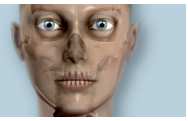
Orthodontics & Craniofacial Research



ORIGINAL ARTICLE

A. J. Ireland
V. Soro
S. V. Sprague
N. W. T. Harradine
C. Day
S. Al-Anezi
H. F. Jenkinson
M. Sherriff
D. Dymock
J. R. Sandy

The effects of different orthodontic appliances upon microbial communities

Authors' affiliations:
A. J. Ireland, V. Soro, S. V. Sprague, N. W. T. Harradine, C. Day, S. Al-Anezi, H. F. Jenkinson, M. Sherriff, D. Dymock, J. R. Sandy, School of Oral and Dental Sciences, University of Bristol, Bristol, UK

Correspondence to:
H. F. Jenkinson
School of Oral and Dental Sciences
University of Bristol
Lower Maudlin Street
Bristol, BS1 2LY, UK
Email: howard.jenkinson@bristol.ac.uk

Date:
Accepted 23 November 2013

DOI: 10.1111/ocr.12037

Ireland A. J., Soro V., Sprague S. V., Harradine N. W. T., Day C., Al-Anezi S., Jenkinson H. F., Sherriff M., Dymock D., Sandy J. R. The effects of different orthodontic appliances upon microbial communities *Orthod Craniofac Res* 2014; **17**: 115–123. © 2013 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

Structured Abstract

Objectives – Orthodontic appliances can promote accumulation of dental plaque, with associated enamel decalcification or gingival inflammation. The aim of this study was to examine longer-term microbiological changes during orthodontic treatment with fixed appliances.

Materials and Methods – Twenty-four orthodontic patients aged 11–14 years undergoing fixed appliance therapy were recruited into the study. Each was randomized for cross-mouth assignment of molar bands and bonded molar tubes to contralateral quadrants of the mouth. All patients received self-ligating brackets, but again using randomization, one upper lateral incisor bracket (left or right) also received an elastomeric ligature. Plaque samples from the molars and upper lateral incisors were obtained at intervals during treatment and up to 1 year after appliance removal. Denaturing gradient gel electrophoresis and 16S rDNA microarray were used to compare plaque microbial fingerprints.

Results – Plaque populations changed within 3 months of commencing treatment at all sites. The greatest differences in plaque composition were seen with self-ligating brackets with an elastomeric ligature. Post-treatment plaque associated with both types of molar attachment contained increased levels of periodontal pathogens *Porphyromonas gingivalis*, *Tannerella forsythia,* and *Eubacterium nodatum*, while *Campylobacter rectus*, *Parvimonas micra*, and *Actinomyces odontolyticus* were also elevated with bonds.

Conclusions – The results suggest that orthodontic treatment may cause sustained changes in plaque microbiotas and that molar bond-associated plaque may have raised disease potential.

Key words: bands and bonds; dental plaque; gingivitis; microarray; periodontal disease

Introduction

Although orthodontic treatment outcomes are mostly successful, it is acknowledged that treatment is not without risks (1). For example, the wearing of fixed appliances increases likelihood of plaque retention (2–4) and can lead to significant increases in plaque index (PI) and gingival index (GI) within 3 months of appliance placement. Enamel decalcification (seen as white spot lesions) during treatment is also an issue (5). Although gingival inflammation subsides and white spot lesions fade when fixed appliances are removed, it is unclear whether there are associated longer-term risks involved, such as greater predisposition to periodontal diseases in adulthood.

Potential longer-term adverse outcomes for orthodontic intervention could be improved if there was better understanding of the microbial perturbations associated with specific treatment regimens and appliances. The use of orthodontic bonds, as opposed to bands, may help subjects to maintain more satisfactory levels of oral hygiene (6). However, there is little evidence to show that banding leads to more adverse microbial or clinical effects than bonding, for some species at least (7). Subjects who are unable to maintain good levels of oral hygiene during orthodontic treatment may benefit from the use of ligatures, rather than elastomeric modules (8). Appliance materials may also be important because cariogenic streptococci have been shown to adhere more readily to plastic than monocrystalline brackets (9).

Although a number of studies have reported that the clinical measures such as plaque index (PI), gingival index (GI), probing depth (PD), and bleeding on probing (BOP) became elevated following placement of fixed or removable orthodontic appliances (4, 7, 10, 11), associated microbiological data are less consistent. Some studies suggest that overall increases in the levels or proportions of anaerobic pathogenic bacteria may occur within supra- or subgingival plaque during the early stages of treatment (7, 12). In one longer-term study, the percentage of subjects with higher counts of six pathogenic bacterial species appeared to return to pre-treatment levels following removal of appliances (13). In other studies, levels of periodontopathogens remained higher after orthodontic treatment (4, 14), perhaps indicating greater potential for the development of periodontal diseases.

Molecular approaches have indicated orthodontic-associated increased detection of periodontopathogens adjacent to and within gingival tissues. The technique of denaturing gradient gel electrophoresis (DGGE) (15, 16) separates DNA fragments on the basis of both size and melting temperature, and provides a fragment profile or 'fingerprint' representative of the mixed microbial sample analyses. More recently, microarray technology has allowed the expression of genes throughout the genome to be simultaneously quantified, and microbial ecologists have been able to produce microarrays of individual nucleotide sequences from different bacteria (17–19). Such advances in bacterial molecular diagnostic techniques have facilitated the investigation of the microbial composition of the necrotic root canal (20) and changes in the oral microbiota associated with orthodontic treatments (21). To date, about 300 species have been identified.

In this study, we have utilized both DGGE and microarrays to determine bacterial community structures and incidence of specific species in plaque samples during orthodontic treatment, and up to 1 year after appliance removal. The objectives were to compare the effects of different types of orthodontic attachment, molar bands or molar bonds, and self-ligating brackets with or without elastomeric modules, on clinical and microbiological parameters that might be

associated with detrimental effects on oral tissues.

Materials and methods

Subjects

The aim of this investigation was to determine how the plaque microbiota might change as a result of orthodontic treatment using fixed appliances. In particular, the effect of molar bands vs. bonded molar tubes was investigated along with the effect of the use of self-ligating brackets with or without elastomeric ligation. Twenty-four subjects (aged 11–14 years) beginning orthodontic treatment with upper and lower fixed orthodontic appliances at Bristol Dental Hospital participated in this study. Ethical approval was granted by UBHT Ethics Committee (04/Q2006/53), and written parental consent was obtained in each case. Eligible patients were all patients aged 11–14 years undergoing a course of full upper and lower fixed appliances, with or without extractions, provided neither molars nor upper lateral incisors were being extracted. Specific exclusion criteria were subjects with known systemic diseases or who had taken antibiotics within 3 months of commencing treatment and those requiring arch expansion or distalization of molars with auxiliary appliances, as these might interfere with oral hygiene practices.

For each patient, bonded molar tubes and molar bands were fitted to contra lateral quadrants of the mouth, with random assignment using sealed envelopes to ensure equal numbers in each group. Therefore, molar bonds were either placed on the lower right and upper left, or on the lower left and upper right, and molar bands were placed in the other quadrants as shown in Fig. 1. All of the remaining teeth were bonded using Damon 2 0.022" self-ligating brackets to test the effect of self-ligation vs. elastomeric ligation on plaque composition around the upper lateral incisor brackets. These brackets, once bonded in position, were randomly assigned to receive, or not, an elastomeric ligature. Randomization was once again performed using a system or sealed envelopes, and either the upper left or upper right lateral incisor bracket clip was closed, as is normal practice, or the clip was not closed and instead was replaced by an elastomeric module.

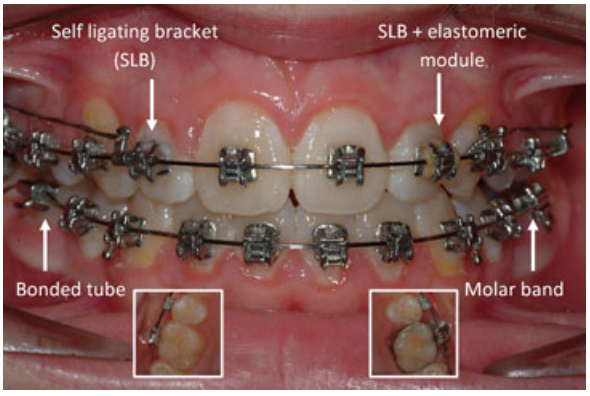


Fig. 1. The orthodontic fixed appliance used in this study and the contralateral assignment of the bonded molar tubes and molar bands, along with the self-ligating brackets (SLB) with or without elastomeric ligation.

In all cases, the brackets and bonded molar tubes were bonded using a composite (Transbond XT; 3M Unitek, St. Paul, MN, USA), and the molar bands cemented using a glass ionomer cement (Intact, Ortho-care Ltd, Bradford, UK). No molar attachments needed to be replaced during the study period although the protocol allowed for any molar bond or band that became detached or needed repositioning to be replaced with an identical attachment.

Clinical measurements and plaque sampling

Oral hygiene instruction and necessary scaling were provided by a hygienist 1 month pre-treatment and 2 weeks after any tooth extractions. Supragingival plaque samples were obtained on the molars (bands and bonds) using sterile curettes and subgingivally using sterile paper points. In the case of the upper lateral incisors, with or without elastomeric ligation, supragingival plaque was collected adjacent to the bracket margins. Two clinical parameters of periodontal disease, namely the plaque index (PI) and bleeding on probing (BOP), were also made on the first molar teeth. These samples and measurements were taken at each of four time points throughout the treatment, namely: pre-bond-up

at the molar separator appointment (T_0); 3 months into treatment (T_1); just prior to appliance removal on the day of debond (T_2); 3 months post-debond (T_3); and finally, 1-year (T_4) post-debond.

Each plaque sample, once collected, was dispersed in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) and stored at 70°C. DNA was extracted with GeneElute PCR DNA Purification Kit (Sigma-Aldrich, Gillingham, UK) according to the manufacturer's instructions.

Denaturing gradient gel electrophoresis

16S rRNA gene V2-V3 regions were PCR amplified using universal primers 2 and 3 (15) within a 50-μl reaction mixture containing: 1.25 U Go*Taq* DNA polymerase (Promega, Madison, WI, USA), 10 μl supplied buffer (with 1.5 mM $MgCl_2$), 0.2 mM each dNTP, 1.0 mM each primer, and approximately 20 ng template DNA. A touchdown protocol was utilized, with a pre-denaturation step of 94°C for 2 min, 15 cycles of 94°C for 30 s, primer annealing steps of 30 s starting at 65°C and descending 1°C each cycle, and extension at 72°C, followed by 10 cycles of 94, 50, 72°C for 30 s each, and a final extension step of 72°C for 10 min. The PCR products were analyzed by electrophoresis through 1.2% agarose. PCR amplimers were separated through 10% polyacrylamide containing a linear gradient of 40–70% urea, in half-strength Tris-acetate-EDTA (Sigma, Gillingham, UK), initially at 30 V (1 h), then at 250 V for 4 h, at 60°C. DNA bands were stained with SYBR green nucleic acid gel stain (10^4 dilution) and imaged under UV transillumination.

Microarray analysis

The molecular diagnostics microarray system ParoCheck 20 (Greiner Bio-One, Stonehouse, UK) was used to detect plaque bacteria. DNA extracted from samples was subjected to PCR amplification of a specific 16S rDNA region, present in all bacteria. Microarray hybridization was performed according to the manufacturer's instructions. Slides were fluorescently scanned and analyzed using an Axon GenePix 4000 scanner at the Transcriptomics Facility, University of Bristol.

Determination of *Treponema denticola*

Treponema denticola ATCC 35405 was grown as previously described (22) and DNA extracted using GenElute. Two universal real-time oligonucleotide primers, Uni-F (5′-CCTACGGGAGGCAGCAG-3′) and Uni-R (5′-ATTACCGCGGCTGCTGG-3′) (15), were used to target conserved sequences in the 16S rDNA of all bacteria, while primers Tre-1F (5′-AAGGCAACGATGGGTATCC-3′) and Tre-R (5′-GCGTCGCTCCGTCAGACT-3′) were employed for specific detection of *T. denticola* (23).

Statistical analyses

The clinical data recorded were plaque index, probing depths, and BOP. The experimental design was repeated measures, and the data were analyzed using Stata version 12 (StataCorp LP, College Station, TX, USA) by means of linear mixed models, with a predetermined significance level of $\alpha = 0.05$.

For the DGGE fragment profiles, a ladder consisting of PCR amplimers (with primers 2 and 3) from *Actinomyces naeslundii*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *Streptococcus constellatus,* and *Tannerella forsythia* was used for standardization of image analyses. Plaque sample amplimer profiles were analyzed using TotalLab TL120 and DM software packages. Similarity matrices were calculated based on the absence or the presence of common amplified DNA fragments. Means for the percentage similarities were obtained, standard errors were calculated, and their significance was obtained using Student's *t*-test (Excel, Microsoft, Redmond, WA, USA)

Results

Clinical measures

With the upper lateral incisors, elastomeric vs. non-elastomeric, there was no statistically significant effect of type of ligation on the three clini-

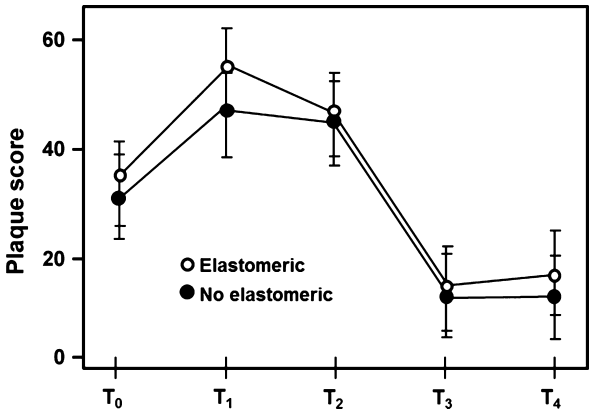


Fig. 2. Estimated means and 95% confidence intervals of the mean for plaque scores on the upper lateral incisor teeth (elastomeric vs. non-elastomeric) at T_0, pre-treatment; T_1, 3 months; T_2, end of treatment; T_3, 3 months after de-bond; T4, 1 year after de-bond.

cal measurements (see Fig. S1). However, utilizing a linear mixed effects model, there was a statistically significant effect of time on plaque score (time $p = 0.001$, elastic $p = 0.759$) as shown in Fig. 2. Three months post-debond, the plaque scores were significantly lower, confirming previous findings that orthodontic appliances increase plaque retention (4, 7).

On the first molars, there were no statistically significant effects of bonded molar tubes vs. molar band on the pre-treatment and 1-year post-debond probing depths. There were some differences at T_2, T_3, and T_4, with PDs increasing at these time periods, but these were not statistically significant. A similarly inconsistent effect was noted with BOP on the molars with time (for all data, see Fig. S1).

Plaque community composition

Denaturing gradient gel electrophoresis, as applied to 16S rDNA fragments PCR amplified from bacteria with universal oligonucleotide primers, has been utilized to assess shifts in ecology within diverse microbial communities (15, 16, 24). Denaturing gradient gel electrophoresis provides a fragment profile (fingerprint) representative of the main microbial community components (Fig. S2). Accordingly, we obtained fingerprints of plaque samples taken from gingival margins adjacent to molar bands or bonded molar tubes, and supragingival sites adjacent to self-ligating brackets with or without an elastomeric module. Denaturing gradient gel electrophoresis fragment profiles for all individual samples were obtained, analyzed digitally, and similarity matrices were constructed for comparison (all raw data available in Fig. S3).

The general trend within samples from the different sites was for fingerprints to become progressively more different from T_0 to T_1 to T_2 and then to shift further at completion of treatment (Fig. 3). The biggest difference in the first 3 months occurred with the elastomeric module on self-ligating bracket (Fig. 3C). At T_3, there was a statistically significant return toward T_0 microbiota for plaque at the gingival margin adjacent to molar bands (Fig. 3A) and bonded molar tubes (Fig. 3B). The plaque microbiotas appeared to stabilize at completion of treatment for supragingival plaque adjacent to self-ligating brackets (Fig. 3C) independent of elastomeric module (Fig. 3D).

There was a small, but statistically significant, return toward a T_0 plaque fingerprint at the gingival margin adjacent to molar bands, at T_4, 1 year after completion of treatment (Fig. 3A). None of the other sites showed this trend. There were no significant changes in plaque microbiota profiles after T_2 for self-ligating brackets with elastomeric module (Fig. 3C). Overall, the results suggest that the microbiota shifted more in plaque adjacent to the self-ligating bracket with elastomeric module and that the new plaque microbiome remained for at least 1 year (T_4) after treatment (Fig. 3C). Conversely, there was lesser change in the subgingival plaque microbiota associated with molar bands as opposed to molar bonds, and there was a shift back toward T_0 microbiota after 1 year (Fig. 3A).

Changes in periodontal bacteria

We then investigated whether there were changes in specific components of the plaque microbiota associated with two types of orthodontic appliances: bonded molar tubes and molar bands. Microarray data (shown in detail in Fig. S4) were translated into a heat map for 20 components of the oral microbiota over the

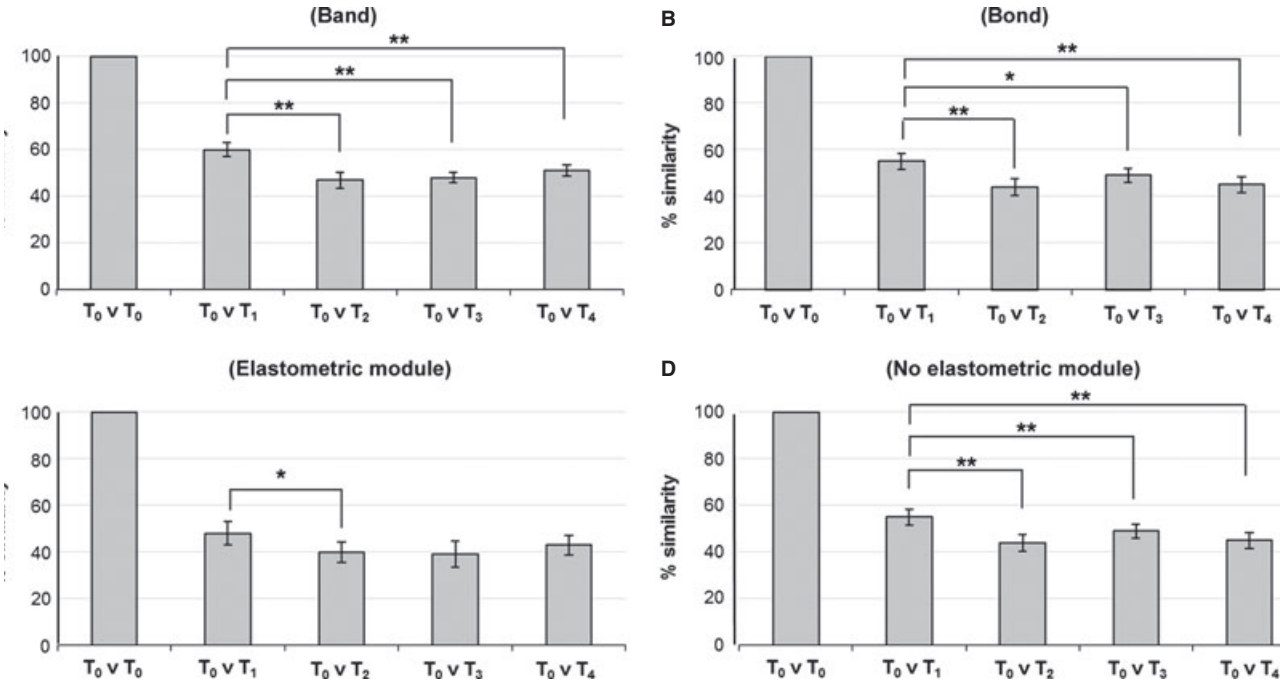


Fig. 3. Shifts in denaturing gradient gel electrophoresis profiles of subgingival plaque samples taken during or after orthodontic treatment for molar bands and bonds, and supragingival plaque samples for elastomeric vs. non-elastomeric ligatures on the upper lateral incisors. Profiles are expressed as mean percentage similarities to T_0 sample profiles. Significant differences are indicated as $*p < 0.05$ and $**p < 0.01$.

treatment period (Fig. 4). More significant changes to detection levels of the 20 bacteria were apparent for bonded molar tubes (6 increasing, 2 decreasing) than molar bands (3 increasing, 2 decreasing) during the first 3 months of treatment. Both showed significant increases in detection of *T. denticola* and *P. nigrescens*, which are associated with periodontal disease, and decreases in *A. actinomycetemcomitans* during this initial phase of treatment. Additionally, molar bond samples showed higher detection of *F. nucleatum* and *C. gracilis*, this being the only significant difference ($p < 0.05$) at T_1 between molar bonds and bands. From 3 months to end of treatment (T_1 vs. T_2), there were few changes, although *S. mitis* detection significantly decreased. Samples obtained 3 months post-treatment (T_3) generally showed reductions in the detection of microarray bacteria, with more changes for molar bonded tubes than for molar bands, including the periodontopathogen *T. denticola* (Fig. 4). Judging by the changes occurring within plaque associated with bonds, these microbiotas were more unstable than those associated with bands.

One-year post-treatment, the plaque associated with both methods of molar retention contained increased levels of *Porphyromonas gingivalis*, *T. forsythia,* and *E. nodatum*, all recognized as periodontal pathogens. In addition, potential pathogens *C. rectus*, *P. micra*, and *A. odontolyticus* were increased in plaque associated with molar bonds (Fig. 4).

Analysis of *Treponema denticola*

Treponema denticola is emerging as an important periodontal pathogen, and therefore, PCR detection was utilized to qualify the microarray data. It was of interest that microarray detection levels increased during treatment, with bonds or bands, but decreased after removal of appliances (Fig. 4). As an independent check on this finding, *T. denticola* was quantified by RT-PCR as percentage of the total bacteria within plaque samples from 20 subjects (results are fully presented in Fig. S5). *Treponema denticola* significantly increased (** <0.05; *<0.01) from T_0 (pre-bond-up) to T_1 (3 months), while levels at T_3 (3 months post-treatment) had mostly returned

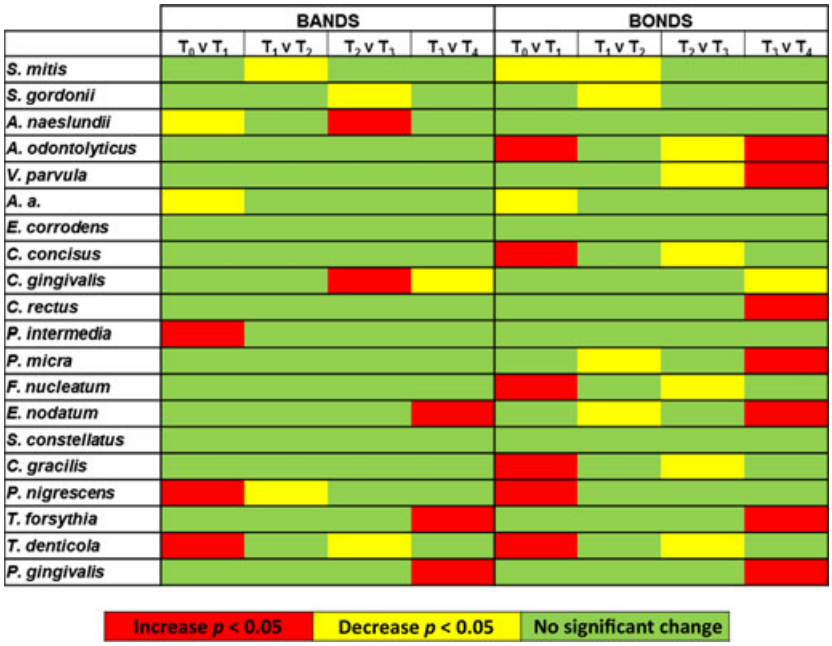


Fig. 4. Heat map illustrating changes in the levels of some periodontal bacterial species at the first permanent molar bands vs. bonded tubes from T_0 to T_4 with orthodontic treatment.

to T_0 levels. These data were consistent with the microarray results.

Discussion

Molecular surveys to assess microbial diversity in plaque, and to associate the presence of various microorganisms with different oral conditions, have utilized 16S rRNA gene sequence analyses and comparisons (25–27). Of particular interest in this study was the shift in populations associated with gingival margins, as opposed to determining levels of bacteria specifically associated with dental caries (e.g., *S. mutans*). We considered that to utilize this sequencing approach for the longitudinal study described here would be too labor intensive for the information likely to be gained. Accordingly, we used DGGE to monitor shifts in plaque ecology during orthodontic treatment. To date, two studies have successfully employed DGGE in the analysis of bacteria present in subgingival plaque (28, 29). Denaturing gradient gel electrophoresis is very suitable for evaluating major shifts in microbial population composition as in response to extrinsic influences. Commercial microarrays give a pattern of indicator or marker microorganisms covering both pathogenic and less pathogenic species.

While there is some evidence that orthodontic treatment efficiency is affected by different fixed appliances (30), there is little information on the longer-term effects of different orthodontic treatments on the composition of oral microbial communities. As clinical treatment proceeded in this study, there were no significant changes in BOP and PD, but the plaque score reduced as expected following removal of the active appliances. However, during and after the treatment, there were progressive shifts in microbial community composition away from pre-treatment plaque ecology. These changes were observed at the gingival margins for both molar bands and bonded molar tubes samples, to a threshold level at treatment completion of around 40–45%, which then remained stable. In supragingival plaque samples adjacent to self-ligating brackets, both with and without elastomeric modules, a progressive shift away from the pre-treatment plaque ecology was observed to a threshold minimum of ~40%. There were no indications of restoration to pre-treatment plaque microbiotas. This was especially evident for bonded molar tubes, and a new microbial community structure was still present 1 year after cessation of treatment.

In a recent longitudinal study of the effects of orthodontic appliances on oral microbial ecology, it was shown by cultivation techniques that the aerobe/anaerobe ratio decreased during treatment and only partially normalized 3 months after appliance removal (14). However, cultivation methods have the potential to underestimate anaerobic bacteria numbers, as many of these organisms are currently uncultivable. In support of our data showing increased levels of several periodontal pathogens following appliance removal, Liu et al. (4) showed increased levels of *P. gingivalis* in plaque by the end of orthodontic treatment, and these remained higher for at least 6 months.

Conclusions

In summary, our work has demonstrated that progressive shifts in dental plaque composition occur during orthodontic treatment, with no indication of restoration to pre-treatment microbiota, even 1 year after cessation of treatment. This is the only full year follow-up study to date showing potential longer-term changes in microbial community structure.

The results suggest that molar bonds are more likely to elicit changes in the normal plaque microbiota toward a microbiota with raised disease potential. There were sustained increases in levels of microbial species not present at the start of treatment, and not present in plaque associated with molar bands. Although these results do not necessarily indicate that subjects may be more prone to gingivitis or periodontal diseases later in life, there should be concern over the potential for extended-term clinical effects associated with these microbiological changes.

Acknowledgements: We thank the British Orthodontic Society Foundation for funding this study.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Supporting clinical data for the information provided in the text.

Fig. S2. DGGE profiles from a single subject prior to (T_0) and three months after start of treatment (T_1).

Fig. S3. Example of how the software packages were utilized to produce dendograms of microbial community relatedness profiles.

Fig. S4. Signal intensities for microarray detection of 20 oral bacterial species from dental plaque at the gingival margin adjacent to molar bands (grey bars) and molar bonds (white bars) for each sample time point through the study.

Fig. S5. Microarray (a) and quantitative RT-PCR (b) data for *T. denticola* in molar band (light grey bars) and molar bond (dark grey bars); plaque samples from 20 patients for microarray and RT-PCR throughout the study.