Oral Microbiology Immunology 2007: 22: 73–79
Printed in Singapore. All rights reserved

Copyright © Blackwell Munksgaard 2007

ORAL MICROBIOLOGY
AND IMMUNOLOGY

Caries-related plaque microcosm biofilms developed in microplates

S. K. Filoche, K. J. Soma, C. H. Sissons
Dental Research Group, Department of Pathology and Molecular Medicine, Wellington School of Medicine, Wellington, New Zealand

Filoche SK, Soma KJ, Sissons CH. Caries-related plaque microcosm biofilms developed in microplates.
Oral Microbiol Immunol 2007: 22: 73–79. © Blackwell Munksgaard, 2007.

In vivo dental plaque biofilms consist of complex communities of oral bacteria that are a challenge to replicate *in vitro*. The aim of this investigation was to establish human dental plaque microcosms in microplates to reflect conditions that are relevant to dental caries. Microcosm plaque biofilms were initiated from the saliva of two different donors, grown for up to 10 days in 24-welled microplates on Thermanox™ coverslips in various types of artificial saliva with and without sucrose, which were replaced daily. Microbiota composition of 40 species associated with oral health and dental caries was monitored in the plaques using Checkerboard DNA–DNA hybridization analysis. pH was measured as an indicator of cariogenic potential. The composition of the saliva inocula was different, and yielded plaque microcosms with different composition and growth responses to sucrose. Artificial saliva type and presence of sucrose, and the resulting growth and pH conditions, modified the growth of individual species and hence the ecological profile of the microplate plaques during development. Complex population shifts were observed during development, and older plaques comprised predominantly facultative anaerobic species. Sucrose supplementation limited the decline of Streptococci over time but did not increase the abundance of mutans Streptococci. Sucrose at 0.15% increased levels of caries-associated species including *Lactobacillus fermentum*, *Lactobacillus acidophilus* and *Actinomyces gerensceriae*; these were further increased with sucrose at 0.5%, in addition to *Actinomyces israelii*, *Rothia dentocariosa* and *Capnocytophaga gingivalis*. The microplate plaques demonstrated complex community dynamics that appeared to reflect the maturation of natural plaques, and sucrose induced a cariogenic plaque composition and pH.

Key words: biofilm; caries; microcosm; microplates; plaque

Sara Filoche, Dental Research Group, Department of Pathology and Molecular Medicine, Wellington School of Medicine, Mein Street, PO Box 7343, Wellington 6002, New Zealand
Tel.: +64 4 918 6314;
fax: +64 4 389 5725;
e-mail: sara.filoche@otago.ac.nz
Accepted for publication June 15, 2006

Dental plaque biofilms consist of complex communities of oral bacteria with hundreds of species present (23, 24) and they are a challenge to replicate *in vitro*. Microcosms represent laboratory systems that have been derived from natural ecosystems and which attempt to simulate the conditions prevailing in the environment under study (36). Several continuous flow microcosm models have been developed to study dental plaque biofilms, including chemostat-based systems (3, 19), the constant-depth film fermenter (17, 22), multiple Sorbarod devices (15, 23) and, in our laboratory, the multi-plaque artificial mouth (8, 31). Most of these systems require specialist equipment and their maintenance is labor intensive. However, the plaque biofilms that are generated from these microcosm systems appear to reflect the complexity, diversity and heterogeneity of *in vivo* plaques, and allow their study under controlled conditions (29, 37).

Microtiter plates have been used to generate up to 96 individual biofilms under batch culture (7, 27) and continuous flow (6) conditions, although typically as monocultures. Consortia-based plaque models grown in 24-well microplates include those based on a model of supragingival plaque comprising five species established on hydroxyapatite (12, 28) and these have further advanced our understanding of plaque ecology. However, monoculture biofilms, and consortia biofilms

comprising a small number of species, do not represent the complexity and diversity of natural plaques.

Checkerboard DNA–DNA hybridization (CKB) is a technique that gives a simultaneous and quantitative analysis of up to 28 plaque samples against 40 key microbial species (33, 34). It has been successfully applied to study predominantly gram-negative bacterial complexes associated with periodontal disease *in vivo* (16, 32) and enables a more detailed analysis than would be practicable with culture techniques (10, 35). To focus on bacterial complexes associated with dental caries we have modified the CKB species panel to incorporate a wider range of potential cariogenic gram-positive species and *Candida albicans*, including bacteria associated with oral health and periodontitis, and have successfully applied this system in a study of childhood caries *in vivo* (35).

The aims of this study were to establish dental plaque microcosm biofilms in microplates with various types of artificial saliva supplemented with and without sucrose, and to characterize their growth and microbiota composition using CKB analysis; with the view to achieving growth conditions relevant to those in caries development.

Materials and methods

Growth conditions and experimental protocol

Two sets of experiments were carried out. Each experiment had a different salivary donor. In Experiment I, three media types (1.8 ml in each well) were used, all with and without 0.15% sucrose. These media were a chemically defined saliva analog [defined medium enriched with mucin (DMM)] and a basal medium mucin (yeast extract and peptones) with and without mucin (BMM and BM, respectively) (38). DMM contains ions, mucin, amino acids, vitamins and growth factors at concentrations based on those in saliva, whereas BMM contains mucin and higher concentrations of yeast extract and peptones (38). BM contains yeast extract and peptones. In Experiment II only DMM was used as the growth medium. The effect of different volumes (0.9, 1.8 and 2.5 ml) of DMM supplemented with and without 0.15% sucrose on growth was evaluated. A subset of this experiment was designed to examine the effect of 0.5% sucrose on microplate plaques grown in 1.8 ml DMM.

Approximately 40 ml stimulated saliva was collected from each donor, who had refrained from oral hygiene for 24 h. Ethical approval was granted by the Wellington Ethics Committee, New Zealand. Dithiothreitol was added (0.5 mM final concentration) and the saliva was filtered through sterile glass wool and collected in a sterile glass beaker and mixed (38). The prepared saliva (400 µl) was inoculated onto 13-mm diameter sterile Thermanox™ coverslips (Nunc Inc., Naperville, IL) placed in each well of the 24-well microplates (Sarstedt, Sarstedt Australia Pty Ltd, Ingle Farm, Australia). After 1 h, the saliva was gently aspirated from the coverslips and the base of the wells, and growth media were added. The plates were then incubated in an anaerobic hood in an atmosphere of 80% N_2, 10% CO_2 and 10% H_2 (Coy™, Laboratory Products Inc., Grass Lake, MI) for up to 10 days at 35°C without shaking. The artificial saliva medium for each well was replaced daily. The plate was gently shaken, the supernatant removed from each replicate well was pooled and its pH was recorded (CD 660 Digital pH meter; Walden Precision Apparatus, Linton, Cambridge, UK).

Analysis of plaque growth

In addition to daily pH measurements of the supernatant, growth of the microplate plaques was measured as wet-weight accumulation (mg). In Experiment I, wet-weight measurements were taken after 3, 7 and 10 days of growth. In Experiment II, wet-weight measurements were taken after 3, 4 and 7 days of growth. A more detailed early time course was investigated for the microplate plaques grown in 1.8 ml DMM with and without 0.15% sucrose with measurements also taken after 1 and 2 days of growth.

After removal of the supernatant, the coverslip and adherent biofilm were removed using sterile tweezers, placed on a sterile tissue to absorb any excess liquid and biofilm biomass was measured as wet weight (mg). The biofilm suspensions were then prepared for CKB analysis by placing the coverslip and adherent biofilm in sterile water and mixing vigorously to remove the adherent biofilm. Aliquots of the samples were dispensed, the cells were harvested and the pellets were stored at −80°C until analysis.

Microbiota profiling of the microplate plaques using CKB analysis

The CKB assay was carried out as described previously (35). Briefly, triplicate homogenized plaque samples (0.25 mg), and DNA standards (10^5 and 10^6 cells/ml, comprising a mixture of all probe species) in 200 µl 0.25 M alkali were denatured and lysed at 95°C for 5 min, cooled and neutralized with 800 µl 5 M NH_4 acetate. Each of the DNA samples and the standards was deposited onto a 15 × 15-cm positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany) using one of 30 parallel lanes of a multichannel immunoblotter (Minislot 30; Immunetics, Cambridge, MA). The membrane was UV cross-linked. Probes were prepared from whole cell chromosomal DNA by random-primed digoxigenin

Table 1. The CKB panel species and their key groupings

Key group	Species
Streptococci	*Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus parasanguis*, *Streptococcus mitis1*, *Streptococcus oralis*, *Streptococcus intermedius*, *Streptococcus vestibularis*, *Streptococcus mitis 2*, *Streptococcus gordonii*, *Streptococcus sanguis*, *Streptococcus anginosus*
Lactobacilli and Bifidobacter	*Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Bifidobacterium dentium*
Candida albicans	*C. albicans*
Actinomyces	*Actinomyces israelii*, *Actinomyces gerencseriae*, *Actinomyces naeslundii*, *Actinomyces odontolyticus*, *Rothia dentocariosa*
Microaerophiles	*Actinobacillus actinomycetemcomitans*, *Eikenella corrodens*
Aerobes	*Neisseria mucosa*, *Haemophilus parainfluenzae*
Anaerobes 1	*Fusobacterium nucleatum* ss *nucleatum*, *Campylobacter rectus*, *Veillonella parvula*, *Capnocytophaga gingivalis*, *Peptostreptococcus asaccharolyticus*, *Gemella morbillorum*, *Prevotella melaninogenica*, *Leptotrichia buccalis*, *Eubacterium saburreum*, *Corynebacterium matruchotii*, *Prevotella nigrescens/intermedia*
Anaerobes 2	*Porphyromonas gingivalis*, *Selenomonas noxia*, *Micromonas micros*

(DIG) labelling according to the manufacturer's protocol (Roche Diagnostics). The 40 species probe panel comprised a selection of caries and periodontal pathogens and other major plaque species (Table 1). The 30 DNA lanes were then cross-hybridized with 40 DIG-labelled probes, stringency washed and signals were detected using CDP-*Star* chemiluminescent substrate and quantified by digital image capture (GeneSnap v. 6; Syngene, Cambridge, UK). Spot intensities were compared to internal standards using software given by Dr S. Socransky. The spot intensities were converted to absolute counts and analysed as the mean percentage of the DNA probe count. Failure to detect a signal (sensitivity $\sim 10^4$ cells) was recorded as zero.

Statistical analysis

All treatments were arranged in triplicate, descriptive statistics were calculated and results were analysed for significance ($P < 0.05$) using a general linear model univariate analysis of variance and the post-hoc Tukey's Honestly Significantly Different (HSD) test (SPSS, v12.0.1 for Windows; SPSS Inc., Chicago, IL). Principal component analysis (PCA) was used to identify the compositional relationships, based on the 40 CKB species, between the microplate plaques in response to the different growth conditions, and relative to the composition of the initiating saliva (SAS® version 8.02; SAS Institute Inc., Cary, NC). PCA is a technique that simplifies a dataset by identifying underlying patterns, providing a concise overview of the data (9). The data are transformed to a coordinate system so that the variance of the data is attributed to a number of different principal components (linear components of the original variables), the first principal component usually accounts for the greatest amount of variance. In this way, the dataset is dimensionally reduced while retaining characteristics that contribute most to its variance (9).

Results

Growth of the microplate plaques

In Experiment I, BMM without sucrose promoted early growth of the microplate plaques and by day 3 the biomass was almost double that of the biofilms grown in DMM ($P < 0.05$) (Fig. 1A). However, after 10 days of growth, all the media types yielded comparable plaque biomasses ($P > 0.05$) (Fig. 1A). The biomass of the microplate plaques was considerably lower in Experiment II than in Experiment I (Fig. 1B). Neither media volume nor 0.15% or 0.5% sucrose supplementation significantly influenced biofilm biomass ($P > 0.05$; Fig. 1B).

In Experiment I the pH, measured every 24 h, of both the BMM and BM supernatants was $\sim$ 7.5. For the first 4 days, the pH of both BMM and BM supplemented with sucrose was $\sim$ 5.4, which then rose on subsequent days to $\sim$ 7.2. The supernatant pH of DMM was $\sim$ 7.4 and with 0.15% sucrose was $\sim$ 6.4 (data not shown). Similar 24-h pH values for DMM and DMM supplemented with 0.15% sucrose were obtained in Experiment II. Sucrose supplementation at 0.5% caused a substantial decrease in the pH of the supernatant to $\sim$ 3.9 (Fig. 2).

Microbiota composition of the microplate plaques

In Experiment I, the majority of the 40 CKB species were detected in the microplate plaques (Figs 3 and 4). During development, microbiota composition shifted from mainly aerobic and facultative anaerobic species to comprise predominantly facultative anaerobes.

Individual species responded differentially to media composition and sucrose supplementation, yielding some major and some subtle variations in the microbial profiles (Figs 3 and 4). The composition relationships analysed by PCA showed that the plaques differentiated according to media type and sucrose supplementation, and were quite different from the saliva inoculum (Fig. 5A).

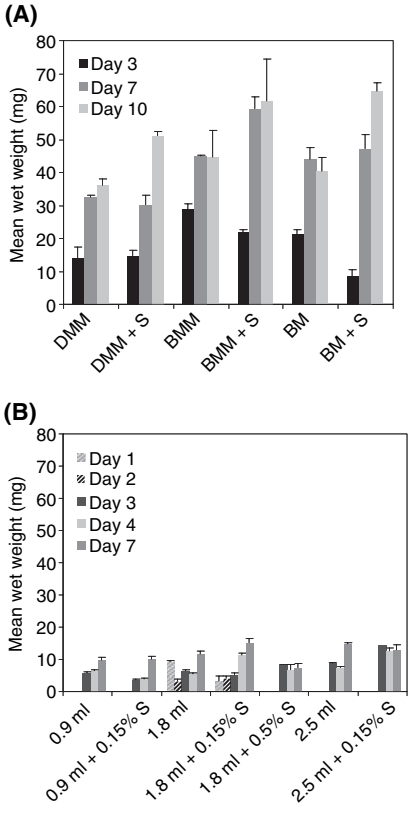

Fig. 1. Growth of the microplate plaques in (A) BMM, BM and DMM with and without 0.15% sucrose (S) after 3, 7 and 10 days, Experiment I and in (B) different volumes (0.9, 1.8 and 2.5 ml) of DMM supplemented with 0.15% and 0.5% sucrose in 1.8 ml only, during 7 days of growth, Experiment II. The error bars represent SE.

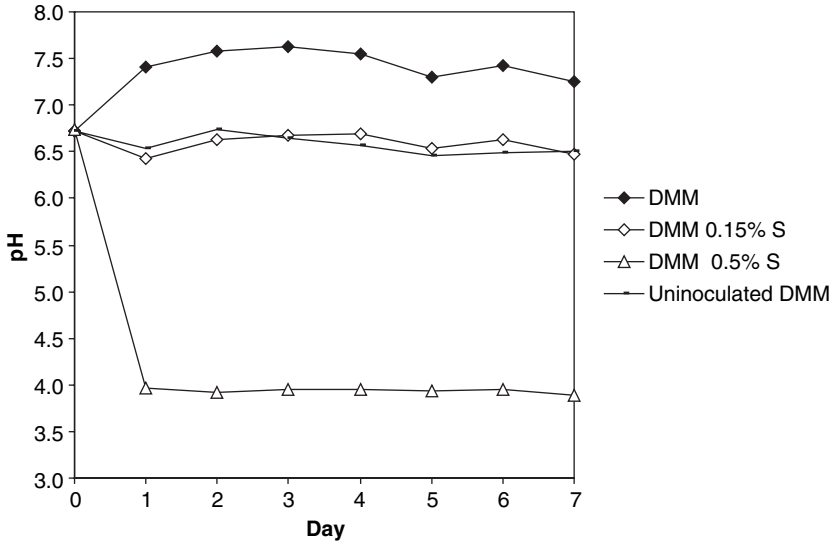

Fig. 2. Effect of sucrose (S) supplementation on the 24-h supernatant pH of DMM supplemented with both 0.15% and 0.5% sucrose in Experiment II.

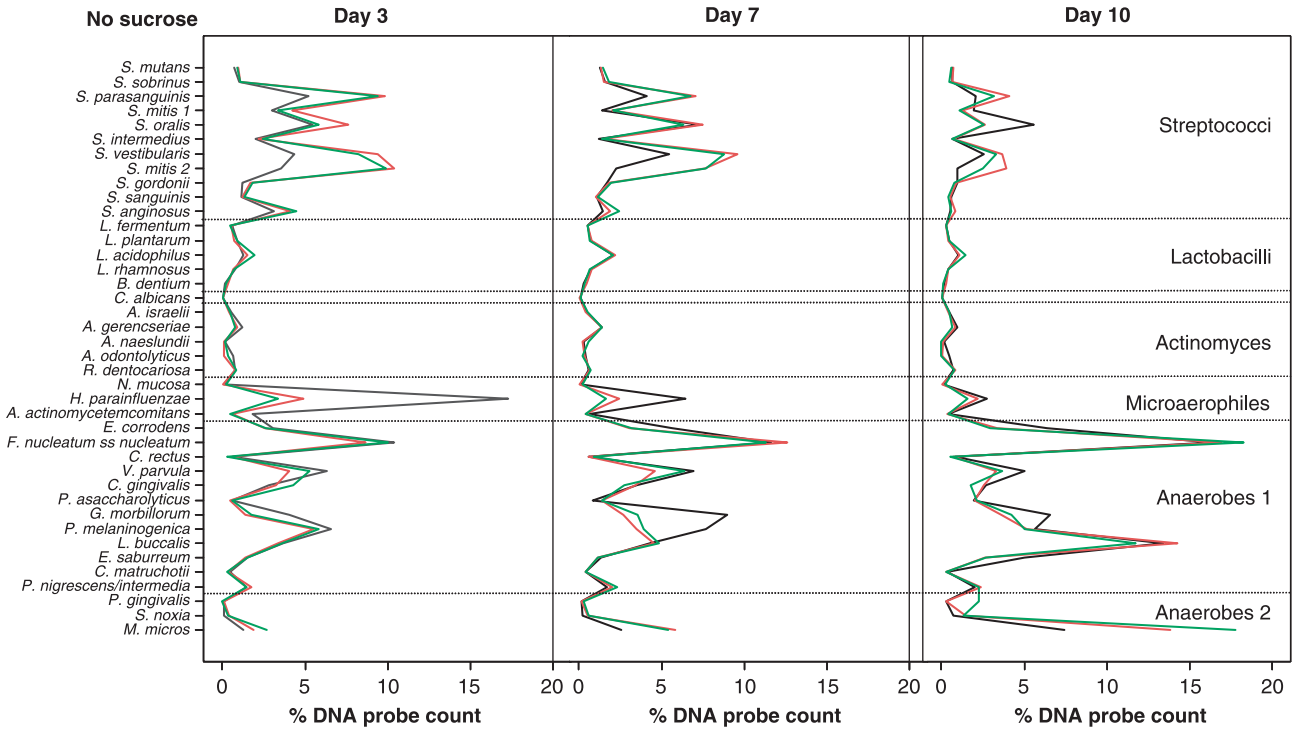

Fig. 3. Composition profiles of the microplate microcosm plaques (monitored over 10 days using CKB analysis) grown without sucrose in the artificial saliva DMM (black lines), BMM (red lines) and BM (green lines).

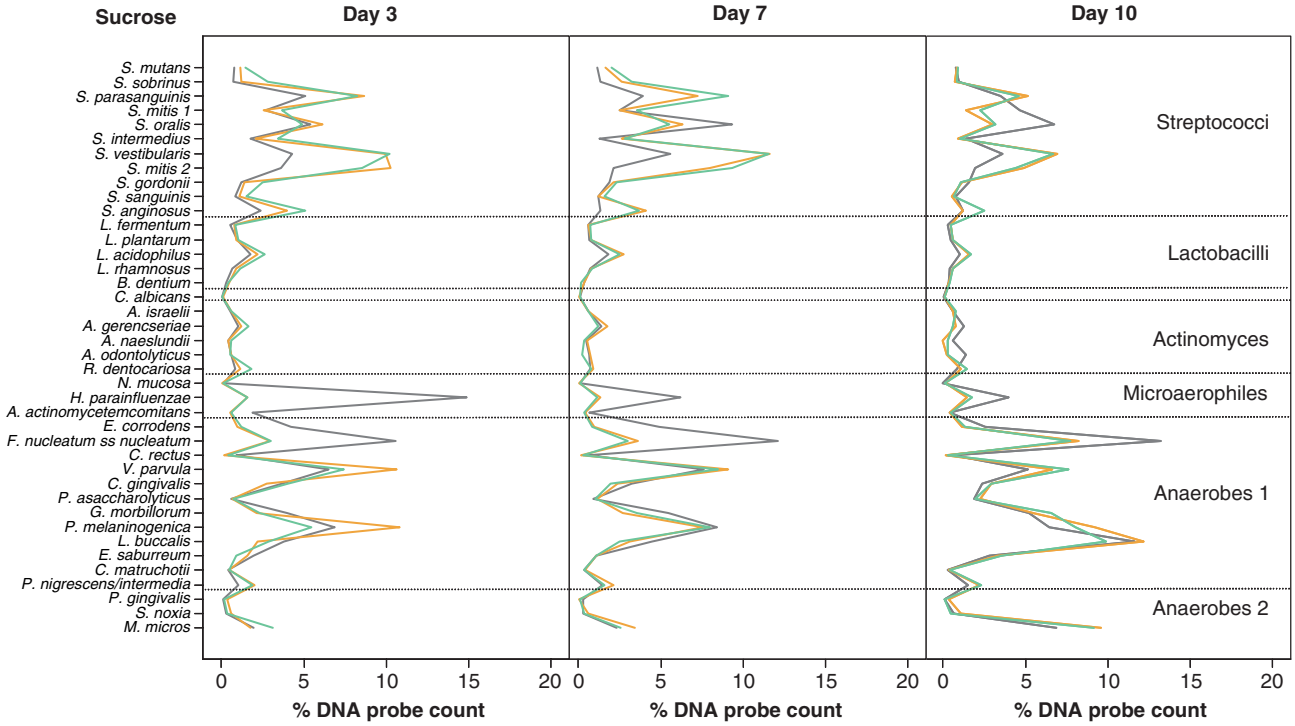

Fig. 4. Composition profiles of the microplate microcosm plaques (monitored over 10 days using CKB analysis) grown with 0.15% sucrose in the artificial saliva DMM (grey lines), BMM (yellow lines) and BM (green lines).

In Experiment II the microplate plaques demonstrated complex population shifts, as in Experiment I. Sucrose concentration appeared to have the greatest effect on the microbiota composition of the microplate plaques, which were quite different to the saliva inoculum, with media volume having some effect on the relative abundance of species present (Fig. 5B). Similar to

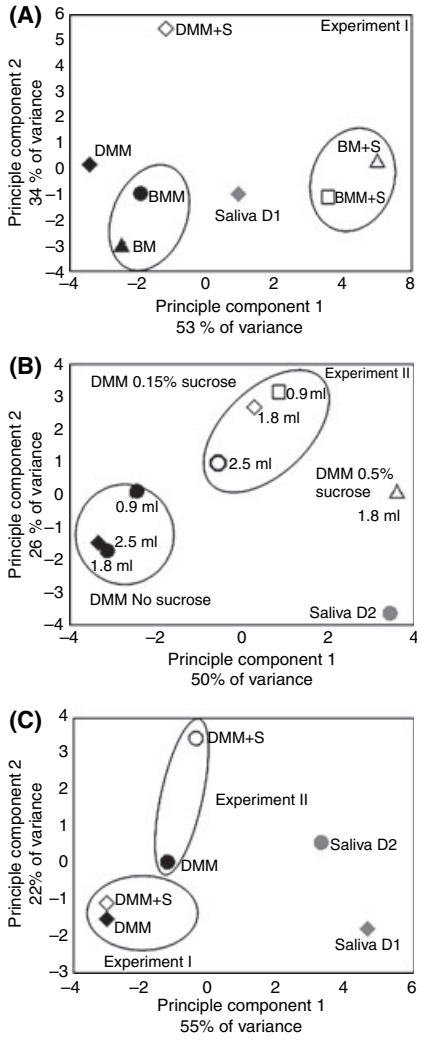

Fig. 5. PCA of the compositional relationships of the CKB species between the microplate microcosm plaques. (A) Effect of different artificial saliva media and 0.15% sucrose supplementation on microbiota composition after 10 days of growth, in relation to the saliva inoculum (D1) in Experiment I. The plaques differentiated according to media type and sucrose supplementation, and were quite different to the saliva inoculum (D1); (B) Effect of artificial saliva (DMM) volume, supplemented with either 0.15% or 0.5% sucrose, on microbiota composition after 4 days of growth, in relation to saliva inoculum (D2), in Experiment II. The plaques differentiated according to sucrose supplementation, with some differences associated with the volume of DMM; (C) Comparison of microbiota composition of the microplate plaques grown in the artificial saliva medium DMM from Experiment I and II after 7 days of growth, in response to 0.15% sucrose supplementation and in relation to the initiating saliva (D1 and D2 respectively). The plaques differentiated according to the initiating saliva, and plaques from Experiment II showed a greater degree of response to sucrose supplementation. In both Experiments, composition of the plaques was quite different from the initiating saliva (D1 and D2).

Experiment I, the composition profiles were influenced by the different growth responses of individual species to sucrose (Fig. 6).

In both experiments the presence of sucrose in the growth medium appeared to limit the decline of Streptococci over time, but did not appear to increase the relative abundance of mutans Streptococci (Figs 3, 4 and 6). Supplementation of DMM with 0.15% sucrose yielded increased abundance of *Lactobacillus fermentum*, *Lactobacillus acidophilus*, *Bifidobacterium dentium*, *Actinomyces gerensceriae* and *Actinomyces naeslundii*. In addition to these species, supplementation of DMM with 0.5% sucrose resulted in increased levels of *Streptococcus vestibularis*, *Actinomyces israelii*, *Rothia dentocariosa*, *Capnocytophaga gingivalis* and *Gemella morbillorum* ($P < 0.05$).

The composition of the two saliva inocula used in the two experiments was different (Fig. 5C) and yielded differences in the microbiota composition of the microplate plaques and in the degree of response to 0.15% sucrose supplementation (Fig. 5B). For some species, such as *Streptococcus parasanguinis*, *Haemophilus parainfluenzae*, *Eikenella corrodens* and *Veillonella parvula*, levels were almost double in DMM with 0.15% sucrose from Experiment I ($P < 0.05$), whereas with 0.15% sucrose supplementation in Experiment II, levels of some species, including *Streptococcus sobrinus*, *L. acidophilus* and *Actinobacillus gerensceriae*, were nearly double ($P > 0.05$). For both experiments, development was comparable, and the effect of sucrose supplementation on microbiota composition was most apparent in the first few days.

Discussion

The microbial composition of the microplate plaques initiated from a one-time saliva inoculation established in both Experiment I and II, as evaluated by CKB analysis, demonstrated a degree of similarity in patterns of ecological development and complex population dynamics. Reproducible plaque biofilms were established from each saliva donor, although differences were observed in the amount of plaque formed, which was probably related to host-specific factors (2, 17).

In a continuous culture environment, such as the oral cavity, the development of *in vivo* plaque biofilms is generally thought of as a sequential process of species attachment to the tooth and/or the extant plaque, and growth (13, 20). In this present study, it has been shown that with a one-time saliva inoculation, under batch culture conditions and daily renewal of the artificial saliva growth medium, it was also possible to replicate the population dynamics of natural plaque development; progressive shifts from mainly aerobic and facultative anaerobic species to predominantly facultative anaerobic and obligate anaerobe species as described for natural plaque (20). It would be expected that ecological shifts in plaque would exist under continuous culture growth conditions, and these have been previously demonstrated (14, 22, and Sissons et al., submitted for publication), but not described for a batch culture model, especially aimed at generating caries-related plaques.

Drawing direct comparisons with other plaque model systems of different hydrodynamic characteristics is difficult; other plaque microcosm studies are either carried out under nutritionally different culture conditions (22, 28), involve continuous biomass removal, as with the constant depth film fermenter (19, 21), or even consortia models comprised of several oral laboratory species (12, 28). However, the key feature of the experimental microcosm systems is ecological complexity. Of particular interest is inter-individual variation, and divergence in microbiota composition from the initiating saliva during growth, representative of the inherent heterogeneity of plaque composition *in vivo*. In this investigation the microbiota composition of the saliva inocula from the two individual donors was different, illustrating that the oral microbiota and ecology of individuals is intrinsically different. Such differences are implicit in the ecological hypothesis of dental disease (17, 18). Compositional differences in the initiating saliva have been shown to persist during the development of complex plaque microcosm communities under the same environmental and nutritional growth conditions (14, 26). Similarly, in this investigation, compositional differences in the saliva inocula yielded plaque microcosms of different composition and properties, in particular, the degree of response to sucrose supplementation.

Sucrose and frequencies of sucrose exposure and associated acidic conditions have been shown to significantly affect the species composition of *in vivo* and *in vitro* plaque biofilms (5, 15, 30, Sissons et al., submitted for publication). Similarly, in this investigation sucrose exposure had a substantial effect on the microbiota com-

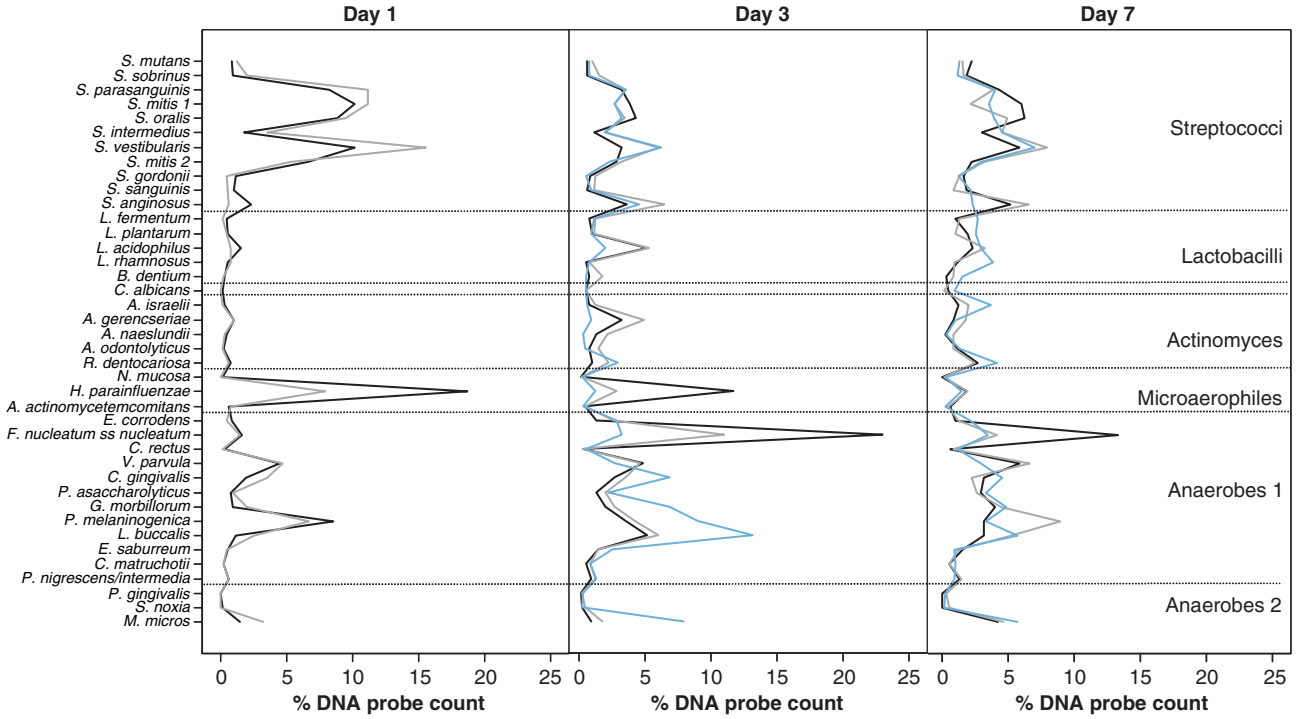

Fig. 6. Composition profiles of the microplate microcosm plaques monitored over 7 days using CKB analysis grown in 1.8 ml DMM with no sucrose (black lines), 0.15% sucrose (grey lines) or 0.5% sucrose (blue lines). Only the microplate plaques grown in 1.8 ml DMM with and without 0.15% sucrose were analysed after 1 and 2 days (2-day data not shown).

position throughout plaque development. Supplementation of DMM with 0.15% and 0.5% sucrose yielded repeatable 24-h pH values of 6.5 and 3.9 respectively. Tooth demineralization is a complicated process, involving factors such as plaque calcium and inorganic phosphate levels, as well as plaque pH. Lowering plaque pH by increasing the frequency of sugar application reduces the mineral protection capacity of plaque (25). Supplementation of DMM with 0.5% sucrose yielded a stable 24-h pH of 3.9, well below the critical pH conditions for demineralization of tooth enamel (~ pH 5.5) (15). The plaque pH environments generated from 0.15% and 0.5% sucrose supplementation in DMM reflect differences in the degree of cariogenicity of the microplate plaques.

Analysis of the bacterial species associated with childhood caries, using several molecular analysis techniques including reverse CKB analysis, indicated that although there was a relationship between *Streptococcus mutans* and caries, many other species appeared to be involved in the disease (1). *A. gerencseriae*, *Bifidobacterium*, *Veillonella*, *Rothia*, *Streptococcus salivarius*, *Streptococcus constellatus*, *S. parasanguinis* and *L. fermentum* were also strongly associated with caries (1). *A. gerencseriae* was the most numerous species in white spot lesions and hence is potentially associated with caries initiation, along with other *Actinomyces* sp. (1). The results from this present investigation indicated that DMM supplemented with 0.15% sucrose appeared to promote the growth of these, and other cariogenic bacteria. DMM supplemented with 0.5% sucrose, and associated acidic conditions appeared to support the growth of *A. israelii* associated with root caries (4), and *C. gingivalis* and *Leptotrichia buccalis* associated with necrotizing gingivitis (11).

Plaque-induced diseases are complex, and appear to be related to the increased levels of particular species in the oral microbiota, which relate to differences in the oral environment (2, 18). The results from this investigation have shown that it is possible to manipulate growth conditions and sucrose exposure to modulate plaque composition and prevalence of species linked to dental caries within a complex plaque-like community in a microplate system. The ability to achieve this will be invaluable for studying the etiology and development of dental diseases, potentially yielding a more realistic laboratory model than consortia and monospecies biofilm systems.

Acknowledgments

This research was funded in part by the Health Research Council (New Zealand), Wellington Medical Research Foundation, New Zealand Dental Research Foundation and Lottery Health Research. The authors thank Lisa Gellen and Megan Coleman for their invaluable technical assistance.

References

1. Becker MR, Paster BJ, Leys EJ et al. Molecular analysis of bacterial species associated with childhood caries. J Clin Microbiol 2002: **40**: 1001–1009.
2. Beighton D. The complex oral microflora of high-risk individuals and groups and its role in the caries process. Community Dent Oral Epidemiol 2005: **33**: 248–255.
3. Bradshaw DJ, Marsh PD, Hodgson RJ, Visser JM. Effects of glucose and fluoride on competition and metabolism within *in vitro* dental bacterial communities and biofilms. Caries Res 2002: **2**: 81–86.
4. Brailsford SR, Shah B, Simons D et al. The predominant aciduric microflora of root-caries lesions. J Dent Res 2001: **80**: 1828–1833.
5. Burne RA, Marquis RE. Alkali production by oral bacteria and protection against dental caries. FEMS Microbiol Lett 2000: **193**: 1–6
6. Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A. The Calgary biofilm

device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J Clin Microbiol 1999: **37**: 1771–1776
7. Das JR, Bhakoo M, Jones MV, Gilbert P. Changes in the biocide susceptibility of *Staphylococcus epidermis* and *Escherichia coli* cells associates with rapid attachment to plastic surfaces. J Appl Microbiol 1998: **84**: 852–858
8. Filoche SK, Anderson SA, Sissons CH. Biofilm growth of *Lactobacillus* species is promoted by *Actinomyces* species and *Streptococcus mutans*. Oral Microbiol Immunol 2004: **19**: 322–326.
9. Fromin N, Hamelin J, Tarnawski S et al. Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. Environ Microbiol 2002: **4**: 634–643.
10. Gellen LS, Wall-Manning GM, Sissons CH. Checkerboard DNA:DNA hybridization technology using DIG detection. In: Hilario E, Mackay JF, ed. *DNA analysis by non-radioactive probes*. Totowa, NY: Humana Press, 2007. in press.
11. Gmür R, Wyss C, Xue Y, Thurnheer T, Guggenheim B. Gingival crevice microbiota from Chinese patients with gingivitis or necrotizing ulcerative gingivitis. Eur J Oral Sci 2004: **112**: 33–41.
12. Guggenheim B, Giertsen W, Schupbach P, Shapiro S. Validation of an *in vitro* biofilm model of supragingival plaque. J Dent Res 2001: **80**: 363–370.
13. Kolenbrander PE, Andersen RN, Moore LV. Intrageneric coaggregation among strains of human oral bacteria: potential role in primary colonization of the tooth surface. Appl Environ Microbiol 1990: **56**: 3890–3894.
14. Ledder RG, Gilbert P, Pluen A, Sreenivasan PK, De Vizio W, McBain AJ. Individual microflora beget unique oral microcosms. J Appl Microbiol 2006: **100**: 1123–1131.
15. Lingström P, van Ruyven FO, van Houte J, Kent R. The pH of dental plaque in its relation to early enamel caries and dental plaque flora in humans. J Dent Res 2000: **79**: 770–777.
16. Mager DL, Ximenez-Fyvie LA, Haffajee AD, Socransky SS. Distribution of selected bacterial species on intraoral surfaces. J Clin Periodontol 2003: **30**: 644–654.
17. Marsh PD. Are dental diseases examples of ecological catastrophes? Microbiol 2003: **149**: 279–294.
18. Marsh PD. Dental plaque: biological significance of a biofilm and community lifestyle. J Clin Periodontol., 2005: **32** (suppl 6): 7–15.
19. Marsh PD, Hunter JR, Bowden GH et al. The influence of growth rate and nutrient limitation on the microbial composition and biochemical properties of a mixed culture of oral bacteria grown in a chemostat. J Gen Microbiol 1983: **129**: 755–770.
20. Marsh PD, Nyvad B. The oral microflora and biofilms on teeth. In: Fejerskov O, Kidd E, ed. *Dental caries, the disease and its clinical management*. Munksgaard: Blackwell, 2003: 29–48.
21. McBain AJ, Bartolo RG, Catrenich CE, Charbonneau D, Ledder RG, Gilbert P. Effects of Tricolsan-containing rinse on the dynamics and antimicrobial susceptibility of *in vitro* plaque ecosystems. Antimicrob Agents Chemother 2003: **47**: 3531–3538.
22. McBain AJ, Sissons CH, Ledder RG, Sreenivasan PK, De Vizio W, Gilbert P. Development and characterization of a simple perfused oral microcosm. J Appl Microbiol 2005: **98**: 624–634.
23. Moore WEC, Moore LVH. The bacteria of periodontal diseases. Periodontol 2000 1994: **5**: 60–77.
24. Paster BJ, Boches SK, Galvin JL et al. Bacterial diversity in human subgingival plaque. J Bacteriol 2001; **183**: 3770–3783.
25. Pearce EI, Sissons CH, Coleman M, Wang X, Anderson SA, Wong L. The effect of sucrose application frequency and basal nutrient conditions on the calcium and phosphate content of experimental dental plaque. Caries Res 2002: **36**: 87–92.
26. Rasiah IA, Wong L, Anderson SA, Sissons CH. Variation in bacterial DGGE patterns from human saliva: over time, between individuals and in corresponding dental plaque microcosms. Arch Oral Biol 2005: **50**: 779–787.
27. Roberts SK, Wei G-X, Wu CD. Evaluating biofilm growth of two oral pathogens. Lett Appl Microbiol 2002: **35**: 552–556.
28. Shapiro S, Guertsen E, Guggenheim B. An *in vitro* model for comparing the efficacy of antimicrobial mouthrinses. Caries Res 2002: **36**: 93–100.
29. Sissons CH. Artificial dental plaque biofilm model systems. Adv Dent Res 1997: **11**: 110–126.
30. Sissons CH, Cutress TW, Faulds G, Wong L. pH responses to sucrose and the formation of pH gradients in deep "artificial mouth" microcosm plaques. Arch Oral Biol 1992: **37**: 913–922.
31. Sissons CH, Wong L, An YH. Culture and analysis of bacterial biofilms. In: Friedman RJ, ed. *Handbook for studying bacterial adhesion*. Totowa: Humana Press, 2000: 133–169.
32. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. J Clin Periodontol 1998: **25**: 134–144.
33. Socransky SS, Haffajee AD, Smith C et al. Use of checkerboard DNA–DNA hybridization to study complex microbial ecosystems. Oral Microbiol Immunol, 2004: **19**: 352–362.
34. Socransky SS, Smith C, Martin L, Paster BJ, Dewhirst FE, Levin AE. "Checkerboard" DNA-DNA hybridization. Biotechniques 1994: **17**: 788–792.
35. Wall-Manning GM, Sissons CH, Anderson SA, Lee M. Checkerboard DNA–DNA hybridization technology focused on the analysis of Gram-positive bacteria. J Microbiol Methods 2002: **51**: 301–311.
36. Wimpenny JWT (ed.). Introduction, models and microcosms. In: *Handbook of laboratory model systems for microbial ecosystems*. Boca Raton, FL: CRC Press Inc., 1988: 1–17.
37. Wimpenny JWT. The validity of models. Adv Dent Res 1997: **11**: 150–159.
38. Wong L, Sissons CH. A comparison of human dental plaque microcosm biofilms grown in an undefined medium and a chemically-defined artificial saliva. Arch Oral Biol 2001; **46**: 477–486.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.