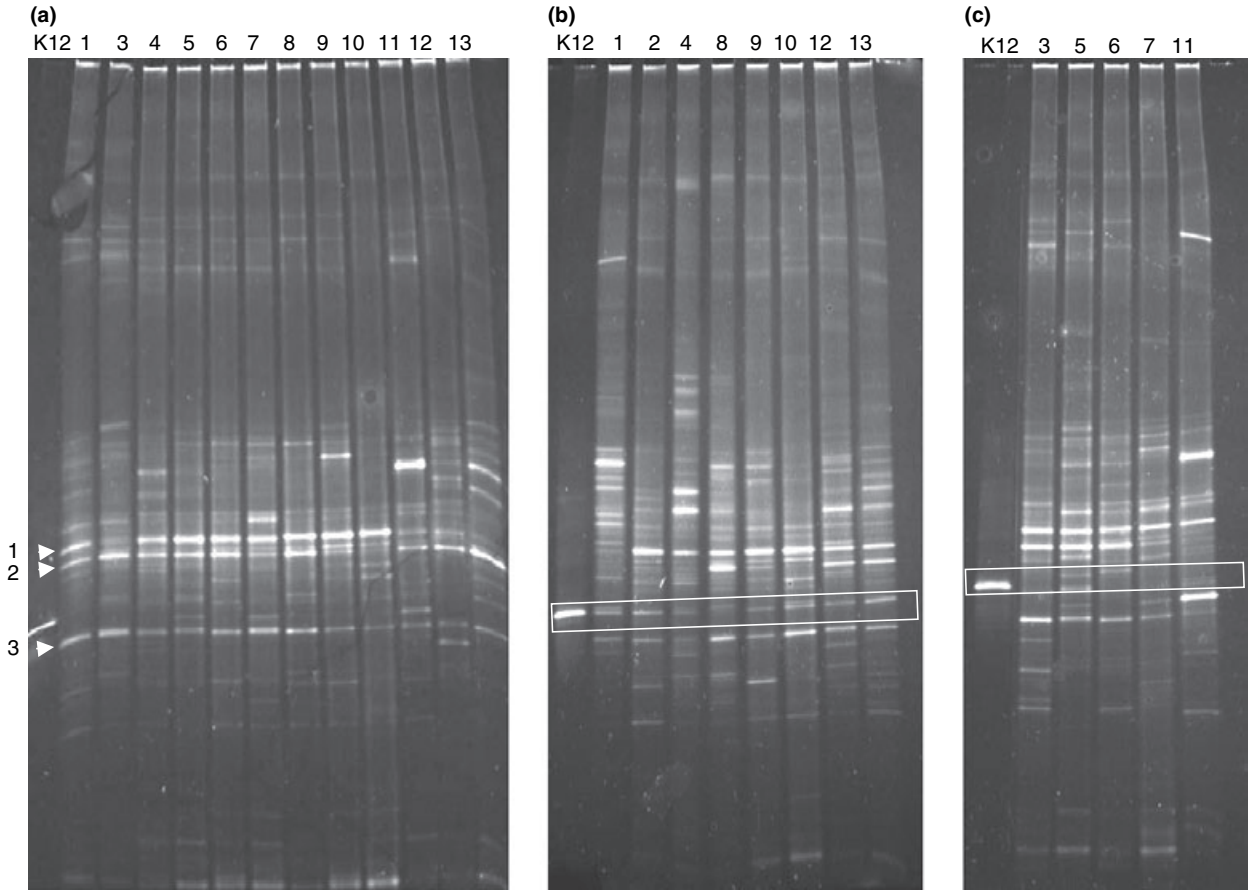

Figure 3 Denaturing gradient gel electrophoresis (DGGE) of 16S rRNA amplicons from bacterial DNA in subjects' saliva samples: (a) prior to treatment, (b) eight subjects exhibiting a decrease of volatile sulphur compounds at day 14 and (c) five subjects not having a decrease in volatile sulphur levels at day 14. Numbers on gel figure denote fragments excised, re-amplified and sequenced. Closest DNA homologies are presented in Table 3.

Table 3 BLAST analysis of bacterial V2-V3 16S rRNA sequences of excised fragments from denaturing gradient gel electrophoresis gels

Fragment in gels	Most related bacterial species match	% identity and bp searched	Accession number
1	*Prevotella veroralis* ATCC 33779	99 (150)	L16473
2	*Prevotella melaninogenica* ATCC 25845	97 (154)	L16469
3	*Veillonella dispar*	98 (172)	AF439639
4	*Prevotella veroralis* ATCC 33779	99 (150)	L16473
5	*Prevotella melaninogenica* ATCC 25845	100 (152)	L16469
6	*Veillonella dispar*	95 (172)	AF439639
7	*Neisseria pharyngis* NCTC 4590	98 (152)	X74893
8	*Prevotella pallens*	98 (167)	Y13106
9	*Prevotella veroralis* ATCC 33779	99 (152)	L16473
10	*Prevotella melaninogenica* ATCC 25845	100 (152)	AY323525
11	*Veillonella dispar*	99 (171)	AF439639

tained low readings typically had a more predominant band in the position corresponding to *S. salivarius* amplicons. Other than for subject 23, who had a different profile in each sample, the subjects in the control group did not exhibit any substantial changes in their DGGE profiles when tested 7 days after completion of the treatment protocols (data not shown).

The DGGE profiles of subjects 1 and 3 were tracked for 28 days (Fig. 4). Subject 1 did not show any immediate profile change after the CHX and K12 treatment, other than the appearance of a fragment presumed to be *S. salivarius* K12. However, the profile of this subject changed dramatically in the day 11 and day 14 samples. After this, the DGGE profile returned to one which was similar to that detected originally. By contrast, the DGGE profiles of subject 3 changed immediately after the treatment commenced. A fragment which migrated similarly to that representative of *S. salivarius* K12 became predominant at this time. However, as with subject 1, there appeared to be major changes in the composition of the microbiota at days 11 and 14. By days 21, 28 and in the post-K12 treatment sample the microbiota appeared to have changed again, to a profile more similar to that present at day 7.

Inhibitory activity of salivaricin-producing *S. salivarius* against halitosis-associated species

Streptococcus salivarius strains known to produce salivaricin A and/or salivaricin B showed inhibitory activity only against Gram-positive halitosis-associated species in deferred antagonism tests (Table 4). The strongest inhibition was given by strain K12, which produces both salivaricin A and salivaricin B. *Streptococcus salivarius* strain NR (salivaricin B producer) exhibited similar activity to strain K12, except for its somewhat weaker activity against *Eubacterium saburreum*. *Streptococcus salivarius* 20P3 (salivaricin A producer) differed from the salivaricin B producers in that it failed to inhibit the *Streptococcus anginosis* indicator strain. *Streptococcus salivarius* MU (salivaricin A-negative, salivaricin B-negative) did not inhibit any of the indicator strains tested.

Inhibitory activity of salivaricin-producing *S. salivarius* against black-pigmented bacteria present in saliva

Blood agar vancomycin medium (semiselective for black-pigmented bacteria) in which one half of the agar had been impregnated with both salivaricin A and salivaricin B was used to detect inhibitory action against black-pigmented bacteria. Dilutions of saliva from two subjects were spiral plated onto the various selective media. The nonbacteriocin-impregnated half of the agar acted as a control. The numbers of black-pigmented bacteria grown from the saliva were decreased by 92% for subject A and 62% for subject B (compared to control counts), on agar that had been impregnated with both salivaricin A and salivaricin B (Table 5, Fig. 5). By contrast, the pregrowth of a salivaricin-negative strain on the test agar effected only a small decrease in the numbers of colonies of black-pigmented colonies in the samples from subject A and even appeared to effect an increase in the numbers of these colonies for subject B. Five representative black-pigmented colonies were picked from the control half of one of the bacteriocin-impregnated plates and the DNA was extracted, amplified by PCR and sequenced. The sequences of the 16S rDNA PCR products from each of these corresponded most closely to *Prevotella* sp. oral clone BE073 (closely related to *Prevotella melaninogenica*, 99% 152 bp, AF385551).

Discussion

It is evident that the use of antimicrobials for treatment of dental infections may also nonspecifically deplete the

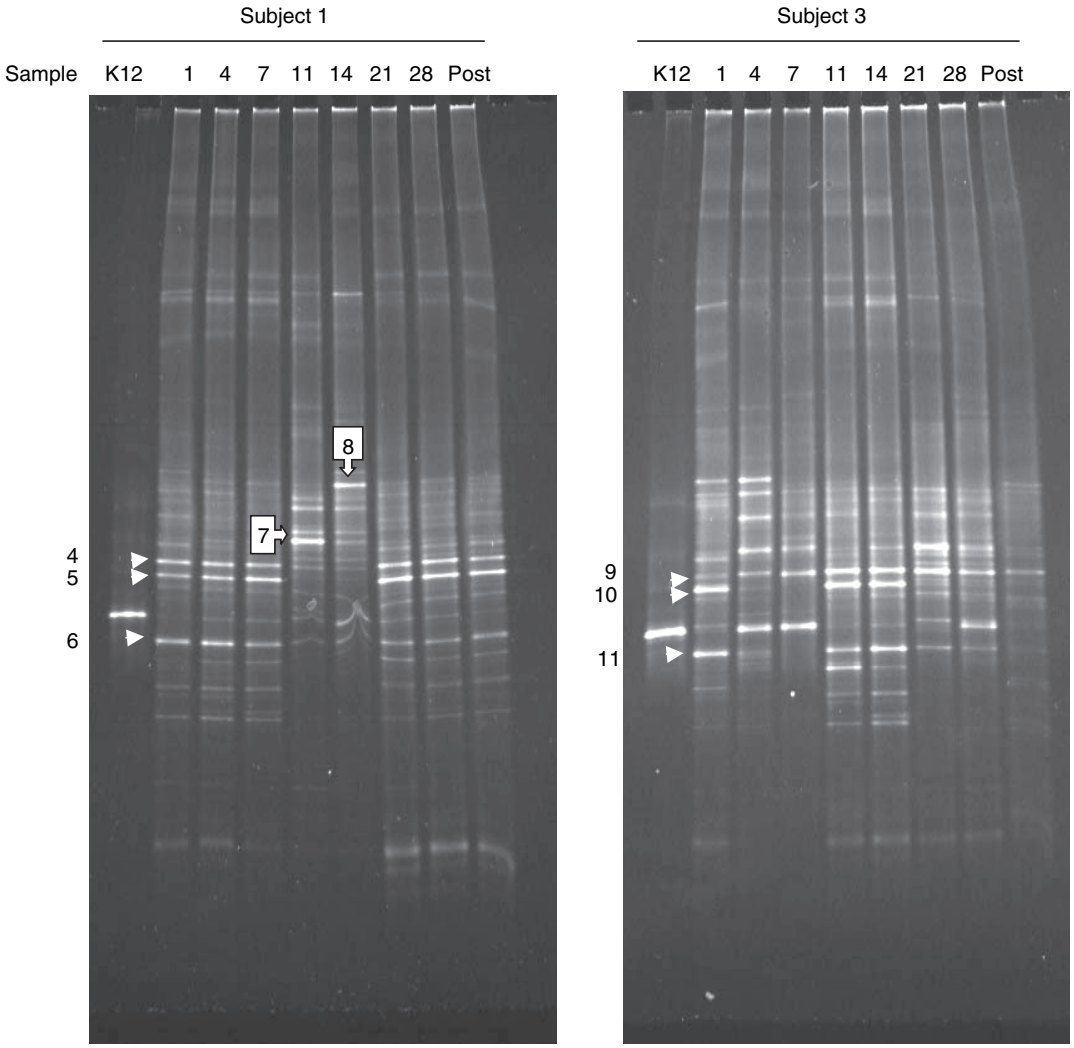

Figure 4 Denaturing gradient gel electrophoresis (DGGE) of 16S rRNA amplicons from two subjects (S1 and S3). Numbers on gel figure denote fragments excised, re-amplified and sequenced. Closest DNA homologies are presented in Table 3.

Table 4 Deferred antagonism inhibition of representatives of certain bacterial species implicated in halitosis by bacteriocin-producing *Streptococcus salivarius*

		Inhibition of species implicated in halitosis						
S. salivarius strain	Bacteriocin/ s produced	*Micrococcus luteus* I1 (control)	*Streptococcus anginosis* T29	*Eubacterium saburreum* ATCC 33271	*Micromonas micros* ATCC 33270	*Porphyromonas gingivalis* ATCC 33277	*Porphyromonas gingivalis* W50	*Prevotella intermedia* ATCC 25611
K12	Salivaricin A Salivaricin B	+++	+++	+++	+++	–	–	–
NR	Salivaricin B	+++	+++	++	+++	–	–	–
20P3	Salivaricin A	+++	–	++	–	–	–	–
MU Neg	Nonproducer	–	–	–	–	–	–	–

levels of some bacterial species perceived to be of benefit to the 'balance' of the ecosystem. In such postantimicrobial states the replenishment of the bacterial micro-environment typically is left to chance as the melee of residual microbes start to repopulate their depleted environment. Sometimes harmless commensals will prosper.

Table 5 Effect of bacteriocin-producing *vs* nonproducing *Streptococcus salivarius* on growth of black-pigmented anaerobes in saliva specimens

	S. salivarius strain and CFU ml^{-1} of black-pigmented colony morphology			
Subject	K12	Control half	MU Neg	Control half
A	$1{\cdot}4 \times 10^5$	$1{\cdot}7 \times 10^6$	$1{\cdot}8 \times 10^6$	$2{\cdot}2 \times 10^6$
B	$3{\cdot}0 \times 10^6$	$7{\cdot}9 \times 10^6$	$5{\cdot}4 \times 10^6$	$2{\cdot}6 \times 10^6$

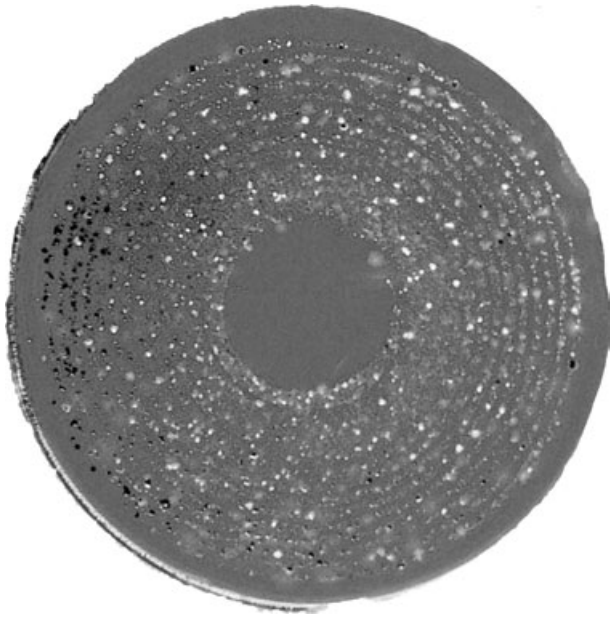

Figure 5 Example of inhibitory effect of *Streptococcus salivarius* K12 (right half) on colonies of black-pigmented bacteria grown from a saliva sample.

However, under certain circumstances, potentially pathogenic bacteria may flourish. In the present study, a CHX mouth rinse pretreatment was used. Chlorhexidine is generally considered to be an effective, though relatively indiscriminate, antimicrobial for the short-term relief of halitosis. However, rather than relying solely upon natural microbial succession to repopulate the mouth, instillation of a harmless oral commensal (*S. salivarius* K12) was used to exert some control over the process (De Boever and Loesche 1995; Kazor *et al.* 2003; Tagg and Dierksen 2003). The attempted introduction of probiotic strains to other established microbial habitats, such as the colon, rarely achieves long-term persistence or colonization by the candidate micro-organisms (Tannock *et al.* 2000). Often, however, even the brief presence and putative metabolic activity of the transitory probiotic micro-organisms appears to aid the restoration of a 'beneficial' microenvironment (Burton *et al.* 2003). In the present study, persistence of *Streptococcus salivarius* K12 was detected in the post-treatment saliva samples (days 7 and 14) obtained from each subject.

When considering the use of probiotics as infection preventatives, favourable characteristics include occupation of tissue sites that would otherwise be taken up by potentially undesirable organisms, production of antibacterial compounds and (possibly) stimulation of the immune defences of the host (Hinode *et al.* 2003). Strain K12 produces at least two lantibiotic bacteriocins that are inhibitory to representative strains of several species of Gram-positive bacteria implicated in halitosis. Although strain K12 did not appear to inhibit reference strains of *Prevotella* and *Porphyromonas in vitro*, when fresh saliva was plated onto media on which strain K12 had previously been grown (leading to the deposition of salivaricin A and salivaricin B), the growth of black-pigmented bacteria identified as *Prevotella* species was inhibited. Studies in our laboratory have shown that salivaricin A is bacteriostatic, whereas, salivaricin B is bacteriocidal in its mode of action (Ross *et al.* 1993; Upton *et al.* 2001; Tagg and Dierksen 2003; Tagg 2004). The limited data presented here with pure isolates and with bacteria present in saliva indicate that inhibition is probably greater when the two salivaricins are present in combination. The production of each of these salivaricins appears to be auto-inducible and moreover they both seem capable of stimulating homologous bacteriocin production by indigenous oral *S. salivarius* populations *in vivo*. Thus, administration of strain K12 may also boost salivaricin levels in a subject's saliva by cross-stimulating salivaricin production by pre-existing members of the indigenous microbiota and may have benefit without actually colonizing the host.

Measurement of VSC levels showed that most subjects displayed significant improvement when tested 7 and 14 days after the commencement of K12 treatment. Two subjects (S1 and S3) who kept using K12 lozenges for 28 days generally maintained lower VSC readings than they had prior to commencing treatment, indicating that lowered VSC levels might be achieved by persistent administration of K12 lozenges (Fig. 2). Although only two subjects were studied in depth, the data obtained indicated that if *S. salivarius* was adopted as a halitosis treatment, the optimal dosing requirements could differ from subject to subject. Some may require only occasional dosing, while others may require a more regular maintenance regimen, as is common practice with other probiotics. In this study, we cannot exclude the possibility that some of the malodour was from primary sources other than the tongue. Indeed, it is conceivable that some of the subjects who showed little change in their malodour parameters may have had nonoral halitosis. Additionally, those subjects who did not experience any significant change in their halitosis parameters may

require a more potent antimicrobial treatment (e.g. a longer or stronger CHX treatment or use of a broad-spectrum antibiotic) to achieve adequate initial depletions of their populations of halitosis-associated bacteria (Pratten *et al.* 1998; McBain *et al.* 2003).

Chlorhexidine is a potent antimicrobial compound and has been shown to be extremely effective when used daily for the treatment of halitosis (Rosenberg *et al.* 1992; van Steenberghe *et al.* 2001; Quirynen *et al.* 2002; Winkel *et al.* 2003; Young *et al.* 2003). Previous studies on the effect of CHX on the oral microbiota have shown in some cases that bacterial populations recover to pretreatment levels within 24 h (Schiott *et al.* 1970). The control group subjects (using bacteria-free lozenges) had a slight, though nonsignificant reduction in their malodour parameters indicating that there may have been a slight residual benefit either from CHX treatment or from the sucking of lozenges. Interestingly, CHX treatment of subject 1 had not altered the VSC level when tested 1 day after ceasing treatment. However, the VSC levels had decreased by day 7 (3 days of K12 treatment only) but returned to high levels when tested 2 weeks after ceasing *S. salivarius* K12 treatment. Also, three recalled subjects from the study who had experienced successful resolution of symptoms during the treatment protocol, failed to show any decrease in their VSC levels when tested 4 days after using just CHX.

Microbial culture analysis was performed on saliva samples, as previous studies have shown that the composition of the salivary microbiota is representative of that found on the dorsal and lateral surfaces of the tongue (Mager *et al.* 2003). More than 600 species of bacteria have been detected in the oral microbiota (Kazor *et al.* 2003). The DGGE profiles obtained in the present study represent only some of the most prevalent members of the salivary populations and indeed similar fragment patterns were obtained for each subject prior to treatment. Fragments corresponding to *Prevotella veroralis*, *P. melaninogenica* and *Veillonella dispar* sequences predominated in these subjects. Although there were changes in the DGGE profiles after treatment, it was difficult to determine whether there was a correlation between banding pattern and a clinical state indicative of halitosis or health. One striking observation, however, was the more prominent appearance of DNA fragments representing *S. salivarius* in samples from the subjects who experienced a reduction in VSC levels (Fig. 3). For the two subjects who were sampled more frequently, some interesting associations were noted. For example, both subjects had VSC levels that temporarily 'spiked' at either day 14 (S3) or day 21 (S1) corresponding to saliva samples in which *S. salivarius* fragments were less pronounced in the DGGE gels (Fig. 4).

For a certain group of bacteria to be detected by DGGE amongst a complex population it must represent a substantial proportion of that population (Muyzer and Smalla 1998). Thus, some 'minority' micro-organisms which may nevertheless exert considerable influence on the composition or metabolism of the microbiota may not be detected. Indeed the species reported here appear to be constituents of the 'normal' oral microbiota (Kazor *et al.* 2003; Mager *et al.* 2003). However, a wide variety of micro-organisms have been implicated in halitosis (Loesche and Kazor 2002) and while some aetiologically significant species may be identified, it is also likely that other organisms may be collectively responsible for the shift from a normal saccharolytic bacterial population to one which is largely proteolytic (De Boever and Loesche 1995). The microbial agents of halitosis may also be minor components of a so-called 'healthy' oral microbiota. Alternatively, a consortium of phylogenetically diverse, but metabolically similar organisms may change the micro-environment. Given the complexity of the oral microbiota implicated in halitosis (Kazor *et al.* 2003), it appears that tools such as DGGE, generally considered suitable for monitoring complex microbial consortia may not have sufficient sensitivity to detect some of the critical bacterial changes. Further studies from information obtained with gene libraries for most oral bacterial species or strains, cultured or not (Kazor *et al.* 2003) may enable more in depth analysis.

Although the majority of the small number of subjects in the active group in this pilot study had a favourable outcome, more extensive double blind, placebo-controlled studies are now required. From the data presented here, it appears, however, that the application of probiotic therapy using *Streptococcus salivarius* K12 following antimicrobial pretreatment may alleviate some forms of halitosis. Also, given that *Streptococcus salivarius* has a numerical, yet benign predominance in the mouth, the impact of competitive (bacteriocin-producing) *S. salivarius* on other oral diseases merits further investigation.

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