Qualitative and quantitative changes in the oral bacterial flora occur shortly after implementation of fixed orthodontic appliances

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Introduction: In this pilot study, we aimed to determine qualitative and quantitative microbiological changes after the implementation of orthodontic appliances. **Methods:** A total of 10 healthy patients aged 12-15 years were recruited who needed to undergo orthodontic treatment with buccal fixed appliances. Gingival conditions were assessed by the Gingival Index, Periodontal Screening Index, and Sulcus Bleeding Index. Microbiological samples were collected before and 1 week after the start of therapy at premolars and molars of the right upper quadrant. Bacterial species were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. **Results:** The total number of bacteria increased. Six bacterial species were identified that are involved in the development of caries and other infectious processes. The bacteria selectively adapted more efficiently to the new oral milieu compared with the general oral microbial background. There was a significant increase in *Streptococcus* spp at the premolars and molars. In all individuals, symptoms of inflammation and gingivitis were detected as a response to the bacterial challenge. **Conclusions:** Orthodontic treatment induces significant changes in the oral microbial flora associated with gingivitis and an enhanced risk for cariogenic reactions within the first days of orthodontic treatment. To prevent or reduce infectious side effects, oral hygiene instructions and control of patients are necessary before and during the beginning of the therapy. (Am J Orthod Dentofacial Orthop 2019;156:735-44)

During orthodontic therapy, 2 clinical problems occur in most cases: (1) formation of bacterial plaque, followed by demineralization and caries at the site where orthodontic appliances are fixed on the tooth surface; and (2) increased accumulation of bacterial plaque with the placement of orthodontic appliances, leading to inflammatory reactions of the gingiva that affect approximately 90% of young patients.[1] Poor oral hygiene enhances plaque formation, thus modifying the oral environment with regard to the amount, flow, and composition of saliva, including its pH and buffer ability, and may induce gingiva hyperplasia and bleeding on probing. The work presented here deals primarily with the problem of bacterial plaque formation potentially resulting in caries, which affects an even greater number of patients than gingivitis and periodontitis. Furthermore, demineralization caused by bacteria may leave white spot lesions on the tooth surface after the orthodontic appliances are removed at the completion of therapy. Bacterial plaques are infectious biofilms on the tooth surface resulting in the formation of an adaptive microbial community. Species composition can change qualitatively and quantitatively depending on the size and roughness of the surfaces to which

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All authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest, and none were reported.
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The study was supported by the Medical Faculty of the University of Jena, Department of Orthodontics and Medical Microbiology, University Hospital Jena, Germany.
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Submitted, January 2018; revised and accepted, December 2018.
0889-5406/$36.00

https://doi.org/10.1016/j.ajodo.2018.12.018

bacteria adhere.[2] Because initial colonization, adhesion, and persistence in biofilms represent a complex interaction of species, we examined all bacterial isolates from emerging plaques at the loci where the orthodontic appliances were fixed. The work focused on the dynamics of the process during the initial phase after insertion (ie, the first week). Although known cariogenic strains were especially interesting, considering the potential interplay of the microbes, complete molecular identification of all species and their colony numbers was anticipated to occur in the probes collected from plaque.[3]

Several studies showed that orthodontic attachments caused an increase in occurrence of cariogenic *Streptococcus mutans*. The number of bacteria was found to be 4 times higher compared with the number at the beginning of the treatment.[4,5] Furthermore, some studies showed that nonoral, opportunistic pathogens such as *Staphylococcus* spp and *Candida* spp can be found on retainers and on the superficial mucosa of the mouth.[6] Periodontitis results from dynamic interactions between the highly complex oral microbial community and the immune system of the host; its etiology is still not fully understood.[7]

Specific subgingival colonization with *Aggregatibacter actinomycetemcomitans* was confirmed and validated in a study in which young patients obtained orthodontic attachments.[8]

The importance of monitoring periodontal-pathogenic bacteria was demonstrated in a recent study.[9] The authors found the contribution to inflammation of an "accessory pathogen" (ie, the naturally harmless *Prevotella nigrescens*). In addition, in an in-vitro study, the same group showed that interactions existed between the cariogenic bacterium *S mutans* and the pathogenic yeast *Candida albicans* via the quorum-sensing system in the biofilm. An earlier study concluded that these complex interactions can extensively influence the occurrence of *S mutans*, and hence its cariogenic potential.[10] The inflammatory reaction of the gingiva at the beginning of treatment can develop into chronic periodontitis.[11] Chronic periodontitis affects nearly 750 million people and is thus 1 of the most prominent infections worldwide.[7] It leads to decay of bone matrix and collagen and subsequently a loss of tooth attachment. It is assumed that in 80% of infectious diseases, biofilms are involved.[12]

The aim of this study was to analyze qualitative and quantitative changes in the bacterial species pattern collected from plaque located in cavities at the tooth surface where the orthodontic appliances are fixed. In parallel, oral health parameters were monitored by the clinical indexes Gingival Index (GI), Periodontal Screening Index (PSI), and Sulcus Bleeding Index (SBI). This analysis considered the early phase of potential biofilm formation during the first week. Several studies also investigated the early treatment period by analyzing supragingval and subgingival samples and gingival conditions.[13-15] It seemed especially interesting to see whether during the initial phase, anaerobic conditions in the emerging biofilms would be sufficient to promote growth of anaerobic key bacteria that can cause periodontitis: *Tannerella forsythia*, *Treponema denticola*, and *Porphyromonas gingivalis*.[1] Samples were taken from plaque developing in the niches formed by the metal surface of the fixed orthodontic appliances and the adjacent tooth surface. Periodontal pockets were not considered. Furthermore, we aimed to investigate whether the findings would be different in the anterior bracket or posterior orthodontic band region. Identification of the microbial species was performed by matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF) of each colony appearing after cultivation of the collected samples, thus allowing quantitative determination of the species. In contrast to deoxyribonucleic acid (DNA)–based techniques, in which dead or lysed bacteria and DNA fragments fixed in the biofilm would also result in signals, only living bacteria with the capacity to grow appeared in the results. It was hypothesized that after the first week of treatment, important clinical and microbiological changes in oral health parameters might already have occurred.

MATERIAL AND METHODS

Patients and indexes

We recruited 10 patients, with an average age of 14.8 years, who needed to undergo orthodontic treatment with buccal fixed appliances at the Department of Orthodontics, University Hospital of Jena, Germany. Before enrollment into the study, the gingival condition of subjects was verified to be healthy (periodontal pockets $\leq$ 3.4 mm, PSI $\leq$ 2). Individuals with significant systemic disease, antimicrobial and anti-inflammatory therapy within the past 6 months, periodontal pockets $>$ 3.4 mm, or PSI $>$ 2, and smokers were excluded. Gingival conditions were assessed by GI, PSI, and SBI (modified after Löe and Silness).[16] Gingival indexes were determined at every time point of microbial sample collection.[17]

For the orthodontic treatment, metal brackets (Dentaurum, Ispringen, Germany) were bonded directly with composite resin (Transbond, 3M Unitek, 3M Dental Products, Monrovia, Calif) on incisors and premolars.

Before fixation, the orthodontic appliances were chemically sterilized by treatment with ethanol to

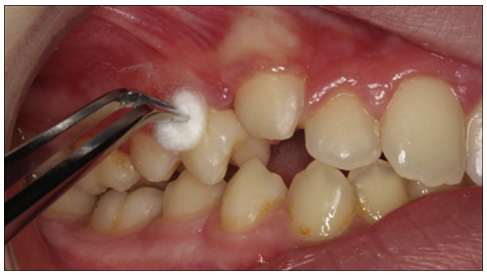

Fig 1. Sample collection with sterile cotton pads before insertion of fixed orthodontic appliances.

prevent resp. minimize the introduction of external bacterial species into the patient, which could potentially influence resident bacterial flora at the beginning of the measurement at time point T1. After disinfecting with ethanol and drying the molar bands (Ormco, Orange, Calif), the bands were cemented (Ketac Cem, 3M Company, St. Paul, MN) on the first and second molars. The archwires were ligated using elastics (3M Unitek).[1] The study protocol was prepared in accordance with the declaration of Helsinki. Ethical approval was obtained from the ethics committee of the University of Jena (4769-04/16).

Sample collection

Sampling was performed during normal orthodontic treatment. Microbial samples were collected from the mesiobuccal side of the maxillary right first molar and the buccal side of the maxillary right first premolar immediately before (time point T1) and 1 week after (time point T2) insertion of orthodontic appliances (Fig 1).[1] Plaque samples were collected from the tooth surface at T1 and from niches (ie, the border formed by the metal, the composite [Transbond] resp. cement [Ketac Cem], and the tooth surface at T2. The size of the sterile cotton tips and the pressure limited the location of sample removal to a specific constant area. Because we were interested in microbial colonization of the niches, further differentiation of sample removal separately from the 3 surfaces (metal, composite–cement, and tooth surface), which are closely adjacent to each other, was not within the scope of our study. Bacterial cell densities were determined on orthodontic molar bands and compared with those at the premolar metal bracket of a quadrant. Samples for analysis of aerobic bacteria were collected from the indicated loci with sterile cotton pads, quickly dried, and immediately transferred into sterile Eppendorf tubes containing 150 µL buffered saline (phosphate-buffered saline). Samples for analysis of anaerobic bacteria were transferred instead into 150-µl thioglycolate medium (Oxoid, Thermo Fisher Scientific Inc., Waltham, MA). To ensure equal sample sizes, sterile standard cotton tips were used, and the tooth surface area was swabbed once.

Isolation of bacterial strains and analysis by mass spectroscopy

For analysis of aerobic bacteria 100µl of the bacteria-containing phosphate-buffered saline samples were plated in dublicate on sheep blood agar (Oxoid) after a series of appropriate dilutions (10^{-1}-10^{-5}) at time point T1 and T2. Samples taken at T1 served as control. After 24 hours in a 37°C/5% CO_2 incubator, the agar plates were evaluated, and the colony-forming units (CFU) were counted (Fig 2).[18] For analysis of anaerobic bacteria 100µl of the thioglycolate medium taken at time point T1 and T2 were plated on Schaedler agar plates (Oxoid). Incubation was performed under strict anaerobic conditions (AnaeroGen2.5L, Thermo Fisher Scientific, Waltham, MA) for 48 hours.[19] To ensure that the cultivation procedure was sufficient to enable the growth of strict anaerobic bacteria, *Veillonella parvula* was added as a representative for oral anaerobic bacteria from stock cultures to control samples and was processed as described. Individual bacterial species were identified from this initial plate in a second round of agar plating: each single colony from the initial agar plates (Fig 2) was plated again on sheep blood agar and incubated for 24 hours in a 37°C/5% CO_2 incubator.

Afterward, the samples were analyzed with MALDI-TOF. This method allows the direct analysis of protein profiles of individual bacterial species.[20] Identification of bacterial species was performed by a 3-step process. Most measurements identified positively charged ribosomal proteins, resulting in a species-specific pattern of informative peaks. This "molecular fingerprint" was compared with an integrated reference database and evaluated with the help of algorithms, resulting in a score that presented the best match with 99.9% accuracy (Fig 3). With growing experimental experience, the bacterial species could be identified on the initial sheep agar plate by colony appearance; in any case, identification was verified by MALDI-TOF.[20]

Identification of bacterial species by MALDI-TOF required 2 cultivation steps to obtain a sufficient amount of the isolated bacteria originating from a single colony. Oral anaerobic bacteria (eg, *Fusobacterium nucleatum* was expected) could not be detected, but the anaerobic gram-negative control species *V parvula* added to the samples was identified

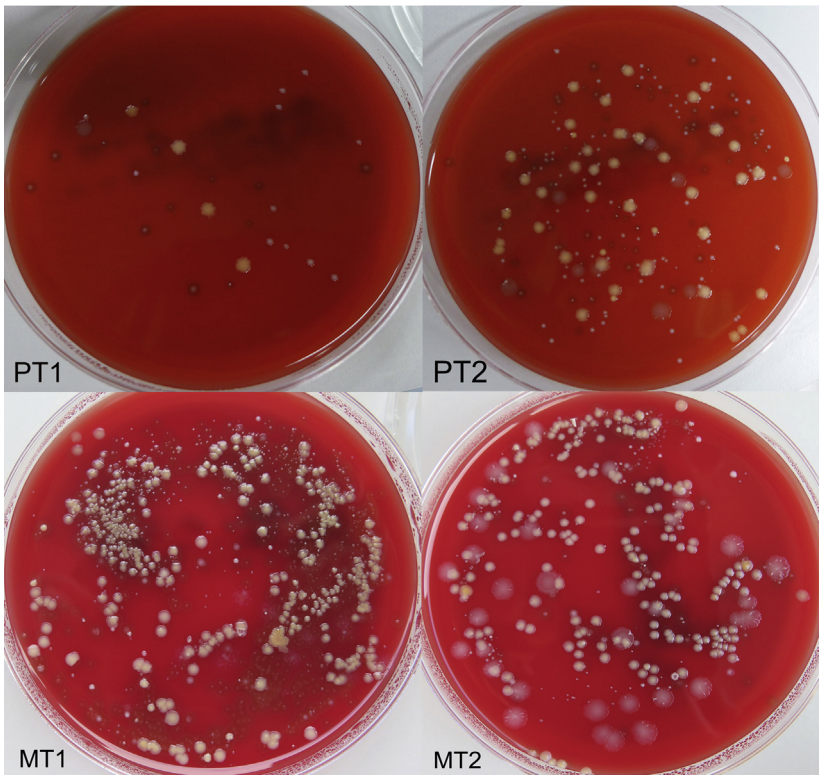

Fig 2. Total bacterial count at premolars and metal brackets (P) and molars and molar bands (M) before (T1) and after (T2) insertion of fixed orthodontic appliances.

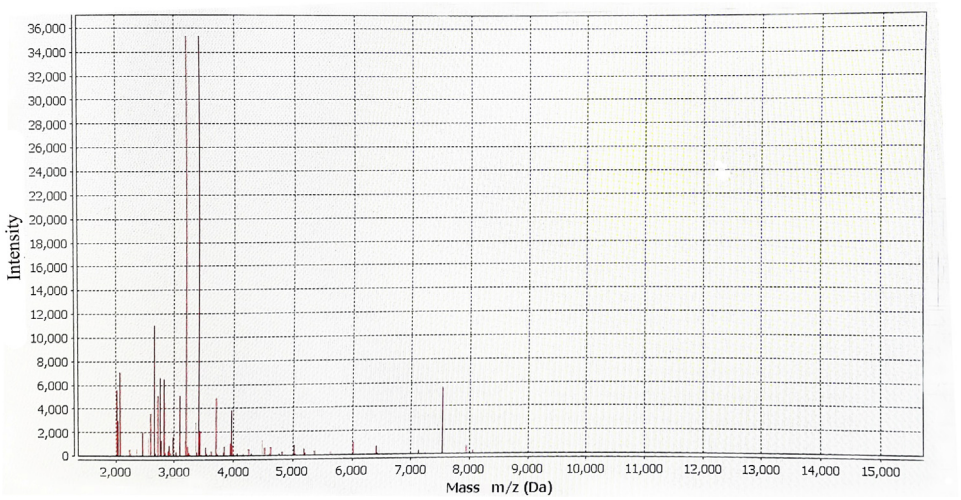

Fig 3. Identification of bacterial species by MALDI-TOF.

by MALDI-TOF. Thus, this control indicated that the procedure was suitable to find strict anaerobic gram-negative species as well. Although the colony appearance on sheep blood agar differed from that on the Schaedler Agar plates in the MALDI-TOF analysis of colonies from both media, only the facultative anaerobic species could be identified. Cultivation on Schaedler medium for 48 hours is generally recommended as a standard procedure for that purpose; incubations longer than 48 hours were not tested. Within the sensitivity limits of methods applied in this work, in all experiments only bacterial species

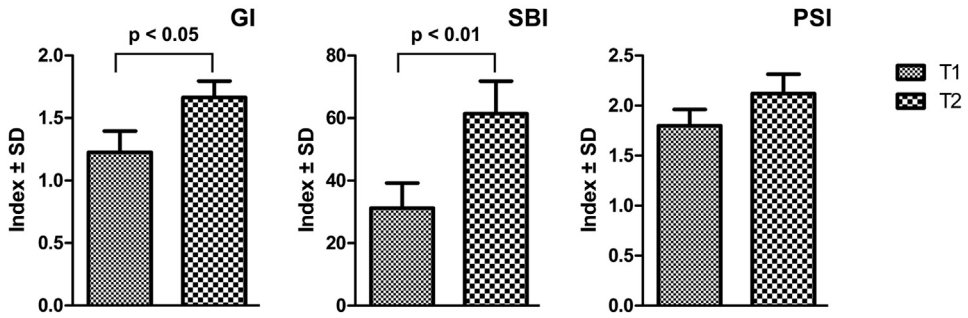

Fig 4. Increase in GI, SBI, and PSI before and after insertion of fixed orthodontic appliances (T1 and T2). GI: $P < 0.05$, SBI: $P < 0.01$. *SD*, standard deviation.

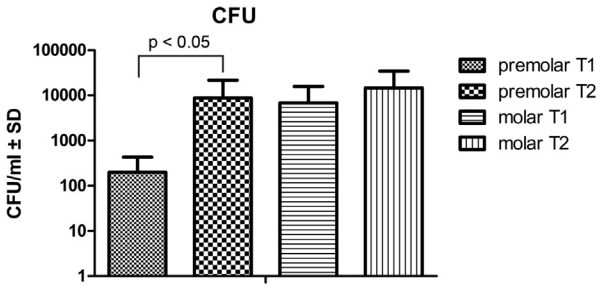

Fig 5. Bacterial count (CFU/mL mean). There was a significant increase in bacterial colonization at the premolars ($P < 0.05$) after the insertion of fixed orthodontic appliances. *SD*, standard deviation.

indicated in the Results section could be identified. Gram-negative anaerobic species did not occur at the loci from which the samples were collected.[19]

Statistical analysis

The *t* test (Wilcoxon) was used to analyze paired samples. Differences were considered significant at $P < 0.05$. All statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc, San Diego, CA).

Statistical justification

For CFUs, a Poisson distribution with a range of 20% measured in the experiments justified statistical analysis. Sample size was proven by Wilcoxon rank sum test comparing 2 groups (n = 20, 10 samples per tooth surface and time point), which resulted in $P < 0.05$ and a power of > 0.8.[21]

RESULTS

Determination of gingival indexes

After the insertion of fixed orthodontic appliances, we determined the typical inflammatory symptoms according to the clinical parameters established for gingival observation. Within 1 week, we observed an increase in plaque accumulation (GI), bleeding on probing, and pocket depth (PSI). Five patients showed an enhanced PSI until code 2, and 3 until code 3. The SBI was enhanced with a statistical significance 30% to 60% ($P < 0.01$) (Fig 4). In comparison, a 2-fold–enhanced SBI was detected in patients without bleeding at the beginning of the study at T1 and in patients with generally poorer oral hygiene. In addition, the GI increased statistically significantly ($P < 0.05$), and PSI increased from the first to the second time point (Fig 4).

Effects on total bacteria count

In parallel, the occurrence of biofilm-forming bacteria was determined. A higher number of bacterial species was detected after the insertion of the fixed orthodontic appliances. This increase was higher in the anterior region than in the posterior area (Fig 5).

There was a significantly lower bacterial count at the premolars than at the molars at T1. After 1 week of fixed orthodontic appliance insertion (T2), we also noticed an increase in bacteria at the molars compared with the premolars.

Microbiological investigation showed a significant increase in bacterial colonization from 1×10^2 CFUs/mL to 1×10^4 CFUs/mL at the premolars ($P < 0.05$) after insertion of fixed orthodontic appliances. The better initial conditions at the metal bracket worsened greatly, as demonstrated by a pronounced increase in CFUs, which reached the same level seen on the molar bands after 1 week. Between T1 and T2, an increase at the metal bands fixed on the molars was also observed.

Analysis of bacterial species

Dental plaque–forming bacteria showed a characteristic distinction in the bacterial spectrum (Fig 6).

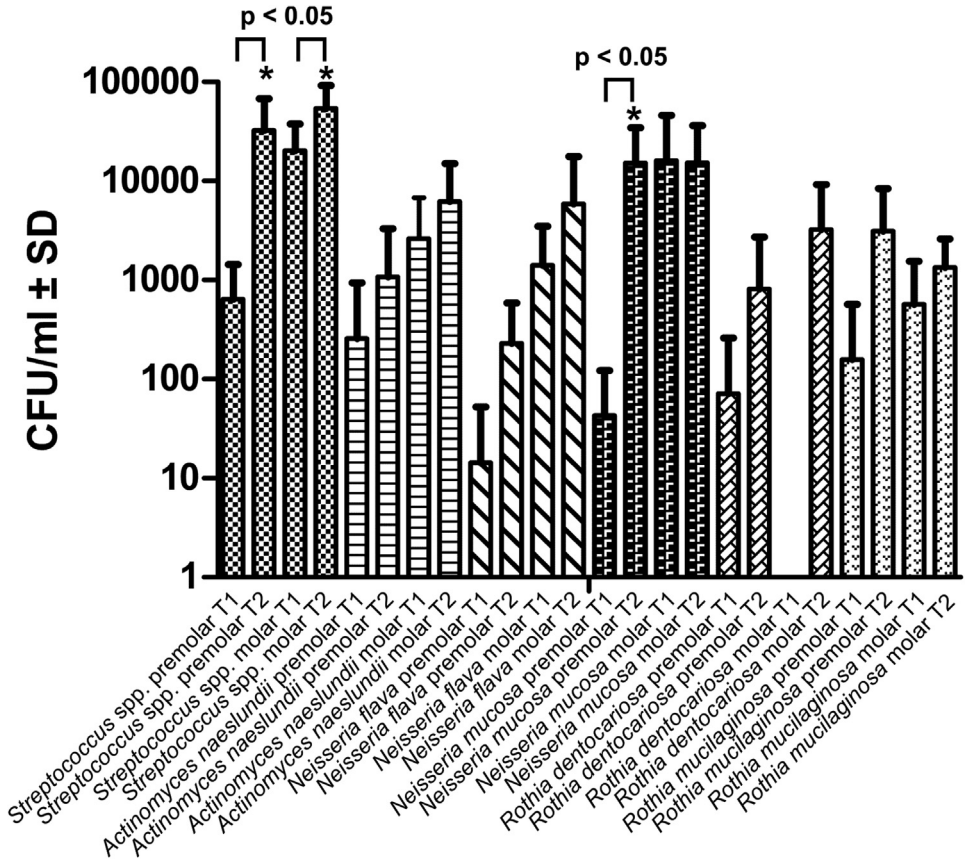

Fig 6. Occurrence of bacterial species before (T1) and after (T2) the insertion of fixed orthodontic appliances at premolars and molars detected by MALDI-TOF. There was a significant increase of Streptococcus spp at premolars and molars and of Neisseria mucosa at premolars after insertion of fixed orthodontic appliances (*P <0.05). *SD*, standard deviation.

Colonization resulting from individual CFU of cariogenic *Streptococcus* spp doubled at the molar band (from 2×10^4 CFU/mL to 3.7×10^4 CFU/mL) and increased exponentially from 0.06×10^4 CFU/mL to 3×10^4 CFU/mL at the premolars after insertion of fixed orthodontic appliances (statistically significant at P <0.05: premolar T1 vs premolar T2, molar T1 vs molar T2, premolar T1 vs molar T1, and premolar T1 vs molar T2). An approximately equal number of *Streptococcus* spp was found at the premolars and molars after 1 week of therapy with fixed orthodontic appliances (Fig 6).

Actinomyces naeslundii are gram-positive, rod-like bacteria and are facultatively anaerobic. They represent 1 of the early colonizers of dental plaque and are important in the succession stages of biofilm formation.[22] *A naeslundii* are also part of the resident and transient normal flora and have been implicated in periodontal diseases as well as in various tooth cavities.[23] They were found in large numbers in every subject. During treatment, this species increased approximately 3-fold at both the premolar bracket (from 0.26×10^3 CFU/mL to 1.07×10^3 CFU/mL) and the molar band (from 2.6×10^3 CFU/mL to 7.7×10^3 CFU/mL), although at the latter location, a larger number of bacteria were found at T1 (Fig 6).

Gram-negative *Neisseria* were detected in each sample. These diplococci normally occur in the upper respiratory tract (*Neisseria flava* or *subflava* and *Neisseria mucosa* or *sicca*). Nevertheless, they had a different distribution after the insertion of fixed orthodontic appliances (Fig 6): there was a significant increase at the premolars (from 0.014×10^3 CFU/mL to 2.9×10^3 CFU/mL; P <0.05) and an increase that was especially visible at the molar band (from 1.4×10^3 CFU/mL to 5.8×10^3 CFU/mL) compared with the starting point at this location.

N mucosa or *N sicca* are aerobic bacteria that colonize mucosal surfaces of humans and animals.[24] In this study, we observed a sharp increase on the premolar

bracket (from 0.042 × 10^3 CFU/mL to 15.1 × 10^3 CFU/mL) and an even distribution on the molar bands (from 16 × 10^3 CFU/mL to 15.3 × 10^3 CFU/mL). Possibly, there was an adhesion of *N mucosa* or *N sicca* before the insertion of fixed orthodontic appliances (Fig 6).

The species *Rothia*, in this case *Rothia dentocariosa* and *Rothia mucilaginosa*, were found in every subject. *R dentocariosa* was increased on the metal bracket (from 0.07 × 10^3 CFU/mL to 0.81 × 10^3 CFU/mL) compared with the molar band (from 0 to 3.2 × 10^3 CFU/mL) where *R dentocariosa* was newly found 1 week after insertion (Fig 6). *R mucilaginosa* occurred from the beginning in every subject at both locations. At the premolar brackets, this bacterial species showed a 3-fold increase (from 0.15 × 10^3 CFU/mL to 3.1 × 10^3 CFU/mL) during orthodontic treatment, whereas there was only a 2-fold increase at the molar band (from 0.57 × 10^3 CFU/mL to 1.34 × 10^3 CFU/mL).

DISCUSSION

This study examined the effects of orthodontic therapy on the oral bacterial milieu during the early treatment period. Our results indicated that as early as 1 week after the insertion of fixed orthodontic appliances, significant changes occurred in the microbial plaque milieu at the metal surfaces and the adjacent tooth surface. At this location, the fixation of bands and brackets led to a prominent change in the microbial environment: a massive but different surface extension, depending on the design of orthodontic brackets and bands as well as a change in roughness caused by the composite resp. cement. The impact of the metal and the composite/cement material on the adjacent tooth surface, potentially facilitating plaque formation, was of major interest in our study.

Bands were fixed by cement in the posterior region, and brackets were fixed by composite in the anterior region. Both substantially enlarged the surface size, potentially supporting bacterial colonization. Although cement causes enhanced roughness compared with composite, brackets provide numerous niches so that microbial biofilm and plaque development benefits even more in the anterior location. This was reflected by our results in a comparison of the CFU (Fig 5). Our finding is supported by Hu and Featherstone,[25] who reported that plaque accumulation is especially higher at brackets and approximal regions and around the gingival region. Because of hyperplasia of the gingiva that covered the tooth surface, it was not possible to collect supragingival samples only from the tooth surface at premolars and molars. In the study by Sukontapatipark et al,[26] extracted premolars that were bonded with metal brackets or not were analyzed by scanning electron microscopy at 1, 2, and 3 weeks after bracket bonding. Similar to our results, the 1-week group showed areas of plaque accumulation especially underneath the bracket wings and ligatures and on the composite surface. Thus, plaque accumulation occurred not only on the metal surface of the brackets but also on the ligatures and neighboring tooth surface. This can lead to demineralization and white spot lesions followed by caries. Gorton and Featherstone[27] as well as O'Reilly and Featherstone[28] found demineralization by 4 weeks after the insertion of fixed orthodontic appliances. Øgaard et al[29] reported that 50%-70% of orthodontic patients developed an initial carious lesion during orthodontic therapy. In addition to changes in the microbial milieu, the determined clinical indexes defining gingival inflammation were significantly increased in our study. One week after the fixed orthodontic appliances were inserted, clear symptoms of gingivitis were observed in all patients. This clinical observation is in accordance with the findings of Deinzer et al,[30] who demonstrated that in contrast to experimental gingivitis, established chronic gingivitis in a natural setting appears as a steady-state inflammation reaction 4 weeks after implementation of fixed orthodontic appliances. According to our observation, there may be 4 reasons for the enhanced adhesion of bacteria: (1) enlarged metal surfaces have more niches on the fixed orthodontic appliances, where biofilms may adhere more easily; (2) oral hygiene is more difficult and laborious; (3) bacterial species adhere to other bacterial cells, thus forming special population structures in biofilm communities (ie, cells of *S mutans* adhere to other immobilized oral bacteria); and (4) biofilms with mixed microbial communities are often thicker and more stable than monospecies biofilms.[2] From the data presented in this study and those mentioned earlier, it can be assumed that after a rapid quantitative and qualitative change in the microbial composition at the beginning of therapy, a new microbial equilibrium is built. This remains active as long as the orthodontic appliances are fixed (lasting ≥2 years), causing clinical findings (enhanced occurrence of *S mutans*) as reported by Rosenbloom and Tinanoff.[5]

Despite comprehensive instructions given to patients before starting treatment, as was reported in other studies, most patients did not carefully conduct oral hygiene.[30] The young adults showed an additional deficit concerning tooth brushing in the molar region, which was also seen in a study of Boyd and Baumrind,[31] who compared plaque accumulation and gingival inflammation between adolescents and adults at molar bands. One aim of this study was to investigate differences between the anterior bracket and the posterior orthodontic

band region because we observed a reduced manual skill to brush sufficiently especially in the premolar regions. Overall, the total bacterial count significantly increased in the anterior regions compared with to the posterior ones (Fig 5). Accordingly, more microbial cells accumulated at the premolar brackets at T2 than at the molar bands (Fig 5). This result is in agreement with the findings of Hu and Featherstone,[25] who demonstrated that the highest risk for demineralization during orthodontic therapy occurs at incisors in the maxillary arch and canines and premolars in the mandibular arch. The demineralization is correlated with more plaque accumulation.[32] Øgaard et al[29] therefore recommended clinical observation for patients because the authors found an increase in plaque accumulation accompanied by an increased occurrence of *S mutans* and low patient compliance during the first 3 months of orthodontic therapy. Thus, during the early period, and as shown in our study, by the first week it is important to assess "the orthodontic higher-risk patients."

Interestingly, we found that the number of individual bacterial species did not increase proportionally. Instead, the growth of some species was more accelerated than for others. Notably, among them were potentially cariogenic species (such as *S mutans*) among the *Streptococcus* ssp, which developed predominantly on the metal brackets (Fig 6). Most importantly, species analysis by MALDI-TOF of the bacterial colonies showed specific changes in the bacterial spectrum in the selected surface sites. The *Streptococcus* spp showed the highest colonization on the investigated teeth bonded resp. cemented with brackets and bands. Thus, at T2, the level of colony counts of *Streptococcus* spp was similarly high at the premolars and molars (Fig 6). This result confirms the observation of other authors who found that orthodontic attachments caused an increase in the occurrence of cariogenic *S mutans*.[5,33]

Accordingly, our results showed that the formation of biofilms promoted by fixed orthodontic appliances appeared to support the growth of *Streptococcus* species selectively by 1 week of treatment. This may lead to a higher risk for the formation of caries (and later, white spot lesions) in patients with orthodontic appliances.

Neisseria ssp is commonly isolated from infections such as endocarditis, bacteremia, and meningitis and from biofilms on catheters.[24] Usually, the occurrence of this species is harmless, although it can cause problems in immunosuppressed patients or in episodes representing multiple infection. In our study, *N mucosa* was found predominantly at molars with fixed metal bands (Fig 6). Starting from a preexisting high colonization, the posterior regions did not show a significant increase in CFU compared with samples from premolar regions. Presumably, there was an adhesion of *N mucosa* at the molars before the insertion of fixed orthodontic appliances. After 1 week of therapy with fixed orthodontic appliances, similarly high numbers of *N mucosa* were detected on the premolars and molars. Interestingly, the bacterial species *N flava* showed a distinct increase at the molar band (Fig 6). These results measured after 1 week are in principal accordance with a study by Koopman et al,[34] in which a significant increase in *Neisseria* colonization was still present after 12 weeks of orthodontic therapy.

The cariogenic *Rothia* spp are commonly isolated from caries lesions and occasionally from abscesses, septicemia, or endocarditis of patients with immunodeficiency.[35,36] In our study, *R dentocariosa* and *R mucilaginosa* were strongly increased in the anterior as well as the posterior regions (Fig 6). These results show that the colonization dynamics of the different bacterial species of the oral flora generally needs to be analyzed specifically.

This was also the case for *A naeslundii,* which showed an increase during 1 week of orthodontic therapy, especially in the molar region (Fig 6). *Actinomyces* are gram-positive bacteria that can be involved in dental caries and periodontitis and cause actinomycosis.[23]

In all subjects included in this study, poor oral hygiene was observed. This was only partly because of increasing difficulties in cleaning the tooth surfaces properly with the fixed orthodontic appliances in place. In addition, a lack of compliance regarding oral hygiene was stated in most patients. Hence, the rapid change in the bacterial milieu can be attributed to some extent to this avoidable shortcoming although it cannot sufficiently explain the disproportional colonization by different bacterial species.

Because oral biofilms on the tooth present a complex interaction of different bacterial species, we analyzed the dynamics of all species occurring in the samples by cultivating the microorganisms, followed by identifying species of all isolated colonies with MALDI-TOF. Thus, solely living microorganisms appear in the results. We chose this experimental approach to exclude signals originating from the DNA of dead or lysed cells resp. DNA fragments that might be fixed in the rapidly emerging biofilms and thus be present in the collected samples. It seemed especially interesting to analyze solely living microbes qualitatively and quantitatively because supragingival plaque is capable of releasing bacteria.[37] The released species may include anaerobic periodontal pathogens that occur deep in the plaque. If present, they may be released via water channels in the biofilm structure. Kim et al[1] demonstrated that by 1 week of orthodontic therapy, the clinically important

periodontal species *T forsythia*, *Campylobacter rectus*, and *P nigrescens* were enhanced in the periodontal pockets. Thus, it seemed important to determine whether these gram-negative species could be released from the developing plaques we analyzed. Despite many attempts including controls with *V parvula* to find anaerobic gram-negative pathogens in the samples with established anaerobic cultivation methods, only the facultative anaerobic gram-positive species appeared. We assume that in the emerging biofilm, the composition and thickness of the matrix was insufficient to provide anaerobic conditions at that depth, at least in the first 7 days. According to our results, it seems unlikely that during the early phase of orthodontic treatment, periodontal clinical pathogens would invade the pockets originating from supragingival plaque. Because they could be therapeutically influenced, it remains an interesting problem whether the release of metabolites or toxins from plaques induced by fixed orthodontic appliances can cause the accumulation of periodontal bacteria and gingival crevicular fluid observed in the pockets.[1,15]

We were able to demonstrate that quantitative and qualitative changes in bacterial composition affected clinical biofilm formation and subsequently inflammation after insertion of orthodontic appliances, especially in the anterior region of patients. Based on these findings, it could be shown that orthodontic treatment induced significant changes in the oral microflora and elicited periodontal reactions and caries risk by the first days of treatment. Thus, the fast-adapting bacterial milieu together with inadequate oral hygiene forms a mutual interacting system that can have adverse clinical consequences after a short time.[38]

Limitations of the study

In this study, we measured a small sample size because only a limited number of patients were available who corresponded to criteria for inclusion given under Material and Methods. A larger sample size needs to be addressed in future studies. Nonetheless, the results are justified by the following observations. First, the data are consistent. Interestingly, all patients had an inflammation reaction 1 week after the fixed orthodontic appliances were inserted, which was determined according to the defined GIs. Second, the sample size was apparently sufficient for statistical analysis because the difference at the 2 time points resp. premolars and molars in the Wilcoxon test ($P = 0.05$; power > 0.8 for CFU) was lower than we had expected and reached a value of about 20% (detailed statistical justifications are given in Material and Methods). Third, measurement was performed 1 week after bracket resp. orthodontic band placement because numerous reports studied the effects on the microbiome solely after long-term treatment.

CONCLUSIONS

Orthodontic therapy induces significant changes in the oral bacterial milieu associated with gingivitis and an enhanced risk of cariogenic reactions within the first days of orthodontic treatment. Our results show that 1 week after the insertion of fixed orthodontic appliances, the total number of microbes significantly increased. All species isolated from the plaque were analyzed by MALDI-TOF. Quantitative analysis of the 6 individual species that could be identified revealed that growth of some bacteria more quickly accelerated under the ecologic conditions of orthodontic therapy. In particular, the cariogenic *Streptococcus* spp appeared to be significantly enhanced in microbial plaque isolated from niches between the metal surfaces and the neighboring tooth surface. This rapid process clearly enhances the risk for developing caries. In addition, an increase in bacterial-forming units isolated from premolars with fixed brackets was significantly more pronounced than that isolated from molars with fixed bands. Together with the observed poorer oral hygiene in the posterior region conducted by patients, the risk for developing caries was further enhanced. One week after fixed orthodontic appliances were inserted, clear symptoms of gingivitis were observed in all patients. In addition to changes in the microbial milieu, the determined clinical indexes GI, SBI, and PSI defining gingival inflammation were significantly increased in our study. To reduce the side effects of orthodontic therapy, it is highly important to provide patients with oral hygiene instructions and control patients' oral hygiene before and during therapy, especially in the early phase.

ACKNOWLEDGMENTS

The authors thank the Medical Faculty of the University Jena, Department of Orthodontics, for financial support, Professor W. Reichardt (Jena, Germany), Dr J. Hirschfeld (University of Birmingham, United Kingdom), and Dr R. Benndorf (Ann Arbor, Mich) for critical reading of the manuscript.

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