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Molecular characterization of intact cell-derived and cell-free bacterial DNA from carious dentine samples

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ABSTRACT

Microbial analyzes of carious dentine samples, especially in terms of interventions, represent a challenge due to difficulties in carious dentine sampling particularly with small bacterial DNA contents. Therefore, information about the quantitative reduction of bacterial DNA as well as microbial shifts and differences in diversity correlating with treatment interventions are scarce. In this study, carious dentine samples were collected in a first step in the course of a selective caries excavation at two different deep dentine caries lesions in three patients. Second, after selective caries excavation and sampling of carious dentine, an intervention was performed by applying dental materials onto the remaining carious dentine followed by a restoration of the study teeth with composite fillings. After 8 weeks, remaining carious dentine was sampled and analyzed as described above. The microbial community before and after therapy was analyzed by conventional culture compared to bacterial DNA analyses using 16S rRNA gene based real-time PCR and terminal restriction fragment length polymorphism (T-RFLP) for fingerprinting community changes within carious dentine samples. An ultra-pure workflow allowed the valid comparison of even small carious dentine samples with low DNA contents and the differentiation between intact cell-derived and cell-free bacterial DNA. Intra- and inter-subject related differences in the bacterial DNA content and its composition in deep dentine caries were determined considering the first visits. The ratio of cell-free bacterial DNA and DNA from intact cells decreased in two of three subjects included in the current study from visit 1 to visit 2 with the test substance (1:200 to 1:17) and the control substance (1:82 to 1:7). T-RFLP revealed changes in the bacterial diversity and composition shifts after treatment as well as between cell-free bacterial DNA and DNA derived from intact cells.

The approach of differentiation and quantification of cell-free and intact cell-derived bacterial DNA is reasonable within the investigation of carious dentine samples, especially when considering the effect of an intervention. T-RFLP is principally suitable for the analysis of microbial shifts within carious dentine samples.

1. Introduction

Dental caries is a polymicrobial biofilm disease driven by diet-microbiota interactions that cause the destruction of the mineralized tooth tissue (Bowen et al., 2018; Lamont et al., 2018; Takahashi, 2015; Takahashi and Nyvad, 2011). With the progression of the carious lesions, the demineralized dentine is infiltrated by the bacteria and enzymatically dissolved (Takahashi, 2015).

Deep carious dentine lesions represent a challenge for the treating dentist due to increased risk of pulp exposure, postoperative symptoms and, subsequently, need of endodontic treatment (Bjørndal et al., 2010). Current literature reveals that, in deep carious lesions, selective caries excavation is supposed to show many advantages revealing this therapy strategy to be recommended (Schwendicke et al., 2013a; Maltz et al., 2012; Maltz et al., 2018). Thus, carious infected dentine has to be maintained within the cavity which is a matter of controversial discussion in the scientific as well as practical dental community (Schwendicke et al., 2013a; Schwendicke et al., 2013b; Schwendicke et al., 2015).

For a better understanding of differences within the microbiome of

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carious dentine and possible implications of leaving bacteria underneath dental restorations, microbiological analyzes of carious dentine samples are needed. As was stated by Nyvad et al. in 2013, there is a lack of molecular studies of caries at the site level (Nyvad et al., 2013). This is, firstly, due to difficulties in sampling of carious dentine, especially in concerns of standardization and quantification of sampled material. Secondly, dealing with small bacterial samples, represents a challenge when working with molecular techniques (Nyvad et al., 2013). Especially regarding interventional studies with differing, partially low bacterial DNA contents, false positive PCR results due to ubiquitous bacterial contaminations are challenging (Salter et al., 2014). Consistently, information about quantitative reduction of bacterial DNA as well as microbial shifts and differences in diversity, especially in concerns of treatment interventions are scarce.

Recent studies are available dealing with the correlation between oral biofilm microbiome and caries development which reveal compositional shifts during caries progression (Bowen et al., 2018; Lamont et al., 2018; Takahashi and Nyvad, 2011; Eriksson et al., 2018; Nascimento et al., 2017; Tanner et al., 2018). Microbiota change to more acidogenic and aciduric bacteria (*Streptococcus mutans* and *non-mutans, Actinomyces, Lactobacilli, Bifidobacteria, Scardovia* spp.) and extracellular-polysaccharide production is enhanced, subsequently causing further acidification. This results in a further loss of diversity and a reduction in the levels and metabolic activity of beneficial bacteria. When dentine is exposed, microenvironmental changes occur with bacteriolytic bacteria thriving (Lamont et al., 2018). Therefore, composition of microbiota within carious lesions is mainly different compared to oral biofilm, e.g. plaque (Nyvad et al., 2013). Former studies based on culture-dependent and real-time PCR methodology showed that, due to anaerobic conditions, microbial composition in deep carious dentine mainly consists of anaerobic gram-positive rods (*Propionibacterium, Eubacterium, Lactobacillus, Bifidobacterium*, and *Actinomyces*) (Hoshino, 1985; Martin et al., 2002). Culture-dependent methods have been shown to be limited for evaluating the microbial composition of carious dentine (Nadkarni et al., 2002; Wolff et al., 2010). By culture-independent studies, which were mainly based on the 16S rRNA gene, caries-associated bacteria were found to be diverse (*S. mutans, non-mutans* streptococci, and members of the genera *Actinomyces, Bifidobacterium, Lactobacillus, Propionibacterium, Veillonella, Selenomonas* and *Atopobium*) (Aas et al., 2008; Gross et al., 2010). Kianoush et al. propose a 'substantial core model' for dentine caries including nearly 60% of the taxa associated with caries (Kianoush et al., 2014). This model was confirmed by the findings of other studies (Johansson et al., 2016; Nasidze et al., 2009; Zaura, 2012) but may be ethnicity-specific (Mason et al., 2013). *Lactobacillus* sp., *Prevotella* sp., *Atopobium* sp., *Olsenella* sp. and Actinomyces sp. were found to be the most abundant taxa associated with dentinal caries. pH was shown to be a crucial factor considering microbiome of dentinal caries (Kianoush et al., 2014). *S. mutans* as a primary caries pathogen is questionable in populations which undergo preventive programs compared to lacking caries treatment and prevention (Johansson et al., 2016).

In 2005, Paddick et al. exclusively used bacterial and yeast cultivation to determine the microbial composition under dental restorations. Baseline microbiota predominantly showed bacterial species of the genera *Lactobacilli, Streptococci* and *Actinomyces* that could not be detected in samples after restoration (Paddick et al., 2005). Additional studies exist on carious dentine, using matrix-assisted laser-desorption/ionization-time-of-flight mass-spectrometry (MALDI-TOF-MS) for phenotypic differentiation of bacteria to species and subspecies level. However, they exclusively refer to *Streptococcus mutans* (Rupf et al., 2008) or *Lactobacillus* species (Kneist et al., 2010).

In 2002, Nadkarni et al. established a 16S rRNA gene based real-time PCR method with a broad specificity for the universal detection of bacteria in carious dentine samples (Nadkarni et al., 2002). Primers and probes used in their assay showed highest coverage of the bacterial taxa and have thus been adapted to a variety of clinical specimens (Horz et al., 2005; Krohn et al., 2014). Preza et al. used 16S rRNA based microarray analyses of root caries lesions in elderly (Preza et al., 2009; Preza et al., 2008) which showed inter-individual differences considering the bacterial community in root caries (Preza et al., 2008). Munson et al. analyzed the microflora within dentinal carious lesions using 16S rRNA gene sequence analysis and found it to be diverse (Munson et al., 2004). In carious dentine lesions of a Japanese population bacterial composition with predomination of *Atopobium* or *Propionibacterium* was found by pyrosequencing (Obata et al., 2014). T-RFLP, over 20 years ago applied for the investigation of community structures in environmental samples (Liu et al., 1997; Kitts, 2001), has been spread in clinical investigations of the gut (Andoh et al., 2014; Koido et al., 2014), the skin (Camarinha-Silva et al., 2012; Dekio et al., 2007), the lung (Jung et al., 2016) but also the oral microbiome (Sakamoto et al., 2005; Abe et al., 2018) and is nowadays by and by replaced by high-throughput sequencing techniques (de Vrieze et al., 2018). Surprisingly, it has not been used for analyzing the microbial composition of carious dentine so far.

Aim of the present study was the combination of ultra-pure bacterial DNA isolation with its subsequent quantification for the valid analyzation and comparison of even small carious dentine samples with low DNA contents. Additionally, differentiation between cell-free DNA and DNA from intact bacterial cells was performed to assess a possible antibacterial effect of dental materials on the remaining carious dentine. Furthermore, T-RFLP based on ultra clean PCR was established as a fingerprinting method to enable analyzing shifts of the carious bacterial community due to treatment therapy. By this, changes in the bacterial quantity and composition of carious dentine samples before and 8 weeks after selective caries excavation were monitored.

2. Material and methods

2.1. Subjects

In this analysis, three participants of a clinical study (patient inclusion was performed between March 2016 and May 2017 in the Department of Cariology, Endodontology and Periodontology, University of Leipzig) were included. The study was designed as a randomized controlled interventional study and was approved by the ethics committee of the Medical Faculty of the University of Leipzig (368–15-05-10-2015). Patients with deep primary carious lesions at two teeth (molars and/or premolars) with radiographic extent up to the inner third of the dentine but with a radiopaque band representing an intact dentin barrier to the pulp were considered to be included. No pulpal symptoms and given sensitivity (proven by testing with CO_2 snow) were mandatory required for inclusion. Patients were informed verbally and in writing about the study course and scientific use of the study. Written informed consent was obtained.

Trouble shooting Extension of carious lesions, finding of two asymptomatic but extended carious lesions within one patient.

2.2. Clinical workflow

The study protocol is given in Fig. 1. The study teeth as well as their neighboring teeth were cleaned before starting caries therapy. Caries therapy was performed in local anesthesia. In each patient both of the lesions were treated under application of rubber dam for the purpose of controlling contamination by saliva or biofilm from the oral cavity. All patients were treated by the same experienced clinician (LB) with solid knowledge of caries excavation and after a pre-study training on sampling carious dentine. The treatment was performed under 2.3 × magnification. Following preparation to a caries-free enamel-dentine junction by a water cooled sterile diamond bur (diameter 012, Komet Gebr. Brasseler GmbH& CO. KG, Lemgo, Germany), in a red handpiece (30.000 rounds per minute), and the removal of the very superficial caries layer, which was discarded, a sample of the mediate

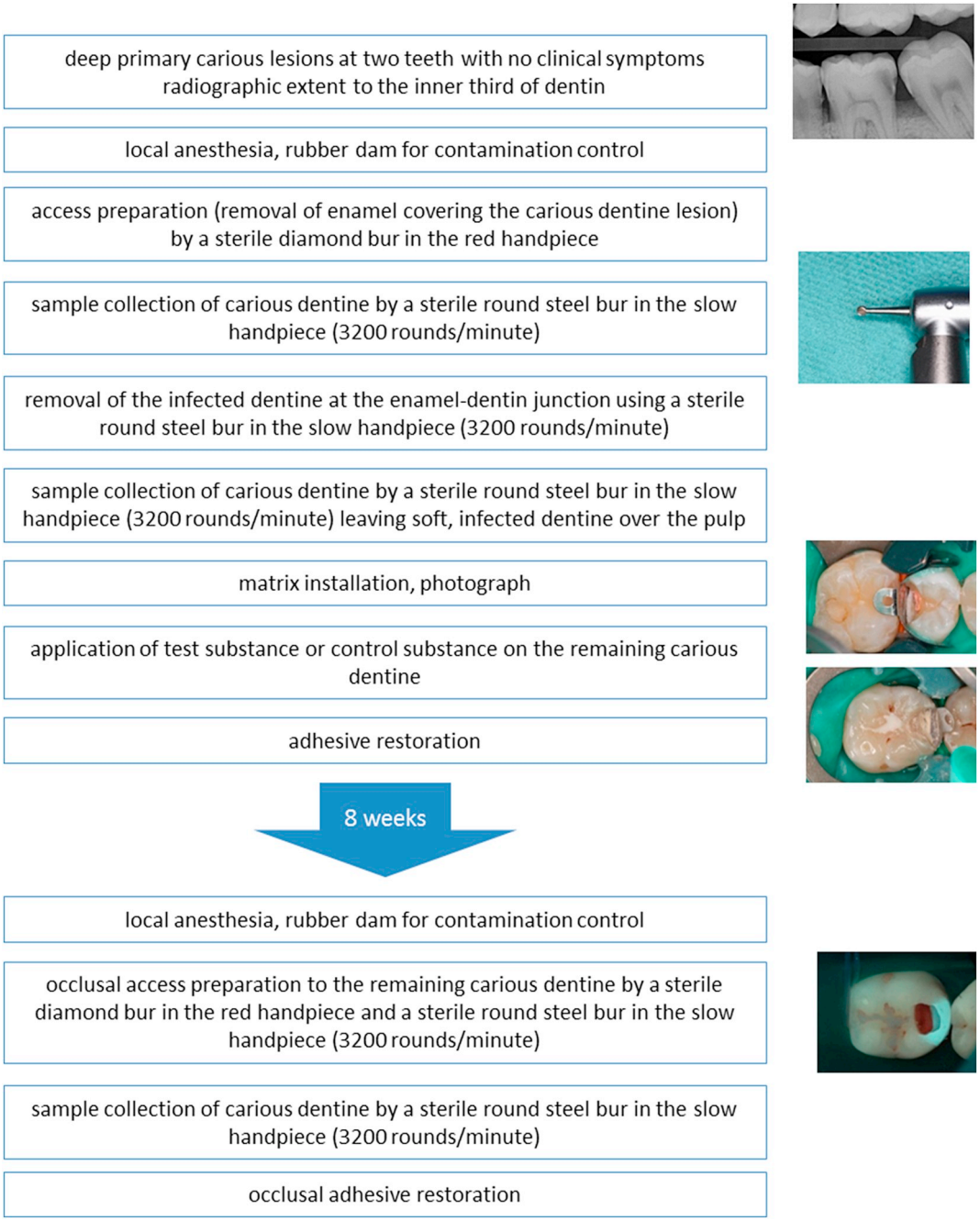

Fig. 1. Clinical workflow for the treatment of the dentine caries lesions, including sample collection and intervention.

caries (***MC***) layer was taken with a sterile standardized round bur (size 018, Komet, Gebr. Brasseler GmbH & CO. KG, Lemgo, Germany). A sample of the deep caries (***DC***) layer was collected in the center of the cavity, again with a sterile round bur of the same standardized size. It was assured that there was remaining soft carious dentine in the center of the cavity following sampling of DC. After randomization, the remaining carious dentine was covered with a calcium-silicate based test substance (***TS***, Biodentine, Septodont, Niederkassel, Germany) in one lesion and in the other one with a control substance (***CTR***, thermo-plasticized gutta-percha) as follows: For the application of Biodentine, the product was processed as recommended by the manufacturer. Subsequently, residual soft carious dentine was covered with TS and, as recommended by the manufacturer, 12 min setting time was kept before further treatment of the cavity. For treating the control cavity, gutta-percha was thermo-plasticized at 170 °C within the Beefill device (VDW, Munich, Germany). Out of the cartridge (0.45 mm diameter Beefill cartridge; VDW, Munich, Germany) it was applied on a spatula and then adapted on the residual soft carious dentine. In both cavities, Optibond FL (Kerr, Rastatt, Germany) was used as adhesive system for composite restauration (SDR, Ceram X; DENTSPLY DeTrey, Constance, Germany) of the lesions according to manufacturers' recommendation.

Trouble shooting: Differences in initial bacterial load due to intra-individual and inter-individual differences of carious lesions; ensuring the adaption of gutta-percha to the residual carious dentine, especially considering the risk of replacement during the sealing process.

2.2.1. Sample collection

For each lesion, two carious dentine samples were taken within visit I. The first sample was collected after preparation of the entrance cavity (mediate layer of carious dentine) and the second one before covering

the residual carious dentine (deep layer of carious dentine) with either TS or CTR as described above. In accordance to a stepwise caries removal (Innes et al., 2016), after 8 weeks, re-entry was performed in each cavity and a sample of the remaining carious dentine was obtained.

The carious dentine samples were collected with standardized round burs (size 018, Komet, Lemgo, Germany), according to a method which was introduced, validated and applied in former studies (Paddick et al., 2005; Kidd et al., 1993; Ratledge et al., 2001). For each lesion a new, sterile round bur of the same size and manufacturer was used (slow handpiece, 3200 rounds/min) when sampling the carious dentine. The bur was carefully taken out of the handpiece with sterile tweezers and transferred into a COPAN ESwab transport system (Copan Diagnostics Inc., Murrieta, CA) containing 1 ml preservation medium. By this, approximately the same volume of carious dentine was obtained in each sampling series.

Trouble shooting: Different proportions of water, bacterial biomass and degraded dentine lead to differences of carious dentine consistency and composition due to degree of mineralization and fluid content of the carious dentine (lesion consistency variances, especially after 8 weeks) (Kidd et al., 1993); risk of pulpal exposure at re-entry (Schwendicke et al., 2013b; Bjørndal et al., 2017; Bjørndal et al., 2017), no sample volume assessable due to sampling method of carious dentine by round bur.

2.3. Laboratory workflow

After caries sampling tubes were transferred to microbial cultivation and molecular analyses. In all samples cell-free and cell-derived bacterial DNA was detected using qualitative PCR and bacterial DNA positive samples were further analyzed for their DNA content. T-RFLP was used to determine changes in the bacterial community structure of the carious dentine sample (Fig. 2).

2.3.1. Sample processing and bacterial culture

The tube containing the bur was vigorously vortexed for 1 min to dislodge the adherent carious dentine bringing it into solution.

For cultural differentiation 20 µl suspension were used. For aerobic growth 10 µl were pipetted and streaked onto a ready-to-use Columbia blood plate (containing special peptone 23 g, starch 1 g, sodium choride 5 g, agar 11 g, defibrinated sheep blood 70 ml per litre; Oxoid, Wesel, Germany) and for anaerobic growth same was done on ready-to-use Brucella plates with hemin and vitamin K1 (containing pancreatic digest of casein 10 g, peptic digest of animal tissue 10 g, yeast extract 2 g, glucose 1 g, sodium chloride 5 g, sodium bisulfite 0.1 g, hemin 0.005 g, vitamin K1 0.01 g, agar 15 g, defibrinated sheep blood 5% per litre; BD™, Heidelberg, Germany). Plates were incubated at 35 °C for 4 days, aerobic growth was performed under 5% CO_2. For pure isolates, colonies were transferred to a fresh Columbia blood and Brucella plate, respectively, and incubated at 35 °C for max 48 h. Isolated strains were identified using the Vitek 2 system (bioMérieux, Marcy l'Étoile, France).

Trouble shooting: Small volume (10 µl) for inoculation leads to misdetection of low abundant bacteria, in rare cases limited growth after inoculation onto fresh agar plates, species not identified by the Vitek 2 system or identification solely upon genus level (e.g. *Streptococcus mitis/oralis*).

2.3.2. Sample processing and avoidance of DNA contaminations

Further sample processing and all molecular analyses from this time point were done using ultra-clean DNA free plastic material including reaction tubes, filter tips and PCR plates to avoid DNA contamination from exogenous sources. Handling was performed under a daily UV-irradiated Class II biological sample safety cabinet. A fresh lab coat, sterile protective gloves and a disposable mask were worn for each sample processing.

For molecular analyses residual suspension in the ESwab tube was vortexed and split into two samples of approximately 400 µl to ensure additional analyses in case DNA-isolation was not successful. Each solution was transferred into a 1.5 ml tube. Tubes were centrifuged for 10 min at 27.000 g at room temperature. For analysis of (i) cell-free DNA, supernatant was transferred into a new 1.5 ml tube and stored at −20 °C until further processing. Cell pellet for analysis of (ii) bacterial DNA from intact cells was resuspended in 100 µl supernatant and 50 µl of 100% glycerin was added to avoid lysis of bacterial cells due to

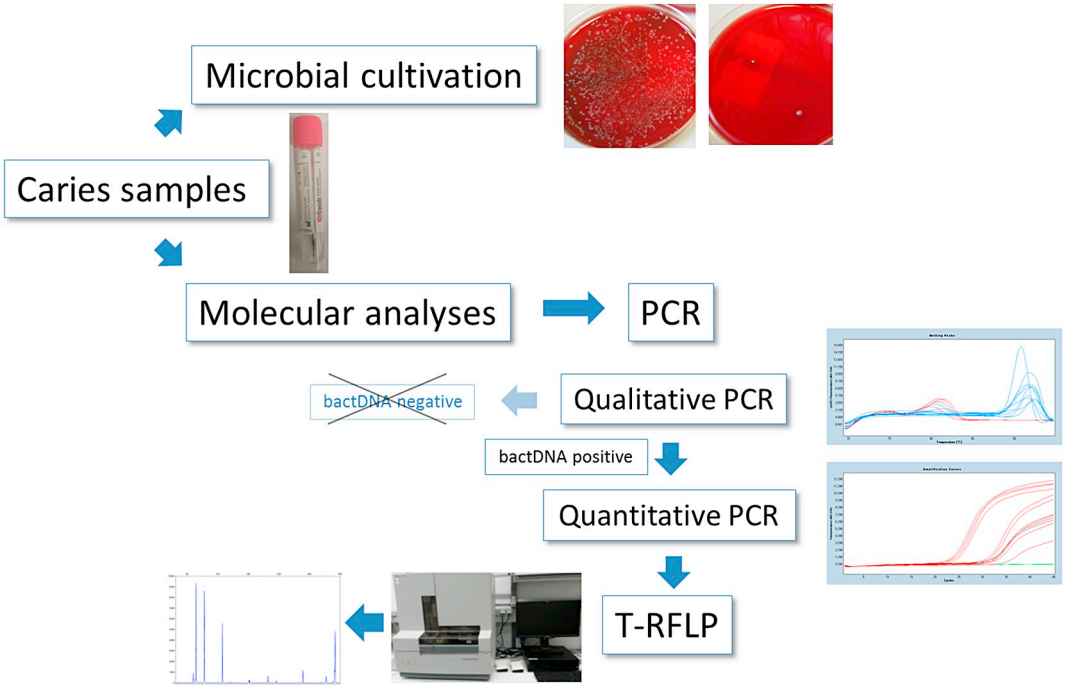

Fig. 2. Laboratory workflow of carious dentine samples.

storing. Samples were stored at −20 °C.

Trouble shooting: Aerosol formation leading to cross contamination between samples (heterogeneity of samples in concerns of bacterial load), incomplete transfer of sample supernatant for determination of bacterial load of cell-free bacterial DNA, fast preparation to avoid cell pellet to go into solution.

Reagents: UV laminar flow workstation, racks, reaction tubes (Biosphere® safe seal tube, Sarstedt, Nümbrecht, Germany), filter tips (Biosphere® Filter tip, Sarstedt); PCR plates (LightCycler® 480 Multiwell Plate 96, Roche, Mannheim, Germany), vortexer, microcentrifuge (> 12.000 × g).

2.3.3. Isolation of bacterial DNA

In order to discriminate bacterial DNA from intact cells compared to cell-free DNA we used two different isolation protocols. For both isolation steps the ultra-clean DNA isolation kit MolYsis Complete5 (Molzym, Bremen, Germany) was used. Here, a DNase treatment prior actual DNA isolation is generally used which depletes free bacterial DNA in the sample. This ensures isolation and quantification of bacterial DNA from intact cells without the detection of already existing cell-free DNA.

Bacterial DNA from resuspended cell pellets was isolated according to manufacturers' instructions. Briefly, each sample was adjusted up to an initial volume of 1 ml with supplied ultra clean buffer (approximately 850 µl). Then samples were treated with a DNase to degrade cell-free bacterial DNA. After enrichment of bacterial cells by centrifugation, cells were enzymatically lysed and DNA was isolated by a bind-wash-elute protocol.

For the isolation of cell-free bacterial DNA, DNase treatment and enzymatic lysis were skipped and 300 µl supernatant was directly used for binding to the silica matrix. DNA isolation started by adding 250 µl chaotropic CS buffer to the sample as recommended in the manufacturers' protocol.

Fetal calf serum (Merck Biochrom, Berlin, Germany) was heated for 20 min at 56 °C and served as negative control. Human serum spiked with *Escherichia coli* at a concentration of 100 cells ml^{-1} was used as positive control in each isolation series.

Spiking experiments with plasmid DNA and/or E.coli cells confirmed on the one hand the efficiency of cell-free bacterial DNA degradation by DNase for quantification of cellular DNA and binding of cell-free DNA to the spin column within the modified isolation version on the other hand.

Trouble shooting: Avoidance of cross contamination of the negative control and elution buffer by handling the reagents directly after UV-irradiation and closing the vials immediately after each working step.

Reagents and supplies: UV laminar flow workstation, racks and cooling rack for 1.5 ml tubes, precision pipettes and DNA-free filter tips for volumes up to 20 µl, 100 µl, 200 µl and 1000 µl, vortexer, bench top microcentrifuge (> 12.000 × g), thermomixer.

2.3.4. Detection of bacterial DNA

The existence of bacterial DNA in carious samples was detected using a 16S rRNA gene-based PCR with SYBR Green and melting curve analysis (Krohn et al., 2014). The ultra-clean PCR mastermix 16S Complete (Molzym) was used, containing broad-range primers covering the V3/V4 variable region of the 16S rRNA gene. Each 25 µl-reaction contained 10 µl mastermix, 0.8 µl Taq-polymerase, 2.5 µl SYBR Green, 1.7 µl PCR-grade water and 10 µl template DNA. Real-time PCR was performed on a LightCycler 480II instrument (Roche) with amplification steps as follows: initial denaturation at 95 °C for 1 min, 45 cycles of 5 s at 95 °C, 10 s at 55 °C and 25 s at 72 °C with a fluorescence acquisition at the end of each 72 °C step, followed by a melting curve analysis program with 95 °C for 1 s, 70 °C for 1 s and heating to 95 °C with a ramp rate of 0.11 °C/s and continuous fluorescence acquisition. All samples were analyzed in duplicates and a PCR no-template control as well as a PCR-positive control containing *Escherichia coli* plasmid DNA was included in each PCR run.

All PCR reactions were subsequently subjected to agarose gel electrophoresis. BenchTop 100 bp DNA ladder in 100 bp increments (Promega, Mannheim, Germany) was used as 100 bp DNA size marker. PCR-positive samples with the appropriate size of approximately 480 bp were purified using the Zymo Clean Gel Recovery Kit (HISS Diagnostics, Freiburg, Germany) and directly sanger-sequenced (GATC-Eurofins, Constance, Germany) to exclude possible contaminations during sample processing.

Trouble shooting: Appropriate material for DNA isolation negative control.

Reagents and supplies: UV laminar flow workstation, racks and two cooling racks for a 96 well PCR plate and 1.5 ml tubes, precision pipettes and DNA-free filter tips for volumes up to 20 µl, 100 µl, 200 µl and 1000 µl, DNA-free reaction tubes for mastermix, vortexer, mini spin microcentrifuge, real time PCR cycler, PCR mastermix 16S complete.

2.3.5. Quantification of bacterial DNA

Bacterial DNA of carious samples with a melting peak at the appropriate temperature and gel band was used for quantitative real-time PCR. The ultra-clean PCR mastermix 16S Complete was used again for quantification of bacterial 16S rRNA genes with addition of a 6-carboxyfluorescein (FAM)-labelled universal hydrolysis probe (Krohn et al., 2014) as described by Nadkarni et al. (Nadkarni et al., 2002). Since the probe was not a component of the ultra-clean mastermix, it was synthesized by Eurogentec (Köln, Germany) and purified using high performance liquid chromatography.

Each 20 µl-reaction contained 8 µl mastermix, 0.64 µl Taq-polymerase, 0.3 µl probe (5 µM), 6.06 µl PCR-grade water and 5 µl template DNA. Amplification steps on the LightCycler 480II instrument were as follows: initial denaturation at 94 °C for 4 min, 45 cycles of 15 s at 94 °C and 60 s at 66 °C with a fluorescence acquisition at the end, followed by a cooling step of 10 s at 40 °C.

A ten-fold diluted plasmid carrying *Escherichia coli* DNA was used to generate standard curves for accurate quantification of the amplified target. All samples were analyzed in duplicates and a PCR-no template control was used.

The limit of quantification in the real-time PCR was 520 copies ml^{-1} which was experimentally determined and confirmed using probit analysis for a different sample set (Engelmann et al., 2016). Since the overall bacterial load will be influenced by the variation of 16S rRNA operons per species, quantification of DNA copy numbers per bur were used instead (Nadkarni et al., 2002).

However, DNA quantities in spiking experiments did show slight discrepancies regarding the isolation/PCR efficiency especially due to insufficient sampling of supernatant for free bacterial DNA quantification, incomplete degradation of free DNA by DNase and lysis of pelleted bacterial cells prior to DNA isolation. These discrepancies, even so all carious dentine samples are affected, should not be disregarded when evaluating cell-free and cellular bacterial DNA quantities.

Trouble shooting: PCR contaminations in negative controls due to 6-carboxyfluorescein (FAM)-labelled universal hydrolysis probe.

2.3.6. Terminal restriction fragment length polymorphism

In order to establish a T-RFLP that discriminates most prominent caries species in our cohort we used cultivated strains from original caries samples (partly not included in this study) for the evaluation of appropriate restriction endonucleases. Most prevalent genera were *Lactobacillus* spp. (*L. casei, L. paracasei, L. gasseri, L. fermentum*), *Streptococcus* spp. (*S. mutans, S. mitis/oralis, S. anginosus, S. constellatus*), *Actinomyces naeslundii, Staphylococcus hominis, Micrococcus luteus, Rothia dentocariosa* and *Veillonella dispar*. DNA from isolated strains was extracted based on microwave exposure. Colonies were picked and resuspended in collection tubes with 50 µl of aqua bidest. For cell lysis, tubes were heated for 56 s at 1050 W in a microwave oven and subsequently chilled on ice. Procedure was repeated once more and followed

Table 1
Results from sequence analysis as well as in silico and measured T-RF from isolated caries species.

Isolated strains	Base pairs	Query coverage [%]	Identity [%]	Accession#	T-RF *FaiI* (measured/in silico) [bp]	T-RF *MluCI* (measured/in silico) [bp]	T-RF *MseI* (measured/in silico) [bp]
Lactobacillus paracasei	471	98	99	MK144803	26.3/37	467.3/471	184.1/187
Lactobacillus fermentum	468	100	99	MK144807	26.4/36	467.2/468	184.6/186
Lactobacillus gasseri	468	100	100	MK144810	44.5/36	172.7/175	466.5/468
Lactobacillus casei	471	100	99	MK144812	44.5/37	467.5/471	184.3/187
Staphylococcus hominis	469	100	99	MK144806	92.9/98	123.4/128	209.3/211
Streptococcus mutans	467	100	100	MK144814	93.2/98	123.4/128	467.0/467
Streptococcus mitis/oralis	468	100	99	MK144805	93.8/98	125.0/128	154.7/157
Streptococcus anginosus	468	100	99	MK144804	92.9/98	124.4/128	154.1/157
Streptococcus constellatus	468	100	99	MK144808	92.8/99	127.2/129	154.4/158
Micrococcus luteus	449	100	99	MK144813	46.3/37	125.2/130	193.6/187
Veillonella dispar	468	100	99	MK144815	467.3/468	123.4/129	177.7/185
Rothia dentocariosa	447	100	100	MK144811	44.5/35	125.3/128	183.5/185
Actinomyces naeslundii	464	100	99	MK144809	461.9/464	241.7/244	187.8/185

by a centrifugation at 4 °C and 21.000 g for 20 min. Supernatant was transferred into a new collection tube. For 16S rRNA gene based sequence analysis of bacterial isolates, DNA was amplified using the primer pair 331f and 797r, for T-RFLP analysis of the isolated strain 5'-FAM-(5-carboxyfluorescein)-labelled 797r reverse primer (Nadkarni et al., 2002) (Eurogentec) was used. PCR amplification was performed on the Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA) using 12.5 µl of the MyTaq™ Mix (Bioline, London, Great Britain), 1 µl of each primer (10 µM) and 5 µl DNA template in a 20 µl-reaction volume. Amplification steps were as follows: initial denaturation at 95 °C for 1 min, 35 cycles of 15 s at 95 °C, 15 s 55 °C and 10 s of 72 °C and a final extension for 10 min at 72 °C. Purified PCR products from 331f-797r amplification were used for sanger-sequencing (Eurofins GATC Biotech). Sequence alignment was performed using the Sequencher 5.4.6 software (Ann Arbor, MI). By using the analytical web program REPK (restriction endonuclease picker) (Collins and Rocap, 2007) we chose *FaiI, MluCI* and *MseI* as the best restriction endonucleases to discriminate diverse caries samples especially with Lactobacilli and Streptococci as dominant genera (Table 1). Purified PCR products from 331f and 5'-FAM-(5-carboxyfluorescein)-labelled 797r were used for T-RFLP analysis with *FaiI* (SibEnzyme, Ludwigshafen, Germany), *MluCI* and *MseI* (New England Biolabs, Frankfurt, Germany) to determine the actual size of the terminal restriction fragment (T-RF) compared to the theoretical size generated by *in-silico* digest of DNA sequences. In-silico and measured terminal restriction fragments (T-RF) showed comparable base pair lengths (Table 1).

Bacterial 16S rRNA gene fragments were amplified with the forward primer 331f and the 5'-FAM-(5-carboxyfluorescein)-labelled 797r reverse primer (Nadkarni et al., 2002) (Eurogentec) at a final concentration of 400 nM each in 25 µl-reactions using the mastermix 16S Basic reagents (Molzym) with 5 µl DNA template. Bacterial DNA positive carious samples showed a DNA content varying from 7.92E + 03 to 3.17E + 08 copies ml^{-1}. In order to receive enough PCR template for performing T-RFLP analysis with three restriction enzymes, a high number of 45 PCR cycles was necessary. The synthesis of the 5'-FAM-(5-carboxyfluorescein)-labelled 797r reverse primer showed contaminated background when performing the 45 cycle PCR for T-RFLP analysis. That is why the Customized Mastermix Purification Service from Molzym GmbH was used based on Mastermix 16S Basic and primers 331f (10 µM) and labelled 797r (10 µM). Using this ultra-clean customized mastermix no contamination could be observed.

PCR-amplification of carious samples was performed on the Veriti 96-well thermal cycler with amplification steps as follows: initial denaturation at 95 °C for 1 min, 45 cycles of 15 s at 95 °C, 15 s at 55 °C and 10 s at 72 °C, followed by a single extension step of 10 min at 72 °C. Purification of PCR products was performed as described above. The DNA content of each PCR product was quantified using the Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

For T-RFLP profiling, 30 ng of PCR product were digested for 3 h with 2 IU of the restriction endonucleases *MluCI* and *MseI* at 37 °C with subsequent heat inactivation of 65 °C for 20 min. Additionally, samples were digested with 0.4 IU of the restriction enzyme *FaiI* for 1 h at 50 °C followed by a heat inactivation of 80 °C for 20 min. All pipetting steps were performed on ice. DNA was precipitated with sodium acetate pH 5.5 and ethanol. DNA pellets of each sample were suspended in 15 µl HiDi Formamide and 0.3 µl MapMarker® X-rhodamine labelled 50–1000 bp standard (BioVentures, Murfreesboro, TN). Samples were denatured at 95 °C for 10 min and subsequently chilled on ice. Using the ABI Prism 3100 genetic analyzer (Applied Biosystems), terminally labelled 16S rRNA gene fragments were separated by capillary electrophoresis. The lengths of labelled terminal restriction fragments (T-RF) within the range of 50 to 500 bp were determined with the Genemapper V3.7 software (Applied Biosystems). Raw T-RFLP signals were separated from noise with a standard deviation of 3 (Abdo et al., 2006). Normalized data were used for creating heat maps with the Bray-Curtis distance (Bray and Curtis, 1957). All analyses were done using the statistical computational R version 2.10.0 (R Development Core Team, 2011) and R package 'gplots' (Warnes et al., 2011).

Trouble shooting: Ensure complete digestion by maintaining the exact incubation time of each enzyme in order to circumvent star activity of restriction enzymes (*FaiI)*.

Reagents and supplies: UV laminar flow workstation, racks and two cooling racks for a 96 well PCR plate and 1.5 ml tubes, precision pipettes and DNA-free filter tips for volumes up to 20 µl, 100 µl, 200 µl and 1000 µl, DNA-free reaction tubes for mastermix, vortexer, mini spin microcentrifuge, real-time PCR cycler, PCR mastermix 16S complete.

3. Results and discussion

3.1. Clinical workflow

In three different patients carious dentine was collected from two different layers of deep dentinal caries at a first visit and 8 weeks after selective caries excavation and coverage of the remaining carious dentine with either the test product or the control substance as illustrated in Fig. 1 and described in the Material and Methods part. Sample collection was performed by standardized round burs as described in literature before (Paddick et al., 2005; Kidd et al., 1993; Vaidyanathan et al., 2009). Bacterial DNA from carious dentine samples was extracted, its DNA content was quantified using 16S rRNA gene based PCR and the bacterial composition within the samples was analyzed using T-RFLP.

The sampling method has to be critically discussed. Due to differences in proportions of water, bacterial biomass and degraded dentine, the quantification of carious dentine is nearly impossible in general. Therefore, quantitative analyses considering bacterial DNA load have to

be interpreted carefully. The sampling procedure applied in the current study was standardized as much as possible by using identical round burs to ensure collection of an equivalent volume of carious dentine within all grooves. In addition, examination and sample collection was performed by the same professional dentist (LB) and under 2.3 × magnification. Alternative sampling methods for gaining carious dentine have been described, especially using hand-excavators (Nadkarni et al., 2002; Munson et al., 2004; Obata et al., 2014). Nadkarni et al. also standardized the carious dentine by weighting the samples and preparing a standard suspension of 10 mg wet wt dentine (ml RTF)-1. However, they analyzed caries samples of extracted teeth (ex-vivo) without any intervention (Nadkarni et al., 2002). Even with this method an exact quantification is limited for the reasons mentioned above. Within the current study, in-vivo investigations at vital teeth under different treatment strategies were performed and sampling with the round bur was chosen for practical reasons.

Regarding the risk of contamination by saliva, sulcus fluid, subgingival plaque or bacteria from the mucosa, rubber dam was applied. At the first visit, due to cavity extension and for reasons of establishing proper restorations using matrix-systems, the study tooth as well as the neighboring teeth had to be included into the rubber dam. However, all tooth surfaces in the operation area were cleaned before preparation and no additional carious lesions with enamel breakdown and treatment indication were available at study patients except of the lesions included into the study.

3.2. Microbiota in carious dentine samples

3.2.1. Culture

Culture was considered as an additional methodological device in concerns of proving bacterial reproducibility within the sample. Culture-dependent methods have been shown to be limited considering gain of information about microbiology, not only in carious lesions but in all biofilms (Nyvad et al., 2013; Wolff et al., 2010; Munson et al., 2004). However, there have been many studies in the past dealing with selective media for growing caries specific bacteria, e.g. *Streptococcus mutans* and *Lactobacilli*, also in the context of interventional studies (Kidd et al., 1993; Vaidyanathan et al., 2009; Wicht et al., 2004; Rolland et al., 2011). Several selective agar media and growth conditions have been introduced when studying certain bacteria from dental carious lesions, e.g. Mitis salivarius agar + 5% sucrose (MSA) and Lactobacillus-selective agar (MRS) (Rolland et al., 2011). In the current study, Columbia blood plates for aerobic and Brucella plates with hemin and vitamin K1 for anaerobic growth were used. Munson et al. analyzed carious dentine samples of teeth with advanced pulpitis because of carious lesions detecting a 6-fold higher growth on non-selective plates compared to microaerophilic conditions (Munson et al., 2004). Within culture performed in this study, different *Lactobacillus* spp., *Actinomyces* spp., *Streptococcus* spp., *Bifidobacterium* spp. and gram-positive bacteria were cultivated which is in line with the findings of studies dealing with culture-dependent methods (Bjørndal and Larsen, 2000).

In Table 2 the qualitative results from bacterial culture are given and found to be negative in three samples, all of them collected in visit II. However, in one of the culture-negative samples culture-independent methods detected bacterial DNA (Subject 2, Control) in contrary to the microbial culture result. Remaining samples showed bacterial growth in the culture. Negative culture results within samples of hard/dry (P3, visit II) and medium/dry (P2, BD) consistency, respectively, are in line with the results of Kidd et al. recovering significantly more bacteria within samples of soft and wet carious dentine compared to medium, hard or dry consistency (Kidd et al., 1993). This finding was also published by other authors who showed that retained demineralized dentine had changed into a harder tissue, and the cultivable flora had declined 4–6 months after initial treatment (Bjørndal and Larsen, 2000).

3.2.2. Qualitative PCR

There have been several studies analyzing carious dentine from a molecular microbiological perspective (Martin et al., 2002; Preza et al., 2008; Munson et al., 2004). However, methodological aspects have to be considered. Within the current investigation, quality assurance and contamination control was assured by the usage of ultra-pure reagents and performing ultra-clean DNA isolation as well as PCR. Bacterial DNA

Table 2
Distribution of isolated bacterial strains and corresponding colony forming units (cfu) from carious dentine.

Subject – Treatment	Visit I – MC		Visit I – DC		Visit II	
	Bacterial strain	cfu ml^{-1}	Bacterial strain	cfu ml^{-1}	Bacterial strain	cfu ml^{-1}
1 – CTR	*Lactobacillus spp.*	10^3	*Streptococcus anginosus*	10^2	*Lactobacillus plantarum*	10^2
	Lactobacillus casei	10^2	*Streptococcus mitis/oralis*	10^2	*S. mitis/oralis*	10^2
	Lactobacillus paracasei	10^2	*Staphylococcus hominis*	10^2	*Actinomyces naeslundii*	10^2
	Bifidobacterium spp.	10^4	*Actinomyces odontolyticus*	10^2	*Peptoniphilus asaccharolyticus*	10^3
	gram + bacteria	10^2	*Lactobacillus spp.*	10^2	*Eggerthella lenta*	10^3
1 – TS	*Lactobacillus acidophilus*	10^4	*L. acidophilus*	10^3	*Arcanobacterium haemolyticum*	10^3
	L. casei	10^4	*L. casei*	10^5		
	L. paracasei	10^4	*L. paracasei*	10^4		
2 – CTR	*S. hominis*	10^2	*A. odontolyticus*	10^3	No growth	–
			A. naeslundii	10^3		
			Actinomyces meyeri	10^3		
2 – TS	*Bifidobacterium spp.*	10^2	*S. anginosus*	10^2	*A. naeslundii*	10^3
	S. anginosus	10^2	*Streptococcus constellatus*	10^2	*S. mitis/oralis*	10^3
	S. mitis/oralis	10^2	Gram+ bacteria	10^2		
3 – CTR	*L. paracasei*	10^4	*L. paracasei*	10^4	No growth	–
	A. odontolyticus	10^4	*A. odontolyticus*	10^4		
	gram+ bacteria	10^4	gram+ bacteria	10^4		
			Rothia dentocariosa	10^3		
3 – TS	*L. paracasei*	10^4	*L. paracasei*	10^5	No growth	–
	A. odontolyticus	10^4	*A. odontolyticus*	10^4		
	R. dentocariosa	10^3	*R. dentocariosa*	10^3		
	Neisseria cinerea	10^3	*N. cinerea*	10^3		

MC: mediate caries layer; DC: deep caries layer; TS: test substance; CTR: control substance.

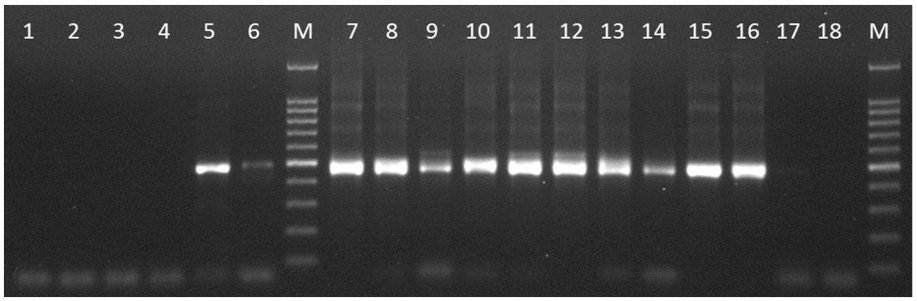

Fig. 3. A representative agarose gel electrophoresis of PCR products with an approximate size of 480 bp for the qualitative detection of bacterial DNA in caries samples. Only caries samples from the deep layer of dentine is exemplarily represented. Lane 1–2: negative PCR control; 3–4: negative control DNA isolation; 5: positive control DNA isolation; 6: positive PCR control; 7: subject 1 test substance (TS) visit I; 8: subject 1 control (CTR) visit I; 9: subject 1 TS visit II; 10: subject 1 CTR visit II; 11: subject 2 TS I; 12: subject 2 CTR I; 13: subject 2 TS II; 14: subject 2 CTR II; 15: subject 3 TS I; 16: subject 3 CTR I; 17: subject 3 BD II; 18: subject 3 CTR II, M: 100 bp DNA size marker.

positive samples were distinguished from bacterial DNA negative samples using melt curve analysis. In an exemplary way, Supplementary Fig. 1 shows the melting peaks for subject 2. Primer dimers are visualized by melting peaks between 78 and 83 °C which are particularly present in negative isolation and PCR controls. A specific melting peak between 89 and 94 °C shows a positive PCR result. Carious samples containing a varying bacterial composition elicit differences in melting peak temperatures. Negative controls do not show a melting peak indicating no background contaminations. Results from qualitative PCR, particularly the correct band size, are confirmed by agarose gel electrophoresis (Fig. 3) also revealing the absence of contaminations. The variations in fluorescence signals between samples indicate differences in bacterial DNA quantities between lesions within the same patient at one time, especially when considering visit II. Within subject 1, there is a loss in fluorescence intensity comparing visit I and visit II revealing a decreased DNA content in the carious dentine 8 weeks after selective caries excavation.

3.2.3. *Quantitative PCR*

Quantitative real time PCR (qPCR) was used to define differences in the bacterial load due to antimicrobial effects of the dental material (Fig. 4, Supplementary Fig. 2). Up to 3.17E + 08 copies of bacterial DNA per bur from intact cells were detected in the middle layer of carious dentine at visit I (Fig. 5). Additionally, intra- and inter-individual differences in the content of bacterial DNA in carious dentine were observed when comparing visits I in subject 1 to 3.

Interestingly, subject 1 and 2 show that the ratio of cell-free bacterial DNA in comparison to DNA from intact cells decreases when comparing visit I and visit II using the test substance (visit I: free DNA vs DNA from intact cells = 1:200; visit II: free DNA vs DNA from intact

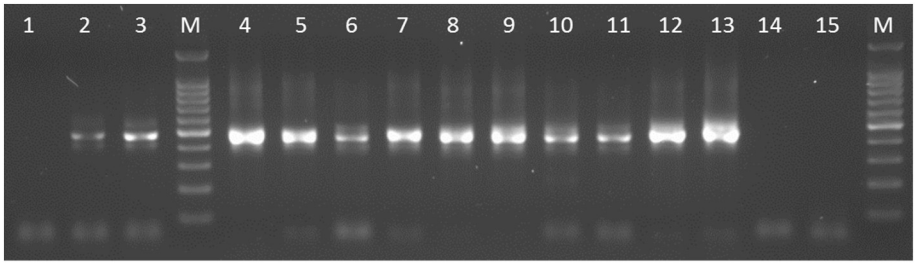

Fig. 4. A representative agarose gel electrophoresis of PCR products with an approximate size of 480 bp for the quantification of bacterial DNA in caries samples. Lane 1–2: negative PCR control; 2: PCR standard 10E + 01; 3: PCR standard 10E + 03; 4: subject 1 test substance (TS) visit I; 5: subject 1 control (CTR) visit I; 6: subject 1 TS visit II; 7: subject 1 CTR visit II; 8: subject 2 TS I; 9: subject 2 CTR I; 10: subject 2 TS II; 11: subject 2 CTR II; 12: subject 3 TS I; 13: subject 3 CTR I; 14: subject 3 TS II; 15: subject 3 CTR II, M: 100 bp DNA size marker.

				Subject 1		Subject 2		Subject 3	
Treatment	Visit	Caries	Bacterial DNA	Bacterial DNA [copies/bur]	Culture result	Bacterial DNA [copies/bur]	Culture result	Bacterial DNA [copies/bur]	Culture result
Test substance	I	MC	intact cells	3.17E+08	positive	1.17E+07	positive	1.02E+07	positive
			free	2.86E+05		2.71E+05		1.05E+06	
		DC	intact cells	3.48E+07	positive	4.74E+06	positive	1.49E+07	positive
			free	5.78E+04		1.40E+05		1.26E+06	
	II	DC	intact cells	1.32E+04	positive	8.95E+05	positive		negative
			free	1.36E+04		4.11E+04			
Control	I	MC	intact cells	4.92E+06	positive	5.31E+06	positive	1.05E+06	positive
			free	1.27E+06		5.94E+04		3.51E+05	
		DC	intact cells	3.61E+06	positive	9.07E+06	positive	2.22E+06	positive
			free	7.97E+04		7.51E+04		3.25E+05	
	II	DC	intact cells	6.59E+05	positive	3.85E+04	negative		negative
			free	9.14E+04		7.92E+03			

Fig. 5. Bacterial DNA quantification in carious dentine samples in comparison to microbial culture results. Bar charts depicting the results from bacterial DNA quantification from the mediate (MC) and deep layer (DC) of carious dentine were presented as logarithmic values. For a simplified overview, bacterial DNA values were indicated as copy numbers per bur.

cells = 1:17) as well as in the control (visit I: free DNA vs DNA from intact cells = 1:82; visit II: free DNA vs DNA from intact cells = 1:7) which means that the number of intact bacterial cells decreases while cell-free DNA numbers persist. Cell-free bacterial DNA might trigger the activation of matrix-metalloproteinases. Therefore, this part of DNA within carious lesions might have an impact on adhesive bond of composite restorations as well as pulpal inflammation and symptoms.

However, quantification particularly when differentiating free from cellular bacterial DNA might be influenced during sampling processing and by incomplete degradation using DNase during isolation steps. Spiking experiments showed slight differences of spiked and quantified bacterial DNA copy numbers but results differed marginally (less than one log level).

There have been few studies evaluating the effects of different caries treatment strategies considering microbiota in carious dentine before and after intervention with different observation times (Paddick et al., 2005; Vaidyanathan et al., 2009; Wicht et al., 2004; Bjørndal and Larsen, 2000; Gu et al., 2010). However, most of them are using culture-dependent methods in evaluation of quantitative reduction in bacteria.

Considering the quantitative PCR results, it has to be mentioned that the transferability of the results of the current study to already existing data is limited due to differences in the sampling procedure. As mentioned above, sterile round burs of the same, standardized size were used for sampling of carious dentine. In most of the recent studies, focusing on molecular microbiological analysis of carious dentine, sterile spoon excavators were used for sampling. However, round burs have also been described by several authors (Paddick et al., 2005; Kidd et al., 1993) as a possibility to assure an approximate similarity considering sampling of carious dentine.

Generally, results based on the use of 16S rRNA gene PCR assays are controversially discussed. Nowadays, DNA isolation and subsequent

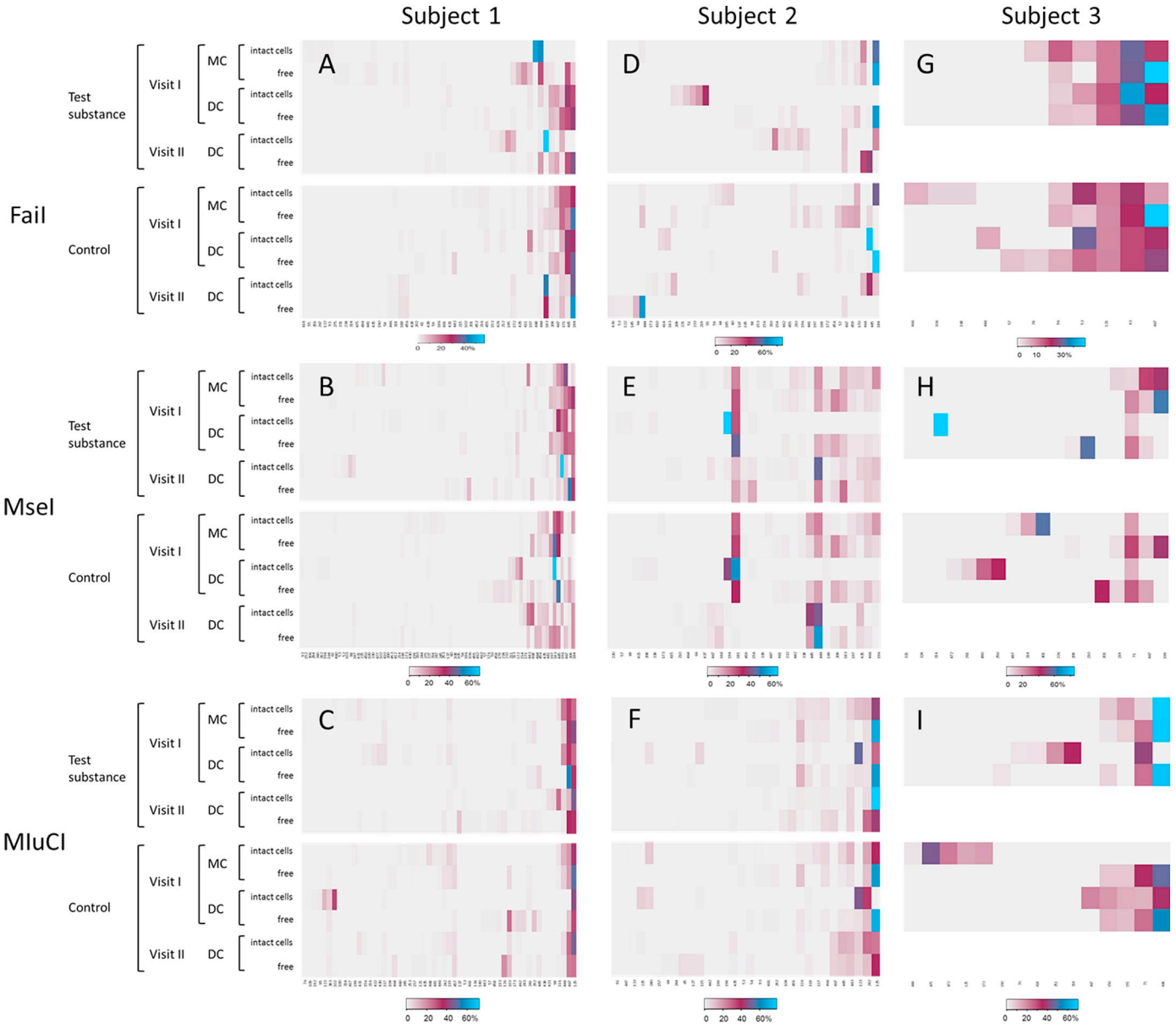

Fig. 6. T-RFLP profiles of bacterial DNA from carious dentine samples of three subjects displayed as heat map using the restriction endonucleases *FaiI, MseI* and *MluCI*. Each square displayed in the heat map represents the relative abundance of a single detected terminal restriction fragment (T-RF; in base pairs) using Bray-Curtis index (deviation = 3). The bacterial composition of carious dentine depending on the therapy (test substance vs control) at different time points (visit I and II) is displayed by rows. Co-occurring T-RF from each sample are clustered and displayed in columns. The color key for the relative abundance of T-RF is shown beneath each individual heat map.

assays to detect and quantify bacterial DNA must be performed under sterile working conditions and with ultra-clean reagents in order to circumvent the ubiquitous sources for bacterial contaminations. In fact, Salter et al. revealed by quantifying ten-fold dilution series of *Salmonella bongori* that bacterial DNA plateaus at 50.000 copies ml^{-1} indicating background levels of contaminating DNA (Salter et al., 2014). This leads particularly in samples with a low DNA content to an erroneous quantification of 16S rRNA genes and a misinterpretation of sequencing results. However, the avoidance of contaminations in our assay (Fig. 4, Supplementary Fig. 2) allows application of 45 cycles in qPCR to also quantify samples with even smaller contents of bacterial DNA than presented in this study (lowest sample containing 7.92E + 03 copies per bur).

3.2.4. T-RFLP

T-RFLP was applied to monitor changes in the microbial composition of bacterial DNA positive carious dentine samples after treatment with a potentially antibacterial dental material. By the use of three restriction endonucleases with rare- and frequent-cutting activity (*FaiI* vs *MluCI* and *MseI*) we were able to observe changes in this bacterial community which is partly composed of closely related species. Thus, subject 2 showed differences in its microbial composition regarding *FaiI* but not when analyzing its bacterial DNA content with *MseI* alone (Fig. 6).

Independent from the therapy applied, we revealed intra-individual changes considering the bacterial diversity of carious dentine samples and its bacterial composition when comparing three participating individuals (Fig. 6). The number of T-RF decreased from subject 1 to subject 3 throughout the set of enzymes used in this study. Additionally, inter-individual changes of particularly deep carious dentine, as shown in subject 2 with *FaiI* and *MseI* (Fig. 6 D, E), were observed which challenges future microbiome based analyses. Especially longitudinal studies analyzing therapy effects on microbial community structure have to be performed in large cohorts to neglect inter- and intra-individual effects. Instead of T-RFLP, Illumina sequencing technology generates additional information on the identification of the community (de La Fuente et al., 2014) and is meanwhile faster and equal in costs. However, in order to produce unbiased NGS data without common laboratory contaminants such as B*radyrhizobium, Sphingomonas* or *Ralstonia* (Laurence et al., 2014) attention should be paid on ultra-pure reagents as it was applied in this entire study.

Interestingly, T-RFLP also revealed changes in the composition of cell-free bacterial DNA compared to DNA derived from intact cells as seen in subject 2 (*FaiI, MseI*; Fig. 6 D, E) and subject 3 (*MseI, MluCI*; Fig. 6 H, I). Hereby, differences in community structure are based on the existence or absence of a restriction site. The choice of the right endonuclease is crucial for a maximal differentiation of species in the sample. Although we used an enzyme set instead of a single enzyme to observe structural changes of the community, several species are still difficult to distinguish e.g. *S. mitis/oralis* and *S. anginosus* as well as *L. casei* and *L. fermentum*.

4. Conclusions

The results of the current study reveal that differentiation and quantification of DNA from intact bacteria and cell-free DNA is reasonable when considering carious dentine samples, especially after intervention. Furthermore, it could be shown that ultra-clean working conditions are essential for the purpose of differentiation and correct quantification of samples with low as well as high bacterial DNA content due to the broad range of bacterial DNA quantities in carious dentine samples.

Future studies evaluating cell-free bacterial DNA may apply the here proposed analyses to investigate triggering of inflammatory cascades compared to cell-derived bacterial DNA. Considering T-RFLP, the results point out that this method is principally suitable in concerns of the analysis of microbial shifts after intervention with potentially antimicrobial components according to a fingerprint analysis. However, it is limited with regard to the identification of possible key players of the bacterial community. Next generation sequencing is a potent alternative method which provides holistic investigation of the microbiome within carious dentine. However, in case the research purpose is solely focused on changes within the microbial community which are represented by shifting patterns, sequencing is not required when T-RFLP is performed. Perspectively, the methodology showed here could also be applicable in concerns of detection of microbial fingerprints which are predictive for future endodontic symptoms.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.01.012.

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