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Non-oral bifidobacteria and the aciduric microbiota of the denture plaque biofilm

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SUMMARY

The microbiota of the denture plaque biofilm colonizing the fitting surface of dentures in edentulous subjects with healthy palates (*n* = 20) and in edentulous subjects with denture stomatitis (*n* = 20) was studied. The numbers of bacteria colonizing the dentures of healthy subjects was significantly less than the numbers colonizing the dentures of stomatitis subjects. The proportions and frequency of isolation of mutans streptococci, lactobacilli, bifidobacteria and yeasts were significantly ($P < 0.05$) greater in the subjects with denture stomatitis. The proportions of these organisms in the denture plaque biofilm of the subjects with denture stomatitis were similar to those found in carious lesions, indicating that the site is a low pH environment. The predominant bifidobacterial species in the mouths of dentate subjects is *Bifidobacterium dentium* but in the edentulous subjects wearing dentures *B. dentium* was isolated from only one of the 20 subjects with denture stomatitis and from none of the 20 subjects with healthy palates. Instead, *Bifidobacterium breve*, *Bifidobacterium scardovii* and *Bifidobacterium longum* subsp. *longum* were isolated. Only a single non-oral bifidobacterial species was isolated from each individual and repetitive extragenic palindromic- and BOX-polymerase chain reaction typing methods indicated that the same genotypes were shared between subjects. Using deferred antagonism spot plate assays, interspecies inhibition was demonstrated between oral isolates of *B. dentium*, *B. breve*, *B. scardovii* and *B. longum* subsp. *longum*. Here we have shown that bifidobacteria and caries-associated microbiota are present in denture plaque at levels similar to those of carious lesions and *B. dentium* cannot be maintained in an edentulous mouth.

INTRODUCTION

The oral cavity contains a wide range of niches from hard non-shedding tooth surfaces to the desquamating epithelial surfaces of the cheek and tongue. The dentition plays a significant role in determining the diversity of the oral flora. The removal of all the teeth results in changes in the oral microbiota including the loss of *Streptococcus mutans* (Carlsson *et al.*, 1969; Theilade *et al.*, 1983), which re-colonize the mouth if dentures, with hard non-shedding surfaces, are inserted. The insertion of a full upper denture creates a new, potentially acidic, environment between the palate and the fitting surface of the denture. This site has limited salivary access and denture plaque, a biofilm on the fitting surface of the denture, will form if the denture is not subjected to regular hygiene procedures. Denture plaque is a biofilm that shows increasing complexity and increased numbers and proportions of aciduric

organisms, including mutans streptococci, lactobacilli and yeasts, *Candida albicans* in particular, with increasing age of the denture plaque biofilm (Budtz-Jörgensen, 1974; Theilade *et al.*, 1983; Wright *et al.*, 1985; Koopmans *et al.*, 1988; Könönen *et al.*, 1991; Sumi *et al.*, 2003; Mizugai *et al.*, 2007).

Bifidobacterium species are members of the microbiota of the gastrointestinal tracts of humans and other mammals (Felis & Dellaglio, 2007). Bifidobacteria are acidogenic and aciduric, gram-positive, pleomorphic, branched, non-motile, non-spore-forming and non-filamentous rods. Bifidobacteriaceae consists of seven genera (*Bifidobacterium*, *Aeriscardovia*, *Falcivibrio*, *Gardnerella*, *Parascardovia*, *Scardovia* and *Alloscardovia*) and about 36 species, the majority of which have been described and isolated from the intestinal and caecal flora. The range of taxa reported to be commensal to the oral cavity is apparently restricted to *Bifidobacterium dentium*, *Scardovia inopicata* and *Parascardovia denticolens*. More recently, *Alloscardovia omnicolens* (Huys *et al.*, 2007), isolated from human infections and from human saliva (Beighton *et al.*, 2008), was described as a new closely related genus. '*Scardovia* genomospecies C1' (Modesto *et al.*, 2006) was reported from carious dentine, '*Scardovia* sp. T01-04' (Hooper *et al.*, 2006) was identified, on the basis of 16S ribosomal RNA (rRNA) sequencing, from cellular samples taken from oral cancer cells and two clones described as Bifidobacteriaceae (G-1 and G-2) spp. are reported in the Human Oral Microbiome Database (HOMD; http://www.homd.org/).

A recent comprehensive analysis of the adult intestinal and faecal cultivable bifidobacterial population showed that the main phylogenetic taxa were *Bifidobacterium longum*, *Bifidobacterium pseudocatenulatum*, *Bifidobacterium adolescentis*, *Bifidobacterium pseudolongum*, *Bifidobacterium breve* and *Bifidobacterium bifidum* with *B. dentium* and *Bifidobacterium animalis* subsp. *lactis* being primarily isolated from faecal samples (Turroni *et al.*, 2009). A similar study in which taxon-specific primers were used to amplify fragments of 16S rRNA genes of bifidobacteria in faeces showed the presence of novel bifidobacterial phylotypes, which had not been found in earlier studies of the cultivable bifidobacteria and may therefore represent novel taxa within the genus *Bifidobacterium* (Turroni *et al.*, 2009). Breast-fed infants also harbour a complex bifidobacterial flora but their faeces are characterized by the presence of *B. longum* subsp. *infantis*. This has the ability to degrade and utilize the oligosaccharides of milk glycoproteins as the result of the presence of intracellular glycosidases, including sialidase (Sanyal & Russell, 1978).

Bifidobacteria in the oral cavity are reported sporadically in association with dental caries (Edwardsson, 1974; Moore *et al.*, 1984; Maeda, 1988; van Houte *et al.*, 1994; Becker *et al.*, 2002; Hoyles *et al.*, 2002; Sumi *et al.*, 2003; Chavez de Paz *et al.*, 2004; Chhour *et al.*, 2005; Sela *et al.*, 2008) but not from healthy mouths (Aas *et al.*, 2005). However, recently we used a mupirocin-based selective medium and reported the isolation of bifidobacteria from 94% of 196 independent stimulated saliva samples (Beighton *et al.*, 2008). The predominant taxa were *B. dentium*, *Scardovia inopinata*, *B. longum*, *P. denticolens* and *A. omnicolens*. When we examined the microbiota of root caries lesions we found that the proportion of bifidobacteria was associated with the clinical severity of root caries surfaces sampled and that *B. dentium* was the predominant *Bifidobacterium* (Mantzourani *et al.*, 2009). Similarly, when we examined the bifidobacteria isolated from occlusal carious lesions in adults and children, bifidobacteria represented approximately 8% of the cultivable microbiota and *B. dentium* was the predominant bifidobacterial taxon (Mantzourani *et al.*, 2009). On sound occlusal enamel surfaces the frequency of isolation of bifidobacteria was significantly less than in the carious lesions but again *B. dentium* was the predominant bifidobacterium (Mantzourani *et al.*, 2009).

To gain a better understanding of the distribution of *B. dentium* in the mouth we investigated the microbiota of denture plaque in edentulous subjects. As this site permits the proliferation of *C. albicans*, an organism that grows and survives in a low pH environment, we hypothesized that other aciduric oral microbiota, mutans streptococci, lactobacilli, yeasts and bifidobacteria would also proliferate. Therefore we investigated the bifidobacterial microbiota of the denture plaque of edentulous patients with healthy palates and with palates exhibiting clinical signs of denture stomatitis.

METHODS

Clinical status of subjects

Subjects selected for the study were adult patients of the Prosthodontics Department of King's College

London Dental Institute. They were all medically stable with no acute medical conditions and receiving no antibiotic treatment. The procedure was fully explained to the patient and verbal consent was obtained. Ethical approval was obtained by a local Research Ethics Committee for collection of the plaque samples from the fitting surface of the upper complete dentures. Twenty denture plaque samples were collected from patients with Newton type II denture stomatitis (24; a generalized mucosal inflammation of the denture-bearing area) and 20 plaque samples were collected from healthy complete denture wearers with no sign of palatal inflammation.

Collection of samples and microbiological processing

Denture plaque samples were obtained in a standardized manner using a sterile, microbiological 5-μl loop (Cheshire Scientific Ltd, Ellesmere Port, UK) and running it once across the fitting surface of the upper complete denture from left to right on the level of the maxillary ridge. The sample represented the total number of organisms on this area of the denture and in most instances the 5-μl loop was not filled. The samples were immediately suspended each in 1.0 ml of anaerobically pre-reduced Fastidious Anaerobic Broth (Oxoid, Basingstoke, UK), placed on ice and processed within 2 h. In addition, paraffin-wax-stimulated whole saliva samples, which provide a representative sample of all intra-oral biofilms, were obtained from 25 dentate adults, not wearing dentures (Beighton *et al.*, 2008).

The denture plaque samples were dispersed by thoroughly vortexing each sample for 10 s with sterile glass beads and one in 10 dilutions were prepared in Fastidious Anaerobic Broth (LabM, Bury, UK). Aliquots of 100 μl of appropriate dilutions of the denture plaque samples were spread on to selective media, modified trypticase–phytone–yeast (MTPY) (Rada & Petr, 2000; Mantzourani *et al.*, 2009) for bifidobacteria, Rogosa Agar for lactobacilli, Sabauraud–Dextrose Agar for yeasts and tryptone–yeast–cysteine–sucrose–bacitracin agar for mutans streptococci, in each sample as previously described (Beighton *et al.*, 2008; Mantzourani *et al.*, 2009). The diluted stimulated saliva samples were only inoculated on to MTPY for the enumeration of bifidobacteria. The total number of cultivable bacteria in the plaque samples was determined by plating appropriate dilutions onto a non-selective enriched culture medium, Fastidious Anaerobe Agar (FAA; LabM), supplemented with 5% (volume/volume) defibrinated horse blood as described previously (Mantzourani *et al.*, 2009). The microbial counts of bifidobacteria in saliva were expressed as colony-forming units (CFU) per ml of saliva and counts of bifidobacteria, lactobacilli, yeasts and mutans streptococci in denture plaque samples were expressed as a percentage of the total cultivable colony count. The detection limit for all taxa was 10 CFU in both saliva and biofilm samples.

Identification of bifidobacteria

To determine the numbers of bifidobacteria in each sample the total number of presumptive bifidobacterial colonies on the MTPY medium was counted and the number of each colony type determined. On MTPY medium presumptive colonies of bifidobacteria appear as white, domed colonies with entire or erose edges up to 2 mm in diameter. For each colony type typically 15–20 colonies were sub-cultured from each sample and tested for fructose-6-phosphate phosphoketolase (Orban & Patterson, 2000) and examined by Gram staining. To identify the presumptive bifidobacteria each isolate was grown overnight on TPY medium (Rada & Petr, 2000) and a suspension of each was prepared in 50 μl of sterile deionized water and used as the DNA template. A partial sequence of the 16S rRNA gene was obtained by amplifying the gene fragment using the genus-specific polymerase chain reaction (PCR) primers Bif164mod: 5′-GGGTGGTAA TRCCSRATG-3′ and Bif662mod: 5′-CCACCGTTACA CCGRGAA-3′ modified from those reported previously (Venema & Maathuis, 2003). The PCR amplification was achieved using the following reaction mixture (25 μl final volume) which comprised 4 μl $MgCl_2$ 25 mM, 2.5 μl 10 × buffer, 1 μl dNTPs (3.125 mM), 1 μl Bif164mod forward primer, 1 μl Bif662mod reverse primer, 0.5 μl *Taq* DNA polymerase (ABGene, Epsom, UK), 14 μl distilled H_2O and 1 μl DNA extract. The thermal cycling conditions were an initial denaturation at 94°C for 5 min, denaturation at 94°C for 60 s., annealing at 58°C for 30 s., extension at 72°C for 1.3 min, repeated for 34 cycles and final extension at 72°C for 7 min. The PCR amplicons were cleaned by polyethyleneglycol precipitation and sequenced in reactions containing

2 μl PCR product, 0.5 μl BigDye v3.0 (Applied Biosystems, Warrington, UK), 1.75 μl of 5 × solution buffer (Applied Biosystems), 1.75 μl sterile UHQ water and 4 μl primer (3 pmol). The cycling protocol and the cleaning of sequencing reaction products were as described in the manufacturer's protocols and sequencing was performed using an ABI 3730xl DNA Analyzer (Applied Biosystems). The 16S rRNA sequence data were manually corrected and edited using BIOEDIT (Hall, 1999) and the isolates were identified using the Ribosomal Database Project (Cole *et al.*, 2007) or by BLAST (Altschul *et al.*, 1997) searching.

For better characterization of the isolates identified by 16S rRNA sequencing as *B. longum* a representative sample ($n = 16$; two from each subject harbouring *B. longum*) was tested for their ability to produce acid from the following carbohydrates: glucose, L-arabinose, melezitose, ribose and D-glucuronate to assist in the differentiation of the three subspecies of *B. longum* (Mattarelli *et al.*, 2008). Additionally, we tested for the production of intracellular sialidase activity produced by *B. longum* subsp. *infantis* and not by the other two subspecies (Sanyal & Russell, 1978). Organisms were grown on TPY and FAA for 2–3 days and suspended in 1 ml of 50 mM *N*-Tris (hydroxymethyl)methyl-2-aminoethanesulphonic acid buffer (pH 7.5) (TES buffer; Sigma, Poole, Dorset, UK) and permeabilized by vigorous vortexing with 25 μl toluene. Assays were set up using 200 μl permeabilized cell suspension and 20 μl of a 100 μg/ml solution of 2′-4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid (Sigma) in TES buffer. The assays were incubated at 37°C for 3 h and release of methylumbellyferone was determined using a plate-reading fluorimeter (Fluoroskan Ascent FL; Thermo Fisher Scientific, Loughborough, UK). *B. longum* subsp. *longum* (NCC 2705) and *B. longum* subsp. *infantis* (DSM 20088) were used as control organisms.

Genotyping of isolates

To determine if the same strains of *B. longum*, *Bifidobacterium scardovii* or *B. breve* were being isolated from the denture plaque samples of different subjects we compared two isolates from each subject using repetitive extragenic palindromic PCR (REP-PCR) and BOX-PCR as previously described (Masco *et al.*, 2003; Beighton *et al.*, 2008). These two PCR-based genotyping methods use different conserved genomic sequences to generate amplicons. The amplicons are separated by electrophoresis, providing a fingerprint for each isolate. The amplicons were examined after staining with Gel Red Nucleic Acid Stain (Cambridge BioScience Ltd, Cambridge. UK) on an AlphaImager HP (GRI Ltd., Essex, UK) and compared visually. Isolates were considered identical if they exhibited the same REP-PCR patterns and the same BOX-PCR patterns, otherwise they were considered non-identical.

Testing for interspecies inhibition

Test organisms, *B. dentium* (OC02_B09 and 12A_B14) isolated previously from occlusal caries lesions (Mantzourani *et al.*, 2009), selected at random, and strains of *B. longum* subsp. *longum* (1_B08 and 8_B13), *B. breve* (DS2-5_B03 and DS2-18_B02) and *B. scardovii* (DS2-23_A02 and DS2-34_B04) isolated from denture plaque samples of different individuals in the present study were used in these experiments. No other isolates were used or screened for inhibitory activity. These eight isolates were screened for inhibitor production against each other using a deferred antagonism spot plate procedure (O'Riordan & Fitzgerald, 1998). The test strains were grown on the surface of TPY agar, containing 2% (weight/volume) (3-[*N*-morpholino] propanesulphonic acid, MOPS), as 5-mm diameter spots inoculated using a sterile plastic loop. Following 48 h of growth at 37°C anaerobically, plates were overlaid with the indicator bacteria. The indicator lawn was prepared by adding 0.5 ml of 48-h grown culture in Brain–Heart Infusion Broth (Oxoid) supplemented with 0.5 g/l L-cysteine.HCl, to 5 ml of molten overlay medium [TPY agar, containing 2% (W/V) MOPS] which was poured gently over the surface of the inoculated test plates. Plates were re-incubated for 48 h at 37°C anaerobically. Inhibition was apparent as clear zones of growth inhibition around producer strains and recorded as present or absent.

Statistical analysis

To compare the total number of bacteria [as $\log_{10}$(CFUper sample)], frequency of isolation and proportional representation of individual taxa, data were analysed using chi-square tests (Preacher,

2001) or appropriate non-parametric statistical tests within SPSSPC$^+$ (Version 15, Chicago, IL).

RESULTS

The mean age of the patients with Newton Type II denture stomatitis was 64 years, 12 were men and eight were women while the mean age of the subjects with no denture stomatitis was 69 years and eight were men and 12 were women. The total number of bacteria, expressed as $\log_{10}$(CFU per sample), recovered using FAA, the non-selective medium, from the dentures of the subjects with denture stomatitis was significantly greater than from the dentures of the healthy subjects (6.43 ± 0.26 and 4.87 ± 0.28, respectively, Mann–Whitney *U*-test, $P < 0.001$). The detection limit for the organisms isolated from the selective culture media is 10 CFU and when expressed as a percentage of the total number of bacteria recovered on FAA this will be approximately < 0.0002% for the samples from patients with denture stomatitis and < 0.01% for the healthy subjects.

Bifidobacteria were isolated from 18 out of 20 samples from the patients who presented with Newton's Type II denture stomatitis, from only one of the 20 samples from healthy denture wearers ($\chi^2 = 28.97$; $P < 0.0001$) and from 100% of the saliva samples from the dentate subjects. The bifidobacteria formed 6.88 ± 3.12% of the total bacterial count from the denture plaque in subjects with denture stomatitis (Table 1). The frequency of isolation of bifidobacteria from subjects with denture stomatitis was significantly greater than from the healthy subjects ($\chi^2 = 28.972$; $P < 0.001$). Lactobacilli were isolated from 19 of the 20 individuals with denture stomatitis and from six of the 20 healthy subjects ($\chi^2 = 18.03$; $P < 0.0001$) and formed 8.74 ± 2.74% and 1.63 ± 1.15%, respectively, of the total bacterial count ($P < 0.01$). Mutans streptococci were present in 14 of the 20 subjects with denture stomatitis and in six healthy patients ($\chi^2 = 6.40$; $P < 0.011$) and formed 2.48 ± 1.05% and 0.05 ± 0.02%, respectively, of the total bacterial count ($P < 0.01$). Yeasts were isolated from 18 out of the 20 subjects with denture stomatitis and from six

Table 1 Proportional representation, as percentage of the total anaerobic colony count, of bifidobacteria isolated from individual denture plaque samples in healthy complete denture wearers ($n = 20$) and in complete denture wearers with denture stomatitis ($n = 20$)

Sample number	Total bifidobacteria	*Bifidobacterium dentium*	*Parascardovia denticolens*	*Bifidobacterium scardovii*	*Scardovia* genomosp. C1	*Bifidobacterium longum* subsp. *longum*	*Bifidobacterium breve*
Denture plaque samples from healthy edentulous patients[1]							
1	4.9	–	–	–	–	4.8	–
Denture plaque from edentulous patients with denture stomatitis[2]							
1	1.4	–	–	–	–	1.4	–
2	0.4	–	–	–	–	–	0.4
3	5.9	–	–	–	–	5.9	–
4	25.7	–	–	25.7	–	–	–
5	20.0	–	0.002	–	–	–	20.0
6	2.3	–	–	–	–	–	2.3
7	1.1	–	–	–	–	1.1	–
8	2.7	–	–	2.7	–	–	–
9	0.01	–	–	0.01	–	–	–
10	0.9	0.2	–	–	–	–	0.7
11	6.9	–	–	6.93	–	–	–
12	1.7	–	–	–	–	1.7	–
13	0.1	–	–	–	–	0.1	–
14	58.7	–	–	–	–	58.7	–
15	4.1	–	–	–	–	4.1	–
16	1.4	–	–	1.44	–	–	–
17	4.3	–	0.9	3.4	–	–	–
18	0.02	–	0.004	–	0.02	–	–

[1]Bifidobacteria were isolated from one of 20 healthy subjects.

[2]Bifidobacteria were isolated from 18 of 20 subjects with denture stomatitis.

of the 20 healthy subjects ($\chi^2 = 15.0$; $P < 0.0001$) and they formed 2.27 ± 0.85% and 0.11 ± 0.07%, respectively, of the total cultivable flora ($P < 0.05$).

A total of 227 presumptive bifidobacteria were subcultured and identified from the 19 denture plaque samples from which bifidobacteria were isolated and 102 were identified as *B. longum*, 69 as *B. scardovii*, 39 as *B. breve*, eight as *Scardovia* genomosp. C1, five as *P. denticolens* and four were identified as *B. dentium*. *B. longum* was the predominant bifidobacterium in seven out of the 18 subjects with denture stomatitis and was the only bifidobacterial species isolated from the single healthy subject from whom bifidobacteria were isolated (Table 1). The *B. longum* isolates fermented glucose, L-arabinose, melezitose and ribose and did not produce sialidase, on either medium, indicating that they were *B. longum* subsp. *longum*. From the subjects with denture stomatitis *B. scardovii* was isolated from six, *B. breve* from four, *P. denticolens* from three and *Scardovia* genomosp. C1 and *B. dentium* were each isolated from one subject. The frequency of isolation of *B. dentium* from the saliva samples of the dentate subjects was significantly greater than from all the denture wearers (76 and 2.5%, respectively $\chi^2 = 41.038$; $P < 0.001$).

The non-oral bifidobacteria (*B. longum*, *B. scardovii* and *B. breve*) were isolated as the only bifidobacterium from most subjects. However, in four subjects they were isolated in conjunction with oral bifidobacteria (*B. dentium*, *P. denticolens* and *Scardovia* genomosp.C1). This high frequency of isolation of non-oral bifidobacteria from the subjects (18/20) with denture stomatitis was significantly greater than the isolation of non-oral bifidobacteria from the saliva samples of the dentate adults ($\chi^2 = 23.467$; $P < 0.001$) but the frequency of isolation from the healthy denture wearers and the saliva samples was not significantly different [$\chi^2 = 0.543$; $P = 0.461$ (Yate's correction)].

The REP-PCR amplicons patterns obtained for the *B. longum* isolates, two each from each of the eight subjects from which *B. longum* was isolated, were found to form only four distinct patterns (Fig. 1) whereas with the BOX-PCR only two patterns were obtained for the same 16 strains (not shown). In seven subjects the two *B. longum* isolates examined had the same REP-PCR and BOX-PCR patterns, but in the eighth subject the REP-PCR, but not the BOX-PCR, patterns were different. The BOX-PCR patterns obtained with the *B. scardovii* strains indicated that multiple genotypes were present in the same subject and that all four subjects harboured different genotypes. The REP-PCR was not discriminatory for the *B. scardovii* isolates. Consideration of the REP-PCR and BOX-PCR patterns obtained with the *B. breve* isolates indicated that both strains from an individual were the same and that two subjects each had unique genotypes while the remaining two subjects harboured the same genotype.

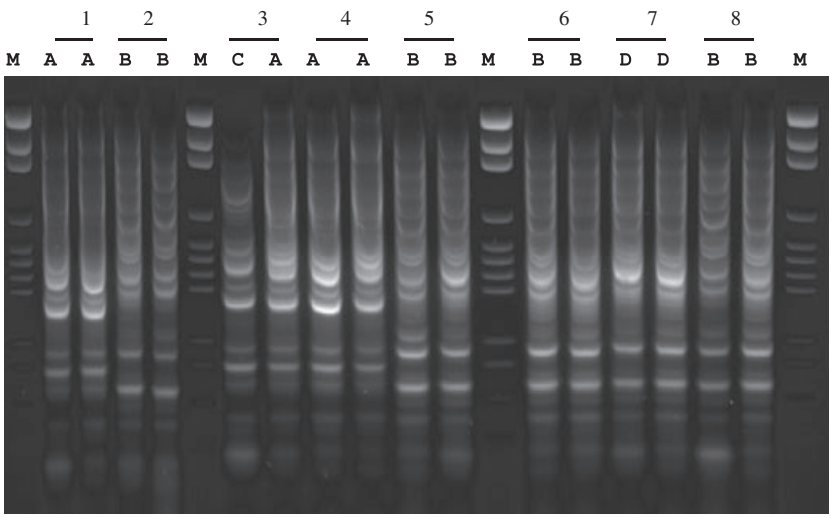

Figure 1 Repetitive extragenic palindromic polymerase chain reaction (REP-PCR) patterns obtained with *Bifidobacterium longum* isolates. Two isolates from each subject were examined and isolates with the same REP-PCR pattern were assigned letters (A–E); lanes marked M are pGEM DNA marker maximum size 2,645 bp (Promega, Madison, WI).

We found that the two *B. dentium* isolates from occlusal caries were each inhibited in deferred antagonism spot plate assays by the *B. longum*, *B. scardovii* and *B. breve* isolates, as indicated by zones of reduced growth around the producer strains. However, we also found that the *B. longum*, *B. breve* and *B. scardovii* were all inhibited by the *B. dentium* isolates. A representative example of inter-species inhibition is shown in Fig. 2.

DISCUSSION

The oral cavity presents numerous surfaces for microbial colonization. Changes in the oral environment including tooth loss and denture wearing result in changes in the oral microflora (Mizugai *et al.*, 2007; Sela *et al.*, 2008). Many studies have concentrated previously on the role of oral yeasts as a contributory factor to denture stomatitis (Cawson, 1965; Budtz-Jörgensen, 1974; Wright *et al.*, 1985) although a few other studies have suggested a role for denture plaque bacteria (Theilade *et al.*, 1983; Koopmans *et al.*, 1988). There have been few studies on the microbiota of edentulous patients with complete dentures but *S. mutans* was shown to re-establish in the mouths of edentulous subjects after the placement of complete dentures (Carlsson *et al.*, 1969; Theilade *et al.*, 1983). In this report we have confirmed that mutans streptococci may be isolated from denture plaque. We have also demonstrated that the frequency of isolation and proportional representation of the aciduric, caries-associated taxa in denture plaque are related to the numbers of organisms cultured from the denture surface and the clinical status of the palate. The more severe clinical condition was related to the accumulation of the biofilm on the fitting surface of the denture. We did not measure the pH of the denture plaque but the isolation of the oral microbiota at such high levels at this site indicates the acidic environment of the biofilm. These same taxa proliferate in active open occlusal carious lesions that have a mean pH of 4.9 (Hojo *et al.*, 1994). The greater numbers and proportions of aciduric microbiota in the denture plaque biofilm associated with the more severe denture stomatitis may be a consequence of poor denture hygiene and limited salivary access to the site so that the normal functioning of saliva to remove bacterially generated acids (Mandel, 1987) from the mouth is diminished. The proportions of mutans streptococci, lactobacilli, bifidobacteria and yeasts in denture plaque are similar to those we have previously reported for active root caries in adults (Mantzourani *et al.*, 2009) and active occlusal caries in adults and children (Mantzourani *et al.*, 2009).

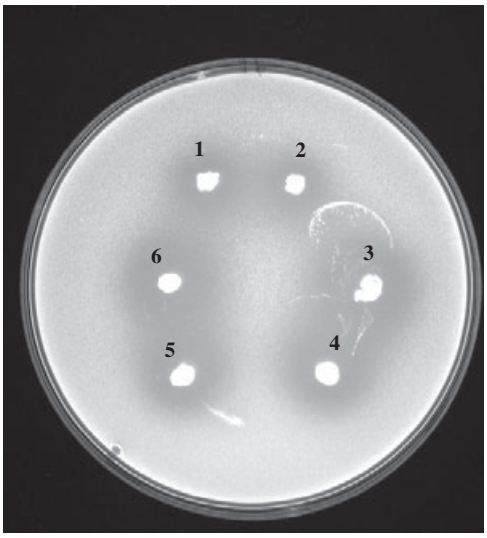

Figure 2 Example of inter-species inhibition identified using the deferred antagonism spot plate assay. Producer strains *Bifidobacterium dentium* (1 = OC02_B09 and 2 = 12A_B14), *Bifidobacterium breve* (3 = DS2-5_B03 and 4 = DS2-18_B02) and *B. longum* subsp. *longum* (5 = 8_B13 and 6 = 1_B08) were grown anaerobically for 48 h as 5-mm spots, overlaid with indicator strain *Bifidobacterium scardovii* DS2-23_A02 and reincubated anaerobically for a further 48 h to permit growth of bacterial lawn and the formation of zones of inhibition.

B. longum has been reported to be the most prevalent bifidobacterial species in the human gut (Turroni *et al.*, 2009). *B. longum*, *B. infantis and B. suis* were unified (Sakata *et al.*, 2002) into the single species *B. longum*, on the basis of DNA–DNA hybridization but later it was proposed that three biotypes of *B. longum* should be re-classified as three subspecies, which should be recognized on the basis of their different phenotypic properties (Mattarelli *et al.*, 2008). On the basis of the phenotypic characteristics of the isolates identified as *B. longum* by 16S rRNA sequencing these isolates were identified as *B. longum* subsp. *longum*.

In previous studies bifidobacteria have been isolated from carious teeth but studies of the oral microbiota using 16S rRNA cloning and sequencing procedures have usually not reported the presence of bifidobacteria in the healthy adult mouth (Aas *et al.*, 2005). We had expected to find that the predominant

bifidobacteria isolated from the denture plaque would be *B. dentium* because this was the predominant bifidobacterium isolated from dentate subjects (Beighton *et al.*, 2008). However, *B. dentium* was only isolated from one of 40 edentulous denture-wearing subjects. Clearly *B. dentium* does not follow the same pattern as *S. mutans* (Theilade *et al.*, 1983) because it does not simply require a hard intra-oral surface to colonize but may need a different intra-oral habitat, which is lost upon the removal of the dentition. As the proportions and numbers of *B. dentium* from sound enamel tooth surfaces are low (Mantzourani *et al.*, 2009) it may be that *B. dentium* preferentially colonizes exposed dentine present in carious lesions and the dentine of exposed root surfaces.

Non-oral bifidobacteria readily gain access to the oral cavity as 10% of dentate adults harboured *B. longum* (Beighton *et al.*, 2008) and here the majority of bifidobacteria isolated from the edentulous subjects were colonized by *B. longum* and *B. breve*. It is likely that these species gain access to the mouth via a faecal–oral route. Such a possibility has been suggested for the oral lactobacilli (Caufield *et al.*, 2007) and it has been shown that food-borne *Enterococcus faecalis* may be isolated from endodontic infections (Razavi *et al.*, 2007). It may also be that they may colonize the oral cavity as a result of food regurgitation or vomiting but these possibilities were not explored in this population group. It is apparent that further investigations are required to understand the ecology of the bifidobacterial flora of the human oral cavity both in dentate and edentulous individuals.

There have been few reports of the isolation of *B. scardovii*; the initial description of the species (Hoyles *et al.*, 2002) was based on five independent clinical isolates obtained from urine samples, blood and a hip infection. Another isolate (CCUG 55376) from human blood has been deposited in the Culture Collection, University of Göteborg, Sweden and we isolated *B. scardovii* from the oral cavity of two of 196 dentate subjects (Beighton *et al.*, 2008). The major habitat of *B. scardovii* is therefore not known but clearly the denture plaque environment is suitable for its proliferation. Detailed studies of the faecal bifidobacteria and mucosally associated bifidobacteria failed to detect *B. scardovii* (Turroni *et al.*, 2009, 2009) suggesting that the human gut is not a major habitat for this species. Clearly both *B. breve* and *B. longum* have the human gut as their major habitat but insufficient independent strains of *B. scardovii* have been isolated from defined habitats to support the hypothesis that the oral cavity is its major habitat. It is also of interest that the three non-oral bifidobacterial species isolated from denture plaque are phylogenetically closely related, being present in the same cluster when compared using 16S rRNA homology (Hoyles *et al.*, 2002). This may suggest that the species had a common ancestor and may share traits relevant to their ability to colonize denture plaque and perhaps other human anatomical sites.

We had expected that the *B. longum* isolates from the denture plaque would be different in each subject when examined using REP-PCR as we had previously found that *B. longum* isolates from dentate subjects were different in each individual (Beighton *et al.*, 2008). No other data are available on genotyping of *B. scardovii* or *B. breve* isolated from the oral cavity. The *B. breve* isolates were more diverse than the *B. longum* but even so, shared genotypes were observed in only four subjects. This may suggest that the ability to colonize denture plaque is restricted to certain genotypes of *B. longum* and *B. breve*.

B. longum, *B. breve* and *B. scardovii* were each isolated in the absence of the other two non-oral species and it may therefore be that they exhibit mutual antagonisms. Bifidobacteria have been tested in numerous assays, usually for their ability to inhibit human pathogenic bacteria (Cheikhyoussef *et al.*, 2008) but here we have tested oral isolates for their ability to inhibit each other. Such a possibility was provided by an earlier study (O'Riordan & Fitzgerald, 1998) in which isolates of *B. breve* and *B. longum* were shown to be mutually antagonistic. We have not investigated the mechanisms underlying these interactions but we used a buffered medium (O'Riordan & Fitzgerald, 1998) to reduce the possibility of pH-mediated inhibition nor have we demonstrated the production of this inter-genus inhibitory activity in vivo. However, the data here suggest that inhibition of *B. dentium* in the denture plaque by the other species may be more potent than the inhibitory activity of *B. dentium* and that colonization by one of the non-oral bifidobacteria prevents colonization of the denture plaque by other non-oral bifidobacteria.

In conclusion we have evidence based on the isolation and enumeration of caries-associated microbiota that denture plaque associated with denture

stomatitis has a microflora similar to that of active caries lesions. The predominant bifidobacteria in the oral cavity of dentate adults, *B. dentium*, may be replaced by non-oral bifidobacterial species, *B. longum* subsp. *longum*, *B. breve* and *B. scardovii* in the oral cavity of edentulous denture wearers.

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