Contribution of fluorescence *in situ* hybridization (FISH) for the study of oral biofilms

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“And once the storm is over, you won’t remember how you made it through, how you managed to survive. You won’t even be sure, whether the storm is really over. But one thing is certain. When you come out of the storm, you won’t be the same person who walked in. That’s what this storm’s all about.”

Haruki Murakami

To Tiago and

Helena

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Abstract

The composition of the microbial community in the oral cavity is rich and highly diverse. Many of these microorganisms can associate to form the oral biofilm commonly called "*plaque*". This structure is a well-organized community of microorganisms agglomerated in a polysaccharide matrix and presents different stages of formation.

In the past, conventional culture methods have been used to characterize the oral microbiota; however, a large proportion of microbes remains uncultivable.

Because this limitation, culture-independent molecular-based approaches have been developed to allow a more comprehensive assessment of the presence of bacterial communities. One powerful technique used is fluorescence *in situ* hybridization (FISH).

Since its origins, this technique has become an invaluable tool and has spawned several diversifications from the original FISH protocol contributing to significantly improve our understanding of oral microbiology.

The aim of this review is to describe the contribution of FISH for the knowledge of the complex structure of oral biofilm. For that, a bibliographic search was performed in PubMed, Cochrane, ISI Web of Knowledge and Scopus using Medical Subject Heading (MeSH) terms.

In conclusion, as a consequence of the continuous improvements of FISH, there is the potential to elucidate the organization and microbial succession in biofilms and their interaction with the host and, therefore, to clarify the role of bacterial invasion in the etiopathogenesis of periodontal disease.

Keywords: Fluorescence *in situ* hybridization (FISH), PNA-FISH, CLASI-FISH, CLSM-FISH, EL-FISH, oral biofilm, oral microbiota, dental plaque.

Resumo

A composição da comunidade microbiana na cavidade oral é rica e altamente diversificada. Muitos desses microrganismos podem associar-se para formar o biofilme oral, comumente chamado de "placa". Esta estrutura é uma comunidade cooperativa, bem organizada, de microrganismos aglomerados em uma matriz polissacarídica e apresenta diferentes estágios de formação.

No passado, métodos convencionais de cultura foram utilizados para caracterizar a microbiota oral; no entanto, uma grande proporção de microrganismos permanece incultivável.

Devido a esta limitação, foram desenvolvidas abordagens moleculares independentes de cultura que fornecem uma avaliação mais abrangente da presença de comunidades bacterianas. Uma técnica poderosa usada é a hibridização fluorescente *in situ* (FISH).

Desde as suas origens, esta técnica tornou-se uma ferramenta inestimável e gerou várias variantes do protocolo FISH original, contribuindo significativamente para a nossa compreensão da microbiologia oral.

O objetivo desta revisão é descrever a contribuição da técnica de FISH para o conhecimento da complexa estrutura do biofilme oral. Para tal, foi realizada uma pesquisa bibliográfica no PubMed, Cochrane, ISI Web of Knowledge e Scopus, utilizando termos MeSH (Medical Subject Heading).

Em conclusão, como conseqüência da melhoria contínua do FISH, há potencial para elucidar a organização e a sucessão microbiana em biofilmes e sua interação com o hospedeiro e, assim, esclarecer o papel da invasão bacteriana na etiopatogénese da doença periodontal.

Palavras-chave: Hibridização fluorescente *in situ* (FISH), PNA-FISH, CLASI-FISH, CLSM-FISH, EL-FISH, biofilme oral, microbiota oral, placa dentária.

Contents

List of abbreviations

CLASI - Combinatorial Labelling and Spectral Imaging

CLSM - Confocal Laser Scanning Microscopy

CP - Chronic Periodontitis

DNA - Deoxyribonucleic Acid

EL - Element Labelling

ELISA - Enzyme-Linked Immunosorbent Assay

FISH - Fluorescence *in situ* Hybridization

GAP - Generalized Aggressive Periodontitis

LPS - Lipopolysaccharide

MeSH - Medical Subject Headings

NAMs - Nucleic Acid Mimics

NanoSIMS - Nanoscale Secondary Ion Mass Spectrometry

PCR – Polymerase Chain Reaction

NUG - Necrotizing Ulcerative Gingivitis

PNA - Peptide Nucleic Acid

qPCR - Quantitative Polymerase Chain Reaction

rRNA - Ribosomal Ribonucleic Acid

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Chapter I

Introduction

The oral cavity harbours a rich and diverse microbial population. Microbiological and molecular researches reported more than 700 species of bacteria that inhabit the oral cavity (1-3), attached on tooth surfaces, tongue, gingival sulcus, hard and soft palate, dental appliances, dental implants and mucous membranes (3).

The oral microbiota plays an important role in health, since it contributes to actively modulate the development of the immune system and protect against diseases (4, 5). Most oral bacteria are commensal species, but one subgroup consists of opportunistic pathogens (5, 6). The imbalance of the bacterial community contributes to the development of oral diseases such as caries, periodontitis or endodontic infections (7), and systemic diseases, such as cardiovascular disease, stroke, preterm birth, diabetes and pneumonia (3, 6, 7).

Over the past 50 years, the characterization and understanding of oral microbiota has undergone significant evolution (4). Research has led to the recognition of biofilm as a cooperative, well organized community of microorganisms agglomerated in a polysaccharide matrix that exhibits different stages of maturation (1, 5). This matrix provides protection against aggressive environment, resistance to the antibiotic and nutrients (1, 5). Studies of these communities may elucidate interactions among microbes, as well as host-microbe interactions of ecological and clinical relevance.

The culture-dependent methods for studying the oral microbiome are often selective, particularly for fastidious or yet-to-be cultured bacteria (8). The selectivity of the nutrient medium and culture conditions favours only a fraction of the bacterial community, which dramatically underestimates the microbial number and composition of the samples under study (9). Approximately 280 bacterial species from the oral cavity have been isolated in culture and formally named (3). It has been estimated that a third to a half of oral microbes cannot be cultivated using standard anaerobic media and

techniques (3, 6). Therefore, the introduction of molecular techniques in this field offered new opportunities for the analysis of the structure and the diversity of oral microbial species (3).

The most commonly used techniques include Polymerase Chain Reaction (PCR), DNA sequencing and DNA hybridization. Hybridization techniques comprise checkerboard DNA–DNA hybridization, DNA microarray technology and Fluorescence *in situ* Hybridization (FISH).

For the checkerboard DNA-DNA techniques, cultivable bacteria are needed in order to provide the genomic DNA to design the probes and lack of probes for unknown bacteria limits the use of DNA microarray technology (10).Regarding to PCR and DNA sequencing techniques, it is necessary to extract nucleic acids from bacteria. However, the bacterial cells are destroyed which means that the spatial organization of samples is disrupted. Consequently, important information about the location and function of microbes is lost.

Hence, the application of FISH has revolutionized our knowledge on microbial communities. The first application of FISH in microbiology was described in 1980 when Giovannoni and colleagues (11) used radioactively labeled rRNA-directed oligonucleotide probes for the microscopic detection of bacteria. Delong and co-workers (12) used oligonucleotide probes as fluorescent markers for detecting bacterial cells. The FISH technique combined with epifluorescence microscopy, confocal laser scanning microscopy (CLSM), allows an *in situ* detection and location of individual cells within their natural microhabitat (8).

Since its origins, FISH has become an invaluable tool for the detection/identification of microorganisms and many different variants have been developed to improve this technique's efficiency. An advance in FISH technology is the replacement of DNA/RNA probes with a class of DNA/RNA analogues, named nucleic acid mimics (NAMs), which have a higher affinity to complementary DNA or RNA (13).

In 1991, Nielsen and colleagues developed a new molecule, peptide nucleic acid (PNA), and it has been used in the detection of microorganisms (14)

since. Because of its neutral polyamide backbone, PNA molecules are uncharged DNA analogues that bind to nucleic acids much more strongly than oligonucleotides because there is no electrostatic repulsion between the PNA probe and the negatively charged sugar-phosphate backbone of the target molecule (15-17). Other NAMS such as LNA and 2'OME have also shown promising results in the identification/location of bacteria, mainly due to its great target affinity and biostability. They are both RNA mimics with modifications in the sugar-phosphate backbone: LNA has a ribonucleoside linked between the 2'-oxygen and the 4'-carbon atom with a methylene unit; and, 2'OME displays a C3´-endo furanose ring conformation (18). The NAM-FISH procedure also increases the possibility of simultaneous identification of different organisms (multiplex identification) using probes labelled with different fluorophores (19).

New variations of FISH techniques were applied to identify oral microbiome. Behrens and co-workers (20) applied Element Labelling FISH (EL-FISH) to microbial aggregates obtained from the gingival sulcus. The new method allowed to link identity and function of members of the natural microbial communities. Another recent variation, known as Combinatorial Labelling and Spectral Imaging FISH (CLASI–FISH), combines combinatorial labelling and spectral imaging (CLASI) with fluorescence *in situ* hybridization (FISH), a method in which fluorescent labels, or probes, are attached to specific DNA sequences. Conventional FISH imaging has limitations in the number of different fluorophores (up to 3) that could be used at one time by limiting bacteria taxa that can be identified in each sample. However, the CLASI-FISH technique overcome this problem by using many different fluorophores (6 or more) at once that greatly expand the number of different kinds of bacteria distinguishable in a field of view (21). The new technique allows researchers to discover highly organized bacterial consortia in dental plaque that may lead to diseases such as periodontitis (22).

The aim of this work is to review the contribution of fluorescence *in situ* hybridization to provide a better understanding of these bacterial communities of the oral cavity.

Chapter II

2.1. Literature search strategy and study selection

Electronic database search on PubMed, Cochrane, ISI Web of Knowledge and Scopus was performed using MeSH (Medical Subject Headings) terms – oral biofilm, oral microbiota, oral microbiome, oral bacteria, dental plaque, fluorescence *in situ* hybridization, FISH, FISH tecniques, PNA-FISH, CLASI-FISH, Multiplex-FISH, EL-FISH and CLSM-FISH. Furthermore, we included MeSH synonyms, related terms, and free terms. The Boolean operators "AND" and "OR" were applied to combine the keywords. The searches were complemented by screening the references of selected studies to find any study that did not appear in the database search. Articles published between years 1989-2019 were reviewed and were included based on inclusion and exclusion criteria.

2.2. Inclusion and exclusion criteria

The inclusion criteria were human studies and written in English. Excluded were case reports describing ecological studies and experimental animal studies. In addition, published studies showing repeated results from the same original study were also excluded.

2.3. Study selection

Initially, all literature from different databases was combined in EndNote Library. In this software, the "*Find Duplicates*" filter was applied and the duplicate studies were automatically identified. Redundant articles were excluded. In the next step, the articles were selected by title and abstracts according to the search strategy described previously. In those cases, in which the abstract and the title were not clear, the study was fully read in order to minimize the possibility of disregarding important studies. Finally, 101 articles were selected after applying the exclusion criteria (Figure 1).

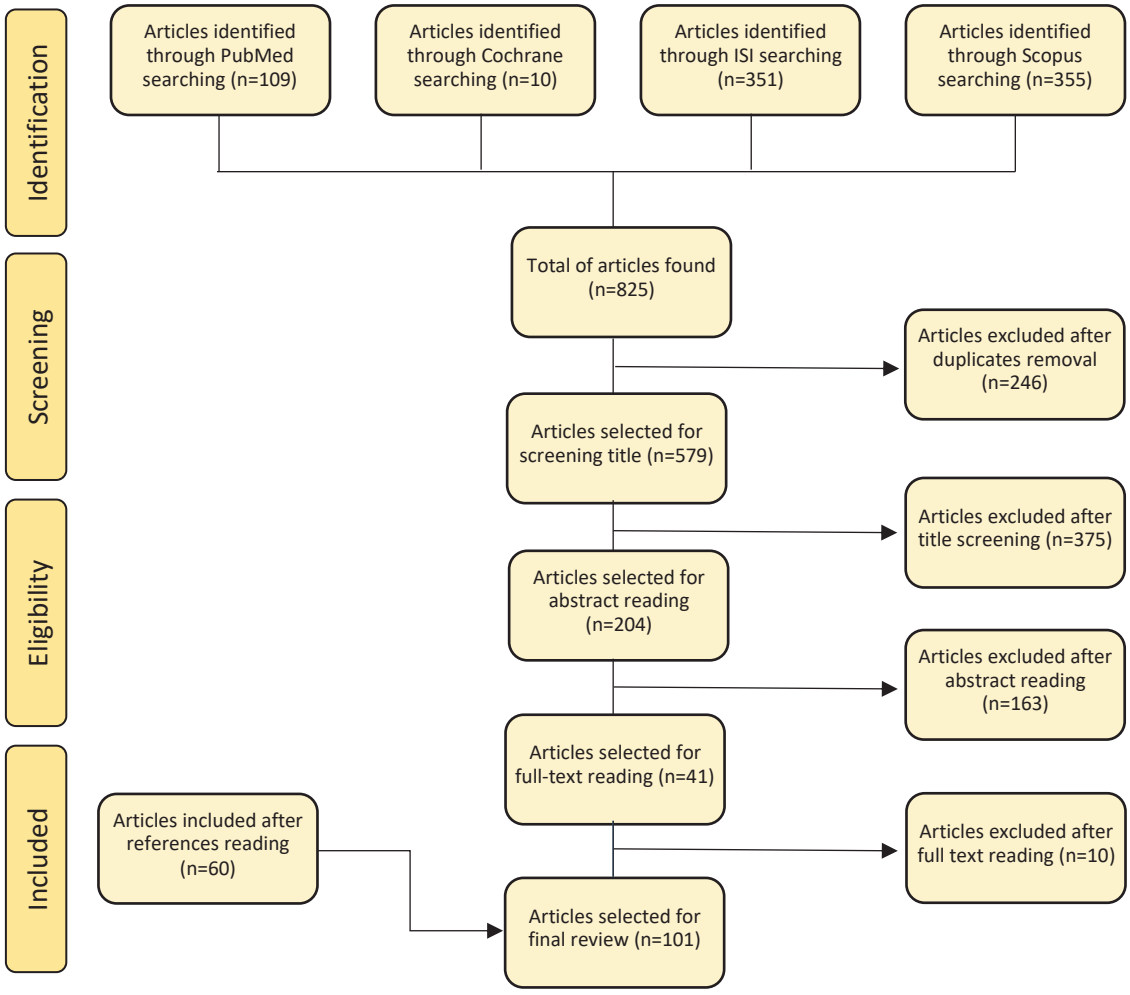

Figure 1 – Flow chart of literature search and study selection process.

Chapter III

3.1. Oral microbiota in health and disease

3.1.1. The healthy oral microbiota

Shortly after birth, the new-born comes in contact with a wide variety of microorganisms. Some of the commensal microorganisms are transient, while others become permanent colonizers. This simple community gradually develops into a highly diverse ecosystem during host growth (23).

During the first months of life, the microorganisms increases in quantity and diversity. With the eruption of deciduous teeth, new types of surfaces and microenvironments are provided (23, 24). Among the species that adhere with great affinity to teeth, we can find *Streptococcus sanguinis*, *Streptococcus mitis* and *Streptococcus oralis* (25), and some species of the genus *Actinomyces* spp. (1, 25).

The complexity of the microbiota increases until it reaches stability during childhood (24). Microorganisms are organized in two types of biofilm on the surface of the tooth: the supra-gingival plaque and the sub-gingival plaque, which differ significantly in the composition of the bacterial flora (23). Supra-gingival plaque is composed of Gram-positive bacteria such as *Streptococcus mutans*, *Streptococcus salivarius*, *S. mitis* and *Lactobacillus* spp., while sub-gingival plaque is formed by Gram-negative anaerobic bacteria, such as *Actinobacillus* spp, *Campylobacter* spp., *Fusubacterium nucleatum*, *Porphyromonas gingivalis* (23).

In the oral mucosa there are essentially facultative anaerobic cocci, with *S. mitis* being predominant. The presence of *Veillonella* spp., *Prevotella* spp. and *Haemophilus* spp. is also significantly observed. On dorsal surface of the tongue *S. salivarius*, *S. parasanguinis*, *N. flavescens* predominate, as well as some strict anaerobes, such as *Veillonella spp.* (7).

Studies have shown that Streptococci possess the ability to produce bacteriocins. For example, *S. salivarius* K12 produces the bacteriocins

salivaricin B and salivaricin A2 which shows inhibitory activity against *Streptococcus pyogenes* (26). Wescombe *et al.* (27) recognized the potential of *S. salivarius* to control diverse bacterial consortia infections including otitis media, for treatment of oral candidiasis and prevention of halitosis and dental caries.

Doel *et al*. (28) found a significant reduction in caries in patients with high salivary nitrate and high nitrate-reducing ability when compared to control subjects. The activity of orally ingested inorganic nitrate is thought to lie in its conversion to nitrite by facultative bacteria found on the dorsal surface of the tongue (29). The author suggested that responsible bacteria for nitrate reduction are Gram-negative *Veillonela* spp. and Gram-positive *Actinomyces* spp.

Kapil *et al.* (29) related nitrate metabolism with cardiovascular health. Oral bacteria reduce nitrate to nitrite which is taken up into the bloodstream *via* gastric absorption. Once within the circulation, nitrite is thought to be converted to the potent vasodilator nitric oxide (NO). NO is essential for vascular health and helps to keep blood vessels pliant and supple and thus has an anti-hypertensive effect (29).

The oral microbiota includes a group of bacteria common to most people; however, it should be regarded as in dynamic equilibrium with the host, alternating phases of greater or lesser stability depending on environmental conditions. Inter-individual variation in microbiota composition results from differences in the environment, genetics, age, and lifestyle of the host (24). The symbiotic relationship among microbiota and host is crucial for individual's oral health throughout life.

3.1.2. The dysbiosis on oral microbiota

In a healthy oral cavity, there is a homeostatic balance between the microbial community and the host. However, when this equilibrium is disturbed, a transition to a pathogen rich population occurs, activating the immune response of the host (30). This change in the microbiota, called dysbiosis, is the cause of oral microbial diseases (31).

There are multiple ways which can disturb the structure of the oral community, such as pH changes, alterations in oxygen tension, variability of the salivary flow, nutrient availability, dietary habits, smoking, immune status or long-term use of antibiotics which will cause a transition from the commensal resident flora to a population rich in agents which induces the immune response of the host (23, 31). Another changing factors such as poor oral hygiene, tooth extractions, caries cavities, dental fillings, dentures and sometimes loss of teeth may affect the ecosystem of the oral microbiota (23).

Several studies have demonstrated that the oral microbiota plays an important role in the pathogenesis and development of oral and systemic diseases.

Dental caries are one of the most common oral health diseases and result from the complex interaction between acid-producing bacteria and fermentable carbohydrates (32). The cause of dental caries is usually the supra-gingival microbiota (23). It was believed that *S. mutans* was a primary pathogen in the etiology of dental caries (7, 23). However, recent studies suggest that *S. mutans* may not be as dominant as it was previously supposed (23, 32). Aas *et al.* (33) found low levels or absence of *S. mutans* in several samples of dental caries and species such as *Veillonella* spp., *Lactobacillus* spp., *Bifidobacterium* spp., *Propionibacterium* spp., low-pH non-*S. mutans* streptococci, *Actinomyces* spp. and *Atopobium* spp. also may play an important role on caries progression. These results indicate that caries occur due to a shift in the balance of the resident microflora driven by changes in local environmental conditions (33).

The most common cause of gingivitis is the accumulation of dental plaque in tooth surface at the level of gingival margin. Gingivitis is characterized by clinical inflammation and bleeding confined to the gingival tissues (34). The sub-gingival microbiota is associated with gingivitis and periodontal disease (35). Plaque-induced gingivitis is characterized by the switch from Gram-positive aerobic to Gram-negative anaerobic bacteria. The predominant taxa associated with gingivitis include TM7, *Leptotrichia* spp*., Selenomonas* spp*.*, *Streptococcus* spp*.*, *Veillonella* spp*.*, *Prevotella* spp*.*, *Lautropia* spp., and *Haemophilus* spp.(32). The removal of plaque will result in a reversal to a

clinically healthy status. However, if gingivitis is not adequately treated, it may progress into periodontitis.

Periodontitis is a chronic inflammatory disorder, in which vascular proliferation and destruction of connective tissue and alveolar bone eventually leads to loss of teeth. *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* are associated with periodontitis. However, recent evidence suggests an ecological disruption of the commensal oral biofilm to a pathologic community mediated by "keystone pathogens" (30). These pathogens, as *P. gingivalis* have the ability to impair the host's immune surveillance elevating the virulence of the entire microbial community and causing a dysbiosis (30). Some studies have expanded the range of disease-associated organisms, including *Filifactor alocis*, *Peptostreptococcus stomatis*, *Prevotella* spp., *Synergistes* spp., *Megasphaera* spp., *Selenomonas* spp., and *Desulfobulbus* spp. (32). Kumar *et al.* (36) found an elevated number of *Filifactor* spp., *Peptostreptococcus* spp., *Megasphera* spp. and *Desulfobulbus* spp. in subjects with periodontitis. This suggests that periodontitis is the result of a global imbalance of the oral bacterial ecology rather than a disease-site specific microbial shift.

Periodontitis is often associated with patients with poorly controlled diabetes mellitus. Diabetes significantly contributes to the severity, prevalence, and progression of periodontal disease and also changes the oral environment, which results in a different periodontal bacteria community than that in non-diabetic subjects (37). Casarin *et al.* (38) found significant differences in subgingival microbiota between diabetic and non-diabetic subjects. Diabetic subjects presented higher percentages of total clones of TM7, *Aggregatibacter* spp., *Neisseria* spp., *Gemella* spp., *Eikenella* spp., *Selenomonas* spp., *Actinomyces* spp., *Capnocytophaga* spp., *Fusobacterium* spp., *Veillonella* spp. and *Streptococcus* genera, and lower percentages of *Porphyromonas* spp., *Filifactor* spp., *Eubacterium* spp., *Synergistetes* spp., *Tannerella* spp. and *Treponema* genera than non-diabetic individuals. Moreover, some phylotypes, including *F. nucleatum*, *V. parvula*, *V. dispar* and *E. corrodens*, were found more often in diabetic subjects than in non-diabetic subjects. Aly

et al. (39) reported higher levels of *Candida* in subjects with poorly controlled diabetes than in subjects with well-controlled diabetes or in healthy subjects.

Several studies have reported that there is an association between periodontitis, inflammation in the gingiva and cardiovascular diseases (e.g. as arteriosclerosis), regardless of the risk factors for these diseases, including obesity, smoking habits, hypercholesterolemia, hypertension and even diet. In a recent study, it was observed that an improvement in the health of the oral cavity results in a decrease in the progression of cardiovascular disease in humans (40). The study by Koren *et al.* (41) showed that the abundance of *Veillonella* spp. and *Streptococcus* spp. in atherosclerotic plaques was correlated with their abundance in the oral cavity. In another study, Ohki *et al.* (42) detected three species of periodontal bacteria in the thrombi of patients with acute myocardial infarction. The detection rates of periodontal bacteria by PCR were 19.7% for *A. actinomycetemcomitans*, 3.4% for *P. gingivalis*, and 2.3% for *T. denticola*. They suggested the possibility that such bacteria are latently present in plaque and that these bacteria might have a role in plaque inflammation and instability. Several mechanisms have been suggested to explain the relationship between periodontitis and cardiovascular diseases. Microorganisms access the circulatory system through oral tissue and make their way to arteries, where they secrete lipopolysaccharide (LPS) and inflammatory mediators, resulting in cardiovascular complications (32).

3.2. Methods for studying the oral microbiota

Typically, the identification and enumeration of microorganism has relied on three main types of analysis: phenotypic, immunological and genetic. Although phenotypic techniques are relatively inexpensive and easy to perform, they are time-consuming and are accompanied by certain limitations. The conventional culture-based methods use the ability of the bacteria to grow under artificial conditions using selective or differential media. For the identification of a microorganism it is necessary culture, colony counting steps, biochemical tests and Gram staining to characterize the bacteria in more detail. The drawback of this methodology are

microorganisms that are not able to grow in artificial media (43, 44). This limitation have encouraged the development of alternative techniques capable of allowing microbial identification in a more rapid and accurate way. Hence, molecular methods have revolutionized microbiological studies because they are more specific, precise and do not require prior culture of microorganisms. Molecular methods can be subdivided into immunological and nucleic acid-based ones.

The development of immunological technologies allowed a rapid detection and identification of microorganisms without culturing. Immunofluorescence techniques were applied to the identification of some bacterial species in plaque samples and gingival tissues (45, 46). Immunological methods rely on binding of antibodies to specific antigens in target bacteria (44). Usually used procedures are serological assays and enzyme-linked immunosorbent assay (ELISA). ELISA uses an enzyme-mediated colour change reaction to detect and often also quantify antibody binding as a measure of microbial presence. Immunological methods have the advantage of being non-dependent on the culture times, thus becoming faster and less expensive. However, these techniques have some limitations, such as reduced specificity and sensitivity due to the difficulty to generate selective antibodies and a large amount of antigens required for quantification (44).

Nucleic acid-based technologies include hybridization, PCR, sequencing and DNA microarray. These molecular methods use molecules that have highly conserved regions between different organisms and variable regions, specific for each one, to detect and identify microorganisms (47). A biomarker commonly used to infer identity from organisms is the ribosomal RNA molecule (rRNA). The rRNA is an integral part of the ribosome and it is considered an ideal biomarker (48). The 16S region composes the minor subunit of ribosomes present in prokaryotic organisms and sequence variation in 16S rRNA has been explored for inferring phylogenetic relationships among microorganisms, for designing specific nucleotide probes for detection of individual microbial groups in natural habitats, determining the genetic diversity of microbial communities and identifying non-cultivable microorganisms (49).

PCR was originally developed in 1983 by Kary Mullis and it is a method for amplifying a fragment of DNA quickly and accurately (50). Based on the natural process cell uses to replicate a new DNA strand, PCR assay requires the presence of a DNA template, primers (short stretches of DNA that initiate the PCR reaction), nucleotides (adenine, thymine, cytosine, and guanine), and DNA *Taq* polymerase (enzyme) (43, 51). There are three main stages that involve a process of heating and cooling called thermal cycling, which is done on an automated thermocycling apparatus: denaturing, anneling and extending. These processes are repeated 20 to 40 times to produce many copies of the DNA sequence of interest (43, 51). After the cycles, the PCR products are stained with a chemical dye and visualized in an agarose gel electrophoresis, which separates DNA products based on size and charge (51). This technique is very fast, sensitive and highly specific for microorganism whose sequence is already known. Several authors have used the PCR technique in the study of periodontal microbiota. Okada *et al.* (52) detected the presence of five putative periodontal pathogens in plaque samples from children. Kumar *et al.* (53) observed associations with chronic periodontitis for some new species or phylotypes, including non-cultivable clones: *Deferribacteres* spp. and *Bacteroides* spp., as well as the species called *E. saphenum*, *P. endodontalis*, P. *denticola* and *Cryptobacterium curtum*. Ashimoto *et al.* (54) determined the prevalence of eight putative periodontal pathogens in sub-gingival plaque of gingivitis and advanced periodontitis lesions.

Although PCR is an extremely sensitive technique, it has a number of limitations. Difficulties can be encountered when studying small quantities of DNA, limit capacity for multiplexing and susceptibility of the process to contamination (55). In order to overcome some of these limitations, variations of the original PCR technique have been developed. Multiplex PCR allows the amplification of several target regions, placing several primers sets in one single reaction (55). However, the use of different primers with the same annealing temperature and the production of amplicons with different molecular weights makes the optimization process slow and complex. Quantitative PCR (qPCR) allows the quantification of all DNA fragments detected by PCR using specific controls of known quantity. In fact, qPCR has

been used to identify some of the major periodontal pathogens such as *P. gingivalis*, *A. actinomycetemcomitans*, *T. denticola* and *T. forsythia* (55, 56). Nevertheless, qPCR has a limited capacity for multiplexing unknown species that cannot be detected.

On the other hand, sequencing technologies provide the most detailed information about microorganisms that are difficult to identify by conventional techniques and they are also used to reveal uncultivable microorganisms. The most common sequencing method is Sanger-based sequencing technology, but other techniques such as pyrosequencing and sing-cell approaches are alternatives for applications in microbiology (44). However, this technique is cost-intensive and time-consuming. Multiplex sequencing reduces the costs but decreases the sensitivity of the analysis, which might be an issue when pathogens have low abundance in the sample (44).

DNA–DNA hybridization technology has been performed to measure the degree of similarity between the genome of different species. The method is based on the hybridization between a probe (usually labelled with fluorescent or radioactive molecules) and the DNA target. A positive hybridization signal indicates the presence of target species (57). While the technique has advantages, it also has several important drawbacks such the need for high amounts of DNA of good quality, difficulty in comparing results between different laboratories (because small changes in experimental conditions give different results), non-discrimination between closely related species can occur due to cross hybridizations and the fact that it is a time-consuming procedure and labour-extensive (57, 58). However, these basic methods have the advantage of enabling the simultaneous detection of multiple species in a sample with the use of two or more specific probes (57).

Socransky *et al.* (59) developed checkerboard DNA-DNA hybridization technology for the detection of 40 bacterial species commonly found in the oral cavity. The assay uses whole genomic and DNA probes labelled with a molecule capable of producing a measurable signal (55). Several studies reported the use of checkboard DNA-DNA hybridization (33, 60-68) in the oral cavity. However, the method requires sophisticated laboratory equipment and expertise, and it is highly specific.

The microarray technology is a tool for global analysis of gene expression, and it allows the investigation of thousands of genes in a sample. It is frequently used to explore genome-wide transcriptional profiles. However, its applications have extended into the fields of environmental microbiology and microbial ecology (69). This technology is based on hybridization between labelled targets derived from biological samples and an array of many DNA probes immobilized on a solid matrix, representing the genes of interest (70). DNA microarray technology has been applied on the evaluation of genes involved in head and neck squamous cell carcinoma from the oral cavity (71-73) and has been used to study infectious diseases of the oral cavity (74, 75). This technique is medium-expensive and time-consuming. The drawbacks of this technique are associated with the limit of detection for organisms with low abundance and unknown species cannot be detected.

FISH proved to be as attractive method for rapid and reliable detection and identification of microorganisms. The classical FISH technique relies on a hybridization reaction between a specific fluorescent labelled probe and a complementary target RNA or DNA sequence (13), while preserves cell integrity and morphological details (8). The procedure includes the following steps: sample fixation, permeabilization, hybridization with the respective probes, detection and analysis of the target cell by epifluorescence microscope (13, 76). In fact, this technique has already used to visualize and locate species in oral diseases, such as periodontitis or caries (2, 77, 78). However, the most important contribution of the FISH technique is related with the *in situ* analyses of the spatial distribution of microorganisms in the supra- and sub-gingival biofilm (1), when FISH was combined with confocal analysis.

3.3. Showing the unknown using FISH technique

As already showed, FISH provides information about the morphology, number and spatial distribution of various microorganisms in dental plaque, without disturbing the samples. In addition, this method provides direct quantitative results with no need for prior culture (79). 16S rRNAs are present at a high

copy number in bacteria, are relatively stable and include both variable and highly conserved sequence domains (80).

Gersdorf *et al.* (46, 81) identify by FISH Gram-negative anaerobes, such as *Bacteroides forsythus*, *Porphyromonas gingivalis* and *Prevotella intermedia*, directly in subgingival plaque samples from patients with advanced periodontitis.

In another study, FISH was used to identify a diversity of cultivable and uncultivable spirochetes. Surprisingly, an unexpected diversity of uncultivable spirochetes (genus Treponema) was found in a subgingival plaque sample from a single patient with destructive periodontitis (73). The number and different morphologies of the identified oral spirochetes have significantly increased as a result of the FISH technique (8). Furthermore, organization and spatial arrangement of oral treponemes could be demonstrated in periodontal biofilms (78).

The candidate phylum TM7 is originally found in natural environmental habitats and is often associated with human inflammatory mucosal diseases. In a study by Brinig *et al.* (82), it was concluded that members of the TM7 are found in 96% of samples from the oral flora of both healthy and diseased sites. But although its prevalence, TM7 phylum remains recalcitrant to cultivation (3). FISH was used to visualize the biofilm architecture of the mixed-species communities while confirming the dominance *in situ* of streptococci in initial dental plaque bacterial populations (2). FISH also revealed that, in the undisturbed plaque, not only *Streptococcus* spp. but also the rarer *Prevotella* spp. were usually seen in small multigeneric clusters of cells (2).

With this technique, Kolenbrander and colleagues (83) concluded that spirochetes and gram-negative bacteria predominate in deeper regions of the pocket, whereas streptococci were abundant in the shallow regions. Fluorescence *in situ* hybridization and electron microscopy were performed to analyse the spatial arrangement of *Selenomonas* spp. in subgingival biofilms collected from patients with generalized aggressive periodontitis (GAP) (actual stage III, grade C, generalized periodontitis). In the samples from

patients, *Selenomonas* spp. showed a lower prevalence in both diseased groups compared with other putative pathogens, and a relatively high prevalence in the periodontitis-resistant group (84).

Using FISH, Zuger *et al.* (85) provided first evidence that uncultivable human *Tannerella* phylotypes consist of elongated filamentous and segmented rods which colonize both supra- and sub-gingival plaque in patients with severe inflammatory periodontal diseases. They are present only in low numbers, do not proliferate to high densities, and therefore are not considered relevant to disease development. According to Teles *et al.* (86) these findings illustrate two important points: phylogeny may not always be used to infer pathogenicity; and quantification is important to determine the relevance of oral microorganisms and their association with health and disease.

FISH have facilitated the observation of the several bacteria. One such organism is *Filifactor alocis*. *F alocis* is a fastidious, Gram-positive, obligate anaerobic rod found to be highly prevalent and abundant in patients with GAP (actual stage III, grade C, generalized periodontitis) and chronic periodontitis (CP) (actual stage II, grade B, generalized periodontitis) (87, 88). Schlafer *et al.* (88) suggested that this species might be involved in co-aggregation events during the establishment and maturation of the biofilms and, therefore, it may play an essential role in biofilm formation. The authors used dot blot hybridization to investigate the prevalence of *F. alocis* in subjects with GAP and CP. In addition, FISH was employed to analyse the spatial arrangement and the architectural role of *F. alocis* in periodontal pockets. The analysis was performed in 11 subgingival biofilms from patients with GAP. It was found that *F. alocis* seemingly prefers the apical parts of the pocket facing the soft tissue and is involved in numerous structural arrangements that point to its potential role as one of the architects of structural organisation within periodontal biofilms. *F. alocis* should be considered an important periodontal pathogen.

Synergistetes spp. is a novel bacterial phylum consisting of Gram-negative anaerobes (89), with evidence of presence in biofilms associated with periodontal and endodontic infections (34). Phylogenetically, the oral *Synergistetes* spp. are divided principally into cluster A and cluster B (90).

Using FISH for bacterial quantification Baumgartner *et al.* (34) found that *Synergistetes* cluster A bacteria were present at higher numbers in necrotizing ulcerative gingivitis (NUG) than in plaque-induced gingivitis. In another study, FISH was used to compare the presence and levels of *Synergistetes* clusters A and B, in saliva of patients with CP (actual stage III, grade B, generalized periodontitis), GAP (actual stage IV, grade C, generalized periodontitis) and non-periodontitis subjects. The authors demonstrated that this phylum, especially cluster A is associated with periodontal diseases and is found at higher prevalence, numbers, and proportions in saliva from patients with periodontitis than in non-periodontitis subjects (89).

3.4. Understanding complexity

Regarding the knowledge of the composition of the biofilm, this information is scarcer. Even when all bacteria can ultimately be cultured, which is quite unlikely, progress in the understanding of the ecology of complex microbial communities will still require studies on the activity and distribution of microbes directly in minimally disturbed samples (91).

The sample preparation for FISH method leads to destruction of the biofilm matrix and it cannot be utilized to study the dynamic changes occurring in live biofilms (92). The absence of detailed spatial information represents a fundamental gap in knowledge that precludes a full understanding of the assembly and interactions of complex microbial communities (22). To identify interactions between bacterial cells in biofilms it is necessary the use of non-destructive methods that provide information of the spatial distribution of individual species (8).

The introduction of confocal laser scanning microscopy (CLSM) combined with FISH has stimulated a renewed interest in studies of intact natural biofilms (93). In combination, this methodology has been established as a valuable tool for obtaining high-resolution images and three-dimensional non-invasive

visualization of cells and the computational reconstruction of mature biofilms without distortion of their structure of a variety of biological samples (94, 95).

Thurnheer *et al.* (95) applied the method to analyse the spatial distribution of a six-species biofilm formed *in vitro* (Figure 2). Thus, it has been shown that it is possible to perform multiple staining of gram-negative and gram-positive organisms simultaneously.

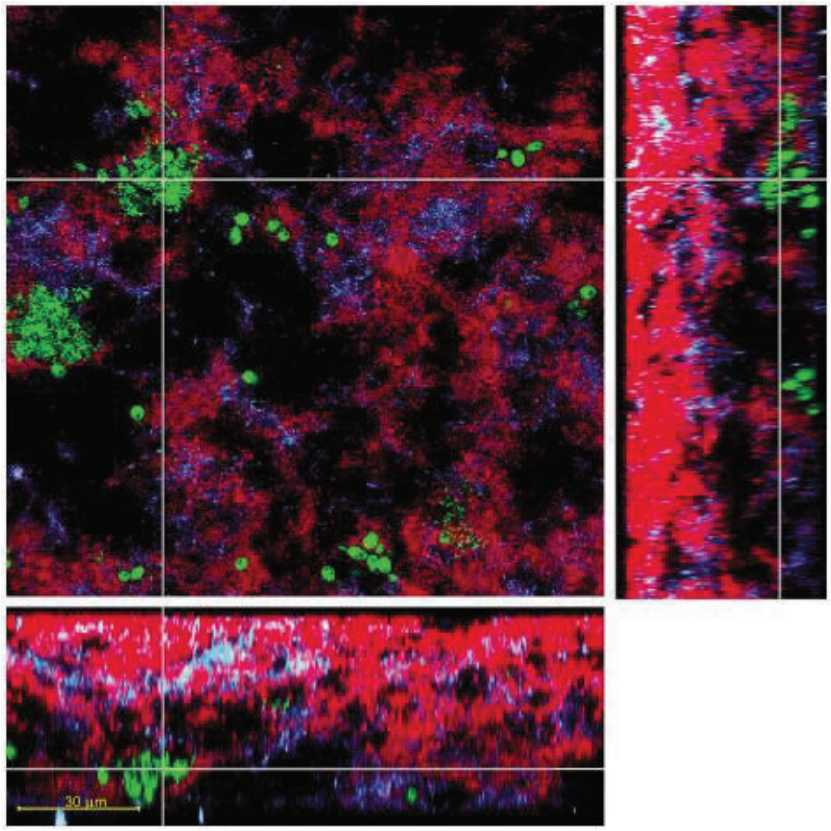

Figure 2 – CLSM images showing a single biofilm stained for all six bacteria species. (Thurnheer *et al.*, 2004)

Some studies of dental biofilms have taken advantage of this combination for studying temporal shifts in the bacterial composition of biofilm architecture *in vivo*. These studies have mainly focused on streptococci. For example, Palmer *et al.* (96) have shown for the first time *in vivo* that initial biofilm formation was the result of co-aggregation and adhesion between *Streptococcus* spp. and *Actinomyces* spp.

Al-Almand *et al.* (97) presented the results of a five-colour FISH of the microbial distribution and population dynamics. After seven days, the proportion of streptococci decreased and the proportion of *Fusobacterium nucleatum* increased in vivo dental biofilm.

Another study, which examined the initial plaque development in three humans wearing retrievable enamel chips, demonstrated that early colonization (after 4 and 8 h) was dominated by *Streptococcus* spp. belonging to the *Streptococcus oralis*/*Streptococcus mitis* group. Other commonly identified genera were *Actinomyces* spp., *Gemella* spp., *Granulicatella* spp., *Neisseria* spp., *Prevotella* spp., *Rothia* spp., and *Veillonella* spp. (2). The authors also proposed that, due to the repetitive and distinctive community composition within subjects, the spatiotemporal interactions and ecological shifts that accompany biofilm maturation also occur in a subject-dependent manner.

A study by Dige *et al.* (77) demonstrated the predominance of streptococci in biofilm during the first 6 to 48 h. The approach enables differentiation of streptococci from other bacteria, including their spatiotemporal organization in young developing biofilms. In a later study with the same technique, Dige *et al.* (98) highlighted the temporospatial relationship and the population dynamics of *Actinomyces* spp. relative to streptococci in the initial stages of biofilm formation. A notable observation of the study was the preferential colonization of *Actinomyces naeslundii* in the deeper regions of the biofilm.

The combination of FISH and CLSM technologies showed that dental biofilm, *in vivo*, is involved in interspecies interactions, which may be of importance in the establishment of functioning microbial communities (2, 96).

Despite these promising features, the classic FISH protocol suffers from some limitations. A major drawback is the often very low signal intensity (99). Apart from permeability issues (FISH requires mechanical disturbance of the cell in order to introduce probes), the main reason for weak fluorescence signals is the low ribosome content found in very small or metabolically inactive cells in environmental samples (99).

To partially solve this problem, researchers began to search for another way to improve the robustness of FISH methodologies using NAMs molecules (100). The development of PNA, that is NAMs, improved FISH methods and has opened new possibilities in many fields in microbiology research. PNA probes have been considered useful in overcoming the variable and sometimes insufficient penetration of probes into bacteria because the PNA molecule is of hydrophobic nature which facilitates cell penetration and diffusion through the biofilm matrix, hybridization could be performed efficiently under low salt concentrations. As a result, the secondary structures of the rRNA become destabilized thereby also improving access to the target sequences. Finally, because it is a synthetic molecule, it presents greater resistance to nucleases and cellular proteases (100). In oral microbiology, Mendes *et al.* (14) reported the development of highly-specific and sensitive PNA probes for the identification and localization of *Aggregatibacter actinomycetemcomintans* and *Porphyromonas gingivalis* in sub-gingival plaque and gingival biopsies. In their research, microorganisms can hybrid to their PNA probes simultaneously (PNA-FISH multiplex) thus making possible to detect microorganisms in the gingival and sub-gingival plaque samples from some patients who suffer from severe periodontitis.

Behrens *et al.* (20) developed a new approach to facilitate further studies of the ecophysiology of known and uncultured microorganisms in complex environment. The novel technique allows species identification and simultaneous analysis of metabolic activity of single cells through stable isotope labels. The method combined EL-FISH with Nanoscale Secondary Ion Mass Spectrometry (NanoSIMS) and was applied to study single cells in complex microbial communities obtained from the human gingival sulcus. Microbial aggregates from oral biofilms were sampled from the gingival sulcus of a healthy individual after two days without tooth brushing. The study provides information of the metabolic activity of single cells and offers insights into the distribution which can be applied to microbial activities in and among individual cells of probe-identified populations (20).

Valm *et al.* (101) have developed a variation of FISH, named Combinatorial Labelling and Spectral Imaging based on Fluorescence *in situ* Hybridization

(CLASI-FISH). In this variation, standard emission wavelengths were substituted by the whole fluorescence spectrum, giving a finer resolution to distinguish among markers even if their emissions overlap. They use eight fluorophores that can form 28 different colours. The newer methods allowed simultaneously identification of 15 different taxa in a human dental plaque sample (Figure 3). The community was dominated by early colonizers, including species of *Streptococcus*, *Prevotella*, *Actinomyces*, and *Veillonella*. The genera Prevotella and Actinomyces showed the most interspecies associations, suggesting a central role of these genera in establishing and maintaining biofilm complexity.

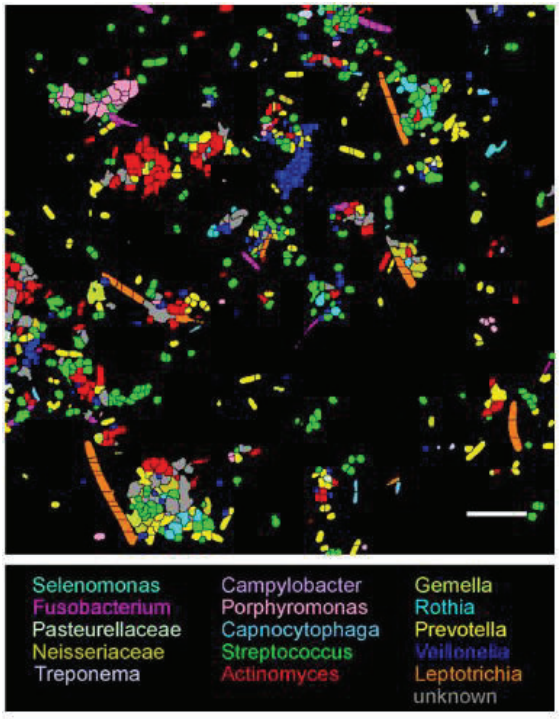

Figure 3 – Images of CLASI-FISH-labelled semi-dispersed human dental plaque. Color in the image represents one of each of the 15 probed taxa. Cells that are ambiguous in their label type because of errors in image segmentation are declared unknown and colored gray. (Valm *et al.,* 2012)

Welch and collaborators (22) employed CLASI-FISH to investigate the spatial organization of the oral biofilm samples as well as the microbiome on the tongue and other oral surfaces. They marked bacteria with up to ten different fluorophors. In dental plaque, they found a surprisingly complex, highly organized microbial consortium consisting of a radially arranged, nine-taxon

structure, around cells of filamentous *Corynebacterium* spp. Within the structure, individual taxa are localized at the micron scale in ways suggestive of their functional niche in the consortium. The nine taxa included *Corynebacterium* spp., *Streptococcus* spp., *Porphyromonas* spp., *Haemophilus* spp./*Aggregatibacter* spp., *Neisseriaceae* spp., *Fusobacterium* spp., *Leptotrichia* spp., *Capnocytophaga* spp., and *Actinomyces* spp.. The CLASI-FISH approach is another way to observe microbial communities to the micron scale and detects interspecific interactions and structural features.

The application of CLASI-FISH to the biofilm and the further increase in the number of labelled organisms allowed by the technique will help to unravel the microbial interactions in natural environment.

Chapter IV

Conclusion and future directions

FISH has proven to be a very useful tool for studies on oral microbiology. Through the use of specific probes, it is possible to identify different microorganisms in complex microbial communities, giving knowledge of inter-species interaction. In fact, the information about the spatial distribution of microbial populations allows the study of the contribution of each specie to the organization and their ecological function in the bacterial communities.

The use of FISH in the dental field provided improvements on the diagnosis, prevention and monitoring methods. Thus, this technique contributed to an understanding of structure, localization and biofilm formation and the role of individual bacterial species in the community.

Based on a literature review, there is still many work to be done to better understand the development of oral diseases so these can be controlled or treated successfully.

The application of the FISH technique *in vitro* can help to understand how oral infections can be controlled or treated. The method can also be applied to real samples, in patients under treatments. This way, there could be the possibility of monitoring the real evolution of the oral microbiota.

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Attachments

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DECLARAÇÃO

Declaro que o presente trabalho, no âmbito na Monografia de Investigação/Relatório de Atividade Clínica, integrado no MIMD, da FMDUP, é da minha autoria e todas as fontes foram devidamente referenciadas

A Investigadora

Izabela Luiza Reis Fonseca

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Porto, 17 de Maio de 2019

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Declaro que o Trabalho de Monografia desenvolvido pela estudante Izabela Luiza Reis Fonseca, do 5º ano do curso de Mestrado Integrado em Medicina Dentária da Universidade do Porto, subordinado ao tema: *"Contribution of fluorescence in situ hybridization (FISH) for the study of oral biofilms"* está de acordo com as regras estipuladas na FMDUP, foi por mim conferido e encontra-se em condições de ser apresentado em provas públicas.

A Orientadora

Prof.ª Doutora Luzia da Conceição Martins Mendes Gonçalves

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