Effects of calcium silicate cement on microbial community in carious dentin

Abstract

Objectives: Nowadays, leaving soft carious affected dentin underneath composite restorations is commonly performed in deep caries lesions. Therefore, sealing agents with antibacterial effect might be advantageous for the long-term therapy outcome. This in-vivo study compared the antibacterial effect of a self-etch adhesive with and without the brominated monomer 12-methacryloyloxydodecyl-pyridinium bromide (MDPB) on carious dentin after selective caries removal.

Methods: 10 patients showing deep primary carious lesions at two posterior teeth without pulpal symptoms were included. At visit I, carious tissue was selectively removed and carious dentin was sampled with a sterile roundbur (Komet No. 18). One cavity was restored with composite (SDR, Ceram X; DENTSPLY DeTrey) using a MDPB-containing self-etch adhesive (Clearfil Protect Bond, Kuraray Noritake; ***CP***). The other restoration served as a control (Clearfil SE Bond II, Kuraray Noritake; ***CB***). At visit II after 8 weeks, carious dentin was sampled again. Bacterial growth in carious dentin was analyzed using cultural differentiation. Bacterial DNA from intact cells and cell-free DNA was quantified using 16S rRNA gene based real time PCR and microbial community was analyzed by amplicon deep-sequencing. Wilcoxon test was applied for statistical analysis.

Results: Both treatments showed a decrease of intact bacterial cells in carious dentin at visit II compared to visit I (CP: visit I=$1.1*10^6$, visit II=$1.7*10^5$ (p=0.03); CB: visit I=$1.1*10^7$, visit II=$2.4*10^5$ (p=0.002)). No statistically significant reduction of cell-free bacterial DNA was detected (CP: BL=$6.1*10^5$, RE=$1.6*10^5$ (p=0.08); CB: BL=$5.3*10^5$, RE=$2.9*10^5$ (p=0.10)). The decrease of intact cell-derived (p=0.309) and cell-free DNA (p=0.330) did not differ significantly between CP and CB. *Lactobacillus* was most abundant within the microbial community at both visits. Alpha-diversity was not affected by treatment and samples showed high intra- and interindividual diversity.

Conclusion and Clinical Significance: Both self-etch adhesives, Clearfil Protect Bond and Clearfil SE Bond, seem to have an antibacterial effect after selective caries removal. However, the results do not reveal an additional effect of MDPB.

The study is officially registered with German Clinical Trials Register (DRKS00011532).

1. Introduction

Treatment of deep carious lesions is challenging. Especially in consideration of keeping pulp vitality and avoiding post-operative symptoms selective (Ricketts et al. 2019) caries removal is recommended as conservative therapy strategy (Schwendicke et al. 2013; Schwendicke et al. 2015) which gives the opportunity to preserve dental structures as much as possible. The impact of the resulting bacterial load can be discussed controversially (Maltz et al. 2018). It is for sure that even after complete caries removal (CCR) bacteria remain in the cavity (Orhan et al. 2008; Lula et al. 2009), though to a lesser extent than after selective caries removal (SCR). Several research groups are focusing the question if and how the microbial community of the remaining carious dentin might be influenced by sealing (Damé-Teixeira et al. 2014; Simón-Soro et al. 2013; Maltz et al. 2012). There is evidence that sealing of the cavity and isolation of the remaining bacteria from nutrient supply of the oral cavity, mainly resulting in carbohydrate starvation, leads to lesion arrest (Maltz et al. 2002; Bjørndal und Larsen 2000).

On the other hand, residual bacteria might have an impact on long-term success of this therapy strategy. Proteolytic strains which were found in dentin carious lesions (e.g. *Prevotella, Propionibacterium,* (Aas et al. 2008; Schmidt et al. 2020; Schmidt et al. 2019) are supposed to be able to metabolize partly degraded dentin matrix (Takahashi 2015). Thus, it might be of importance to consider changes of the microbial community which is left and sealed under a composite restoration. Not only quantitative changes but also alterations in the composition of the microbiome within the carious dentin seem to be detrimental in terms of degradation of the dentin matrix and, subsequently, the adhesive bonding.

When selective caries removal was introduced as therapeutic approach antibacterial dentine bonding agents (DBAs) were developed. Especially in case of self-etching primers the DBA is applied directly on the left carious dentine. Subsequently, it might have antimicrobial activity before and after light-curing (Vaidyanathan et al. 2009). Until 2015, Clearfil Protect Bond™ (Kuraray Co. Ltd., Japan) was the only adhesive system which incorporated antimicrobial monomer 12-methacryloyloxydodecyl-pyridinium bromide (MDPB) (Vaidyanathan et al. 2009). There are several in vitro as well as ex vivo studies which showed antibacterial effects of Clearfil Protect Bond using different methods and bacterial strains (Cocco et al. 2015; Banzi et al. 2014). However, for a holistic understanding clinical studies are necessary (Cocco et al. 2015) which consider natural carious lesions and investigate changes in carious dentin's microbiota resulting after adhesive restoration using DBA with and without antibacterial monomer.

The aim of the present study was to determine the effect of the MDPB-containing self-etch adhesive Clearfil Protect Bond ***(CP)*** on carious dentin left in the cavity in SCR therapy and to compare it to the self-etch adhesive Clearfil SE Bond 2 ***(CB)*** which shows the same chemical ingredients but non-brominated MDP. The hypothesis was that there would be a higher reduction in intact bacterial cells when sealing the cavity with CP compared to CB. We present qualitative as well as quantitative data considering the bacterial DNA (bactDNA) content present in deep dentin caries lesions, before and eight weeks after SCR and sealing the cavity with either Clearfil Protect Bond or Clearfil SE Bond II. Furthermore, the microbial community in carious dentin was analyzed in both instances by amplicon deep-sequencing of 16S rRNA genes.

2. Materials and Methods

Study Design, Patient Recruitment, and Randomization

This trial was performed as a two-arm double blinded interventional clinical study. It is approved by the ethics committee of the Medical Faculty of the University of XXX (XXX) and registered in the German Clinical Trials Register (DRKSXXX). Ten patients were included into the study according to the criteria given in the consort diagram (Figure 1). Each study participant presented 2 posterior teeth (molars, premolars) with deep primary carious lesions. Subsequently, 20 lesions were considered, reaching a power of > 80 % according to a preliminary calculation based on an in-vitro study on the antibacterial effect of CP compared to CB (Banzi et al. 2014). Study participants gave written informed consent after being informed verbally and in writing about the aim of the study and its course. The study started in January 2017, last patient's second visit was in April 2018. Study center was the Department of Cariology, Endodontology and Periodontology, University of Leipzig. In each participant, CP and CB were randomly assigned to the lesions by a study assistant. Operator and patient were blinded considering the treatment strategy. Blinding was also performed in laboratory as well as statistical analyses.

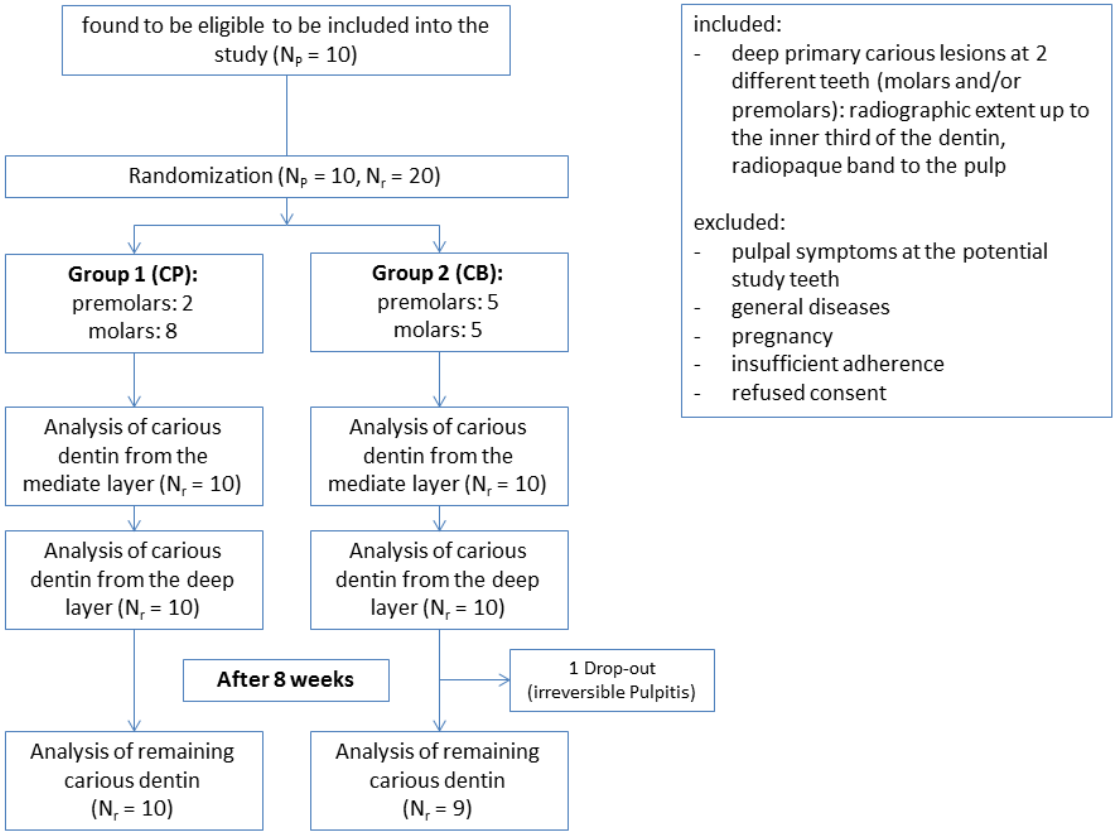


Figure 1: Consort diagram of patient inclusion and treatment within the clinical study course. CP, Clearfil Protect Bond; CB, Clearfil SE Bond 2.

Clinical Procedures

The workflow of the presented study as well as the sampling method is mainly modelled on the protocol which was recently described by the authors (Schmidt et al. 2019; Schmidt et al. 2020). In ***visit I***, after access preparation and caries removal at the enamel-dentin junction a sample of carious dentin from the middle caries layer (***MC I***) was collected. In the zone next to the enamel-dentin junction (minimum width of 1.5 mm) non-selective caries removal was performed for proper adhesive sealing in caries-free dentin. This was followed by selective caries removal in the center of the cavity (Schwendicke et al. 2016; Schmidt et al. 2019) with sampling of carious dentin from the deep caries layer (***DC I***). Carious dentin was sampled with standardized sterile roundburs (Komet no. 18). After selective etching of enamel with phosphoric acid (37 %, sealing of the cavity was performed according to manufacturer's recommendation. There was no difference in the sealing protocol between CP and CB. For easier finding of the sampling region at re-entry, this region was covered with thin film of a

pink compomer (Twinky Star Flow, pink; Voco, Cuxhaven, Germany). Subsequently, cavities were restored with composite (SDR, Ceram X; DENTSPLY Sirona).

Eight weeks later (***visit II***) access was achieved by an occlusal cavity while preserving the marginal ridge if possible. After a sample of the remaining carious dentin (***DC II***) was obtained, the access cavity was adhesively restored according to the current recommendations for the repair of composite fillings (Blunck 2013).

Microbial Cultivation of Bacteria

As has been described in detail in previously published papers (Schmidt et al. 2020; Schmidt et al. 2019), the round burs containing the samples of carious dentin were transferred into COPAN ESwab transport systems (Copan Diagnostics Inc., Murrieta, CA). After carious dentin was dislodged from the burs, classical culture-dependent identification was performed under differentiation for aerobic (Columbia blood agar) and anaerobic (Brucella agar) growth. Bacterial isolates were identified by Vitek 2 system (bioMérieux, Marcy l'Étoile, France).

Molecular Analysis

Differentiation between cell-free and cellular bacterial DNA (bactDNA) was performed in the same way as has been described in detail before (Schmidt et al. 2019). In summary, cell pellet (containing cellular bactDNA) and supernatant (containing free bactDNA) were separated by centrifugation. Subsequently, MolYsis Complete5 kit (Molzym, Bremen, Germany) was used for bactDNA isolation but with a modification in the protocol for cell-free bactDNA neglecting the use of a DNase pre-treatment (Schmidt et al. 2019). Molecular analyses were performed under ultra-clean conditions and with ultra-pure DNA-free reagents and plastic materials.

16S rRNA gene-specific PCR and subsequent melting-curve analysis identified bactDNA positive samples which were subsequently quantified with a 16S rRNA gene-specific real-time PCR based on a hydrolysis probe and primers targeting the V3/V4 regions of the 16S rRNA gene.

Amplicon Deep-sequencing of 16S rRNA Genes

Amplification of the V3/V4 regions of bacterial 16S rRNA genes including the incorporation of Illumina sequencing adapters was recently described in detail (Klindworth et al. 2013), 16S Metagenomic Sequencing Library Preparation protocol from Illumina was used for library preparation. Libraries were sequenced on an Illumina MiSeq system using the 600 cycles V3 chemistry. GenXPro GmbH (Frankfurt, Germany) performed PCR and sequencing.

Statistical Analysis and Computational Analysis of Sequencing Data

Qualitative and quantitative 16S rRNA gene-specific PCR was statistically analyzed by SPSS 22 software (SPSS Inc., Chicago, IL). Variables are given as median and range or frequency (%). Wilcoxon signed rank test was performed for analysis of quantitative PCR results' significance between visits. CP and CB groups were compared by Mann-Whitney U test and chi-squared test. All p-values were performed two-sided and statistical significance was assumed for $p < 0.05$.

Primer sequence clipping, low quality read trimming as well as dereplication were done with DADA2 (Callahan et al. 2016). The resulting amplicon sequence variants (ASVs) were further processed for chimera removal and taxonomic assignment using the QIIME2 pipeline (Bolyen et al. 2018). The 16S rRNA gene SILVA 128 database (Quast et al. 2013) was used as a taxonomic reference with a similarity threshold of 97 %. Statistical analyses of microbiomes were performed in R using the phyloseq (McMurdie und Holmes 2013), microbiome (Leo Lahti, Sudarshan Shetti et al.) and ampvis2 (Skytte Andersen et al. 2018) packages.

3. Results

Demographic and Clinical Characteristics of Study Participants and Teeth

10 symptom-free patients and, subsequently, 20 teeth were included into this study. The participants' demographic and clinical characteristics are presented in Table 1. All cavities were class II, with 80 % and 50 % at molars in the experimental and control group, respectively. One study participant (age of 39 years) reported postoperative symptoms one week after visit I at the CB study tooth which were diagnosed as irreversible pulpitis. Therefore, one drop-out is reported in the CB group. No other reasons for drop-outs occurred in the study course.

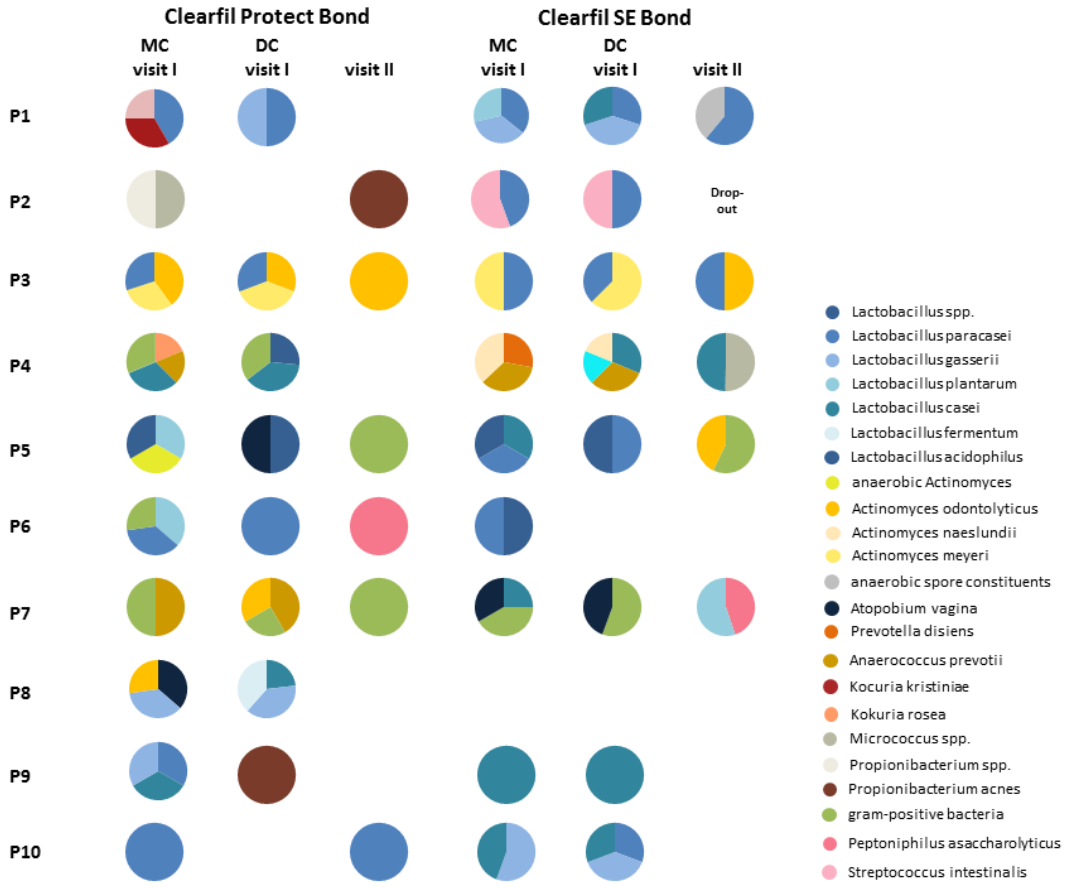
Table 1. Demographic and Clinical Characteristics of Study Participants and Teeth

Variables	**Experimental group** (CP) (n = 10)	**Control group** (CB) (n = 10)	**Total across both groups** (n = 20)
Sex, *n* (%)			
Male	7 (70)		
Females	3 (30)		
Median age, y (Min – Max, y)	23.5 (19 – 39)		
Tooth type, *n* (%)			
Premolars	2 (20)	5 (50)	7 (35)
Molars	8 (80)	5 (50)	13 (65)
Restorations, *n* (%)			
Class I	0 (0)	0 (0)	0 (0)
Class II	10 (100)	10 (100)	20 (100)
Carious dentin consistency at visit I, n (%)			
soft	2 (20)	8 (80)	10 (50)
medium	8 (80)	2 (20)	10 (50)
hard	0 (0)	0 (0)	0 (0)
Carious dentin consistency at visit II, n (%)			
soft	0 (0)	0 (0)	0 (0)
medium	9 (90)	7 (70)	16 (80)
hard	1 (10)	2 (20)	3 (15)
Postoperative symptoms, *n* (%)	0 (0)	1 (10)	1 (5)
Dropouts, *n* (%)	0 (0)	1 (10)	1 (5)

Cultural Differentiation of Bacterial Species in Carious Dentin Samples

Carious dentin showed differences between lesions within one patient as well as between different patients by cultural analysis. All cavities showed changes in microbial composition from visit I to visit II. The available cultural results are given in Appendix Figure 1. At visit II, no bacteria were isolated in 4 out of 10 samples (40 %) in the CP group, whereas 4 out of 9 samples (33 %) did not show bacterial growth in CB.

Considering paired MC/DC samples, 16 out of 20 samples (80 %) from visit I showed differences in the occurrence and/or abundance of bacteria, e.g. in patient 1 (P1) in both teeth. In CB, MC and DC samples of one patient and DC sample of another patient did not show bacterial growth in visit one. In 2 DC samples of the CP group no bacteria grew under given conditions. All patients (10 out of 10; 100 %) showed intraindividual differences in the bacterial composition at visit I between both teeth analyzed for this study (CP vs. CB).



Supplementary Figure 1: Semiquantitative description of the bacterial composition in carious dentin samples from microbial cultivation. The diagrams refer to proportions of bacterial strains in log10 (CFU). Empty spaces represent no bacterial growth of the respective carious dentin sample. Each color represents an isolated bacterial strain.MC: sample from the middle caries layer, DC: sample from the deep caries layer.

Quantification of Cellular and Cell-Free Bacterial DNA in Carious Dentin Samples

Independent of the treatment strategy, quantity of cellular bactDNA in carious dentin of the deep layer (DC I) was lower compared to the middle layer (median 6.92 log10 copies per bur in MC I vs. 6.24 log10 copies in DC I; p = 0.076) as shown in Figure 2A.

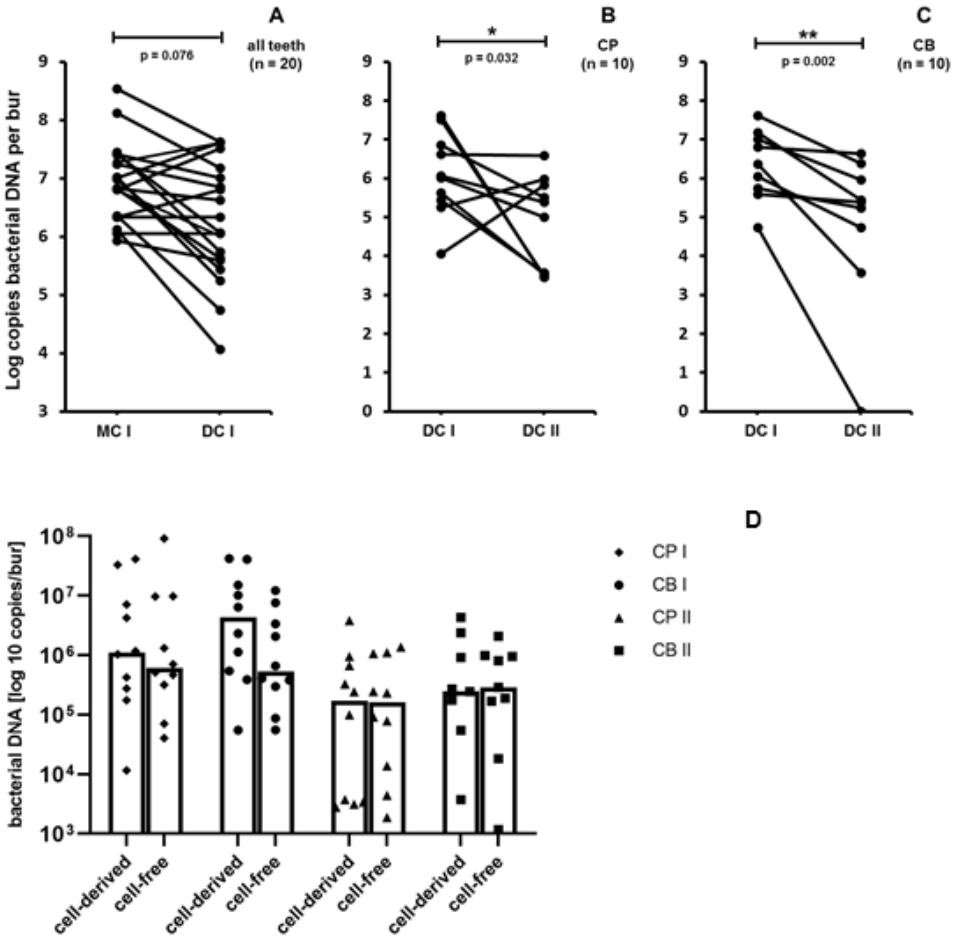


Figure 2: Bacterial DNA quantity in carious dentin samples. (A) Comparison of bacterial DNA quantities of the middle caries (MC) and deep caries (DC) layer at visit I. (B) Reduction of bacterial DNA in carious dentin samples treated with Protect Bond (CP) and Clearfil SE Bond II (CB) at visit I and II; (D) Comparison of cell-free and cell-derived bacterial DNA content in carious dentin samples depending on the therapy and the sampling visit. Bars represent median values

Eight weeks after intervention, there was found a significant reduction of copy numbers detected from intact bacterial cells (CP visit I vs. II: median 6.04 log10 copies per bur vs. 5.23 log10 copies; p = 0.032 and CB visit I vs. visit II: 6.64 log10 copies vs. 5.38 log10 copies, p = 0.002) (Figure 2B and 2C, Appendix Table 1). Furthermore, we also found reduced copy numbers of cell-free bactDNA at visit II in both

treatment groups. However, the differences did not reach statistical significance (p_{CP} = 0.08, p_{CB} = 0.102) (Appendix Table 1). There were no statistically significant differences in the decrease of bacterial DNA quantity between the adhesives Clearfil Protect Bond and Clearfil SE Bond 2 (with and without brominated MDPB) included in the current study (p = 0.309).

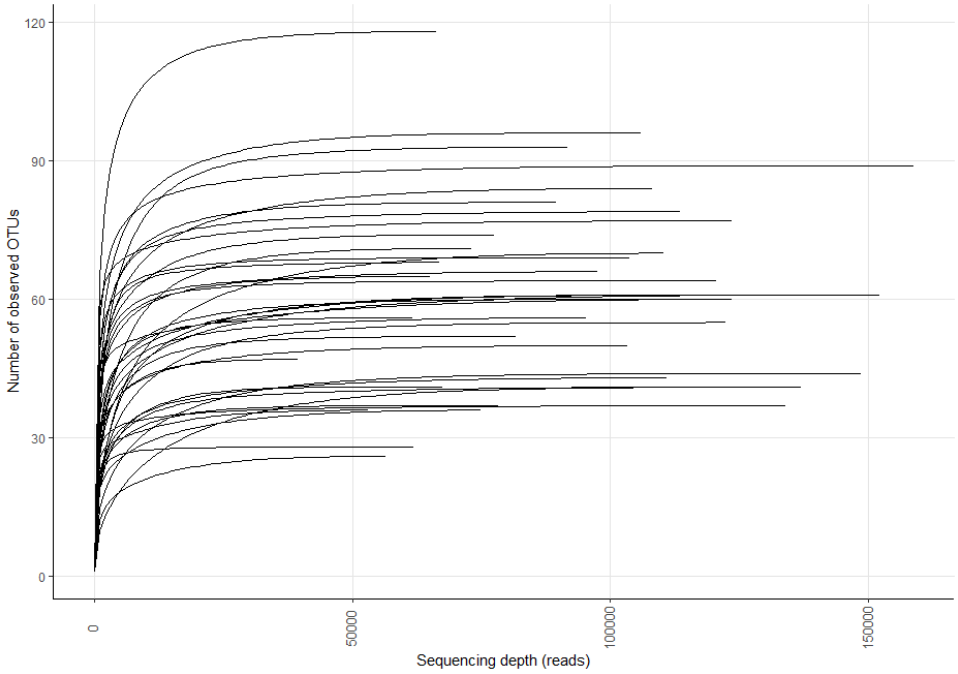
Considering the median ratio between cellular and cell-free bactDNA (log10 copies per bur), there were found comparable results for CP at visit I (6.04 cellular DNA to 5.79 cell-free DNA, ratio 1.04, Figure 2D) and visit II (5.23 cellular DNA to 5.20 cell-free DNA, ratio 1.01, Figure 2D). In the CB group the proportion slightly shifted in favor of cell-free DNA from visit I (6.64 cellular DNA to 5.72 cell-free DNA, ratio 1.16) to visit II (CB: 5.38 cellular DNA to 5.46 cell-free DNA, ratio 0.99, Figure 2D).

Appendix Table 1: Quantification of cell-free and cell-derived bacterial DNA in the middle and the deep layer of carious dentin

		Visit I		Visit II	p_{DC} Visit I vs. Visit II
		MC I	DC I	DC II	
Protect Bond (CP)	Culture positive (%)	10/10 (100)	8/10 (80)	6/10 (60)	0.314
	bactDNA of intact cells; PCR positive (%), median log10 copies/bur (range)	10/10 (100) 6.91 (6.12-7.41)	10/10 (100) 6.04 (4.06-7.62)	10/10 (100) 5.20 (3.44-6.58)	0.032
	cell-free bactDNA; PCR positive (%), median log10 copies/bur (range)	11/11 (100) 6.32 (5.21-7.71)	11/11 (100) 5.78 (4.61-7.96)	7/11 (63.6) 5.16 (3.28-6.13)	0.080
Clearfil SE Bond 2 (CB)	Culture positive (%)	11/11 (100)	10/ 11 (90.9)	5/11 (45.5)	0.259
	bactDNA of intact cells; PCR positive (%), median log10 copies/bur (range)	11/11 (100) 6.82 (5.93-8.54)	11/11 (100) 6.59 (4.74-7.62)	9/11 (81.8) 5.39 (3.57-6.64)	0.002
	cell-free bactDNA; PCR positive (%), median log10 copies/bur (range)	11/11 (100) 5.88 (5.36-8.26)	11/11 (100) 5.72 (4.74-7.09)	10/11 (90.9) 5.46 (3.07-6.32)	0.102
	$p_{bactDNA\ intact\ cells}$ (DNA content$_{CP}$ vs. DNA content$_{CB}$)	0.315	0.398	0.330	

Microbial Composition of Carious Dentin Using Amplicon Deep-Sequencing of 16S rRNA Genes

Amplicon deep-sequencing of 16S rRNA genes was performed for all samples from the deep layer of carious dentin that were bactDNA positive in previous qualitative PCR. Rarefaction analysis demonstrated a sufficient sequencing depth for all samples (Appendix Figure 3).



Supplementary Figure 2: Rarefaction curve for bacterial 16S rRNA sequence analysis of 41 carious dentin samples. ASV, amplicon sequence variants

The bacterial composition for all samples is summarized in Figure 4B and Supplementary Figure 2. In total, … ASVs were detected in 38 carious dentin samples belonging to … different genera with *Lactobacillus* as the most abundant genus. For both groups, alpha-diversity analyses (Figure 4A) revealed no differences considering ASV number between visit I and visit II (p_{CP} = 0.96 vs. p_{CB} = 0.79). Species evenness did not change from visit I to visit II, independent of the adhesive used (p_{CP} = 0.96 vs. p_{CB} = 0.79). Relative abundance of most abundant taxa (top 20) varied between groups and visits (Figure 3B). We found *Lactobacillus* to be the most abundant genera in all samples. However, in P2 and P9 no *Lactobacillus* were found, which is in line with the cultural results (Supplementary Fig. 1). There are several more taxa within the samples which are less abundant such as *Streptococcus, Olsenella, Delftia, and Stenotrophomonas* (Figure 3B).

When samples obtained in visit I were compared to visit II, there was found an increase in abundance of the Proteobacteria *Delftia*, and *Stenotrophomonas*, and *Pelomonas* in visit II compared to visit I in all patients' cavities except for patient 10 in CP (in this case the abundance of all three species decreased from visit I to visit II) (Figures 4A, 4B). *Lactobacillus* abundance changes between the visits differed among patients. P3, P4, P5, P6 and P10 showed increase in abundance in visit II compared to visit I, whereas no changes appeared in the CB cavities of P1, P7 and P8 and both cavities of P2 and P9.

Decrease of *Lactobacillus* abundance in visit II was shown in CP cavities of P1 and P8 (Figure 4A). Overall, there was found a tendency of decreased abundance of *Lactobacillus sp*. CP at visit II compared to CB (Figure 4B). Overall, a high range of ASC abundance was found between different patients and treatments (Figure 4A).

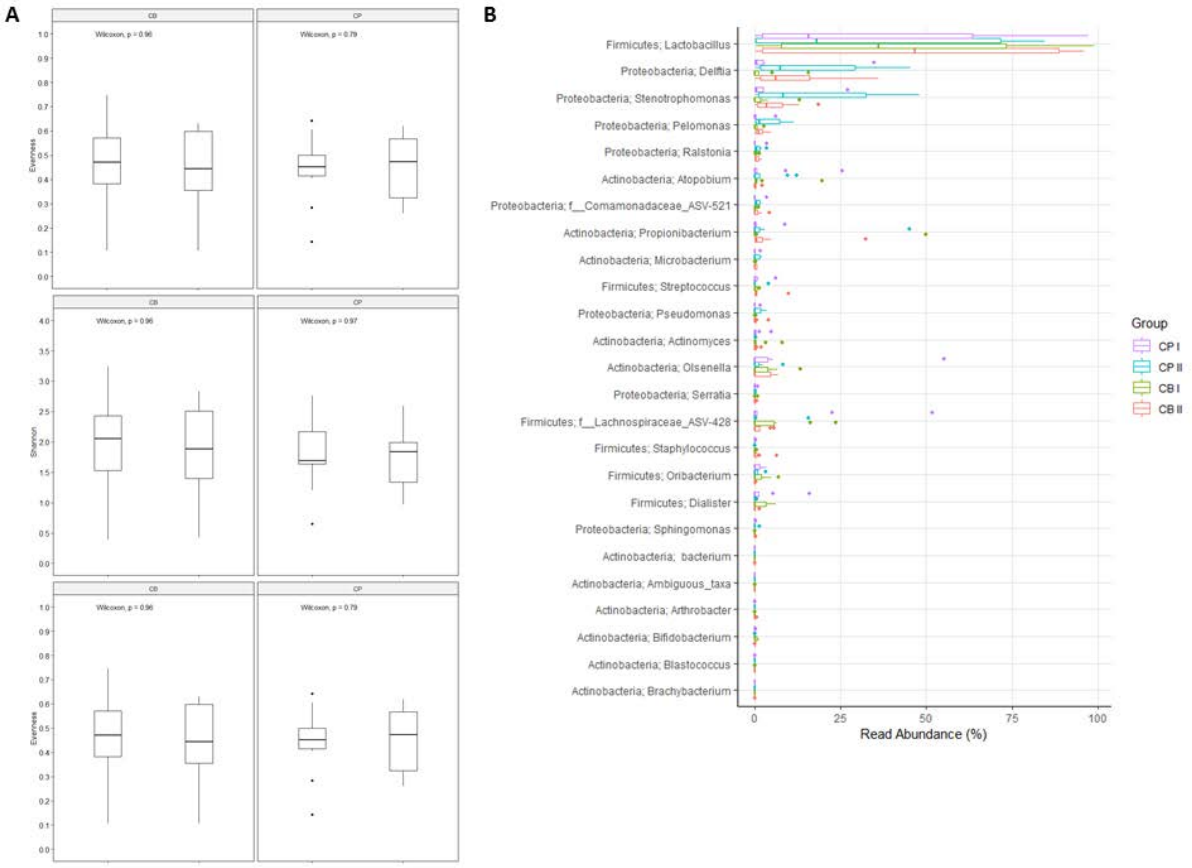
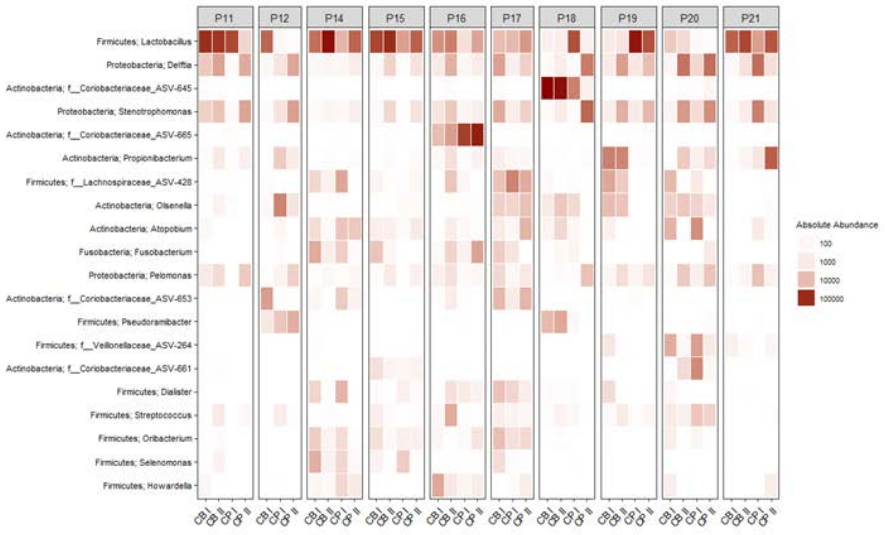


Figure 3: Microbial community analyses. (A) Alpha-diversity measures for CP and CB groups at visit I and II. (B) Relative abundance of 20 most abundant genera.



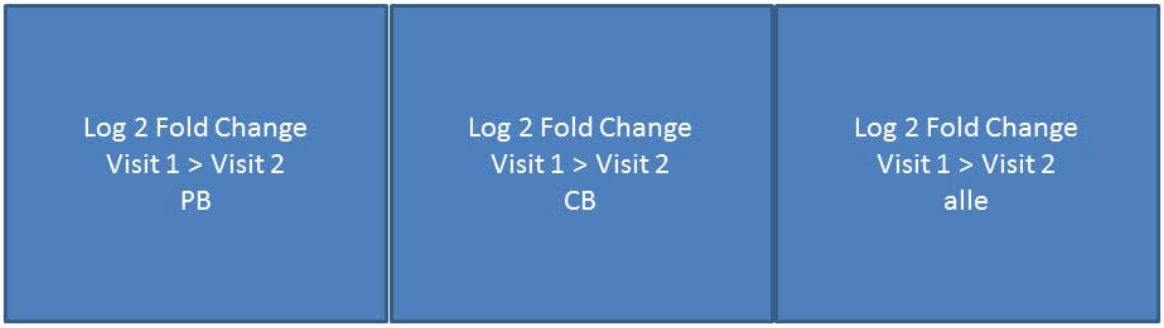


Figure 4: Microbial community analyses. (A) Heatmap of absolute abundance of bacterial species in carious dentin samples; (B) Log2-fold change of amplicon sequence variants (ASVs) with significant difference (Wald test alpha < 0.01) in abundance after the second visit.

4. Discussion

By analogy with a recently published *in-vivo* study considering the additional antibacterial effect of sealing the remaining soft carious dentin with the tricalcium silicate cement Biodentine (***BD***) after selective caries removal (Schmidt et al. 2020), the current investigation addressed the same question about antibacterial as well as microbiome-modifying effects for a self-etch adhesive with and without the brominated monomer 12-methacryloyloxydodecyl-pyridinium bromide (MDPB).

Showing 1 log stage reduction of bactDNA from intact cells, a significant decrease in quantity of bactDNA was found in visit II, eight weeks after sealing remaining soft carious dentin, for both adhesives. The antibacterial effect did not differ between CP and CB. No statistical relevant increase in cell-free bacterial DNA, representing an effective lysis of bacterial cells (Schmidt et al. 2019), was observed in visit II, neither in CP nor in CB. This is in contrast with in-vitro studies which reveal pronounced antibacterial effect of CP, but not CB (Banzi et al. 2014; Imazato et al. 2006). However, a recent study found CB to be effective against *Streptoccus sobrinus* (almost no survival one week after sealing) as well as *Lactobacillus rhamnosus* (5 % survival after 2 weeks sealing), showing antibacterial effectiveness of CB which is in line with the findings of the present study. Additionally, studies report much higher antibacterial efficiency of CP (Rolland et al. 2011; Polydorou et al. 2006) than we found. Interestingly, some authors report no antibacterial effect of CP after 2 weeks (Polydorou et al. 2013; Feuerstein et al. 2007).

Comparability of the study results is limited because most studies were performed in vitro, considered bacterial culture results and selected bacterial strains in suspension.

Starting with the first point, the present study was performed in natural caries lesions. Therefore, demineralized dentin was involved. This is supposed to have an impact on neutralization effects of the MDP anion, pH and, therefore, MDPB effectiveness (Rolland et al. 2011) and might be a possible explanation for the lack of difference between CP and CB in our findings.

Considering the other aspects, Banzi et al. even showed a significant reduction in antibacterial effectiveness of CP when multi species instead of single species were investigated (Banzi et al. 2014). Due to (metabolic) interactions, decreased effectiveness of antibacterial agents in biofilms and bacterial communities has to be assumed (Gao et al. 2016). Thus, it seems reasonable that a microbial community contained within demineralized dentin may be influenced differently by antibacterial substances than single species as well as multi species bacteria in suspension. Another limitation of most studies investigating antibacterial effectiveness of sealing agents is the choice of bacteria to be considered when using culture-dependent methods as well as selected bacterial strains, favoring

Lactobacilli and *Streptococci* (Banzi et al. 2014; Imazato et al. 2006; Rolland et al. 2011; Polydorou et al. 2006; Marggraf et al. 2018; Krishnamurthy et al. 2018). The natural bacterial spectrum is much more diverse and, additionally, varies among patients and even lesions within the same patient as has been shown by culture-independent methods (Schmidt et al. 2020; Aas et al. 2008; Banerjee et al. 2002). Sequencing data reveal presence of *Streptococcus* DNA in several caries samples within this study. However, there was only one cavity at visit I showing growth of *Streptococcus intestinalis* using microbial culture. These findings support other studies (Wolff et al. 2019; Munson et al. 2004; Aas et al. 2008; Schmidt et al. 2020) showing that *S. mutans* does not seem to be necessarily dominant or even present in deep dentin carious lesions. Our NGS analysis, in accordance with the results of bacterial culture, reveal *Lactobacilli* as the predominant bacteria within carious dentin (Figure 3 B) which confirms previous findings (Simón-Soro et al. 2014; Martin et al. 2002; Gross et al. 2010; Schmidt et al. 2020). Changes in *Lactobacilli* from visit I to visit II were inconsistent among patients and treatments. This stands in contrast to other studies which revealed a significant decrease after sealing with CP, but not CB (Imazato et al. 2006; Banzi et al. 2014). As mentioned above, demineralisation degree of carious dentin might be detrimental. However, in comparison to CB, there was a tendency of decreased *Lactobacillus* abundance in CP at visit II (Figure 4B). That might reflect a slightly higher effectiveness of CP against *Lactobacillus sp*., but should be interpreted with caution. Overall, both adhesives seem to have less influence on *Lactobacilli* compared to Biodentine which has been recently considered with the same methods (Schmidt et al. 2020). With comparable baseline copies of bactDNA, the reduction of bactDNA by CP and CB is less pronounced compared to Biodentine (4 log stages reduction of intact bactDNA). For Biodentine, NGS results reveal a significant reduction in *Lactobacillus* abundance (Schmidt et al. 2020). Differences of the findings of the current study could be due to decreased pH-value by acidic self-etching adhesives compared to BD sealing and are in accordance with findings of Kianoush et al. who showed pH dependence of bacteria's abundance (Kianoush et al. 2014; Imazato et al. 2006). CP as well as CB induce a decrease of pH by acidic monomers (pH < 2.5) (Banzi et al. 2014). The pH of the cured DBA in a clinical situation is hard to presume and might differ individually, as neutralization effects due to dentin occur in dependence of dentin demineralization degree (Rolland et al. 2011). This again reflects the high variability within the clinical situation. The importance of the presence of certain microorganisms for therapy outcome seems to be worth to be discussed.

The present study has a number of strengths, considering its in vivo set-up and, therefore, reflection of the clinical situation. The research question is of growing interest, especially with regard to minimally invasive dentistry and, additionally, lacks clinical studies (Cocco et al. 2015). However, reasonable concerns considering the standardization of carious lesions as well as sampling of carious

dentin *in-vivo* have previously been discussed in our recent publications (Schmidt et al. 2020; Schmidt et al. 2019). The observational period was quite short with 8 weeks until re-opening. Therefore, a follow-up visit for clinical evaluation of therapy success is reasonable.

Another point worth to discuss is a possible contamination during amplicon deep-sequencing of 16S rRNA genes because PCR reagents for this step were not ultra-clean. Our results reveal an increase in abundance of *Proteobacteria* in visit II, especially considering *Delftia, Stenotrophomonas* and *Pelomonas* (Figures 3B, 4A, 4B). *Pelomonas* is a genus mainly found in water (Gomila et al. 2007; Lerch et al. 2017), subsequently, most likely due to contamination. Considering *Delftia* and *Stenotrophomonas,* Kinanoush et al. have found both to be carious lesion-associated in a pH range of 6.0 to 6.5 (Kianoush et al. 2014). However, due to the focus on the 16S rRNA gene the present study did not differentiate up to species level. For the given reasons, results based on amplicon deep-sequencing should be interpreted with reservation considering environment-associated genera which are not specified to species-level.

In conclusion, the results reveal comparable antibacterial effects of CP and CB, but to a lesser extent than has been shown for CP by in vitro studies. Thus, addition of MDPB does not show clear advantages considering antimicrobial effectiveness in clinical set-up. With regard to microbial composition, *Lactobacilli* persisted with high abundance but with an emerging increase in *Proteobacteria* 8 weeks after SCR and sealing remaining carious dentin. The changes in microbial profile differed on patient and cavity level. Consequently, it seems reasonable also to consider the influence of MMP activity and inflammatory reaction of the pulp-dentin-complex in evaluation of therapy strategies in minimally invasive therapy of deep dentin carious lesions.

Figure 1. Consort diagram of patient inclusion and treatment within the clinical study course. CP, Clearfil Protect Bond; CB, Clearfil SE Bond 2

Figure 2. Bacterial DNA quantity in carious dentin samples. (A) Comparison of bacterial DNA quantities of the middle caries (MC) and deep caries (DC) layer at visit I. (B) Reduction of bacterial DNA in carious dentin samples treated with Biodentine and (C) gutta-percha at visit I and II; (D) Comparison of cell-free and cell-derived bacterial DNA content in carious dentin samples depending on the therapy and the sampling visit. Bars represent median values.

Figure 3. Microbial community analyses. (A) Alpha-diversity measures for BD and CTR groups at visit I and II. (B) Relative abundance of 20 most abundant genera.

Figure 4. Summary of microbial community analyses. (A) Alpha-diversity measures for CP and CB groups at visit I and II. (B) Relative abundance of 20 most abundant genera. (C) Log2-fold change of amplicon sequence variants (ASVs) with significant difference (Wald test alpha < 0.01) in abundance after the second visit.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards (ethics committee vote number XXX).

Informed consent

Informed consent was obtained from all individual participants included in the study.

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