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ORIGINAL ARTICLE

Detection of nine microorganisms from the initial carious root lesions using a TaqMan-based real-time PCR

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OBJECTIVE: The purpose of this study was to quantify nine selected cariogenic bacteria in plaque from sound root surfaces and initial carious root lesions using TaqMan PCR and to analyse a putative dependence on the kind of initial periodontal treatment.

MATERIAL AND METHODS: Fifty-four subjects with generalized chronic periodontitis were randomly allocated to one of the following initial periodontal therapies: full-mouth disinfection, full-mouth scaling and root planing or scaling and root planing within 7 days. Plaque samples were taken before and after periodontal treatment and analysed by TaqMan PCR.

RESULTS: The quantity of the cariogenic bacteria *Actinomyces* spp., *Streptococcus mutans*, *Streptococcus sobrinus*, *Lactobacilllus* spp., *Rothia dentocariosa*, *Parvimonas micra*, *Propionibacterium acnes* and *Neisseria mucosa* were significantly higher, while the quantity of *Veillonella parvula* was significantly lower on initial carious lesions than on the sound surfaces both before and after periodontal therapy. No significant differences could be found in any of the tested bacteria except *P. micra* on initial carious lesions and sound surfaces for both examinations between the groups.

CONCLUSION: All the nine species analysed were found to be present in initial carious root lesions as well as sound root surfaces but in different quantities, independent of the different periodontal therapies.

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Keywords: cariogenic; microorganisms; PCR; root; caries

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Introduction

Epidemiological studies have shown that root caries (RC) as well as periodontitis are common in adults (Hellyer *et al*, 1990; Papapanou, 1996; Micheelis and Schiffner, 2006). Gingival recession as a result of periodontal disease increases the risk for RC (Lawrence *et al*, 1995; Ravald and Hamp, 1981). A number of other factors, for example ageing or periodontal treatment regimens, can attribute to RC (Spratt, 2003). Poor oral hygiene is also known to be associated with an increased risk for RC (Kitamura *et al*, 1986). The most critical role in this multifactorial aetiology of RC is played by microorganisms (Marsh and Martin, 2003; Shen *et al*, 2002; Spratt, 2003).

The microbiological nature of dental plaque associated with RC was shown to differ from that of coronal caries, although it is technically still regarded as supragingival plaque (Marsh and Martin, 2003).

There is no consensus on the microbial aetiology of RC in the elderly and as to which microbes may cause the disease. Some studies carried out by culturing have demonstrated that *Streptococcus mutans*, *Lactobacillus* spp. and *Actinomyces* spp. are involved in the aetiology of RC (Sumney and Jordan, 1974; Syed *et al*, 1975; Ellen *et al*, 1985; Emilson *et al*, 1993), while other studies have failed to implicate specific bacterial species in its aetiology (Van Houte *et al*, 1994; Schupbach *et al*, 1996; Shen *et al*, 2002).

Munson *et al* found nearly hundred cultivable and not-yet-cultivated bacteria including *Actinomyces* spp., *Streptococcus* spp., lactobacilli, *Propionibacterium acnes*, *Parvimonas micra* (previously named as *Peptostreptococcus micros* or *Micromonas micros*), *Rothia dentocariosa* and *Neisseria mucosa* in carious dentine lesions (Munson *et al*, 2004). However, culture-independent studies have so far not been published to describe the quantity of distinct microorganisms in the biofilm of sound root surfaces in comparison with those in initial RC lesions.

While the working groups of Quirynen and van der Reijden reported relatively high detection frequencies of *S. mutans* after periodontal therapy (Quirynen *et al*, 1999; Van der Reijden *et al*, 2001), another study observed a significant decrease in the detection frequency of *S. mutans* (De Soete *et al*, 2005) in groups where antiseptics (fluoride or CHX, chlorhexidine) had been used in initial periodontal therapy.

Little data are available in the literature on the microbiology of RC using PCR; however, there is none on the microbiology of initial carious root lesion. Therefore, the aim of this study was to quantify nine selected cariogenic bacteria, namely *Actinomyces* spp., *S. mutans, Streptococcus sobrinus*, *lactobacilli, R. dentocariosa, P. micra, P. acnes, Veillonella parvula* and *N. mucosa*, in plaque from sound root surfaces and initial carious root lesions. The primary purpose of this investigation was to assess the prevalence of these species in initial root carious lesions and in sound root surfaces. These species have frequently been found in dental plaque associated with normal oral flora and RC (Filoche *et al*, 2007; Preza *et al*, 2008), and they are acidogenic and aciduric (Marsh and Martin, 2003).The second purpose of the investigation was to examine the hypothesis that the dental microbiota associated with RC may be modulated to a certain extent, by the kind of periodontal treatment implemented.

Material and methods

Subjects

Fifty-four subjects (23 women and 31 men) with an average age of 54.15 (±11.65) years participated in this study. Of the above subjects, twelve were smokers. They were recruited from the Department of Periodontics of the University of Duesseldorf. To have an 80% chance of detecting a difference of 0.2 as significant (at the two-sided 5% level), a sample size of 15 subjects was calculated. Based on the assumption of a follow-up dropout of 20%, 18 subjects per group were required. All the participants suffered from moderate to severe generalized chronic periodontitis. Inclusion criteria for the subjects were moderate to severe generalized periodontitis, the presence of at least one tooth with one initial carious root lesion and one sound root surface. Further, the subjects were required to be healthy and to possess at least 20 natural teeth and a stimulated salivary secretion rate of ≥ 0.7 ml min^{-1}. Exclusion criteria were the use of systemic medications interfering with the condition of periodontal tissues or influencing the risk of caries development, the use of systemic antibiotics or an antibacterial mouthrinse <2 months prior to commencement of the study and during the study, or local application of fluoride or antibacterial agents 3 months prior to start of the study. Third molars were not included in the study and likewise teeth with a furcation involvement of grade II and III. The demography of subjects and the number of single-rooted and multi-rooted teeth from which the samples were collected are shown in Tables 1 and 2. The study design was approved by the ethics committee of the University of Duesseldorf (Protocol number 2906/2006). All patients received a detailed description of the proposed treatment and gave their informed and written consent.

Table 1 Descriptive statistics of the patient population ($n = 54$) sorted by treatment methods

Methods (n = *18*)	*Females/ Males*	*Smoker/ Non-smoker*	*Age (mean ± s.d.)*
FMD	7/11	4/14	47.99 ± 11.94
FMT	8/10	3/15	59.32 ± 11.90
SRP Q–Q	6/12	5/13	55.34 ± 9.40

FMD, full-mouth disinfection; FMT, full-mouth treatment; SRP Q–Q, quadrantwise scaling and root planing.

Table 2 Number of single-rooted and multi-rooted teeth per sample collection

	Sound root surfaces		*Initial carious root lesion*	
Methods	*Single-rooted teeth*	*Multi-rooted teeth*	*Single-rooted teeth*	*Multi-rooted teeth*
FMD	7	11	2	16
FMT	9	9	3	15
SRP Q–Q	4	14	8	10

FMD, full-mouth disinfection; FMT, full-mouth treatment; SRP Q–Q, quadrantwise scaling and root planing.

Clinical examination

All examination procedures were performed by one experienced examiner (B.I.E.) to avoid inter-examiner differences. The real-time PCR conducted subsequently was blinded. Before commencement of the study, the examiner was calibrated on a series of patients to ensure validity of the clinical measurements. Intra-examiner reliability data of probing pocket depth (PPD), bleeding on probing (BOP) and plaque index showed high mean percentages of agreement (80–100%). Plaque records were assessed according to the plaque index (Quigley & Hein Index, QHI) (Quigley and Hein, 1962). Gingivitis was recorded using the Papillary Bleeding Index (PBI, scored according to the criteria of Saxer and Muehlemann) (Saxer and Muhlemann, 1975). Clinical parameters were recorded at baseline (E_0) and 2 months after initial periodontal treatment (E_1) with a standardized periodontal probe (PCPUNC 15; Hu-Friedy, Chicago, IL, USA). PPD and BOP were recorded for each tooth at four sites (mesial, distal, buccal and oral).

Sample collection

Plaque samples were collected from all subjects before and 2 months after initial periodontal treatment. The subjects were asked not to brush their teeth on the day of examination and to refrain from eating 1 h prior to the examination. Root surface lesions seen as incipient (grade I) or shallow (grade II) were coded as initial (Billings *et al*, 1985). The teeth were isolated with cotton

rolls, and supragingival plaque samples were taken from the initial carious root lesion and sound root surface, respectively, using sterile Gracey curettes. Each plaque sample was placed in a 1.5-ml tube (Eppendorf, Hamburg, Germany) containing 400 μl of sterile phosphate-buffered saline (PBS [0.12 M NaCl, 0.01 M Na_2HPO_4, 5 mM KH_2PO_4, pH 7.5]), transported on ice to the laboratory and stored at −80°C for later analysis. A standardized procedure was followed throughout the study. A special accuracy weighing machine (Mettler Toledo, Greifensee, Switzerland) was used to calculate the weight of each plaque sample. This was determined by weighing the tube containing PBS before and after addition of the plaque sample.

Periodontal treatment

All subjects used the same fluoride toothpaste (Meridol, GABA, Basel, Switzerland), toothbrush and interdental brush (Curadent, Stutensee, Germany) throughout the study period. Additionally, the full-mouth disinfection (FMD) group used an antibacterial mouthrinse, while its usage was prohibited for the other groups during the course of the study. Prior to periodontal treatment, professional tooth cleaning and polishing with a fluoride-containing paste were carried out and oral hygiene instructions were given. Scaling and root planing was performed using periodontal hand instruments (Gracey curettes, scaler; Hu-Friedy) and Cavitron Select SPS (Dentsply, York, PA, USA).

According to age, gender and smoking habit (Table 1), one dentist (not the examiner) randomly assigned the patients into one of the following three initial periodontal treatment groups:

1. Group A (n = 18): FMD – one-stage full-mouth root planing and FMD (Quirynen *et al*, 1995), followed by the use of antiseptic agents for 2 months: Curasept 0.2% CHX (Curadent, twice a day), Curasept 0.5% CHX-gel (Curadent, once a day) and Curasept 0.5% CHX-Spray (Curadent, once a day)
2. Group B (n = 18): full-mouth treatment (FMT within 24 h) – one-stage full-mouth root planing without the use of an antimicrobial agent except for Meridol® toothpaste
3. Group C (n = 18): quadrantwise scaling and root planing (SRP Q–Q within 7 days) – scaling and root planing (in quadrants) within 1 week and without adjunctive products except for Meridol® toothpaste

All subjects in Group A were given new mouthwash bottles against their empty ones at each visit. This allowed the investigator to monitor the use of mouth rinse throughout the study.

Bacterial strains and DNA extraction

The bacterial strains used in this study are listed in Table 3. Total DNA of the 200 μl plaque resuspension was extracted in 100 μl elution buffer using the Qiagen DNA kit on a BioRobot EZ1 machine (Qiagen, Hilden, Germany).

Table 3 Bacterial strains used in the present study

Bacterial species	*Strain number*
Actinomyces viscosus	DSM 43329
Lactobacillus casei	_[a]
Lactobacillus rhamnosus	_[a]
Parvimonas micra	DSM 20468
Neisseria mucosa	DSM 4631
Propionibacterium acnes	_[a]
Rothia dentocariosa	DSM 43762
Streptococcus sobrinus	DSM 20742
Streptococcus mutans	_[a]
Veillonella parvula	DSM 2007
Escherichia coli	DH5α

[a]Microorganism derived from diagnostic laboratory (Institute of Medical Microbiology and Hospital Hygiene, Clinical Centre of Heinrich-Heine University, Duesseldorf, Germany).

PCR primers. Alignment of 16S rDNA gene sequences by the software program MegAlign (DNASTAR, Madison, WI, USA) facilitated the identification of species-specific regions useful as targets for the PCR. As TaqMan primers and probe sets could be taken from literature for the detection of *Pa* (*P. acnes*) (Eishi *et al*, 2002), *Sm* (*S. mutans*) (Price *et al*, 2007), *Ss* (*S. sobrinus*) (Price *et al*, 2007), *La* (*Lactobacillus* spp.) (Byun *et al*, 2004), *Pm* (*P. micra*) (Price *et al*, 2007) and *Vp* (*V. parvula*) (Price *et al*, 2007), only TaqMan PCRs targeting the 16S rDNA of *Rd* (*R. dentocariosa*), *Nm* (*N. mucosa*) and *Ac* (*Actinomyces* spp.) had to be newly designed (Table 4). For the final design of specific TaqMan primers and probes, the Primer Express Software (Applied Biosystems, Foster City, CA, USA) was used (Table 4). All primers and probes were synthesized by Metabion (Martinsried, Germany).

PCR assessment. The real-time PCR assay was carried out in a total volume of 25 μl consisting of 1× Eurogentec MasterMix without ROX (Eurogentec, Seraing, Belgium), 0.3 μM each of forward and reverse primers, 0.2 μM of labelled probes and 2.5 μl of template DNA. The human GAPDH (glycerinaldehyde-3-phosphate-dehydrogenase) gene was used as a positive control to rule out an inhibition of the PCR. Thermal cycling conditions for all real-time PCR assays were as follows: 1 cycle at 50°C for 10 min, 1 cycle at 95°C for 10 min followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. Cycling, fluorescent data collection and analysis were carried out on an iCycler from BioRad (Munich, Germany) according to the instructions of the manufacturer.

For the construction of quantification standards, conventional PCR was conducted in 55 μl 1× Biorad iQ-Supermix with 3 mM $MgCl_2$ and 1 μM of each primer pair, under the following cycling conditions: 1 cycle at 95°C for 5 min, followed by 40 cycles at 95°C for 1 min, 52°C for 1 min and 72°C for 1 min. The amplicons were isolated using the High Pure PCR Purification Kit (Roche Diagnostics, Mannheim, Germany); approximately, 2.5 ng amplicon was ligated into 25 ng pGEM-T-vector using the Rapid DNA

Table 4 TaqMan primers and probes targeting specific regions of the 16S rRNA gene

Species	*Acc. No.*	*Primer/Probe (5′–3′)*
Parvimonas micra	D14143	Mm-F: TCG AAC GTG ATT TTT GTG GAA A
		Mm-R: GGT AGG TTG CTC ACG TGT TAC TCA
		Mm-T: FAM-CCC GTT CGC CAC TT-BHQ1
Rothia dentocariosa	AJ717364	Rd-F: CTG GGA TAA GCC TGG GAA ACT
		Rd-R: CTC CAT AAC GCT TTC CAC CAA
		Rd-T: TEX- ATA CCG GAT ACG ACC AAT CTC CGC ATG-BHQ2
Streptococcus mutans	AJ243965	Sm-F: TGG GAC GCA AGG GAA CA
		Sm-R: CCC GTT CGC GAC TCA AGA
		Sm-T: HEX-CTT GCA CAC CGT GTT T-BHQ1
Streptococcus sobrinus	AJ243966	Ss-F: GGA CTT GCT CCA GTG TTA CTA ATG AG
		Ss-R: CCG CTA TCA GGC AGG TTA CC
		Ss-T: HEX-CTC ACC CGT TCG CGA-BHQ2
Lactobacillus spp.	AY196975	La-F: TGG AAA CAG ATG CTA ATA CCG
		La-R: CGT CCA TTG TGG TAG ATT CCC T
		La-T: FAM- CTG AGA CAC GGC CCA WAC TCC TAC GG-BHQ1
Neisseria mucosa	AJ239279	Nm-F: GGG TGA GTA ACA TAT CGG AAC GTA
		Nm-R: CGC AAG GCC CGA AGG T
		Nm-T: HEX- TAC CGC ATA TTC TCT GAG GAG GAA AGC AGG-BHQ1
Actinomyces spp.	AJ635359	Ac-F: GRC CCC CCA CAC CTA GTG
		Ac-R: TGG CCC CCA CAC CTA GTG
		Ac-T: FAM- CTG GTA GTC CAC GCC GTA AAC GTT GG BHQ1
Veillonella parvula	X84005	Vp-F: TGC TAA TAC CGC ATA CGA TCT AAC C
		Vp-R: GCT TAT AAA TAG AGG CCA CCT TTC A
		Vp-T: HEX-CTA TCC TCG ATG CCG A-BHQ1
Propionibacterium acnes	DQ672261	Pa-F: GCG TGA GTC ACG GTA ATG GGT A
		Pa-R: TTC CGA CGC GAT CAA CCA
		Pa-T: TEX-RED-AGC GTT GTC CGG ATT TAT TGG GCG BHQ2

Ligation Kit (Roche Diagnostics) following the manufacturer's instructions and subsequently propagated in *Escherichia coli* DH5α. After cultivation on LB-Amp agar plates, insert-positive clones were identified by restriction analysis and subsequent sequencing carried out on an ABI sequencer by the method of Sanger *et al* (1977).

Sensitivity and the lower limits of the TaqMan PCRs were measured using the pGemT-cloned amplicons as template DNA in 10-fold dilutions (2.5×10^5 to 2.5×10^1 copies/PCR). All values were measured in duplicates, and linearity was reproduced in a second run.

The specificity of each TaqMan PCR set was proven *in silico* by a Blast homology search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and using DNA from different oral bacteria. Blast analysis revealed that in each TaqMan PCR, only DNA of the respective species that was homologous was amplified and testing of the DNA templates of oral bacteria affirmed the specificity.

Data analysis

Statistical analysis was performed with SPSS, version 12.0 for Windows. The distributions were analysed using the Kolmogorov–Smirnov test. Data analysis was accomplished using the non-parametric Wilcoxon test, Kruskal–Wallis test and Mann–Whitney U-test. The dependent variable was the change in the bacterial load, as compared to baseline. Tests were performed with $\alpha = 0.05$ to test for significant differences between baseline (E0) and 2 months after periodontal treatment (E1) and differences between the groups from E0 to E1 (E01) ($P < 0.05$).

Results

The dropout rate was 0%.

DNA copies/mg plaque from initial carious root lesions and sound root surfaces

The copy numbers measured for each sample, in each of the nine real-time PCRs normalized to 1 mg plaque, are presented for baseline examination before initial periodontal treatment (E0) and 2 months after initial periodontal treatment (E1) in Table 5.

Significant decreases in values between initial carious root lesions and sound root surfaces for both examinations (E0 and E1) ($P < 0.001$) were found for *P. micra, R. dentocariosa, S. mutans, S. sobrinus, Lactobacillus* spp., *N. mucosa, Actinomyces* spp. *and P. acnes*, whereas *V. parvula* showed a significant increase. The species with the highest values were *R. dentocariosa*, *Actinomyces* spp. and *N. mucosa* (10^6) for initial carious root lesions and *V. parvula* (10^6) for sound root surfaces at the E0 and E1 examinations. The species with the lowest values were *S. mutans, S. sobrinus* and *P. acnes* for sound root surfaces for both E0 and E1 examinations (Table 5).

A significant increase in values between baseline and 2 months after treatment for initial carious root lesions was found for *Actinomyces* spp., *V. parvula* and *P. acnes, while Lactobacillus* spp. *showed a* significant decrease. *Streptococcus sobrinus* showed a significant increase and *Lactobacillus* spp a significant decrease in values for sound root surfaces ($P < 0.05$) (Table 5).

The frequency of each species within the total nine species is shown in Table 6. The microorganisms

Table 5 The DNA copies/mg plaque for nine species from the initial carious root lesions and sound root surfaces at baseline and 2 months after periodontal treatment

DNA copies/mg plaque	*Examination*	*Initial carious* *Mean (Standard deviation)*	*Sound* *Mean (Standard deviation)*	*Significance*
Parvimonas micra	Baseline	2.28×10^6 (6.90×10^6)	2.74×10^5 (1.14×10^6)	***
	After treatment	7.40×10^5 (2.71×10^6)	2.40×10^4 (5.65×10^4)	
Rothia dentocariosa	Baseline	3.71×10^7 (1.64×10^8)	2.59×10^6 (7.63×10^6)	***
	After treatment	2.69×10^8 (1.54×10^9)	4.66×10^7 (2.83×10^8)	
Streptococcus mutans	Baseline	1.73×10^7 (6.20×10^7)	1.06×10^6 (5.28×10^6)	***
	After treatment	7.97×1^6 (4.11×10^9)	4.52×10^4 (1.70×10^5)	
Streptococcus sobrinus	Baseline	1.01×10^5 (3.66×10^5)	7.10×10^3 (3.19×10^4)[a]	***
	After treatment	1.57×10^6 (1.11×10^7)	1.22×10^4 (7.44×10^4)[a]	
Lactobacillus spp.	Baseline	2.66×10^6 (1.72×10^7)[b]	1.08×10^4 (2.20×10^4)[c]	***
	After treatment	3.30×10^5 (1.70×10^6)[b]	3.48×10^3 (1.06×10^4)[c]	
Neisseria mucosa	Baseline	3.39×10^{10} (2.49×10^{11})	3.61×10^6 (1.47×10^8)	***
	After treatment	1.04×10^{15} (7.66×10^{15})	2.78×10^{13} (2.04×10^{14})	
Actinomyces spp.	Baseline	3.61×10^7 (1.47×10^8)[d]	2.71×10^6 (5.78×10^6)	***
	After treatment	3.81×10^7 (1.52×10^8)[d]	6.17×10^6 (2.63×10^7)	
Veillonella parvula	Baseline	2.55×10^6 (4.11×10^6)[e]	1.45×10^7 (3.51×10^7)	***
	After treatment	1.14×10^7 (7.24×10^7)[e]	6.29×10^7 (2.58×10^8)	
Propionibacterium acnes	Baseline	6.32×10^1 (1.68×10^2)[f]	2.41×10^1 (9.7×10^1)	***
	After treatment	3.05×10^2 (5.88×10^2)[f]	2.66×10^1 (7.94×10^1)	

Values with the same letter (a–f) as superscript within the column indicate significant differences between baseline and after treatment.
Significant differences between initial carious root lesion and sound root surfaces ($P < 0.001$).

Table 6 The frequency of each species within the total nine species from initial carious root lesions and sound root surfaces, at baseline and 2 months after periodontal treatment

DNA copies/Sum nine microorganisms	*Examination*	*Initial carious* *Mean (standard deviation)*	*Sound* *Mean (standard deviation)*
Parvimonas micra	Baseline	4.81 (13.40)	1.94 (7.12)
	After treatment	4.77 (13.24)	0.94 (2.46)
Rothia dentocariosa	Baseline	19.96 (26.23)	13.15 (24.27)
	After treatment	24.82 (34.62)	26.54 (33.87)
Streptococcus mutans	Baseline	6.30 (19.69)	2.19 (10.88)
	After treatment	8.28 (23.16)	1.28 (5.64)
Streptococcus sobrinus	Baseline	1.22 (7.62)	0.06 (0.37)
	After treatment	0.42 (2.40)	0.54 (3.63)
Lactobacillus spp.	Baseline	2.45 (13.15)	0.14 (0.28)
	After treatment	2.76 (14.29)	0.18 (1.04)
Neisseria mucosa	Baseline	22.93 (28.63)	17.16 (26.00)
	After treatment	27.61 (35.57)	19.67 (30.20)
Actinomyces spp.	Baseline	33.30 (32.83)	19.77 (22.02)
	After treatment	24.85 (32.10)	18.14 (24.13)
Veillonella parvula	Baseline	9.03 (13.28)	45.58 (35.19)
	After treatment	6.49 (14.00)	32.70 (34.50)
Propionibacterium acnes	Baseline	0.0002 (0.0005)	0.0001 (0.0004)
	After treatment	0.002 (0.007)	0.004 (0.29)

R. dentocariosa, *N. mucosa*, *Actinomyces* spp. and *V. parvula* were found to have higher frequencies than the others in both initial carious root lesions and sound root surfaces, at both examinations (E0 and E1).

DNA/mg plaque for different periodontal treatment groups

The differences for the nine species between E0 and E1 (E01) of DNA copies/mg plaque from initial carious root lesions and sound root surfaces are shown for the different treatment groups in Table 7. Significantly higher E01 values of *P. micra* for FMD in comparison with SPR Q–Q treatment were found independent of the sampling region, i.e. plaque from initial carious root lesions or sound root surfaces. Additionally, *P. micra* showed significantly higher E01 values for FMT in comparison with SRP Q–Q from the plaque of the sound root surfaces. A significant decrease in E01 values for *Lactobacillus* spp. (FMT and SRP Q–Q), for *V. parvula* (FMT) and for *P. acnes* (FMD) was found between initial carious root lesions and sound root surfaces ($P < 0.05$) (Table 7).

Clinical parameters

All three groups showed a significant decrease in the PPD, BOP and PBI between baseline and after

Table 7 The DNA copies/mg plaque for nine species from initial carious root lesions and sound root surfaces between baseline and 2 months after periodontal treatment, between the groups

DNA copies/mg plaque	*Treatment*	*E01 Initial carious Mean (standard deviation)*	*E01 Sound Mean (standard deviation)*	*Significance*
Parvimonas micra	FMD	1.13×10^6 $(4.39 \times 10^6)^a$	1.85×10^3 $(2.21 \times 10^4)^b$	
	FMT	-2.53×10^6 (8.85×10^6)	2.32×10^4 $(9.33 \times 10^4)^c$	
	SRP Q–Q	-3.22×10^6 $(8.30 \times 10^6)^a$	-7.74×10^5 $(1.86 \times 10^6)^{b,\ c}$	
Rothia dentocariosa	FMD	6.49×10^8 (2.66×10^9)	1.24×10^8 (4.89×10^8)	
	FMT	-6.29×10^6 (2.90×10^8)	7.16×10^6 (2.60×10^7)	
	SRP Q–Q	5.30×10^7 (2.05×10^8)	7.88×10^5 (9.41×10^6)	
Streptococcus mutans	FMD	9.13×10^8 (3.67×10^9)	-1.57×10^6 (6.64×10^6)	
	FMT	-7.99×10^5 (3.41×10^6)	6.48×10^3 (4.27×10^4)	
	SRP Q–Q	1.43×10^9 (6.13×10^9)	-1.48×10^6 (6.33×10^6)	
Streptococcus sobrinus	FMD	-3.84×10^4 (2.20×10^5)	-2.68×10^3 (6.59×10^3)	
	FMT	4.55×10^6 (1.91×10^7)	3.02×10^4 (1.28×10^5)	
	SRP Q–Q	-1.00×10^5 (6.86×10^5)	-1.23×10^4 (5.87×10^4)	
Lactobacillus spp.	FMD	6.18×10^5 (2.69×10^6)	-6.33×10^3 (1.20×10^4)	
	FMT	-7.05×10^6 (2.97×10^7)	-1.15×10^4 (3.14×10^4)	**
	SRP Q–Q	-5.68×10^5 (2.50×10^6)	-4.21×10^3 (2.92×10^4)	*
Neisseria mucosa	FMD	8.35×10^{10} (9.18×10^{11})	5.19×10^9 (2.18×10^{10})	
	FMT	3.13×10^{15} (1.33×10^{16})	8.33×10^{13} (3.54×10^{14})	
	SRP Q–Q	5.35×10^7 (1.33×10^8)	2.04×10^6 (1.46×10^7)	
Actinomyces spp.	FMD	3.41×10^7 (1.22×10^8)	8.20×10^5 (5.04×10^6)	
	FMT	-1.80×10^7 (3.61×10^8)	1.29×10^7 (4.52×10^7)	
	SRP Q–Q	-1.01×10^7 (1.79×10^7)	-3.37×10^6 (7.56×10^6)	
Veillonella parvula	FMD	-9.04×10^5 (5.41×10^6)	8.52×10^7 (2.66×10^8)	
	FMT	-2.34×10^6 (5.05×10^6)	-9.18×10^6 (2.48×10^7)	*
	SRP Q–Q	2.98×10^7 (1.25×10^8)	6.91×10^7 (3.40×10^8)	
Propionibacterium acnes	FMD	3.72×10^2 (8.30×10^2)	-3.50×10^1 (1.75×10^2)	**
	FMT	2.66×10^2 (5.37×10^2)	3.81×10^1 (1.32×10^2)	
	SRP Q–Q	8.58×10^1 (4.27×10^2)	4.27×10^0 (2.92×10^1)	

FMD, full-mouth disinfection; FMT, full-mouth treatment; SRP Q–Q, quadrantwise scaling and root planing, values with the same letter (^{a,b,c}) as superscript within the column indicate significant differences between the groups.
*, ** significant differences between initial carious root lesion and sound root surfaces (*$P < 0.05$; **$P < 0.01$).

treatment. A statistically significant reduction in QHI between E0 and E1 was found only for FMT. No statistically significant difference in the PPD, BOP and oral hygiene indices (QHI, PBI) could be observed between the different treatment groups ($P < 0.05$) (Table 8).

Discussion

The outcome of a number of studies on the microbiota of RC has demonstrated that several microorganisms play a decisive role in the development of RC (Schupbach *et al*, 1996; Sumney and Jordan, 1974; Syed *et al*, 1975; Ellen *et al*, 1985; Shen *et al*, 2002; Preza *et al*, 2008; Van Houte *et al*, 1994). Preza *et al* (2008) investigated the bacterial profiles of RC and showed that the microbial communities are more complex than previously presumed. The study suggested that the putative aetiological agents of RC include not only *S. mutans*, lactobacilli *and Actinomyces* spp. but also species of *Atopobium, Olsenella, Pseudoramibacter, Propionibacterium* and *Selenomonas*. Another study showed that *S. mutans*, lactobacilli and *Actinomyces* spp. play a major role, while other microorganisms such as *Staphylococcus*, *Peptostreptococcus*, *Veillonella, Clostridium, Eubacterium, Bifidobacterium, Capnocytophaga, Prevotella and Candida* play a minor role in RC (Shen *et al*, 2002). Additionally, this data suggested that *Bifidobacterium* spp. and periodontal pathogens such as *Prevotella* spp., *Bacteroides* spp. and *Fusobacterium* spp. were not involved in the initial RC lesion. In a longitudinal study, Ellen *et al* (1985) selected bacterial species from 45 subjects with RC over a 32-month period and isolated *Actinomyces* spp. from all lesions and *Lactobacillus* spp. from 73% of lesions. Similar results from other studies also indicate that the predominant cultivable flora are streptococci, staphylococci, lactobacilli and *Actinomyces* spp. (Van Houte, 1994; Keltjens *et al*, 1987). Based on the above observations and the fact that presently there are no data on the quantity of cariogenic bacteria in the initial carious lesion and on sound root surfaces, the nine oral bacteria (genera), namely *Actinomyces* spp., *S. utans*, *S. sobrinus*, *Lactobacillus* spp., *R. dentocariosa*, *P. micra*, *P. acnes*, *V. parvula* and *N. mucosa*, which are found in dental plaque, were selected for this investigation.

Till date, very few studies have used real-time PCR for the quantification of cariogenic microorganisms. Almost all the studies that have carried out so have examined coronal and not RC. Martin *et al* (2002), who examined 65 coronal lesions for anaerobic bacterial species by real-time PCR, identified *P. micra* in 60% of the carious samples. They found that the mean of DNA copies for *P. micra* was 1.5×10^6 per mg plaque. This result is in accordance with our findings, where the mean of DNA copies for *P. micra* for initial carious root

Table 8 Pocket probing depth, BOP and oral hygiene indices (QHI, PBI) at baseline and 2 months after periodontal treatment, within the groups

	Methods	*Baseline Mean (standard deviation)*	*After treatment Mean (standard deviation)*	*Significance*
PPD	FMD	4.60 (0.44)	2.71 (0.52)	***
	FMT	4.63 (0.37)	2.84 (0.36)	***
	SRP Q–Q	5.04 (0.49)	3.13 (0.46)	***
BOP	FMD	0.34 (0.19)	0.08 (0.11)	***
	FMT	0.35 (0.25)	0.07 (0.09)	***
	SRP Q–Q	0.39 (0.24)	0.12 (0.10)	***
QHI	FMD	0.78 (0.35)	0.63 (0.38)	
	FMT	0.92 (0.34)	0.68 (0.33)	**
	SRP Q–Q	1.02 (0.59)	0.85 (0.56)	
PBI	FMD	0.86 (0.43)	0.44 (0.28)	***
	FMT	0.78 (0.37)	0.40 (0.29)	***
	SRP Q–Q	1.13 (0.67)	0.52 (0.46)	***

FMD, full-mouth disinfection; FMT, full-mouth treatment; SRP Q–Q, quadrantwise scaling and root planing; PPD, probing pocket depth; BOP, bleeding on probing; QHI, Quigley & Hein Index; PBI, Papillary Bleeding Index.
*, **, *** significant differences between baseline and after treatment (*$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$).

lesion is 2.28×10^6 DNA copies per mg. Nevertheless, it seems to be difficult to compare the results of different studies because of bias in methodology, plaque sampling and analysis. The bacterial load for lactobacilli with $0–10^8$ copies/per mg plaque that we found on initial lesions corresponded well to those detected by Byun *et al* (2004) on teeth with coronal caries. Their result showed the mean (standard deviation) of DNA copies/mg plaque for total lactobacilli as 1.9×10^7 (2.8×10^7) from carious lesions. Yoshida *et al* (2003) examined the plaque from 10 patients with coronal caries and found the following results: DNA copies/mg for *S. mutans* $0–4.82 \times 10^6$ and for *S. sobrinus* $0–4.76 \times 10^6$. The results of the present study reveal $0–3 \times 10^8$ for *S. mutans* and $0–2.6 \times 10^6$ for *S. sobrinus,* indicating that *S. mutans* may play a major role in the development of initial RC. The range of cell numbers and percentages of these bacteria in dental plaque are similar to previous results with cell culture method (Bowden *et al*, 1975).

Veillonella spp. utilize lactate, which may ameliorate the caries process (Delwiche *et al*, 1985). It has been shown that lesser caries occurred when rats were co-infected with *S. mutans* and *V. alcalescens* than when infected with *S. mutans* alone. A culture-based study supported the correlation between the numbers of *Veillonella* and the number of *lactobacilli, mutans streptococci* and *Actinomyces* spp., which ferment carbohydrates to lactate, leading to an acidification (Toi *et al*, 1999). The results of the present study show a higher value of *V. parvula* on sound root surfaces (1.45×10^7) as compared to the initial carious root lesions (2.55×10^6).

Of the nine organisms analysed in this study, the highest bacterial load was represented by *R. dentocariosa*, *N. mucosa* and *Actinomyces* spp. The total quantity being approximately 76% at E0 and 77% at E1 in initial carious root lesions and approximately 50% at E0 and approximately 65% at E1 on sound root surfaces. The frequency of *V. parvula* was approximately 9% at E0 and approximately 6% at E1 in initial carious root. With approximately 45% *V. parvula* at E0 and approximately 33% at E1 on sound root surfaces, the frequency of *V. parvula* was higher than that of the others on sound root surfaces at both examinations (E0 and E1). These results suggest that *V. parvula* may be a good indicator for initial RC lesions. Furthermore, these findings lead us to hypothesize that an increase in the load of *V. parvula* or elimination or reduction in the bacterial load of *R. dentocariosa*, *N. mucosa* and *Actinomyces* spp. could lead to a reduction in initial RC. Within the limitation of this study, it can be proposed that these microorganisms may play a role in the preventive strategy for initial RC lesions.

The hypothesis that the amount and composition of the dental microbiota may be modulated by the kind of periodontal treatment was not affirmed as significant differences in bacterial load of the different species were not found between the groups A and C (FMD, FMT or SRP Q–Q) in the time interval, before and 2 months after periodontal treatment. Using cultivated plaque, De Soete *et al* (2005) showed that an initial periodontal therapy could result in a shift from periodontal pathogenic towards cariogenic species. Our findings did not reveal any significant difference between the groups, in the bacterial load for most of the bacteria tested (except *P. micra*). The SRP Q–Q design and the (0.2–1%) CHX applied in our study were, however, different from those used by de Soete and co-workers. Variations in the research design, method of collection of the bacterial samples, application of different treatment protocols and time and method of outcome assessment may be factors affecting the different results obtained. In contrast to the present study, the bacterial samples in the study of De Soete *et al* were taken from supra- and subgingival plaque, saliva and tongue.

Parvimonas micra is found in ulcerating infections as well as in the active phase of periodontitis (Van Dalen *et al*, 1998). Rams *et al* (1992) reported a higher prevalence of *P. micra* in disease-active than in disease-inactive periodontitis. The results of the present study showed a significant decrease in the number of *P. micra* after FMT and FMD in comparison with SRP Q–Q. This might be attributed to the less active periodontal pockets after FMT and FMD than after SRP Q–Q.

In the present study, clinical parameters at baseline and 2 months after initial treatment showed no statistically significant difference between the three periodontal treatment groups. These results are confirmed by some studies (Mongardini *et al*, 1999; Quirynen *et al*, 2000), but also contradicted by others (Apatzidou and Kinane, 2004; Koshy *et al*, 2005; Wennström *et al*, 2005; Jervoe-Storm *et al*, 2006). The difference in the results between the studies could be explained on the basis of the methods of allocation, concealment and examiner masking, which affect the magnitude of

clinical outcomes in periodontal trials (Fenwick *et al*, 2008). The present study design was different from the others in respect of the SRP Q–Q design and the concentrations of CHX. Furthermore, all subjects in the study used toothpaste (Meridol®) with known anti-caries activity. The resulting antimicrobial effect of Meridol® is however considered irrelevant in this study as all three groups were compared with each other. Meridol® was applied in this study, in patients with periodontitis, for ethical reasons.

Systematic reviews (Tonetti, 1998; Bergstrom, 2006) have further strengthened the evidence that smoking is an important risk factor for periodontitis. Therefore, to avoid bias, the distribution of smokers in our study was similar in all three groups.

Our study had the following limitations: (i) The nine microorganisms selected for this study may not completely represent all of the microorganisms present on the root surfaces. However, because these microorganisms are present in the biofilm, our data are valid and substantial enough to meet the aim of our study. (ii) The small sample size ($n = 56$) can be regarded as another weakness. (iii) The duration of the study was short (2 months).

To reduce the above-mentioned limitations, it is recommended that well-designed animal investigations be conducted to determine the causality between these microorganisms and the development of initial carious root lesions.

In conclusion, on the basis of the results presented here and considering the limitations of this study, the nine microorganisms, *Actinomyces* spp., *S. mutans, S. sobrinus*, *Lactobacilli, V. parvula, R. dentocariosa*, *P. micra*, *P. acnes* and *N. mucosa*, quantitatively analysed were shown to be present in initial carious root lesions as well as on sound root surfaces, but the number of *Actinomyces* spp., *S. mutans, S. sobrinus*, *Lactobacilli, R. dentocariosa*, *P. micra*, *P. acnes* and *N. mucosa* was higher in initial carious root lesions than on sound root surfaces, and the number of *V. parvula* was higher on sound root surfaces than in initial carious root lesions. These data suggest that in addition to the known RC microorganisms, *R. dentocariosa*, *N. mucosa* and *Actinomyces* spp. may also play a role in the aetiology of initial carious root lesions. This thesis has, however, to be confirmed with the help of future studies. Secondly, no significant increase in the investigated cariogenic bacteria on initial carious root lesions and sound root surfaces were observed 2 months after the periodontal treatment.

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Author contribution

M.B. was responsible for, conception and design of the study, acquisition of data, analysis and interpretation of data and writing the manuscript. B. I. E. carried out the study. D. P., W. H-M R., P.S., and T.B. helped to draft the manuscript. B.H. was responsible for conception and design of the PCR and helped to draft the manuscript. SZ participated in its design and helped to draft the manuscript.

Conflict of interest

The authors declare that they have no conflict of interests. The study was supported by Heinrich-Heine-University Duesseldorf. Curadent AG Switzerland and GABA GmbH Germany provided free materials for the study.

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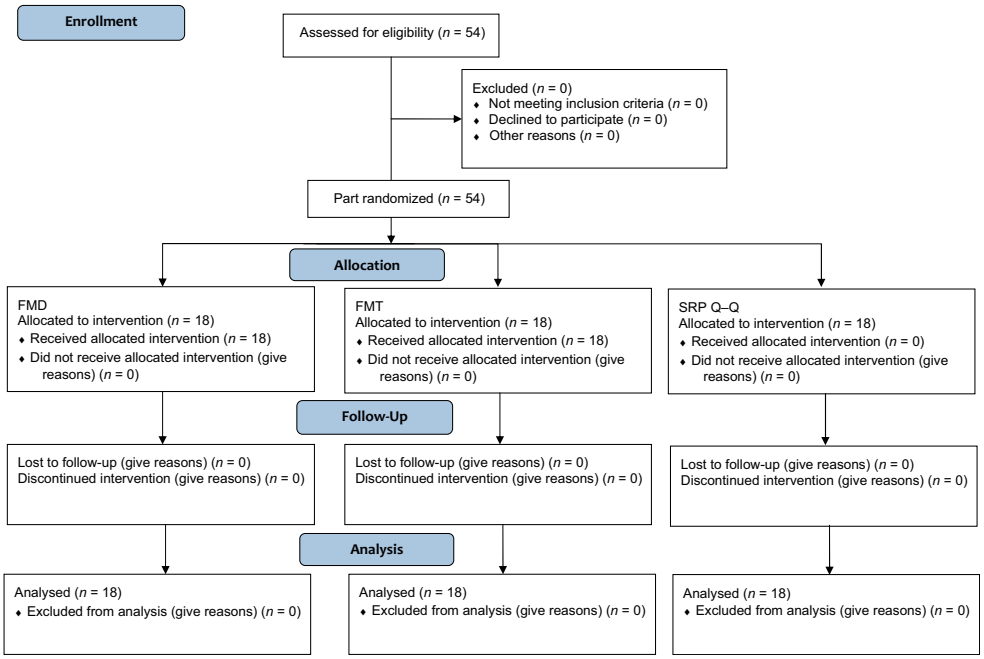
Appendix A: CONSORT 2010 Flow Diagram



Appendix B: CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	*Item no*	*Checklist item*	*Reported on page no*
Title and abstract			
	1a	Identification as a randomised trial in the title	None
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	3
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	4
	2b	Specific objectives or hypotheses	4
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	5–6
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	None
Participants	4a	Eligibility criteria for participants	5
	4b	Settings and locations where the data were collected	5–6
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	5–7
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	5–7
	6b	Any changes to trial outcomes after the trial commenced, with reasons	None
Sample size	7a	How sample size was determined	5
	7b	When applicable, explanation of any interim analyses and stopping guidelines	5
Randomisation: sequence generation	8a	Method used to generate the random allocation sequence	None
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	16
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	None

Appendix B: Continued

Section/Topic	*Item no*	*Checklist item*	*Reported on page no*
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	5–6
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	5–6
	11b	If relevant, description of the similarity of interventions	5–6
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	7
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	7
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	8
	13b	For each group, losses and exclusions after randomisation, together with reasons	None
Recruitment	14a	Dates defining the periods of recruitment and follow-up	8
	14b	Why the trial ended or was stopped	None
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	16, 18–21
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	8
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	8, 18–21
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	None
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	None
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	None
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	9–11
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	None
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	9–11
Other information			
Registration	23	Registration number and name of trial registry	5
Protocol	24	Where the full trial protocol can be accessed, if available	5
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	11

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see http://www.consort-statement.org.