COMPARISON OF CURETTE AND PAPER POINT SAMPLING OF SUBGINGIVAL PLAQUE BACTERIA AS ANALYZED BY REAL-TIME PCR

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CONTENTS

ZUSAMMENFASSUNG

Aufgrund ihrer wichtigen ätiologischen Bedeutung bei der Parodontitis hat der mikrobiologische Nachweis subgingival lokalisierter parodontalpathogener Bakterien eine wichtige Bedeutung in der klinischen Diagnostik. Nur wenige Studien haben bisher allerdings Entnahmetechniken subgingivaler Plaquebakterien systematisch untersucht. Diese Studie diente dem erstmaligen Vergleich von zwei häufig verwendeten Probeentnahmetechniken (Papierspitze bzw. Kürette) mit Hilfe der Methode der quantitativen Real-time PCR.

Zwanzig Patienten mit chronischer Parodontitis nahmen an einer prospektiven Studie im cross-over Design teil. Bei jedem Patienten wurde eine Tasche tiefer als 6 mm an einem Frontzahn zur Probeentnahme ausgewählt. Bei der Gruppe A wurde die erste Probe mit einer Papierspitze und die zweite mit einer Gracey-Kürette entnommen. Bei der Gruppe B wurde die Probeentnahme in der umgekehrten Reihenfolge durchgeführt. Die Proben wurden in einem mikrobiologischen Fachlabor durch einen geblindeten Untersucher mit einem auf Real-Time PCR basierten Test analysiert. Dies erlaubte die quantitative Bestimmung von *Actinobacillus actinomycetemcomitans, Fusobacterium nucleatum ssp., Porphyromonas gingivalis, Prevotella intermedia, Treponema denticola, Tannerella forsythia* sowie der Gesamtbakterienzahl. Acht Wochen nach antiinfektöser Therapie der Patienten wurden in jeweils derselben Reihenfolge nochmals Proben entnommen. Die statistische Auswertung erfolgte mit t-Test, Kappa und Spearman Korrelation.

In dieser Studie konnten mit Küretten mehr Gesamtbakterien als mit Papierspitzen entnommen werden. Die Verhältnisse zwischen Papierspitze und Kürette lagen in Gruppe A vor Therapie bei 1:4 und nach Therapie bei 1:1, in Gruppe B waren sie 1:4 vor und 1:3 nach Therapie. Hingegen waren die relativen Anteile der Zielbakterien an der Gesamtprobe vergleichbar. Beide Entnahmetechniken zeigten übereinstimmend sowohl eine Reduktion der Gesamtbakterienzahl als auch des relativen Anteils der Parodontalpathogene an der Gesamtprobe nach Therapie. Insgesamt bestand eine relativ gute Übereinstimmung der Ergebnisse nach den untersuchten Entnahmetechniken zur Analyse subgingivaler Plaque-Bakterien, so dass beide für die klinisch-mikrobiologische Diagnostik bei Patienten mit Parodontitis geeignet erscheinen.

1. INTRODUCTION

1.1. Plaque formation and structure in general

Bacterial accumulation on the hard and soft surfaces is assumed to be the main cause of inflammatory diseases in the oral cavity. The unique site at which soft and hard surfaces join each other at the gingival and periodontal connection represents a weak point, which easily can be colonized and attacked by the microorganisms of the dental plaque. The accumulation of plaque at these sites initially causes gingivitis and if an irreversible attachment loss develops, gingivitis has transformed into periodontitis. Therefore, investigating the formation and structure of dental plaque is very important to understand and treat periodontitis (Lang et al. 2003).

The initial plaque formation on both natural and artificial hard surfaces in the oral cavity is similar (Siegrist et al. 1991). Few minutes after cleaning a hard surface in the oral cavity a very thin layer of macromolecules (acquired pellicle) adsorbs on the hard surface changing its electrical charge and free energy enabling the bacteria to adhere on its surface. The resulting microbial community is defined as a biofilm, which includes very large amounts and different types of microorganisms, some of which are considered as periodontopathogenic and able to cause periodontitis (Lang et al. 2003).

Gram-positive facultative anaerobic cocci are dominant species in the first phase of plaque formation followed by enhancement in Gram-positive rods. As a next step Gram-negative microorganisms bind to Gram-positive cocci and rods. These Gram-negative microorganisms are able to digest proteins of the gingival or periodontal exudates and to survive without external dietary sources. Such microorganisms do not produce extracellular polymers; they form the loose top layer of plaque in the periodontal pocket (Lang et al. 2003).

Generally there are no big differences between supra- and subgingival plaque. Remains of gingival originating cells and a characteristically differentiated microbial community can be found composing the subgingival plaque. In the layer of subgingival plaque on the tooth surface mostly filamentous microorganisms dominate all in the coronal portion, while the layer facing the soft tissues contains more spirochetes and flagellated

microorganisms with decreased number of Gram-negative cocci and rods (Listgarten 1976).

A close relationship between periodontitis and some microbial species has been strongly suggested for *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*, *T. denticola*, *F. nucleatum ssp.* and *P. intermedia* (Zambon 1985, Eisenmann et al. 1983, Johnson et al. 1993, van Winkelhoff et al. 2002, Ezzo & Cutler 2003).

1.2. Microbiological examination methods

The search for causes of periodontitis led to many methods in examining the microbiological composition of subgingival plaque. Dark field and phase contrast microscopy was the first method used to demonstrate plaque microorganisms (Rosebury et al. 1950); hereby some plaque microorganisms can be morphologically differentiated. However, an exact quantitative testing of plaque samples with such a technique is not possible. The information about the vitality of the plaque is limited. Only motile bacteria can be distinguished as alive (Listgarten 1986, Lange et al. 1983, Müller et al. 1989).

Another identification method is bacterial cultivation, which represents the golden standard in microbiological diagnosis, although it possesses some disadvantages. Target microorganisms must survive sampling and transportation and stay vital in order to be able to colonize. Several putative periodontopathogenic microorganisms require anaerobic growth conditions. Therefore, for diagnostic procedures problems of sampling, transport and cultivation have to be taken into consideration. Limitations with respect to detecting non-viable bacteria, the inability of some species to grow reliably on selective media as well as high costs narrow the use in periodontal microbiological diagnostics (Loomer 2004). If an antibiotical sensitivity test is made at the same time; the growth of the sampled bacteria could be inhibited and lead to false negative results (Slots 1986).

Enzyme tests are very fast methods and mostly used as a chair side diagnostic. As a reaction between the microbiological enzymes and the testing agent occurs, the color of the containing medium changes to reflect the detection of microorganisms. The disadvantage of such tests is their inability of detecting very important periodontal pathogens like *A. actinomycetemcomitans*. The BANA test (N-Benzoyl-DL-Arginine-2-Napthylamide) is an example for this method. This test detects microbial groups rather than species and does not give quantitative results (Jervøe-Storm 1992, Loomer 2004, Loesche 1986).

Polymerase chain reaction (PCR) based methods are more specific and sensitive than cultivation, based on detection of gene specific DNA sequences, thus a possibility to distinguish close related bacteria is given. A PCR assay for the identification of *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *T. forsythensis* and *T. denticola* has recently been described (Eick and Pfister 2002). The authors advocated this test as a highly sensitive and specific method for the analysis of the subgingival plaque.

Many efforts have been made to quantify target DNA molecules initially serving as template in a PCR reaction. Standard PCR lacks the ability for precise quantification because only an endpoint determination can be analysed. Real-time PCR overcomes these problems by direct monitoring of the increasing amount of PCR products throughout the enzymatic assay. The amount of newly synthesised PCR product molecules is directly dependent on the amount of template molecules. The data for quantification are collected in exponential phases of the PCR. This allows a precise quantification of the target DNA copy number, when using internal and external standards (Bustin 2000).

The accumulation of a PCR product is monitored by the addition of fluorescent dyes to the PCR reaction. Although conventional PCR is a rather sensitive method, the sensitivity of real-time PCR is mostly enhanced due to the fluorescence based detection of the PCR product. Up to date, basically two different methods for the generation of the fluorescent signal are available: the so-called SYBR®Green assays use unspecific fluorescent dyes that exhibit an increased light emission, when intercalating into double-stranded DNA (Bustin 2000). The fluorescent probe assays with TaqMan®- probes use specific fluorescent oligonucleotide probes. The highly specific binding of the probe to

the target DNA, or to the increasing amount of specific PCR product, is a prerequisite for the emission of fluorescent light (Kuboniwa et al. 2004). Due to this third specific probe, in addition to the two specific primers that are also needed for PCR, these assays show a significantly increased specificity in comparison to the SYBR®Green assays (Bustin 2000, Malinen et al. 2004).

An example of a real-time PCR based test is Meridol® Perio Diagnostics (GABA International, Münchenstein, Switzerland). This test allows the detection and quantification of *A. actinomycetemcomitans*, *F. nucleatum ssp.*, *P. gingivalis*, *P. intermedia*, *T. denticola*, *T. forsythia* as well as the total bacterial load. A comparison between real-time PCR and cultivation was made and could prove that real-time PCR is a very sensitive and promising method for periodontal diagnostic and research (Jervøe-Storm 2005).

1.3. Sampling of subgingival plaque bacteria

1.3.1. Indications

Periodontal pathogens play an important role in etiology and pathogenesis of periodontitis; although they are not the only deciding factor, their absence in the periodontal pocket indicates more stability and better prognosis (Socransky & Haffajee 1992). Microbiological examination of subgingival plaque is used at the present time in etiological research as well as in clinical treatment of periodontitis to select the appropriate antibiotic agent if indicated. Examples of such conditions are aggressive periodontitis, advanced chronic periodontitis, refractory periodontitis, moderate and advanced chronic periodontitis in combination with systemic diseases or conditions that affect the immune system (Beikler et al. 2004).

1.3.2. Sampling techniques

The outcome of microbiological sampling depends on the used technique. The commonly used sampling devices were discussed by Tanner and Goodson (1986). They described the sampling tools as “dental approved” devices. Sampling using

curettes, scalers, paper points, barbed broaches within cannulas, irrigation of periodontal pockets etc. were reported. A 10 µl automatic pipette was used for sampling in a study of Strand et al. (1987).Another method called pocket-out-method based on collecting biological materials of non-viable periodontal pathogens originating from the pocket was described by Smola et al. (2003).

Tanner and Goodson (1986) stated that paper points are used by an increasing number of investigators mostly for cultural studies; hereby the loosely adherent tissue associated microorganisms in the periodontal pocket were sampled. They reported that all sampling methods depend on the performing person, curette samples being the most sensitive. They declared that curette sampling needs a high training and should not be preferred if the microbial ecosystem is not to be disturbed, because curettes could disturb the ecosystem much more than paper points. Kornman (1986) stated that paper points do not remove enough bacteria for microbiological analysis after periodontal therapy, and up to then no adequate technique which would not alter the ecosystem. Loomer (2004) reported that paper point samples differ from curette samples and that curette collects plaque from the entire pocket whereas paper points collect plaque from the outer layer of the plaque, which contains more pathogens. At the same time paper points are less successful at sampling the apical part of the pocket, where more pathogens are expected to be. This means that by paper point samples the most coronal and outer portion of the plaque is sampled. This result was also partially confirmed by Baker et al. (1991) in their in vitro study testing if paper point sampled homogenous and non homogenous plaque equally and from all parts of the pockets. They concluded that paper points could misrepresent the microbiological composition in the apical part of the pocket. In another study by Renvert et al. (1992) paper point samples were compared with scaler samples both before and after treatment. This study concluded that paper points collected more colony forming units and spirochetes before and after therapy. To validly compare techniques, the technique itself should be the only variable. Unfortunately, few published studies have been performed which meet this requirement.

Comparisons between currently used sampling devices can be made based on their relative ability to access defined zones in a pocket, sample size, and sample

composition when the same site is re-sampled by the same or different devices (Tanner and Goodson 1986).

1.4. Aims of the study

Because of the importance of subgingival plaque bacteria in the etiology, diagnosis, and treatment of periodontitis reliable sampling methods are needed. Even though curettes and paper points are the most commonly used sampling devices, previous evaluations were limited by microbiological methods that would preclude quantification and/or sensitive and specific identification of target pathogens. The novel method of quatitative real-time PCR can overcome these shortcomings.

Therefore, it was the aim of the present study to compare curette and paper point sampling techniques of subgingival plaque bacteria by real-time PCR.

2. MATERIALS AND METHODS

2.1. Patients

Twenty patients with chronic periodontitis (Age range: 35 - 60 years) took part in the study, after informed consent had been obtained. The study had been approved by the International Ethic´s Committee in Freiburg. All patients had at least 1 periodontal pocket with a probing depth more than 6 mm on a front tooth. The patients had no systemic or local factors, which could influence their gingival or periodontal health, interfere with the healing procedure after periodontal therapy (like diabetes) or inhibit the performance of the adequate oral hygiene (like crowns or restorations with improper margins). Patients had not received any antibiotic or periodontal therapy within a period of 6 months before initiation of the study. Each patient was given repeated oral hygiene training until a low plaque score (< 20 %) was obtained.

2.2. Grouping according to sampling sequence

To be able to compare two different sampling methods it was necessary to use both methods at the same time and at the same site. In order to balance the effect of the first sample on the second one, samples were taken in 2 opposite sequences in two patient groups (Cross-over Design). Depending on the sequence of sampling patients were randomized into two groups (A and B) each with 10 patients.

In group A patients the first sample was taken using one sterile paper point ISO #40 taper 0.02 mm/mm (Co. Roeko, Langenau, Germany), which was inserted as deep as possible into the pocket and left there for 20 sec. The second sample was taken with a sterile Gracey-curette 5-6 (Hu-Friedy, Chicago, USA) with one light strike on the root surface. In group B patients samples were taken in the opposite sequence, i.e. the first sample was taken with a sterile Gracey-curette 5-6 (Hu-Friedy, Chicago, USA), while the second one with one sterile paper point ISO #40 taper 0.02 mm/mm (Co. Roeko, Langenau, Germany), which was inserted in the pocket as deep as possible and left there for 20 sec.

2.3. Chronological design of the study

The baseline of the study was the first clinical assessment, which was performed in all patients before scaling and root planing and after the patients had demonstrated good personal oral hygiene. At the 2nd week the first sampling took place. A mechanical anti-infective periodontal therapy consisting of deep scaling and root planing followed. All periodontal diseased teeth were treated in the Department of Periodontology, Operative and Preventive Dentistry at the University of Bonn in one or more visits depending on the number of the pockets which should be treated. The therapy had to be completed within 2 weeks i.e. before the end of the 4th week. 6 weeks later i.e. at the 10th week after baseline a second clinical assessment was performed to determine the probing pocket depths. At the 12th week the same sampling procedure was repeated.

Figures 1 and 2 show the chronological design of the study, while Figure 3 shows the used sampling techniques.

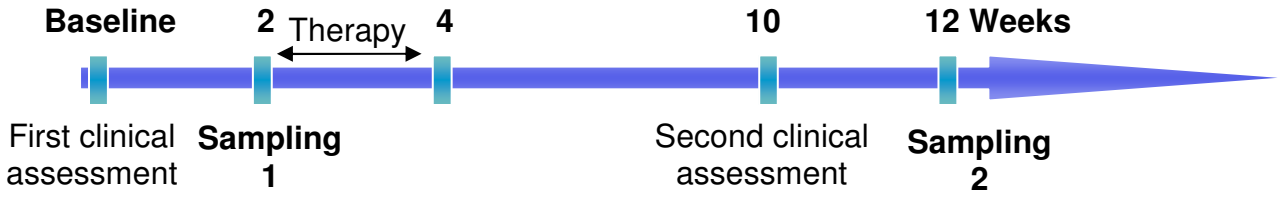

Figure 1:
The chronological design of the study showing the time points of clinical assessment, treatment and plaque sampling.

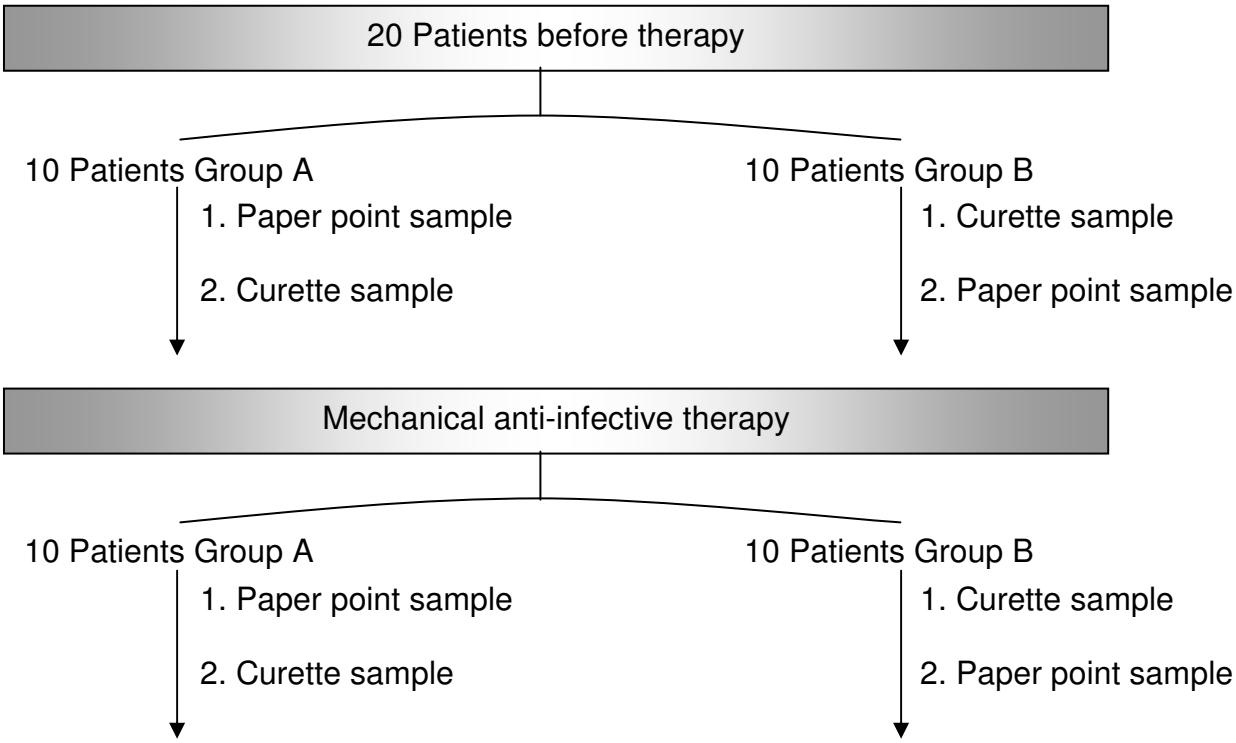

Figure 2:
The study design before and after therapy; the numbers 1 and 2 refer to the sequence of sampling.

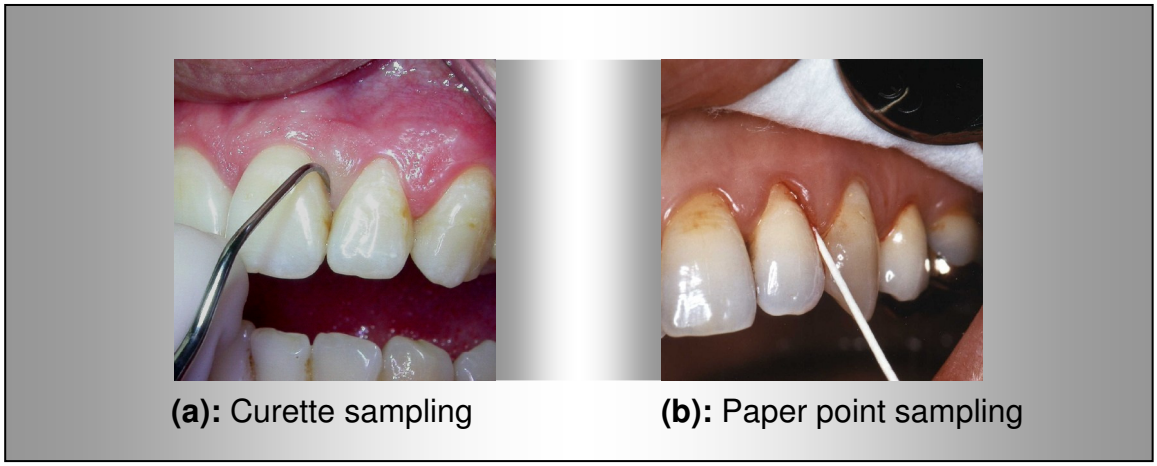

Figure 3:
The used techniques of sampling of subgingival plaque bacteria with (a) curette and (b) paper point.

2.4. Sampling conditions

All samples were taken by the same dentist in order to standardize the sampling procedure. Before sampling, selected lesions and the adjacent teeth were isolated with cotton rolls, supragingival plaque was careful removed with a sterile scaler to prevent the contamination of the samples with saliva or supragingival plaque. For sampling with paper points one paper point ISO #40 taper 0.02 mm/mm (Co. Roeko, Langenau, Germany) was inserted slowly with a sterile dental tweezer into the pocket to the predetermined depth until tissue resistance. The paper point was left for 20 sec., then it was carefully removed without touching the adjacent unrelated tissues and placed into a special sterile container and sent for microbiological examination. For curette sampling Gracey-curettes Nr. 5/6 (Hu-Friedy, Chicago, USA) were used. The curette was inserted slightly as deep as possible into the pocket without applying any pressure on the tooth surface, in order to avoid a dislocation of subgingival plaque with the curette into the depth of the pocket. As soon as the curette met tissue resistance at the apical part of the pocket, subgingival sampling was performed with one single vertical stroke. For transport of the sample, the working end of the curette was striped with one sterile paper point ISO #40 taper 0.02 mm/mm (Co. Roeko, Langenau, Germany). The paper point was sent for microbiological examination in a similar sterile container as used for the paper point sample. Care was always taken to harvest the second sample from the same site as the first one.

2.5. Laboratory methods

The samples were sent to Carpegen GmbH (Münster, Germany) for real-time PCR analysis. There the cells were harvested by centrifugation (15.000 g at 4°C) for 10 min and immediately subjected to the automated process of the meridol® Perio Diagnostics (GABA International, Basel, Switzerland) analysis. This Real-time PCR based analysis was developed and validated by Carpegen GmbH (Münster, Germany). Specificity of meridol® Perio Diagnostics had been verified with purified genomic DNA from several bacterial and fungal species as well as with human DNA. Even closely related species, such as *P. intermedia* and *P. nigrescens*, had not shown any cross-reactivity.

In addition, PCR products obtained from positive patients´ samples had been sequenced. Sensitivity and linearity had been determined by reactions containing serial dilutions of purified genomic DNA and with plasmids containing the appropriate PCR amplicons. The measurement range had then been calculated according to the official guidelines of the international organization for standardization and the German industry standards (ISO 17025 and DIN 32645 standards). A standard curve prepared with these dilutions was used in every experiment for each pathogen.

To test whether contaminations within the sample DNA preparation inhibited the PCR reaction, each sample DNA preparation was added to a reaction mixture which contained a defined amount of an artificial DNA sequence (no natural occurrence). This artificial DNA sequence had to be amplified as efficiently as a control reaction containing the same primer / probe set and artificial DNA, but no sample DNA.

The main validated test parameters of meridol® Perio Diagnostics are:

- the detection limit for each of the five pathogens is 100 bacteria within a patient's sample
- the linear range for quantification comprehend 7 orders of magnitude for each pathogen
- the coefficient of variation is 15%

The test method detects and quantifies six periodontal pathogens (*A. actinomycetemcomitans , F. nucleatum ssp., P. gingivalis, P. intermedia, T. forsythensis and T. denticola*) and the total bacterial load.

The bacterial genomic DNA was isolated and purified with the AGOWA® mag DNA Isolation Kit Sputum (AGOWA GmbH, Berlin, Germany). The protocol followed the manufacturer´s instructions with minor changes to adjust the procedure to the automated isolation with a pipetting robot (Tecan, Genesis Workstation; Tecan Schweiz AG, Switzerland). Primers and probes for meridol® Perio Diagnostics were designed to match highly specifically to ribosomal DNA (rDNA) of the six bacterial pathogens. The exact primer and probe sequences were selected with the Primer Express software (Applied Biosystems, Foster City, California, USA), which checks the primer and probe sets for matching the guidelines that are recommended for real-time PCR with TaqMan® probes. The primers and probes were purchased from Applied Biosystems (Foster City,

California, USA). Real-time PCR was carried out with 2 µl of the isolated DNA as template in a reaction mixture containing the appropriate primer probe sets and the TaqMan® Universal PCR Mastermix. The PCR was carried out in an ABI 7900 HT (Applied Biosystems, Foster City, California, USA) real-time PCR cycler in 384 well plates (Jervøe-Storm et al. 2005).

2.6. Statistical analysis

Plaque sampling before as well as after therapy was performed in a cross-over design randomly allocating to possible sequences: curette - paper point and paper point - curette randomly to equal sized groups of patients. Before analysis the bacterial counts were transformed to natural logarithms adding one to each count before transformation to avoid problems with zero counts. To analyze the differences between the bacterial counts with curette and paper point sampling, standard techniques for cross over analysis were used. The comparison between the two sampling techniques (C - P) was made by a two sample t-test applied to the period differences divided by two, compared between the two sequence groups. Point estimators for the difference between both techniques and confidence limits were also revealed from this test. Carry over was checked by comparing the period averages (sums) between both patient groups also using a two sample t-test. In case of a carry over effect, the estimator of the difference may be biased. The correlation between bacterial counts gathered with the two methods was analyzed using Spearman correlation coefficient. The agreement of the qualitative detection of different bacteria with both sampling techniques is judged by Cohen's Kappa.

3. RESULTS

3.1. Clinical results (sampled sites)

In group A probing pocket depths showed a mean of 7.5 $\pm$ 2.0 mm before therapy (baseline), after therapy (10^{th} week) a reduction was obtained (6.1 $\pm$ 2.0 mm). In group B probing pocket depth was 6.9 $\pm$ 0.8 mm before therapy and 5.3 $\pm$ 1.7 mm after therapy. With respect to bleeding on probing in group A 100% of the sampled pockets showed bleeding before therapy and 40% after therapy. Bleeding on probing was found in all sampling sites of group B before therapy and in 60% of the same pockets after therapy.

3.2. Microbiological results

3.2.1. Total bacterial counts (TBC)

In group A median counts of TBC at first sampling were higher than at the 12^{th} week with paper point as well as with curette (2^{nd} week: paper point: 14,028,200; curette: 51,212,987; 12^{th} week: paper point: 3,142,383; curette: 3,751,742 bacteria / sample) (Tables 1 & 2). The same result was found for group B (2^{nd} week: curette: 34,500,000; paper point: 8,500,000; 12^{th} week: curette: 16,500,000; paper point: 5,524,471 bacteria / sample) (Tables 1 & 2). Figure 4 shows the distribution of total bacterial counts of curette and paper point samples in both groups as box plots. Curettes in both groups sampled always more bacteria than paper points.

With t-test a significant difference between curette and paper point samples before therapy was calculated (log mean of difference: -0.947, CI: [-0.283, -1.611], SD: 0.707, p= 0.008) (Table 10 app.). This significance could not be recorded after therapy (log mean of difference: -0.444, CI: [0.136, -1.023], SD: 0.617, p= 0.125).

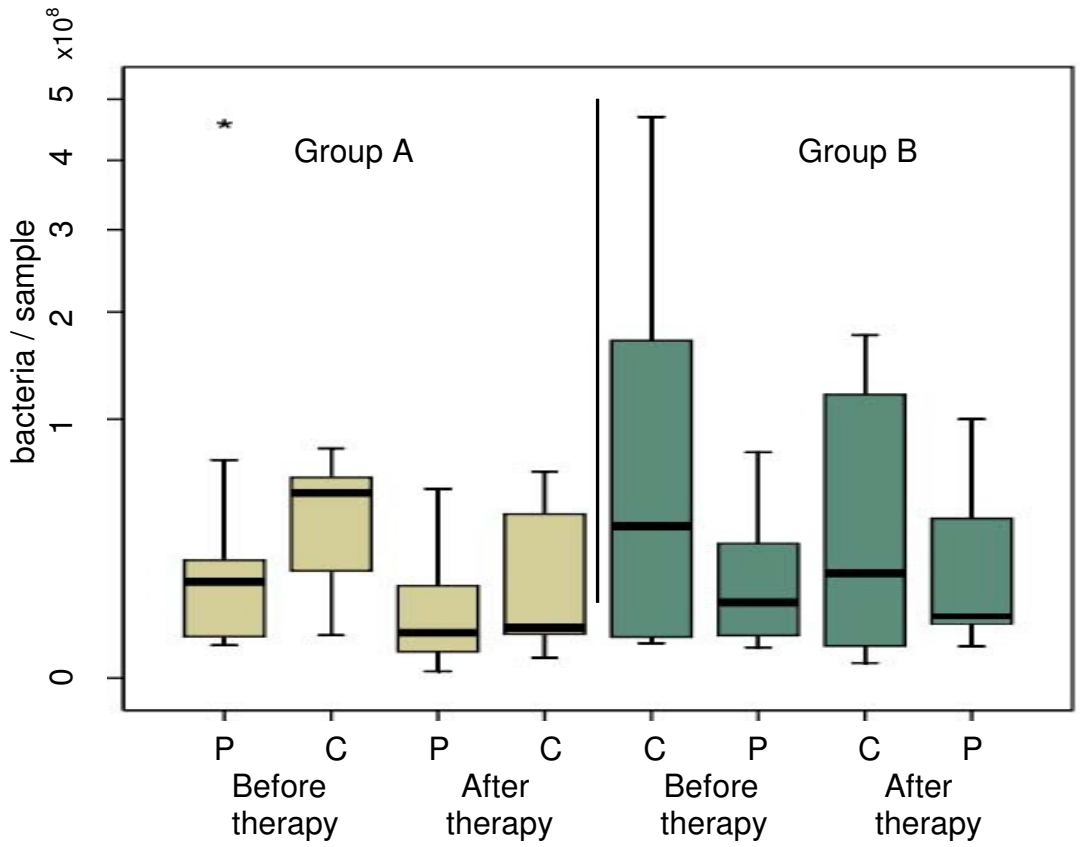

Figure 4:
Total bacterial counts (TBC) of paper point (P) and curette (C) samples before and after therapy in group A and B. Box-plots show: median, interquartile range between 25th and 75th percentile, whiskers indicate maximum and minimum, outliers are shown as asterisk, n : 10 in each group.

3.2.2 Selected bacteria

Actinobacillus actinomycetemcomitans

The median counts of *A. actinomycetemcomitans* in group A at both the 2nd and the 12th week after therapy were low due to the little number of positive sites found (2nd week: paper point: 100; curette: 100; 12th week: paper point 100; curette: 100 bacteria / sample) (Tables 1 & 2). The same was found in group B (2nd week: curette: 175; paper point: 250; 12th week: curette: 618; paper point: 809 bacteria / sample). Figure 5 shows the distribution of total bacterial counts of curette and paper point samples in both groups as box plots.

The number of *A. actinomycetemcomitans* positive sites was the same for paper point and curette in group A before therapy, after therapy paper point found one more positive pocket (2) than curette (1); in group B more positive sites were found with paper point as

with curette at all time points (Table 3). No difference between curette and paper point sampling was determined with t-test (before therapy: log mean of difference: 0.088, CI: [0.505, -0.330], SD: 0.444, p= 0.665; after therapy: log mean of difference: 0.028, CI: [0.840, -0.783], SD: 0.864, p= 0.943). Table 11 (app.) displays these results. However, no firm conclusions could be drawn, because of the small number of sites and patients positive for *A. actinomycetemcomitans* (Table 3).

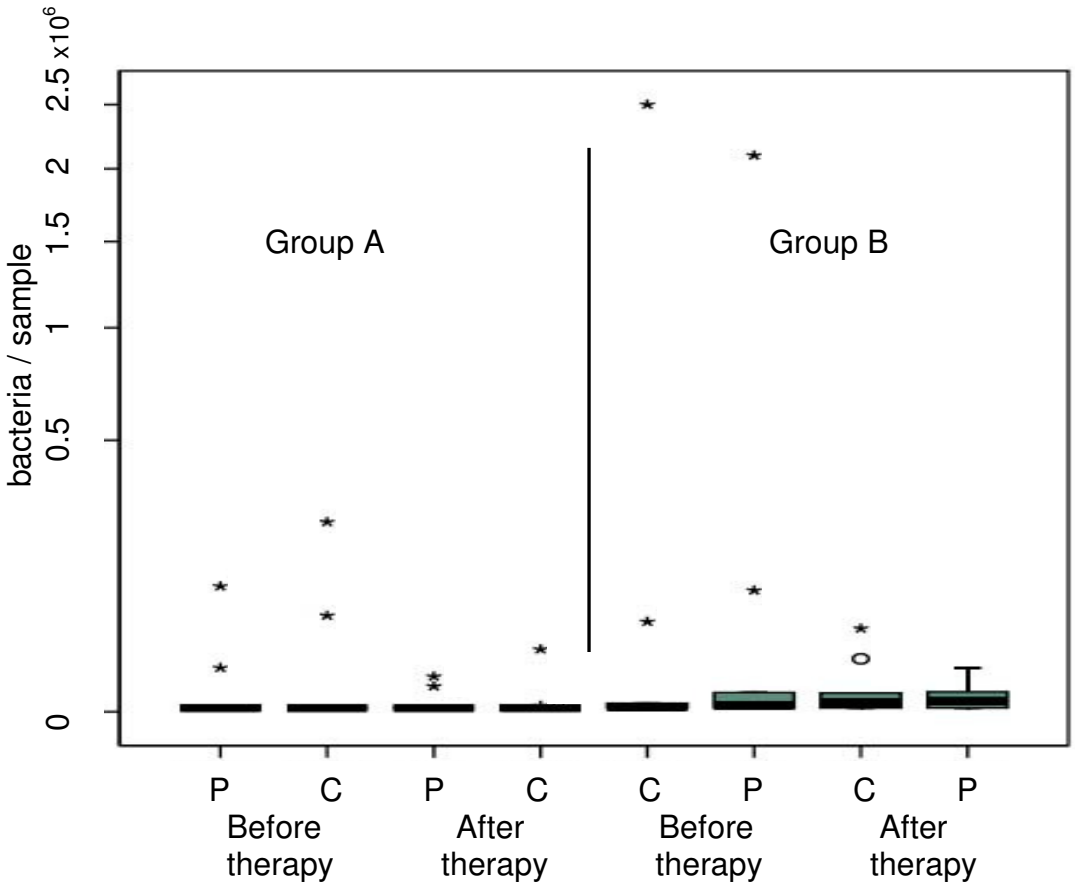

Figure 5:
A. actinomycetemcomitans counts of paper point (P) and curette (C) samples before and after therapy in group A and B. Box-plots show: median, interquartile range between 25th and 75th percentile, whiskers indicate maximum and minimum, outliers are shown as asterisks and circles, n : 10 in each group.

Fusobacterium nucleatum ssp.

The median counts of *F. nucleatum ssp.* in group A were reduced after therapy (2nd week: paper point: 61,045; curette: 708,421; 12th week: paper point: 19,677; curette: 47,260 bacteria / sample) (Tables 1 & 2). In group B almost no change for curette was

found, for paper point samples an increase after therapy could be noticed (2nd week: curette: 279,000; paper point: 45,000; 12th week: curette: 295,000; paper point: 230,000 bacteria / sample). Figure 6 shows the distribution of total bacterial counts of curette and paper point samples in both groups as box plots.

The number of *F. nucleatum ssp.* positive sites was the same for paper point and curette in group A before therapy, almost no change after therapy in *F. nucleatum ssp.* positive sites was recorded; in group B more positive sites were found with paper point as with curette before therapy; after therapy the same number of positive pockets was found (Table 3).

Statistically, no difference between curette and paper point sampling was determined with t-test for *F. nucleatum ssp*. (before therapy: log mean of difference: -0.848, CI: [0.714, -2.410], SD: 1.663, p= 0.269; after therapy: log mean of difference: -0.156, CI: [0.912, -1.225], SD: 1.137, p= 0.762). Table 12 (app.) displays these results.

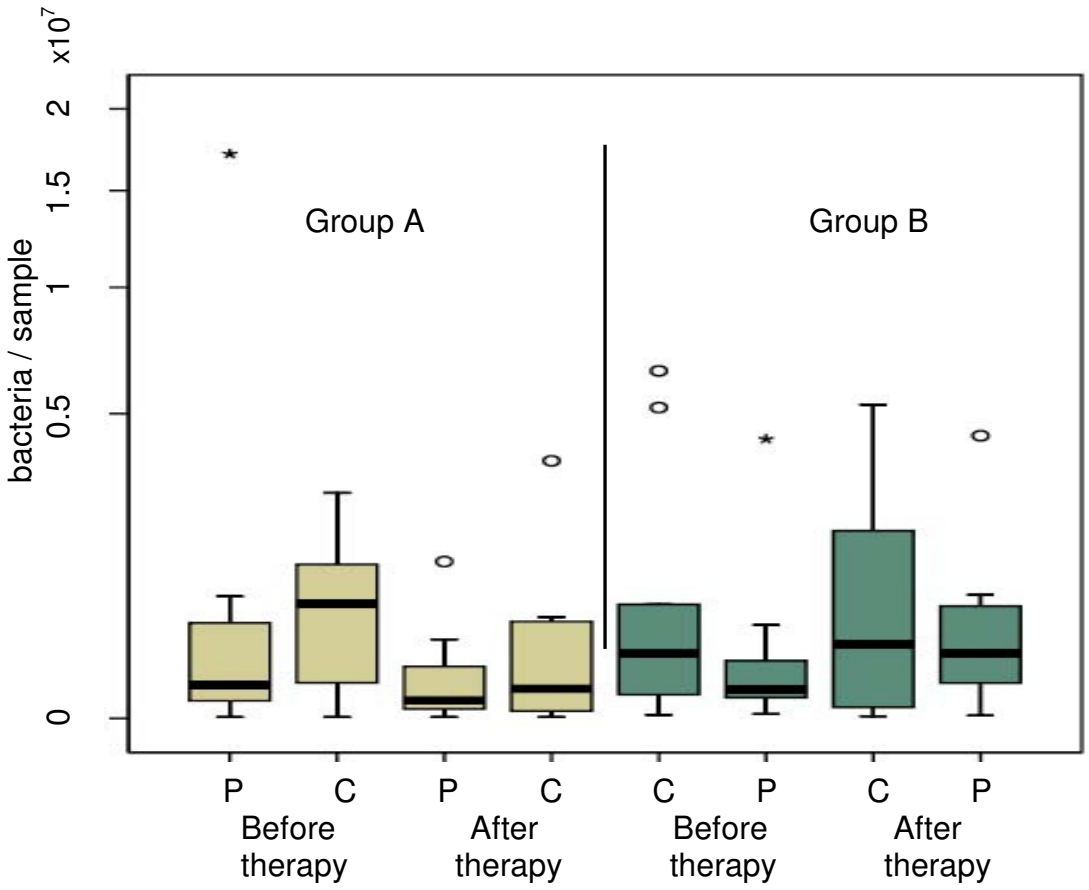

Figure 6:
F. nucleatum ssp. counts of paper point (P) and curette (C) samples before and after therapy in group A and B. Box-plots show: median, interquartile range between 25th and 75th percentile, whiskers indicate maximum and minimum, outliers are shown as asterisks and circles, n : 10 in each group.

Porphyromonas gingivalis

Although the median counts of *P. gingivalis* in group A sampled with curette were higher than sampled with paper point, median counts of both were similar after therapy (2nd week: paper point: 1,886,378; curette: 5,840,647; 12th week: paper point: 252,831; curette: 239,496 bacteria / sample) (Tables 1 & 2). In group B less bacteria could be sampled with both techniques after therapy (2nd week: curette: 1,500,000; paper point: 590,000; 12th week: curette: 340,000; paper point: 165,000 bacteria / sample). Figure 7 shows the distribution of *P. gingivalis* of curette and paper point samples in both groups as box plots.

The same number of *P. gingivalis* positive sites was registered for paper point and curette in group A before and after therapy; in group B one more positive site was found with curette as with paper point before therapy; after therapy the opposite was found (Table 3).

For *P. gingivalis* a difference between paper point and curette samples before therapy was calculated with t-test (before therapy: log mean of difference: -1.262, CI: [-0.487, -2.0367], SD: 0.825, p= 0.003; after therapy: log mean of difference: -0.329, CI: [0.0379, -0.695], SD: 0.390, p= 0.076) (Table 13 app.).

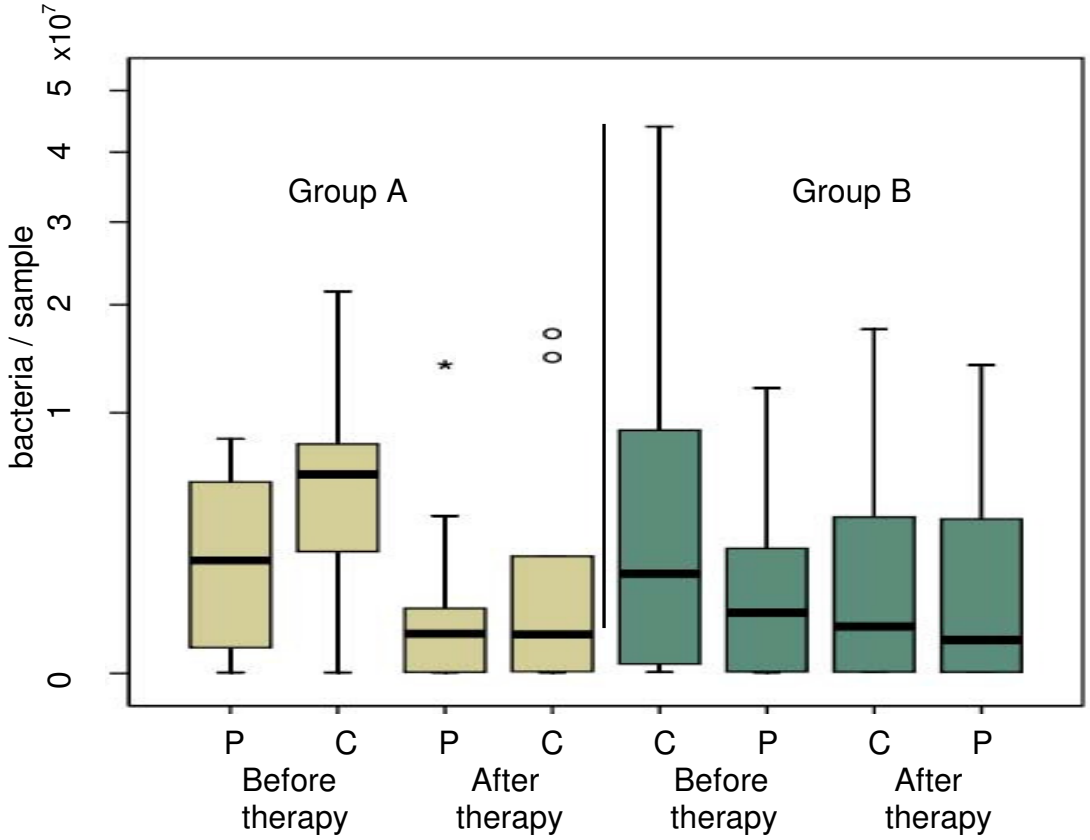

Figure 7:
P. gingivalis counts of paper point (P) and curette (C) samples before and after therapy in group A and B. Box-plots show: median, interquartile range between 25th and 75th percentile, whiskers indicate maximum and minimum, outliers are shown as asterisks and circles, n : 10 in each group.

Prevotella intermedia

The median counts of *P. intermedia* in group A at both the 2nd and the 12th weeks after therapy were low due to the little number of positive sites found (2nd week: paper point: 538; curette: 425; 12th week: paper point: 100; curette: 258 bacteria / sample) (Tables 1 & 2). In group B curette median counts were always higher regardless of sampling time point (2nd week: curette: 312,500; paper point: 57,000; 12th week: curette: 130,500; paper point: 34,415 bacteria / sample). Figure 8 shows the distribution of *P. intermedia* of curette and paper point samples in both groups as box plots.

The number of *P. intermedia* positive sites was the same for paper point and curette in group A, this was also the case in group B before therapy, after therapy in group B a small difference was found (Table 3).

For *P. intermedia* (Table 14 app.) a difference could be found with t-test between paper point and curette samples after therapy (before therapy: log mean of difference: -1.219, CI: [0.0402, -2.478], SD: 1.340, p= 0.057; after therapy: log mean of difference: -0.611, CI: [-0.036, -1.186], SD: 0.612, p= 0.039).

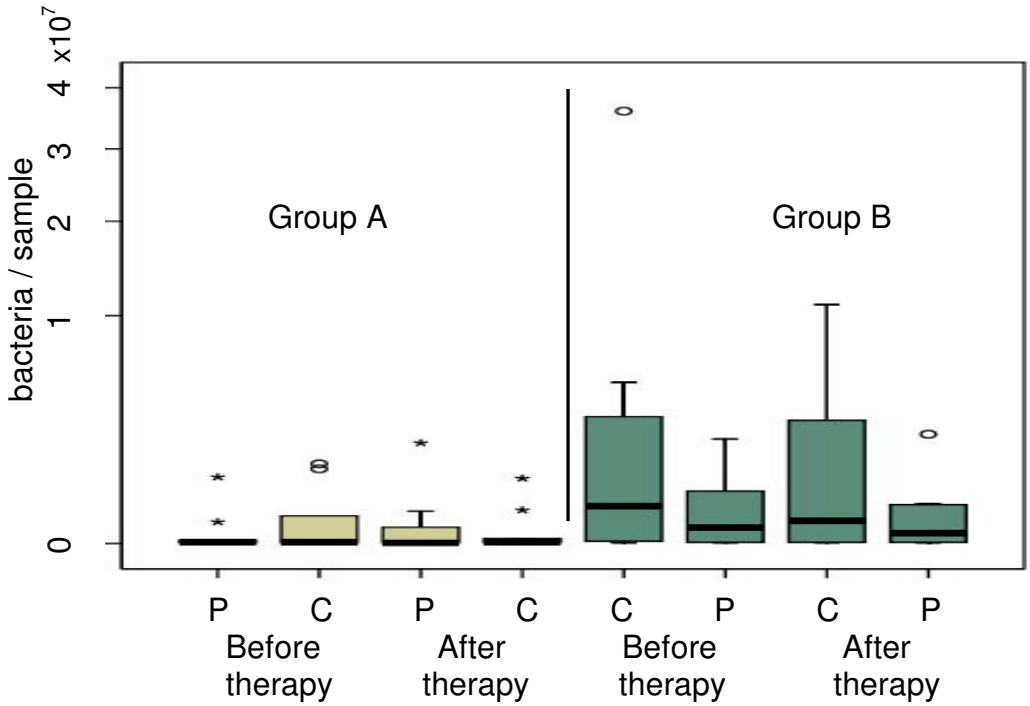

Figure 8:
P. intermedia counts of paper point (P) and curette (C) samples before and after therapy in group A and B. Box-plots show: median, interquartile range between 25th and 75th percentile, whiskers indicate maximum and minimum, outliers are shown as asterisks and circles, n : 10 in each group.

Treponema denticola

Median counts of *T. denticola in* group A and B were reduced after therapy, curette always sampled more bacteria as paper point (group A 2nd week: paper point: 506,577; curette: 2,232,867; 12th week: paper point: 26,359; curette: 85,055 bacteria / sample; group B 2nd week: curette: 1,950,000; paper point: 470,000; 12th week: curette: 215,000; paper point: 149,500 bacteria / sample) (Tables 1 & 2). Figure 9 shows the distribution of *T. denticola* of curette and paper point samples in both groups as box plots.

The same number of *T. denticola* positive sites was registered for paper point and curette in group B before and after therapy; in group A more positive sites were found with paper point as with curette before therapy; after therapy the opposite was found (Table 3).

t-test did not find any significant difference for *T. denticola* (Table 15 app.) between paper point and curette samples before and after therapy (before therapy: log mean of difference: -0.852, CI: [0.194, -1.897], SD: 1.113, p = 0.104; after therapy: log mean of difference: -0.478, CI: [0.364, -1.321], SD: 0.897, p= 0.248).

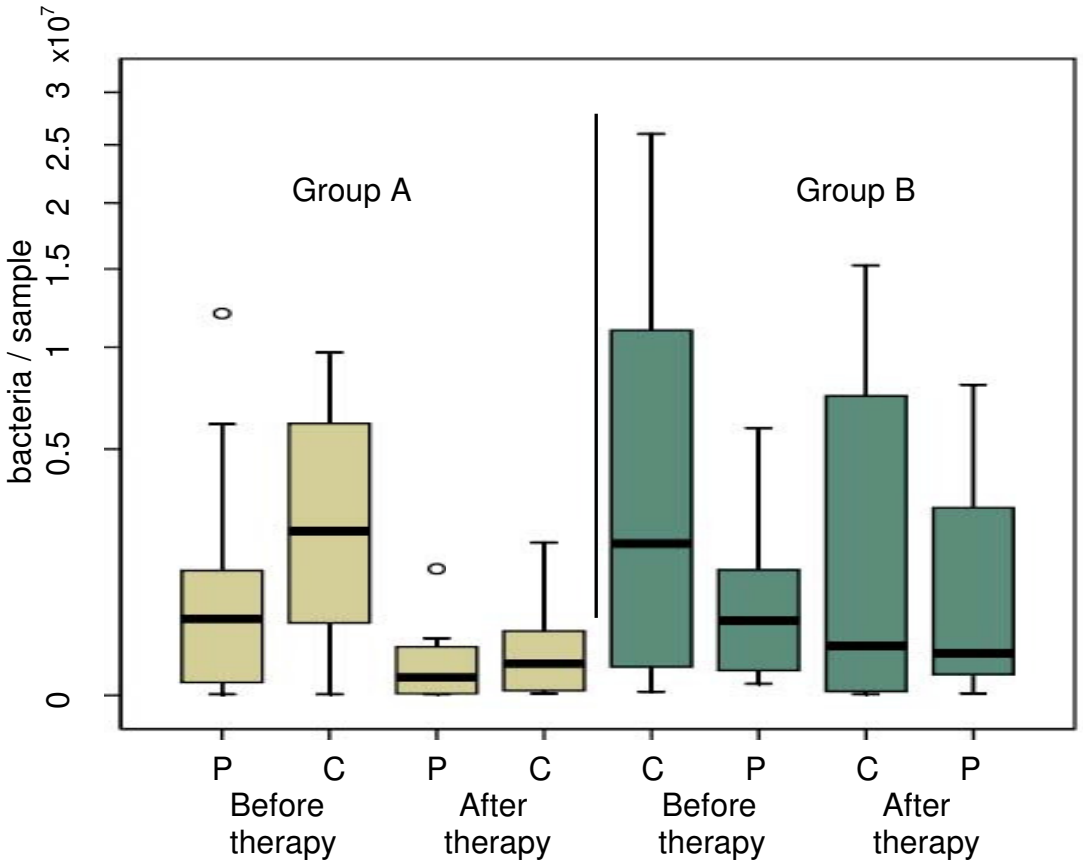

Figure 9:
T. denticola counts of paper point (P) and curette (C) samples before and after therapy in group A and B. Box-plots show: median, interquartile range between 25th and 75th percentile, whiskers indicate maximum and minimum, outliers are shown as circles, n : 10 in each group.

Tannerella forsythia

Median counts of *T. forsythia* in group A and B were reduced after therapy (Tables 1 & 2), curette always sampled more bacteria as paper point (group A 2nd week: paper point: 577,263; curette: 2,744,542; 12th week: paper point: 50,287; curette: 79,938 bacteria / sample; group B 2nd week: curette: 1,330,000; paper point: 250,000; 12th week: curette: 435,000; paper point: 330,000 bacteria / sample). Figure 10 shows the distribution of *T. forsythia* of curette and paper point samples in both groups as box plots.

Before therapy, both sampling techniques found the same number of *T. forsythia* positive sites, while after therapy different results were found (Table 3).

For *T. forsythia* (Table 16 app.) a statistical difference with t-test could be observed only before therapy (before therapy: log mean of difference: -1.206, CI: [-0.164, -2.248], SD: 1.109, p= 0.026; after therapy: log mean of difference: -0.282, CI: [0.673, -1.237], SD: 1.016, p= 0.543).

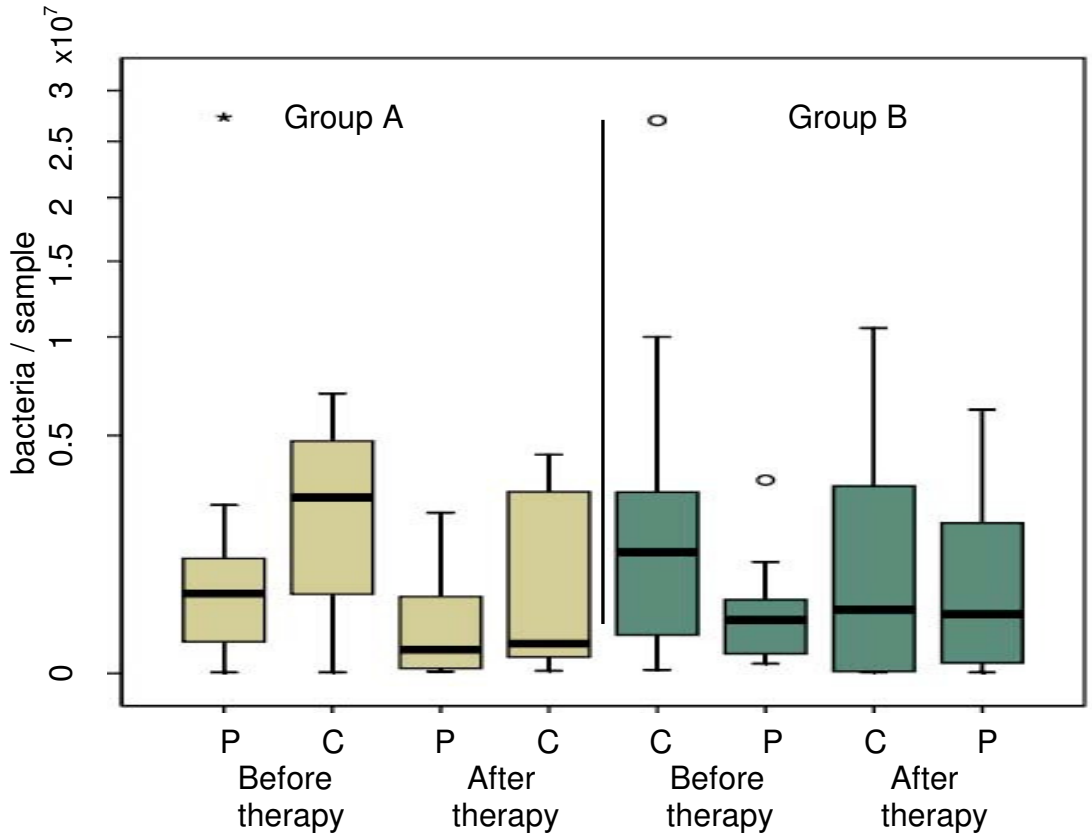

Figure 10:
T. forsythia counts of paper point (P) and curette (C) samples before and after therapy in group A and B. Box-plots show: median, interquartile range between 25th and 75th percentile, whiskers indicate maximum and minimum, outliers are shown as asterisks and circles, n : 10 in each group.

Table 1: Medians (Med), Minimal values (Min), Maximal values (Max), Quartile 25% (Q25) and Quartile 75% (Q75) in group A and B before therapy.

G	T	V	TBC	*Aa*	*Fn*	*Pg*	*Pi*	*Td*	*Tf*
A	P	Med	14,028,200	100	61,045	1,886,378	538	506,577	577,263
		Min	1,601,037	100	100	100	100	100	100
		Max	458,253,279	106,732	17,149,838	8,089,599	851,900	12,028,143	27,306,598
		Q25	3,729,603	100	20,927	149,294	100	31,950	124,942
		Q75	20,236,288	100	403,143	5,232,140	1,851	1,216,632	1,153,800
A	C	Med	51,212,987	100	708,421	5,840,647	425	2,232,867	2,744,542
		Min	2,720,740	100	100	100	100	100	100
		Max	78,755,730	244,341	2,736,895	21,475,650	1,222,900	9,700,393	6,921,940
		Q25	18,511,028	100	123,963	2,458,222	100	494,501	703,063
		Q75	58,883,449	100	1,254,147	7,704,689	112,600	5,524,085	4,643,917
B	C	Med	34,500,000	175	279,000	1,500,000	312,500	1,950,000	1,330,000
		Min	1,800,000	100	690	270	100	1,100	1,100
		Max	470,000,022	2,500,000	6,500,000	44,000,000	36,000,000	26,000,000	26,999,999
		Q25	5,375,000	100	32,500	114,750	4,698	74,250	152,500
		Q75	148,500,000	463	682,500	8,675,000	2,775,000	8,925,000	2,725,000
B	P	Med	8,500,000	250	45,000	590,000	57,000	470,000	250,000
		Min	1,400,000	100	1,000	100	100	11,000	8,300
		Max	76,000,000	2,100,000	4,200,000	12,000,000	2,100,000	5,900,000	3,300,000
		Q25	3,375,000	100	26,750	30,308	438	52,000	65,500
		Q75	23,750,000	2,200	167,500	2,075,000	467,500	1,250,000	477,500

Abbreviations: G: Group, T: Sampling technique, V: Variable, P: Paper point, C: Curette, TBC: Total bacterial counts, *Aa: A. actinomycetemcomitans, Fn: F. nucleatum ssp, Pg: P. gingivalis, ntermedia, Td: T. denticola, Tf: T. forsythia.*

Table 2: Medians (Med), Minimal values (Min), Maximal values (Max), Quartile 25% (Q25) and Quartile 75% (Q75) in group A and B after therapy.

G	T	V	TBC	*Aa*	*Fn*	*Pg*	*Pi*	*Td*	*Tf*
A	P	Med	3,142,383	100	19,677	252,831	100	26,359	50,287
		Min	66,936	100	100	100	100	100	309
		Max	53,363,503	8,401	1,324,115	14,019,924	1,945,883	1,319,555	2,281,664
		Q25	1,076,131	100	4,767	4,448	100	348	5,786
		Q75	11,710,569	100	123,441	563,961	38,136	185,083	420,943
A	C	Med	3,751,742	100	47,260	239,496	258	85,055	79,938
		Min	623,714	100	100	100	100	252	545
		Max	63,653,387	26,408	3,568,819	16,984,133	816,628	1,929,582	4,220,247
		Q25	3,030,977	100	3,069	5,645	100	4,557	30,105
		Q75	34,591,532	100	466,405	1,636,308	3,770	303,499	2,230,372
B	C	Med	16,500,000	618	295,000	340,000	130,500	215,000	435,000
		Min	340,000	100	250	250	100	100	100
		Max	175,399,958	47,000	5,300,000	17,428,442	11,000,000	15,256,595	10,528,009
		Q25	3,068,625	140	13,200	342	437	25,959	4,990
		Q75	97,500,000	2,250	1,696,094	3,575,000	2,346,853	5,950,000	2,535,000
B	P	Med	5,524,471	809	230,000	165,000	34,415	149,500	330,000
		Min	1,500,000	100	412	250	100	340	100
		Max	99,999,996	13,000	4,300,000	13,996,438	2,300,000	7,963,461	6,140,826
		Q25	4,600,000	138	74,500	513	285	38,500	34,700
		Q75	36,750,000	2,625	652,832	2,875,000	275,000	2,800,000	1,635,000

Abbreviations: G: Group, T: Sampling technique, V: Variable, P: Paper point, C: Curette, TBC: Total bacterial counts, *Aa: A. actinomycetemcomitans, Fn: F. nucleatum ssp, Pg: P. gingivalis, Pi: P. intermedia, Td: T. denticola, Tf: T. forsythia.*

Table 3: Number of positive sites in group A and B before as well as after therapy.

G	A before		A after		B before		B after	
T	P	C	P	C	C	P	C	P
TBC	10	10	10	10	10	10	10	10
Aa	2	2	2	1	2	4	4	5
Fn	8	8	9	8	9	10	8	8
Pg	9	9	7	7	8	7	6	7
Pi	4	4	3	3	7	7	6	5
Td	9	8	6	8	10	10	8	8
Tf	9	9	8	9	10	10	7	9

Abbreviations: G: Group, T: Sampling technique, P: Paper point, C: Curette, TBC: Total bacterial counts, *Aa: A. actinomycetemcomitans, Fn: F. nucleatum ssp, Pg: P. gingivalis, Pi: P. intermedia, Td: T. denticola, Tf: T. forsythia*; Positive sites $\geq$ 1000 bacteria / sample of the target bacteria.

3.3. Relative proportions of target bacteria

The mean of the proportion of the total detected pathogens in both patient groups before therapy was for curette before therapy 26.9% (1.1 - 53.2%) and for paper point 28.7% (0.2 - 59.9%), this mean after therapy was for curette 18.6% (0.4 - 47.5%) and for paper point 20.7% (0 - 52.5%).

Group A curette samples contained higher proportions of pathogens with a mean of total pathogens of 32%, while paper point samples contained a mean of total pathogens of only 27% before therapy. The opposite was found after therapy as paper point samples contained a mean of total pathogens of 24% and curette mean of total pathogens of only 19%. In the same group (A) the plaque composition regarding target pathogens before therapy was similar for both sampling techniques (Figures 11 & 12; Table 4), the same was found after therapy (Figures 13 & 14; Table 5).

In group B paper point samples registered a slightly higher mean of total pathogens proportions before (24%) as well as after therapy (16%) compared with curette samples which registered lower proportions of total pathogens with a mean of (23%) before and (14%) after therapy. In the same group (B) the plaque composition with respect to identified bacteria before therapy was similar, slight differences were found for *A. actinomycetemcomitans* and *T. forsythia* (Figures 15 & 16; Table 6). After therapy with paper point higher proportions of pathogens were sampled, except for *P. intermedia* (Figures 17 & 18; Table 7).

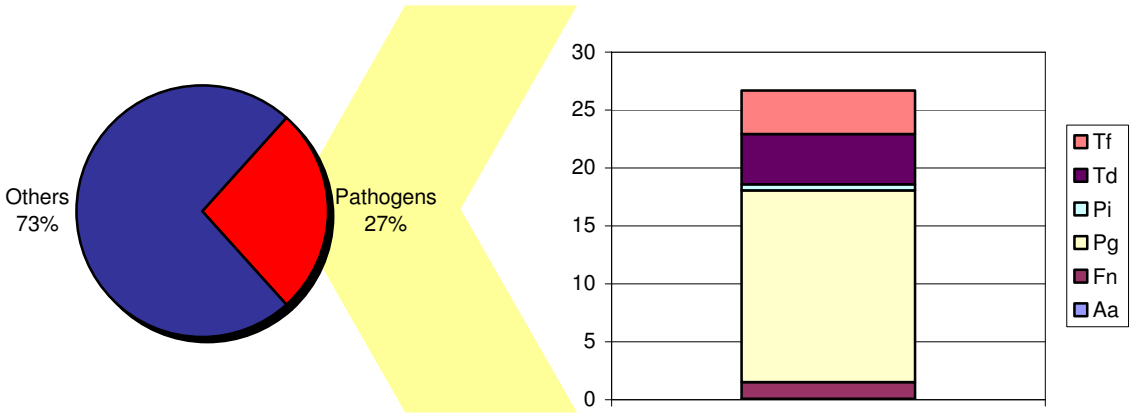

Figure 11:
Means of proportions of paper point samples in group A before therapy. *Aa: A. actinomycetemcomitans, Fn: F. nucleatum ssp., Pg: P. gingivalis, Pi: P. intermedia, Td: T. denticola, Tf: T. forsythia.*

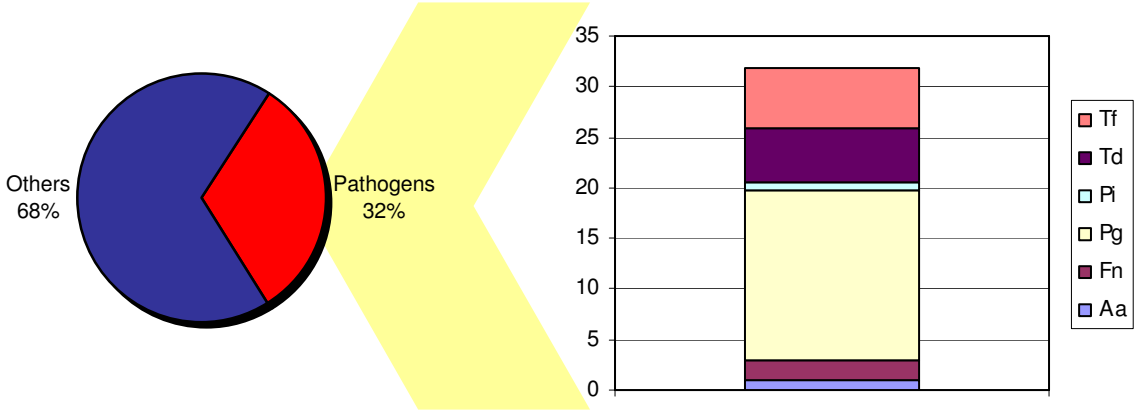

Figure 12:
Means of proportions of curette samples in group A before therapy. *Aa: A. actinomycetemcomitans, Fn: F. nucleatum ssp., Pg: P. gingivalis, Pi: P. intermedia, Td: T. denticola, Tf: T. forsythia.*

Table 4: Means of proportions of sampled plaque in group A before therapy showing the proportions of target periodontopathogens for both sampling techniques.

Target bacteria	**Paper point**	**Curette**
A. actinomycetemcomitans	0.07%	0.91%
F. nucleatum ssp.	1.45%	2.10%
P. gingivalis	16.54%	16.72%
P. intermedia	0.52%	0.85%
T. denticola	4.32%	5.38%
T. forsythia	3.75%	5.85%
Pathogens	≈ 27%	≈ 32%

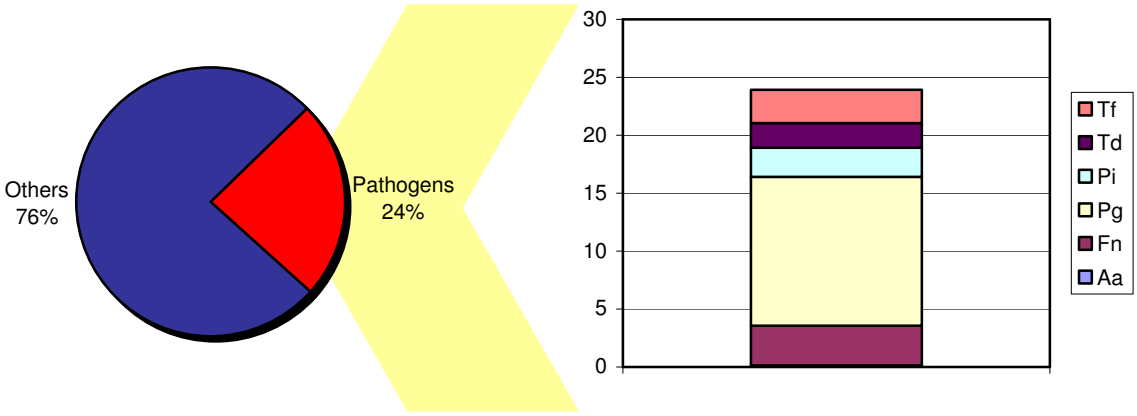

Figure 13:
Means of proportions of paper point samples in group A after therapy. *Aa: A. actinomycetemcomitans, Fn: F. nucleatum ssp., Pg: P. gingivalis, Pi: P. intermedia, Td: T. denticola, Tf: T. forsythia.*

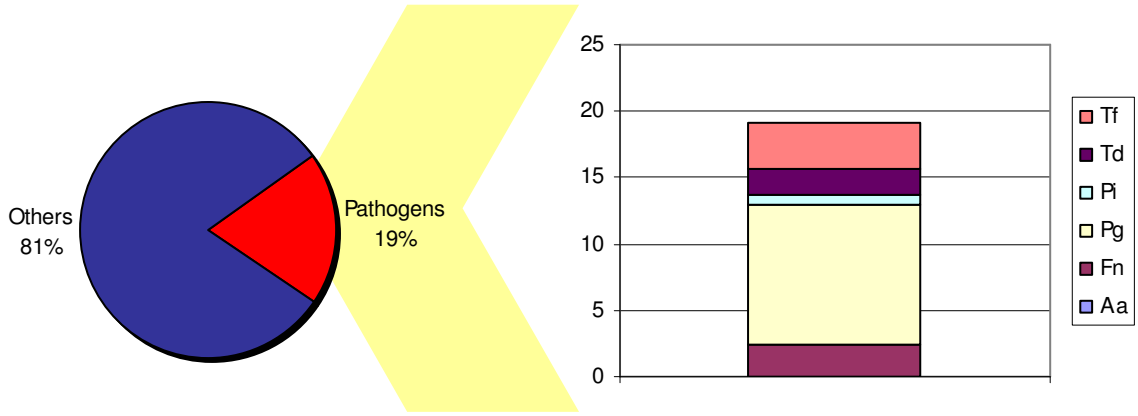

Figure 14:
Means of proportions of curette samples in group A after therapy. *Aa: A. actinomycetemcomitans, Fn: F. nucleatum ssp., Pg: P. gingivalis, Pi: P. intermedia, Td: T. denticola, Tf: T. forsythia*

Table 5: Means of proportions of sampled plaque in group A after therapy showing the proportions of target periodontopathogens for both sampling techniques.

Target bacteria	**Paper point**	**Curette**
A. actinomycetemcomitans	0.13%	0.07%
F. nucleatum ssp.	3.42%	2.37%
P. gingivalis	12.86%	10.45%
P. intermedia	2.51%	0.75%
T. denticola	2.11%	1.99%
T. forsythia	2.89%	3.54%
Pathogens	≈24%	≈19%

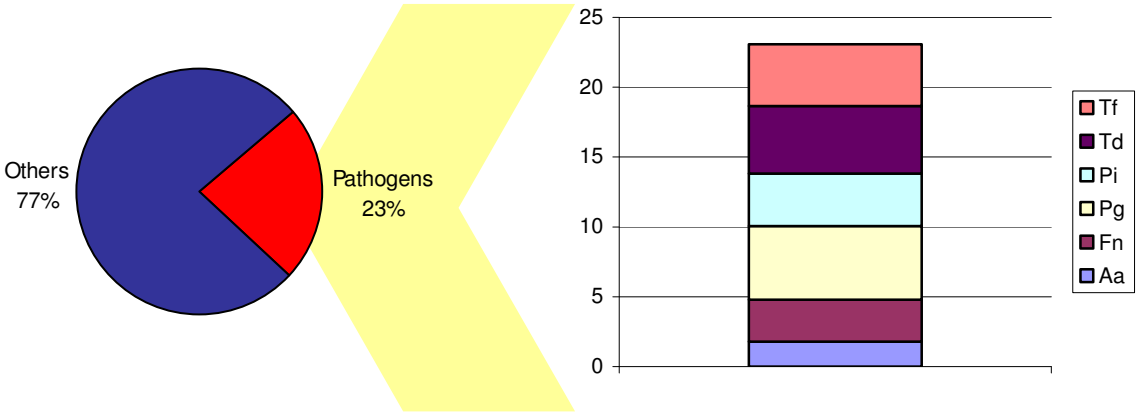

Figure 15:
Means of proportions of curette samples in group B before therapy. *Aa: A. actinomycetemcomitans, Fn: F. nucleatum ssp., Pg: P. gingivalis, Pi: P. intermedia, Td: T. denticola, Tf: T. forsythia.*

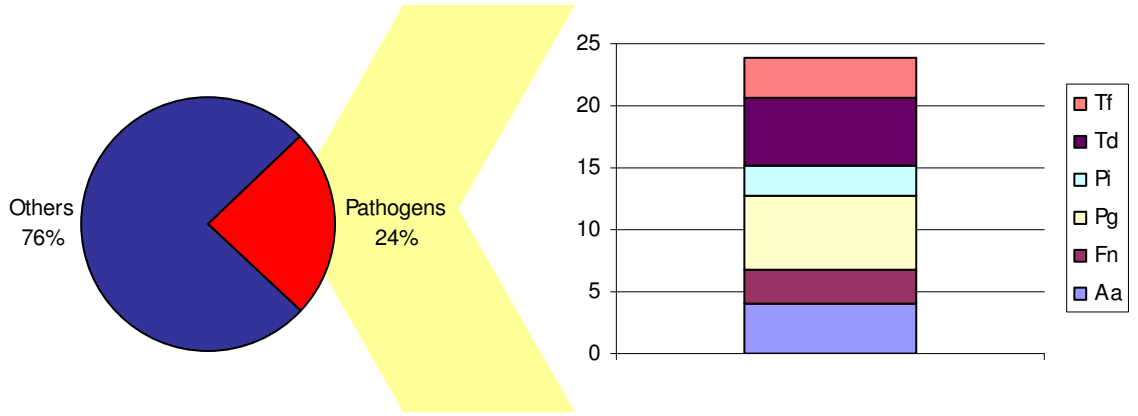

Figure 16:
Means of proportions of paper point samples in group B before therapy. *Aa: A. actinomycetemcomitans, Fn: F. nucleatum ssp., Pg: P. gingivalis, Pi: P. intermedia, Td: T. denticola, Tf: T. forsythia.*

Table 6: Means of proportions of sampled plaque in group B before therapy showing the proportions of target periodontopathogens for both sampling techniques.

Target bacteria	**Curette**	**Paper point**
A. actinomycetemcomitans	1.79%	3.96%
F. nucleatum ssp.	2.99%	2.77%
P. gingivalis	5.29%	5.96%
P. intermedia	3.74%	2.51%
T. denticola	4.83%	5.49%
T. forsythia	4.41%	3.09%
Pathogens	≈23%	≈24%

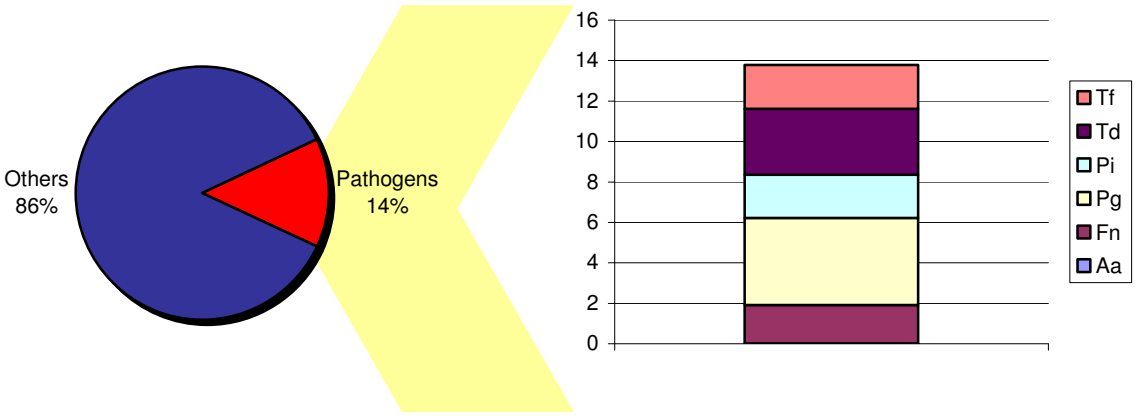

Figure 17:
Means of proportions of curette samples in group B after therapy. *Aa: A. actinomycetemcomitans, Fn: F. nucleatum ssp., Pg: P. gingivalis, Pi: P. intermedia, Td: T. denticola, Tf: T. forsythia.*

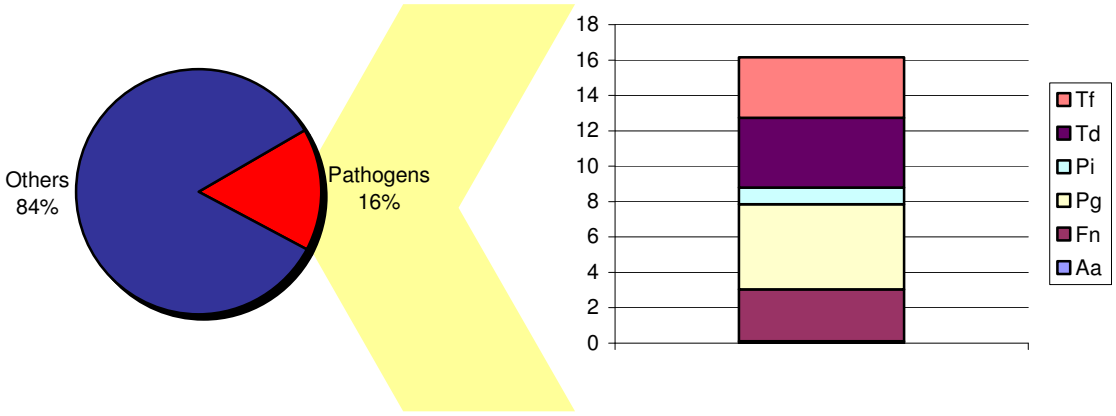

Figure 18:
Means of proportions of paper point samples in group B after therapy. *Aa: A. actinomycetemcomitans, Fn: F. nucleatum ssp., Pg: P. gingivalis, Pi: P. intermedia, Td: T. denticola, Tf: T. forsythia.*

Table 7: Means of proportions of sampled plaque in group B after therapy showing the proportions of target periodontopathogens for both sampling techniques for both sampling techniques.

Target bacteria	**Curette**	**Paper point**
A. actinomycetemcomitans	0.02%	0.10%
F. nucleatum ssp.	1.89%	2.94%
P. gingivalis	4.29%	4.80%
P. intermedia	2.14%	0.95%
T. denticola	3.26%	3.94%
T. forsythia	2.16%	3.41%
Pathogens	≈14%	≈16%

3.4. Agreement between sampling techniques

3.4.1. Correlation

For the description of the agreement of quantitative results of both sampling techniques Spearman correlation coefficients were calculated. Figures 19-25 depict the correlation between paper point and curette samples as scatter plots regardless of the group of patients and time of sampling. The correlation between paper point and curette sampling techniques was analyzed for both groups before as well as after therapy.

As well for TBC as for target bacteria a strong positive correlation was found between curette and paper point samples, correlation coefficients with their significances are listed in Table 8 and presented in Figures 19 - 25.

Table 8: Spearman correlation between paper point and curette samples in group A and B before as well as after therapy (n: 40).

Variable	Spearman coefficient	Significance
Total bacteria counts	0.758	0.000
A. actinomycetemcomitans	0.943	0.000
F. nucleatum ssp.	0.659	0.000
P. gingivalis	0.867	0.000
P. intermedia	0.780	0.000
T. denticola	0.844	0.000
T. forsythia	0.735	0.000

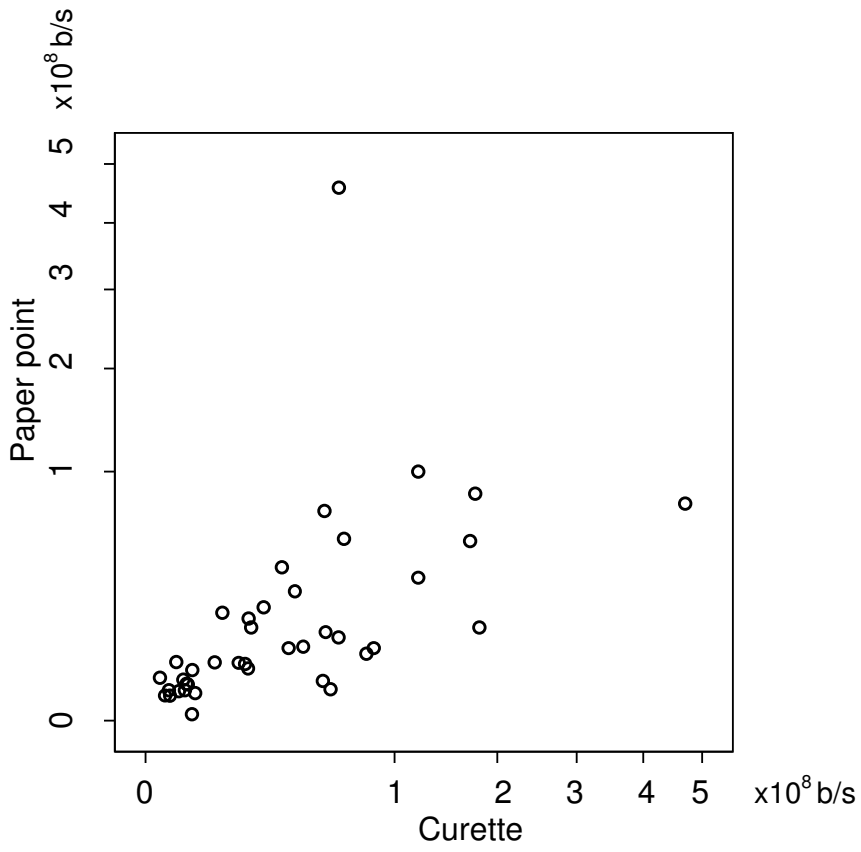

Figure 19:
Correlation of total bacteria counts (TBC) showing the distribution of paper point versus curette samples in group A and B. Spearman coefficient = 0.758, n : 40; b/s: bacteria / sample.

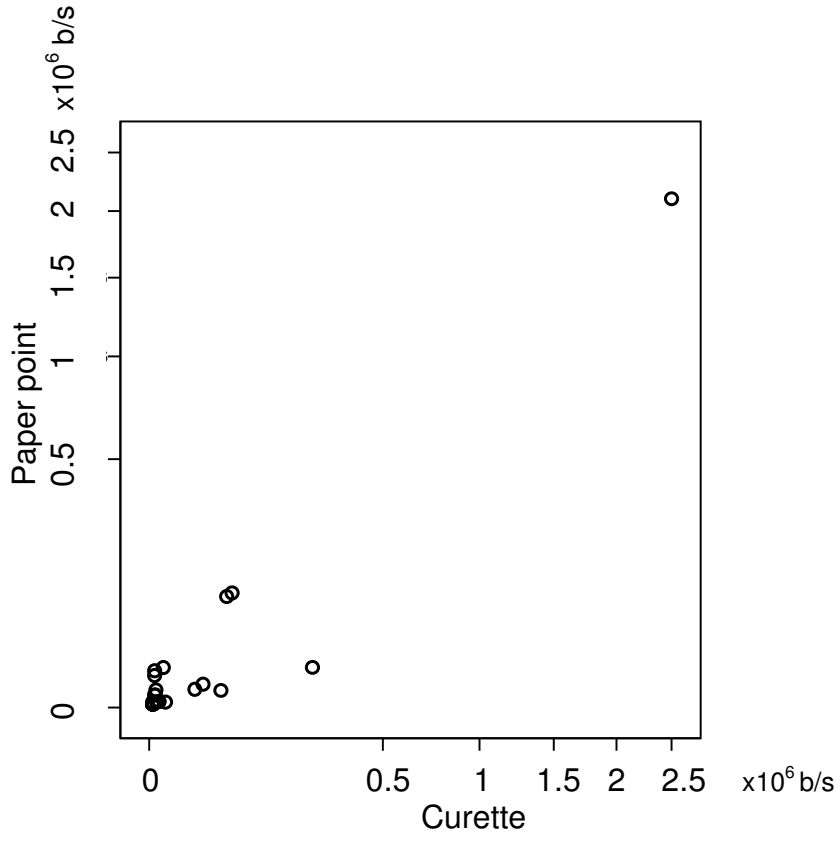

Figure 20:
Correlation of *A. actinomycetemcomitans* (*Aa*) showing the distribution of paper point versus curette samples in group A and B. Spearman coefficient = 0.943, n : 40; b/s: bacteria / sample.

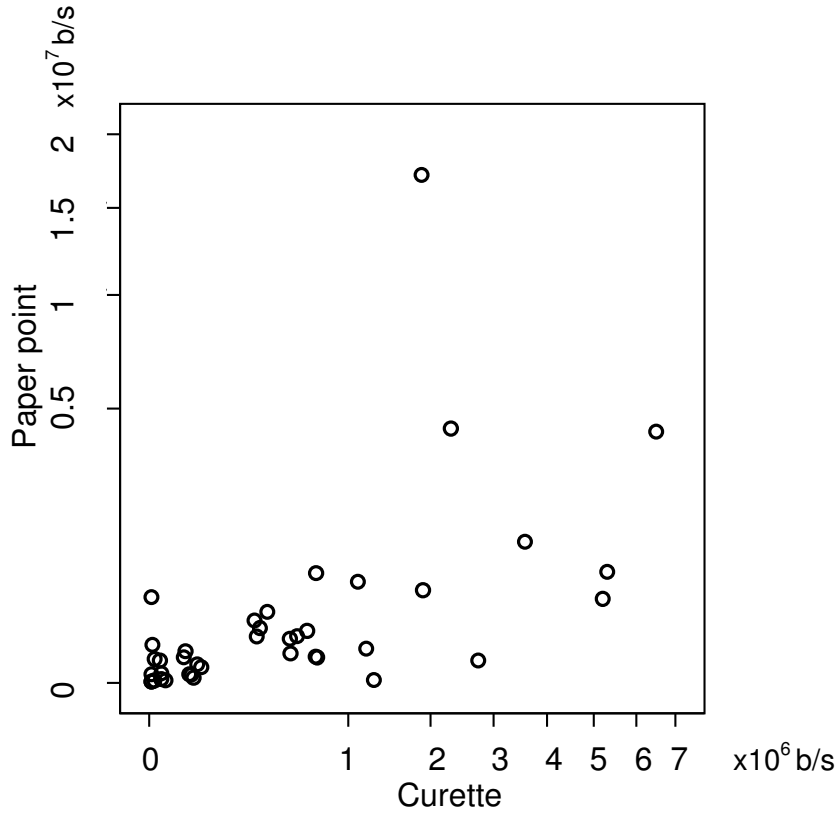

Figure 21:
Correlation of *F. nucleatum ssp.* (*Fn*) showing the distribution of paper point versus curette samples in group A and B. Spearman coefficient = 0.659, n : 40; b/s: bacteria / sample.

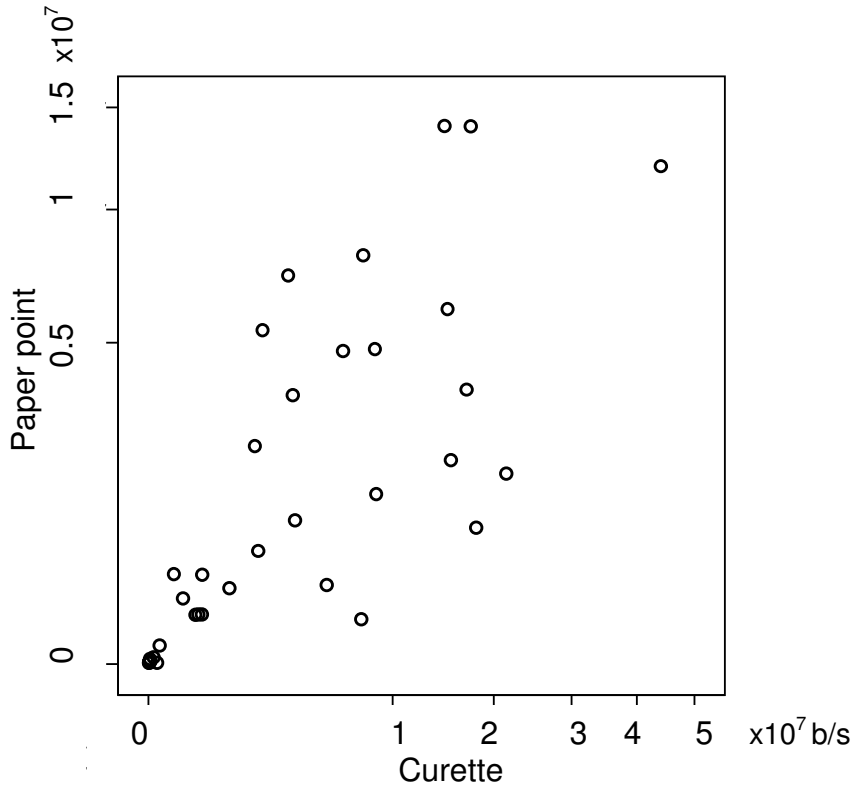

Figure 22:
Correlation of *P. gingivalis* (*Pg*) showing the distribution of paper point versus curette samples in group A and B. Spearman coefficient = 0.867, n : 40; b/s: bacteria / sample.

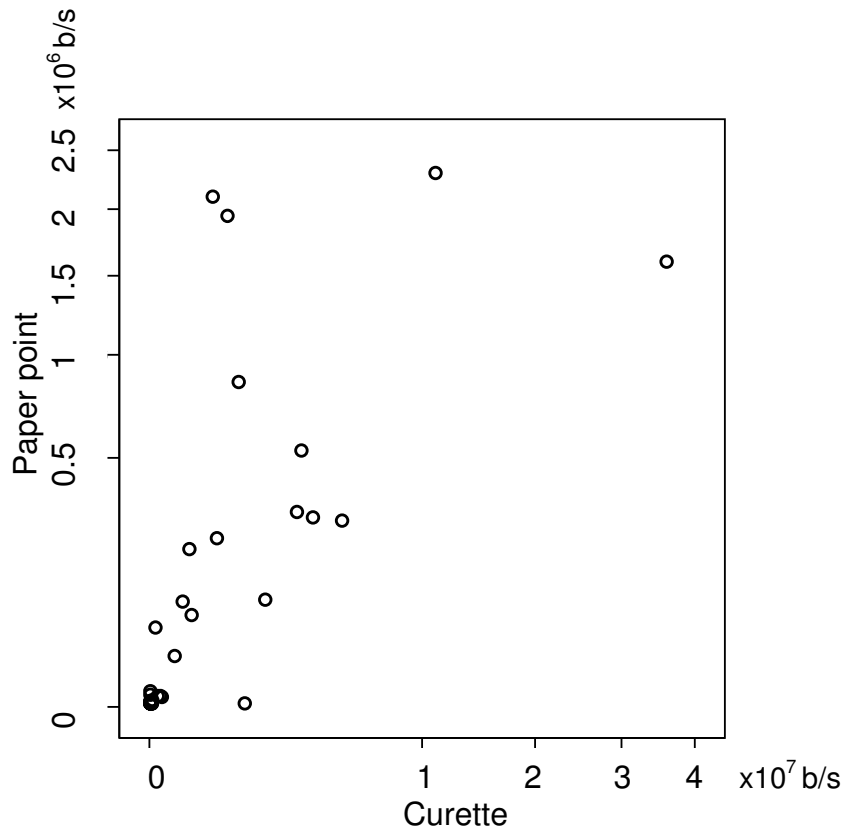

Figure 23:
Correlation of *P. intermedia* (*Pi*) showing the distribution of paper point versus curette samples in group A and B. Spearman coefficient = 0.780, n : 40; b/s: bacteria / sample.

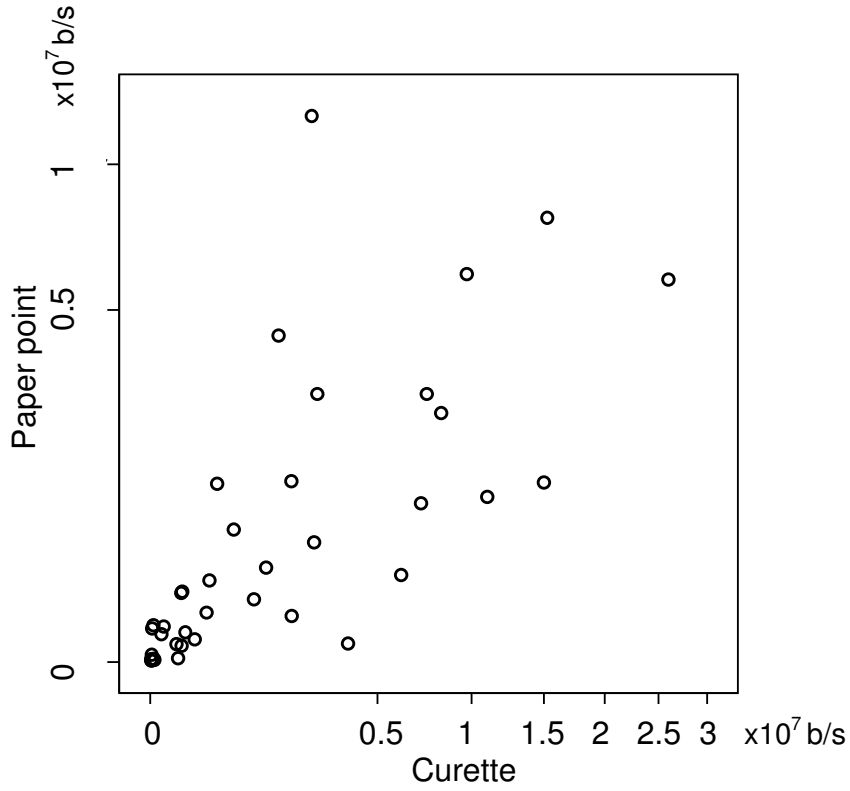

Figure 24:
Correlation of *T. denticola (Td)* showing the distribution of paper point versus curette samples in group A and B. Spearman coefficient = 0.844, n : 40; b/s: bacteria / sample.

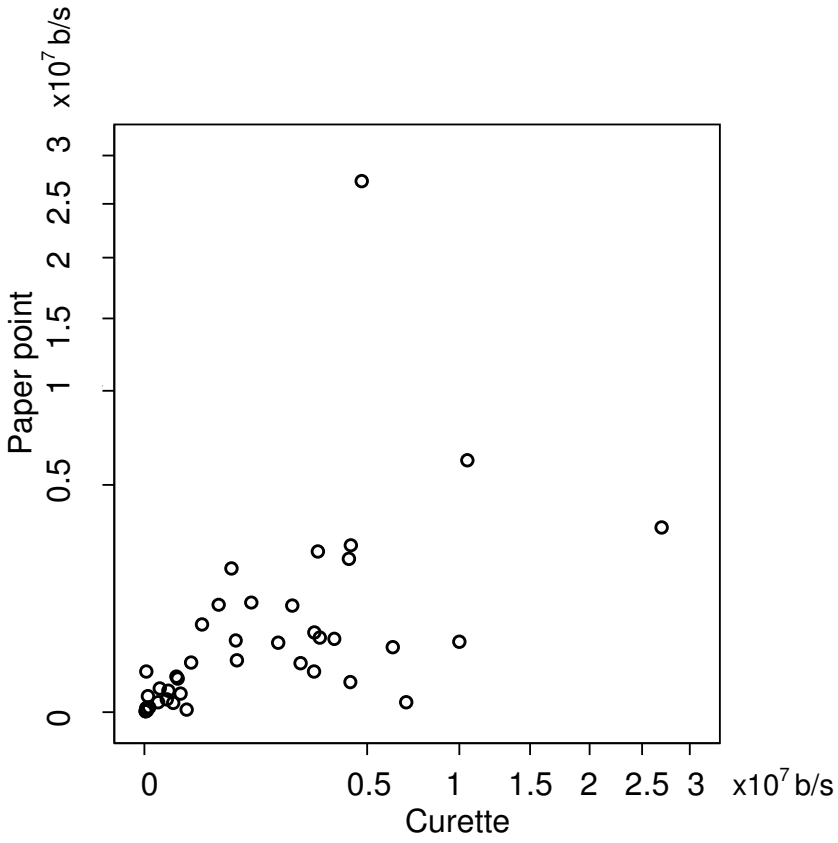

Figure 25:
Correlation of *T. forsythia (Tf)* showing the distribution of paper point versus curette samples in group A and B. Spearman coefficient = 0.735, n : 40; b/s: bacteria / sample.

3.4.2. Kappa

For measuring the agreement between the two sampling techniques kappa was used. Group A and B were tested together before as well as after therapy. The results showed an excellent agreement for *P. gingivalis* (κ = 0.867) and *P. intermedia* (κ = 0.750), a good agreement for *A. actinomycetemcomitans* (κ = 0.629) *and T. forsythia* (κ = 0.625) and a fair agreement for *F. nucleatum ssp.* (κ = 0.415) and *T. denticola* (κ = 0.625). The Kappa results are listed and interpreted in Table 9.

Table 9: Kappa coefficients with evaluation of agreement between curette and paper point samples (n: 40).

Target bacteria	Simple Kappa coefficient	Evaluation
A. actinomycetemcomitans	0.629	Good agreement
F. nucleatum ssp.	0.415	Fair agreement
P. gingivalis	0.867	Excellent agreement
P. intermedia	0.750	Excellent agreement
T. denticola	0.541	Fair agreement
T. forsythia	0.625	Good agreement

4. DISCUSSION

The main finding of the present study was, that curettes collected more subgingival plaque bacteria than paper points at any given time point and irrespective of the sampling sequence. The ratio between curette and paper point median counts before therapy in group A was 4:1, after therapy this relation was approximately 1:1, with slightly more bacteria sampled with curette. In group B the corresponding ratios were 4:1 before and 3:1 after therapy. In both groups, total bacterial counts were found reduced after periodontal therapy, regardless of sampling method. These findings are in agreement with Kiel & Lang (1983) comparing the curette with different sequences of paper points by culture methods. They reported that curettes sampled more colony forming units / ml (cfu/ml) than paper points.

Higher proportions of total pathogens were found with curette than with paper point before therapy in group A, after therapy the opposite was recorded. In group B, where first the curette had been used for sampling, proportions of total pathogens were also reduced due to therapy, both in the curette and in the paper point group. However, in group B differences between the proportions of total pathogenic bacteria between both sampling methods were minimal, before as well as after therapy.

It is of interest to note, that the proportions of the total target pathogens sampled with curettes decreased after treatment more obvious in group A than in group B. Reductions of total target bacteria were observed with paper points in both groups in this study, but more pronounced changes were found in group B than in group A. Tanner and Goodson (1986) expected differences in the outcome of different sampling techniques, because every technique employs a different physical principle. They reported, that paper points sample the loosely adherent bacteria by absorbing pocket fluids and exudates, whereas curettes remove the adherent plaque bacteria (biofilm) from the tooth surface, sampling the more adherent microorganisms. Such compositional differences were also suggested by Kiel and Lang (1983) who isolated higher proportions of black pigmented *Bacteroides* from paper point compared with curette samples.

In group A, paper points were always used before curette, thereby not inducing larger changes in the biofilm, which subsequently was sampled with curette. The differences between the two sampling devices in group A, found after therapy might reflect the impact on the subgingival microflora due to anti-infective therapy.

In group B, curettes always removed the biofilm from the root surface before the use of paper points, thereby increasing the relative proportions of planctonic subgingival bacteria at both sampling time points. As a consequence, some of the sessile bacteria of the biofilm could have become planctonic-like, and would have been easier to collect subsequently with paper points, resulting in the slightly larger proportions of the target bacteria with paper point compared to curette samples.

In both groups at the 6 weeks re-evaluation a mean reduction of sampling site probing pocket depth was found, indicating that treatment was effective. A concomitant reduction in bleeding on probing was found, demonstrating favourable clinical outcomes of the anti-infective therapy, as reflected by the reduction of total bacteria. These results are in agreement with recent reviews describing the influence of subgingival scaling and root planing on the subgingival microflora (Petersilka et al. 2002, Umeda et al. 2004).

The analysis of the sampled plaque composition suggests that plaque collected with curettes or paper points included the same proportions of periodontopathogens before as well as after therapy. This finding is in agreement with the results of Renvert et al. (1992), who found only slight differences in the composition of sampled plaque using both techniques. In the present study the agreement between both methods with respect to the quantitative results was calculated with the Spearman correlation coefficient. As well for total bacterial counts as for single target bacteria a strong positive correlation was found between curette and paper point, regardless of the group of patients and time of sampling. For measuring the agreement between the two sampling techniques Kappa was used. The results showed an excellent agreement with *P. gingivalis* and *P. intermedia*, a good agreement with *A. actinomycetemcomitans* and *T. forsythia* and a fair agreement with *F. nucleatum ssp.* and *T. denticola*.
Analysing the individual six target pathogens, in group A, curettes found target bacteria in higher proportions than paper points before therapy. After therapy, except for *T. forsythia*, the opposite was found. Paper point samples found proportion of *P. intermedia* and *F. nucleatum* increased after periodontal therapy. These findings indicate an alterated biofilm composition, due to changed ecological conditions after therapy (Socransky & Haffajee 2002).

Despite the small differences in proportions of total pathogens between both types of samples in group B, paper points collected higher proportions of A. *actinomycetemcomitans*, *P. gingivalis*, *T. denticola* than curettes before therapy. After therapy this was found for *A. actinomycetemcomitans*, *F. nucleatum ssp.*, *P. gingivalis*, *T. denticola* and *T. forsythia*. Curettes in group B sampled always higher proportions of the six analysed pathogens before than after therapy. With paper points *F. nucleatum ssp.* and *T. forsythia* were harvested in higher proportions after therapy than before, the other four target pathogens were found reduced after therapy, indicating treatment effects on subgingival biofilm. Interestingly, comparing group A with B before therapy, *A. actinomycetemcomitans* was found in considerably higher proportions with paper points, when curette was used first as in group B. Under the assumption that *A. actinomycetemcomitans* adheres very well to the biofilm, it might be necessary to disrupt the biofilm with curettes. Thereby *A. actinomycetemcomitans* presumably changes from a sessile to a planctonic form. As a result of this, paper points can sample *A. actinomycetemcomitans* more easily. This is supported by the findings of Kaplan et al. (2003) describing the ability of A. *actinomycetemcomitans* to form extremely tenacious biofilms.

Any comparison of two different subgingival plaque sampling techniques has inherent problems. Due to the fact that the bacterial content of the sampled periodontal pocket before sampling is unknown, once one sampling technique has been performed in a site, the content of the pocket has been changed. The influence on the outcome of the succeeding sampling cannot be estimated. As it would be expected that the first sampling inevitably influences the succeeding one, it was attempted to compensate for these problems with the present study design. A cross over design was chosen in order to determine, whether the sequence would have an impact and the sampling techniques would interfere with each other. It was expected, that paper point sampling would not affect the outcome of succeeding curette sampling as much as the opposite way. On the other hand, a curette would possibly change the relations between loosely and adherent plaque, thereby changing the outcome of paper point sampling. In fact, statistical

analysis revealed a sequence effect; however, it could be compensated by the cross over design.

The possible effect of sampling sequence was previously tested by Renvert et al. (1992), who sampled 3 sites per patient at baseline and a week later with different procedures. At the first site, 3 parallel inserted paper points always preceded scaler, at the second site scaler was used before paper points at baseline, a week later the sequence was inverted. At the third site paper points preceded scaler at baseline, a week later the opposite was performed. They reported that at all instances paper points sampled more bacteria than scalers. Their study did not register any effect of the sampling sequence on the obtained results. In the present study curettes always collected more bacteria as paper points. But, in contrast to Renvert et al. (1992), only one paper point instead of 3 parallel inserted paper points was used. Strand et al. (1987) compared washing and curette sampling techniques with inverted sequences. They did not find any influence of the sampling sequence on the results. In the present study, likewise comparable results in terms of composition of the sampled plaque for the two methods were found, supporting the findings of Renvert et al. (1992) and Strand et al. (1987).

Scalers or curettes are traditionally used devices for the collection of subgingival plaque bacteria. Due to their size, curettes can remove a major portion of total pocket microbiota (Tanner & Goodson 1986). A problem bound to curette technique is the reproducibility of the samples. Sixou et al. (1991) compared curette and paper point sampling techniques. They found curette sampling to be an efficient technique both quantitatively and qualitatively. Difficulties in standardizing this method, however, were encountered and they failed to achieve reproducible results. For this reason the technique of paper point was preferred, this method was found to be more reliable and reproducible. In the present study the same type of curette, a Gracey curette number 5-6 was always used. It has a shape, which allows the instrument to glide in the pockets of single rooted teeth easily.

Mombelli et al. (1989) tested the reproducibility of paper point samples. The results did not indicate a general bias of the results of the second sampling by the previous

sampling. The present study was not aimed to test the reproducibility of curette and paper point techniques. Nevertheless, the ability of paper points in the present study to sample comparable or even higher proportions of bacteria directly after curettes, suggests that curettes do not harvest the same bacterial content of the pocket as paper points. Mousques et al. (1980) demonstrated the effects of repeated sampling on the subgingival flora. Plaque was collected at baseline and then either after 3, 7, 14, 21, 28 or 42 days. Only small changes up to 3 days occurred, suggesting only minor changes in the composition of subgingival plaque due to sampling. The study, however, did not perform a repeated sampling within one session as in the present study.

Baker et al. (1991) found paper points unable to sample deeper regions of sampling sites. This finding was based upon an in vitro study with multiple layers of liquid cultures of periodontopathogenic species. The complex composition of the periodontal pocket however, cannot be reflected by a culture medium with only few bacterial species, and caution has to be exercised when extrapolating these results to in vivo conditions. Still, paper point sampling might have its limitations, due to the fact that the absorbent material starts to absorb gingival fluid containing bacteria as soon as the paper point has been inserted into to the pocket. The paper point may be saturated before it reaches the apical portion of the pocket. As a consequence the paper point sample may only be representative for the more coronal bacterial content of the site. This assumption, however, was not confirmed in the present in vivo study, as paper point samples yielded the same plaque composition as curette samples.

Another important aspect for the comparison of different studies evaluating sampling techniques is the identification method used for microbiological analysis. Most of the studies referred to by Tanner & Goodson (1986) have used traditional methods such as cultivation, dark field or phase contrast microscopy for the analysis of plaque samples. These previous evaluations were limited by microbiological methods that would preclude quantification and/or sensitive and specific identification of target pathogens. Novel molecular biological methods could overcome these shortcomings. The advantages of a PCR based identification method are well known (Eick & Pfister 2002, Jervøe-Storm et al. 2005). The real-time PCR based identification method used in the present study, cannot only detect periodontopathogenic species, which are difficult to differentiate from

close related taxa. It also provides the possibility to estimate the relative proportions of the target bacteria on the basis of the total bacterial counts. This gives a much more precise basis for evaluation of two different sampling techniques.

In conclusion, the results of the present study have demonstrated that even though curette samples harvested significantly more total bacteria, the composition of the plaque samples with respect to selected target pathogens were quite similar for both sampling techniques. Thus, both techniques can be recommended for clinical use for microbiological testing of periodontal lesions.

5. SUMMARY

Because of their important etiological role in periodontitis the microbiological identification of subgingival plaque bacteria is essential for clinical diagnostics. However, only few studies have systematically evaluated microbiological sampling methods of periodontal bacteria. The present study has been conducted to compare for the first time two widely used sampling techniques (paper point and curette) using the novel method of quantitative real-time PCR.

Twenty adult patients with chronic periodontitis participated in a prospective study using a cross-over design. In each patient one periodontal pocket with a probing depth of more than 6 mm was selected for microbial sampling. In patients of group A the first sample was obtained with a paper point and the second with a Gracey-curette. In group B the sampling sequence was reversed. Samples were analysed by a blinded examiner in a specialised microbiological laboratory using real-time PCR technology. The analysis enabled the quantitative evaluation of *Actinobacillus actinomycetemcomitans, Fusobacterium nucleatum ssp., Porphyromonas gingivalis, Prevotella intermedia, Treponema denticola, Tannerella forsythia* as well as total bacterial counts. Eight weeks after anti-infective periodontal therapy the sites were sampled again, using the same sampling sequence as before. Statistical analysis included t-test, Kappa and Spearman correlation.

In this study higher total bacterial counts could be harvested by use of curettes than by paper points. The ratios between paper point and curette samples in group A were 1:4 before and 1:1 after therapy, in group B the respective values were 1:4 before and 1:3 after therapy. In contrast, the relative proportions of target bacteria in the total sample were similar. Following therapy, both sampling techniques showed a reduction of total bacterial counts as well as of the relative proportion of periodontopathogens.
Overall, there was a relatively good agreement for the results of the investigated sampling techniques for the analysis of subgingival plaque bacteria. Thus, both techniques appear to well suited for microbiological diagnostics of patients with periodontitis.

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7. APPENDIX

Table 10: t-test results of total bacterial counts comparing paper point and curette sampling.

Total bacterial counts	**Before**	**After**
Mean of difference	-0.947	-0.444
Standard deviation	0.707	0.617
Upper confidential level	-0.283	0.136
Lower confidential level	-1.611	-1.023
t-value	-3.000	-1.610
p-value	0.008	0.125

Table 11: t-test results of *A. actinomycetemcomitans* comparing paper point and curette sampling.

A. actinomycetemcomitans	**Before**	**After**
Mean of difference	0.088	0.028
Standard deviation	0.444	0.864
Upper confidential level	0.505	0.840
Lower confidential level	-0.330	-0.783
t-value	0.440	0.070
p-value	0.665	0.943

Table 12: t-test results of *F. nucleatum ssp.* comparing paper point and curette sampling.

F. nucleatum ssp.	**Before**	**After**
Mean of difference	-0.848	-0.156
Standard deviation	1.663	1.137
Upper confidential level	0.714	0.912
Lower confidential level	-2.410	-1.225
t-value	-1.140	-0.310
p-value	0.269	0.762

Table 13: t-test results of *P. gingivalis* comparing paper point and curette sampling.

P. gingivalis	**Before**	**After**
Mean of difference	-1.262	-0.329
Standard deviation	0.825	0.390
Upper confidential level	-0.487	0.038
Lower confidential level	-2.037	-0.695
t-value	-3.420	-1.880
p-value	0.003	0.076

Table 14: t-test results of *P. intermedia* comparing paper point and curette sampling.

P. intermedia	**Before**	**After**
Mean of difference	-1.219	-0.611
Standard deviation	1.340	0.612
Upper confidential level	0.040	-0.036
Lower confidential level	-2.478	-1.186
t-value	-2.030	-2.230
p-value	0.057	0.039

Table 15: t-test results of *T. denticola* comparing paper point and curette sampling.

T. denticola	**Before**	**After**
Mean of difference	-0.852	-0.478
Standard deviation	1.113	0.897
Upper confidential level	0.194	0.364
Lower confidential level	-1.897	-1.321
t-value	-1.710	-1.190
p-value	0.104	0.248

Table 16: t-test results of *T. forsythia* comparing paper point and curette sampling.

T. forsythia	**Before**	**After**
Mean of difference	-1.206	-0.282
Standard deviation	1.109	1.016
Upper confidential level	-0.164	0.673
Lower confidential level	-2.248	-1.237
t-value	-2.430	-0.620
p-value	0.026	0.543

Table 17: Original data of group A and B patients for the total bacterial counts (TBC) before and after therapy.

	Before therapy		After therapy	
Patient	**Paper point**	**Curette**	**Paper point**	**Curette**
A1	70,973,556	51,666,316	1,231,064	3,966,371
A2	458,253,285	60,298,468	66,936	3,483,188
A3	7,226,162	78,755,729	2,121,856	2,880,240
A4	2,564,083	50,759,658	8,844,180	40,122,042
A5	1,601,037	55,340,112	14,000,000	18,000,000
A6	20,719,123	22,571,053	4,162,911	3,537,112
A7	16,879,006	17,157,686	53,363,504	63,653,389
A8	18,787,781	9,545,072	12,666,032	52,341,457
A9	2,199,096	2,720,740	1,022,598	964,844
A10	11,177,394	60,064,561	1,024,487	623,714
Patient	**Curette**	**Paper point**	**Curette**	**Paper point**
B1	2,300,000	2,700,000	7,700,000	5,500,000
B2	14,000,000	5,400,000	890,000	1,500,000
B3	170,000,000	52,000,000	17,000,000	4,400,000
B4	2,500,000	1,500,000	340,000	3,000,000
B5	1,800,000	1,400,000	16,000,000	5,200,000
B6	33,000,000	8,500,000	120,000,000	100,000,000
B7	36,000,000	27,000,000	30,000,000	38,000,000
B8	180,000,000	14,000,000	120,000,000	33,000,000
B9	470,000,000	76,000,000	175,399,954	83,200,130
B10	84,000,000	8,500,000	1,524,833	5,548,942

Table 18: Original data of group A and B patients demonstrating *A. actinomycetemcomitans* (*Aa*) results (bacteria/sample) before and after therapy.

	Before therapy		**After therapy**	
Patient	**Paper point**	**Curette**	**Paper point**	**Curette**
A1	106,732	62,735	4,398	26,408
A2	100	100	100	100
A3	100	100	100	100
A4	100	100	100	100
A5	100	100	100	100
A6	100	100	100	100
A7	100	100	100	100
A8	100	100	100	100
A9	13,096	244,341	8,401	250
A10	100	100	100	100
Patient	**Curette**	**Paper point**	**Curette**	**Paper point**
B1	100	100	1,800	13,000
B2	2,500,000	2,100,000	260	11,000
B3	480	250	2,400	250
B4	100	100	100	100
B5	100	100	100	100
B6	100	250	47,000	2,400
B7	250	1,300	340	1,300
B8	55,000	100,000	19,000	2,700
B9	100	100	100	100
B10	410	2,500	897	318

Table 19: Original data of group A and B patients demonstrating *F. nucleatum ssp.* (*Fn*) results (bacteria/sample) before and after therapy.

	Before therapy		**After therapy**	
Patient	**Paper point**	**Curette**	**Paper point**	**Curette**
A1	16,613	68,086	4,967	100
A2	17,149,838	1,875,903	1,854	49,521
A3	33,868	2,736,895	336,060	351,940
A4	43,307	713,172	57,762	504,560
A5	569	1,275,291	4,700	45,000
A6	143,668	291,592	100	100
A7	489,635	100	145,334	551,931
A8	801,217	703,671	1,324,115	3,568,819
A9	100	100	33,249	2,870
A10	78,783	1,190,715	6,104	3,665
Patient	**Curette**	**Paper point**	**Curette**	**Paper point**
B1	3,600	1,000	33,000	67,000
B2	630,000	180,000	6,600	440
B3	40,000	5,000	280,000	260,000
B4	690	38,000	250	97,000
B5	58,000	23,000	310,000	200,000
B6	30,000	44,000	1,100,000	680,000
B7	6,500,000	4,200,000	2,300,000	4,300,000
B8	5,200,000	470,000	5,300,000	820,000
B9	500,000	130,000	1,894,792	571,326
B10	700,000	46,000	635	412

Table 20: Original data of group A and B patients demonstrating *P. gingivalis* (*Pg*) results (bacteria/sample) before and after therapy.

	Before therapy		**After therapy**	
Patient	**Paper point**	**Curette**	**Paper point**	**Curette**
A1	8,089,599	7,740,745	117,389	369,906
A2	2,214	4,841	100	100
A3	1,756,212	21,475,650	388,273	488,401
A4	302,902	5,332,783	620,872	2,018,944
A5	98,092	7,596,520	17,000	21,000
A6	4,748,350	6,348,511	264	527
A7	5,393,404	2,185,778	14,019,923	14,701,969
A8	7,306,858	3,275,556	3,647,846	16,984,132
A9	100	100	250	250
A10	2,016,544	15,342,861	393,229	109,086

Patient	**Curette**	**Paper point**	**Curette**	**Paper point**
B1	13,000	100	200,000	210,000
B2	420,000	120,000	250	250
B3	8,600,000	4,800,000	480,000	120,000
B4	550	410	400	1,300
B5	270	310	250	250
B6	1,100,000	280,000	3,500,000	3,500,000
B7	1,900,000	2,300,000	3,600,000	1,000,000
B8	8,700,000	1,400,000	15,000,000	6,100,000
B9	44,000,000	12,000,000	17,428,441	13,996,438
B10	18,000,000	900,000	322	250

Table 21: Original data of group A and B patients demonstrating *P. intermedia* (*Pi*) results (bacteria/sample) before and after therapy.

	Before therapy		After therapy	
Patient	**Paper point**	**Curette**	**Paper point**	**Curette**
A1	89,767	147,185	200,995	214,810
A2	1,218	100	100	852
A3	100	250	100	100
A4	100	600	100	100
A5	976	8,847	250	100
A6	2,063	100	100	100
A7	851,900	1,072,221	1,945,883	816,628
A8	100	100	100	100
A9	100	100	100	415
A10	100	1,222,900	50,764	4,742
Patient	**Curette**	**Paper point**	**Curette**	**Paper point**
B1	85,000	21,000	240,000	68,000
B2	16,000	1,000	21,000	830
B3	100	100	100	100
B4	930	100	250	100
B5	100	250	100	250
B6	1,800,000	93,000	3,600,000	290,000
B7	540,000	2,100,000	610,000	230,000
B8	36,000,000	1,600,000	11,000,000	2,300,000
B9	3,100,000	530,000	2,925,803	306,993
B10	5,000,000	280,000	998	391

Table 22: Original data of group A and B patients demonstrating *T. denticola* (*Td*) results (bacteria/sample) before and after therapy.

	Before therapy		After therapy	
Patient	**Paper point**	**Curette**	**Paper point**	**Curette**
A1	6,069,392	9,700,393	20,956	193,995
A2	12,028,142	2,526,049	643	75,240
A3	1,016,283	7,100,681	193,820	94,869
A4	86,307	1,939,686	158,870	1,041,885
A5	13,830	3,791,870	270,000	340,000
A6	707,347	678,345	250	1,921
A7	1,283,415	433,219	1,319,555	1,929,582
A8	2,276	250	250	252
A9	100	100	100	321
A10	305,806	6,101,491	31,762	12,464
Patient	**Curette**	**Paper point**	**Curette**	**Paper point**
B1	18,000	51,000	310,000	99,000
B2	96,000	11,000	100	340
B3	1,300,000	360,000	120,000	36,000
B4	1,100	55,000	400	46,000
B5	67,000	13,000	100,000	200,000
B6	2,600,000	580,000	7,400,000	2,900,000
B7	2,700,000	2,900,000	1,600,000	4,300,000
B8	15,000,000	1,300,000	8,200,000	2,500,000
B9	26,000,000	5,900,000	15,256,595	7,963,461
B10	11,000,000	1,100,000	1,279	447

Table 23: Original data of group A and B patients demonstrating *T. forsythia* (*Tf*) results (bacteria/sample) before and after therapy.

	Before therapy		**After therapy**	
Patient	**Paper point**	**Curette**	**Paper point**	**Curette**
A1	2,502,119	3,032,209	16,343	49,727
A2	27,306,598	4,765,740	614	179,484
A3	409,095	6,225,017	123,891	102,876
A4	88,653	4,278,449	519,961	3,636,183
A5	9,910	6,921,940	45,000	57,000
A6	1,165,738	1,148,358	2,267	2,187
A7	1,117,986	554,631	2,281,664	4,220,246
A8	745,430	334,225	614,615	2,914,001
A9	100	100	309	545
A10	233,807	2,456,874	55,574	23,565
Patient	**Curette**	**Paper point**	**Curette**	**Paper point**
B1	220,000	240,000	110,000	110,000
B2	130,000	34,000	100	100
B3	1,800,000	470,000	19,000	9,600
B4	1,100	25,000	320	160,000
B5	83,000	8,300	840,000	500,000
B6	860,000	260,000	4,300,000	2,700,000
B7	2,200,000	1,100,000	760,000	2,000,000
B8	2,900,000	160,000	3,100,000	540,000
B9	27,000,000	3,300,000	10,528,009	6,140,826
B10	10,000,000	480,000	307	1,790

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