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FULL PAPER

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

Sara Bernardi[1], Maria Adelaide Continenza[1], Ali Al-Ahmad[2], Lamprini Karygianni[3], Marie Follo[4], Andreas Filippi[5], Guido Macchiarelli[1]

[1]*Department of Life, Health and Environmental Sciences, University of L'Aquila, Italy;*
[2]*Department of Operative Dentistry and Periodontology, Center of Dental Medicine, Faculty of Medicine, University of Freiburg, Germany;*
[3]*Clinic for Preventive Dentistry, Periodontology and Cariology, Center of Dental Medicine University of Zurich, Switzerland;*
[4]*Department of Hematology, Oncology and Stem Cell Transplantation, Core Facility, Medical Center - University of Freiburg, Faculty of Medicine, University of Freiburg, Germany;*
[5]*Department of Oral Sugery, University Center for Dental Medicine Basel, University of Basel, Basel, Switzerland*

SUMMARY

The present study involved a qualitative and quantitative evaluation of tongue dorsum biofilms sampled from halitosis patients and healthy volunteers. The aim of the study was to quantify the distribution of *Streptococcus* spp. and *Fusobacterium nucleatum* within the oral halitosis biofilm in order to highlight the role of these bacterial members in halitosis. Tongue plaque samples from four halitosis-diagnosed patients and four healthy volunteers were analyzed and compared. The visualization and quantification of the tongue dorsum biofilm was performed combining fluorescence *in situ* hybridization (FISH) and confocal laser scanning microscopy (CLSM). *Eubacteria, Streptococcus* spp. and *Fusobacterium nucleatum* were stained using specific fluorescent probes. For a comparison of the two tested biofilm groups the Wilcoxon rank-sum test was used.

Morphological analysis by CLSM illustrated the distribution of the species which were tracked. *Streptococcus* spp. appeared to be enclosed within the samples and always associated to *F. nucleatum*. Furthermore, compared to the control group the biofilm within the halitosis group contained significantly higher proportions of *F. nucleatum* and *Streptococcus* spp., as revealed by the FISH and CLSM-analysis. The total microbial load and relative proportions of *F. nucleatum* and *Streptococcus* spp. can be considered as causative factors of halitosis and thus, as potential treatment targets.

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INTRODUCTION

The microbial biofilm is represented by a collection of microbial communities enclosed by a matrix of extracellular polymeric substance (EPS) and separated by a network of open water channels (Brown *et al*., 2015). The bacterial population within a biofilm is protected from environmental factors such as ultraviolet (UV) light and dehydration, in addition to host immune cells such as neutrophils and other phagocytes, as well as microbicidal substances (Hall-Stoodley *et al*., 2004). This highly hydrated shield is provided by the EPS, which is composed of polysaccharides and proteins (Flemming and Wingender, 2010). Moreover, bacteria within a biofilm exhibit a phenotype which is different from that of suspended bacterial cells of the same genotype (Dufour *et al*., 2010).

An oral biofilm which is still not well understood is the one coating the tongue, although various reports have associated its presence with halitosis in patients (Amou *et al*., 2014). The morphological structure of the dorsal tongue surface allows the formation of a unique and complex bacterial biofilm and tongue coating has been considered one of the most complex ecological biofilm niches in the mouth (Bernardi *et al.* 2013; Neu *et al.* 2014; Bernardi *et al*., 2016). The presence of such a tongue coating was found to be associated with overall enhanced bacterial load, as well as the presence of bacteria able to hydrolyze benzoyl-DL-arginine-naphthylamide, associated with the pathological subgingival plaque in case of periodontitis, on the tongues of halitosis patients (De Boever and Loesche, 1995). The anaerobic microorganisms mainly associated with volatile sulfur compounds (VSCs) were *Centipeda periodontii, Eikenella corrodens, Fusobacterium nucleatum, Fusobacterium periodonticum, Porphyromonas gingivalis, Prevotella melaninogenica, Prevotella intermedia, Solobacterium moorei, Tannerella forsythia and*

Key words:
Tongue dorsum biofilm, fluorescence *in situ* hybridization (FISH), confocal laser scanning microscopy (CLSM), oral bacteria, halitosis.

Corresponding author:
Sara Bernardi
E-mail: sara.bernardi@univaq.it

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Treponema denticola (Amou *et al*., 2014). In earlier studies *Streptococci* and *Fusobacterium nucleatum* were shown to be main members of oral biofilms, stressing their role for the structure and stability of the supragingival plaque. Furthermore, the proportion of these two components changes with increasing age of the oral biofilm towards *F. nucleatum* which correlates with increasing the anaerobic environment maturing process of the biofilm. Additionally, a comprehensive large-scale analysis of the oral biofilm regarding influences of the oral location inside the oral cavity revealed significant differences in the biofilm composition regarding *oral streptococci* and *F. nucleatum* (Karygianni *et al*., 2012). However, the focus of most studies regarding these components was on the supragingival oral biofilm. Hence, we focused in the present study on analyzing these two key microbial components of the halitosis biofilm using fluorescence in situ hybridization to clarify the role of *Streptococci* and *F. nucleatum* in the tongue coating biofilm without further destruction of its structure.

Brightfield microscopy was used initially for the study of adherent oral bacteria (Hartley *et al*., 1996). However, this method gave valid results only as long as the thickness of the samples was suitable. Interestingly, the study of biofilms has changed the methodological approach to improve the sensitivity of microscopical techniques in such biological samples. Electron microscopy methods can be challenging and produce artifacts resulting from sample preparation (Hannig *et al*., 2010, D'Ercole *et al*., 2015, Bernardi *et al*., 2018). Consequently, the three-dimensional reconstruction of biofilms remained limited (Neu *et al*., 2014). Confocal laser scanning microscopy (CLSM) has radically changed the structural investigation of microbiological samples since the 1990s. The key study for the use of this tool in the study of biofilms was performed by Lawrence in 1991 (Lawrence *et al*., 1991). The authors demonstrated the potential use of CLSM for monitoring microbial biofilms. Indeed, due to the three-dimensional characteristics and immobilization of cells within microbial biofilms, CLSM is ideal for visualizing a wide range of microbial biofilms. The combination of fluorescent *in situ* hybridization (FISH) and CLSM have been shown to be useful for visualization and quantification of key bacterial members of the initial and mature oral biofilm (Al-Ahmad *et al*., 2007; Hess *et al*., 2008; Al-Ahmad *et al*., 2009). As a result of a literature search using several database sources (MEDLINE, SCOPUS, EBSCO-Host), to date, a study of the human tongue dorsum biofilm of health and halitosis-affected subjects by means of imaging techniques has not been performed. Therefore, the aim of this pilot study was to visualize and quantify *Streptococcus* spp., as representatives of aerobic species, and *F. nucleatum*, as representatives of anaerobic species, within the halitosis biofilm samples by means of FISH and CLSM.

MATERIALS AND METHODS

Subjects and Samples

Since tongue-coating biofilm has been shown to be related to halitosis, four samples from healthy volunteers and four samples from halitosis-diagnosed patients were analyzed and compared. All subjects gave their written informed consent to the study protocol, which was reviewed and approved by the Ethics Committee of the Albert-Ludwigs-University of Freiburg (74/15). All procedures involving human participants were performed in accordance with the ethical standards of the institutional and/or national research committee as well as with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from all individuals included in the study.

The subjects were not enrolled in the study if any of the following criteria were present:

1) pregnancy or lactation,
2) severe systemic diseases,
3) use of any antibiotics within the past 30 days,
4) use of local antimicrobial mouth rinses such as chlorhexidine (CHX) within the last 30 days,
5) use of tongue brush and tongue scraper,
6) participation in another clinical study during the previous 3 months. All subjects kept their daily oral health practice (tooth brushing) during the study.

Halitosis in the subjects was assessed and confirmed by measuring exhaled air using a sulfide monitor (Halitmeter, Interscan Corporation, Chatsworth, CA, USA). Furthermore, an accurate medical and dental anamnesis and a periodontal clinical assessment was performed. Due to the controversial etiologic association between daily eating habits and halitosis (Migliaro *et al.,* 2011, Kim *et al.,* 2015), diet was not considered a primary parameter and was therefore not recorded in the medical history of the halitosis patients. The tongue dorsum biofilm was collected using a 0.1 ml sterile inoculating loop: the loop was rubbed in three different location of the middle surface of tongue dorsum. No air-drying was necessary to remove excessive saliva. The loop was placed in vials containing 0.75 ml reduced transfer fluid (RTF), and kept at -80°C prior to use. The visualization of the tongue dorsum biofilm was performed combining fluorescent *in situ* hybridization (FISH) and confocal laser scanning microscopy (CLSM).

Fluorescent in situ *hybridization (FISH) and confocal laser scanning microscopy (CLSM)*

The FISH technique was performed according to the protocol first described by Amman (1990) and modified by Al-Ahmad *et al*. (2007) (Amman 1990; Al-Ahmad *et al*., 2007). Briefly, the collected samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, 1.7 mM KH_2PO_4-5 mM Na_2HPO_4 with 0.15 M sodium chloride, pH 7.2) for 12 h at 4.8°C. After this initial fixation the specimens were washed with phosphate-buffered saline (PBS) and fixed again in an ethanol solution (50% in PBS, v/v) for 12 h. In order to minimize cell loss during the following hybridization and washing steps, the samples were embedded in agarose (PeQLab Biotechnologie GmbH, Munich, Germany). For this purpose, the fixed plaque materials were spotted onto microscope slides (Erie Scientific Company, Portsmouth, UK). The spotted samples were allowed to dry at 46°C. Afterwards, the slides were immersed in molten 0.5% agarose at 37°C for 3 s. The slides were then refrigerat ed at 4°C until the agarose had solidified. At this point the probes were washed twice with PBS, followed by incubation in a solution containing 7 mg of lysozyme per ml of 0.1 M Tris-HCl-5 mM EDTA, pH 7.2, for 10 min at 37.8°C, in order to permeabilize adherent cells.

Dehydration was carried out using a series of ethanol washes containing 50%, 80%, and 100% ethanol for 3 min each. The specimens were then incubated with the specific oligonucleotide probes at a concentration of 50 ng each per 20 ml of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 25% formamide (v/v), and 0.01% sodium dodecyl sulphate (w/v). All of the High Performance Liquid Chromatography (HPLC) purified oligonucleotide probes used in this study were synthesized commercially and 5'-end-labelled with different fluorochromes (Thermo Electron). The EUB 338 probe was used to visualize the entire bacterial population within the plaque specimen (Amman 1990; Al-Ahmad et al., 2007). The FUS 664 probe was used to visualize *F. nucleatum* and the STR 405 probe to target *Streptococcus* spp.

Image acquisition and quantification of bacterial targets

After washing, the labeled biofilms were analyzed by CLSM (Leica TCS SP2 AOBS) using a 63× water immersion objective (HCX PL APO/bd.BL 63.061.2 W; Leica) and a zoom setting of 1.7. Excitation of the FISH probes was carried out using the following wavelengths: 488 nm (fluorescein), 543nm (Cy3), and 633 nm (Cy5). Fluorescence emission of the probes was measured at the following wavelengths: 495–565 nm (fluorescein), 552-592 nm (Cy3), and 644-703 nm (Cy5). To minimize spectral overlap between the probes, confocal scanning was carried out sequentially for each image. The tongue dorsum biofilm was examined at three different locations. Within each area the thickest point was measured by determination of the upper and lower boundaries of the biofilm. This procedure was repeated twice so that a mean biofilm thickness could be determined from the three measurements. Biofilms were scanned from these three starting points, generating sections of a thickness of approximately 0.5 µm each at 2 µm intervals throughout the biofilm layers, in order to avoid overlaps.

The quantification of the two bacterial species in the confocal biofilm image stacks was performed using the image analysis program MetaMorph 6.3r7 (Molecular Devices Corporation). The EUB 338 (named *Eubacteria* to indicate all bacterial cells) corresponding fluorescent volume was set as 100% of bacterial biomass in the biofilm. All other targets were calculated as percentage of the biomass calculated by EUB 338. The program was used to calculate the biofilm composition from stacks of three-channel images by measuring voxel intensities. Fluorescence intensity thresholds were manually set for each of the fluorescent colors (Al-Ahmad *et al.,* 2007).

Statistical analysis

Three different locations from each biofilm sample taken from each patient were analyzed. The results of 12 analyzed biofilm fields which consisted of different sections were included in the statistical analysis. For a descriptive analysis median, mean and standard deviation (SD) were computed. Boxplots were used for graphical presentation of the data. For a comparison of the two tested groups the Wilcoxon rank-sum test was used. All calculations were done with STATA 14.1.

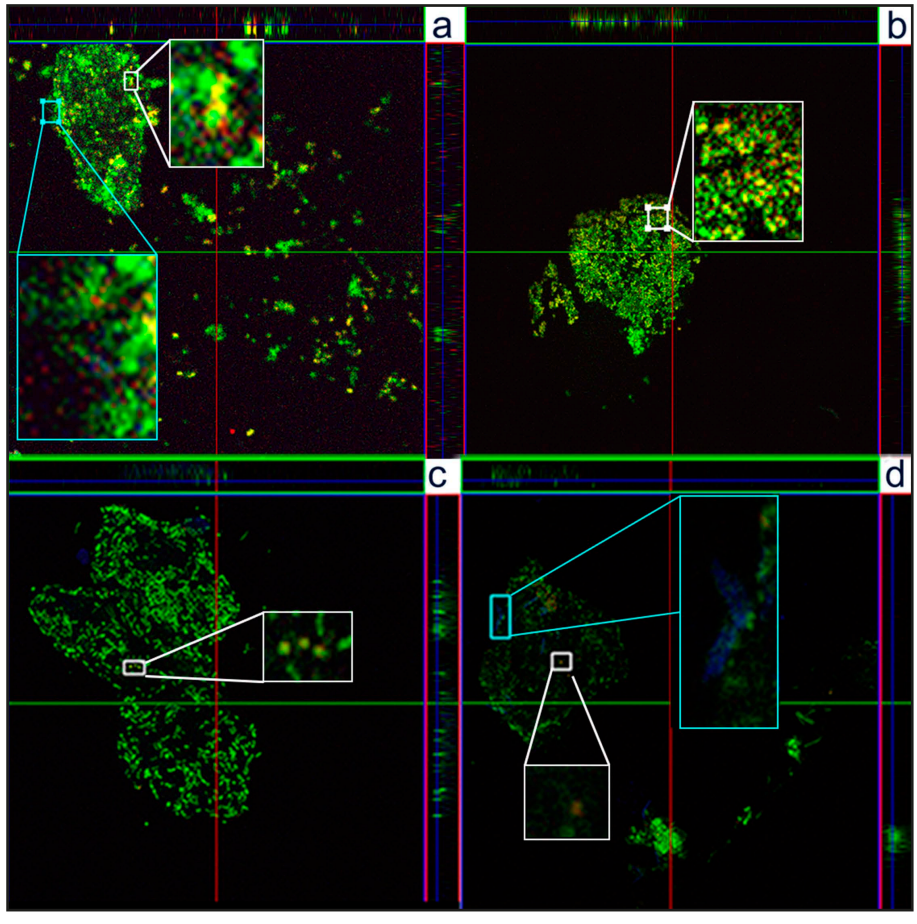

Figure 1 - *a.b. Representative imaging picture of halitosis samples: All bacterial cells (green), Streptococcus spp. (red), representatively framed in white and* F. nucleatum *(blue), framed in pale-blue. c.d.. Healthy Sample: All bacterial cells (green) and* Streptococcus *spp. (red), representatively framed in white and* F. nucleatum *(blue), framed in pale-blue. Standard images were made with a zoom setting of 1.7 corresponding to physical dimensions of 140x140 µm for each image (640x). The area of each section was transformed into a digital image containing 1024x1024 pixels. The magnifications of the areas within the rectangles are set at 1280x."*

RESULTS

Halitosis assessment results

The four halitosis patients reported values of VSCs part per billion (ppb) ranging from 122 ppb to 226 ppb, and with a Periodontal Screening and Recording (PSR) index scored as 1. For the four healthy volunteers, the Halimeters values were scored as 0 and the PRS index scored as 0.

Halitosis and health-related biofilms had a comparable distribution of Streptococcus spp. and F. nucleatum

Figure 1 (a-d) contains CLSM images of FISH-stained microorganisms in the sampled biofilms, allowing for a qualitative representation of the microflora distribution within halitosis- and health-related biofilms. FISH allowed us to visualize *Streptococcus* spp. (shown here in red), with its typical coccoid round shape, *F. nucleatum* (shown in blue), with its fusiform shape, and *all bacterial cells* (shown in green) with various configurations. In both groups, *Streptococcus* spp. and *F. nucleatum* were always detected in association with each other.

Despite their comparable thickness halitosis-related biofilms have a higher bacterial load of Streptococcus *spp. and* F. nucleatum *than healthy ones*

Figure 2 dotplot demonstrates the distribution of the FISH-targeted *Streptococcus* spp. and *F. nucleatum* within halitosis-related and healthy biofilms. A higher bacterial load of *Streptococcus* spp. and *F. nucleatum* could be exhibited within the halitosis-related biofilms compared to the health-related ones. Although the halitosis-related biofilm was as thick (7 μm) as the biofilm isolated from the healthy volunteers, it was more densely colonized by both *Streptococcus* spp. and *F. nucleatum*.

Halitosis-related biofilms may contain up to two or three times higher levels of Streptococcus *spp. and* F. nucleatum *than healthy biofilms, respectively*

Streptococcus spp. proportion in the halitosis group ranged between 1.6% and 73.2% (median: 7.2%) and was significantly higher ($p<0.0001$) than in the healthy group, in which the proportion of *Streptococcus* spp. ranged between 0.8% and 35.9% (median: 4.2%).

The frequency of *F. nucleatum* in the healthy group ranged between 0.8% and 6.7% (median: 2.8%). In the halitosis samples the *F. nucleatum* proportion ranged between 0.2% and 18.3% (median: 1.5%). In addition, the proportion of *F. nucleatum* in the halitosis group turned out to be significantly higher ($p < 0.0001$) than that of the healthy group.

DISCUSSION

Oral biofilm studies have in general focused on the internal distribution of the different bacterial species, the adhesion of the microbes on hard and soft oral tissues, as well as on biofilm formation processes (Hanning *et al*., 2010; Karygianni *et al*., 2012). Each of these characteristics helps us to understand biofilm formation *in situ* and to justify the use of oral care products and anti-microbial molecules. Indeed, consistent therapeutic results with regard to oral biofilm have been found to be achievable through a combination of mechanical and chemical cleaning. The use of antimicrobial products may result in the selection of resistant microorganisms, although this topic has not been studied in the field of halitosis research yet.

This study is the first to report on the combination of morphological and microbiological aspects of the tongue dorsum biofilm. The combined use of FISH and CLSM allows for visualization and quantification of two of the most important bacterial members of the tongue dorsum biofilm, which had not been reported to date.

If biofilm formation on hard oral tissues or artificial surfaces can be easily reproduced *in vitro*, the biofilms formed on soft tissues such as the tongue are more difficult to monitor and analyze, especially using common imaging techniques (Hanning *et al*., 2010). The visualization and quantification of microbial biofilms is crucial, due to the need to illustrate and understand initial bacterial adhesion to surfaces, as well as the distribution and position of the predominant bacterial species within the biofilm (Al-Ahmad *et al*., 2013).

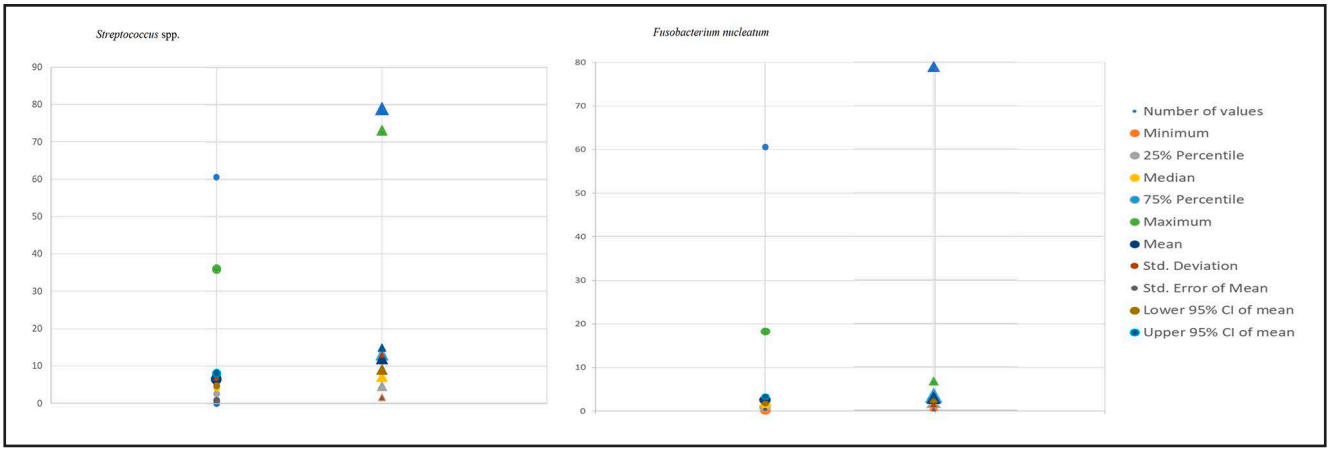

Figure 2 - *Dotplots demonstrating the distribution of* Streptococcus *spp, and* F. nucleatum*, as detected by FISH in halitosis and control tongue dorsum biofilms. The density of the bacterial presence is shown to be higher in the halitosis group than in the healthy volunteers' group. The percentages of bacterial cells stained with oligonucleotide probes specific* Streptococcus *spp. and for* F. nucleatum *were calculated in relation to all bacterial cells (stained with an oligonucleotide probes specific for* Eubacteria*).*

The tongue dorsum biofilm has been frequently studied for the investigation of particular features of tongue dorsal morphology. It has been found to contain primarily anaerobic bacteria (du Toit, 2003), and has been associated with periodontal pathogens (Allaker *et al*., 2008) and with pathologic situations such as halitosis (Loesche *et al*., 2002). The tongue coating is a visible white-brownish pellicle adhering to the dorsum of the tongue, together with desquamated epithelial cells, blood cells, metabolites, nutrients and bacteria. The tongue biofilm can vary in its composition, dependent on different factors such as age, salivary flow, oral hygiene, and periodontal status (Kullaa-Mikkonen, Järvinen 1998). The presence of a tongue coating has been reported to be normal in both healthy, gingivitis and periodontitis patients, where a thin coating was spotted in 40% of the patients and a thick coating in 52% (Mantilla Gómez *et al*., 2001). The degree of tongue coating has been quantitatively evaluated by the use of different indexes: Miyazaki *et al*. (1995) scored tongue coatings as present or absent in three areas, while no indication of thickness was registered (Miyazaki *et al*., 1995). Roldán *et al*. (2003) divided the tongue into six areas, scoring each one independently as 0 (no coating), 1 (light coating), and 2 (heavy coating) (Roldan *et al*., 2003). The final value of this so-called Winkel Tongue Coating Index is obtained by adding all six scores together.

With its large surface area, the tongue microflora represents a unique niche within the oral cavity. The dorso-posterior surface of the tongue hosts a high biomass of adherent bacteria, typically reaching 10^9 or 10^{10} colony forming units (CFU) per cm^2 (Hartley *et al*., 1996). In particular, the microbial population related to oral malodor has been found to be located in the zone from the dorsal posterior to the circumvallatae papillae, a region that cannot be easily reached by regular oral hygiene procedures (Allaker *et al*., 2008). Even though it has been shown that the microbial population of the tongue is highly diverse, some anaerobic species such as *Prevotella intermedia* and *Fusobacterium nucleatum* are known to be associated with the production of volatile sulfur compounds, which are responsible for bad oral breath (Amou *et al*., 2014; Krespi *et al*., 2006). The techniques used for biofilm analysis in previous reports have always focused on the microbial and biochemical points of view, using culture techniques, molecular techniques, air exhalation monitors and models (Hess *et al*., 2008; Flemming and Wingender, 2010; Bollen and Beikler, 2012; Becker *et al*., 2002; Anesti *et al*., 2005; Ademovski *et al*., 2012). High-resolution microscopy techniques allow a detailed insight into the smallest niches of bacterial biofilms and their surrounding environment (Hannig *et al*., 2010). In particular, the use of CLSM in combination with FISH has been particularly useful for microbial biofilm visualization (Neu *et al*., 2014). CLSM allows for the visualization of thick microbial plaque samples, the elimination of out of focus haze and a three-dimensional digital reconstruction of the biofilm from the optical sections (Lawrence *et al*., 1991). Fully hydrated living biological samples can be examined using this microscopy technique, and if it is used in combination with fluorescent stains, even initial bacterial adhesion can be visualized.

The use of other microscopy techniques for biofilm ultrastructure analysis can be more time-intensive and modify the native structure of the biofilm to a greater extent than CLSM. Nevertheless, the analysis of biofilm morphology is fundamental to identify contributing components or to perform an *in situ* quantitative analysis of the sample. Indeed, due to the intrinsic difficulty of sampling biofilm and its fragility, biofilms have primarily been visualized whether *in vivo, in situ, or in vitro,* by exploiting different types of surfaces. The combination of FISH and CLSM has been shown to be one of the best available methods for studying oral biofilm formed *in situ,* without necessitating the destruction of its native structure (Karygianni *et al*., 2014). FISH allows for molecular characterization and reveals the localization of the tracked bacterial species within a microbial biofilm, as well as how they coexist immersed in the common oral microflora (Al-Ahmad *et al*., 2007).

In the present study the use of FISH/CLSM has allowed us to visualize and quantitatively describe the composition of tongue dorsum biofilm, and to compare biofilm from healthy subjects and halitosis-suffering patients. Even though the parameter of the region of the tongue of the sampling was not annotated due to difficulties in accurately identify the area and in repeating the procedure in all of the subjects, such data could give more accurate information, as shown by a previous own study on the supragingival oral biofilm from different sites within the oral cavity (Karygianni *et al*., 2012). This point should be considered in future studies. As early colonizers *Streptococcus* spp. seem to be the most prevalent among all bacterial species in the samples and reside within the eubacteria in association with *F. nucleatum,* which serves as a bridge between early and late colonizers within the oral biofilm. The higher proportions of streptococci in the halitosis biofilm samples stress the dense structure of dorsum tongue biofilm. This density may contribute to an anaerobic environment which itself increases the fraction of *Fusobacterium nucleatum* which has been shown to be associated with halitosis. Furthermore, the halitosis samples also showed a greater microbial presence of both type species than the healthy controls. This last finding confirms the data in the literature, suggesting that *F. nucleatum* is a microorganism producing volatile sulfur compounds in halitosis patients. Furthermore, the association of *F. nucleatum* with the biofilm samples from the tongue dorsum indicates a shift of the microbial population towards anaerobes in halitosis patients. These results have to be confirmed in future studies, which should include a higher number of patients. Within the limitation of this study, these findings help to better understand the composition of associated microorganisms in sampled flocs of ecological niches present within the tongue dorsum biofilm in halitosis patients, aiding the improvement of current mechanical and chemical therapies for halitosis. Further molecular analysis on a larger size of samples is required to confirm the data regarding the microbial population on tongue biofilm.

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