

Combining culture and culture-independent methods reveals new microbial composition of halitosis patients' tongue biofilm

Sara Bernardi¹ | Lamprini Karygianni² | Andreas Filippi³ |
Annette Carola Anderson⁴ | Andrea Zürcher³ | Elmar Hellwig⁴ | Kirstin Vach⁵ |
Guido Macchiarelli¹ | Ali Al-Ahmad⁴

¹Department of Life, Health and Environmental Sciences, University of L'Aquila, L'Aquila, Italy

²Clinic for Preventive Dentistry, Periodontology and Cariology, University of Zurich, Zurich, Switzerland

³Department of Oral Surgery, Center of Dental Traumatology, University Center for Dental Medicine Basel UZB, Basel, Switzerland

⁴Department of Operative Dentistry and Periodontology, Center for Dental Medicine, Faculty of Medicine, University of Freiburg, Freiburg, Germany

⁵Institute for Medical Biometry and Statistics, University of Freiburg, Freiburg, Germany

Correspondence

Sara Bernardi, Molecular and Ultrastructure Imaging European Ph.D., Dept. Of Life, Health and Environmental Sciences, University of L'Aquila, Via Vetoio, Coppito 2, 67100, L'Aquila, Italy.
Email: sara.bernardi@univaq.it

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Abstract

Background: Oral malodor is a very discomforting condition deriving from the presence of volatile sulfur compounds in the expired air. In halitosis of intraoral etiology, the volatile sulfur compounds are metabolic products of the oral microorganisms within the biofilm coating the tongue dorsum as well as other tissues in the oral cavity. The aim of this study was to characterize and compare the microbial composition of tongue biofilm in volunteers suffering from halitosis and healthy volunteers by means of both the culture method and culture-independent cloning technique.

Results: A high bacterial variety (more than 80 different species) was detected using the combination of both methods. A distinct bacterial composition was revealed in the halitosis-associated biofilms compared with the health-associated biofilms. *Actinomyces graevenitzii* was shown to be significantly associated with the halitosis condition. The culture method identified 47 species, included *Veillonella rogosae*, never isolated from the tongue biofilm of halitosis patients so far. In the healthy condition, the culture-dependent method showed that the most frequent species were *Streptococcus parasanguinis* among the aerobes and *Veillonella* spp. among the anaerobes. The culture-independent cloning method detected more than 50 species. *Streptococci*, in particular *S. mitis/oralis*, *S. pseudopneumoniae*, and *S. infantis* as well as *Prevotella* spp., were found most frequently in halitosis patients. *Streptococcus salivarius* and *Rothia mucilaginosa* were found more frequently in the healthy condition.

Conclusions: The combination of the culture-dependent and culture-independent cloning techniques allowed for a widespread analysis of the tongue biofilm in halitosis patients. The results can support further pharmacological research for new antimicrobial agents and halitosis therapy strategies.

KEYWORDS

culture-independent cloning technique, halitosis, microbial culture, tongue biofilm

The present study was carried out at the Department of Operative Dentistry and Periodontology, Faculty of Medicine, Center for Dental Medicine, University of Freiburg, and at the Department of Oral Surgery, Oral Radiology and Oral Medicine and Centre of Dental Traumatology, University of Basel.

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1 | INTRODUCTION

Halitosis is widely known as malodor deriving from exhaled breath due to the presence of volatile sulfur compounds (VSCs) arising from the oral cavity or from the upper airways (Scully & Greenman, 2008). The VSCs include hydrogen sulfide, methyl mercaptan, and dimethyl sulfide (Scully & Porter, 2008). The volatile products causing intraoral halitosis derive from the interaction of oral microbiota with specific substrates, such as the amino acid cysteine, methionine, tryptophan, arginine, and lysine that are metabolized into the different VSCs (Dzink & Socransky, 1990).

Clinical halitosis is classified according to the primary source. We can therefore distinguish between intraoral halitosis, with the oral cavity as etiological source, and extraoral halitosis, usually a symptom of a pathological disease (Tangerman & Winkel, 2010), such as an organ dysfunction or systemic disease. In that context, respiratory disorders or respiratory tract inflammations, as well as diseases of the gastrointestinal system, can result in the release of smelly gases within the oral cavity and the nose. Concerning the gastrointestinal apparatus, gastroesophageal reflux disease (GERD) and *Helicobacter pylori*-related diseases are also associated with bad breath. Systemic diseases such as diabetes, renal failure, liver disease, trimethylaminuria, hypermethioninemia, and cystinosis can also have a specific malodor as a clinical manifestation (Madhushankari et al., 2015; Scully & Porter, 2008; Tangerman & Winkel, 2010).

The organoleptic difference between the intraoral and extraoral halitosis consists in the composition of the VSCs. Indeed, hydrogen sulfide and methyl mercaptan have been found to be the main contributors to intraoral halitosis, whereas dimethyl sulfide is more associated with extraoral, "blood-borne" halitosis (Tangerman & Winkel, 2010). Intraoral halitosis is associated with periodontal diseases, poor oral hygiene, salivary flow alterations, cancerous lesions, and bone necrosis (Dzink & Socransky, 1990). It is etiologically related to the microbiota of the dorsal tongue biofilm (Roldán, Herrera, & Sanz, 2003; Yaegaki & Coil, 2000), and in particular to the presence of anaerobic microorganisms responsible for the production of VSCs, such as *Centipeda periodontii*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *F. periodonticum*, *Porphyromonas gingivalis*, *Prevotella melaninogenica*, *P. intermedia*, *Solobacterium moorei*, *Tannerella forsythia*, and *Treponema denticola*. Due to its papillary structure that creates an ecological niche for microorganisms, the tongue biofilm represents an oral microenvironment which is well distinguished from the supragingival biofilm, also known as dental plaque, and the subgingival biofilm (Amou, Hinode, Yoshioka, & Grenier, 2014; Bernardi et al., 2018; Bernardi, Marzo, & Continenza, 2016; Bernardi, Zeka, Mummolo, Marzo, & Continenza, 2013).

To date, the halitosis-relevant literature comprises many studies on the microbial characterization of the biofilm using *in vitro* models, culture technique, species-specific PCR (Brunner, Kurmann, & Filippi, 2010; Mashima, Kamaguchi, & Nakazawa, 2011), confocal laser scanning microscopy study (Bernardi et al., 2019), and quantitative PCR assays (Vancauwenberghe et al., 2013), allowing for the study of the targeted species, as well as a few studies applying

high-throughput sequencing to tongue biofilm (Hall et al., 2017; Ren et al., 2016; Seerangaiyan et al., 2017).

Up to now, over 300 bacterial species have been found inhabiting the tongue (Yang et al., 2013), revealing a high bacterial diversity within this biofilm (Mashima et al., 2011; Mashima & Nakazawa, 2013; Vancauwenberghe et al., 2013).

The aim of this study was to characterize the *in vivo* biofilm on the dorsal tongue surface combining molecular and culture techniques in healthy volunteers and halitosis patients, in order to understand which microbial taxa contribute to the halitosis-associated tongue biofilm. So far, this combination of methods has not been used to study this particular biofilm. The open-end approach of the molecular cloning technique in addition to the culture method represents a valid contribution to the research in this field.

2 | METHODS

2.1 | Subjects and samples

According to the study protocol six patients affected by oral malodor and six healthy volunteers were recruited. The presence of halitosis was assessed by the instrumental measurement of exhaled air, using a sulfide monitor (Halimeter, manufactured by Interscan Corporation). Furthermore, the medical history and dental history were comprehensively checked as well as periodontal clinical investigations performed: Periodontal probing and gingival bleeding were assessed. Subsequently, the tongue dorsum biofilm was collected using 0.1 ml sterile inoculating loops. The sampling was performed with two loops. The pooled samples were divided and stored in two vials containing 0.75 ml Reduced Transfer Fluid (RTF) (Syed & Loesche, 1972) and kept at -80°C prior to use.

2.2 | Clinical halitosis assessment

A total of 12 patients and volunteers were recruited at the Dental Clinic of the University of Basel, Switzerland. The patients included in the study suffered from intraoral halitosis. The exclusion criteria were (a) presence of extraoral halitosis, (b) diagnosis of a mental illness, (c) patients aged under 18 years, (d) the intake of antibiotics in the previous 3 months before the start of the study and/or the use of antiseptics one month before study start, and (e) poor general health with reference to American Society of Anesthesiologists Physical Classification System. Prior to the sampling procedure, a general medical history questionnaire was submitted to the participants of the study (Table 1). The periodontal status of each participant was then assessed and documented, using the Periodontal Screening and Recording (PSR) Index, recommended by the American Dental Association as an established stage of oral diagnostic examinations for all dental patients (Periodontology, 1993). The presence of VSCs was determined by means of a Halimeter (Brunner et al., 2010), and the results were recorded. Lastly, the tongue dorsum biofilm samples were collected as described above.

TABLE 1 Anamnestic questionnaire

Patient number
Anamnestic questionnaire
Age
Gender
Current health Status
Do You suffer from chronic gastroesophageal reflux?
Do You suffer from diabetes?
Do You suffer from renal disease (chronic kidney failure)?
Did You undergo antibiotic treatment during the last 3 months?
If so, do You remember the medication?
Habits
Do You drink alcohol regularly? (more than three times a week)
Do You smoke?
Do You brush Your tongue? If yes, with what frequency?
Periodontal health status
Does the patient wear a removable prosthetic device?
Number of present teeth
Number of missing teeth
PSR INDEX
PSR™
Code 0 indicated periodontal health (neither bleeding on probing nor defective restoration margins and gingival sulcus depths <3.5 mm)
Code 1 indicated bleeding on probing, no defective restoration margins and a gingival sulcus depth <3.5 mm at a minimum of one site within the sextant
Code 2 indicated bleeding on probing, the presence of supra- or subgingival calculus, defective restoration margins, and a gingival sulcus depth <3.5 mm at a minimum of one site within the sextant
Code 3 indicated bleeding on probing and a pocket depth of 3.5–5.5 mm at a minimum of one site within the sextant
Code 4 indicated that a pocket depth >5.5 mm was present at a minimum of one site within the sextant (American Dental Association and American Academy of Periodontology, 1992)
VSCs Analysis result

2.3 | Culture method

The culture method was performed as described in detail previously (Schirrmeister et al., 2009). The vials containing the samples in RTF were thawed at 36°C in a water bath and vortexed for 30–45 s. For the isolation and identification of the microorganisms, 100 µl of the undiluted sample and serial dilutions thereof were cultivated. The serial dilutions (10^{-1} – 10^{-7}) were prepared in peptone yeast medium (PY). Each dilution was plated on yeast cysteine blood agar plates (HCB) to cultivate anaerobic bacteria at 37°C for 10 days and on Columbia blood agar plates (CBA), incubated at 37°C and 5%–10% CO₂ atmosphere for 5 days to cultivate aerobic species. The resulting colony types were phenotypically evaluated and counted to calculate the number of colony-forming units (CFUs) per ml in the

original sample. All colony types were subcultivated to obtain pure cultures which were analyzed by MALDI-TOF (MALDI Biotyper, Bruker Daltonik GmbH), as described in detail by our own group (Anderson et al., 2014).

2.4 | DNA Isolation

The biofilm samples were centrifuged at 16,000 g for 10 min, and the supernatant was discarded. Lysis of microbial cells was then performed using a Precellys 24 bead mill homogenizer (PEQLab Biotechnologie GmbH) in ATL buffer (QiaAMP Micro Kit; Qiagen, Hilden). The vials were shaken twice at 3,500 rpm for 30 s. The DNA was subsequently purified by means of QiaAMP Micro Kit (Qiagen, Hilden) according to the manufacturer's protocol for tissue samples. The total microbial DNA was eluted twice with 50 µl AE buffer (Qiagen) and then stored at –20°C.

2.5 | PCR Amplification of 16S rRNA Genes

Bacterial 16S rRNA genes were amplified using the following universal primers: 27F-YM (5'-AGAGTTTGATYMTGGCTCAG-3') and 1492R (5' TACGGYTACCTTGTTACGACTT-3') (Frank et al., 2008). The PCR amplification was performed in a total volume of 50 µl. The reaction mixture contained 1 × PCR buffer (Qiagen), 0.2 mM each of the four deoxyribonucleoside triphosphates (dNTPs; PEQLab Biotechnologie), 0.5 µM of forward and reverse primers, 2 µl Utaq-Polymerase (Qiagen), and 5 µl of the isolated sample DNA. The PCR cycling conditions consisted of a denaturation step at 94°C for 2 min, followed by 35 cycles with denaturation at 94°C for 1 min; annealing at 55°C for 1 min; extension at 72°C for 1.5 min; and a final extension step at 72°C for 10 min.

A no-template control and a positive control were included in each set of PCRs. PCR reaction products were analyzed by electrophoresis in a 1.5% agarose gel and positive reactions were used to prepare clone libraries.

2.6 | Cloning of PCR products and analysis of clone libraries

The 16S rDNA amplification products were ligated into the PCR[®]2.1-TOPO[®] plasmid vector using the TOPO TA Cloning[®] Kit (Invitrogen, Life Technologies) according to the manufacturer's protocol and as described in detail earlier (Anderson et al., 2012). Fifty white clones from each library were picked, and the presence of inserts was confirmed by PCR amplification with their respective primers, followed by gel electrophoresis. PCR products of all recombinants were subjected to a restriction enzyme digestion with Hha I, Rsa I, and Hinf I (New England Biolabs GmbH). Fragment length patterns were compared and grouped if they were similar. One representative clone was selected from each group and used for sequencing. Sequencing was performed on an automated ABI 3,730 × I DNA Analyzer (Applied Biosystems, Life Technologies GmbH).

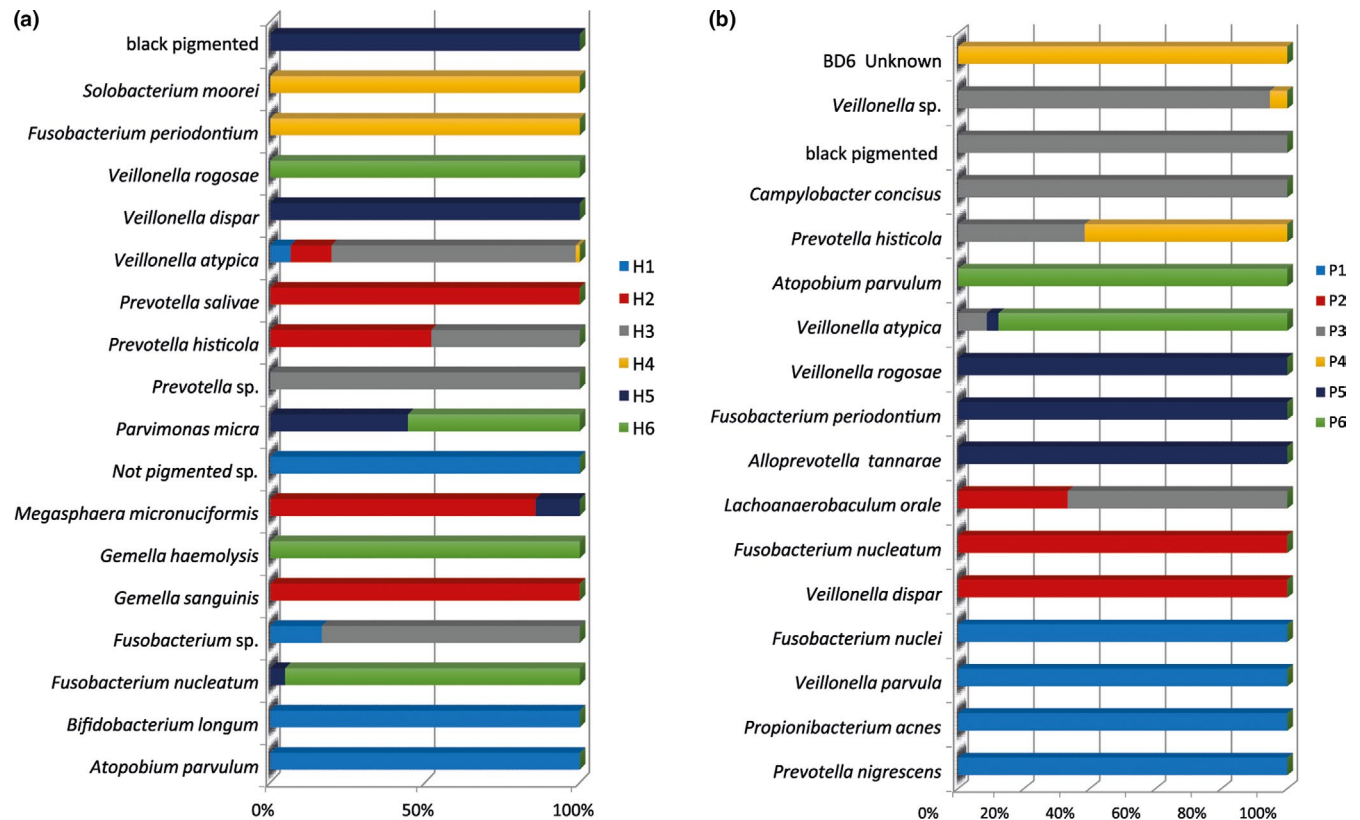


FIGURE 1 Culture technique: (a) Relative distribution (in % CFU) of anaerobic bacteria among the halitosis patients and (b) relative distribution (in % CFU) of anaerobic bacteria among the healthy volunteers

almost 47, the culture-independent cloning method detected 55 species.

3.3 | Culture analysis revealed a distinct bacterial composition of halitosis-associated biofilms compared to the health-associated biofilms

By means of MALDI-TOF analysis, it was possible to identify 47 different microbial species overall. A total of 36 different species were identified in the halitosis condition, and 36 different species were identified in the samples derived from the healthy condition. The culture analysis of the microflora disclosed distinguishable differences in the abundance distribution of the aerobic and anaerobic species within the tongue dorsum biofilm of healthy volunteers and halitosis patients (Figures 1,2). In particular, in the halitosis condition 18 aerobic and 18 anaerobic species were identified; similarly, in the healthy group 19 aerobic species and 17 anaerobic species were detected. The highest percentage of CFUs among aerobic species (1.9×10^8 CFU/ml) in the halitosis volunteers was found for *Streptococcus mitis* (Figures 3,4); in the healthy volunteers, the highest percentage of CFUs among aerobic species was found for *Streptococcus parasanguinis* (1.11×10^8 CFU/ml). Among the anaerobic species, the highest percentage was found for *Veillonella atypica* (7.6×10^7 CFU/ml) in the halitosis group and for *Veillonella sp.* (9×10^6 CFU/ml) in the healthy group (Figures 3,4). A statistically significant association was found between the presence of *Actinomyces*

graevenitzii and the halitosis condition ($p < .05$) (Figure 3). In addition, the culture analysis allowed the identification of *V. rogosae* in the tongue biofilm also of halitosis patients.

3.4 | Analysis of the 16S rDNA clone libraries disclosed a high bacterial diversity within the halitosis-associated biofilms

The molecular identification confirmed the presence of the bacterial species detected by the culture method, and it allowed us to detect even more species including various *Streptococcus* and other taxa including *Haemophilus parainfluenzae*, *Okadaella gastrococcus*, and *Tannerella forsythia* (Figures 5 and 6).

More specifically, the other species detected in halitosis samples were *Streptococcus anginosus*, *S. cristatus*, *S. gordonii*, *S. lactarius*, *S. oligofermentans*, *S. thermophilus*, *S. tigurinus*, *S. pseudopneumoniae*, *S. australis*, *Okadaella gastrococcus*, *Prevotella sp.*, *P. histicola*, *P. pallens*, *P. melaninogenica*, *P. veroralis*, and *Veillonella parvula* (Figure 5).

The adjunctive taxa detected in the samples derived from the healthy volunteers were *Gemella sanguinis*, *Streptococcus thermophilus*, *Porphyromonas sp.*, *Prevotella pallens*, *Haemophilus parainfluenzae*, *Abiotrophia para-adiacens*, and *Selenomonas sp.*

The most abundant species found in the halitosis condition was *Streptococcus mitis* (Figure 6). The most abundant species among the samples derived from the healthy condition was *Streptococcus salivarius* (Figure 6). The statistical analysis revealed a significant

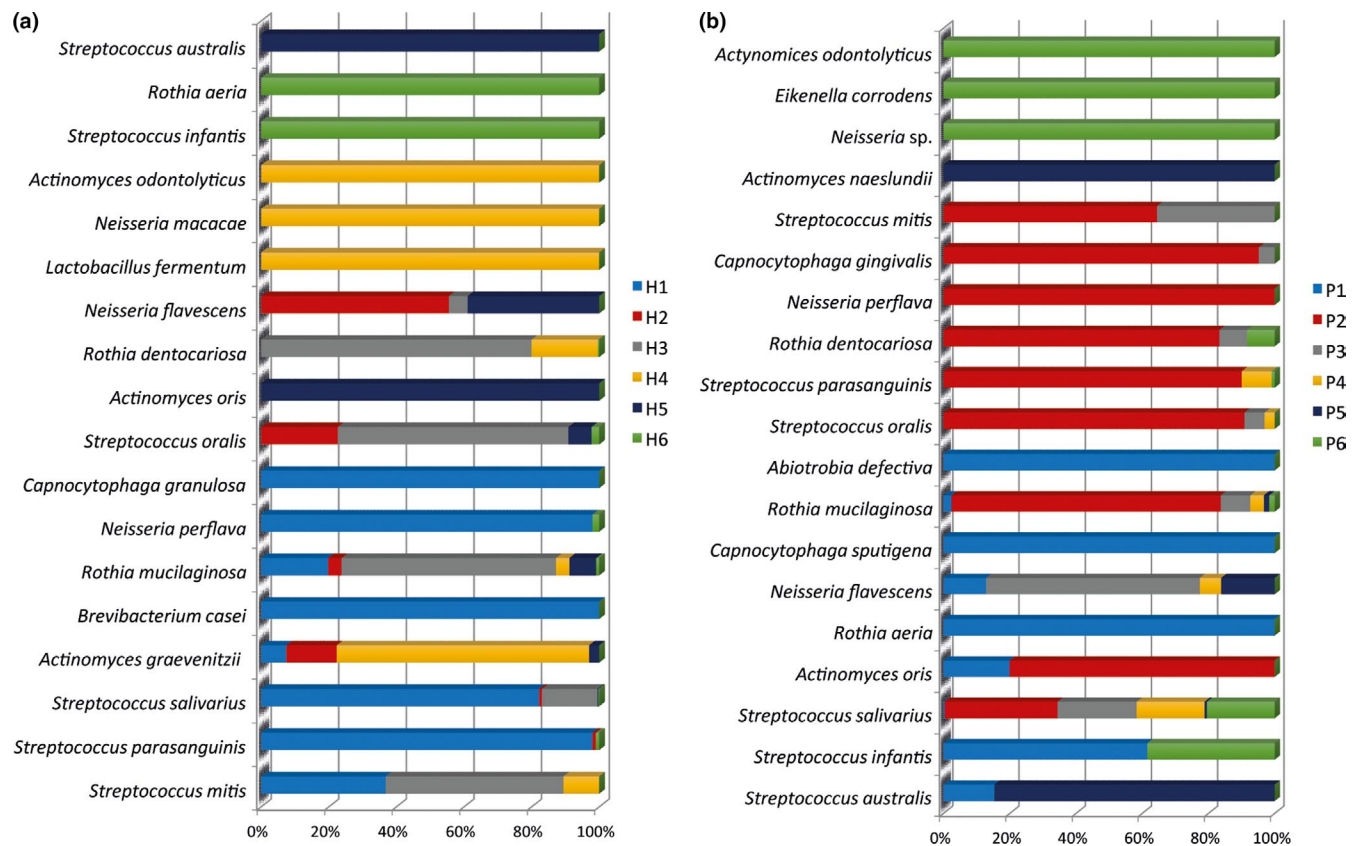


FIGURE 2 Culture technique: (a) Relative distribution (in % CFU) of aerobic bacteria among the halitosis patients and (b) relative distribution (in % CFU) of aerobic bacteria among the healthy volunteers

association (p value $< .05$) of *S. mitis* and *S. pseudopneumoniae* with the halitosis condition (Figure 6). Some taxa were only found in the halitosis patients, but not in the healthy controls, for example, *Okadaella gastrococcus* (4% abundance), *Leptotrichia* sp. (1% abundance), and *Tannerella forsythia* (1% abundance).

4 | DISCUSSION

Intraoral halitosis is predominantly caused by bacteria. According to literature, it is widely accepted that the microbial composition of the dorsal tongue surface correlates with the VSCs' production as stated in different studies (Amou et al., 2014; Aylıkcı & Colak, 2013; Bosy, Kulkarni, Rosenberg, & McCulloch, 1994; De Boever & Loesche, 1995; Hess, Greenman, & Duffield, 2008; Kazor et al., 2003; Yang et al., 2013). The VSCs produced by the dorsal tongue microbiota are the molecules directly responsible for the oral malodor.

In clinical practice, patients affected by this health issue address their dentist or dental hygienist in order to solve it (Thoppay et al., 2019). The first steps for a correct diagnosis are to obtain data using a general medical history questionnaire and to clinically evaluate the oral health status and the detection of the VSCs (Seemann et al., 2014). The detection of VSCs is a crucial step and topic of debate. Indeed, as reported by Scully C et al. the clinical assessment can be performed using portable gas chromatography

or a sulfide monitor or organoleptic assessment, performed by the nose of the clinicians (Scully & Greenman, 2012). The last method is considered the gold standard in the clinical practice, but the clinician sniff can present many side effects such as the transmission of diseases or subjectivity level (Falcão et al., 2017). The portable gas chromatography can be preferred if the clinical situation requires a differentiation of the VSCs. The sulfide monitor instead can be sufficient for an initial objective assessment of halitosis (Scully & Greenman, 2012). In our clinical assessment, the general medical history questionnaire revealed the absence of mechanical tongue scraping among the adopted oral hygiene habits. The clinical examination allowed for the documentation of the periodontal status, and the objective assessment of VSCs by means of the sulfide monitor enabled the diagnosis of halitosis associated with the tongue coating. However giving the limit of the sulfide monitor, we were not able to assess the degree of the halitosis condition. The periodontal status was found to be in good condition in the healthy volunteers' group and with signs of disease in the halitosis group. Two patients belonging to the halitosis group also showed GERD, which can be a primary cause of oral malodor. Indeed, the GERD lowers the pH in the oral cavity and therefore influences the microbial composition of the oral biofilm of teeth, mucosa, and tongue dorsum. However, the microbial composition of the tongue biofilm belonging to these two particular patients did not show any taxa significantly predominant. Among the aerobes, the most

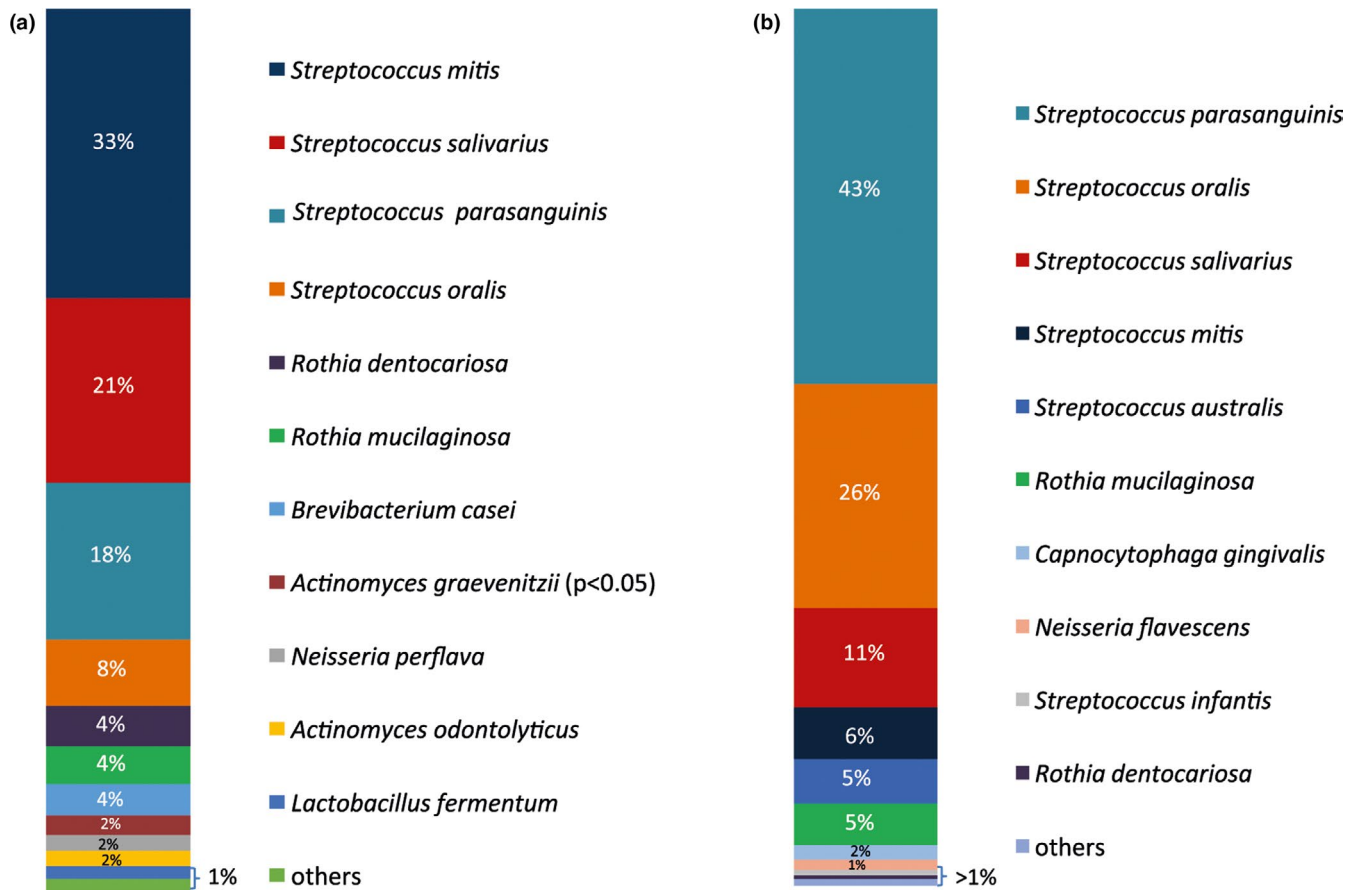


FIGURE 3 Culture technique: (a) Microbial composition (in % CFU) of aerobic bacteria in biofilm samples of halitosis patients and (b) bacterial concentration composition (in % CFU) of aerobic species in biofilm samples of healthy volunteers. The significantly associated species (p value < 0.05) are marked

abundant species were *Streptococcus parasanguinis* and *Okadella gastrococcus*, while among the anaerobes the most abundant species were *Veillonella atypica*, *Prevotella histicola*, and *Veillonella Rogosae*. Interestingly, the patient suffering from GERD presented as most abundant species the *V. Rogosae*. As stated before, the source of the oral malodor is found in the microbial metabolism. Many studies have reported that the composition of the microflora is characterized by a great diversity and accompanied by the presence of high proportions of anaerobic bacteria (Anesti et al., 2005; Loesche & Kazor, 2003; Mantilla Gómez et al., 2001; Roldán et al., 2003).

The combination of culture and culture-independent methods applied in the present study confirmed this trend, showing a high variability of the microbial population of the biofilm, and a higher proportion of the aerobic taxa in the halitosis group.

In particular, we were able to detect the main species associated with oral malodor so far, including *Prevotella melaninogenica*, *Fusobacterium periodonticum*, *Tannerella forsythia*, and *Solobacterium moorei*.

Previous studies profiled the microbiota in halitosis patients and healthy individuals by means of culture-dependent and culture-independent techniques in order to understand the microflora dominating this pathological biofilm microenvironment (De Boever &

Loesche, 1995; Hess et al., 2008; Kato et al., 2005; Kazor et al., 2003; Mantilla Gómez et al., 2001; Seerangaiyan et al., 2017). In 1966, Gordon and Gibbons were the first to report the prevalence of bacterial species on the tongue surface using culture methods (Gordon & Gibbons, 1966). They found streptococci, *Veillonella* spp., micrococci, staphylococci, *Bacteroides* spp., *Neisseria* spp., *Fusobacterium* spp., and unidentified Gram-negative rods and cocci. Later, De Boever and Loesche made a first effort to determine which of the bacterial species colonizing the tongue surface correlated with oral malodor (De Boever & Loesche, 1995). In that context, they isolated cultivable bacteria from tongue plaque from halitosis patients and found that the prevalent Gram-positive halitosis-associated bacterial species were *Actinomyces* spp., *Streptococcus salivarius*, *S. sanguinis*, and *Rothia dentocariosa*, whereas the prevalent Gram-negative halitosis-associated bacterial species were *Prevotella intermedia*, *Capnocytophaga* spp., and *Fusobacterium* spp. Our study confirmed the presence of these aerobic species associated with halitosis condition,

Since the detection of uncultivable bacteria is not possible using solely culture-dependent methods, the available information on the microbiota situated on the tongue surface was limited. After applying culture-independent methods, namely the amplification, cloning, and sequencing of 16S rRNA cistrons, Kazor et al. managed to determine

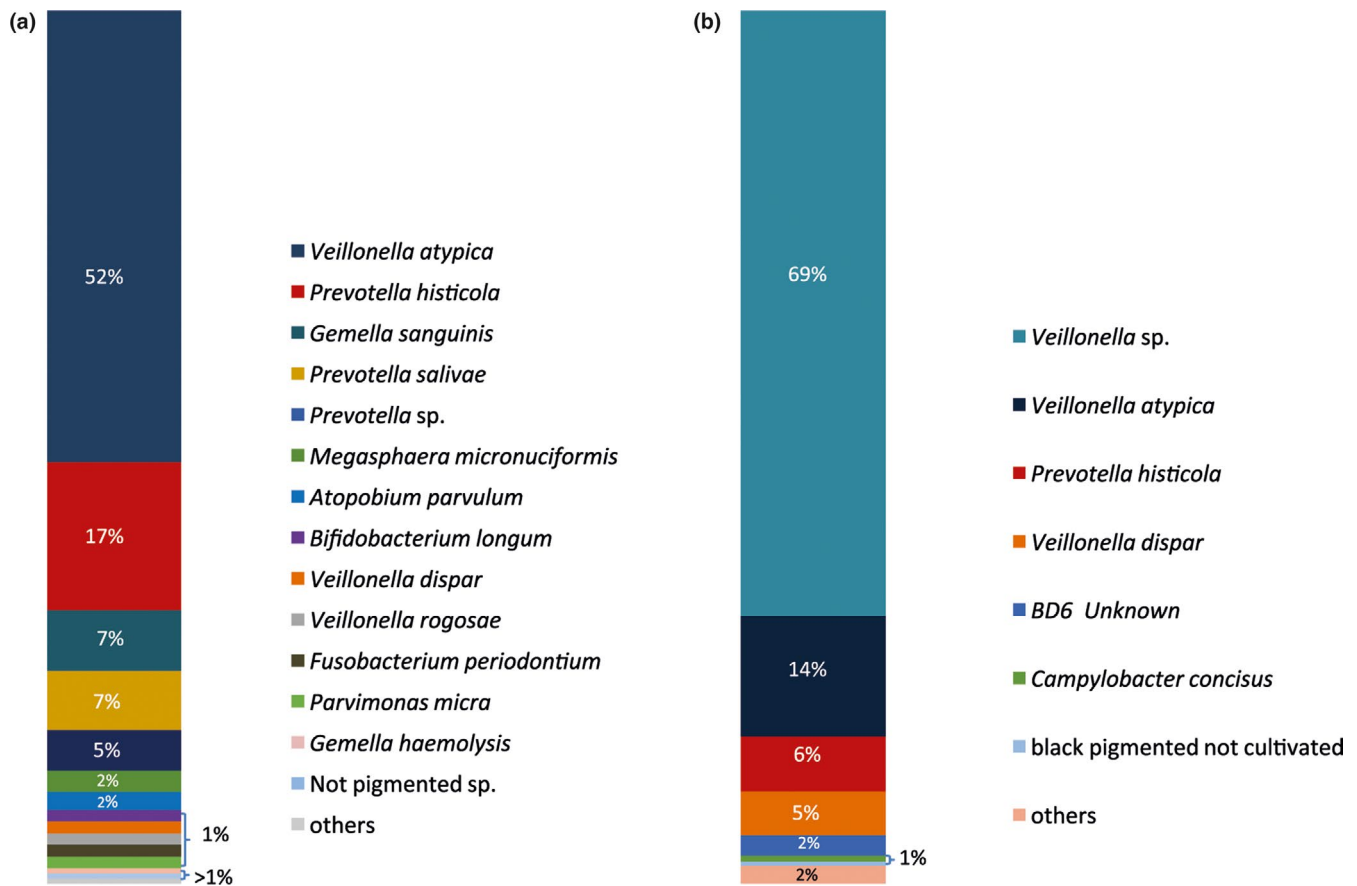


FIGURE 4 Culture technique: (a) Microbial composition (in % CFU) of anaerobic bacteria in biofilm samples of halitosis patients and (b) microbial composition (in % CFU) of anaerobic bacteria in biofilm samples of healthy volunteers. The significantly associated species (p value $<.05$) are depicted

the bacterial composition on the tongue surface in halitosis patients more comprehensively (Kazor et al., 2003). Interestingly, the author found the most prevalent bacterial species were *Atopobium parvulum* and *Solobacterium moorei*. In contrast, other bacterial species such as *Streptococcus salivarius* and *Rothia mucilaginosa* were predominant in healthy subjects (Kazor et al., 2003). This finding was confirmed in the present study, in which *S. salivarius* and *R. mucilaginosa* were also found in healthy. In healthy subjects *R. mucilaginosa* comprise 5% CFU, in halitosis 4%. In another study, Haraszthy et al. applied the combination of the anaerobic culture and direct amplification of 16S ribosomal DNA using an open-ended method similar to the one in the present study, in an attempt to overcome the limits of the culture technique (Haraszthy et al., 2007). They found *Streptococcus salivarius* and *Campylobacter concisus* as the most prevalent species in the control group. These species were found in the control group of our study, too. In addition, *Actinomyces graevenitzii*, statistically associated with the halitosis condition in the present study, was also one of the most prevalent species in halitosis group in the Haraszthy et al. study (Haraszthy et al., 2007).

Moreover, the present results revealed, in accordance with these earlier findings, the presence of *Actinomyces odontolyticus*, *Solobacterium moorei*, *Streptococcus oralis*, and *S. sanguinis* in halitosis patients. These bacterial species were often detected in halitosis

biofilm in literature (Haraszthy et al., 2007). Riggio et al. profiled and compared the microbiota on the tongue dorsum by means of culture-independent techniques, using PCR amplification, cloning, and sequencing of 16S rRNA genes (Riggio et al., 2008). The authors concluded that the tongue dorsum presents a higher microbial diversity in halitosis samples compared with the controls. According to the authors' findings, *S. salivarius* was present in high concentrations both in the halitosis and control groups (Riggio et al., 2008). The present study confirmed these findings. Consequently, it can be assumed that this microorganism does not play an etiological role in the development of oral malodor.

Recently, Yang et al. used pyrosequencing in a cross-sectional and longitudinal study for a comparison of the microbial communities in halitosis patients and in healthy volunteers (Yang et al., 2013). They found that *Prevotella* spp. and *Leptotrichia* spp. were positively linked to hydrogen sulfide (Yang et al., 2013). Similarly, Ren et al. found members of the genera *Prevotella* and *Leptotrichia* (and *Actinomyces*, *Selenomonas* etc.) in halitosis with pyrosequencing (Ren et al., 2016). Seerangaiyan et al. using Illumina MiSeq high-throughput sequencing found *Leptotrichia*, *Prevotella*, *Selenomonas*, and *Tannerella* taxa abundant in halitosis, whereas several *Streptococcus* species were more abundant in the control (Seerangaiyan et al., 2017).

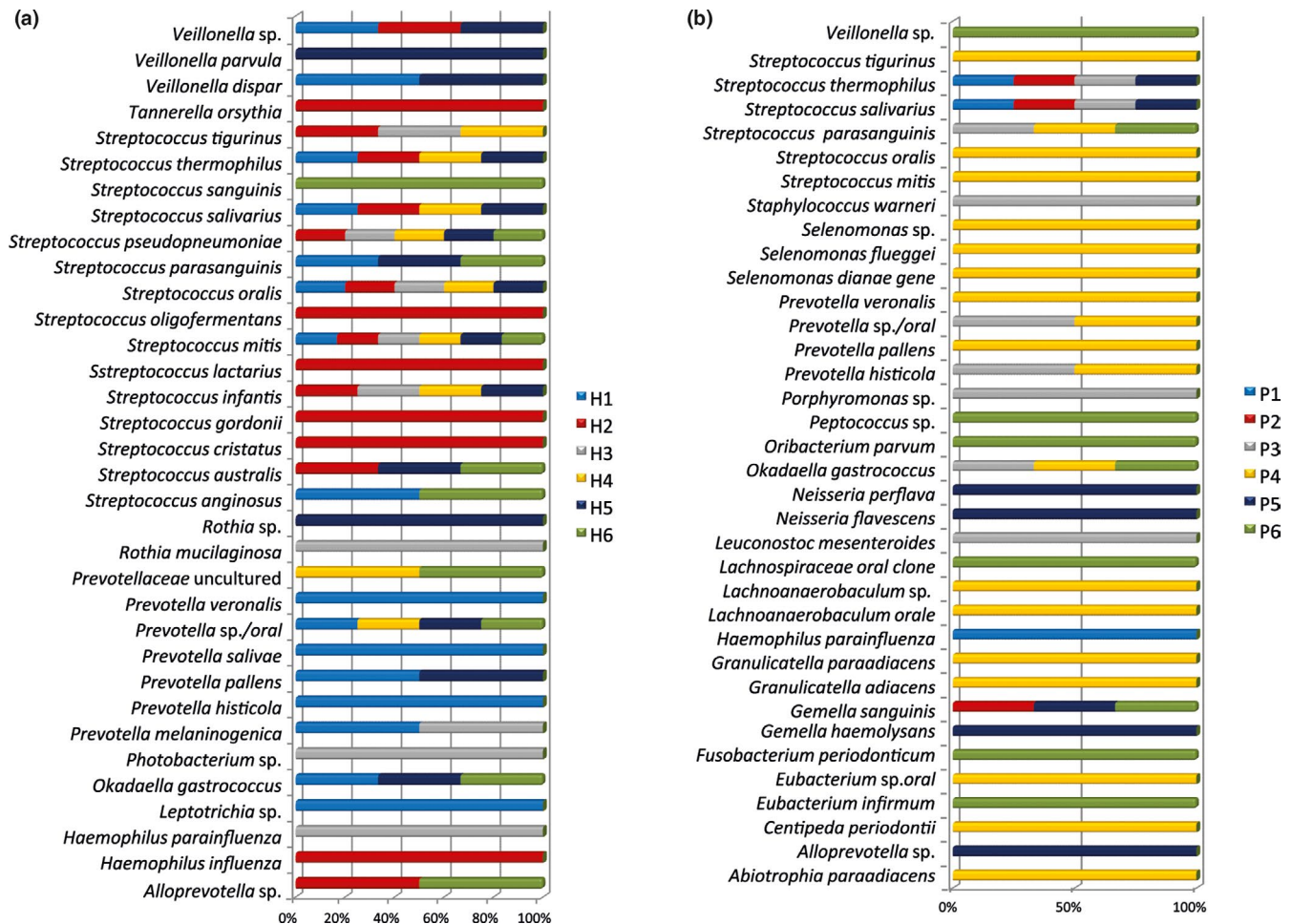


FIGURE 5 Cloning technique: (a) Relative distribution of all bacteria among the halitosis patients (in %). (b) Relative distribution of all bacteria among the healthy volunteers (in %)

The results deriving from our culture-independent “open-ended” technique in combination with the culture technique confirmed the presence of the taxa found in these high-throughput sequencing studies, specifically the detection of several *Prevotella* species with both methods, for example, *P. histicola*, which was found in high concentrations in samples from the halitosis patients with culture technique. Moreover, our methods revealed the significant presence of *S. mitis* and *S. pseudopneumoniae* in the halitosis samples which might indicate their role in the adhesion to the tongue surface during the biofilm formation. In contrast to the high-throughput sequencing studies, with our methodological approach by means of sequencing full-length 16S rDNA fragments, we were able to differentiate the many *Streptococcus* species that were detected. Both Seerangayian K et al. and Yang et al. found certain OTUs (operational taxonomic units) of the genus *Streptococcus* associated with healthy study participants, yet they were not able to achieve a clear species-level analysis (Seerangayian K et al., 2017; Yang et al., 2013).

The low number of participants in our study is an obvious limitation; however, other reports draw conclusions regarding the etiological flora for halitosis using similar study populations, for example, the study by Kazor CE et al. using a culture-independent approach

on six halitosis patients and five healthy controls, or the study by Ren W et al. comparing five halitosis patients with five controls (Kazor et al., 2003; Ren et al., 2016). In our study, the results of the combination of culture-dependent and culture-independent “open-ended” cloning techniques highlighted the most prevalent bacterial species within the halitosis biofilms and the bacterial species influencing the healthy biofilms. This had not been performed yet. The aerobic and anaerobic cultivable species from the halitosis group corresponded to the taxa reported by many authors: All of those species except for *Veillonella rogosae* were previously found on the tongue dorsum of halitosis subjects. This species had previously been isolated from supragingival dental plaque and from the tongue biofilm of healthy individuals (Arif et al., 2008; Mashima et al., 2011; Mashima & Nakazawa, 2013). *Veillonella rogosae* is a Gram-negative, nonmotile, nonsporulating coccoid and appears as a single cell or in short chains. It is strictly anaerobic and oxidase-negative. It exhibits pyroglutamic acid arylamidase and variable alkaline phosphatase activity. Major acid end products are acetic and propionic acids (Arif et al., 2008). *Veillonella* genus has always been connected with the production of VSCs and is therefore responsible for malodor (Mashima et al., 2011), but to our knowledge, *V. rogosae* was never associated with halitosis so far.

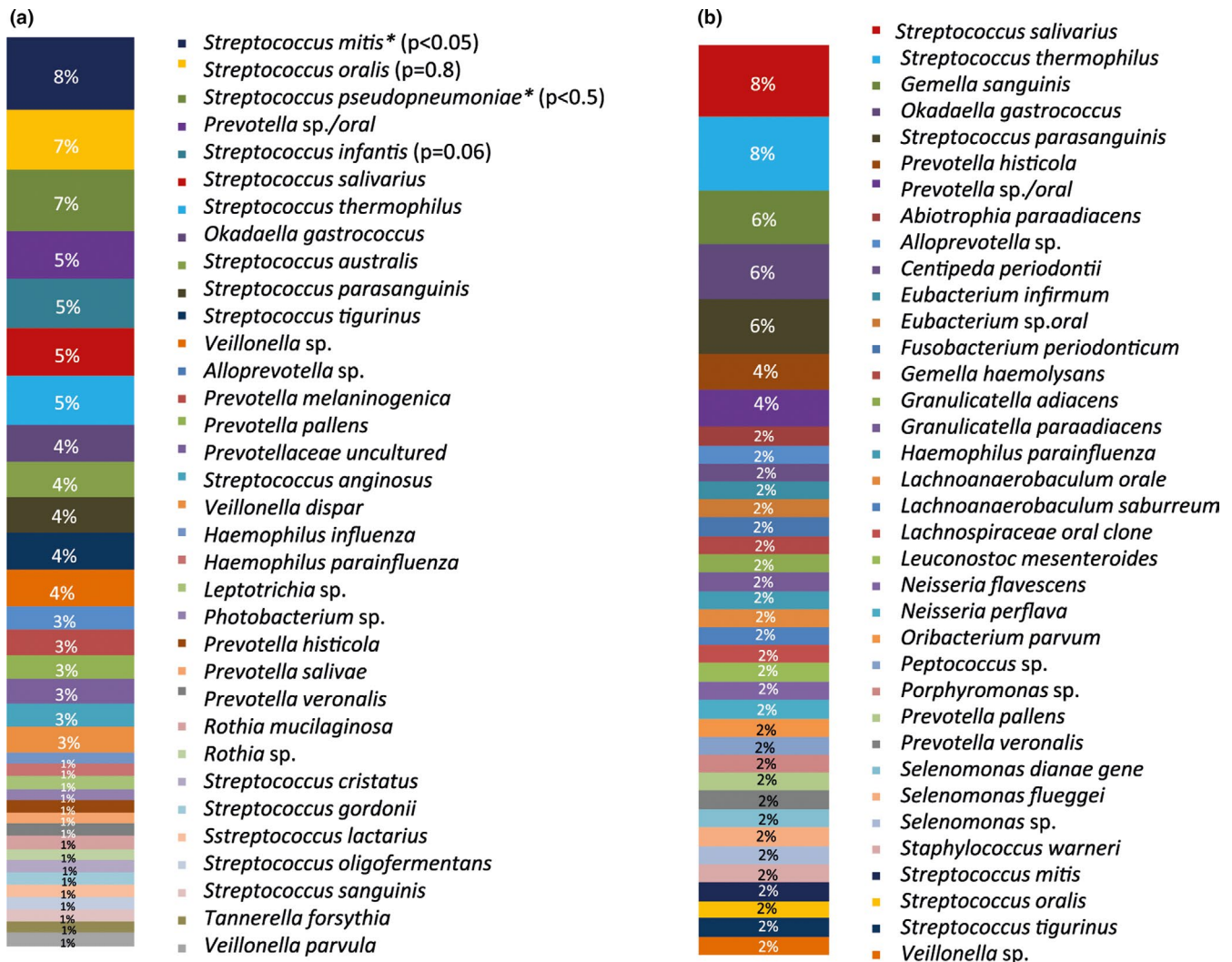


FIGURE 6 Cloning technique: (a) Relative abundance (in %) of all bacteria in biofilm samples of halitosis patients. The significantly associated species (p value < 0.05) are marked. (b) Relative abundance (in %) of all bacteria in biofilm samples of healthy volunteers

The cloning method, exploiting a “hypothesis-free” approach to achieve a greater overview of the total microbial diversity, showed a high variability among the detected species between the two groups. Particularly in the halitosis group, it allowed for the detection of different *Streptococcus* spp, *Haemophilus parainfluenzae*, *Prevotella pallens*, *P. veronalis*, *Photobacterium* spp. *Leptotrichia wadei*, and *Tannerella forsythia*, in line with the results obtained by (Riggio et al., 2008; Yang et al., 2013). In the control group, using the cloning method, we were able to detect *Abiotrophia para-adiacens*, *Granulicatella* spp., *Lachnoanaerobaculum saburream*, *Selenomonas* spp., and *Staphylococcus warneri*, which were not detected by means of culture-dependent methods. Particularly in the control group, two interesting species were noted: *Selenomonas* is a genus which is generally taken to be a volatile sulfur compounds producer (Persson et al., 1990). In general, *S. mitis*, *S. oralis*, and *S. pseudopneumoniae* are rather seen as belonging to the healthy physiological flora than associated with any oral disease. However, the 16S rRNA gene of *S. mitis*

and *S. pseudopneumoniae*, as shown by the recent study of Tze et al., has a 98% correspondence with a new isolated species from the tongue dorsum in a halitosis patient: the *S. halitosis*. Hence, it might be possible that these taxa would provide favorable conditions in the microenvironment of the tongue biofilm for other, halitosis-associated taxa to thrive.

5 | CONCLUSION

In conclusion, in combining the culture method and culture-independent cloning technique this study confirmed the wide variety of the tongue microbiota in halitosis patients, including new species that had not been detected so far. A combination of different microbial techniques is recommended to analyze the etiological microflora associated with halitosis. Increased knowledge of the microbiota of the tongue biofilm is essential for further research to develop new antimicrobial agents for halitosis therapy strategies.

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CONFLICT OF INTERESTS

None declared.

AUTHORS' CONTRIBUTIONS

Sara Bernardi and Annette Anderson contributed to conceptualization; Kirstin Vach contributed to formal analysis; Sara Bernardi contributed to investigation; Lamprini Karygianni, Ali Al-Ahmad, Elmar Hellwig, and Guido Macchiarelli contributed to supervision; Andreas Filippi, Andrea Zurcher, and Ali Al-Ahmad facilitated the resources; Sara Bernardi, Annette Anderson, Kristin Vach, Lamprini Karygianni, Andreas Filippi, Andrea Zurcher, and Ali Al-Ahmad contributed to writing—original draft preparation; Elmar Hellwig, Guido Macchiarelli, Lamprini Karygianni, Ali Al-Ahmad, Annette Anderson, Andreas Filippi, Andrea Zurcher, and Kristin Vach contributed to writing—review and editing; all authors read and approved the final manuscript.

ETHICS STATEMENT

The study design was reviewed and approved by the Ethics Committee of the Albert-Ludwigs-University of Freiburg (74/15).

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ORCID

Sara Bernardi  <https://orcid.org/0000-0001-6130-8533>

Lamprini Karygianni  <https://orcid.org/0000-0002-7893-0393>

Annette Carola Anderson  <https://orcid.org/0000-0002-4489-1939>

Kirstin Vach  <https://orcid.org/0000-0001-9278-2203>

Guido Macchiarelli  <https://orcid.org/0000-0002-9182-0586>

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