FRIENDS WITH BENEFITS

AN INSIDE LOOK OF PERIODONTAL MICROBES' INTERACTIONS

Guilherme José Vieira de Melo Esteves

U.PORTO

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TECHNICAL DATA SHEET

TITLE
Friends with benefits: an inside look of periodontal microbes' interactions – scoping review

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FRONT COVER
Image adapted from Mark Welch, J. L., *et al.* (2016)

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RESUMO

Introdução: A periodontite é resultado da inflamação crónica dos tecidos de suporte dos dentes, que pode levar à perda dentária. O método de hibridação *in situ* fluorescente (FISH) tem provado ser útil na descrição da organização espacial de populações microbianas, fornecendo informação sobre a morfologia, localização e ecossistema celular dos microrganismos envolvidos no seu habitat natural. Nem todos estes microrganismos estão diretamente associados à periodontite, mas podem criar as condições necessárias para que outros se desenvolvam e comprometam o equilíbrio com o hospedeiro. A presente *scoping review* tem como objetivo identificar e mapear todas as interações documentadas entre microrganismos periodontais no interior das bolsas periodontais pela técnica de FISH.

Materiais e métodos: Três bases de dados eletrónicas foram consultadas em busca de artigos que convergissem com o objetivo da revisão: MEDLINE (via *PubMed*), *Scopus* (*Elsevier*) e *Web of Science* (*Clarivate Analytics*). As bases de dados foram consultadas até 7 de novembro de 2020. Estudos que identificaram interações *ex vivo* e *in situ* entre, pelo menos, dois microrganismos foram considerados elegíveis.

Resultados e discussão: Dez artigos foram incluídos, dos quais seis são estudos de caso, três são estudos caso-controlo e um é um estudo transversal, todos com heterogeneidade considerável. Sete estudos analisaram a microbiota residente no biofilme subgengival, dois examinaram a microbiota residente no biofilme supragengival e apenas um analisou ambos os biofilmes. Um estudo não apresentou critérios de exclusão. A técnica de FISH foi desenvolvida nos diferentes estudos com diversos objetivos. Apesar da variabilidade intra e interpessoal, estruturas radiais ou em camadas constituídas por diversos microrganismos são os tipos de consórcios mais comuns. Microrganismos anaeróbios estritos ou facultativos tendem a ser encontrados frequentemente no interior e nas porções mais profundas, enquanto aeróbios são comumente encontrados na periferia das estruturas. Certos produtores e consumidores de metabólitos aparecem espacialmente associados; determinados microrganismos demonstraram a capacidade de apresentar várias configurações dependendo do microrganismo com que estavam agregados nas diferentes áreas do biofilme. Definimos este fenómeno como "mentalidade de grupo dos microrganismos" e acreditamos ser uma vantagem crucial de certos membros da microbiota periodontal.

Conclusões: A técnica de FISH foi capaz de identificar interações entre microrganismos dos três Domínios da Vida, permitindo o desenvolvimento de uma hipótese provável de forma a compreender o papel destas interações no desenvolvimento e progressão das doenças periodontais. Contudo, a evidência científica existente sobre a estrutura, composição e interações entre microrganismos

nos biofilmes periodontais é escassa, impossibilitando uma conclusão precisa. Assumir que este número insuficiente de interações observadas é, de alguma forma, indicativo da microbiota periodontal na saúde e na doença é um salto de fé que necessita de validação em estudos de larga escala. Apesar deste facto, acreditamos que a integração de conceitos de patogénese atuais com as melhoradas técnicas de FISH poderá dar origem a uma nova ferramenta de diagnóstico periodontal, com o desenvolvimento de um kit de sondas oligonucleotídicas que identifiquem agentes patogénicos com capacidade de provocar disbiose.

Palavras-chave: Doença periodontal; método de hibridação *in situ* fluorescente; biofilme oral; microscopia; microbiota oral.

ABSTRACT

Introduction: Periodontitis is the result of the chronic inflammatory loss of the tooth-supporting tissues, leading to tooth loss when untreated. Fluorescence *in situ* hybridization (FISH) has proven to be particularly useful to describe the microbial composition and spatial organization of mixed microbial infections, as it happens in periodontitis, providing information about the morphology, location, and cellular ecosystem of organisms, in time and space within their natural context. Not all these microorganisms are directly associated with periodontitis but may create the necessary conditions for other microorganisms to grow and to disrupt the periodontal balance between host and microbes. This scoping review aims to identify and map all the documented interactions between microbes in periodontal pockets by the FISH technique.

Materials and methods: Three electronic sources of evidence were consulted in search of suitable articles that matched the aim of this review: MEDLINE (via PubMed), Scopus (Elsevier), and Web of Science (Clarivate Analytics) online databases. The databases were consulted up to the 7th of November 2020. Studies that showed *ex vivo* and *in situ* interactions between, at least, two microorganisms were found eligible.

Results and discussion: Ten papers were included, of which six are case studies, three are case-control studies, and one is a cross-sectional study, with considerable heterogeneity. Seven studies focused on the microbiota resident on the subgingival plaque, two studies on the microbiota in supragingival plaque, while only one study collected both supra- and subgingival plaque. One study did not report any exclusion criteria. FISH was performed in each study for a variety of purposes. Although the stock of the oral microbiota has a lot of inter- and intra-individual variation, layered or radially ordered multiple-taxon structures are the most common form of consortium. Strict or facultative anaerobic microorganisms are mostly found in the interior and the deepest portions of the structures, while aerobic microorganisms are mostly found on the periphery. Consumers and producers of certain metabolites tend to be spatially related; some microorganisms exhibited the ability to shape various configurations influenced by their peers in different biofilm areas. We referred to this last phenomenon as "microbes herd mentality behavior" and we believe that it may represent an imperative benefit of certain members of the periodontal microbiota.

Conclusions: The FISH technique was able to detect interactions between microorganisms from the three Domains of Life, allowing for the creation of a potential hypothesis for understanding the role of these interactions in the emergence and progression of periodontal diseases. However, the available evidence regarding the structural composition and interactions of microorganisms within periodontal biofilms is incomplete, and no precise conclusions can be drawn. Assuming that these

limited observable interactions are, somehow, indicative of the periodontal microbiota in health and disease is a leap of faith that needs validation in large-scale studies. Nonetheless, we are confident that by integrating current pathogenesis principles with the novel FISH-based techniques, a new diagnostic tool could arise, in the form of an oligonucleotide probes kit able to target periodontal pathogens that cause dysbiosis.

Keywords: Periodontal disease; fluorescence *in situ* hybridization; oral biofilm; imaging; microbes; oral microbiota.

SCIENTIFIC OUTPUTS

The following list reflects the achievements accomplished during the development of this monograph.

Oral communications in National Scientific Meetings

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Papers in journals

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INDEX OF ABBREVIATIONS

µm	Micrometer
3D	Three-dimensional
AAP	American Academy of Periodontology
ACP	Advanced chronic periodontitis
AEP	Acquired enamel pellicle
AHLs	Acyl-homoserine lactones
AIPs	Autoinducing peptides
ASA	Andreia Sofia Azevedo
BOP	Bleeding on probing
CAL	Clinical attachment loss
CFB	*Cytophaga-Flavobacterium-Bacteroides* cluster
CLASI-FISH	Combinatorial labeling and spectral imaging fluorescence *in situ* hybridization
CLSM	Confocal laser scanning microscopy
CP	Chronic periodontitis
Cy3	Cyanine 3
Cy5	Cyanine 5
D	Domain
DNA	Deoxyribonucleic acid
EFP	European Federation of Periodontology
ePTFE	Expanded polytetrafluoroethylene
F	Family
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate
G	Genus
GAP	Generalized aggressive periodontitis
GCF	Gingival crevicular fluid
GME	Guilherme Melo Esteves
IF-FISH	Immunofluorescence-fluorescence *in situ* hybridization
KPH	Keystone pathogen hypothesis
LM	Luzia Mendes
LNA	Locked nucleic acid
mRNA	Messenger ribonucleic acid
NAI	No available information
NAMs	Nucleic acid mimics
ND	Not determined
NAI	No available information
NUG	Necrotizing ulcerative gingivitis
P	Phylum
PCC	Population concept context
PCR	Polymerase chain reaction
PNA	Peptide nucleic acid

PPD	Probing pocket depth
PR	Periodontitis-resistant
PSD	Polymicrobial synergy and dysbiosis
QS	Quorum sensing
RBL	Radiographic bone loss
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
RPP	Rapidly progressive periodontitis
rRNA	Ribosomal ribonucleic acid
S	Specie
SUBG-FL	Subgingival first layer
SUBG-IL	Subgingival intermediate layer
SUBG-OL	Subgingival outside layer
SUBG-TL	Subgingival top layer
SUBG	Subgingival
SUPG-BL	Supragingival basal layer
SUPG-SL	Supragingival second layer
SUPG	Supragingival

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CHAPTER 1
INTRODUCTION

INTRODUCTION

Periodontitis' profile

Periodontitis is the result of the chronic inflammatory loss of the tooth-supporting tissues, which can lead to tooth loss when untreated (1). It is the consequence of the imbalance between the polymicrobial microbiota, that colonizes the tooth surfaces in form of biofilms, the immune and inflammatory host response within the gingival tissues (1-3), and the individual variations in the stock of these taxa (4). This imbalance, in susceptible individuals (5, 6), results in the loss of attachment between the teeth and its *periodontium*, triggering the formation of periodontal pockets. It is described as a life condition, even if patients follow a successful therapy since periodontitis requires long-term supportive treatment to avoid its recurrence (7).

Periodontal diseases are estimated to affect 20 to 50 percent of the world's population (8). Furthermore, mounting evidence suggests that many chronic disorders, such as diabetes, cardiovascular disease, and tumors, are linked to disruptions in the oral ecosystem (9-11). FIGURE 1 reveals the clinical appearance of periodontitis and a healthy periodontium.

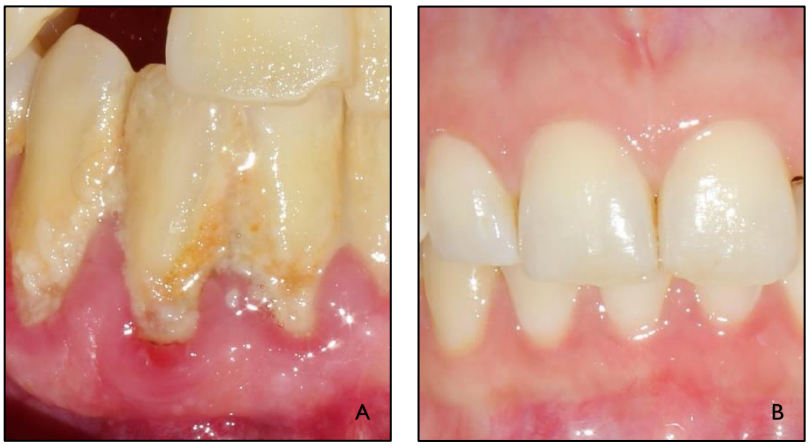


FIGURE 1. (A) Periodontitis; (B) Healthy *periodontium*. Original images taken by Guilherme de Melo Esteves.

A total of 2074 genomes and 529 taxa of microbes (12) are estimated to inhabit the oral ecosystem. Not all these microorganisms are directly associated with periodontitis but may create the necessary conditions (e.g., nutrient supply or oxygen depletion) for other microorganisms to grow and disrupt the periodontal balance between host and microbes. These species are involved in the formation of a sessile and complex polymicrobial structure, extremely well-ordered, surface-associated groupings of multispecies embedded in an extracellular matrix - the oral biofilm (13, 14).

These organisms rarely exist in confinement (15), but instead live in polymicrobial communities, revealing various patterns of gene expression in comparison to planktonic cells. Once they are attached to a substratum surface, a physical, biological, and chemical (16, 17) multistep process starts to establish the biofilms. Even though the structure and composition of biofilms are site-specific, all biofilms are originated from the same process, as represented in FIGURE 2 (14, 17).

We must differentiate two concepts that are sometimes misunderstood: "coaggregation" – the usually reversible (18) interaction between planktonic microorganisms of different species, where the selection of beneficial partners is observed within the biofilm – from "coadhesion" – the casual interaction between a sessile, already adhered organism and planktonic microorganisms from a different species (16, 19). The main difference is that coadhesion is one of the many primary events promoting the development of biofilms (16, 20), while coaggregation may play a central role in the spatial and temporal development of biofilms, moderating the consortia community composition (21).

The concept of "co-localization" has been recently introduced and portrayed as a more passive process, described as the interaction between microbes that encourages the local growth of a beneficial partner by supplying benefits for its development, but without a physical connection (18).

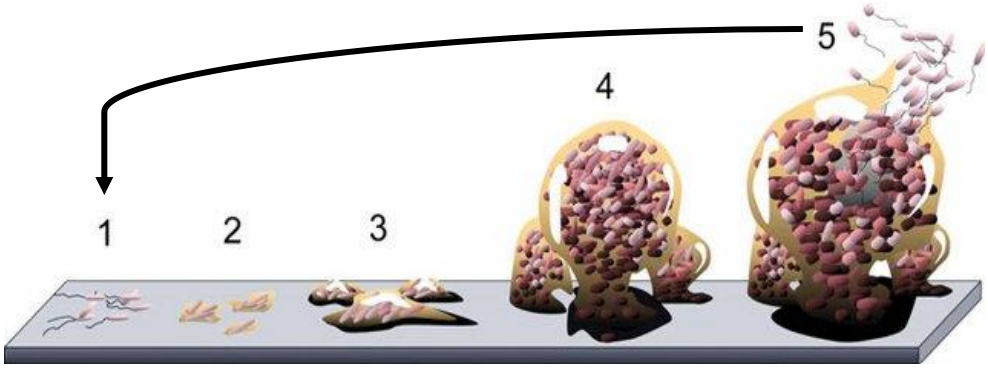


FIGURE 2. Stages of biofilm development. (1) Adsorption of biofilm's components [such as organic molecules], microbial transport, and [reversible] coadhesion; (2) [Permanent] bond of individual organisms and microbial aggregates to the substratum; (3) Coaggregation between microbial pairs; (4) Formation of fixed, stable, and irreversible adhesion through exopolymer manufacture – the extracellular matrix; (5) Growth and detachment of single cells or aggregates, which leads to cell's migration and posterior colonization of other sites. Reprinted and adapted from Unosson, Erik (2015) with the authors' permission (22).

Culture reset – a turning point of molecular techniques

Many types of research concentrated primarily on culturable bacteria during the period from the 1970s to the early 1990s. However, microorganism culture is often overly discerning, time-consuming, and costly (23). The creation of culture-independent methods has allowed the identification of periodontitis-associated uncultured and fastidious species, providing a more detailed look at the bacterial communities in periodontal tissues. The *in situ* and *in vivo* understanding of the structure, function, and composition of dental plaque biofilm may be highly helpful in finding preventive and treatment strategies (24).

Many alternative molecular techniques have been introduced into periodontal microbiology to identify microorganisms in dental plaque specimens without previous cultivation, for example, immunofluorescence and immunohistochemistry (25-28). These methods can detect specific bacteria by antibodies-antigen reactions, available for specific microorganisms, but cannot discriminate between living and dead cells (29), it is dependent on specific antibodies' kits and cannot discriminate specimens arranged by polymicrobial communities (30).

Therefore, the use of nucleic acids-based methods has been preferred to identify microorganisms directly from clinical samples, such as polymerase chain reaction methods (PCR) (31-33) and DNA-DNA hybridization methods such as *in situ* hybridization (34, 35), checkerboard hybridization (36, 37), and 16S rRNA-based microarrays (38, 39). These molecular techniques detect microorganisms by DNA amplification and/or the hybridization of oligonucleotide probes with complementary sequences in the target microorganism (29).

The *in vitro* amplification of nucleic acids by PCR methods creates some limitations (40-43), such as: (i) the high costs and onerous investment required for PCR testing; (ii) the demand for initial technical training; (iii) extensive time-consuming sample preparation with the extraction of DNA; (iv) high prevalence of false positives or negatives outcomes since PCR is a DNA amplification-based technique (many amplified molecules result in a particular PCR cycle, which can potentially contaminate successive amplifications of the same initial target sequence), due to laboratory contamination or to the detection of dead cells; (v) nonexistence of a standard protocol; (vi) and variable quality of reagents and equipment.

The use of molecular techniques like checkerboard DNA-DNA hybridization and 16S rRNA-based microarrays also have its limitations, for instance, the identification of a fairly small number of species for a fairly small number of samples (30), offering little applications in the detection of microorganisms in specimens of multispecies infections, like periodontitis. Additionally, checkerboard hybridization is restricted to species for which probes are available.

FISH for the study of the biofilm's spatial organization

The fluorescence *in situ* hybridization (FISH) technique was developed to overcome some limitations of previous ones. This technique has proven to be particularly useful to describe the microbial composition and spatial organization of mixed microbial infections. This molecular technique relies on the hybridization of single-stranded, fluorescently labeled DNA- or RNA-targeted oligonucleotides probes with fluorescent molecules (e.g., fluorochromes) that hybridize to its complementary conserved 16S or 23S rRNA sequences in the microorganism (23, 44, 45). This imaging approach offers information about the morphology, cellular ecosystem, and spatial organization of organisms, in time and space within their natural context, making it the gold standard for the imaging of complex microbial communities (46).

The conventional FISH technique includes the following steps: (i) cells' fixation; (ii) model's preparation, perhaps containing specific pretreatment steps; (iii) probe hybridization with the respective target sequence; (iv) washing stages to remove unbinding probes; (v) mounting, imaging, and documentation of the results (23).

Although the above-mentioned applications and advantages of the use of FISH, there are reports of some limitations of this technique, especially in prokaryotic cells, where the nucleoid and cytoplasm coexist in the same section of the cell. The reduced cellular volume of the bacteria leads to considerably high RNA densities, causing signal overlapping and limiting the microscopy detection of those organisms with a low RNA abundance. Several authors bypass this problem by estimating mRNA copy numbers from the total fluorescence signal for highly expressed target mRNA molecules in *E. coli* (46) or by using high-resolution techniques able to solve smaller detection volumes, enabling the detection of single mRNA molecules in overcrowded environments.

The autofluorescence of microorganisms and the material surrounding the bacteria in the specimens (caused by the natural fluorescence of biological or inorganic fragments) is another notable challenge of this technique. It is recommended to use narrow-band filter sets and signal amplification methods to overcome this problem (23).

To circumvent the lack of specificity, every FISH experiment should use positive and negative controls (23). Negative controls are created with closely related strains embracing target sequences with few mismatches to the respective positive oligonucleotide probe, increasing the chance of only the positive controls bind to the target sequences.

Conventional FISH uses DNA/RNA probes, which have relatively low affinity to the target, making it tricky for the target probes to access the target site (which leads to low specificity and sensitivity of the probes) (47). To bypass these limitations, nucleic

acid mimics (NAMs), such as peptide nucleic acid (PNA) and locked nucleic acid (LNA) probes have been pioneered into hybridization techniques (5, 23).

Adjustments to nucleic acids may be added in the nucleobase, the sugar ring, or the phosphodiester backbone (48). In PNA probes, the negatively charged sugar-phosphate (PO) backbone of DNA is substituted by an achiral, a noncharged peptide backbone, formed by repetitive units of N-(2-aminoethyl) glycine (49, 50). LNA probes contain 2′-O,4′-C-methylene linked bicyclic ribofuranosyl nucleosides locked in an N-type arrangement (FIGURE 3) (51).

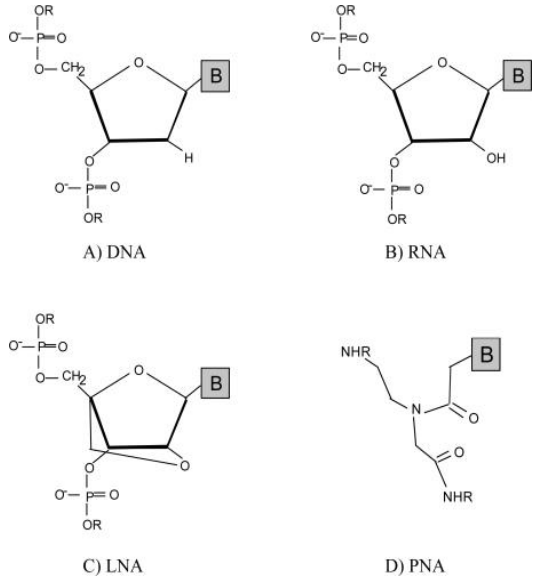


FIGURE 3. Chemical structures of DNA (A), RNA (B), LNA (C), and PNA (D). Reprinted from Cerqueira, Laura, *et al.* (2008) with authors' permission (52).

In PNA probes, this modification allows the probes to avoid electrostatic repulsion, hybridizing more rapidly with the complementary sequences and obeying the Watson-Crick base-pairing rules (53). NAMs are usually formed by 15 bases, shorter than conventional oligonucleotide probes, permitting easier access into the cell and faster results (54).

It has been attempted to replace the normal phosphate (PO) backbone in LNA probes with phosphorothioate (PS), in which a sulfur atom replaces one of the non-bridging oxygen atoms in the probe's internucleoside linkages. This modification makes probes more resistant to nuclease degradation, allowing them to be used in therapeutic applications. (55). LNA probes can be designed in conjunction with DNA monomers or other chemical modifications like alterations at the 2' position of the ribose ring with 2'-OMe-RNAs.

LNA intercalated with 2'-OMe allows monitoring the thermodynamic parameters, such as melting temperature (T_m) and overall Gibbs free energy change ($\Delta G_{overall}$),

detecting microorganisms under standardized hybridization conditions (45). Therefore, this process leads to superior hybridization efficiency for multiplex purposes, by increasing its design flexibility (45, 55).

Even in simple samples where only two or three fluorophores are used, spectral overlap and crosstalk seem difficult to eradicate. Additionally, the use of bandpass filters in fluorescence image acquisition restricts the number of fluorochromes that can be simultaneously distinguished, making it problematic to recognize one signal from another simultaneously with certainty (56, 57) and consequently restricting the microscopic identification of various taxa of microbes in single samples.

Valm, Alex M., *et al.* (2012) (57, 58) developed a strategy to overcome this limitation – the Combinatorial Labeling and Spectral Imaging (CLASI) - and blended it with FISH (CLASI-FISH). This technique relies on the labeling of microbes of interest by FISH with two or more combinations of fluorochromes (57), increasing spectral discrimination of the fluorochromes that have a propensity to overlap in excitation and emission spectra.

This upgrade resulted in a significant advance in diagnosing by fluorescence image, since quite a few atomic line intensities simultaneously may make available numerous measurement techniques (56, 59). CLASI-FISH is currently capable to distinguish unambiguously 120 differently labeled organisms (60) in a specimen of semi-dispersed human dental plaque, resulting in exclusive mixed colors that are distinguished by the application of linear unmixing algorithms.

FISH observations enable the identification of the conventional forms in which microbes are arranged, correlating its spatial organization with the kind of interactions within the consortia. Bacteria are spatially structured in three general ways: single-species microcolonies, bacterial coaggregation, and layered-structured-biofilm (61).

In the first type, the species establish separately alongside microcolonies, resulting in a non-commensal relationship, dependent on the nutritional supplies available. In the second form, the species involved are mixed and can be found together within the biofilm. In the last form, cells are disposed of in layers. These two last forms of biofilm's arrangement can either establish a synergistic interaction, where an effective metabolic interaction is developed as a public good to the whole consortia (62), or a competitive interaction, where resources are limited (61).

Microbes' friendzone

Group behavior is coordinated by the ability to sense and respond to stimuli from neighborhood cells. However, there seems to exist a lack of understanding regarding how the cooperative feature has evolved and how it is maintained (62). Oral

microbiota has been reported to demonstrate inter- and intraspecific mechanisms. It is essential to comprehend these mechanisms to better understand how chronic infections are established.

Intraspecific interactions, biofilm formation, virulence factor production, and carbon utilization are mainly coordinated by quorum sensing (QS), described as the accumulation of self-made signals in proportion to alter gene expression in the environment (15). This process allows bacteria to estimate cell density (63) and to adapt to the environment, which may affect the interactions within a mixed biofilm in a different number of ways, such as the acquirement of a competitive benefit or the capacity to overcome the host defense mechanisms, which is essential for cells' survival and diffusion in natural high-competitive communities, like the oral cavity (14).

QS is predominantly mediated by collectively produced diffusible molecules, which are the expression of quorum-sensing-dependent genes (14, 61), the acyl-homoserine lactones (AHLs) in Gram-negative bacteria, and by small peptides in Gram-positive bacteria, the autoinducing peptides (AIPs). Their production is highly dependent on cellular density (14), and the concentration of these molecules may represent the measurement of cell quantity since bacteria sense signal molecules are proportional to the cell density (15, 64).

The bacterial "cross-talk", cooperative and competitive interactions are examples of types of interspecific interactions (15). The first type is described as the cell's ability to alter gene expression. Cooperative interactions often lead to increased disease severity, boosted tolerance to antibiotics, and make polymicrobial chronic infections difficult to treat and resolve (62). Competitive interactions are often correlated to nutrient restriction, especially during infection, causing infecting bacteria to strive for nutrients, such as iron (15, 65).

These types of interactions lead to tolerance to antibiotics, showing that it can also be beneficial for microbes and result in increased perseverance of the infection (14, 15).

What are the next stages?

Subgingival plaque or crevice fluid samples seem to be appropriate for the study of microorganisms in periodontal pockets (29). Many methods have been described to collect subgingival plaque, such as the use of a sterile paper point inserted into the periodontal pockets (29, 66, 67).

Another sampling method involves the use of carriers consisting of expanded polytetrafluoroethylene (ePTFE) membranes (3, 68, 69). The fixed localization of the carriers allows the topographic association of distinct bacterial morphotypes or species

with the depth of periodontal pockets and the information about the time-dependent development of biofilms can be controlled, which are all advantages of this approach.

The use of epoxy resin crowns (70, 71) and bovine enamel/dentin slabs (72-74) has been employed to collect dental biofilm samples as well. However, these techniques are only useful for studying the supragingival microbiota in a restricted way (68). Since the type of substrate affects the biofilm's spatial characteristics (74), the choice of the carriers' substrate should take into consideration factors particular to the studies' aim. Nevertheless, mounting evidence suggests that the material in which the carriers are made does not have a great influence on the microorganisms' pattern of colonization, (3) possibly due to the similar colonization conditions provided by the salivary pellicle on the surface of each carrier (68, 75).

The gathering of microorganisms with sterile curettes (76-78) may disrupt the spatial organization of subgingival biofilms. However, literature has shown that despite some disruption that may occur, biofilm ultrastructure tends to be preserved if the sample is carefully processed afterward.

A non-invasive alternative solution for the collection and analysis of sub- and supragingival plaque that successfully preserves the biofilm's spatial architecture was described by Mark Welch, J. L., *et al.* (2016) (79). In this technique, dental plaque is carefully fixed in paraformaldehyde and embedded in methacrylate resin, which is later sectioned, these sections are applied to slides and subjected to FISH directly on the slide.

As another option, the atraumatic extraction of teeth by a trained professional tends to be an effective solution that does not disrupt the plaque's arrangement. If possible, the surgery may occur without the use of elevators (80).

Although *in vitro*, animal models, and culture-dependent methods opened the door for much of today's knowledge, it is still unclear if these methods reflect an accurate framework of collective bacterial communities. Microbes' spatial organization in periodontal biofilms, the understanding of the interactions between microbes in biofilm, and how it influences the establishment, development, and the outcome of the chronic infection in periodontal pockets have been emerging subjects in periodontal microbiology.

This review has the purpose to identify and map the existing evidence about *in situ* and *ex vivo* interactions between microbes in periodontal pockets, identified with the FISH technique, as well as to identify and analyze any knowledge gaps that can point to further research directions. We will focus on the mapping, reporting, and discussion of the known evidence and for that reason, we find the scoping review's methodology the most suitable for our purpose (81).

CHAPTER 2
MATERIALS & METHODS

MATERIALS AND METHODS

Focused question

This scoping review was conducted following the guidelines of the Transparent Reporting of Systematic Reviews and Meta-Analyses extension for scoping reviews (PRISMA-ScR) (SUPPLEMENTAL TABLE 1) (82), to answer the focused (PCC) question: "What are the identified interactions between microbes in periodontal pockets by the FISH technique?".

Search strategy and information sources

To prepare this review, three electronic sources of evidence were consulted in search of suitable articles that matched the aim of this review: MEDLINE (via PubMed), Scopus (Elsevier), and Web of Science (Clarivate Analytics) online databases. The databases were consulted up to the 7th of November 2020.

Papers were searched using the following keywords: "Periodontal disease"; "Periodont*"; "Periodontal pockets"; "Microbes"; "Oral biofilm"; "Micro*"; "Oral micro*"; "Bacteria"; "Imaging"; "FISH"; "FISH technique"; "fluorescence *in situ* hybridization" and "hybridization". The keywords were combined with the Boolean operators "AND" or "OR" with the proximity operators ["" and ()] and with the truncation operator (*) used whenever appropriate. The search strategy was personalized according to the different databases (SUPPLEMENTAL TABLE 2).

The electronic database search was supplemented with a hand search across the references of all included papers. The authors of the included papers were contacted to find additional unpublished images that could fulfill our inclusion criteria and were asked permission to reproduce images in this scoping review. Additional permission of all reproduced images was granted by the Copyright Clearance Center.

Eligibility criteria

As inclusion criteria, all studies that described *ex vivo* and *in situ* interactions between, at least, two microorganisms were found eligible. We only incorporated articles applied in humans and written in Portuguese, English, or Spanish.

The exclusion criteria detached experiments using other molecular cytogenetic techniques rather than the FISH technique, articles with no images, or experiments that used manufactured bovine enamel/dentin slabs, acrylic, or epoxy resin appliances

to extract dental biofilm. Restrictions were also made to article type excluding reviews, case reports, or letters.

Screening and selection of sources of evidence

Two independent reviewers (GME, LM) selected papers by evaluating their titles and their abstracts information. Any disagreements in the acquired results were resolved upon discussion with a third reviewer (ASA). Furthermore, the selected articles were then read in full and were not included if did not fulfill the inclusion criteria or if any of the exclusion criteria was detected.

Data extraction and analysis

Data from the included articles were processed for analysis. Information regarding the year of publication, study design, FISH conditions, probes' names and sequences, images, microorganisms found, their location, and their interactions within the biofilm were collected by GME and LM.

Articles found during the updated literature search but not considered suitable or excluded after full-text reading were used later in the introduction and discussion, if pertinent.

Synthesis of results

The studies were categorized based on the collected data. Evidence is reported in a table and a visual representation, that incorporates all the images in the finest resolution acquired from the FISH experiments performed on the included papers.

CHAPTER 3
RESULTS

RESULTS

Selection of sources of evidence

The initial electronic search resulted in 2090 studies, of which 832 located in PubMed, 625 in Web of Science (Clarivate Analytics), and 633 in Scopus (Elsevier). After removing 1144 duplicated studies, 660 studies were rejected after screening articles by title. By reading the abstract, 245 articles were rejected. The remaining 41 studies were obtained and analyzed. After full-text reading, 8 studies met the inclusion criteria. Additional hand searching of the reference lists of the selected papers retrieved 10 additional studies for full-text reading, of which 2 papers met the inclusion criteria. As such, 10 papers (3, 66-69, 76-80) were included in the present scoping review.

The PRISMA flow diagram (FIGURE 4) demonstrates the selection process.

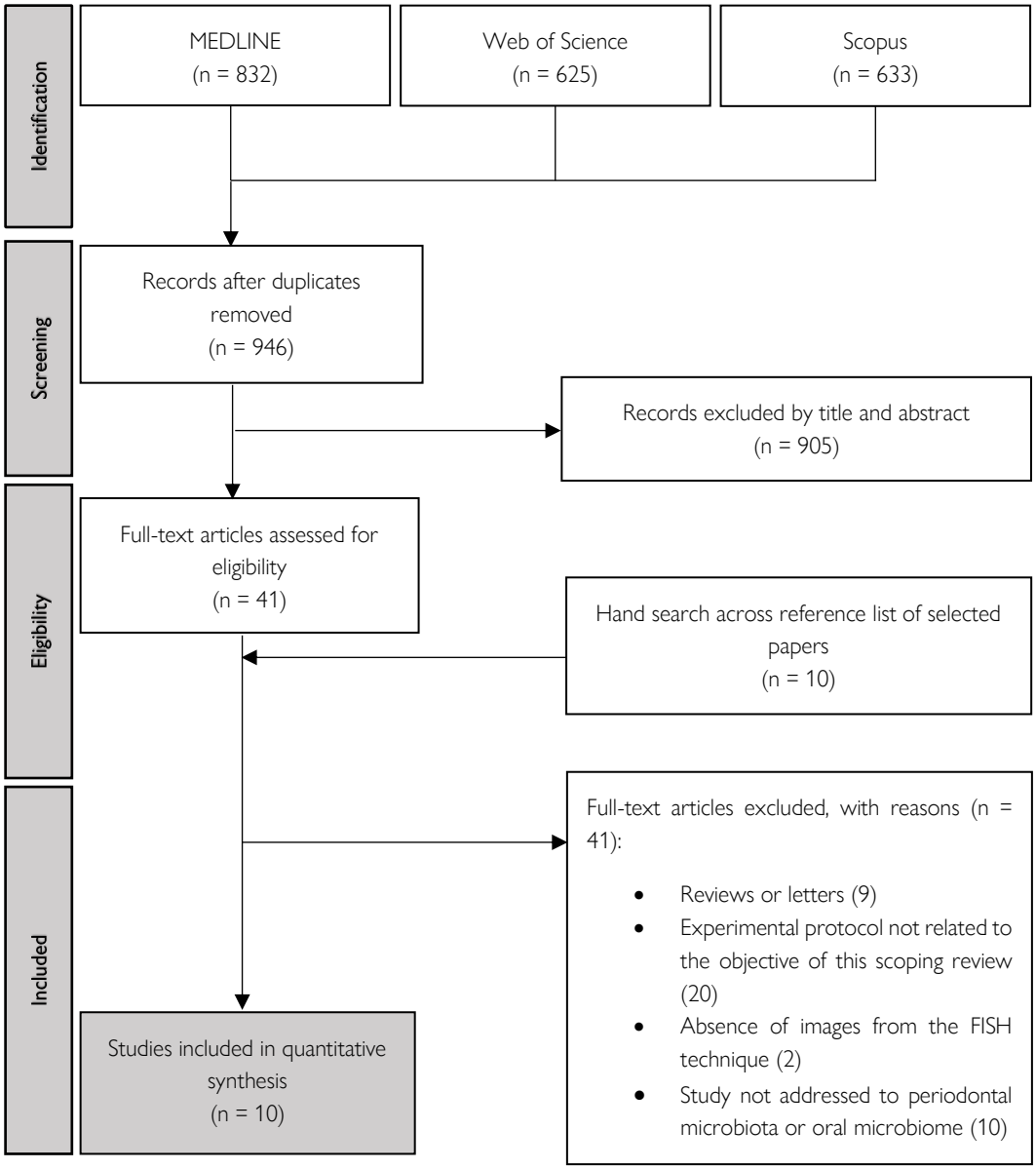


FIGURE 4. Flowchart of literature search and study selection.

Characteristics of sources of evidence

The general characteristics of the six case studies (TABLE 1), the three case-control studies (TABLE 2), and the one cross-sectional study (TABLE 3), are presented in this section.

TABLE 1. Characteristics of the case studies. **PPD**: probing pocket depth; **RPP**: rapidly progressive periodontitis; **BOP**: bleeding on probing; **NUG**: necrotizing ulcerative gingivitis; **NAI**: no available information; **IF-FISH**: immunofluorescence-fluorescence *in situ* hybridization; **ACP**: advanced chronic periodontitis; **GAP**: generalized aggressive periodontitis; **RBL**: radiographic bone loss.

CASE STUDIES (6)					
#	Sample	Exclusion criteria	Cases		Aim
			Characteristics	n	
2	Subgingival	Subjects suffering from chronic diseases; subjects that have received antimicrobial therapy within the past 6 months	PPD means of 8.07±1.63 mm	52	Assess the frequency and spatial organization of specific treponemes in subgingival plaque specimens of patients suffering from RPP
4	Supragingival	Subjects suffering from chronic diseases; subjects that have received antimicrobial therapy within the past 3 months	Gingival pain, gingival ulcers, or necrosis, pseudomembranes, fetid odor, loss of gingival papillae, PPD ≤ 4 mm, having at least 20 natural teeth, BOP, and plaque index	42	Compare the distribution of presumed periodontal pathogens in two groups of patients with gingivitis or NUG
5	Subgingival	NAI	Patients with ACP	NAI	Develop an IF-FISH protocol for identifying distinct cell populations within complex dental plaque samples
7	Subgingival	Subjects suffering from chronic diseases; subjects that have received antimicrobial or anti-inflammatory therapy within the past 6 months; pregnant or lactating women	GAP: having a disease onset estimated at < 30 years and PPD ≥ 6 mm at more than 3 permanent teeth (other than first molars or incisors)	11	Study the incidence, spatial distribution, and architectural function of *Filifactor alocis* in clinical samples of GAP patients
8	Supra- and subgingival	Subjects suffering from chronic diseases; subjects that have received antimicrobial therapy within the past 3 months	PPD > 6 mm RBL > 30%	10	Provide a clear vision of biofilm architecture and the spatial organization of predominant periodontal taxa
10	Supragingival	Subjects suffering from chronic diseases	Subjects refrained from oral hygiene for 12 to 48 hours before sample collection	22	

TABLE 2. Characteristics of the case-control studies. **PPD**: probing pocket depth; **RPP**: rapidly progressive periodontitis; **BOP**: bleeding on probing; **CAL**: clinical attachment loss; **GAP**: generalized aggressive periodontitis; **CP**: chronic periodontitis; **PR**: periodontitis-resistant.

CASE-CONTROL STUDIES (3)							
#	Sample	Exclusion criteria	Cases		Controls		Aim
			Characteristics	n	Characteristics	n	
1	Subgingival	Subjects suffering from chronic diseases; subjects that have received antimicrobial or anti-inflammatory therapy within the past 6 months	PPD ≥ 6 mm BOP	200	Site clinically not affected	44	Assess the frequency of specific treponemes in subgingival plaque specimens of patients suffering from RPP
3	Subgingival	Subjects suffering from chronic diseases; subjects that have received antimicrobial therapy within the past 3 months; pregnant or lactating women; subjects with diabetes or HIV-positive	Gingivitis (BOP; CAL ≤ 1 mm; PPD ≤ 4 mm); Slight periodontitis (BOP; CAL 2-3 mm; PPD ≥ 4 mm); Moderate periodontitis (BOP; CAL 4-5 mm; PPD ≥ 4 mm); Severe periodontitis (BOP; CAL ≥ 6 mm; PPD ≥ 4 mm)	167	Healthy (BOP; CAL ≤ 1 mm; PPD ≤ 3 mm)	67	Identify populations of methanogenic *Archaea* in periodontal pockets
6	Subgingival	Subjects suffering from chronic diseases; subjects that have received antimicrobial or anti-inflammatory therapy within the past 6 months; pregnant or lactating women	GAP: having a disease onset estimated at < 30 years and PPD ≥ 6 mm at more than 3 permanent teeth (other than first molars or incisors); CP: PPD of ≥ 4 mm at 30% or more of the residual teeth	144	PR subjects: (age ≥ 65 years; at least 20 natural teeth; CAL ≤ 2 mm; PPD ≤ 5 mm)	19	Study the spatial organization and possible function of *Selenomonas* sp. in periodontal biofilms in patients with GAP

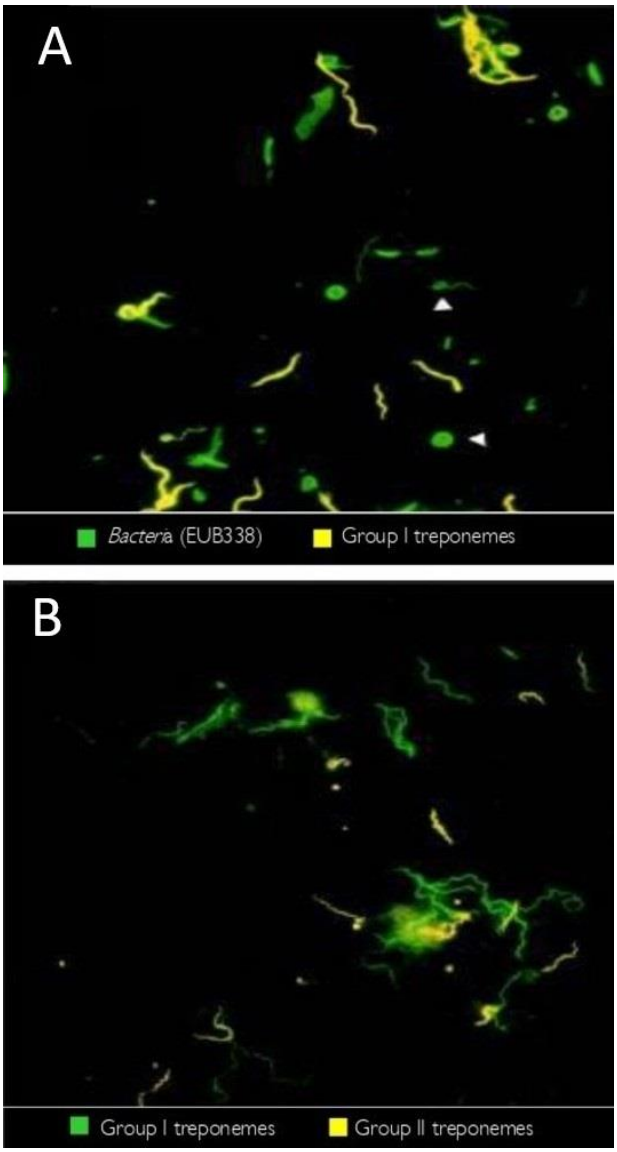
TABLE 3. Characteristics of the cross-sectional study.

CROSS-SECTIONAL STUDY (1)					
#	Sample	Exclusion criteria	Subjects' condition	n	Aim
9	Subgingival	Subjects suffering from chronic diseases; subjects that have received antimicrobial therapy within the past 3 months; subjects that had psychotropic or anticonvulsant medication in the preceding 3 months; subjects that have received professional tooth-cleaning in the previous 6 months or were receiving orthodontic treatment	Pregnant	20	Test the hypothesis of qualitative and quantitative differences of eight periodontal pathogens between pregnant and non-pregnant women
			Non-pregnant	20	

Results of individual sources of evidence

#1 | MOTER, A., *ET AL.* (1998)

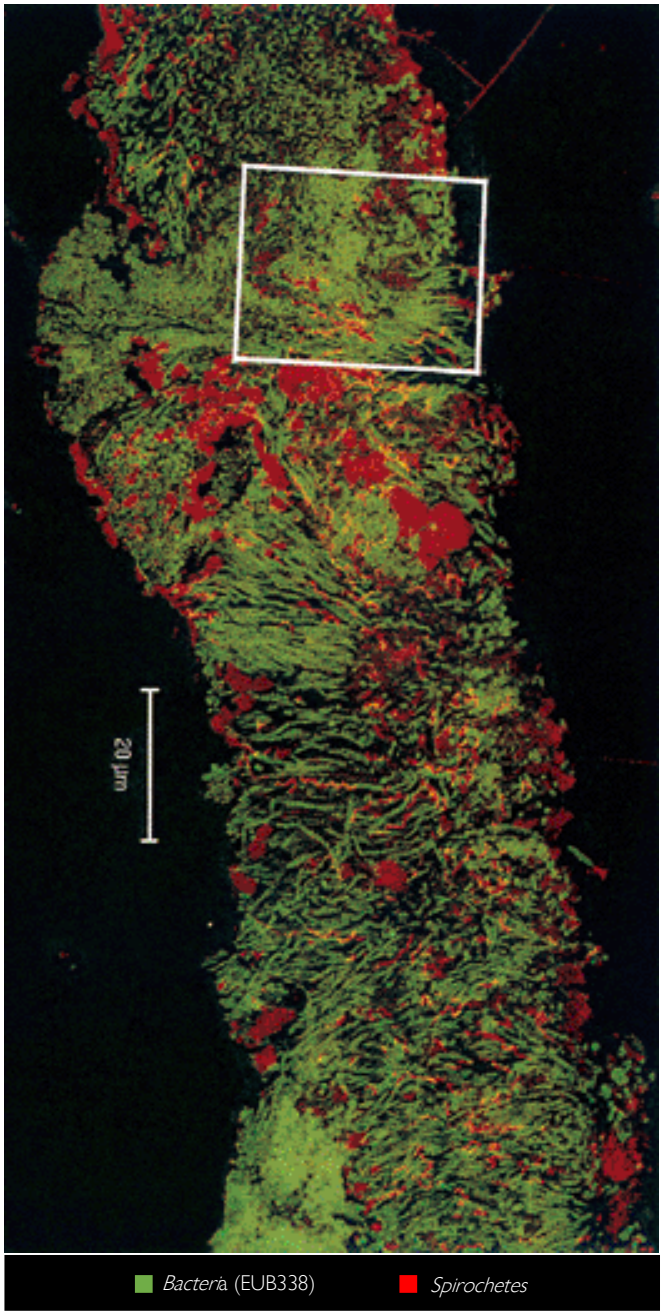
The group I treponemes appeared as large, dense spirochetes, revealing the distinct morphotypes of subgingival plaque bacteria and the spherical bodies of the treponemes (FIGURE 5A). Group II treponemes, on the other hand, were observed as thin, slender, and having many waves (FIGURE 5B). Group II treponemes were less common than group I treponemes. All the treponemes identified predominated at diseased sites but were found infrequently at periodontally stable sites.



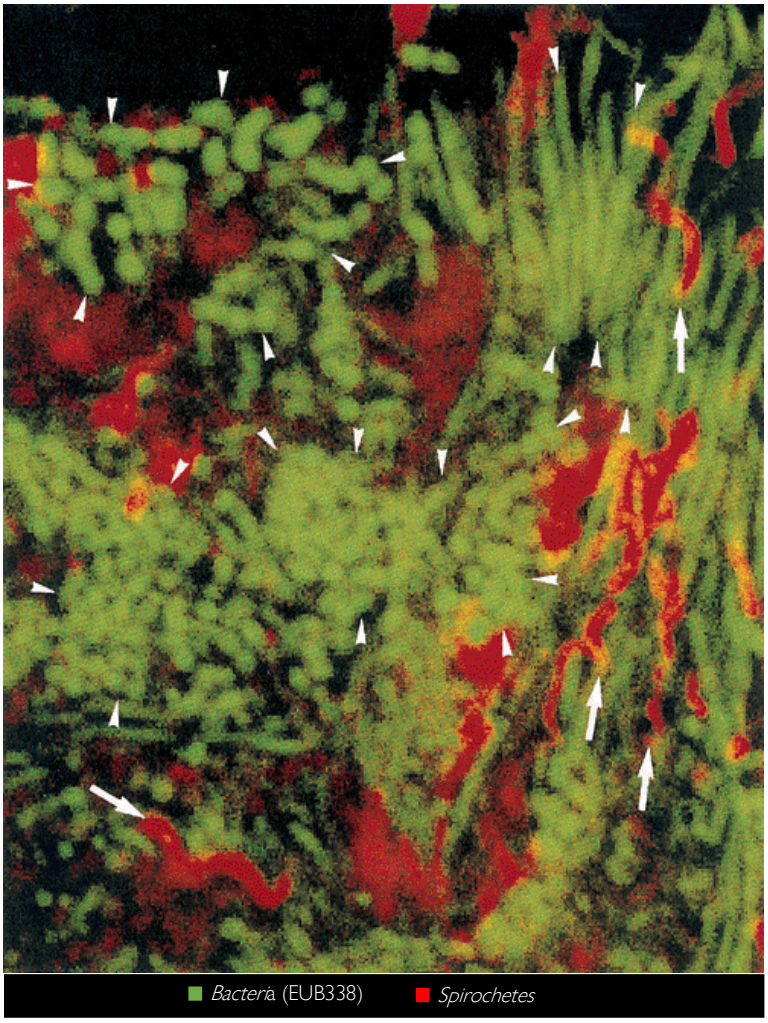
#1 | FIGURE 5. (A) Microphotograph from subgingival plaque material after synchronized hybridization with the probes $EUB338_{FITC}$ (*Bacteria*) (green) and TRE I_{Cy3} (group I treponemes) (yellow). (B) Microphotograph from subgingival plaque material after simultaneous hybridization with TRE I_{FITC} (green) and TRE II_{Cy3} (group II treponemes) (yellow). No scale bar mentioned. Reprinted and adapted with the authors' permission.

#2 | WECKE, J., *ET AL.* (2000)

In this experimental study, only certain portions of the carriers located in the deepest zone of the periodontal pockets had high numbers of undulated group I treponemes and the presence of Gram-negative rods and coccoid cells (FIGURE 6 and FIGURE 7). Gram-positive cocci, on the other hand, were located on the most coronal section of the specimens.



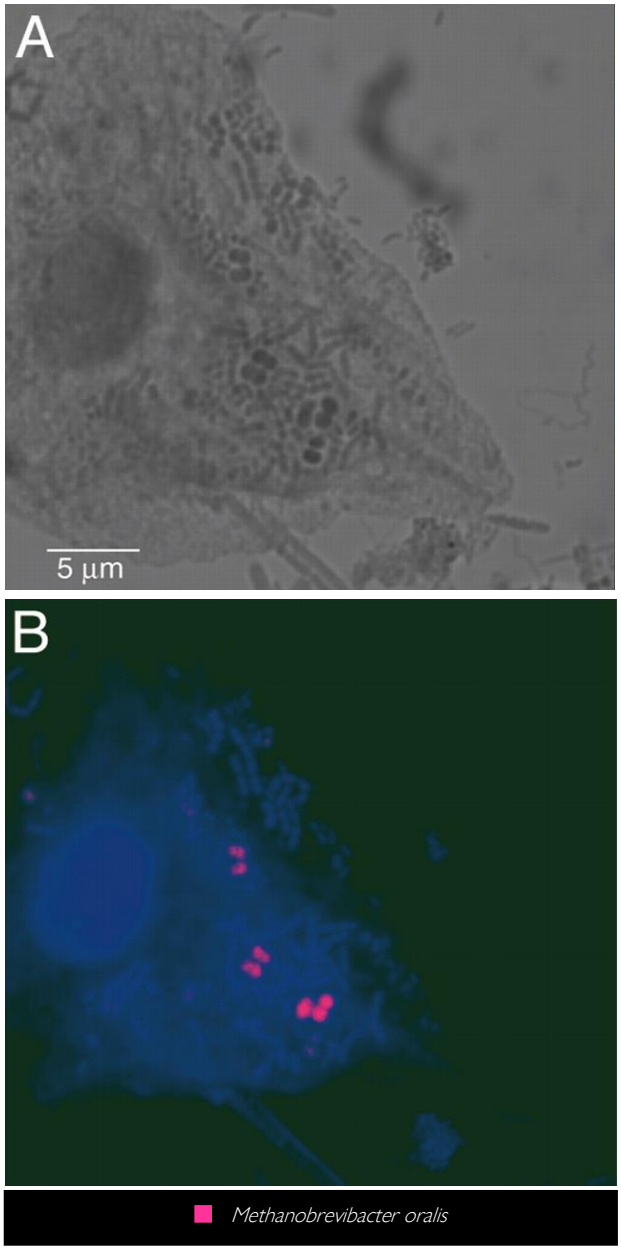
#2 | FIGURE 6. Confocal laser scanning microscopy (CLSM) micrograph of a subgingival specimen. FISH was designed with the group-specific probe TRE I_{Cy3} (reveals the existence of spirochetes), and *Bacteria*-specific probe $EUB338_{FITC}$ (labels all bacteria). Reprinted and adapted with the authors' permission.



#2 | FIGURE 7. Higher magnification of FIGURE 6 white frame revealing different morphotypes of e.g., cocci or rods (green colored), marked with the white arrows head. Additionally, the probe $TREI_{Cy3}$ revealed some large yellow/red spirochetes spread between other bacteria (white arrows). No scale bar mentioned. Reprinted and adapted with the authors' permission.

#3 | LEPP, P. W., *ET AL.* (2004)

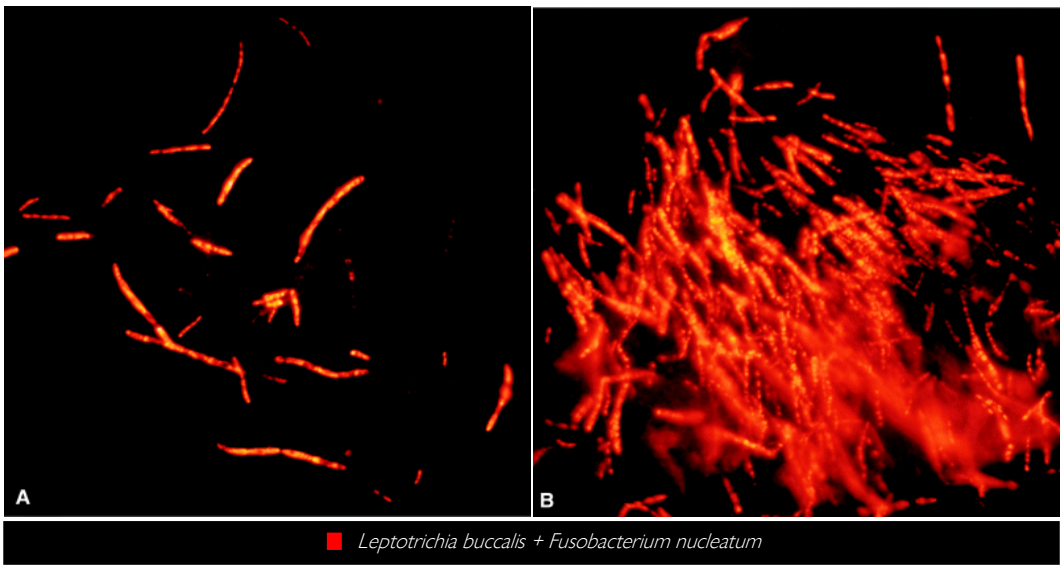
A population of *Methanobrevibacter oralis* consisting mainly of diplococcobacilli was identified in the periodontal pockets (FIGURE 8). *Archaea* were detected in only a subset of patients with severe disease. Treponemal rDNA was found in significantly lower abundance in sites with archaeal rDNA than in sites without archaeal rDNA, suggesting that *Treponema* species may compete with methanogens.



#3 | FIGURE 8. Confocal micrographs of bacteria and archaea in subgingival plaque from a patient with severe periodontitis. (A) "Bright field" micrograph. (B) FISH micrograph of oral *Archaeum* using SBGA-1_{Cy3} (pink), specific for *Methanobrevibacter oralis,* and counterstained with the nucleic acid dye YO-PRO-1 (blue), revealing a population of *M. oralis* consisting mainly of diplococcobacilli. Reprinted and adapted with the authors' permission.

#4 | GMÜR, R., *ET AL.* (2004)

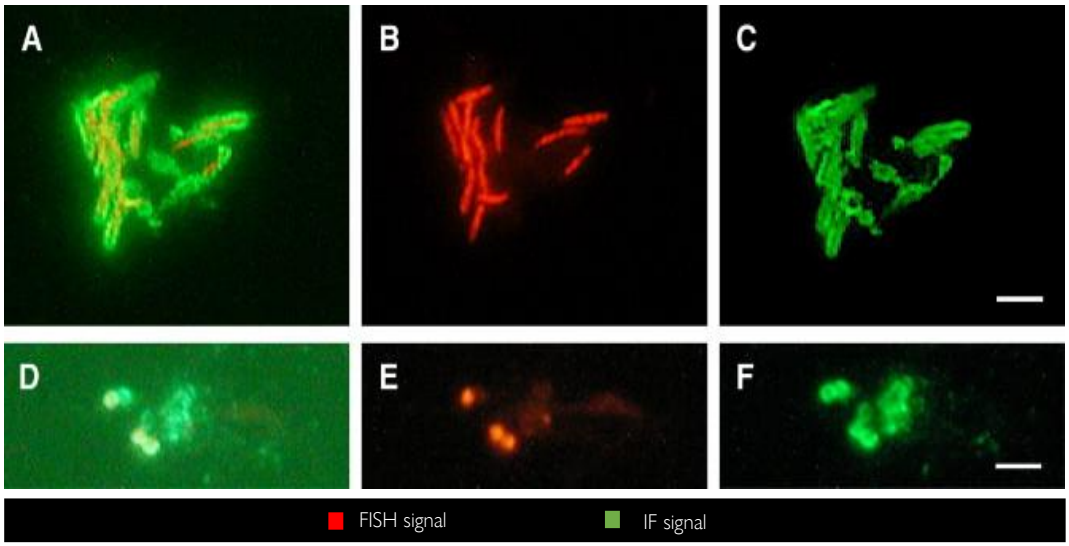
Prevotella intermedia was present in 100% of the samples, while *Prevotella nigrescens* was found in just 71% of gingivitis samples and 81% of NUG samples. In both disease classes, *P. intermedia* outnumbered *P. nigrescens* quantitatively: gingivitis samples contained 9 times more *P. intermedia* than NUG samples, and NUG samples contained 5.9 times more *P. intermedia* than *P. nigrescens*. *Capnocytophaga* sp., *Fusobacterium* sp., *F. nucleatum*, and *Leptotrichia buccalis* and treponemes occurred with a 100% prevalence in both test groups, while *F. necrophorum* was not detected. The probe FUS664, staining almost the entire *Fusobacterium* genus, detected 2.0% of gingivitis and 3.0% of NUG in the total bacterial cell population, almost twice the concentration of cells identified with the *F. nucleatum*-specific probe Fnuc133 (1.3% and 1.5%, respectively). The prevalence of *Capnocytophaga* sp. was very similar to the proportions of *Fusobacteria* (mean values of 2.3% and 3.1% respectively). *L. buccalis* was significantly less common, with mean proportions of 0.7% in the gingivitis group and 0.5% in the NUG group. In NUG samples, *Selemonas sputigena* was found in higher proportions, while *Veillonella* sp. was found in similar proportions in both clinical classes. Treponemes were present in just 1.5% of NUG samples and 0.75% of gingivitis samples on average. In the NUG community, the number of these microorganisms percentages was found to be substantially higher. FIGURE 9 shows a FISH image of a marginal plaque specimen from a NUG patient.



#4 | FIGURE 9. FISH with Lbuc668$_{Cy3}$ probe labeled two cell types in marginal plaque from a patient with necrotizing ulcerative gingivitis (NUG). (A) The probe hybridized with relatively wide and sometimes segmented fusiform rods with the typical morphology of *Leptotrichia buccalis* and also identified fusiform rods of variable length, much smaller, thin, spindle-shaped, and dotted fluorescent cell types that resemble and are indistinguishable from most *Fusobacterium nucleatum*, *Capnocytophaga* sp., and *Fusobacterium periodonticum* cells (B) A small clump of plaque highly and densely colonized by both species labeled by Lbuc668$_{Cy3}$ (*Leptotrichia buccalis* and *Fusobacterium nucleatum*). No scale bar mentioned. Reprinted and adapted with the authors' permission.

#5 | GMÜR, R. AND H. LÜTHI-SCHALLER (2007)

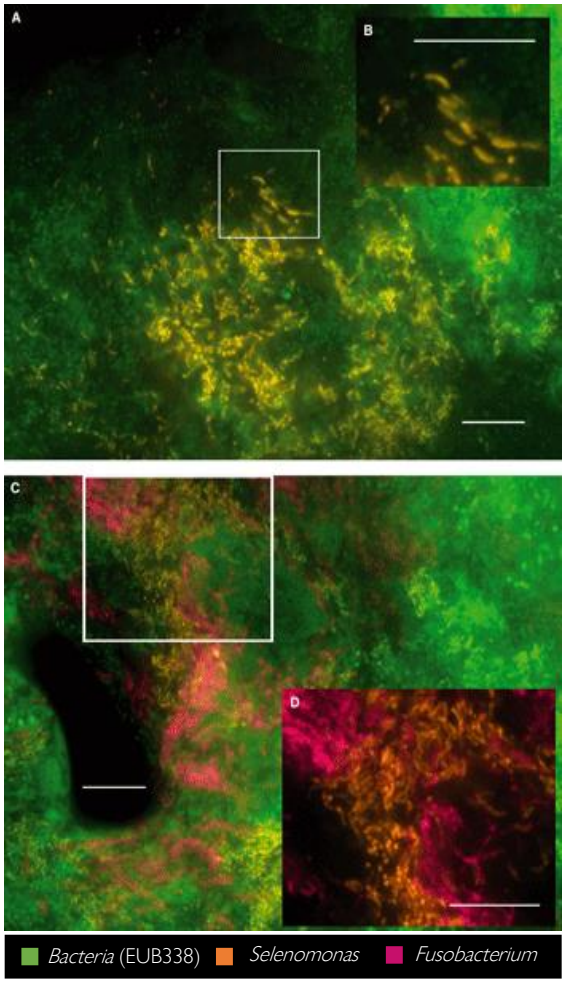
Methods that enable the simultaneous identification of cell populations defined by genetic and phenotypic markers have become a way to better comprehend complex natural biofilm ecologies. That aim can be achieved by combining immunofluorescence (IF) and fluorescence *in situ* hybridization (FISH) to label target cell populations (FIGURE 10), using antibodies and oligonucleotides probes, respectively. In the proposed IF-FISH protocol the findings were higher when IF came first. The best results were obtained in the assay that reduced IF incubation times to 30 minutes (limiting rRNA degradation), increased the second antibody concentration to raise fluorescence intensity, and replaced avidin-FITC with streptavidin-FITC, which resulted in a significant reduction in IF background fluorescence.



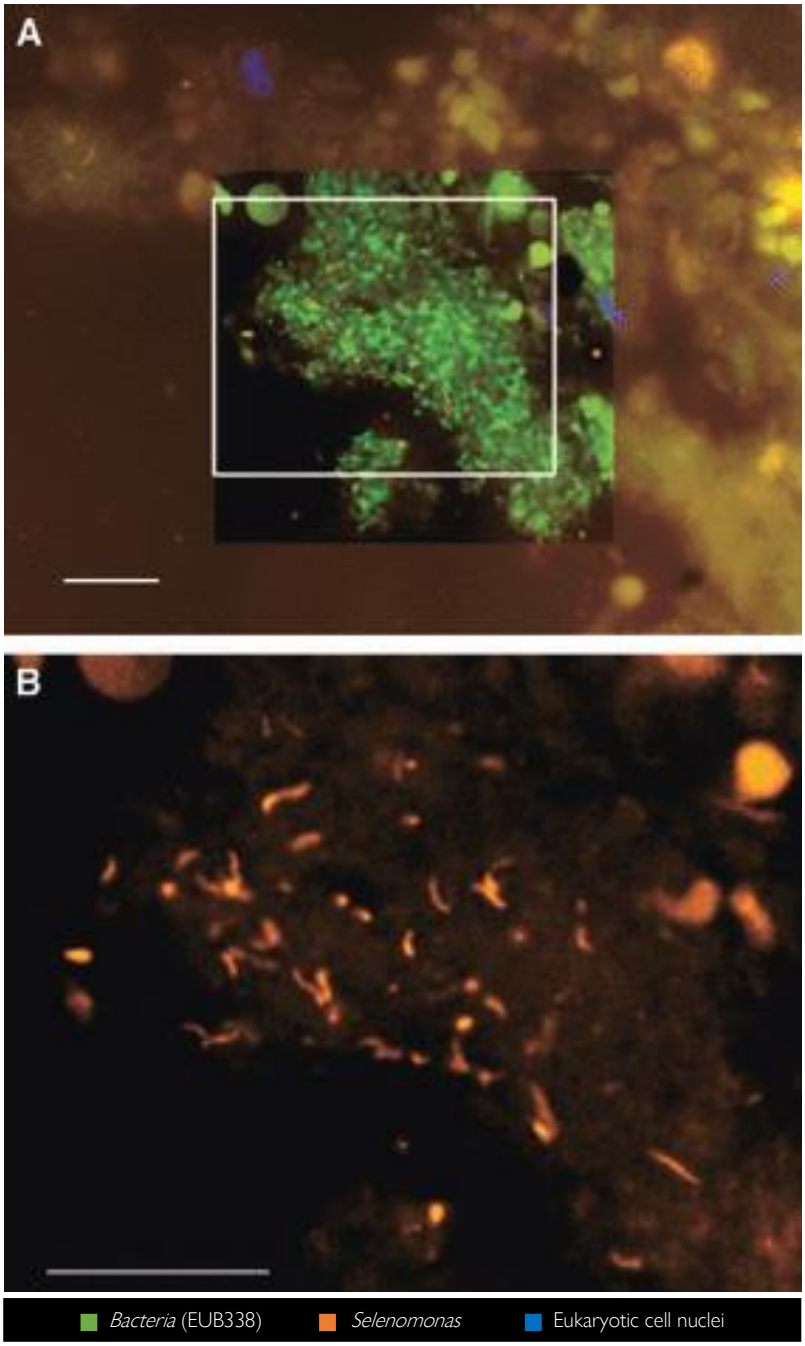
#5 | FIGURE 10. Micrographs of bacterial cells from dental plaque visualized by combined IF-FISH. (A-C) Small clump containing *Tannerella forsythia* labeled by 116BFI.2$_{FITC}$ (IF) and Tfor582$_{Cy3}$ (FISH). (D-F) Cells are stained by 376FN1.2$_{FITC}$ (IF) recognizing surface-bound phosphorylcholine and EUB338$_{Cy3}$ (FISH) with specificity for *Bacteria*. (A and D) Images were made using a fluorescence filter providing the detection of both signals. (B and E) Images with the filters for Cy3 fluorescence. (C and F) Images with the filters for FITC fluorescence. Bars indicate 2.5 µm. Reprinted and adapted with the authors' permission.

#6 | DRESCHER, J., *ET AL.* (2010)

Selenomonas sp. were visualized as abundant and with a crescent-shaped morphology (FIGURE 11A and FIGURE 12). SELE-positive microorganisms (*Selenomonas* sp.) colonized various parts of the membranes from the cervical section to the biofilm portion derived from the pocket's depth and both on the side facing the tooth and the side facing the soft tissue. SELE-positive bacteria may be seen as single dispersed cells, but they were more usually seen as closely packed groups of organisms, (FIGURES 11A and C). A close spatial relationship between the bacteria of two genera was observed when FISH was performed with both SELE and FUSO (*Fusobacterium* sp.) probes (FIGURE 11C and D), revealing SELE-positive organisms and *Fusobacterium* sp. tangled. Images from gingival biopsy subjected to FISH revealed a cell morphology and appearance of SELE-positive organisms like those observed in carrier-grown biofilms.



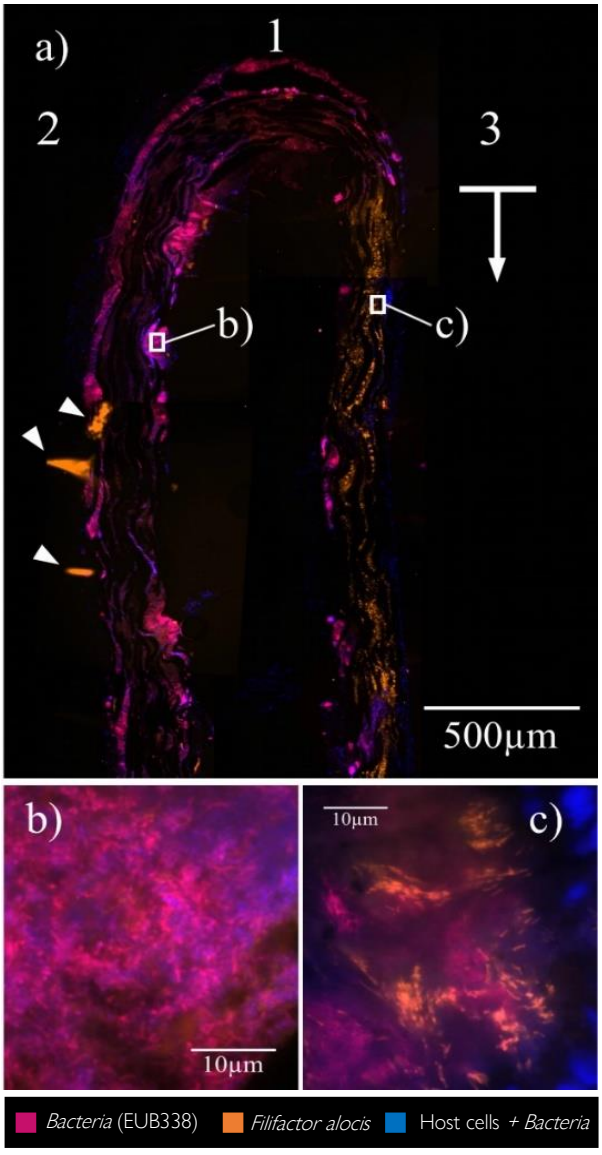
 # 6 | FIGURE 11. (A and C) Organisms stained by the probe SELE appear as densely packed groups in the cervical portion of the subgingival biofilm. (B) Higher magnification shows a crescent-shaped morphology. (C and D) $EUB338_{FITC}$ (green), $SELE_{Cy3}$ (bright orange) and $FUSO_{Cy5}$ (magenta). (D) Higher magnification, with $EUB338_{FITC}$ filter removed for better interpretation. Bars indicate 10 μm. Reprinted and adapted with the authors' permission.



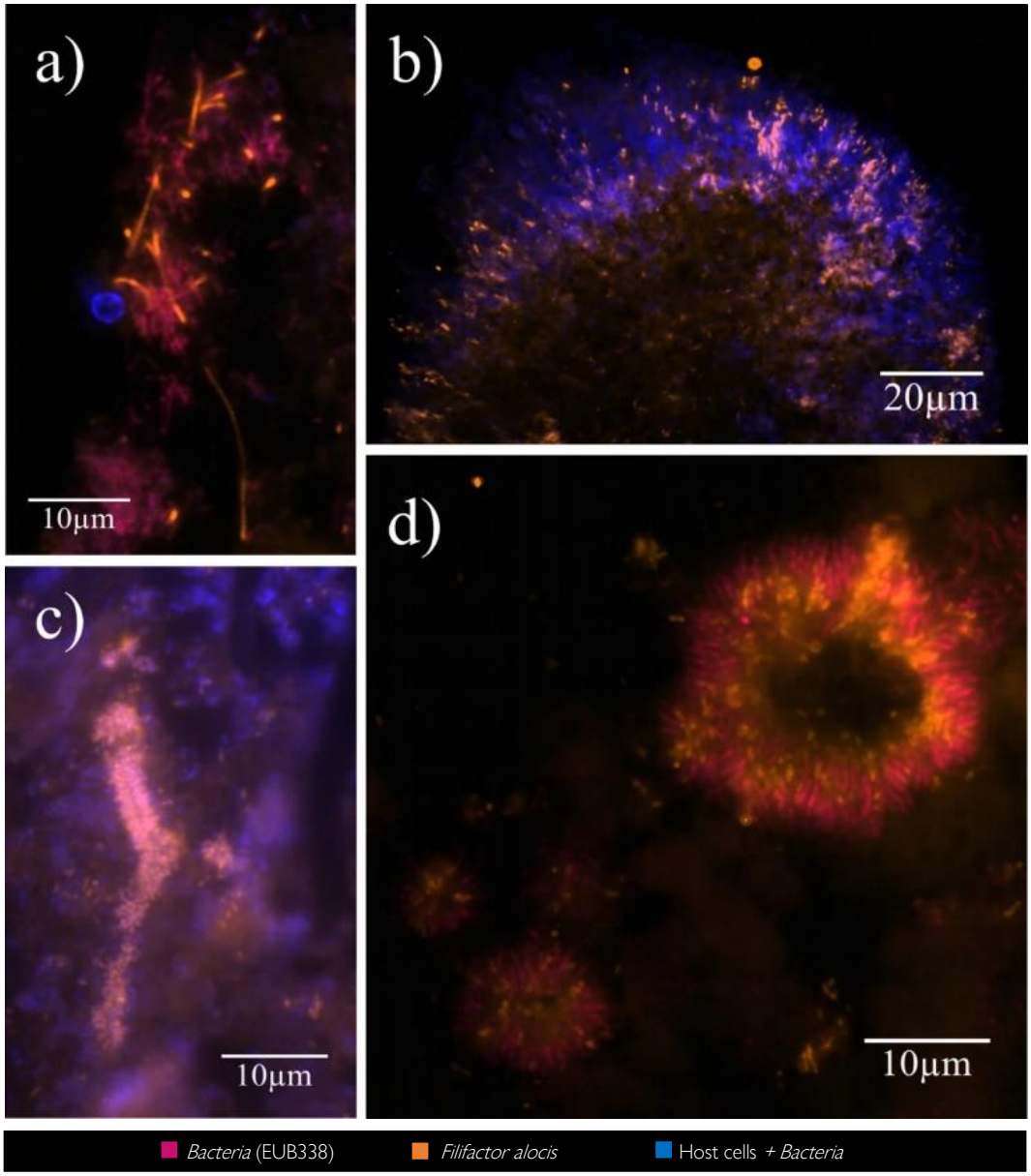
#6 | FIGURE 12. FISH performed on a gingival biopsy. (A) $EUB338_{FITC}$ (green), $SELE_{Cy3}$ (orange), and eukaryotic cell nuclei stained with DAPI (blue). (B) Higher magnification took with the Cy3 filter set only. Bars indicate 10 μm. Reprinted and adapted with the authors' permission.

#7 | SCHLAFER, S., *ET AL.* (2010)

Filifactor alocis was found in 9 out of 11 carrier patients. The organism was present in areas that corresponded to the depth of the pockets, but very occasionally in areas that corresponded to the cervical portion and the carrier's very tip. *F. alocis* colonized the carrier side facing the soft tissue in the majority of cases (FIGURE 13C), and was present in small numbers or not at all on the carrier side facing the root (FIGURE 13B). *F. alocis* was portrayed as a short rod of 1-2 µm of length in several sections of the biofilm, but the organism appeared longer at some locations, stretching to 7-8 µm (FIGURE 14A). *F. alocis* was present in closely packed groups (FIGURE 13C), clustered in concentrical structures (FIGURE 14D), and grouped in "test-tube brush" patterns around signal-free channels on several occasions (FIGURE 14C). The radial orientation of *F. alocis* towards the surface of a mushroom-like protuberance of the biofilm is shown in FIGURE 14B.

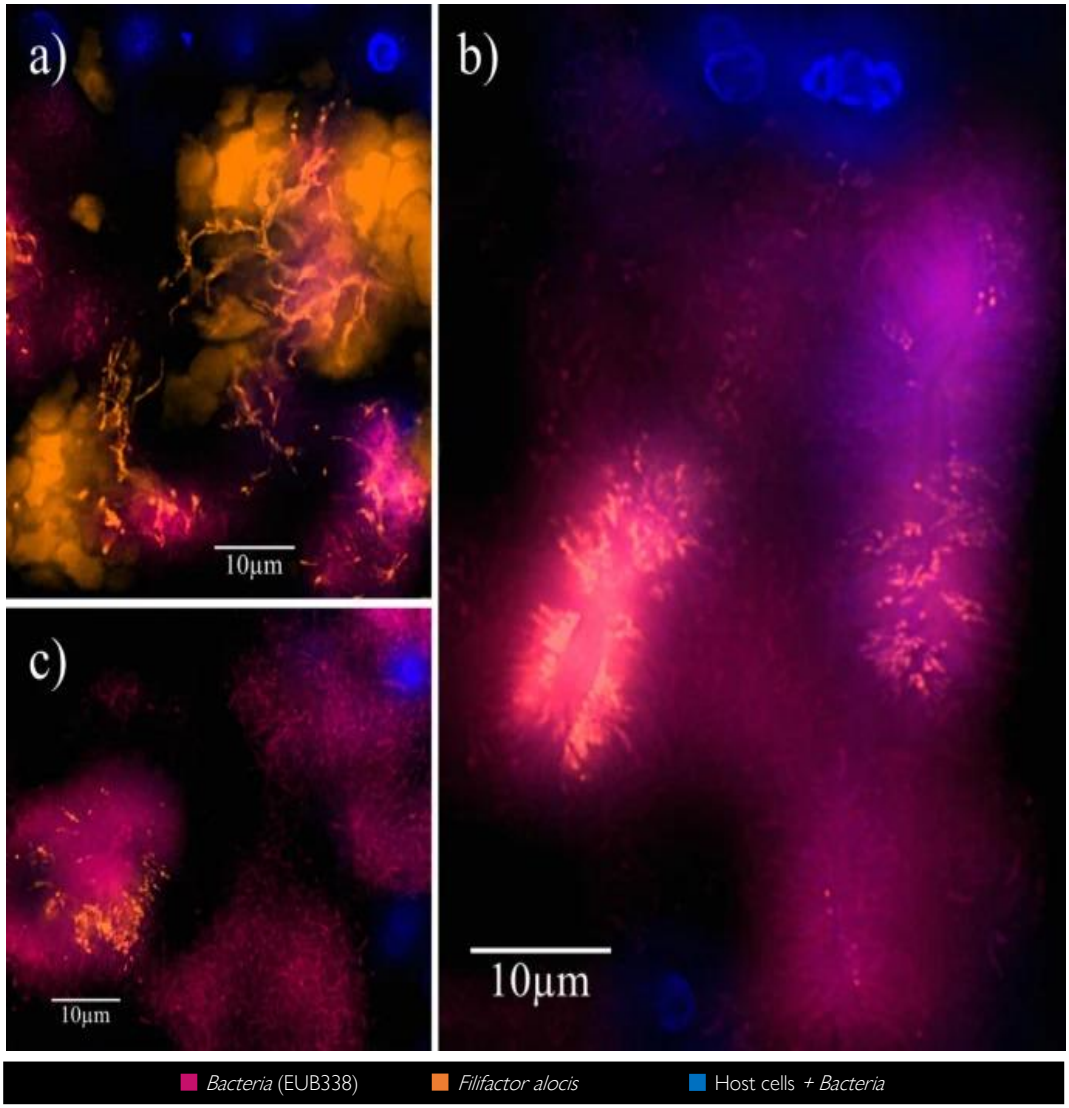


#7 | FIGURE 13. Subgingival biofilm visualized by FISH. $EUB338_{Cy5}$ (magenta), $FIAL_{Cy3}$ (bright orange), and DAPI staining (blue). DAPI stains both host cell nuclei and bacteria. (1) The carrier tip. (2) The carrier side facing the tooth. (3) The carrier side facing the pocket epithelium. (1 and 2) Little or no presence of *Filifactor alocis*. (3) Presence of a bright orange signal, indicating a vindicated presence of *F. alocis* (arrow). The image show artifacts caused by the folding of the embedded carriers (arrowheads). (b and c) Higher magnification. (b) Rare colonization of *F. alocis* amongst the bacteria. (c) *F. alocis* in densely packed groups among the organisms on the carrier side facing the soft tissues and host cell nuclei (blue). Reprinted and adapted with the authors' permission.



#7 | FIGURE 14. Establishment of *Filifactor alocis* in a subgingival biofilm. (a) Overlay of $FIAL_{Cy3}$, $EUB338_{Cy5}$, and DAPI filter sets. In some parts of the biofilm *F. alocis* rods can reach a considerable length. (b and c) Overlay of $FIAL_{Cy3}$ and DAPI filter sets. (b) Radial orientation of *F. alocis* towards the exterior of a mushroom-like protuberance. (c) Test-tube-brush formations of *F. alocis* around signal-free channels. (d) Overlay of $FIAL_{Cy3}$ and $EUB338_{Cy5}$ filter sets. *F. alocis* and fusiform bacteria form concentrical structures. Reprinted and adapted with the authors' permission.

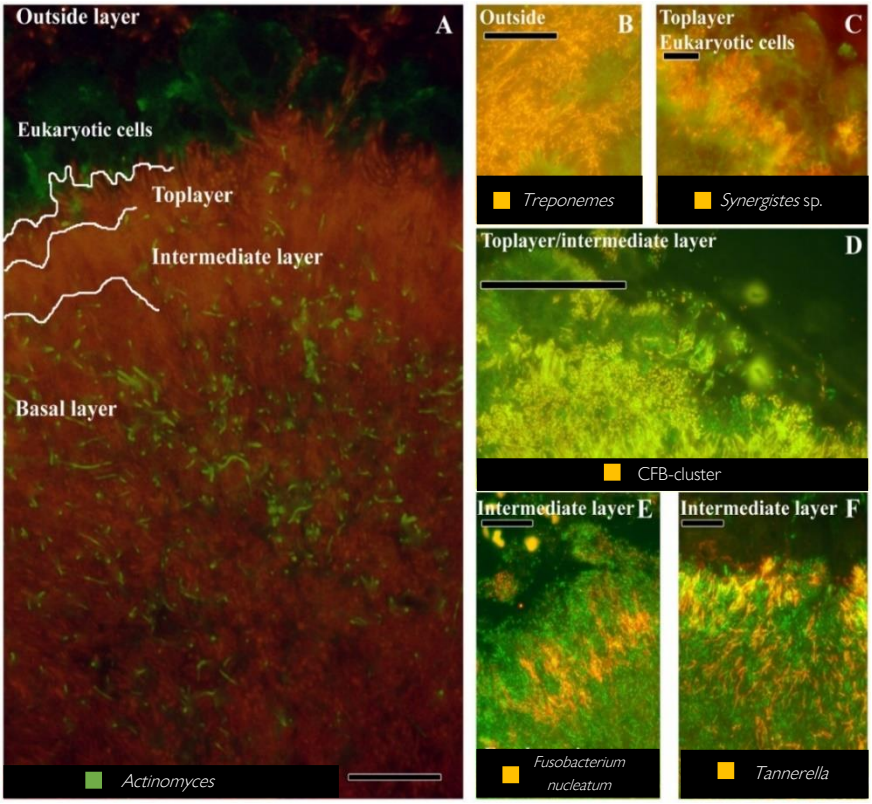
The ultrastructural organization of the biofilm can also be seen in the gingival biopsy. *F. alocis* shaped branch-like structures inside the affected tissue in several locations (FIGURE 15B). *F. alocis* was contained among the species in concentric bacterial aggregations once more (FIGURE 15C).



#7 | FIGURE 15. Establishment of *Filifactor alocis* in periodontal tissue. (a) *F. alocis* forming tree-like structures among coccoid and fusiform bacteria and autofluorescent erythrocytes. (b) *F. alocis* forming palisades with fusiform bacteria around large rod-shaped eubacterial organisms. (c) *F. alocis* being part of concentrical bacterial aggregations like those found in FIGURE 14 (d). Reprinted and adapted with the authors' permission.

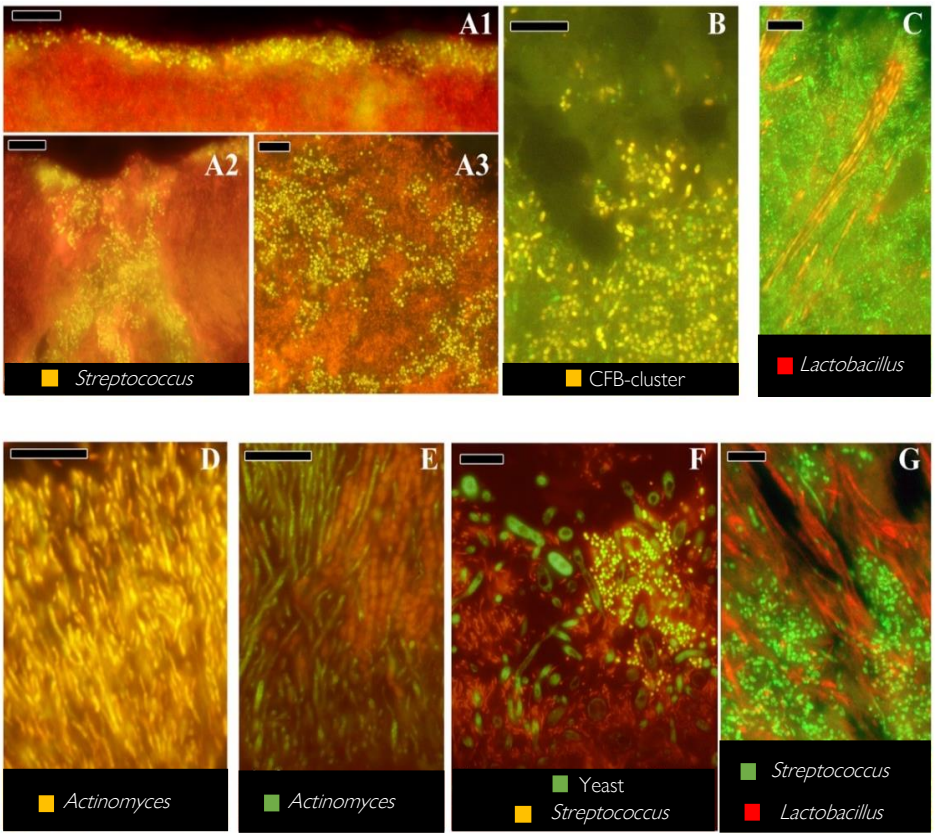
#8 | ZJINGE, V., *ET AL.* (2010)

The authors detected four layers of the subgingival biofilm. Biofilm's first layer is composed exclusively of *Actinomyces* sp. cells exhibiting little fluorescence. The intermediate layer, which was made up of several fusiform cells, including *F. nucleatum*, *T. forsythia*, and probably other *Tannerella sp.* (FIGURE 16E and F). The biofilm's top layer and a portion of the intermediate layer were mostly filamentous, rod-shaped, or even coccoid bacteria from the *Cytophaga-Flavobacterium-Bacteroides* (CFB)-cluster (FIGURE 16D). The most filamentous bacteria corresponded to *Tannerella* sp., while many of the rod-shaped bacteria were *Prevotella* sp. and *Bacteroidetes* species. Authors observed wide cigar-like bacteria belonging to the *Synergistetes* group A in the top layer, which were arranged in a palisade-like lining. These last species appeared nearby eukaryotic cells resembling polymorphonuclear leukocytes (PMN's) (FIGURE 16C). A fourth layer was discovered outside of the biofilm, but it lacked organization. Treponemes were first discovered in this layer nearby bacterial aggregates, known as rough and fine test-tube brushes (FIGURE 16B).



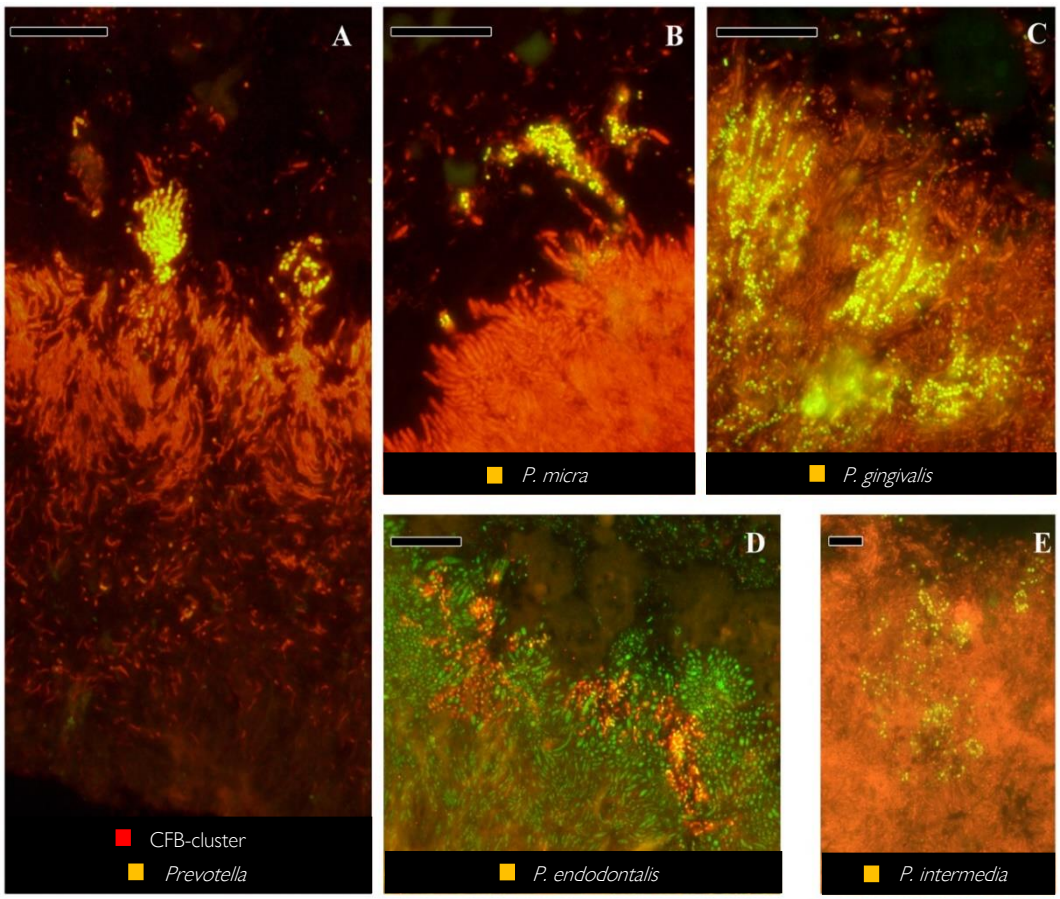
#8 | FIGURE 16. Localization of the most abundant species in subgingival biofilms. (A) Overview of the four layers of subgingival biofilm. *Actinomyces* sp. (green), bacteria (red), and eukaryotic cells (large green cells on top). (B) *Spirochaetes* (yellow) outside the biofilm, without clear organization. (C) Detail of *Synergistetes* (yellow) in the top layer near PMN's (green). (D) Presence of the *Cytophaga-Flavobacterium-Bacteroides cluster* (CFB-cluster) (yellow) in the top and intermediate layer. (E) *Fusobacterium nucleatum* in the intermediate layer. (F) *Tannerella* sp. (yellow) in the intermediate layer. Each panel is double-stained with probe EUB338 labeled with FITC or Cy3. Bars indicate 10 μm. Reprinted with the authors' permission.

Only two layers were found in supragingival plaque: the basal and second layers. The first consists of the initial plaque adhering to the tooth surface, and four different types of biofilm were discovered: a biofilm of rod-shaped *Actinomyces* sp. cells (FIGURE 17D); a mixture of *Actinomyces* sp. and chains of cocci (not streptococci) (FIGURE 17E); a biofilm with filamentous *Streptococcus* sp. forming a distinct colony around yeast and not identified bacteria (FIGURE 17F); a biofilm composed of mainly *Streptococcus* sp. growing near *Lactobacillus* sp. (FIGURE 17G). All these biofilm forms were discovered to be growing perpendicular to the tooth surface. The second layer can be found on top of any form of basal layer biofilm. *Streptococcus* sp. cells were found aligned on top of the second biofilm in different ways: (i) as a thin coat (FIGURE 17A1), (ii) colonizing cracks in the biofilm (FIGURE 17A2), or (iii) without clear organization (FIGURE 17A3). The heterogeneity of scattered CFB-cluster cells with no particular organization was observed (FIGURE 17B). At last, *Lactobacillus* sp. popularized in long string-shape away-oriented from the tooth surface, surrounded by cells with different morphologies (FIGURE 17C).



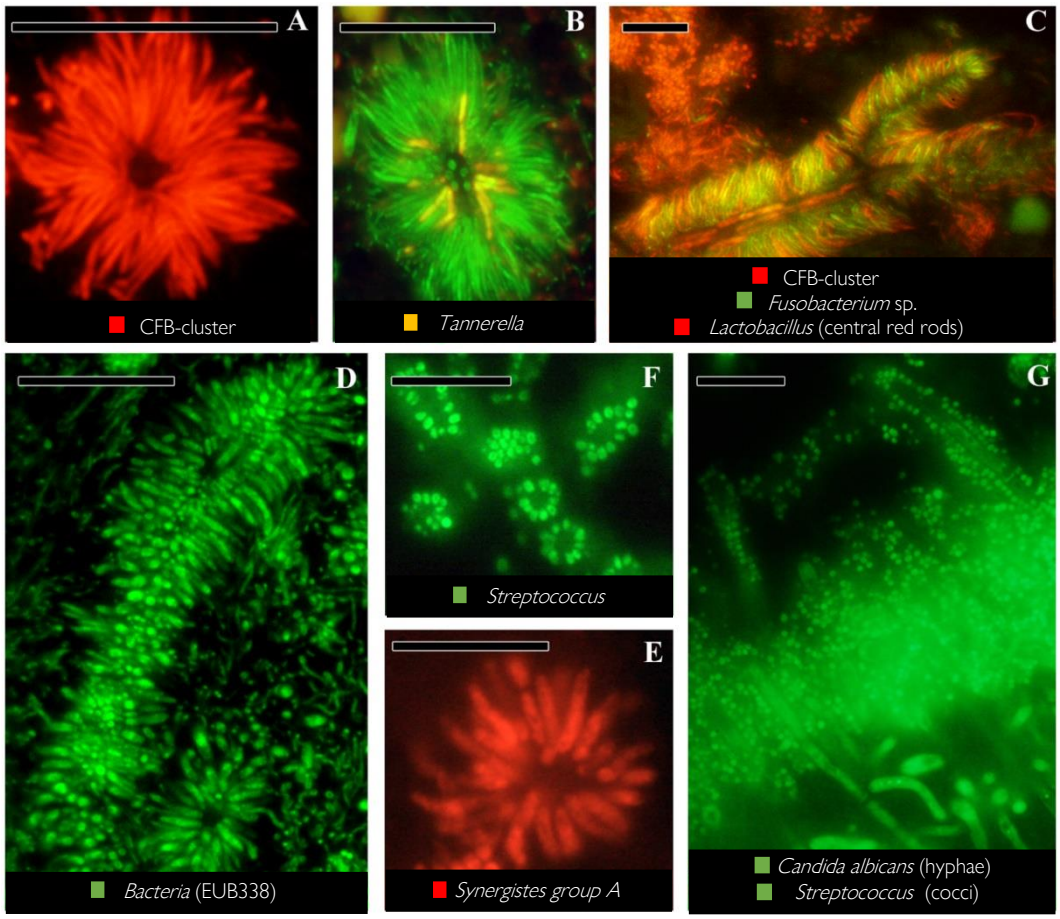
#8 | FIGURE 17. Localization of the most abundant species in supragingival biofilms. (A1-C) The second layer. (D-G) Basal layer. (A1-A3) *Streptococcus* sp. disposed of in different ways on the second layer. (B) Cells from the CFB-cluster stained in the top layer of the biofilm. (C) *Lactobacillus* sp. (red) through the top layer. (D) On the basal layer, A*ctinomyces* sp. cells (yellow). (E) *Actinomyces* sp. (green) and chains of cocci. (F) Colonies of *Streptococcus* sp. (yellow) all-around yeast cells (green) and bacteria unidentified (red). (G) *Streptococcus* sp. (green) growing closely to *Lactobacillus* sp. (red). Black holes might be channels through the biofilm. Panels A, B, C, E, F are double stained with probe EUB338 labeled with FITC or Cy3. Bars indicate 10 μm. Reprinted with the authors' permission.

Porphyromonas gingivalis, *Prevotella intermedia*, *Porphyromonas endodontalis*, or *Prevotella nigrescens* are Gram-negative bacteria, belonging to the CFB-cluster, often found in the top and intermediate layers of the subgingival biofilm. *Prevotella* sp., on the other hand, colonize the biofilm in micro-colonies on top of it (FIGURE 18A), but they also live inside the top layer, as shown by *P. intermedia* (FIGURE 18E). Periodontal pathogens, including *P. gingivalis* and *P. endodontalis* (FIGURE 18C and D), are mainly found in the exterior layers of subgingival biofilm constructing micro-colonies. *Parvimonas micra*, an example of a Gram-positive bacteria associated with periodontitis, is also found in the top layer as micro-colonies (FIGURE 18B). Pathogenic microorganisms can also be present as part of bacterial aggregates found in both sub- and supragingival biofilms.



#8 | FIGURE 18. Localization of presumptive species associated with periodontitis in subgingival biofilms. (A) Colonization of the subgingival biofilm by the CFB-cluster species (red) and *Prevotella* sp. (yellow). Since *Prevotella* sp. are part of the CFB-cluster of bacteria, cells appear in yellow. (B) Top of the biofilm with a micro-colony of *Parvimonas* micra (yellow). (C and D) Micro-colonies of *Porphyromonas gingivalis* (yellow) and *Porphyromonas endodontalis* (yellow) in the top layer, respectively. (E) Micro-colonies of *Prevotella intermedia* in the top layer. Panels B, C, D, and E are double stained with probe EUB338 labeled with FITC or Cy3. Bars indicate 10 μm. Reprinted with the authors' permission.

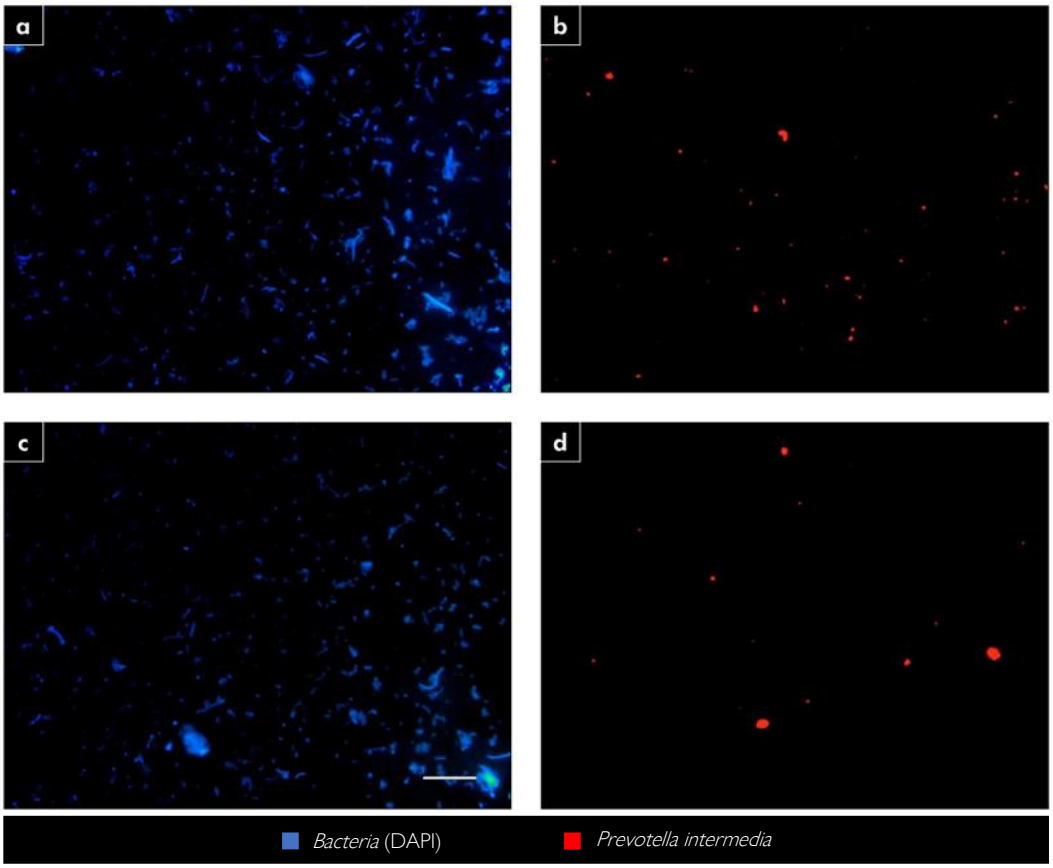
Different aggregates were also found in the outside layer of subgingival plaque. Filaments from the CFB-cluster were found perpendicularly arranged around *Lactobacillus* sp. in a fine test-tube brush shape, morphologically like *T. forsythia* and *F. nucleatum* (FIGURE 19 A-C). Test-tube brushes shapes were also found in a complex mixture of cells containing *T. forsythia*, *Campylobacter* sp., *P. micra*, *Fusobacteria,* and *Synergistetes* group A, among others. *Synergistetes* were found forming aggregates exclusively with themselves (FIGURE 19D and E). In supragingival plaque were observed corncob structures consisting of *Streptococcus* sp. adhering to a central axis of yeast cells or hyphae in the top layer of the biofilm (FIGURE 19G).



#8 | FIGURE 19. Bacterial aggregates detected in both sub- and supragingival plaque. (A) Filamentous cells from the CFB-cluster in the fourth layer of the subgingival plaque. (B) *Tannerella* sp. (yellow) in a test-tube brush. (C) Test-tube brush with *Lactobacillus* sp. (red rods) as central structures. *Fusobacterium nucleatum* (green) and CFB-cluster filaments (red), morphologically identical to *Tannerella forsythia*, perpendicularly radiating around lactobacilli. (D) Test tube brush stained with the eubacterial probe. (E) *Synergistetes* group A species forming aggregates solely with themselves. (F) *Streptococcus* sp. (green) aggregation around a central cell (not stained) in supragingival plaque. (G) Supragingival plaque with *Streptococcus* sp. (green cocci) adhering to a central axis of yeast cells or hyphae, such as *Candida albicans* (green hyphae). Bars indicate 10 µm. Reprinted with the authors' permission.

#9 | MACHADO, F. C., *ET AL.* (2012)

High amounts of the most common periodontal pathogens in the pregnant women group were observed, especially *Prevotella intermedia* (FIGURE 20), but significant microbiological differences were not detected between the pregnant and non-pregnant groups.



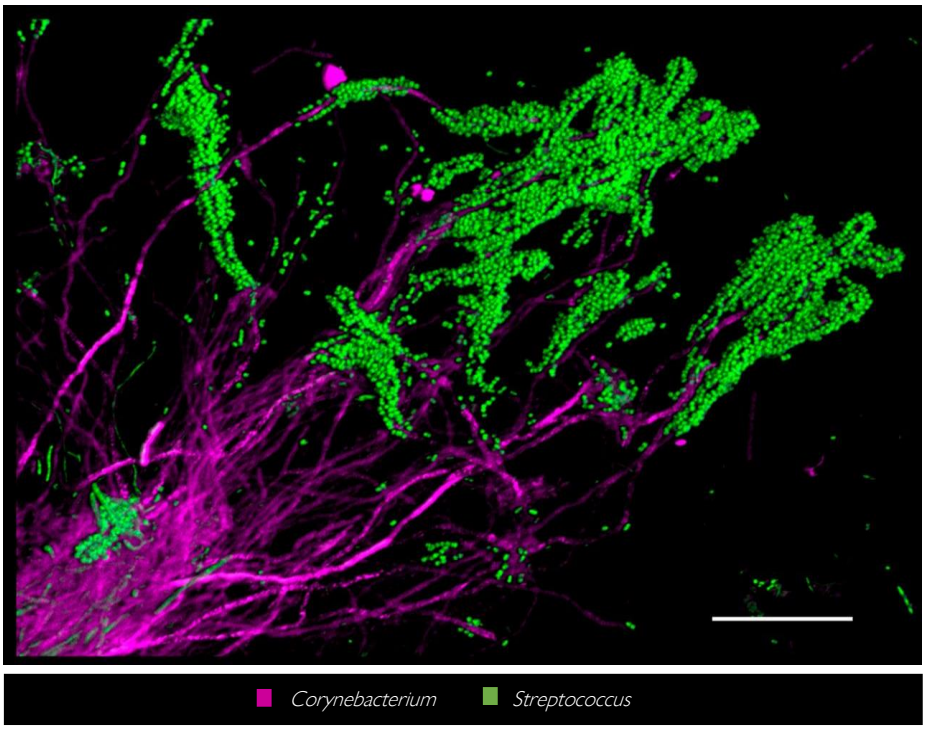
#9 | FIGURE 20. (A and C) Plaque specimen stained for total bacterial cells (with DAPI) in pregnant and non-pregnant women, respectively. **(B and D)** Plaque specimen stained for *Prevotella intermedia* (with probe Pint649) in pregnant and non-pregnant women, respectively. Bars indicate 20 μm. Reprinted and adapted with the authors' permission.

#10 | MARK WELCH, J. L., *ET AL.* (2016)

Only 13 out of 57 genera tested had at least 3% mean abundance and were also prevalent, being identified in more than 90% of supragingival specimens. These 13 genera (*Corynebacterium* sp., *Capnocytophaga* sp., *Fusobacterium* sp., *Leptotrichia* sp., *Actinomyces* sp., *Streptococcus* sp., *Neisseria* sp., *Haemophilus/Aggregatibacter* sp., *Porphyromonas* sp., *Rothia* sp., *Lautropia* sp., *Veilonella* sp., and *Prevotella* sp.) described 85% of the sequencing data from supragingival plaque and the same 13 genera described more than 80% of the subgingival specimens, indicating a close relationship between the two plaque substrates.

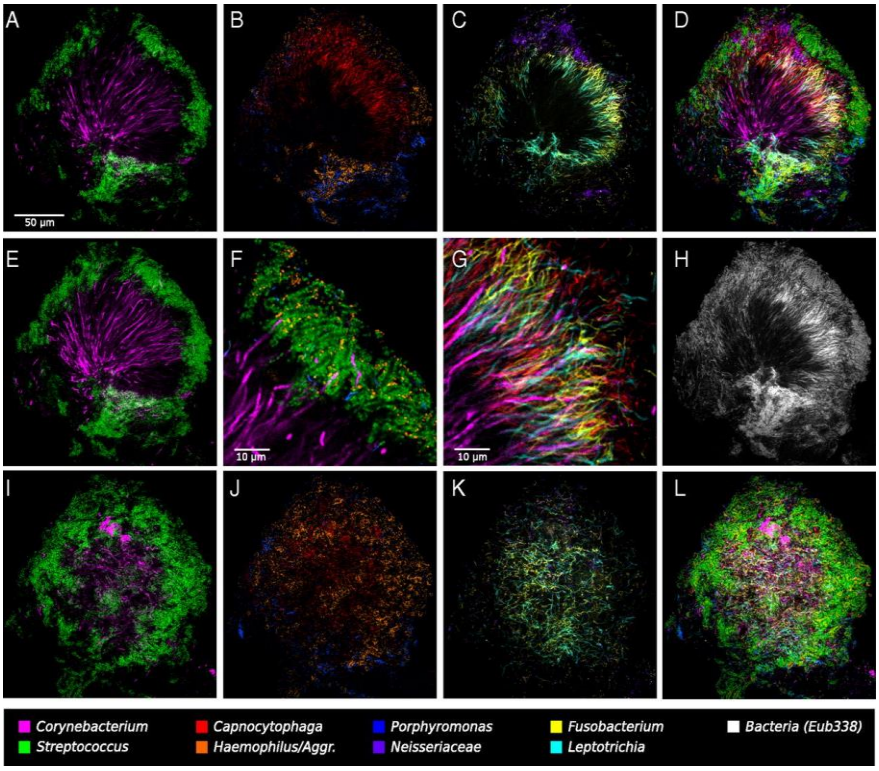
Corynebacterium sp. was remarkably specific to supragingival (12%) and subgingival (8%) plaque. *Corynebacterium matruchotii* and *Corynebacterium durum* were present at significant levels. *Corynebacterium matruchotii* was the dominant species in most individuals.

Besides *Corynebacterium* sp., other genera with plaque specificity were *Capnocytophaga* sp., *Lautropia* sp., and *Rothia* species. By contrast, genera like *Streptococcus* sp., *Veillonella* sp., and *Haemophilus* sp. occupied a wide range of substrates in oral ecosystems. The authors reported associations between cocci and *Corynebacterium* sp. filaments (FIGURE 21).



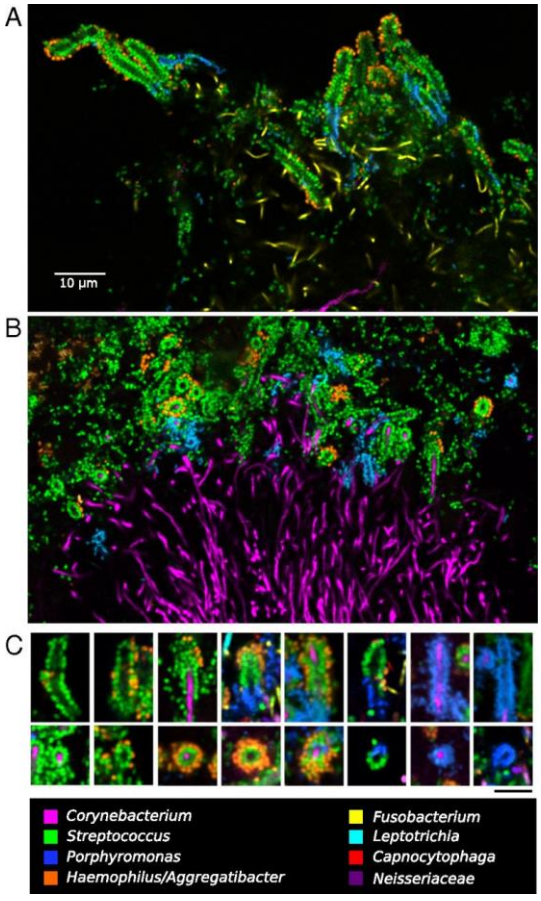
#10 | FIGURE 21. Conventional FISH of partially disrupted plaque hybridized with two probes: one for *Corynebacterium* sp. and a universal bacterial probe. The image revealed corncob-like clusters of *Corynebacterium* sp. cells (magenta), with long filaments, continuous from the base to the top, that were crusted at their tips by brilliantly staining cocci (green). Bars indicate 20 µm. Reprinted and adapted with the authors' permission.

A complex microbial consortium in a hedgehog-shape structure was observed, showing the spatial organization of the plaque microbiota. In short, these hedgehog structures were radially organized, with a multi-taxa consortium composed of a skeleton mainly of *Corynebacterium* sp. with coccoid *Streptococcus* sp. cells arranged around the distal tips, a multi-genus filament-rich halo composed of *Fusobacterium* sp., *Leptotrichia* sp., and *Capnocytophaga* sp. cells, and a periphery of corncobs structures composed by a filamentous core bordered primarily with *Streptococcus* sp., *Porphyromonas* sp. *and Haemophilus*/*Aggregatibacter* sp. cells (FIGURE 22).



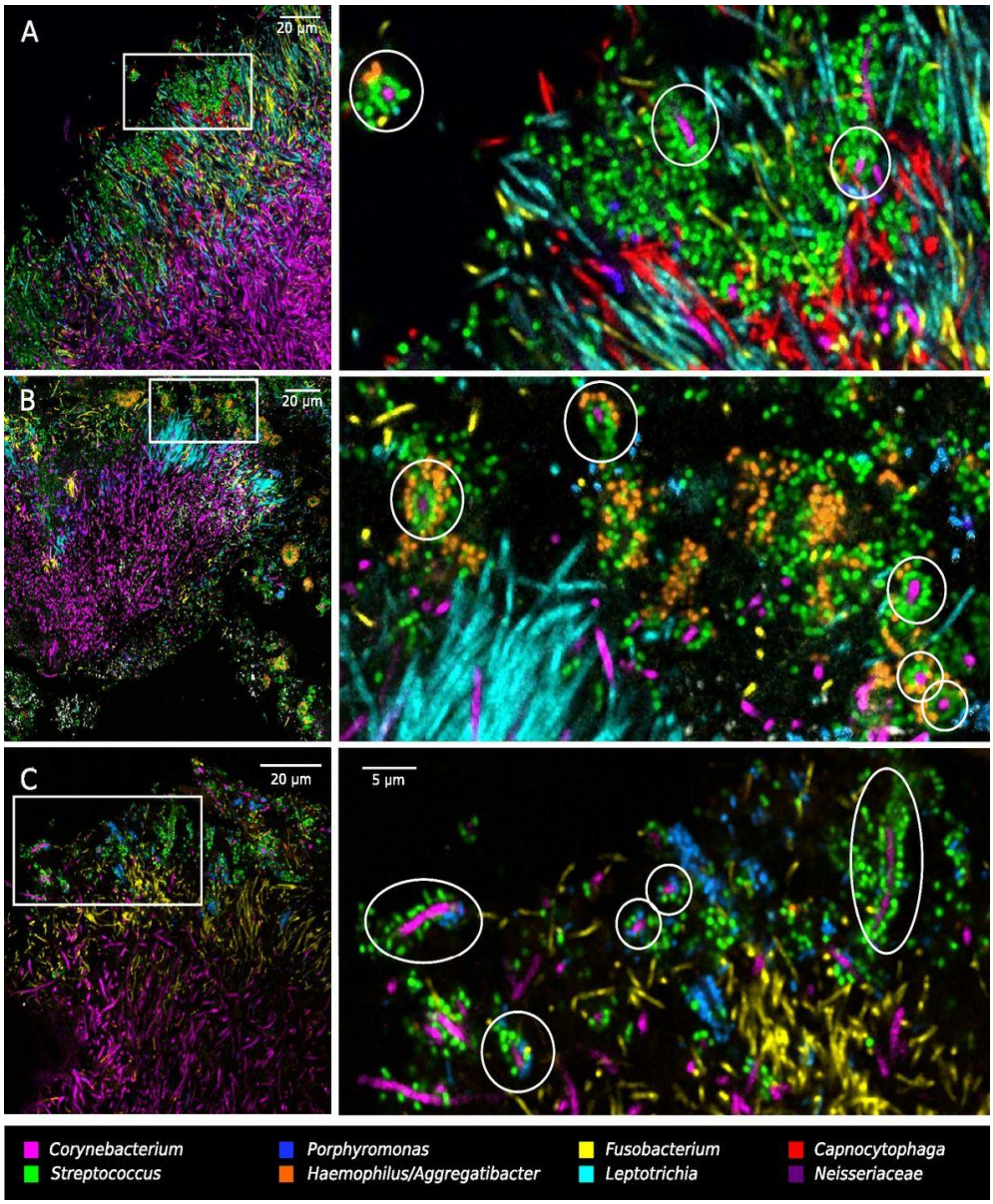
#10 | FIGURE 22. Hedgehog-shape structure. (A-D and F-H) represents a single focal plane nearby the middle part of the structure. (A) *Corynebacterium* sp. filaments radiating outward from near the center of the image and coccoid *Streptococcus* sp. cells arranged around the distal tips of the *Corynebacterium* sp. filaments. (B) Cells of *Haemophilus/Aggregatibacter* sp. and *Porphyromonas* sp. sited at the structure's periphery, in the same region as the *Streptococcus* sp. cells. *Capnocytophaga* sp. also inhabited a wide band inside the periphery. (C) *Fusobacterium* sp. and *Leptotrichia* sp. occupying this band called "filament-rich halo". *Neisseriaceae* establishing constellations in and nearby the periphery. *Actinomyces* sp., represented by a small number of cells located near the structure's base. (D) All taxa overlayed. Many cells from the "filament-rich annulus" overlapped the exterior of the consortia. (E-G) Detail of the *Corynebacterium*'s arrangement relatively to other taxa. (E) The maximum intensity projection of three adjacent optical sections shows that these filaments are continuous from the center to the periphery of the structure for more than 50 µm. Some filaments persist visible within the *Streptococcus* cells. (F) Detail of the periphery. Corncob structures are composed of a filamentous core (sometimes visualized as *Corynebacterium* sp. filaments but often not stained) bordered primarily by *Streptococcus* cells but also by *Porphyromonas* sp. and *Haemophilus/Aggregatibacter* sp., both in proximal contact with *Streptococcus* sp. cells. (G) On the periphery of these corncob structures, *Corynebacterium* sp. filaments pass through the halo that is highly densely colonized with elongated rods of *Fusobacterium* sp., *Leptotrichia* sp., and *Capnocytophaga* species. (H) Fluorescent signal of the universal probe EUB338. (I-L) The exterior of the hedgehog structure, composed mainly of corncobs. (K) The edge of the *Fusobacterium-Leptotrichia* sp. halo. Reprinted with the authors' permission.

The corncobs at the periphery showed that "kernels" (coccoid cells) were composed of different taxonomic types and could be either single or double layered (FIGURE 23). Single-layer corncobs had coccoid cells of both *Streptococcus* sp. or *Porphyromonas* sp. (in some cases *Porphyromonas* sp. kernels coexisted with *Streptococcus* sp. around the same filament), whereas double-layer kernels consisted of a combination of *Streptococcus* sp. in the inner layer and *Haemophilus/Aggregatibacter* sp. in the outer layer (FIGURE 23C). The most common type of kernels visualized were the ones that had a single layer of *Streptococcus* sp. cells surrounded by a partial or complete layer of *Haemophilus/Aggregatibacter* species. *Porphyromonas* sp. cells were only observed organized in single-layer corncobs. On contrary, cells of the genus *Haemophilus/Aggregatibacter* sp. were only found forming double-layer structures, exclusively with *Streptococcus* sp. cells, demonstrating an undoubtedly specific relationship. Competitive, exploitative, or mutualistic interactions between these taxa were detected in this specific biofilm architecture between single- and double-layers corncobs.

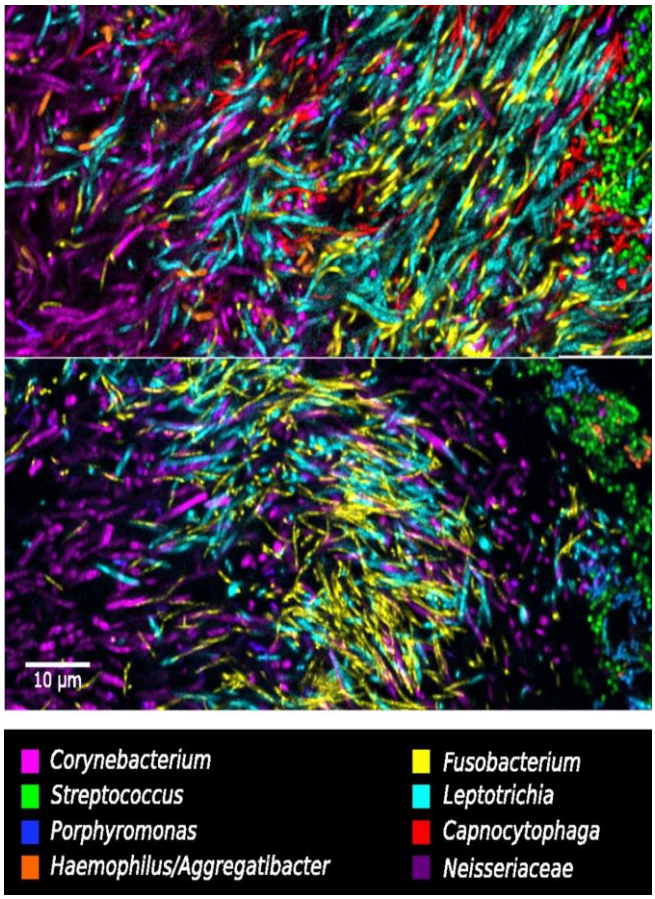


#10 | FIGURE 23. Dense corncob structures in supragingival plaque. (A) The central filament lacked hybridization in whole-mount preparation. (B) The central filament was well visualized in methacrylate-embedded and sectioned preparations. (C) Representative images of types of corncobs found. Bar indicates 5 µm in C. Reprinted with the authors' permission.

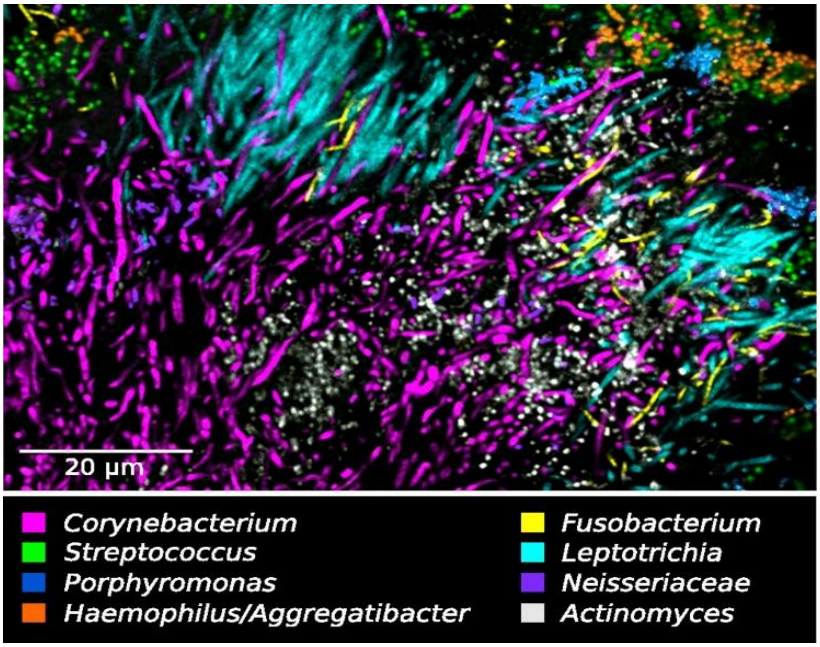
In all cases that the central filament stained, it stained for *Corynebacterium* sp., including in cases where cells from other taxa were present in abundance near the corncobs, such as *Fusobacterium* sp., *Leptotrichia* sp., and *Capnocytophaga* sp. (FIGURE 24). Within hedgehogs, bacteria do not form broad single-taxon groups; rather, cells were observed intermingling with at least four different taxa (FIGURE 25). Authors only did not find this type of interaction between *Actinomyces* sp. and *Corynebacterium* sp. cells (FIGURE 26), where were visualized irregular clumps in the base of the hedgehogs or nearby the hedgehogs, rather than intermixed within this structure.



#10 | FIGURE 24. Long filaments of *Corynebacterium* sp. cells in close specific proximity to coccoid cells. Reprinted with the authors' permission.

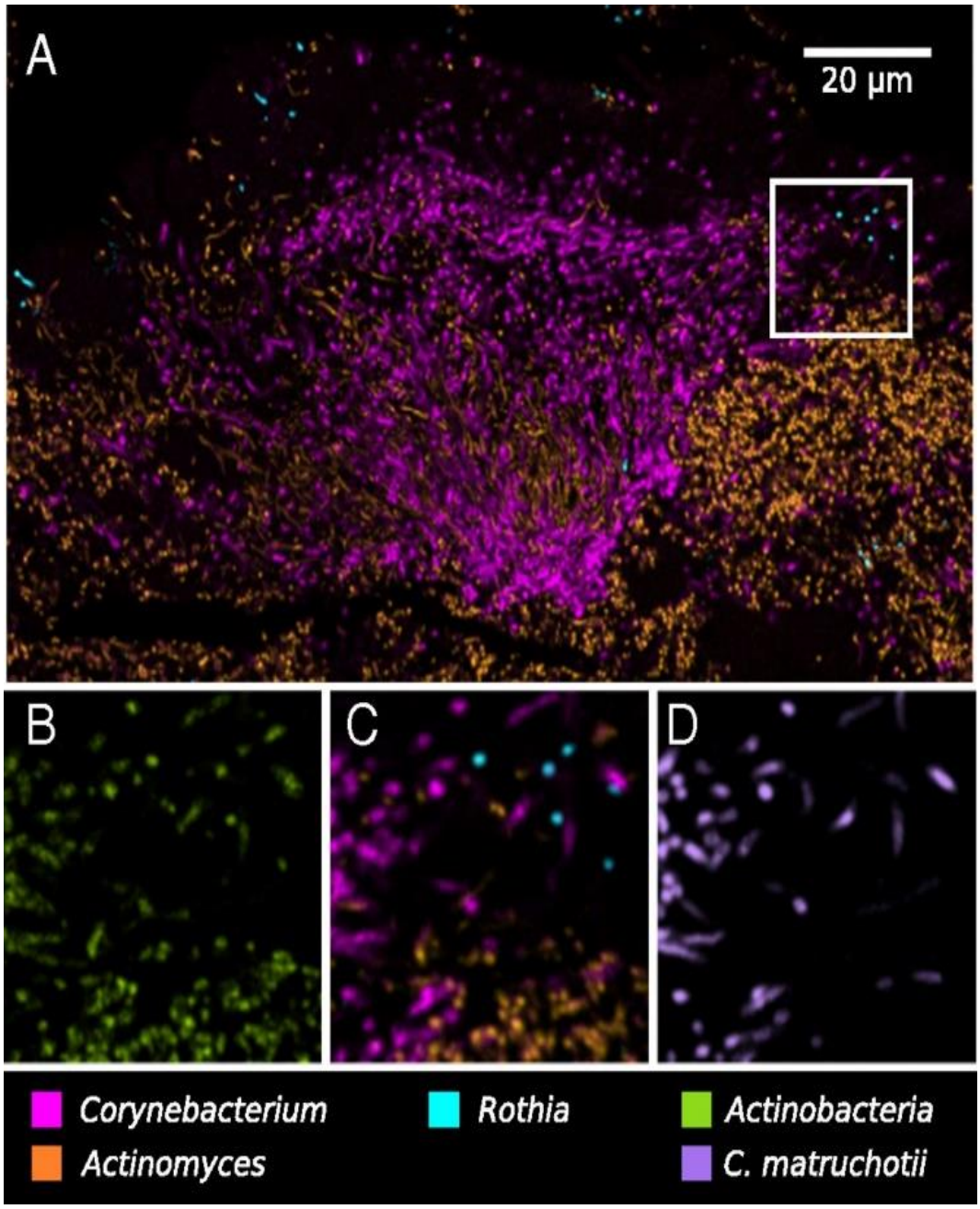


#10 | FIGURE 25. Methacrylate-embedded and sectioned preparations. Bacteria do not form wide single-taxon groups inside hedgehogs, but rather cells of at least four different taxa interact with one another at a micron scale. Reprinted with the authors' permission.

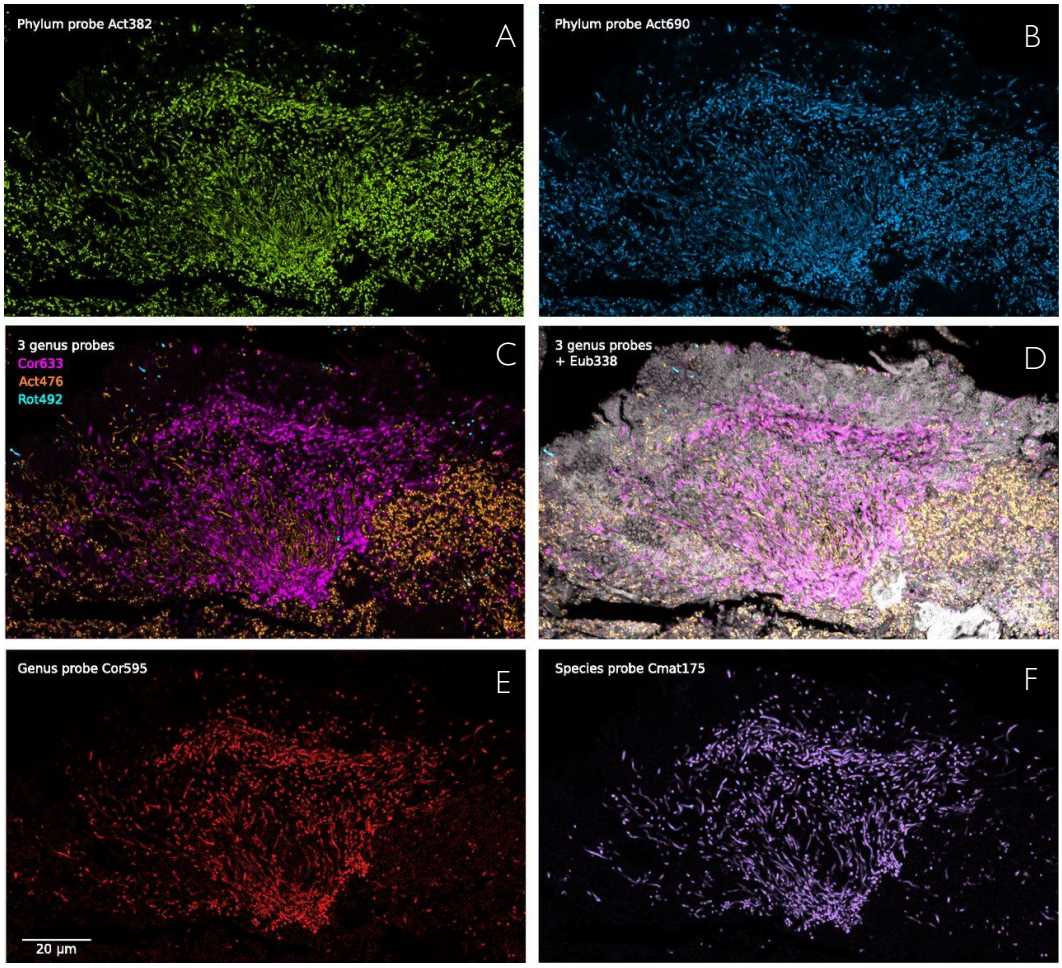


#10 | FIGURE 26. Methacrylate-embedded and sectioned preparation showing *Actinomyces* sp. localization relatively to *Corynebacterium* species. Patchy groups of *Actinomyces* sp. were observed in the base of the hedgehog. Reprinted with the authors' permission.

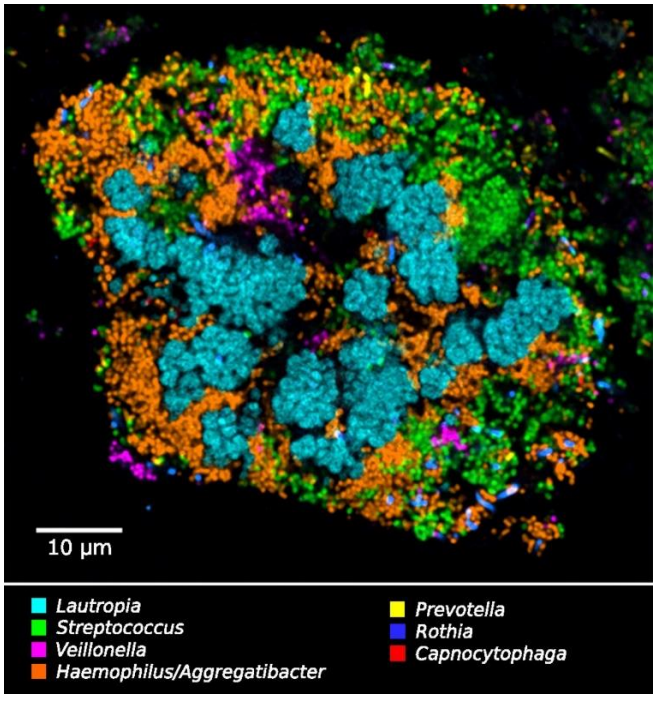
Only three genera (*Corynebacterium* sp., *Actinomyces* sp., and *Rothia* sp.) comprised virtually all the *Actinobacteria* present in plaque (FIGURE 27 and FIGURE 28). *C. matruchotii* contained nearly all the *Corynebacterium* sp. in the hedgehog structure.



#10 | FIGURE 27. Application of a nested probe for species-level identification of *Corynebacterium* species. (A) The low-magnification image shows the three major oral genera of phylum *Actinobacteria*: *Corynebacterium* sp., *Actinomyces* sp., and *Rothia* species. (B) A high-magnification image shows all *Actinobacteria*. (C) The three genera. (D) *C. matruchotii*. Reprinted with the authors' permission.

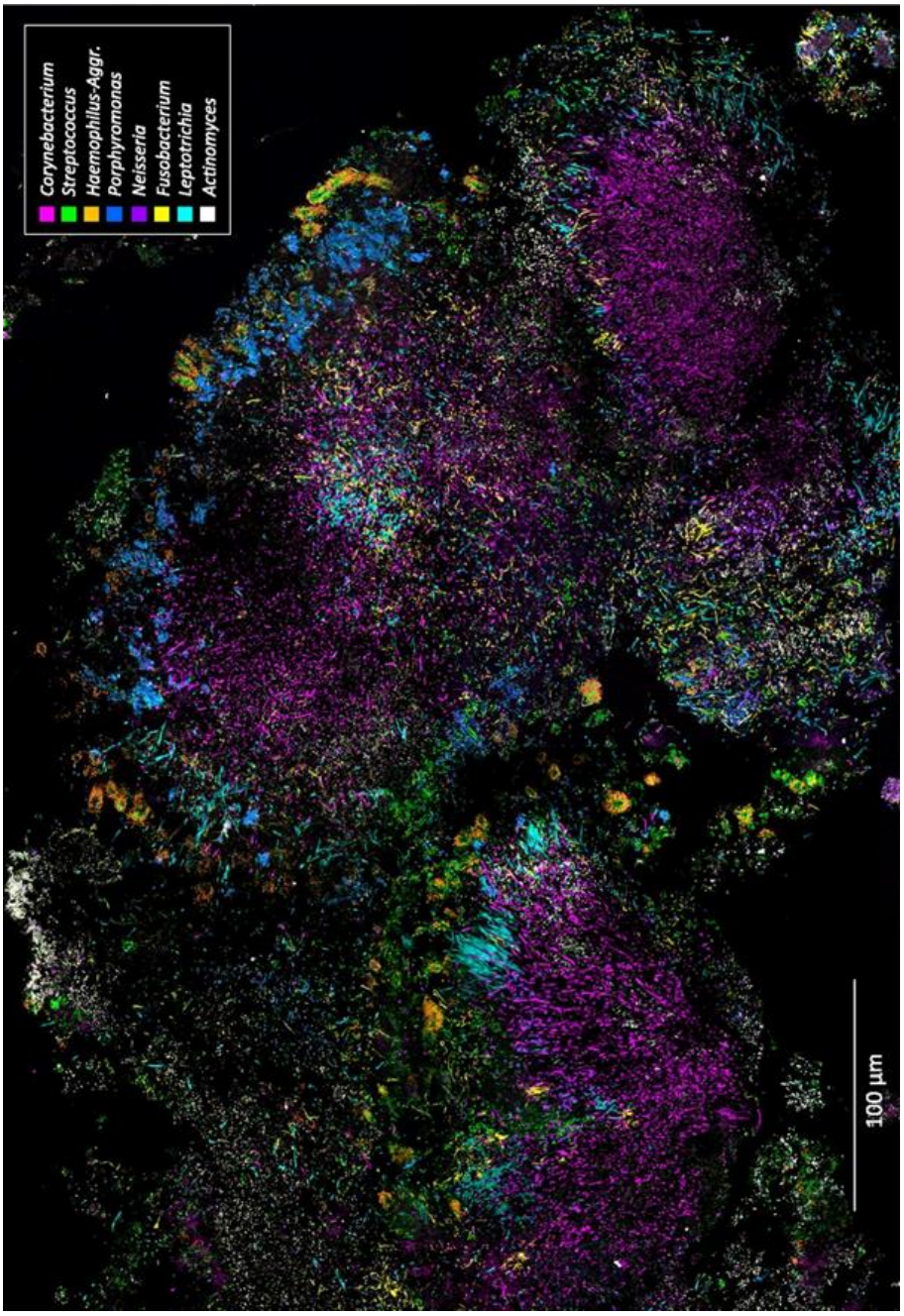


#10 | FIGURE 28. Nested probe set targeting cells at the taxonomic levels of phylum (A and B), genus (C-E), and species (F) identification in methacrylate-embedded and sectioned plaque. (A and B) *Actinobacteria*. (C and D) *Corynebacterium* sp., *Actinomyces* sp., and *Rothia* species. (E) *Corynebacterium* sp. (F) *C. matruchotii*. Reprinted with the authors' permission.



#10 | FIGURE 29. A cauliflower structure in plaque. Reprinted with the authors' permission.

Hedgehogs were the most observed type of structure, being identified in every individual who sampled multiple times and in approximately 80% of individuals who sampled once. Some samples had multiple hedgehogs' structures adjacent to each other (FIGURE 30), especially on the tooth surface on the buccal side and plaque from the gingival margin. Another kind of consortia was also found: a cauliflower structure in plaque (FIGURE 29) constituted by *Lautropia* sp., forming the center of the structure, surrounded by *Streptococcus* sp., *Haemophilus/ Aggregatibacter* sp., and *Veilonella* species. Dispersed cells of *Prevotella* sp., *Rothia* sp., and *Capnocytophaga* sp. were also visible.



#10 | FIGURE 30. Multiple hedgehogs' structures adjacent to each other. Reprinted with the authors' permission.

Synthesis of the results

The findings collected from the included articles in this scoping review are summarized in TABLE 4. We also included a supplemental table in the appendix section, with details on all the oligonucleotide probes and the FISH conditions used in each microorganisms' analysis (SUPPLEMENTAL TABLE 3).

TABLE 4. Synthesis of the results. **SUBG**: subgingival; **SUPG**: supragingival; **CLSM**: confocal laser scanning microscopy; **SUBG – FL**: subgingival first layer; **SUBG – IL**: subgingival intermediate layer; **SUBG – TL**: subgingival top layer; **SUBG – OL**: subgingival outside layer; **SUPG – BL**: supragingival basal layer; **SUPG – SL**: supragingival second layer.

#	Site	Microorganisms	Microorganisms' shape	Relevant interactions	Microscopic technique
1	SUBG	Group I of oral treponemes	Large and dense	These organisms are present in high proportions in subgingival plaque samples and thus represent the predominant flora. Group I treponemes outnumbered group II treponemes	Dark-field
		Group II of oral treponemes	Thin, slender, wavily		
2	SUBG	Group I of oral treponemes	Large and undulated	Treponemes appeared spread between Gram-negative bacteria in the deepest parts of the periodontal pockets	CLSM
		Bacteria	Rods and coccoid		
3	SUBG	*Methanobrevibacter oralis*	Diplococcobacilli	Treponemal rDNA was found in significantly lower abundance in sites with archaeal rDNA than in sites without archaeal rDNA. These species may compete for hydrogen (methanogenesis by *M. oralis* is a hydrogen-consuming process that enables the growth of hydrogen-producing organisms, that comprises some of the known early oral pathogens like facultative anaerobes)	CLSM
4	SUPG	*Leptotrichia buccalis*	Wide and segmented fusiform rods	The fusiform taxa were highly present in both groups (gingivitis and NUG). There was a significantly increased total abundance of periodontal pathogens in the NUG group compared with the gingivitis group	Dark-field
		Indistinguishable *Fusobacterium nucleatum, Capnocytophaga* sp., and *Fusobacterium periodonticum*	Smaller, thin, spindle-shaped, and dotted fusiform rods		
5	SUBG	*Tannerella forsythia*	Clumps	*Tannerella forsythia* was detected in the deepest zones of the periodontal pockets	Epifluorescence

#	Site	Microorganisms	Microorganisms' shape	Relevant interactions	Microscopic technique
6	SUBG	*Selenomonas* sp.	Densely packed groups with a crescent-shaped form	These genera appeared spatially related and tangled with each other, suggesting that this coaggregation plays a fundamental role during the establishment of *in vivo* grown biofilms	Epifluorescence
		Fusobacterium sp.	Densely groups with a fusiform shape		
7	SUBG	*Filifactor alocis*	(i) Short rod clustered in concentrical and radial-orientated structures nearby fusiform bacteria on mushroom-shaped biofilms; (ii) Test-tube brush shapes; (iii) Branch-like structures in gingival tissue; (iv) Palisades structures nearby fusiform bacteria and eubacterial organisms	The findings suggest a strong involvement of *F. alocis* in periodontal disease. The organism was more frequently detected in GAP and CP subjects, and in the deeper pockets (7-9 mm). *F. alocis* shape was different when the microorganism was observed unaccompanied or co-aggregated with a partner	Epifluorescence
8	SUBG – FL	*Actinomyces* sp.	Rod-shaped	Display little fluorescence	Epifluorescence
	SUBG – IL	*Fusobacterium nucleatum*	Fusiform cells	TL and a portion of the IL were mostly made of filamentous, rod-shaped, or even coccoid bacteria from the CFB-cluster (especially *P. gingivalis*, *P. intermedia*, *P. endodontalis*, or *P. nigrescens*)	
		Tannerella forsythia			
		Tannerella sp.			
		CFB-cluster	Filamentous, rod-shaped, coccoid		
	SUBG - TL	CFB-cluster	Filamentous, rod-shaped, coccoid, micro-colonies (*Prevotella*)		
		Synergistetes group A	Wide cigar-like bacteria in a palisade lining		
		Parvimonas micra	Micro-colonies		
	SUBG - OL	Treponemes	Test-tube brush shapes	CFB-cluster cells were found perpendicularly arranged around *Lactobacillus* sp. in test-tube brush shape. Test-tube brushes shapes were also found in a complex mixture of cells containing *T. forsythia, Campylobacter* sp., *P. micra, Fusobacterium* sp., *and Synergistetes* group A	
		CFB-cluster			
		Lactobacillus sp.	Rod-shaped		
		Porphyromonas gingivalis	Micro-colonies		
		Porphyromonas endodontalis			
		Tannerella forsythia	Test-tube brush shapes		
		Campylobacter sp.			
		Parvimonas micra			
		Fusobacterium sp.			
		Synergistetes group A			

#	Site	Microorganisms	Microorganisms' shape	Relevant interactions	Microscopic technique
	SUPG - BL	*Actinomyces* sp.	Rod-shaped	Bacterial deposits made up of early colonizers, growing perpendicularly to the tooth surface	
		Actinomyces sp. + chains of cocci	Rod-shaped and coccoid		
		Streptococcus sp. + yeast and not identified bacteria	Filamentous		
		Streptococcus sp. + *Lactobacillus* sp.			
	SUPG - SL	*Streptococcus* sp.	(i) Thin coat; (ii) Colonizing biofilm's cracks; (iii) Without clear organization	Corncob structures consisting of *Streptococcus* sp. adhering to a central axis of yeast/hyphae cells	
		CFB-cluster	Heterogenous and without clear organization		
		Lactobacillus sp.	Long string-shape		
9	SUBG	*Prevotella intermedia*	Patchy groups	*P. intermedia* was frequently found in the SUBG plaque of pregnant women, but no significant microbiological differences between the pregnant and non-pregnant groups were observed	Epifluorescence
10	SUPG	*Corynebacterium* sp.	Continuous filaments from the base to the periphery of the structure	*Corynebacterium* sp. filaments were crusted at their distal tips by brilliant cocci	CLSM
		Streptococcus sp.	Coccoid		
		Capnocytophaga sp.	Filamentous	Part of a multi-genus halo	
		Fusobacterium sp.			
		Leptotrichia sp.			
		Actinomyces sp.	Patchy groups	Observed in the base of the hedgehogs' structures	
		Haemophilus/ Aggregatibacter sp.	Filamentous	Built a periphery of corncobs structures in addiction with *Streptococcus* sp. cells. Single-layer corncobs had coccoid cells of both *Streptococcus* sp. or *Porphyromonas* sp. whereas double-layer kernels consisted of a combination of *Streptococcus* sp. in the inner layer and *Haemophilus/ Aggregatibacter* sp. in the outer layer	
		Porphyromonas sp.			
		Rothia sp.		Cells of at least four different taxa interact with one another at a micron scale. *Lautropia* sp. was found forming the center of alternative structures (a cauliflower)	
		Lautropia sp.			
		Veilonella sp.			
		Prevotella sp.			
		Neisseria sp.			

CHAPTER 4
DISCUSSION

DISCUSSION

Biofilm imaging allowed a better understanding of complex microbial communities. However, these techniques still present some drawbacks. The visualization of individual cell compounds, how they structurally interact among themselves, and with the entire consortia is beyond the resolution of biofilm imaging (83). High-resolution advanced microscopy techniques with simpler protocols (e.g., Super Resolution Confocal Microscopy) are becoming widely available, allowing researchers to distinguish between microbial subpopulations and examine gene expression in single cells *in situ*, as well as understanding cell organization employing a growing number of fluorescent tools (84).

Biofilms are the most broadly disseminated and effective *modus operandi* of microbial life on Earth (85). Since the first definitions of biofilms in the 1980s (86, 87), our understanding of this mode of life has advanced vastly and this concept has been applied to numerous chronic infections in humans, animals, medical devices, and plants (88).

Summary of evidence

Our findings indicate a paucity of research focusing specifically on the study of the spatial organization of periodontal pathogens within oral biofilms from the *Bacteria* domain and its etiologic significance.

In our results, the biofilm's fluorescent intensity and variety of labeled microorganisms increased as images drifted from the tooth to the epithelium side, and from the pocket's depth to the coronal surface, indicating differences in the physiological activity of the cells. Subsequently, we present three possible explanations: (i) the cell structures and morphologies of supragingival biofilms are much more diverse than those of subgingival biofilms; (ii) the unidentified microorganisms may belong to species for which there are no probes available (iii) prior stages of the biofilm that have been shielded from nutrients, comprising dead/inactive cells with low fluorescence activity, are located in the basal layers (80).

Although the stock of the oral microbiota has a lot of inter- and intra-individual variation, about 16 genera are almost universally found in supragingival dental plaque, including *Corynebacterium* sp., *Capnocytophaga* sp., *Fusobacterium* sp., *Leptotrichia* sp., *Actinomyces* sp., *Campylobacter* sp., *Streptococcus* sp., *Neisseria* sp., *Selenomonas* sp., *Haemophilus/Aggregatibacter* sp., *Porphyromonas* sp., *Rothia* sp., *Lautropia* sp., *Veilonella* sp., and *Prevotella* sp. (69, 79, 80, 89, 90). Bacteria that thrive below the gumline are cut off from high oxygen stress, salivary and dietary nutrients, relying on

gingival crevicular fluid (GCF) for nutrition. As a result, strict or facultative anaerobic proteolytic bacteria become more prevalent in the subgingival microbiota, such as *Filifactor* sp., *Fusobacterium* sp., *Parvimonas* sp., *Porphyromonas* sp., *Prevotella* sp., *Tannerella* sp., and *Treponema* sp. (3, 67, 68, 80, 91, 92), particularly in individuals with periodontitis.

In healthy people, the fungal load is thought to be lower than the bacterial load. However, the size and morphology of fungal cells, as well as their synergistic interactions with bacteria, indicate that these species play an important role in the formation of dental plaque (13, 80, 93).

The adsorbing of salivary proteins and glycoproteins to the tooth's surface triggers the initial plaque establishment, forming the acquired enamel pellicle (AEP) - a conditioning layer that covers all teeth present in the oral cavity and acts as a substrate for bacterial attachment (75, 94). The AEP is formed during the early stages of teeth eruption when saliva contacts the tooth's surface (94) and is never fully removed even if professional dental hygiene is performed.

Planktonic cells, aggregates of cells, and early colonizers (e.g., *Streptococcus* sp., *Lactobacillus* sp., *Actinomyces* sp., and *Candida* sp.) adhere to this pellicle via specialized adhesins on the bacterial cell surface (21, 95). These species do not promiscuously bind to any filament available, but rather engage in a highly specific interaction with already adhered cells, such as *Corynebacterium* sp., *Actinomyces* sp., (79), or yeast/hyphae cells forming the first layer of supragingival plaque (80, 93).

The role of *Actinomyces* sp. as a primary colonizer has already been proved due to its significant role in gingivitis (96). Many *Actinomyces* species, such as *A. naeslundii*, *A. oris*, and *A. johnsonii* (97) have been found in supra- and subgingival plaque specimens from diseased patients, suggesting that these are the most significant plaque initial formers among the *Actinomyces* genus. *Actinomyces* sp. can store intracellular glycogen or search for biofilm material such as extracellular polymeric substances and compounds from dead bacterial cells (80, 98), which can create an important advantage in surviving in the deepest layers of the biofilm community. *Actinomyces* sp. were found in the base of the hedgehogs' structures (79), which also suggests that *Corynebacterium* sp. cells do not directly colonize the tooth surface but on a previous and established biofilm containing *Actinomyces* species.

Possibly, these already adhered microorganisms may create a microenvironment favorable to other colonizers to grow. Therefore, biofilm maturation occurs by the coaggregation of planktonic bacteria to an already adhered biofilm (27).

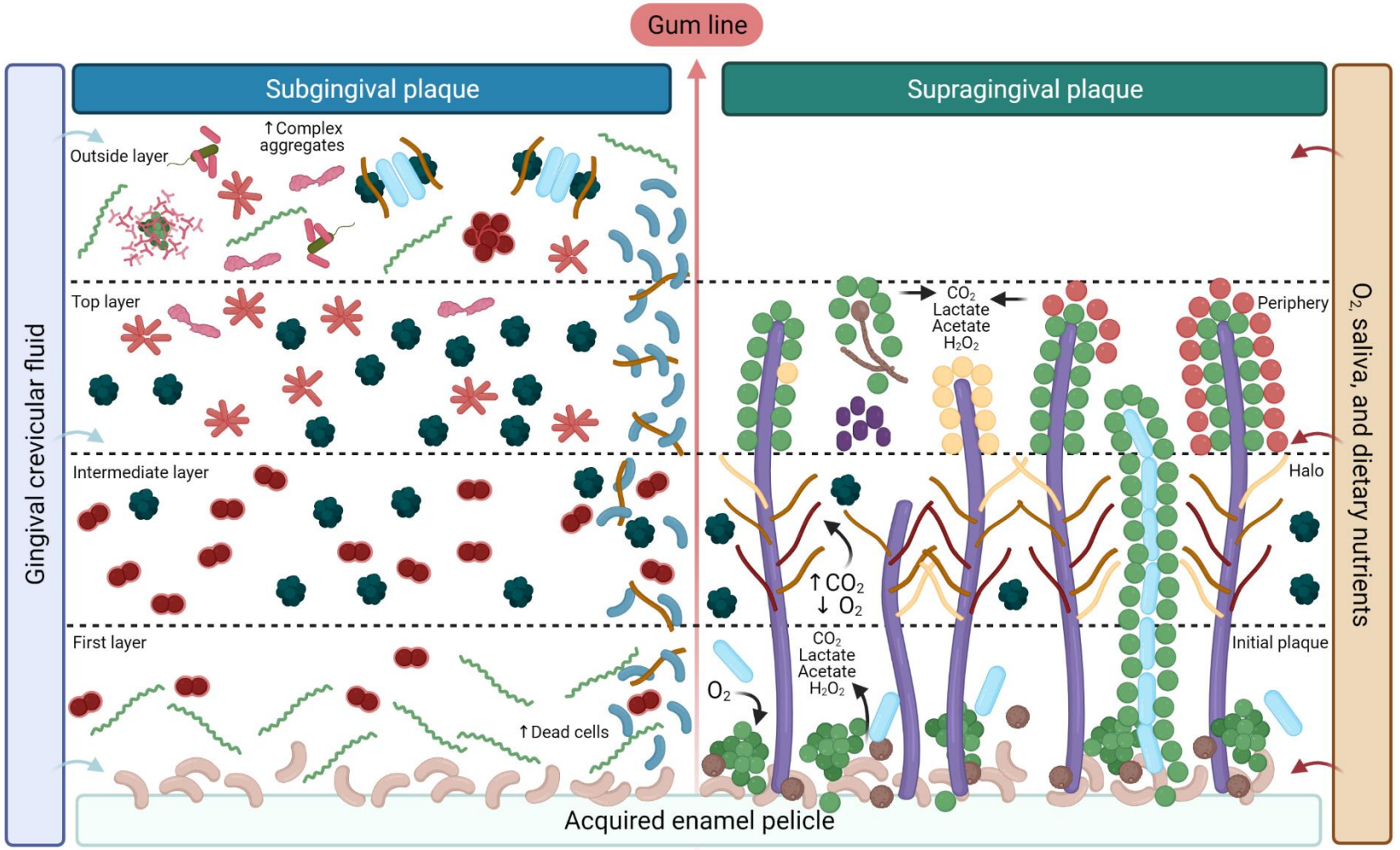
Early colonizers of supragingival dental surfaces are usually facultative anaerobic bacteria, like *Streptococcus* sp. These species produce carbon dioxide (CO_2), lactate, and acetate, containing hydrogen peroxide (H_2O_2) by consuming oxygen (O_2). The

redox potential is lowered, allowing strict anaerobes (e.g., *Fusobacterium* sp., *Leptotrichia* sp., and *Capnocytophaga* sp.) to settle and multiply in the biofilm.

The presence of *Streptococcus* sp. and bacteria from the CFB-cluster in the second layer of supragingival biofilm (80) could indicate a critical transition of a supragingival plaque made of predominantly Gram-positive saccharolytic bacteria to a Gram-negative proteolytic subgingival plaque, which could be caused by nutrient availability (e.g., dietary sugars in the supragingival plaque, or proteins from saliva and GCF in the subgingival plaque). Additionally, the presence of *Streptococcus* sp. in the basal and second layers of biofilm also allows us to infer that these organisms can adapt to a wide variety of environments, settling first as early colonizers but with the ability to expand and prosper. Our hypothesis is summarized in FIGURE 31.

The presence and abundance of *Corynebacterium* sp. in hedgehog structures in a bush-like skeleton, anchored from the presumed tooth surface (79, 99) may also indicate that this genus plays a key role in the plaque community, while its plaque specificity indicates that it occupies a niche that is influenced by tooth surface and/or GCF properties.

By reducing the flow of GCF with the use of anti-inflammatory agents the outgrowth of proteolytic bacteria would be prevented. Another alternative would be to use oxygenating or redox agents to make the gingival crevice less anaerobic, selectively inhibiting the growth of obligate anaerobes (100). Therefore, the conventional periodontal treatment approaches that involve mechanical removal of plaque, physical disruption of biofilm structure, or antibiotic therapies could be enhanced.


Gum line
Subgingival plaque
Supragingival plaque
Gingival crevicular fluid
O2, saliva, and dietary nutrients
↑ Complex aggregates
Outside layer
Top layer
Intermediate layer
First layer
↑ Dead cells
CO2
Lactate
Acetate
H2O2
Periphery
Halo
↑ CO2
↓ O2
O2
Initial plaque
Acquired enamel pelicle

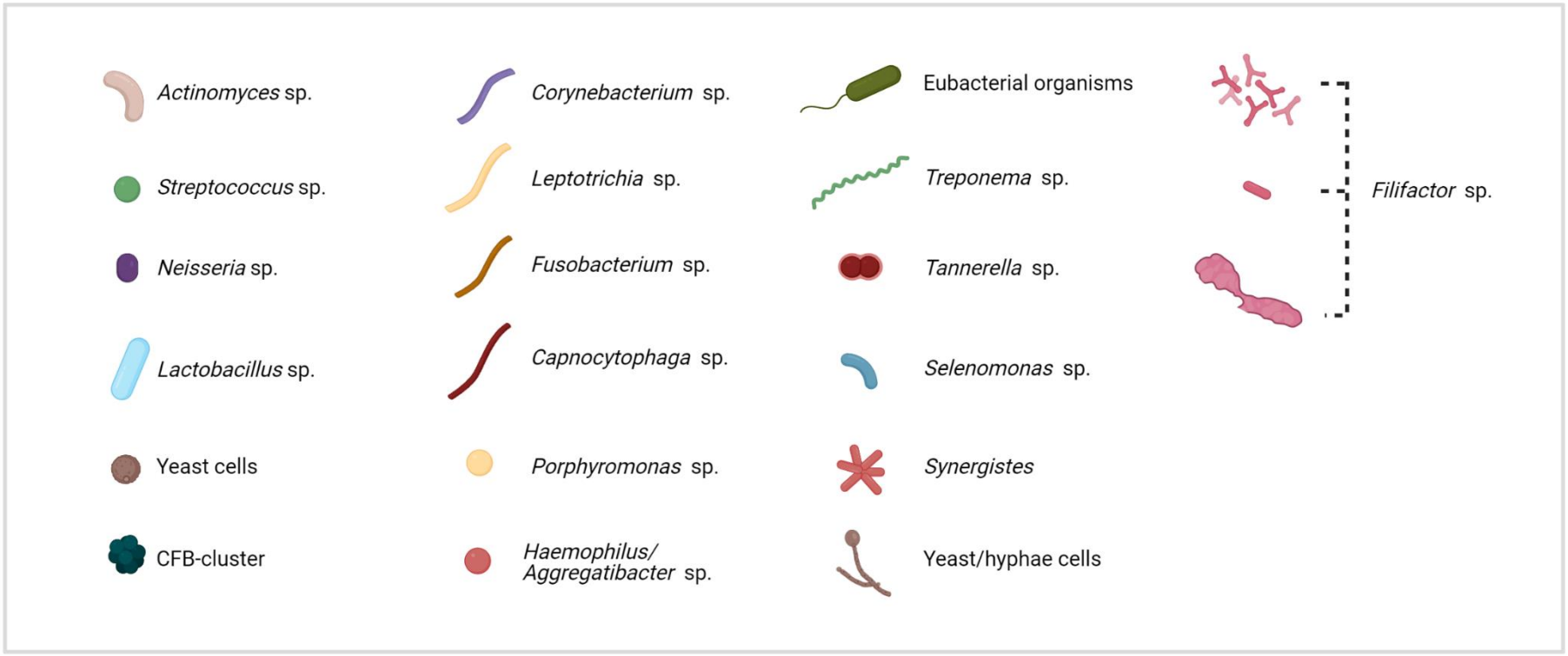

Actinomyces sp.
Streptococcus sp.
Neisseria sp.
Lactobacillus sp.
Yeast cells
CFB-cluster
Corynebacterium sp.
Leptotrichia sp.
Fusobacterium sp.
Capnocytophaga sp.
Porphyromonas sp.
Haemophilus/ Aggregatibacter sp.
Eubacterial organisms
Treponema sp.
Tannerella sp.
Selenomonas sp.
Synergistes
Yeast/hyphae cells
Filifactor sp.

FIGURE 31. Summary hypothesis for the interpretation of the gathered evidence. Microorganisms that thrive below the gumline, in contrast to bacteria in supragingival plaque, are cut off from high oxygen stress, salivary, and dietary nutrients, depending on gingival crevicular fluid (GCF) for nutrition. The adsorbing of salivary proteins and glycoproteins to the tooth's surface triggers the initial plaque establishment, forming the acquired enamel pellicle (AEP) - a conditioning layer for bacterial attachment. In supragingival plaque, *Corynebacterium* sp. filaments bind to an existing biofilm containing *Streptococcus* sp., *Actinomyces* sp., yeast cells, and *Lactobacillus* sp. These species produce carbon dioxide (CO_2), lactate, and acetate, containing hydrogen peroxide (H_2O_2) by consuming oxygen (O_2). The redox potential is lowered, allowing strict anaerobes (such as *Fusobacterium* sp., *Leptotrichia* sp., and *Capnocytophaga* sp.) to settle and multiply in the CO_2-requiring "halo" section. This environment is also favorable to the growth and development of microorganisms from the CFB-cluster, especially, *Prevotella* sp. In the periphery, cells shape single- or double-layered corncobs structures. *Streptococcus* sp. adheres to a central axis of yeast/hyphae, *Corynebacterium* sp., or *Lactobacillus* sp. cells. Subgingival plaque is divided into four layers: (i) the first layer, which is made of early colonizers displaying few fluorescences; (ii) the intermediate and (iii) the top layers are composed of a variety of microorganisms from the CFB-cluster, *Treponema* sp., *Tannerella* sp., *Synergistetes*, and *Fusobacterium* sp. tangled with *Selenomonas* sp. in the cervical portion; (iv) the outside layer, made of Treponemes nearby a complex mixture of CFB-cluster, *Lactobacillus* sp. and *Fusobacterium* sp. cells coaggregated in fine test-tube brushes. *Tannerella* sp. and *Synergistetes* formed aggregates solely with themselves. In this last layer, *Filifactor* sp. form a variety of concentrical-orientated structures, such as tree-like shapes among coccoid cells, palisades around eubacterial organisms, or remote test-tube-brush shapes.

Microbes' herd mentality behavior

Taxa that are present primarily or exclusively in one site may provide clues to the distinctive features of the habitat and the role that those taxa contribute to the site but it's still unclear if pathogens found at the control sites are part of the local/commensal flora or are originated from adjacent periodontal lesion sulcus crevicular fluid (67).

Depending on which partner the microorganism was found coaggregated with, *F. alocis* shaped several conformations, especially with fusiform bacteria, which formed concentrical radial-orientated structures in mushroom-shaped biofilms or palisades structures when the coaggregation occurred with eubacterial organisms additionally (3). When isolated, *F. alocis* formed test-tube brushes shapes around signal-free channels. This pattern and degree of organization can play a role in the events that occur during biofilm growth and maturation and be strongly linked to biofilm formation.

Our findings suggest that other microorganisms adopted different configurations influenced by their peers in different biofilm areas and tissues. Test-tube brushes shapes were once again found in a complex mixture of cells containing *T. forsythia, Campylobacter* sp., *P. micra, Fusobacterium* sp*., and Synergistetes* group A in the outside layer of subgingival plaque (80). On the other hand, *Synergistetes* group A adopted a wide cigar-like shape in a palisade lining when found isolated, forming

aggregates exclusively with themselves. The CFB-cluster also manifested different shapes, especially *Prevotella* sp. that formed micro-colonies in the top layer of subgingival biofilm but were observed as filamentous, rod-shaped, and coccoid on the intermediate layer.

Reliant on which microorganisms they are coaggregated with, some microorganisms appear to "metamorphose". We call this process "herd mentality behavior" and we believe that might be an important advantage of periodontal pathogens. To verify our hypothesis, future research should focus on the spatial organization of periodontal microbiota. The studies should be performed considering sample collection and preparation that preserves as much spatial organization as possible, like whole-mount preparations that permit the imaging of 3D structures (79).

Understanding the periodontal microbiota

Many theories were developed to try to understand the microbiology etiology of periodontal diseases. We will only denote the ones that are considered more modernized since it is not our goal to explain the evolution of these hypotheses, but rather to correlate our findings with the pathogenesis hypothesis, keeping in mind the relevance of previous concepts in the creation of the most actualized theories.

The "keystone pathogen hypothesis" (KPH) suggests that low-abundance keystone species, not necessarily belonging to the "red complex" species (96), can disrupt tissue homeostasis by altering the commensal microbiota quantitatively and qualitatively (101). Keystone pathogens can cause inflammation even when they are present in small numbers, in comparison to dominant organisms. This was observed in studies in mouse models that showed that the very low presence of *Porphyromonas gingivalis* (<0.01% of the total bacterial count in plaque) could alter the plaque composition, remodeling a symbiotic community into a dysbiotic state that triggered inflammatory bone loss (102).

The "polymicrobial synergy and dysbiosis" theory (PSD) is the most recent and extensive pathogenesis concept (6). A complex synergistic and dysbiotic microbiota colonizes the gingival sulcus (103). The host and these communities are in symbiosis. Overgrowth and overt pathogenicity are regulated by the host, even though these microorganisms are proinflammatory and can generate toxic products, such as proteases.

Changes in host immune competence or diet (e.g., systemic and genetic conditions, smoking, aging) can alter community composition, resulting in intensification in virulence factor development (e.g., colonization by *Porphyromonas gingivalis,* elevates the virulence of the entire community following interactive communication with

accessory pathogens, such as *Streptococcus* sp. (104)). Host immune surveillance is weakened, inflowing in a dysbiotic cycle that drives the chronic infection - the provoking-disease population grows, disrupting tissue homeostasis and causing inflammation, triggering the increase of GCF's flow, that facilitates the outgrowth of microorganisms associated with a dysbiotic state. At this stage, eubiotic organisms are unable to benefit from the new environmental conditions and are outcompeted, instigating periodontal tissue loss. The inflammation is exacerbated, and the cycle is repeated.

Periodontitis is caused by a synergistic and dysbiotic microbiota in which various members and gene combinations play distinct roles that combine to form and stabilize a disease-causing microbiota. Hence, the health-disease transition requires a dysbiotic microbiota and a susceptible host (105).

The microbiota associated with a healthy state is, therefore, more generalist, while the disease-provoking microbiota is influenced by keystone microorganisms that have metabolic functions and an elevated virulence capacity that is mostly absent in healthy states (65).

We believe that limited organisms can perform this more specialized dysbiotic role. FISH is a unique tool that combines the molecular information provided by 16S and/or 23S sequencing with the spatial resolution of light microscopy. Using this technique, it would be possible to identify key pathogens from a set of taxa identified by oral microbiota research, as well as spatially characterize the periodontal microbiota in health and disease.

FISH presents the multiplexing capability of using a handful of different probes in any single sample. Out of the 529 taxa of microbes that are estimated to inhabit the oral ecosystem (12), FISH probes should be designed to target the following 18 taxa, which are likely to shape the spatial and metabolic framework of the dental plaque microbiota: *Corynebacterium* sp., *Capnocytophaga* sp., *Fusobacterium* sp., *Leptotrichia* sp., *Actinomyces* sp., *Streptococcus* sp., *Neisseria* sp., *Selenomonas* sp., *Haemophilus* sp., *Aggregatibacter* sp., *Porphyromonas* sp., *Rothia* sp., *Lautropia* sp., *Veilonella* sp., *Prevotella* sp., *Treponema* sp., *Filifactor* sp., and *Tannerella* species.

Limitations

None of the included articles classified periodontal diseases according to current diagnostic criteria. In June 2018, the American Academy of Periodontology (AAP) released a series of reviews in partnership with the European Federation of Periodontology (EFP) (106-110) suggesting significant updates in periodontal diagnose, like combining chronic and aggressive periodontitis in unique-entity periodontitis with

various phenotypes. Another major improvement was the association of the periodontal disease's classification with its progression rate and prognostic factors in a more accurate and reliable staging classification (111).

The results demonstrate that the available evidence relies on a limited number of observations. Assuming that these observable interactions are somehow indicative of the periodontal microbiota in health and disease, is a leap of faith that needs validation in large-scale studies. Some images catch our eye, but in the absence of meticulous cell counting, it may contribute to an overestimation of microbes' population in plaque specimens, contradicting quantitative findings. This raises the question of whether all FISH studies should involve quantitative cell counting or dynamic time lap observations.

Furthermore, all the studies performed FISH with DNA/RNA probes, which has a few disadvantages that we have already discussed, and only one study performed fluorescence spectral imaging. As a result, we believe that the added value of the use of nucleic acid mimics (NAMs) in combination with fluorescence spectral imaging in future research, will allow the identification of multiple microorganisms and open the door for a systems-level view of the periodontal microbiota's spatial structure.

The fact that the bases of the molecular pathology rationale discussed in this scoping review were obtained from *in vitro* studies or performed in animals, is another drawback of this scoping review. To research species networks in a more realistic and holistic setting, complex community-like structures should be used.

Conclusions and future perspectives

Supragingival biofilm seems to present a radially ordered multiple-taxon structure, backboned by facultative anaerobic long rods, such as *Corynebacterium* sp. and *Lactobacillus* sp., bordered by coccoid and/or filamentous cells. On the other hand, subgingival biofilm seems to present a more layered multiple-taxon structure from the tooth's surface to the pocket's epithelium. Anaerobic microorganisms dominate the subgingival environment, especially in the deepest portions. Both sub- and supragingival biofilms exhibited a richer outside layer when compared with the inner layers, and the biggest contrast was observed in the subgingival biofilm. Consumers and producers of certain metabolites tend to be spatially related; some microorganisms exhibited the ability to shape various configurations influenced by their peers in different biofilm areas. We referred to this last phenomenon as "microbes herd mentality behavior" and we believe that it may represent an imperative benefit of certain members of the periodontal microbiota.

In conclusion, and in response to the previous query, the FISH technique was able to detect interactions between microorganisms from the three Domains of Life, enabling the construction of a possible hypothesis for understanding the influence of these interactions in the establishment and development of periodontal diseases. However, the evidence about the structural composition and microorganism's interactions in periodontal biofilms is scarce and no definite conclusion can be drawn.

The enhanced FISH-based techniques provide significant information about the spatial organization of a complex natural polymicrobial community, allowing researchers and dental medicine doctors to better understand how the individual taxa interact within a community in periodontal diseases, and how their relations compromise the whole assemblage.

Identifying keystone and accessory pathogens capable of inducing a dysbiosis condition in periodontal pockets, as well as exploring the metabolic framework of the disease-causing microbiota, should be the subject of future research.

We strongly believe that by combining current pathogenesis concepts and FISH-based techniques, a new diagnostic and therapeutic tool would emerge, with the development of a kit of oligonucleotide probes, targeting generalist, keystone, and accessory pathogens responsible for inducing a dysbiosis condition in periodontal pockets. This will enable researchers to investigate the metabolic framework of the disease-causing microbiota, which could help guide disease onset, progression, or remission, as well as therapy response.

REFERENCES

1. Vartoukian SR, Palmer RM, Wade WG. **Diversity and morphology of members of the phylum "synergistetes" in periodontal health and disease**. Appl Environ Microbiol. 2009;75(11):3777-86.

2. Bostanci N, Bao K, Greenwood D, Silbereisen A, Belibasakis GN. **Periodontal disease: From the lenses of light microscopy to the specs of proteomics and next-generation sequencing.** Advances in Clinical Chemistry. 93: Elsevier; 2019. p. 263-90.

3. Schlafer S, Riep B, Griffen AL, Petrich A, Hübner J, Berning M, et al. **Filifactor alocis - Involvement in periodontal biofilms.** BMC Microbiology. 2010;10.

4. Curtis MA, Diaz PI, Van Dyke TE. **The role of the microbiota in periodontal disease**. Periodontology 2000. 2020;83(1):14-25.

5. Mendes L, Rocha R, Azevedo AS, Ferreira C, Henriques M, Pinto MG, et al. **Novel strategy to detect and locate periodontal pathogens: The PNA-FISH technique.** Microbiol Res. 2016;192:185-91.

6. Hajishengallis G, Lamont RJ. **Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology.** Molecular oral microbiology. 2012;27(6):409-19.

7. Caton JG, Armitage G, Berglundh T, Chapple IL, Jepsen S, Kornman KS, et al. **A new classification scheme for periodontal and peri-implant diseases and conditions–Introduction and key changes from the 1999 classification.** Journal of periodontology. 2018;89:S1-S8.

8. Nazir M, Al-Ansari A, Al-Khalifa K, Alhareky M, Gaffar B, Almas K. **Global prevalence of periodontal disease and lack of its surveillance.** The Scientific World Journal. 2020;2020.

9. Bui FQ, Almeida-da-Silva CLC, Huynh B, Trinh A, Liu J, Woodward J, et al. **Association between periodontal pathogens and systemic disease**. Biomedical journal. 2019;42(1):27-35.

10. Carrizales-Sepúlveda EF, Ordaz-Farías A, Vera-Pineda R, Flores-Ramírez R. **Periodontal disease, systemic inflammation and the risk of cardiovascular disease.** Heart, Lung and Circulation. 2018;27(11):1327-34.

11. Michaud DS, Fu Z, Shi J, Chung M. **Periodontal disease, tooth loss, and cancer risk.** Epidemiologic reviews. 2017;39(1):49-58.

12. Escapa IF, Chen T, Huang Y, Gajare P, Dewhirst FE, Lemon KP. **New insights into human nostril microbiome from the expanded human oral microbiome database (eHOMD): a resource for the microbiome of the human aerodigestive tract.** Msystems. 2018;3(6).

13. Valm AM. **The structure of dental plaque microbial communities in the transition from health to dental caries and periodontal disease.** Journal of molecular biology. 2019;431(16):2957-69.

14. Lazar V. **Quorum sensing in biofilms–how to destroy the bacterial citadels or their cohesion/power?** Anaerobe. 2011;17(6):280-5.

15. Ibberson CB, Whiteley M. **The social life of microbes in chronic infection.** Current opinion in microbiology. 2020;53:44-50.

16. Busscher H, Van der Mei H. **Physico-chemical interactions in initial microbial adhesion and relevance for biofilm formation.** Advances in dental research. 1997;11(1):24-32.

17. Bos R, Van der Mei HC, Busscher HJ. **Physico-chemistry of initial microbial adhesive interactions–its mechanisms and methods for study**. FEMS microbiology reviews. 1999;23(2):179-230.

18. Wolcott R, Costerton J, Raoult D, Cutler S. **The polymicrobial nature of biofilm infection.** Clinical Microbiology and Infection. 2013;19(2):107-12.

19. Marsh PD, Moter A, Devine DA. **Dental plaque biofilms: communities, conflict and control.** Periodontology 2000. 2011;55(1):16-35.

20. Rendueles O, Ghigo J-M. **Multi-species biofilms: how to avoid unfriendly neighbors.** FEMS microbiology reviews. 2012;36(5):972-89.

21. Katharios-Lanwermeyer S, Xi C, Jakubovics N, Rickard A. **Mini-review: microbial coaggregation: ubiquity and implications for biofilm development.** Biofouling. 2014;30(10):1235-51.

22. Unosson E. **Antibacterial strategies for titanium biomaterials:** Acta Universitatis Upsaliensis; 2015.

23. Moter A, Göbel UB. **Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms.** Elsevier; 2000.

24. Al-Ahmad A, Wunder A, Auschill TM, Follo M, Braun G, Hellwig E, et al. **The in vivo dynamics of Streptococcus spp., Actinomyces naeslundii, Fusobacterium**

nucleatum and Veillonella spp. in dental plaque biofilm as analysed by five-colour multiplex fluorescence in situ hybridization. J Med Microbiol. 2007;56(Pt 5):681-7.

25. Gmür R, Strub J, Guggenheim B. **Prevalence of Bacteroides forsythus and Bacteroides gingivalis in subgingival plaque of prosthodontically treated patients on short recall.** Journal of Periodontal Research. 1989;24(2):113-20.

26. Gmür R, Thurnheer T. **Direct quantitative differentiation between Prevotella intermedia and Prevotella nigrescens in clinical specimens**. Microbiology. 2002;148(5):1379-87.

27. Palmer Jr RJ, Gordon SM, Cisar JO, Kolenbrander PE. **Coaggregation-mediated interactions of streptococci and actinomyces detected in initial human dental plaque.** Journal of bacteriology. 2003;185(11):3400-9.

28. Noiri Y, Li L, Ebisu S. **The localization of periodontal-disease-associated bacteria in human periodontal pockets.** Journal of dental research. 2001;80(10):1930-4.

29. Yoshida A, Ansai T. **Microbiological diagnosis for periodontal diseases. Periodontal diseases–A clinician's guide.** 2012:55-66.

30. Socransky SS, Haffajee AD. **Periodontal microbial ecology.** Periodontology 2000. 2005;38(1):135-87.

31. Ashimoto A, Flynn M, Slots J. **Molecular genetic detection of Bacteroides heparinolyticus in adult periodontitis.** Oral microbiology and immunology. 1995;10(5):284-7.

32. Alves ACB, Napimoga MH, Klein MI, Hofling JF, Gonçalves RB. **Increase in probing depth is correlated with a higher number of Prevotella intermedia genotypes.** Journal of periodontology. 2006;77(1):61-6.

33. Colombo AV, Barbosa GM, Higashi D, di Micheli G, Rodrigues PH, Simionato MRL. **Quantitative detection of Staphylococcus aureus, Enterococcus faecalis and Pseudomonas aeruginosa in human oral epithelial cells from subjects with periodontitis and periodontal health. Journal of medical microbiology.** 2013;62(10):1592-600.

34. Bernardi S, Continenza MA, Al-Ahmad A, Karygianni L, Follo M, Filippi A, et al. **Streptococcus spp. and Fusobacterium nucleatum in tongue dorsum biofilm from halitosis patients: A fluorescence in situ hybridization (FISH) and confocal laser scanning microscopy (CLSM) study.** The new microbiologica. 2019;42(2):108-13.

35. Thurnheer T, Gmür R, Giertsen E, Guggenheim B. **Automated fluorescent in situ hybridization for the specific detection and quantification of oral streptococci in dental plaque.** Journal of microbiological methods. 2001;44(1):39-47.

36. do Nascimento C, Paulo DF, Pita MS, Pedrazzi V, de Albuquerque Junior RF. **Microbial diversity of the supra-and subgingival biofilm of healthy individuals after brushing with chlorhexidine-or silver-coated toothbrush bristles.** Canadian journal of microbiology. 2015;61(2):112-23.

37. Tanner A, Maiden M, Macuch P, Murray L, Kent Jr R. **Microbiota of health, gingivitis, and initial periodontitis.** Journal of clinical periodontology. 1998;25(2):85-98.

38. Anaya-Bergman C, Rosato A, Lewis J. **Iron-and hemin-dependent gene expression of Porphyromonas gingivalis.** Molecular oral microbiology. 2015;30(1):39-61.

39. Crielaard W, Zaura E, Schuller AA, Huse SM, Montijn RC, Keijser BJ. **Exploring the oral microbiota of children at various developmental stages of their dentition in the relation to their oral health.** BMC medical genomics. 2011;4(1):1-13.

40. Abubakar I, Irvine L, Aldus C, Wyatt G, Fordham R, Schelenz S, et al. **A systematic review of the clinical, public health and cost-effectiveness of rapid diagnostic tests for the detection and identification of bacterial intestinal pathogens in faeces and food.** Health technology assessment (Winchester, England). 2007;11(36):1-216.

41. Malorny B, Hoorfar J, Hugas M, Heuvelink A, Fach P, Ellerbroek L, et al. **Interlaboratory diagnostic accuracy of a Salmonella specific PCR-based method.** International journal of food microbiology. 2003;89(2-3):241-9.

42. Saroj SD, Shashidhar R, Karani M, Bandekar JR. **Rapid, sensitive, and validated method for detection of Salmonella in food by an enrichment broth culture–Nested PCR combination assay.** Molecular and cellular probes. 2008;22(3):201-6.

43. Yamamoto Y. **PCR in diagnosis of infection: detection of bacteria in cerebrospinal fluids.** Clinical and diagnostic laboratory immunology. 2002;9(3):508-14.

44. Levsky JM, Singer RH. **Fluorescence in situ hybridization: past, present and future.** Journal of cell science. 2003;116(14):2833-8.

45. Azevedo AS, Sousa IM, Fernandes RM, Azevedo NF, Almeida C. **Optimizing locked nucleic acid/2′-O-methyl-RNA fluorescence in situ hybridization**

(LNA/2′ OMe-FISH) procedure for bacterial detection. PloS one. 2019;14(5):e0217689.

46. Rombouts S, Nollmann M. **RNA imaging in bacteria.** FEMS Microbiology Reviews. 2020.

47. Almeida C, Azevedo NF, Santos S, Keevil CW, Vieira MJ. **Discriminating multi-species populations in biofilms with peptide nucleic acid fluorescence in situ hybridization (PNA FISH).** PloS one. 2011;6(3):e14786.

48. Petersen M, Wengel J. **LNA: a versatile tool for therapeutics and genomics**. Trends in biotechnology. 2003;21(2):74-81.

49. Forrest GN. **PNA FISH: present and future impact on patient management.** Expert review of molecular diagnostics. 2007;7(3):231-6.

50. Nielsen PE, Egholm M, Buchardt O. **Peptide nucleic acid (PNA). A DNA mimic with a peptide backbone.** Bioconjugate chemistry. 1994;5(1):3-7.

51. Koshkin AA, Nielsen P, Meldgaard M, Rajwanshi VK, Singh SK, Wengel J. **LNA (locked nucleic acid): an RNA mimic forming exceedingly stable LNA: LNA duplexes.** Journal of the American Chemical Society. 1998;120(50):13252-3.

52. Cerqueira L, Azevedo NF, Almeida C, Jardim T, Keevil CW, Vieira MJ. **DNA mimics for the rapid identification of microorganisms by fluorescence in situ hybridization (FISH).** International Journal of Molecular Sciences. 2008;9(10):1944-60.

53. Egholm M, Buchardt O, Christensen L, Behrens C, Freier SM, Driver DA, et al. **PNA hybridizes to complementary oligonucleotides obeying the Watson–Crick hydrogen-bonding rules**. Nature. 1993;365(6446):566-8.

54. Stender H, Fiandaca M, Hyldig-Nielsen JJ, Coull J. **PNA for rapid microbiology.** Journal of microbiological methods. 2002;48(1):1-17.

55. Fontenete S, Carvalho D, Guimarães N, Madureira P, Figueiredo C, Wengel J, et al. **Application of locked nucleic acid-based probes in fluorescence in situ hybridization.** Applied microbiology and biotechnology. 2016;100(13):5897-906.

56. Dickinson M, Bearman G, Tille S, Lansford R, Fraser S. **Multi-spectral imaging and linear unmixing add a whole new dimension to laser scanning fluorescence microscopy.** Biotechniques. 2001;31(6):1272-8.

57. Valm AM, Welch JLM, Borisy GG. **CLASI-FISH: principles of combinatorial labeling and spectral imaging.** Systematic and applied microbiology. 2012;35(8):496-502.

58. Valm AM, Welch JLM, Rieken CW, Hasegawa Y, Sogin ML, Oldenbourg R, et al. **Systems-level analysis of microbial community organization through combinatorial labeling and spectral imaging.** Proceedings of the National Academy of Sciences of the United States of America. 2011;108(10):4152-7.

59. Waters JC. **Accuracy and precision in quantitative fluorescence microscopy**. The Rockefeller University Press; 2009.

60. Valm AM, Oldenbourg R, Borisy GG. **Multiplexed spectral imaging of 120 different fluorescent labels.** PloS one. 2016;11(7):e0158495.

61. Elias S, Banin E. **Multi-species biofilms: living with friendly neighbors.** FEMS microbiology reviews. 2012;36(5):990-1004.

62. Tarnita CE. **The ecology and evolution of social behavior in microbes.** Journal of Experimental Biology. 2017;220(1):18-24.

63. Brown SP, Buckling A. **A social life for discerning microbes.** Cell. 2008;135(4):600-3.

64. Bjarnsholt T. **The role of bacterial biofilms in chronic infections.** Apmis. 2013;121:1-58.

65. Lamont RJ, Koo H, Hajishengallis G. **The oral microbiota: dynamic communities and host interactions.** Nature Reviews Microbiology. 2018;16(12):745-59.

66. Gmür R, Lüthi-Schaller H. **A combined immunofluorescence and fluorescent in situ hybridization assay for single cell analyses of dental plaque microorganisms.** J Microbiol Methods. 2007;69(2):402-5.

67. Moter A, Hoenig C, Choi B-K, Riep B, Göbel UB. **Molecular epidemiology of oral treponemes associated with periodontal disease.** Journal of Clinical Microbiology. 1998;36(5):1399-403.

68. Wecke J, Kersten T, Madela K, Moter A, Göbel UB, Friedmann A, et al. **A novel technique for monitoring the development of bacterial biofilms in human periodontal pockets.** FEMS Microbiol Lett. 2000;191(1):95-101.

69. Drescher J, Schlafer S, Schaudinn C, Riep B, Neumann K, Friedmann A, et al. **Molecular epidemiology and spatial distribution of Selenomonas spp. in subgingival biofilms.** Eur J Oral Sci. 2010;118(5):466-74.

70. Listgarten MA, Mayo HE, Tremblay R. **Development of dental plaque on epoxy resin crowns in man: a light and electron microscopic study.** Journal of periodontology. 1975;46(1):10-26.

71. Listgarten MA. **The structure of dental plaque.** Periodontology 2000. 1994;5(1):52-65.

72. Zaura-Arite E, Van Marle J, Ten Cate J. **Confocal microscopy study of undisturbed and chlorhexidine-treated dental biofilm.** Journal of Dental Research. 2001;80(5):1436-40.

73. Al-Ahmad A, Follo M, Selzer A-C, Hellwig E, Hannig M, Hannig C. **Bacterial colonization of enamel in situ investigated using fluorescence in situ hybridization.** Journal of medical microbiology. 2009;58(10):1359-66.

74. Guerreiro-Tanomaru JM, de Faria-Júnior NB, Duarte MAH, Ordinola-Zapata R, Graeff MSZ, Tanomaru-Filho M. **Comparative analysis of Enterococcus faecalis biofilm formation on different substrates.** Journal of endodontics. 2013;39(3):346-50.

75. Jakubovics NS. **Saliva as the sole nutritional source in the development of multispecies communities in dental plaque.** Metabolism and Bacterial Pathogenesis. 2015:263-77.

76. Machado FC, Cesar DE, Assis AV, Diniz CG, Ribeiro RA. **Detection and enumeration of periodontopathogenic bacteria in subgingival biofilm of pregnant women.** Braz Oral Res. 2012;26(5):443-9.

77. Gmür R, Wyss C, Xue Y, Thurnheer T, Guggenheim B. **Gingival crevice microbiota from Chinese patients with gingivitis or necrotizing ulcerative gingivitis.** European journal of oral sciences. 2004;112(1):33-41.

78. Lepp PW, Brinig MM, Ouverney CC, Palm K, Armitage GC, Relman DA. **Methanogenic Archaea and human periodontal disease.** Proc Natl Acad Sci U S A. 2004;101(16):6176-81.

79. Mark Welch JL, Rossetti BJ, Rieken CW, Dewhirst FE, Borisy GG. **Biogeography of a human oral microbiome at the micron scale.** Proc Natl Acad Sci U S A. 2016;113(6):E791-800.

80. Zijnge V, van Leeuwen MB, Degener JE, Abbas F, Thurnheer T, Gmür R, et al. **Oral biofilm architecture on natural teeth.** PLoS One. 2010;5(2):e9321.

81. Munn Z, Peters MD, Stern C, Tufanaru C, McArthur A, Aromataris E. **Systematic review or scoping review? Guidance for authors when choosing**

between a systematic or scoping review approach. BMC medical research methodology. 2018;18(1):143.

82. Tricco AC, Lillie E, Zarin W, O'Brien KK, Colquhoun H, Levac D, et al. **PRISMA extension for scoping reviews (PRISMA-ScR): checklist and explanation**. Annals of internal medicine. 2018;169(7):467-73.

83. Coenye T, Kjellerup B, Stoodley P, Bjarnsholt T. **The future of biofilm research–Report on the '2019 Biofilm Bash'.** Biofilm. 2020;2:100012.

84. Haagensen JA, Regenberg B, Sternberg C. **Advanced microscopy of microbial cells.** High Resolution Microbial Single Cell Analytics. 2010:21-54.

85. Flemming H-C, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S. **Biofilms: an emergent form of bacterial life.** Nature Reviews Microbiology. 2016;14(9):563.

86. Costerton JW, Cheng K, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, et al. **Bacterial biofilms in nature and disease.** Annual Reviews in Microbiology. 1987;41(1):435-64.

87. Nickel J, Ruseska I, Costerton J. **Tobramycin resistance of cells of Pseudomonas aerogenosa growing as a biofilm on urinary catheter material.** Antimicrob Agents Chemother. 1985;27:610-24.

88. Høiby N. **A short history of microbial biofilms and biofilm infections.** Apmis. 2017;125(4):272-5.

89. Xu X, He J, Xue J, Wang Y, Li K, Zhang K, et al. **Oral cavity contains distinct niches with dynamic microbial communities.** Environmental microbiology. 2015;17(3):699-710.

90. Bik EM, Long CD, Armitage GC, Loomer P, Emerson J, Mongodin EF, et al. **Bacterial diversity in the oral cavity of 10 healthy individuals.** The ISME journal. 2010;4(8):962-74.

91. Ge X, Rodriguez R, Trinh M, Gunsolley J, Xu P. **Oral microbiome of deep and shallow dental pockets in chronic periodontitis.** PloS one. 2013;8(6):e65520.

92. Li Y, He J, He Z, Zhou Y, Yuan M, Xu X, et al. **Phylogenetic and functional gene structure shifts of the oral microbiomes in periodontitis patients.** The ISME journal. 2014;8(9):1879-91.

93. Koo H, Andes DR, Krysan DJ. **Candida–streptococcal interactions in biofilm-associated oral diseases.** PLoS pathogens. 2018;14(12):e1007342.

94. Chawhuaveang DD, Yu OY, Yin IX, Lam WY-H, Mei ML, Chu C-H. **Acquired salivary pellicle and oral diseases: A literature review.** Journal of Dental Sciences. 2020.

95. Borisy GG, Valm AM. **Spatial scale in analysis of the dental plaque microbiome.** Periodontology 2000. 2020.

96. Socransky S, Haffajee A, Cugini M, Smith C, Kent Jr R. **Microbial complexes in subgingival plaque.** Journal of clinical periodontology. 1998;25(2):134-44.

97. Vielkind P, Jentsch H, Eschrich K, Rodloff AC, Stingu C-S. **Prevalence of Actinomyces spp. in patients with chronic periodontitis**. International Journal of Medical Microbiology. 2015;305(7):682-8.

98. Takahashi N, Yamada T. **Glucose and lactate metabolism by Actinomyces naeslundii.** Critical Reviews in Oral Biology & Medicine. 1999;10(4):487-503.

99. Diaz P, Valm A. **Microbial interactions in oral communities mediate emergent biofilm properties.** Journal of dental research. 2020;99(1):18-25.

100. Marsh PD. **Are dental diseases examples of ecological catastrophes?** Microbiology. 2003;149(2):279-94.

101. Hajishengallis G, Darveau RP, Curtis MA. **The keystone-pathogen hypothesis.** Nature Reviews Microbiology. 2012;10(10):717-25.

102. Hajishengallis G, Liang S, Payne MA, Hashim A, Jotwani R, Eskan MA, et al. **Low-abundance biofilm species orchestrates inflammatory periodontal disease through the commensal microbiota and complement.** Cell host & microbe. 2011;10(5):497-506.

103. Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, et al. **Bacterial diversity in human subgingival plaque.** Journal of bacteriology. 2001;183(12):3770-83.

104. Daep CA, Novak EA, Lamont RJ, Demuth DR. **Structural dissection and in vivo effectiveness of a peptide inhibitor of Porphyromonas gingivalis adherence to Streptococcus gordonii.** Infection and immunity. 2011;79(1):67-74.

105. Hajishengallis G. **Immunomicrobial pathogenesis of periodontitis: keystones, pathobionts, and host response.** Trends in immunology. 2014;35(1):3-11.

106. Caton JG, Armitage G, Berglundh T, Chapple IL, Jepsen S, Kornman KS, et al. **A new classification scheme for periodontal and peri-implant diseases and conditions–Introduction and key changes from the 1999 classification.** Wiley Online Library; 2018.

107. Chapple IL, Mealey BL, Van Dyke TE, Bartold PM, Dommisch H, Eickholz P, et al. **Periodontal health and gingival diseases and conditions on an intact and a reduced periodontium: Consensus report of workgroup 1 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions.** Journal of periodontology. 2018;89:S74-S84.

108. Papapanou PN, Sanz M, Buduneli N, Dietrich T, Feres M, Fine DH, et al. **Periodontitis: Consensus report of workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions.** Journal of periodontology. 2018;89:S173-S82.

109. Jepsen S, Caton JG, Albandar JM, Bissada NF, Bouchard P, Cortellini P, et al. **Periodontal manifestations of systemic diseases and developmental and acquired conditions: Consensus report of workgroup 3 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions.** Journal of clinical periodontology. 2018;45:S219-S29.

110. Berglundh T, Armitage G, Araujo MG, Avila-Ortiz G, Blanco J, Camargo PM, et al. **Peri-implant diseases and conditions: Consensus report of workgroup 4 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions.** Journal of periodontology. 2018;89:S313-S8.

111. Fine DH, Patil AG, Loos BG. **Classification and diagnosis of aggressive periodontitis.** Journal of clinical periodontology. 2018;45:S95-S111.

APPENDIX

APPENDIX

SUPPLEMENTAL TABLE 1. Reporting items from this scoping review. Adapted from "The Preferred Reporting Items for Systematic reviews and Meta-Analyses extension for Scoping Reviews (PRISMA-ScR) Checklist" (82).

SECTION	ITEM	PRISMA-ScR CHECKLIST ITEM	REPORTED ON PAGE
TITLE			
Title	1	Identify the report as a scoping review.	V
ABSTRACT			
Structured summary	2	Provide a structured summary that includes (as applicable): background, objectives, eligibility criteria, sources of evidence, charting methods, results, and conclusions that relate to the review questions and objectives.	IX-XII
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known. Explain why the review questions/objectives lend themselves to a scoping review approach.	2-9
Objectives	4	Provide an explicit statement of the questions and objectives being addressed with reference to their key elements (e.g., population or participants, concepts, and context) or other relevant key elements used to conceptualize the review questions and/or objectives.	9
METHODS			
Eligibility criteria	5	Specify characteristics of the sources of evidence used as eligibility criteria (e.g., years considered, language, and publication status), and provide a rationale.	12
Information sources	6	Describe all information sources in the search (e.g., databases with dates of coverage and contact with authors to identify additional sources), as well as the date the most recent search was executed.	12
Search	7	Present the full electronic search strategy for at least 1 database, including any limits used, such that it could be repeated.	12, 73
Selection of sources of evidence	8	State the process for selecting sources of evidence (i.e., screening and eligibility) included in the scoping review.	13
Data charting process	9	Describe the methods of charting data from the included sources of evidence (e.g., calibrated forms or forms that have been tested by the team before their use, and whether data charting was done independently or in duplicate) and any processes for obtaining and confirming data from investigators.	13
Data items	10	List and define all variables for which data were sought and any assumptions and simplifications made.	13
Synthesis of results	11	Describe the methods of handling and summarizing the data that were charted.	13
RESULTS			
Selection of sources of evidence	12	Give numbers of sources of evidence screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally using a flow diagram.	16
Characteristics of sources of evidence	13	For each source of evidence, present characteristics for which data were charted and provide the citations.	17-19
Results of individual sources of evidence	14	For each included source of evidence, present the relevant data that were charted that relate to the review questions and objectives.	20-43

SECTION	ITEM	PRISMA-ScR CHECKLIST ITEM	REPORTED ON PAGE
Synthesis of results	15	Summarize and/or present the charting results as they relate to the review questions and objectives.	44-46
DISCUSSION			
Summary of evidence	16	Summarize the main results (including an overview of concepts, themes, and types of evidence available), link to the review questions and objectives, and consider the relevance to key groups.	49-54
Limitations	17	Discuss the limitations of the scoping review process.	56, 57
Conclusions	18	Provide a general interpretation of the results with respect to the review questions and objectives, as well as potential implications and/or next steps.	58
FUNDING			
Funding	19	Describe sources of funding for the included sources of evidence, as well as sources of funding for the scoping review. Describe the role of the funders of the scoping review.	III

SUPPLEMENTAL TABLE 2. Database search strategy.

Database	Search strategy
MEDLINE (via PubMed)	(Periodont* OR "Periodontal pockets" OR Microbes OR "Oral biofilm" OR Micro* OR "Oral micro*" OR "Bacteria") AND (Imaging OR FISH OR "FISH technique" OR "fluorescence *in situ* hybridization" OR "hybridization")
Scopus (Elsevier)	(Periodont* OR "Periodontal pockets" OR Microbes OR "Oral biofilm" OR Micro* OR "Oral micro*" OR "Bacteria") AND (Imaging OR FISH OR "FISH technique" OR "fluorescence *in situ* hybridization" OR "hybridization")
Web of Science (Clarivate Analytics)	TS = ((Periodont* OR "Periodontal pockets" OR Microbes OR "Oral biofilm" OR Micro* OR "Oral micro*" OR "Bacteria") AND (Imaging OR FISH OR "FISH technique" OR "fluorescence in situ hybridization" OR "hybridization"))

SUPPLEMENTAL TABLE 3. Oligonucleotide probes' names and sequences, the microorganisms targeted, and the FISH conditions used in the studies included in this scoping review. NIA: no available information. ND: not determined; D: Domain; P: Phylum; F: Family; G: Genus; S: Specie.

#	Probe name	Sequence (5′→ 3′)	Target		FISH conditions			
			Taxon	Microorganisms	% Form. (vol/vol)	Hyb. Temp. (°C)	Hyb. Time (hours)	Ref.
1	EUB338	GCTGCCTCC CGTAGGAGT	D	*Bacteria*	0-20	46	1-3	Amann, Rudolf I., *et al.* (1990)
	TRE I	ACGCAAGCTC ATCCTCAAG	G	Group I of oral treponemes				Choi, B. K., *et al.* (1994)
2	EUB338	GCTGCCTC CCGTAGGAGT	D	*Bacteria*	20	46	3.5	Amann, Rudolf I., *et al.* (1990)
	TRE I	ACGCAAGC TCATCCTCAA G	G	Group I of oral treponemes				Moter, Annette, *et al.* (1998)
3	SBGA-1	NAI	S	*Methanobrevibacter oralis*	0-80	65	8	DeLong *et. al.* (1999)
4	Lbuc668	TACTCGTGC AGTTCCGTCC	S	*Leptotrichia buccalis Fusobacterium nucleatum*	30-40	46	2.5	Manz, W. (1999)
5	EUB338	GCTGCCTC CCGTAGGAGT	D	*Bacteria*	40	46	2	Amann *et al.* (1995)
	Tfor582	GCGGACTTA ACAGCCCAC CT	S	*Tannerella forsythia*				Züger (2006)
6	EUB338	GCTGCCTC CCGTAGGAGT	D	*Bacteria*	0-30	46	3-5	Amann, Rudolf I., *et al.* (1990)
	SELE	TCGGAATGTT GTCCCCATCC	G	*Selenomonas sp. Centipeda sp.*				Schlafer, Sebastian, *et al.* (2008)
	FUSO	CTAATGGGA CGCAAAGCT CTC		*Fusobacterium sp.*				Loy, A., Horn, M., & Wagner, M. (2003)
7	EUB338	GCTGCCTC CCGTAGGAGT	D	*Bacteria*	0-30	46	3-5	Amann, Rudolf I., *et al.* (1990)
	FIAL	TCTTTGTCCA C TATCGTTTTG A	S	*Filifactor alocis*				This study

#	Probe name	Sequence (5′→3′)	Target		FISH conditions			
			Taxon	Microorganisms	% Form. (vol/vol)	Hyb. Temp. (°C)	Hyb. Time (hours)	Ref.
8	EUB338	GCT GCC TCC CGT AGG AGT	D	*Bacteria*	0-50	50	3	Amann, Rudolf I., *et al.* (1990)
	EUK502	ACC AGA CTT GCC CTC C		*Eukarya*	15-45			Amann, Rudolf I., *et al.* (1990)
	CAAL	GCC AAG GCT TAT ACT CGC T	S	*Candida albicans*	30			Kempf, Volkhard AJ, Karlheinz Trebesius, and Ingo B. Autenrieth (2000)
	STR493	GTT AGC CGT CCC TTT CTG G	G	most *Streptococcus* sp . and some *Lactococcus* sp.	0			Franks, Alison H., *et al.* (1998)
	LAB759	CTA CCC ATR CTT TCG AGC C		*Lactobacillus* sp., *Ruminococcaceae* sp. and *Pediococcus* sp.	35	46		This study
	Mmicros1435	GCC GCC GAT CTA ACC GCA	S	*Parvimonas micra*	0	50		Wildeboer-Veloo, A. C. M., *et al.* (2007)
	Sel1469	CCA GTC ACC TTC CCC ACC	G	*Selenomonas* sp. but not *S. sputigena*	30-50	46		This study
	SynA1409	ACA CCC GGC TCG GGT GGT		*Synergistetes* group A	40-50			This study
	ACT218	CGA GCC CAT CCC CCA CCA		*Actinomyces* sp.	0	50		This study
	Fus664	CTT GTA GTT CCG CYT ACC TC		most *Fusobacterium* sp.	40	46		Gmür, Rudolf, *et al.* (2004)
	Fnav1254	CTT CAC AGC TTT GCG ACT C	S	*F. naviforme, F. nucleatum subsp. fusiforme*	30			This study
	Fnuc133c	GTT GTC CCT ANC TGT GAG GC		*F. nucleatum, F. periodonticum*				Gmür, Rudolf, *et al.* (2004)
	SPIRO1400	CTC GGA TGG TGT GAC GGG CG	F	subgroup of the *Spirochaetaceae*	ND	50		Daly, Kristian, and Soraya P. Shirazi-Beechey (2003)
	TrepG1	GAT TCC ACC CCT ACA CTT	S	*T. medium and T. denticola*	20-50	46		This study

#	Probe name	Sequence (5′→3′)	Target		FISH conditions			
			Taxon	Microorganisms	% Form. (vol/vol)	Hyb. Temp. (°C)	Hyb. Time (hours)	Ref.
	Td469	CAT GAC TAC CGT CAT CAA AGA AGC		*T. denticola*	ND	50		Moter, Annette, *et al* (1998)
	Aa829	GGG CTA AAC CCC AAT CCC		*A. actinomycetemcomitans*				This study
	Camp655	CAT CTG CCT CTC CCT YAC	G	*Campylobacter* sp.	30	46		Gmür, Rudolf, and Helga Lüthi-Schaller (2007)
	CFB935	CCA CAT GTT CCT CCG CTT GT		*Cytophaga-Flavobacterium-Bacteroides cluster*	>40	50		Daly, Kristian, and Soraya P. Shirazi-Beechey (2003)
	BAC303	CCA ATG TGG GGG ACC TT	F	*Bacteroidaceae and Prevotellaceae*	0			Manz, Werner, *et al.* (1996)
	Prev394	GCA CGC TAC TTG GCT GG	G	*Prevotella* sp.	25	46		Diaz, Patricia I., *et al.* (2006)
	Pi425	CTT TAC TCC CCA ACA AAA GCA GTT TAC AA	S	*P. intermedia*	20			Sunde, Pia T., *et al.* (2003)
	Pnig657	TCC GCC TGC GCT GCG TGT A		*P. nigrescens*	≤ 40			Gmür, Rudolf, and Thomas Thurnheer (2002)
	Tfor582	GCG GAC TTA ACA GCC CAC CT		*T. forsythia*	40			Züger, Janine, Helga Lüthi-Schaller, and Rudolf Gmür (2007)
	Tfor440	CGT ATC TCA TTT TAT TCC CCT GTA		*T. forsythia*	20			Sunde, Pia T., *et al.* (2003)
	Tfor127	CTC TGT TGC GGG CAG GTT AC		*T. forsythia*	40			Züger, Janine, Helga Lüthi-Schaller,

#	Probe name	Sequence (5′→3′)	Target		FISH conditions			
			Taxon	Microorganisms	% Form. (vol/vol)	Hyb. Temp. (°C)	Hyb. Time (hours)	Ref.
								and Rudolf Gmür (2007)
	Tfor997	TCA CTC TCC GTC GTC TAC		*T. forsythia*	40			Züger, Janine, Helga Lüthi-Schaller, and Rudolf Gmür (2007)
	Pg477	CAA TAC TCG TAT CGC CCG TTA TTC		*P.gingivalis*	20			Sunde, Pia T., *et al.* (2003)
	Pendo740	CAG TGT CAG ACG GAG CCT		*P. endodontalis*	30-40			This study
	Capno371	TCA GTC TTC CGA CCA TTG	G	*Capnocytophaga*	0	50		Kampinga, G. A., *et al.* (2002)
9	Pint649	GCCGCCRC TGAASTCAAG CC	S	*Prevotella intermedia*	40	42	left overnight	Gmür and Thurnheer
10	EUB338	GCTGCCTC CCGTAGGAGT	D	*Bacteria*	20	46	2-4	Amann, Rudolf I., *et al.*
	Act381	CGTCGCTG CATCAGGCTT	P	*Actinobacteria*				This study
	Act692	CTGATATCT GCGCATTCC		*Actinobacteria*				This study
	Act476	ATCCAGCTA CCGTCAACC	G	*Actinomyces*				Gmür, Rudolf, and Helga Lüthi-Schaller.
	Cor595	CCGGAATTT CACAGACGA CG		*Corynebacterium*				This study
	Cor633	AGTTATGCC CGTATCGCCT G		*Corynebacterium*				This study
	Rot491	TAGCCGGC GCTTTCTCTG		*Rothia*				Valm AM, *et al.*
	Cmat175	ACTAAACCA TGGTCCTATC CG	S	*Corynebacterium matruchotii*				This study
	Cap371	TCAGTCTTC CGACCATTG	G	*Capnocytophaga*				Zijnge V, *et al.*
	Prv392	GCACGCTAC TTGGCTGG		*Prevotella*				Diaz PI, *et al.*
	Pg1160	CCTCACGCC TTACGACGG		*Porphyromonas*				Valm AM, *et al.*

#	Probe name	Sequence (5′→3′)	Target		FISH conditions			
			Taxon	Microorganisms	% Form. (vol/vol)	Hyb. Temp. (°C)	Hyb. Time (hours)	Ref.
	Str405	TAGCCGTC CCTTTCTGGT		*Streptococcus*				Paster BJ, Bartoszyk IM, Dewhirst FE
	Vei488	CCGTGGCT TTCTATTCCG		*Veillonella*				Chalmers NI, Palmer RJ Jr, Cisar JO, Kolenbrander PE
	Lmir444	TGGCACAG TCCTTTTCGT TCC	S	*Lautropia mirabilis*				This study
	Nei1030	CCTGTGTTA CGGCTCCCG	F	*Neisseriaceae*				Valm AM, *et al.*
	Pas111	TCCCAAGC ATTACTCACC		*Pasteurellaceae*				Valm AM, *et al*
	Fus714	GGCTTCCC CATCGGCATT	G	*Fusobacterium*				Valm AM, *et al.*
	Lep568	GCCTAGAT GCCCTTTATG		*Leptotrichia*				Valm AM, *et al*

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DECLARAÇÃO
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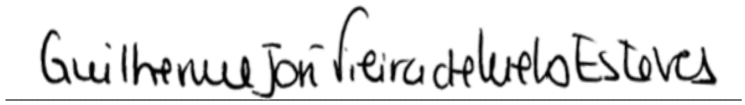

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