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Authors
Schillinger, Claudia
Petrich, Annett
Lux, Renate
et al.

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Co-Located or Randomly Distributed? Pair Cross Correlation of In Vivo Grown Subgingival Biofilm Bacteria Quantified by Digital Image Analysis

Claudia Schillinger¹,², Annett Petrich¹,², Renate Lux³, Birgit Riep⁴, Judith Kikhney¹, Anton Friedmann⁴, Lawrence E. Wolinsky⁵, Ulf B. Göbel¹, Holger Daims⁶, Annette Moter¹*°

¹ Institut für Mikrobiologie und Hygiene, Charité – Universitätsmedizin Berlin, Berlin, Germany, ² UCLA School of Dentistry, University of California Los Angeles, Los Angeles, California, United States of America, ³ Abteilung für Parodontologie und Synoptische Zahnmédizin, Charité – Universitätsmedizin Berlin, Berlin, Germany, ⁴ School of Dentistry, Faculty of Health, University of Witten, Witten, Germany, ⁵ Texas A&M Health Science Center, Baylor College of Dentistry, Dallas, Texas, United States of America, ⁶ Department of Microbial Ecology, Ecology Center, University of Vienna, Vienna, Austria

Abstract

The polymicrobial nature of periodontal diseases is reflected by the diversity of phylotypes detected in subgingival plaque and the finding that consortia of suspected pathogens rather than single species are associated with disease development. A number of these microorganisms have been demonstrated in vitro to interact and enhance biofilm integration, survival or even pathogenic features. To examine the in vivo relevance of these proposed interactions, we extended the spatial arrangement analysis tool of the software daime (digital image analysis in microbial ecology). This modification enabled the quantitative analysis of microbial co-localization in images of subgingival biofilm species, where the biomass was confined to fractions of the whole-image area, a situation common for medical samples. Selected representatives of the disease-associated red and orange complexes that were previously suggested to interact with each other in vitro (Tannerella forsythia with Fusobacterium nucleatum and Porphyromonas gingivalis with Prevotella intermedia) were chosen for analysis and labeled with specific fluorescent probes via fluorescence in situ hybridization. Pair cross-correlation analysis of in vivo grown biofilms revealed tight clustering of F. nucleatum/periodonticum and T. forsythia at short distances (up to 6 µm) with a pronounced peak at 1.5 µm. While these results confirmed previous in vitro observations for F. nucleatum and T. forsythia, random spatial distribution was detected between P. gingivalis and P. intermedia in the in vivo samples. In conclusion, we successfully employed spatial arrangement analysis on the single cell level in clinically relevant medical samples and demonstrated the utility of this approach for the in vivo validation of in vitro observations by analyzing statistically relevant numbers of different patients. More importantly, the culture-independent nature of this approach enables similar quantitative analyses for “as-yet-uncultured” phylotypes which cannot be characterized in vitro.

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* E-mail: annette.moter@charite.de (AM); daims@microbial-ecology.net (HD)

° These authors contributed equally to this work.

† These authors also contributed equally to this work.

Introduction

Periodontal diseases are prevalent bacterial biofilm infections in humans that involve progressive destruction of the tooth-supporting tissues and ultimately tooth loss in the absence of treatment. A strong association between periodontal and systemic diseases as well as unfavorable pregnancy outcomes has also been reported [1,2,3]. The traceability of periodontal bacteria and derived concepts of pathogenesis, however, relate strongly to the methods applied for microbial analysis.

Early electron microscopy studies revealed the complexity and highly organized structure of the microbiota residing in subgingival biofilms [4,5,6,7]. Further efforts to elucidate the etiology of periodontal diseases included: (i) A comprehensive inventory of the oral microbiome: To date more than 1,000 distinct taxa have been identified in the oral cavity and about 400 of these have been so far associated with the colonization of the periodontal pocket [8,9]. In addition to species identification, a number of these culture-independent studies implicated novel periodontal pathogens [10,11,12,13,14,15]; (ii) Arrangement of a large panel of cultivable subgingival flora into microbial complexes based on their co-occurrence and association with health and disease [16]: Reflective of the multitude of microorganisms comprising the oral microbiota, clusters of microorganisms rather than single species have been implicated as indicators for periodontal health or disease. The so-called “red complex” which is strongly correlated with the severity of disease is comprised of Porphyromonas gingivalis,
Tannerella forsythia, and Treponema denticola. These species appear to require the more prevalent “orange complex” species such as Fusobacterium spp. or Prevotella intermedia among others for biofilm integration [17]; (ii) Extensive in vitro examination of the ability of oral species to form aggregates with each other [10,19,20,21,22,23,24,25,26,27]. The multitude of in vitro studies assessing individual interspecies adherence behavior or “co-aggregation” allowed a more detailed picture of the elaborate interactions involved in building the architecturally complex oral biofilm networks. Some of these in vitro interactions were validated in vivo for supragingival biofilm formation [28,29,30]. The subgingival in vivo distribution of several residents of the periodontal pocket has been examined immuno-histochemically [31,32] and most recently via a very comprehensive fluorescent in situ hybridization (FISH)-based study [33]. Taken together these approaches cumulated in our current understanding that periodontal diseases involve complex synergistic and antagonistic bacterial interactions [34].

In contrast to diseases caused by a single etiological agent, polymicrobial biofilm infections are characterized by multiple, often opportunistic pathogens whose virulence features are often enhanced by the interplay with other community members. Therefore, the subgingival in vivo dynamics and bacterial biofilm interactions have become a focus of current periodontal research. Three critical issues remain to be addressed: (i) the casting of main characters is currently incomplete: even though certain oral microbial species have been assigned as periodontal colonizers the disease-association of the majority of species still has to be revealed [10,12,13,35,36]; (ii) The in vivo pathogenic potential of specific bacteria (“casting of good and bad guys”) continues to be under discussion, especially since the complexity of the biofilm network enables mutual interactions that we are just beginning to comprehend [10,14,37]; (iii) Finally, the “leading and supporting players” in the interplay of this lively, interwoven network of subgingival plaque bacteria are hardly determined [38]. Especially co-localization can be indicative of cell-to-cell adherence or synergistic associations. Detailed understanding of these complex relationships is adamant for the development of comprehensive therapeutical concepts targeting key pathogenic species or interactions, one of the central goals to improve the existing therapeutical concepts targeting key pathogenic species or interactions [31].

Our present study provides novel insight into the spatial relationships among bacteria including adhesion-based events as well as those based on metabolic relationships in a naturally grown, subgingival biofilm on a quantitative level. A recently established carrier-based in vivo model [41] enabled sampling of undisturbed subgingival biofilm and hybridized sections allowed high resolution examination on a single cell level. Two pairs of suspected periodontal pathogens were visualized by fluorescence in situ hybridization (FISH) and their distribution relative to each other was digitally quantified. For this proof of concept study, target species were chosen on the basis of the associations defined by Socransky’s microbial complexes in subgingival biofilms [17] and positive interactions determined in vitro [19]. Since T. forsythia, a member of the strongly periodontitis-associated red complex, and F. nucleatum of the orange complex physically and synergistically interrelate in vitro [26], we examined the in vivo relevance of their relationship on a quantitative level. Additionally, the controversial relationship between the suspected periodontal pathogens P. gingivalis (red complex) and P. intermedia (orange complex) was evaluated. These oral bacterial species have been found to adhere to each other by some authors [42], while others imply that they do not interact [23,43].

In the present study the target organism pairs were first visualized by FISH and epifluorescence microscopy for identification and analysis of their localization within the histological context. Second, the pair cross-correlation function (PCC) was quantified to determine whether the pairwise spatial arrangement of the analyzed bacterial populations was random, attractive or repulsive. For this purpose, we extended the spatial arrangement analysis tool of the software daime, “Digital Image Analysis In Microbial Ecology” [44]. For the quantification of spatial arrangement patterns, daime implements a stereological approach to estimate the PCC [45]. The generated PCC curve allows the determination of co-localization, random distribution or rejection (mutual avoidance) of two bacterial populations. This concept has successfully been applied to environmental biofilms [44,46,47] and now for the first time could be applied to validate relationships of oral bacterial species in medical biofilms in situ.

Methods

Ethics Statement

The study was approved by the local Institutional Review board, the Ethikkommission der Charité-Universitätsmedizin Berlin and written consent of the participants was obtained.

Subject population

Ten previously untreated subjects (three male and seven female) with generalized aggressive periodontitis (GAP) selected from a population referred for periodontal treatment to the Department of Periodontology at the University Hospital Charité were included in this institutionally approved study. Subjects ranged in age between 18 and 44 years (mean 35.1, SD 7.3 years). Clinical examination included medical and dental history, intraoral examination, full-mouth periodontal probing as well as a full mouth series of intraoral radiographs. Inclusion criteria for patient selection were based on the diagnosis of GAP according to the criteria of the 1999 International Workshop for Classification of Periodontal Disease and Conditions [49]: disease onset estimated at <30 years based on clinical examination, past radiographs, and/or interview, as well as 6 mm probing pocket depth (PPD) at a minimum of three permanent teeth other than first molars and incisors. Exclusion criteria were previous periodontal treatment, chronic systemic disease, anti-inflammatory or antimicrobial therapy within the last six months as well as pregnant or lactating women.

Sampling

Subgingival biofilms were grown in vivo using a carrier-based model system as described previously [41]. Briefly, carriers were inserted in periodontal pockets of 10 GAP patients in 28 sample sites with a mean periodontal probing depth (PPD) of 7.8 mm, SD 1.3 mm. After 7 days of biofilm development, carriers were fixed, embedded, and sectioned as described previously [50]. Sections (2 µm in thickness) were sliced along the longitudinal axis of the ePTFE carrier [41].
Fluorescence in situ hybridization (FISH)

Probes for detection of \( F. \) nucleatum/periodonticum (FUNU), \( T. \) forsythia (TAFO, formerly named \( B(T) \) AFO), \( P. \) gingivalis (POGI) as well as \( P. \) intermedia (PRIN), and the domain-specific probe EUB338 which recognizes most \( B. \)acteria were synthesized commercially (Biomers, Ulm, Germany). These probes have been published previously and were deposited in probeBase \([51]\). The sequence of probe FUNU matches those of \( F. \) nucleatum, \( F. \) periodonticum, \( F. \) newינוי, and \( F. \) carinatum, the latter two not being relevant for periodontal disease. The species-specific probes were 5’end-labeled with either the Cy3 (indocarbocyanine) or Cy5 (indodicyocyanine) fluorescent dye, while EUB338 contained FITC (Fluoresceinisothiocyanate) as a label to allow combinations with each species-specific probe. FISH procedures were performed as reported previously \([52]\). To confirm the specificity of the probes, fixed cells of the following strains served as positive controls: \( F. \) nucleatum (ATCC 25586), \( P. \) gingivalis (ATCC 33277), \( P. \) intermedia (ATCC 25611) and \( T. \) forsythia (ATCC 43037); species with the lowest number of mismatches at the probe binding site served as negative control; respectively \( F. \) corium (ATCC 8501), \( P. \) gulae (ATCC 51700), \( P. \) bryantii (DSM 11371), \( B. \) suis (ATCC 35419). Vecastech (Vector Laboratories, Orthon Southgate, UK) was applied as mounting medium containing DAPI (4’,6-Diamidino-2-phenylindole) for visualization of all cells including eukaryotic cell nuclei.

Epifluorescence microscopy and image acquisition

Microscopic observations were performed with an epifluorescence microscope (AxioPlan II, Zeiss, Jena, Germany). The microscope was equipped with a 100 W high-pressure mercury lamp (HB0 103W/2; Osram, Munich, Germany) and 10 ×, 40 ×, and 100 × objectives. Narrow band filter sets (AHF Analysentechnik, Tubingen, German) were applied to separate the FITC (ET F46-002), Cy3 (HQ F41-007), Cy5 (HQ-F41-008) and DAPI (HQ F31-000) signals, respectively. For image acquisition, the AxioPlan microscope was combined with an AxiosCam MRm (Zeiss) digital camera controlled by the AxioVision 4.7 software. Multichannel images were captured at 1000 × magnification and a resolution of 1388 × 1040 pixels (16 bit). For each patient and bacterial pair combination, the entire hybridized sections were examined and images were recorded at random positions, for each bacterial pair. Since the data were normally distributed, group comparison of the results was performed by calculating a common PCC curve for each distance \( r \) in \( \mu \)m. Random distribution (the ‘null hypothesis’) is indicated by \( g(r) = 1 \), whereas \( g(r) > 1 \) suggests co-aggregation and \( g(r) < 1 \) mutual avoidance of the populations.

Spatial arrangement analysis with \textit{daime}

Spatial arrangement analysis with \textit{daime} is an Open Source software for digital image analysis of microbial cells in situ. The three sets of binarized TIFF-images obtained per patient and per pair of bacterial populations, which corresponded to the total biomass (FITC-labeled) and the two microbial species of interest (Cy3 and Cy5-labeled), were imported into \textit{daime} for further analysis. The \( \times \) \( \mu \)m size of the images was set to 88.31 \( \times \) 66.92 \( \mu \)m according to the scaling factor of 0.064 \( \mu \)m/pixel indicated by the AxioVision Software. Automatic 2D-segmentation was performed to identify connected components (i.e., objects such as microbial cells and cell aggregates). During this step objects smaller than 28 pixels, which most likely represented noise, were ignored. Remaining artifacts, human or abiotic materials were removed using the object editor options. To perform the spatial arrangement analysis by the “Linar Dipole” algorithm, the distance range was set from 0 to 50 \( \mu \)m and every fifth distance spaced at intervals of \( \sim 0.5 \mu \)m was selected. The analysis was performed in random dipole mode, where the number of random dipoles was adjusted to 200,000 per distance. Finally, the reference space for the analysis was specified, individually for each FOV, by using the EUB338 images as reference space masks. The results were imported into Microsoft Excel (Microsoft Corporation).

The spatial arrangement tool provided with the software \textit{daime} applies a stereological method to estimate the pair correlation (for one microbial population) or the pair cross-correlation (for two populations) functions by analyzing the chance encounters between cells and linear dipole probes. This approach has been described in detail elsewhere \([53]\). The obtained PCC, \( g(r) \), indicates whether two populations co-aggregate, avoid each other, or are randomly distributed at distance \( r \) (in \( \mu \)m). Random distribution (the ‘null hypothesis’) is indicated by \( g(r) = 1 \), whereas \( g(r) > 1 \) suggests co-aggregation and \( g(r) < 1 \) mutual avoidance of the populations.

Statistical analysis

Statistical validation of spatial arrangement analysis was executed by \textit{daime} with \( n = 25 \) images per pair of bacteria and per patient. For \( n \) images the mean PCC \( g(r) \) for each distance \( r \) and 95% confidence interval (CI) was calculated using the standard deviation among the images and the student’s \( t \)-distribution for \( n-1 \) degrees of freedom \([44]\). The data obtained for all patients were then merged individually for TAFO/FUNU and POGI/PRIN, respectively, by statistical evaluations to enable comparison of the results. This consolidation of the PCC results was performed by calculating a common PCC curve for each bacterial pair. Since the data were normally distributed, group mean (\( m \)) and standard error of the mean (\( \text{SEM} \)) were used to calculate CI for the group mean by the formula 95% CI = \( m \pm 1.96 \times \text{SEM} \).

Results

Extension of the spatial arrangement tool of \textit{daime}

The \textit{in vivo} grown subgingival biofilms analyzed in this study were restricted in size and shape by a number of factors (population densities, spatial limits such as the intrinsic margins defined by the periodontal pocket and carrier etc.). Thus, the images of the biofilm sections contained biomass plus variable proportions of empty space (background), the latter mainly beyond the natural borders of the biofilm. The software \textit{daime} \([44]\), which we employed for quantifying the spatial localization of bacterial populations, was previously used in studies of biofilms and activated sludge flocs from wastewater treatment plants \([44,46]\). The abundant biomass in those samples typically filled the entire
FOV, and the recorded FISH images did not contain much (if any) empty space. Hence, the original version of the spatial arrangement analysis tool in daime used the whole images as “reference space” for the Linear Dipole algorithm [44]. This approach would cause biases with the images of subgingival biofilm, because the spatial clustering of all biomass (enforced by the biofilm size and shape) in the non-empty regions of these images would result in high PCC values \( g(r) \) and could not be distinguished from biologically caused co-localization. To overcome this problem, an additional feature was added to daime that allows the user to define those image regions, which actually contain any biomass and should be used as the “reference space” in the analysis. For this purpose, the user can specify a so-called “reference space mask” image that indicates the locations of biomass and background. To overcome this problem, an additional feature was added to daime that allows the user to define those image regions, which actually contain any biomass and should be used as the “reference space” in the analysis. For this purpose, the user can specify a so-called “reference space mask” image that indicates the locations of biomass and background. To overcome this problem, an additional feature was added to daime that allows the user to define those image regions, which actually contain any biomass and should be used as the “reference space” in the analysis. For this purpose, the user can specify a so-called “reference space mask” image that indicates the locations of biomass and background. To overcome this problem, an additional feature was added to daime that allows the user to define those image regions, which actually contain any biomass and should be used as the “reference space” in the analysis. For this purpose, the user can specify a so-called “reference space mask” image that indicates the locations of biomass and background. To overcome this problem, an additional feature was added to daime that allows the user to define those image regions, which actually contain any biomass and should be used as the “reference space” in the analysis. For this purpose, the user can specify a so-called “reference space mask” image that indicates the locations of biomass and background. To overcome this problem, an additional feature was added to daime that allows the user to define those image regions, which actually contain any biomass and should be used as the “reference space” in the analysis. For this purpose, the user can specify a so-called “reference space mask” image that indicates the locations of biomass and background.

Spatial arrangement analysis of target species

The bacterial pairs \( T. forsythia \) with \( F. nucleatum \) and \( P. gingivalis \) with \( P. intermedia \) were chosen for this study. Each pair consists of a representative of the “red complex” (\( T. forsythia \) and \( P. gingivalis \)) and the “orange complex” (\( F. nucleatum \) and \( P. intermedia \)). While positive interactions between \( T. forsythia \) and \( F. nucleatum \) have been consistently reported [26,33,54,55] but has never been confirmed by quantitative spatial analysis using \( in vivo \)-grown subgingival biofilms, the relationship between \( P. gingivalis \) and \( P.
intermedia is less clear [23,42,43]. Their spatial in vivo association was examined via qualitative visual inspection followed by quantitative spatial arrangement analysis to determine, by means of the PCC function, the co-localization, repulsion or randomness of bacterial distribution.

Analysis of T. forsythia and F. nucleatum/periodonticum

Among the 10 subjects, 22 subgingival plaque carriers obtained from eight different patients exhibited strong hybridization signals for the probe combination TAFO/FUNU (detection of T. forsythia/F. nucleatum/periodonticum). FISH results of the remaining patients were either negative for the respective probes, or the fluorescent signals exhibited a low signal to noise ratio which made them unsuitable for further analysis. Visual inspection revealed excellent single cell resolution with typical morphologies of the target species among a variety of different bacterial morphotypes. A typical pattern of colony and cell association was observed in numerous specimens hybridized with TAFO/FUNU; strongly suggesting co-localization of these species (Figure 1).

Individual-related spatial analysis of T. forsythia and F. nucleatum/periodonticum

Separate sets of random images were taken for each of the eight patients positive for T. forsythia and F. nucleatum/periodonticum for spatial analysis of these bacteria. From a total of 476 recorded micrographs, 199 were used for determining PCC values. Seven of the eight PCC curves for T. forsythia and F. nucleatum/periodonticum clearly showed co-localization of these species. The PCC plot of patient 01 (Figure 3A), based on a set of 25 randomly taken images, is described in detail as a representative example. Since in our samples F. nucleatum/periodonticum cells as the largest morphotype reached up to 10 μm in length, the spatial arrangement analysis of daime was set to plot the PCC function $g(r)$ against distances $r$ between 0 and 25 μm. The PCC curve showed a...
pronounced peak and a tight 95\% CI. Following the mean PCC values, the lower CI (\(\sim 95\%\)) remained above the reference line \(g=1\) from 0 to 11 μm. Thus, two important criteria for co-localization were met within short distances between the two populations: (i) A pronounced peak of the mean PCC values along with (ii) a narrow CI whose lower boundary was clearly greater than 1.

These findings indicated tight spatial clustering of \(T.\) forsythia and \(F.\) nucleatum/periodonticum within a distance range from 0–6 μm. Similar PCC values were obtained for six of the other patients (data not shown). For only one patient (Figure 3B), however, a higher variance of the PCC values at short distances was observed and thus, for this specific sample co-aggregation of the two populations could not be confirmed unambiguously.

**Analysis of \(P.\) gingivalis and \(P.\) intermedia**

Hybridization with probes POGI/PRIN resulted in strong hybridization signals suitable for quantitative analysis for six different subjects (18 subgingival plaque carriers). In contrast to the micrographs obtained for \(T.\) forsythia and \(F.\) nucleatum/periodonticum, the qualitative visual assessment of images showing \(P.\) gingivalis and \(P.\) intermedia was ambivalent due to a high variability of the observed distribution patterns. Within the same FOV seemingly repulsive and attractive localization patterns were found in close proximity. As exemplified by an image obtained from patient 03 (Figure 2A), the distribution of both species suggested a nearly constant distance between their microcolonies, which would indicate a repulsive spatial arrangement. Within a distinct region of the biomass, however, \(P.\) gingivalis and \(P.\) intermedia were located in direct vicinity, overgrowing each other (inset in Figure 2A). Furthermore, occurrence of both species in the same FOV was less common than for \(T.\) forsythia and \(F.\) nucleatum/periodonticum. Thus, mere visual observation was insufficient to characterize the spatial distribution of \(P.\) gingivalis and \(P.\) intermedia, and statistical spatial analysis was required.

**Figure 3. Pair cross correlation results.** The mean PCC function \(g(r)\) (continuous line) and the 95\% confidence interval (dotted lines) are plotted against distances \(r\) spaced at intervals of \(\sim 0.5\) μm. The dashed horizontal reference line on the level of \(g(r)=1\) corresponds to the value of randomness and provides an internal ‘null hypothesis’ for testing attraction or repulsion between cellular units. (A) Representative, individual-related PCC of \(T.\) forsythia and \(F.\) nucleatum/periodonticum calculated for 25 images obtained from patient 01. A pronounced peak of 2.5 PCC values at 1.5 μm indicated co-localization of \(T.\) forsythia and \(F.\) nucleatum/periodonticum cells within short distances from 0–6 μm. (B) Outlier evaluation. PCC of \(T.\) forsythia and \(F.\) nucleatum/periodonticum calculated for 25 images obtained from patient 10. An initially prominent peak was in contrast to Figure 3A embedded in a wide CI, which lower boundary (\(\sim 95\%\)) dropped below the reference line, indicating a high variance in PCC values within the first 3 μm. (C) Representative, individual-related PCC of \(P.\) gingivalis and \(P.\) intermedia calculated for 32 images obtained from patient 05. \(P.\) gingivalis and \(P.\) intermedia cells are randomly distributed within the entire distance range. (D) Outlier evaluation. PCC of \(P.\) gingivalis and \(P.\) intermedia was calculated for 30 images obtained from patient 04. Similar to the outlier results of \(T.\) forsythia and \(F.\) nucleatum/periodonticum (Figure 3 B), the high variance of PCC values at distances <4.7 μm allowed no valid analysis for patient 04. Above 4.7 μm the curve of \(P.\) gingivalis and \(P.\) intermedia oscillated around the reference line surrounded by a relatively narrow CI, whose lower limit remained below the PCC value \(g(r)=1\). These characteristics indicated random spatial distribution at distances >4.7 μm in contrast to the curve shown in Figure 3 B, whose PCC values decreased constantly.

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Co-Localization of Subgingival Biofilm Bacteria

Individual-related spatial analysis of \textit{P. gingivalis} and \textit{P. intermedia}

In total 175 images obtained from six different patients were used for spatial arrangement analysis of \textit{P. gingivalis} and \textit{P. intermedia}. The PCC curve for patient 05, whose image set consisted of 32 micrographs (Figure 3C), is representative for most of the image sets analyzed. The mean PCC fluctuated slightly above and below the reference line \([g(r) = 1]\), intersecting this line several times. As the CI limits consistently enclosed the reference line, this curve clearly indicated random spatial distribution of the two bacterial species.

The PCC curves obtained for four of the six patients were consistent with random distribution within the analyzed distance range of 0–25 \(\mu\text{m}\). For patients 01 and 04, however, the curves were different within the first 4 \(\mu\text{m}\). The PCC curve calculated for patient 04 reached a pronounced peak at a distance of 1 \(\mu\text{m}\) with 95\% CI spanning a broad range above and also below the reference line (Figure 3D). The PCC curve obtained for patient 01 was similar (data not shown). Due to the high variance of the PCC values for patient 01 and 04 within the first 4 \(\mu\text{m}\), these curves do not unequivocally indicate co-aggregation of the two populations despite the peak of the mean PCC. In contrast, the CI limits suggest a random distribution also in these two cases. To assess the effect of these outliers on the patient group as a whole, all individual-related PCC curves for \textit{P. gingivalis} and \textit{P. intermedia} were merged by statistical evaluations.

PCC analysis of consolidated patient groups

For a statistical comparison of the results obtained for the \textit{T. forsythia} versus \textit{F. nucleatum}/\textit{periodonticum} and \textit{P. gingivalis} versus \textit{P. intermedia} pairs, we calculated for both group means the respective 95\% CI as described in Methods. Both consolidated PCC curves for \textit{T. forsythia} and \textit{F. nucleatum}/\textit{periodonticum} \((n = 8)\) and \textit{P. gingivalis} and \textit{P. intermedia} \((n = 6)\) were plotted with their respective 95\% CI against a distance range of 0–25 \(\mu\text{m}\) (Figure 4). The two curves were significantly different in terms of peak heights and progression relative to the reference line. The curve for \textit{T. forsythia} and \textit{F. nucleatum}/\textit{periodonticum} exhibited a strong and statistically significant peak within very short distances (peak maximum at 1.46 \(\mu\text{m}\)), clearly suggesting co-localization of these two populations. In contrast, the curve for \textit{P. gingivalis} and \textit{P. intermedia} confirmed random distribution by fluctuating around the reference line without any significant peak. The clear separation of the lower CI of the co-localized bacterial species from the upper CI of the randomly distributed organisms within 0–19 \(\mu\text{m}\) shows that the two curves are significantly different and that the two population pairs follow different spatial arrangement patterns in the biofilm.

Discussion

Bacterial interactions play important roles in the pathogenic potential of polymicrobial medical biofilms such as the subgingival biofilms in periodontal disease. Several decades of periodontal research were characterized by different approaches to reveal the interaction patterns within the microbial community associated with disease. While providing valuable information these previous studies were intrinsically limited by: i) Focusing on pairwise \textit{in vitro} interactions of planktonic cells, ii) employing biofilm models with cultivable species, iii) examining the \textit{in vivo} distribution of subgingival species on a qualitative level only or iv) using disrupted rather than intact biofilm samples. Our present study adds a new level of understanding to these earlier studies by analyzing \textit{in vivo} grown subgingival biofilms of a statistically significant number of patient samples with a novel method to quantify the nature of colonization patterns. This approach enables for the first time the rigorous statistical verification of microbial interactions, in subgingival plaque, that were previously proposed from \textit{in vitro} experiments or from qualitative (intrinsically subjective) microscopic biofilm observations.

The image analysis software \textit{dame} was extended by a new feature of its spatial arrangement tool, which was required for the correct image analysis of sectioned oral biofilms. The added functionality (reference space mask images) is not specific for medical biofilms but extends to all cases where biomass does not cover the whole area of the images to be analyzed. This includes also environmental biofilm samples.

By addressing the aforementioned methodical issues, we successfully analyzed the spatial arrangement patterns of \textit{T. forsythia}/\textit{F. nucleatum}/\textit{periodonticum} and \textit{P. gingivalis}/\textit{P. intermedia} \textit{in vivo} grown specimens obtained from 10 GAP patients. These four oral bacterial species play important roles in a medical context and have been implicated as putative periodontal pathogens.

\textit{T. forsythia} and \textit{F. nucleatum}/\textit{periodonticum}

\textit{T. forsythia} and \textit{F. nucleatum}/\textit{periodonticum} were chosen as one of the test pairs in this study, since they have been proposed to adhere to each other and form synergistic relationships [26,54]. In addition, these two oral species are members of the red and orange complexes, respectively, which indicate disease correlation. According to extensive co-occurrence studies by Socransky and coworkers [17], members of the red complex (such as \textit{T. forsythia}) are strongly correlated with pocket depth and severity of disease, while the orange complex species (such as \textit{F. nucleatum}) precede the red complex and were proposed to facilitate colonization of red complex bacteria. The initial visual assessment (Figure 1) resembled recent findings by Zijnge \textit{et al.} [33] who observed frequent close association of \textit{T. forsythia} and \textit{F. nucleatum}. These authors also observed these species to reside predominantly in the “intermediate layer” of the examined tooth attached biofilms. These qualitative impressions of co-localization of \textit{T. forsythia} and \textit{F. nucleatum} and previous \textit{in vitro} studies suggesting interaction of these species [26,33,54,56] were confirmed by the quantitative evaluation carried out in this study. Altogether, the results of past research and the data reported here strongly suggest a positive biological interaction between these two important disease-related oral bacteria.

\textit{P. gingivalis} and \textit{P. intermedia}

Similar to \textit{T. forsythia} and \textit{F. nucleatum}/\textit{periodonticum}, our second test pair \textit{P. gingivalis} and \textit{P. intermedia} are also classified as members of the red and orange complexes, respectively. In contrast to \textit{T. forsythia} and \textit{F. nucleatum}/\textit{periodonticum}, however, reports about a possible mutualistic relationship of these organisms have been controversial. Based on \textit{in vitro} co-aggregation experiments of \textit{P. gingivalis} vesicles with \textit{P. intermedia} cells, Kamaguchi \textit{et al.} [42] concluded that \textit{P. gingivalis} and \textit{P. intermedia} physically interact via a HPG17 domain protein. In contrast, Kolenbrander and coworkers [23,43] did not observe such interaction between these two species. Consistently, visual inspection of \textit{in vivo} grown subgingival biofilm sections did also not indicate co-aggregation, because \textit{P. gingivalis} and \textit{P. intermedia} appeared to grow predominantly in distinct microcolonies [33] and this study, Figure 2). The quantitative spatial analysis (Figures 3 and 4) has resolved the controversy about \textit{P. gingivalis} and \textit{P. intermedia} by confirming random distribution of these bacteria, relative to each other, in the \textit{in vivo} grown biofilm. Thus, at least the spatial arrangement of
these two species does not point at any specific biological interaction (mutualism or repulsion) between them. We assume that the co-existence of *P. gingivalis* and *P. intermedia* in the same parts of the subgingival biofilm is caused by other and not yet identified factors.

### Possibilities and Limitations

In this study, we show that digital image analysis can be used to objectively quantify and describe medical biofilm architecture. These daime-based analyses of bacterial distribution and interaction patterns in biofilms could be combined with the recently developed CLASIFISH approach [40] to enable simultaneous investigation of the relationships between multiple bacterial species. This would allow for a comprehensive investigation of the interactions among oral bacteria is an important prerequisite for the development of targeted therapeutic concepts. The quantitative characterization of spatial localization patterns has the potential to reveal previously overlooked interactions, whose nature can subsequently be studied by using culture-independent methods that analyze microbial physiology on the single-cell level [57]. This approach is not limited to the analysis of subgingival biofilms, but can efficiently be applied to other medical or environmental samples.

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