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

Bernardi, Sara ; Karygianni, Lamprini ; Filippi, Andreas ; Anderson, Annette Carola ; Zürcher, Andrea ; Hellwig, Elmar ; Vach, Kirstin ; Macchiarelli, Guido ; Al-Ahmad, Ali

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.

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Title Page

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

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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.

71 **Abstract**

72 **Background.** Oral malodor is a very discomforting condition deriving from the presence of volatile
73 sulphur compounds in the expired air. In halitosis of intra-oral etiology, the volatile sulphur
74 compounds are metabolic products of the oral microorganisms within the biofilm coating the tongue
75 dorsum as well as other tissues in the oral cavity. The aim of this study was to characterize and
76 compare the microbial composition of tongue biofilm in volunteers suffering from halitosis and
77 healthy volunteers by means of both the culture method and culture-independent cloning technique.
78 **Results.** A high bacterial variety (more than 80 different species) was detected using the
79 combination of both methods. A distinct bacterial composition was revealed in the halitosis-
80 associated biofilms compared to the health-associated biofilms. *Actinomyces graevenitzii* was
81 shown to be significantly associated with the halitosis condition. The culture method identified 47
82 species, included *Veillonella rogosae*, never isolated from the tongue biofilm of halitosis patients so
83 far. In the healthy condition, the culture-dependent method showed that the most frequent species
84 were *Streptococcus parasanguinis* among the aerobes and *Veillonella* spp. among the anaerobes.
85 The culture-independent cloning method detected more than 50 species. *Streptococci*, in particular
86 *Streptococcus mitis/oralis*, *Streptococcus pseudopneumoniae* and *Streptococcus infantis* as well as
87 *Prevotella* spp. were found most frequently in halitosis patients. *Streptococcus salivarius* and
88 *Rothia mucilaginosa* were found more frequently in the healthy condition. **Conclusions**. The
89 combination of the culture-dependent and culture-independent cloning technique allowed for a
90 widespread analysis of the tongue biofilm in halitosis patients. The results can support further
91 pharmacological research for new anti-microbial agents and halitosis therapy strategies.

92 **Keywords**

93 Halitosis, Tongue Biofilm, Microbial Culture, Culture-independent Cloning Technique

94

95

96 **Introduction**

97 Halitosis is widely known as malodor deriving from exhaled breath due to the presence of volatile
98 Sulphur compounds (VSCs) arising from the oral cavity or from the upper airways (Scully and
99 Greenman, 2008). The VSCs include hydrogen sulphide, methyl mercaptan and dimethyl sulphide
100 (Scully and Porter, 2008). The volatile products causing intra-oral halitosis derive from the
101 interaction of oral microbiota with specific substrates, such as the amino acids cysteine, methionine,
102 tryptophan, arginine and lysine that are metabolized into the different VSCs (Dzink and Socransky,
103 1990).

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

114 The organoleptic difference between the intra-oral and extra-oral halitosis consist in the
115 composition of the VSCs. Indeed, hydrogen sulphide and methyl mercaptan have been found to be
116 the main contributors to intra-oral halitosis, whereas dimethyl sulphide is more associated with
117 extra-oral, "blood-borne" halitosis (Tangerman and Winkel, 2010). Intra-oral halitosis is associated
118 with periodontal diseases, poor oral hygiene, salivary flow alterations, cancerous lesions and bone
119 necrosis (Dzink and Socransky, 1990). It is etiologically related to the microbiota of the dorsal
120 tongue biofilm (Yaegaki and Coil, 2000; Roldán, Herrera and Sanz, 2003), and in particular to the

121 presence of anaerobic microorganisms responsible for the production of VSCs, such as *Centipeda*
122 *periodontii, Eikenella corrodens*, *Fusobacterium nucleatum, Fusobacterium periodonticum*,
123 *Porphyromonas gingivalis*, *Prevotella melaninogenica*, *Prevotella intermedia*, *Solobacterium*
124 *moorei*, *Tannerella forsythia* and *Treponema denticola.* Due to its papillary structure that creates an
125 ecological niche for microorganisms, the tongue biofilm represents an oral microenvironment which
126 is well-distinguished from the supragingival biofilm, also known as dental plaque, and the
127 subgingival biofilm (Bernardi *et al*., 2013, 2018; Amou *et al*., 2014; Bernardi, Marzo and
128 Continenza, 2016) .

129 To date, the halitosis-relevant literature comprises many studies on the microbial characterization of
130 the biofilm using *in vitro* models, culture technique, species-specific PCR (Brunner, Kurmann and
131 Filippi, 2010; Mashima, Kamaguchi and Nakazawa, 2011), confocal laser scanning microscopy
132 study (Bernardi *et al*., 2019) and quantitative PCR assays (Vancauwenberghe *et al*., 2013), allowing
133 for the study of the targeted species, as well as a few studies applying high-throughput sequencing
134 to tongue biofilm (Ren *et al*., 2016; Hall *et al*., 2017; Seerangaiyan *et al*., 2017)

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

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

144

145 **Methods**

146 **Subjects and Samples**

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

156 **Clinical halitosis assessment**

157 A total of twelve patients and volunteers were recruited at the Dental Clinic of the University of
158 Basel, Switzerland. The patients included in the study suffered from intra-oral halitosis. The
159 exclusion criteria were: (i) presence of extra-oral halitosis, (ii) diagnosis of a mental illness, (iii)
160 patients aged under 18 years, (iv) the intake of antibiotics in the previous three months before the
161 start of the study and/or the use of antiseptics one month before study start, and (v) poor general
162 health with reference to American Society of Anesthesiologists Physical Classification System.
163 Prior to the sampling procedure, a general medical history questionnaire was submitted to the
164 participants of the study (Table 1). The periodontal status of each participant was then assessed and
165 documented, using the Periodontal Screening and Recording (PSR) Index, recommended by the
166 American Dental Association as an established stage of oral diagnostic examinations for all dental
167 patients (Periodontology, 1993). The presence of VSCs was determined by means of a Halimeter
168 (Brunner, Kurmann and Filippi, 2010) and the results were recorded. Lastly, the tongue dorsum
169 biofilm samples were collected as described above.

170 **Culture method**

171 The culture method was performed as described in detail previously (Schirrmeister *et al.*, 2009).
172 The vials containing the samples in RTF were thawed at 36°C in a water bath and vortexed for 30–
173 45 s. For the isolation and identification of the microorganisms, 100 µl of the undiluted sample and
174 serial dilutions thereof were cultivated. The serial dilutions (10^{-1} to 10^{-7}) were prepared in peptone
175 yeast medium (PY). Each dilution was plated on yeast-cysteine blood agar plates (HCB) to cultivate
176 anaerobic bacteria at 37°C for 10 days, and on Columbia blood agar plates (CBA), incubated at
177 37°C and 5%–10% CO_2 atmosphere for 5 days to cultivate aerobic species. The resulting colony
178 types were phenotypically evaluated and counted to calculate the number of colony forming units
179 (CFUs) per ml in the original sample. All colony types were sub-cultivated to obtain pure cultures
180 which were analyzed by MALDI-TOF (MALDI Biotyper, Bruker Daltonik GmbH, Bremen,
181 Germany), as described in detail by our own group (Anderson *et al.*, 2014).

182 **DNA Isolation**

183 The biofilm samples were centrifuged at 16.000 g for 10 min and the supernatant was discarded.
184 Lysis of microbial cells was then performed using a Precellys 24 bead mill homogenizer (PEQLab
185 Biotechnologie GmbH, Erlangen) in ATL buffer (QiaAMP Micro Kit; Qiagen, Hilden, Germany).
186 The vials were shaken twice at 3500 rpm for 30 s. The DNA was subsequently purified by means of
187 QiaAMP Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol for tissue
188 samples. The total microbial DNA was eluted twice with 50 µl AE buffer (Qiagen) and then stored
189 at −20°C.

190 **PCR Amplification of 16S rRNA Genes**

191 Bacterial 16S rRNA genes were amplified using the following universal primers: 27F-YM (5′-
192 AGAGTTTGATYMTGGCTCAG-3′) and 1492R (5′ TACGGYTACCTTGTTACGACTT-3′)
193 (Frank *et al.*, 2008). The PCR amplification was performed in a total volume of 50 µl. The reaction
194 mixture contained 1× PCR buffer (Qiagen), 0.2 mM each of the four deoxyribonucleoside

195 triphosphates (dNTPs; PEQLab Biotechnologie, Erlangen, Germany), 0.5 μM of forward and
196 reverse primers, 2 μl UTaq-Polymerase (Qiagen) and 5 μl of the isolated sample DNA. The PCR
197 cycling conditions consisted of a denaturation step at 94°C for 2 min, followed by 35 cycles with
198 denaturation at 94°C for 1 min; annealing at 55°C for 1 min; extension at 72°C for 1.5 min; a final
199 extension step at 72°C for 10 min.
200 A no-template control and a positive control were included in each set of PCR reactions. PCR
201 reaction products were analyzed by electrophoresis in a 1.5% agarose gel and positive reactions
202 were used to prepare clone libraries.

203 **Cloning of PCR Products and Analysis of Clone Libraries**

204 The 16S rDNA amplification products were ligated into the PCR®2.1-TOPO® plasmid vector
205 using the TOPO TA Cloning® Kit (Invitrogen, Life Technologies, Darmstadt, Germany) according
206 to the manufacturer's protocol and as described in detail earlier (Anderson *et al.*, 2012). Fifty
207 white clones from each library were picked and the presence of inserts was confirmed by PCR
208 amplification with their respective primers, followed by gel electrophoresis. PCR products of all
209 recombinants were subjected to a restriction enzyme digestion with Hha I, Rsa I and Hinf I (New
210 England Biolabs GmbH, Frankfurt, Germany). Fragment length patterns were compared and
211 grouped if they were similar. One representative clone was selected from each group and used for
212 sequencing. Sequencing was performed on an automated ABI 3730×l DNA Analyzer (Applied
213 Biosystems, Life Technologies GmbH, Darmstadt, Germany).

214 **Sequence Analysis**

215 The sequence data obtained from the ABI sequencer was visually proofread and edited using the
216 Ridom TraceEdit software (Ridom GmbH, Münster, Germany). The partial and almost full-length
217 16S rDNA sequences were compared to those from public sequence databases, Genbank, EMBL
218 and DDBJ using the BLAST program, which was run through the server hosted by the National
219 Center for Biotechnology Information (http://www.ncbi.nigh.gov/BLAST) (Altschul *et al.*, 1990).

220 The sequences that showed 98% similarity or less with public database sequences were checked for
221 chimeras with the Pintail software (version 1.0) (Ashelford *et al*., 2005). The chimeric sequences
222 were excluded from further analysis. The sequences with a 99–100% match to a database sequence
223 were considered to belong to the same species as the one with the highest similarity and score bits.
224 In addition, all 16S rDNA sequences were compared with the database sequences of the Ribosomal
225 Database Project (http://rdp.cme.msu.edu/) (Cole *et al*., 2009).
226

227 **Statistical analysis**

228 The concentration and the abundance of the species were analyzed with descriptive and associative
229 statistical test (Wilcoxon Rank-Sum and the Fisher's exact test). All calculations were done by the
230 statistical software STATA 14.1.
231

232 **Results**

233

234 **Clinical assessment**

235 The six recruited halitosis patients, four female and two male subjects, were between 25 and 65
236 years old. Two patients claimed to suffer from gastroesophageal disorder within the limit of the
237 physiological disturbance, and one of them was a smoker. Tongue brushing was not performed by
238 any of them as part of normal oral hygiene procedure. The PSR Index was between 0 and 3,
239 indicating a certain degree of periodontal disease and the Halimeter values ranged from 122 to 226
240 parts per billion (Table 2).
241 The ages of the six healthy volunteers ranged between 22 and 33 years. The tongue plaque was
242 sampled from four females and two males. One volunteer consumed alcohol on a regular basis, and
243 two subjects brushed the dorsal tongue surface regularly. The PSR Index and the Halimeter values
244 were 0 for all healthy volunteers (Table 2).

245 **Microbiological analysis**

246 The combination of the culture-dependent methods and the molecular cloning technique revealed a
247 high abundance and diversity of bacterial species in both the halitosis and control groups. A high
248 bacterial variety (more than 80 different species) resulted from the combination of the two methods.
249 While the culture-method identified almost 47, the culture-independent cloning method detected 55
250 species.

251 **Culture analysis revealed a distinct bacterial composition of halitosis-associated biofilms**
252 **compared to the health-associated biofilms**

253 By means of MALDI-TOF analysis it was possible to identify 47 different microbial species
254 overall. 36 different species were identified in the halitosis condition and 36 different species were
255 identified in the samples derived from the healthy condition. The culture analysis of the microflora
256 disclosed distinguishable differences in the abundance distribution of the aerobic and anaerobic
257 species within the tongue dorsum biofilm of healthy volunteers and halitosis patients (Figure 1-2).
258 In particular, in the halitosis condition 18 aerobic and 18 anaerobic species were identified,
259 similarly in the healthy group 19 aerobic species and 17 anaerobic species were detected. The
260 highest percentage of CFUs among aerobic species ($1.9x10^8$ CFU/ml) in the halitosis volunteers
261 was found for *Streptococcus mitis* (Figure 3, 4); in the healthy volunteers the highest percentage of
262 CFUs among aerobic species was found for *Streptococcus parasanguinis* ($1.11x10^8$ CFU/ml).
263 Among the anaerobic species the highest percentage was found for *Veillonella atypica* ($7.6x10^7$
264 CFU/ml) in the halitosis group and for *Veillonella* spp. ($9x10^6$ CFU/ml) in the healthy group (Figure
265 3,4). A statistically significant association was found between the presence of *Actinomyces*
266 *graevenitzii* and the halitosis condition ($p<0.05$) (Figure 3). In addition, the culture analysis allowed
267 the identification of *Veillonella rogosae* in the tongue biofilm also of halitosis patients.

268 **Analysis of the 16S rDNA clone libraries disclosed a high bacterial diversity within the**
269 **halitosis-associated biofilms.**

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

274 More specifically, the other species detected in halitosis samples were *Streptococcus anginosus,*
275 *Streptococcus cristatus, Streptococcus gordonii, Streptococcus lactarius, Streptococcus*
276 *oligofermentans, Streptococcus thermophilus, Streptococcus tigurinus, Streptococcus*
277 *pseudopneumoniae, Streptococcus australis, Okadaella gastrococcus, Prevotella* sp.*, Prevotella*
278 *histicola, Prevotella pallens, Prevotella melaninogenica, Prevotella veroralis and Veillonella*
279 *parvula* (figure 5).

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

283 The most abundant species found in the halitosis condition was *Streptococcus mitis* (Figure 6). The
284 most abundant species among the samples derived from the healthy condition was *Streptococcus*
285 *salivarius* (Figure 6). The statistical analysis revealed a significant association (p value<0.05) of *S.*
286 *mitis* and *S. pseudopneumoniae* with the halitosis condition (Figure 6). Some taxa were only found
287 in the halitosis patients, but not in the healthy controls, e.g. *Okadaella gastrococcus* (4%
288 abundance), *Leptotrichia* sp. (1% abundance) and *Tannerella forsythia* (1% abundance).

289 **Discussion**

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

296 In clinical practice, patients affected by this health issue address their dentist or dental hygienist in
297 order to solve it (Thoppay *et al*., 2019) . The first steps for a correct diagnosis are to obtain data
298 using a general medical history questionnaire, to clinically evaluate the oral health status, and the
299 detection of the VSCs (Seemann *et al*., 2014). The detection of VSCs is a crucial step and topic of
300 debate. Indeed, as reported by Scully C et al. the clinical assessment can be performed using
301 portable gas chromatography or a sulphide monitor or organoleptic assessment, performed by the
302 nose of the clinicians (Scully and Greenman, 2012). The last method is considered the gold standard
303 in the clinical practice, but the clinician sniff can present many side effects such as the transmission
304 of diseases or subjectivity level (Miranda *et al.*, 2017). The portable gas chromatography can be
305 preferred if the clinical situation requires a differentiation of the VSCs. The sulphide monitor
306 instead can be sufficient for an initial objective assessment of halitosis (Scully and Greenman,
307 2012). In our clinical assessment, the general medical history questionnaire revealed the absence of
308 mechanical tongue scraping among the adopted oral hygiene habits. The clinical examination
309 allowed for the documentation of the periodontal status, and the objective assessment of VSCs by
310 means of the sulphide monitor enabled the diagnosis of halitosis associated with the tongue coating.
311 However giving the limit of the sulphide monitor, we were not able to assess the degree of the
312 halitosis condition. The periodontal status was found to be in good condition in the healthy
313 volunteers' group, and with signs of disease in the halitosis group. Two patients belonging to the
314 halitosis group also showed GERD, which can be a primary cause of oral malodor. Indeed, the
315 GERD lowers the pH in the oral cavity and therefore influences the microbial composition of the
316 oral biofilm of teeth, mucosa and tongue dorsum. However, the microbial composition of the
317 tongue biofilm belonging to these two particular patients did not show any taxa significantly
318 predominant. Among the aerobes the most abundant species were *Streptococcus parasanguinis* and
319 *Okadella gastrococcus*, whilst among the anaerobes the most abundant species were *Veillonella*
320 *atypica*, *Prevotella histicola* and *Veillonella Rogosae*. Interestingly, the patient suffering from
321 GERD presented as most abundant species the *Veillonella Rogosae*. As stated before, the source of

322 the oral malodor is found in the microbial metabolism. Many studies have reported that the
323 composition of the microflora is characterized by a great diversity and accompanied by the presence
324 of high proportions of anaerobic bacteria (Mantilla Gómez *et al*., 2001; Loesche and Kazor, 2002;
325 Roldán *et al*., 2003; Roldán, Herrera and Sanz, 2003; Anesti *et al*., 2005).
326 The combination of culture and culture independent methods applied in the present study confirmed
327 this trend, showing a high variability of the microbial population of the biofilm, and a higher
328 proportion of the aerobic taxa in the halitosis group.
329 In particular, we were able to detect the main species associated with oral malodor so far, including
330 *Prevotella melaninogenica, Fusobacterium periodonticum, Tannerella forsythia,* and
331 *Solobacterium moorei*.
332 Previous studies profiled the microbiota in halitosis patients and healthy individuals by means of
333 culture-dependent and culture-independent techniques in order to understand the microflora
334 dominating this pathological biofilm microenvironment (De Boever and Loesche, 1995; Mantilla
335 Gómez *et al*., 2001; Kazor *et al*., 2003; Kato *et al*., 2005; Hess, Greenman and Duffield, 2008;
336 Seerangaiyan *et al*., 2017). In 1966, Gordon and Gibbons were the first to report the prevalence of
337 bacterial species on the tongue surface using culture- methods(Gordon and Gibbons, 1966). They
338 found streptococci, *Veillonella* spp., micrococci, staphylococci, *Bacteroides* spp., *Neisseria*
339 spp., *Fusobacterium* spp. as well as unidentified Gram-negative rods and cocci. Later, De Boever
340 and Loesche made a first effort to determine which of the bacterial species colonizing the tongue
341 surface correlated with oral malodor (De Boever and Loesche, 1995). In that context, they isolated
342 cultivable bacteria from tongue plaque from halitosis patients and found that the prevalent Gram-
343 positive halitosis-associated bacterial species were *Actinomyces* spp., *Streptococcus salivarius*,
344 *Streptococcus sanguinis* and *Rothia dentocariosa*, whereas the prevalent Gram-negative halitosis-
345 associated bacterial species were *Prevotella intermedia*, *Capnocytophaga* spp. and *Fusobacterium*
346 spp. Our study confirmed the presence of these aerobic species associated with halitosis condition,

347 Since the detection of uncultivable bacteria is not possible using solely culture-dependent methods,
348 the available information on the microbiota situated on the tongue surface was limited. After
349 applying culture-independent methods, namely the amplification, cloning and sequencing of 16S
350 rRNA cistrons, Kazor et al. managed to determine the bacterial composition on the tongue surface
351 in halitosis patients more comprehensively (Kazor *et al*., 2003). Interestingly, the author found the
352 most prevalent bacterial species were *Atopobium parvulum* and *Solobacterium moorei.* In contrast,
353 other bacterial species such as *Streptococcus salivarius* and *Rothia mucilaginosa* were predominant
354 in healthy subjects (Kazor *et al*., 2003). This finding was confirmed in the present study, in which *S.*
355 *salivarius* and *R. mucilaginosa* was also found in healthy. In healthy subjects *R. mucilaginosa*
356 comprise 5% CFU, in halitosis 4%. In another study, Haraszthy et al. applied the combination of the
357 anaerobic culture and direct amplification of 16S ribosomal DNA using an open-ended method
358 similar to the one in the present study, in an attempt to overcome the limits of the culture technique
359 (Haraszthy *et al*., 2007) . They found *Streptococcus salivarius* and *Campylobacter concisus* as the
360 most prevalent species in the control group. These species were found in the control group of our
361 study, too. In addition, *Actinomyces graevenitzii,* statistically associated with the halitosis condition
362 in the present study, was also one of the most prevalent species in halitosis group in the Haraszthy
363 et al. study (Haraszthy *et al*., 2007).

364 Moreover, the present results revealed, in accordance with these earlier findings, the presence of
365 *Actinomyces odontolyticus*, *Solobacterium moorei*, *Streptococcus oralis*, and *Streptococcus*
366 *sanguinis* in halitosis patients. These bacterial species were often detected in halitosis biofilm in
367 literature (Haraszthy *et al*., 2007). Riggio et al. profiled and compared the microbiota on the tongue
368 dorsum by means of culture-independent techniques, using PCR amplification, cloning and
369 sequencing of 16S rRNA genes (Riggio *et al*., 2008). The authors concluded that the tongue dorsum
370 presents a higher microbial diversity in halitosis samples compared to the controls. According to the
371 authors' findings *Streptococcus salivarius* was present in high concentrations both in the halitosis
372 and control group (Riggio *et al*., 2008). The present study confirmed these findings. Consequently,

373 it can be assumed that this microorganism does not play an etiological role in the development of
374 oral malodor.
375 Recently, Yang et al. used pyrosequencing in a cross-sectional and longitudinal study for a
376 comparison of the microbial communities in halitosis-patients and in healthy volunteers (Yang *et*
377 *al*., 2013). They found that *Prevotella* spp. and *Leptotrichia* spp. were positively linked to hydrogen
378 sulphide (Yang *et al*., 2013). Similarly, Ren et al. found members of the genera *Prevotella* and
379 *Leptotrichia* (and *Actinomyces*, *Selenomonas* etc.) in halitosis with pyrosequencing (Ren *et al*.,
380 2016). Seerangaiyan et al. using Illumina MiSeq high-throughput sequencing found *Leptotrichia,*
381 *Prevotella, Selenomonas, Tannerella* taxa abundant in halitosis, whereas several *Streptococcus*
382 species were more abundant in the control (Seerangaiyan *et al*., 2017).
383 The results deriving from our culture-independent "open ended" technique in combination with the
384 culture technique confirmed the presence of the taxa found in these high-throughput sequencing
385 studies, specifically the detection of several *Prevotella* species with both methods, e.g. *P. histicola*,
386 which was found in high concentrations in samples from the halitosis patients with culture
387 technique. Moreover, our methods revealed the significant presence of *S. mitis* and *S.*
388 *pseudopneumoniae* in the halitosis samples which might indicate their role in the adhesion to the
389 tongue surface during the biofilm formation. In contrast to the high-throughput sequencing studies,
390 with our methodological approach by means of sequencing full-length 16S rDNA fragments, we
391 were able to differentiate the many *Streptococcus* species that were detected. Both Seerangayian K
392 *et al.* and Yang *et al.* found certain OTUs (operational taxonomic units) of the genus *Streptococcus*
393 associated with healthy study participants, yet they were not able to achieve a clear species-level
394 analysis (Seerangayian K *et al.,* 2017, Yang *et al.,* 2013).
395 The low number of participants in our study is an obvious limitation, however, other reports draw
396 conclusions regarding the etiological flora for halitosis using similar study populations, e.g. the
397 study by Kazor CE et al. using a culture-independent approach on six halitosis patients and five
398 healthy controls, or the study by Ren W et al. comparing five halitosis patients with five controls

399 (Kazor *et al*., 2003; Ren *et al*., 2016). In our study, the results of the combination of culture-
400 dependent and culture-independent "open-ended" cloning techniques highlighted the most prevalent
401 bacterial species within the halitosis biofilms, and the bacterial species influencing the healthy
402 biofilms. This had not been performed yet. The aerobic and anaerobic cultivable species from the
403 halitosis group corresponded to the taxa reported by many authors: all of those species except for
404 *Veillonella rogosae* were previously found on the tongue dorsum of halitosis subjects. This species
405 had previously been isolated from supra-gingival dental plaque and from the tongue biofilm of
406 healthy individuals (Arif *et al*., 2008; Mashima, Kamaguchi and Nakazawa, 2011; Mashima and
407 Nakazawa, 2013). *V. rogosae* is a Gram-negative, non-motile, non-sporulating coccoid and appears
408 as a single cell or in short-chains. It is strictly anaerobic and oxidase-negative. It exhibits
409 pyroglutamic acid arylamidase and variable alkaline phosphatase activity. Major acid end products
410 are acetic and propionic acids (Arif *et al*., 2008). *Veillonella* genus has always been connected with
411 the production of VSCs and is therefore responsible for malodor (Mashima, Kamaguchi and
412 Nakazawa, 2011), but to our knowledge, *V. rogosae* was never associated with halitosis so far.
413

414 The cloning method, exploiting a "hypothesis-free" approach to achieve a greater overview of the
415 total microbial diversity, showed a high variability among the detected species between the two
416 groups. Particularly in the halitosis group it allowed for the detection of different *Streptococcus* spp,
417 *Haemophilus parainfluenzae*, *Prevotella pallens*, *Prevotella veroralis*, *Photobacterium* spp.
418 *Leptotrichia wadei*, and *Tannerella forsythia*, in line with the results obtained by Riggio et al.
419 (Riggio *et al*., 2008) and Yang et al. (Yang *et al*., 2013). In the control group, using the cloning
420 method, we were able to detect *Abiotrophia para-adiacens*, *Granulicatella* spp.,
421 *Lachnoanaerobaculum saburreum*, *Selenomonas* spp. and *Staphylococcus warneri*, which were not
422 detected by means of culture dependent methods Particularly in the control group, two interesting
423 species were noted: *Selenomonas* is a genus which is generally taken to be a volatile sulphur
424 compounds producer (Persson *et al*., 1990). In general, *S. mitis*, *S. oralis* and *S. pseudopneumoniae*

425 are rather seen as belonging to the healthy physiological flora than associated with any oral disease.
426 However, the 16S rRNA gene of *S. mitis and S. pseudopneumoniae,* as shown by the recent study of
427 Tze et al., have a 98% correspondence with a new isolated species from the tongue dorsum in a
428 halitosis patient: the *Streptococcus halitosis.* Hence it might be possible that these taxa would
429 provide favorable conditions in the microenvironment of the tongue biofilm for other, halitosis-
430 associated taxa to thrive.

431 **Conclusion**

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

438 **Declarations**

439 **Data availability statement**

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

442 **Authors' contributions**

443 **Conceptualization:** Sara Bernardi, Annette Anderson

444 **Formal Analysis**: Kirstin Vach

445 **Investigation:** Sara Bernardi

446 **Supervision**: Lamprini Karygianni, Ali Al-Ahmad, Elmar Hellwig, Guido Macchiarelli

447 **Resources:** Andreas Filippi, Andrea Zurcher, Ali Al-Ahmad

448 **Writing – original draft preparation:** Sara Bernardi, Annette Anderson, Kristin Vach, Lamprini

449 Karygianni, Andreas Filippi, Andrea Zurcher, Ali Al-Ahmad

450 **Writing – review and editing**: Elmar Hellwig, Guido Macchiarelli, Lamprini Karygianni, Ali Al-

451 Ahmad, Annette Anderson , Andreas Filippi, Andrea Zurcher, Kristin Vach

452 All authors read and approved the final manuscript.

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458 **Conflict of interests**

459 None declared

460 **Ethics statement**

461 The study design was reviewed and approved by the Ethics Committee of the Albert-Ludwigs-

462 University of Freiburg (74/15).

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600 **Tables**
601 **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 three 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 sub-gingival 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:

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607 **Table 2**. Overview of the outcomes of the anamnestic and clinical assessments.

osis Grou	Age	Gender	Gastro-esophageal reflux	Diabetes	Renal disease	Alcohol	Smoke	Brush tongue	Rem. Prost	PSR Index	HALIMETER

	62	F	YES	NO	NO	NO	NO	NO	NO	1	122 ppb
	38	M	NO	NO	NO	NO	NO	NO	NO	1	133 ppb
	51	M	NO	NO	NO	NO	NO	NO	NO	3	152 ppb
	65	F	NO	NO	NO	NO	NO	NO	NO	1	123 ppb
	43	F	YES	NO	NO	NO	NO	NO	NO	0	125 ppb
	29	F	NO	NO	NO	NO	YES	NO	NO	1	226 ppb
Control Group	33	F	NO	NO	NO	NO	NO	NO	NO	0	0
	26	F	NO	NO	NO	NO	NO	NO	NO	0	0
	25	M	NO	NO	NO	YES	NO	NO	NO	0	0
	23	F	NO	NO	NO	NO	NO	ONCE A WEEK	NO	0	0
	28	F	NO	NO	NO	NO	NO	ONCE A DAY	NO	0	0
	22	M	NO	NO	NO	NO	NO	NO	NO	0	0

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621 **Figure legends**

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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

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

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

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 < 0.05) are depicted

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 %)

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

A

black pigmented
Solobacterium moorei
Fusobacterium periodontium
Veillonella rogosae
Veillonella dispar
Veillonella atypica
Prevotella salivae
Prevotella histicola
Prevotella sp.
Parvimonas micra
Not pigmented sp.
Megasphaera micronuciformis
Gemella haemolysis
Gemella sanguinis
Fusobacterium sp.
Fusobacterium nucleatum
Bifidobacterium longum
Atopobium parvulum

0% 50% 100%

H1
H2
H3
H4
H5
H6

B

BD6 Unknown
Veillonella sp.
black pigmented
Campylobacter concisus
Prevotella histicola
Atopobium parvulum
Veillonella atypica
Veillonella rogosae
Fusobacterium periodontium
Alloprevotella tannarae
Lachoanaerobaculum orale
Fusobacterium nucleatum
Veillonella dispar
Fusobacterium nuclei
Veillonella parvula
Propionibacterium acnes
Prevotella nigrescens

0% 20% 40% 60% 80% 100%

P1
P2
P3
P4
P5
P6

A

Streptococcus australis
Rothia aeria
Streptococcus infantis
Actinomyces odontolyticus
Neisseria macacae
Lactobacillus fermentum
Neisseria flavescens
Rothia dentocariosa
Actinomyces oris
Streptococcus oralis
Capnocytophaga granulosa
Neisseria perflava
Rothia mucilaginosa
Brevibacterium casei
Actinomyces graevenitzii
Streptococcus salivarius
Streptococcus parasanguinis
Streptococcus mitis

0% 20% 40% 60% 80% 100%

H1
H2
H3
H4
H5
H6

B

Actynomices odontolyticus
Eikenella corrodens
Neisseria sp.
Actinomyces naeslundii
Streptococcus mitis
Capnocytophaga gingivalis
Neisseria perflava
Rothia dentocariosa
Streptococcus parasanguinis
Streptococcus oralis
Abiotrobia defectiva
Rothia mucilaginosa
Capnocytophaga sputigena
Neisseria flavescens
Rothia aeria
Actinomyces oris
Streptococcus salivarius
Streptococcus infantis
Streptococcus australis

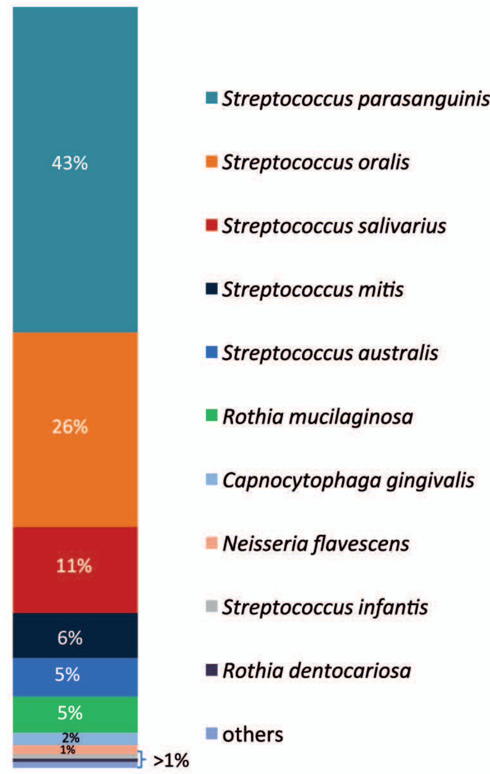
0% 20% 40% 60% 80% 100%

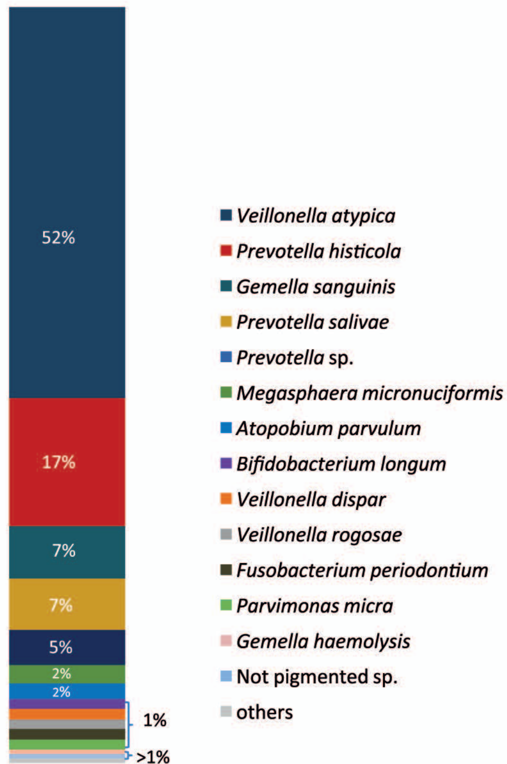
P1
P2
P3
P4
P5
P6

A

33%
21%
18%
8%
4%
4%
4%
2%
2%
2%
1%

Streptococcus mitis
Streptococcus salivarius
Streptococcus parasanguinis
Streptococcus oralis
Rothia dentocariosa
Rothia mucilaginosa
Brevibacterium casei
Actinomyces graevenitzii (p<0.05)
Neisseria perflava
Actinomyces odontolyticus
Lactobacillus fermentum
others





B

69%

14%

6%

5%

2%

1%

2%

Veillonella sp.

Veillonella atypica

Prevotella histicola

Veillonella dispar

BD6 Unknown

Campylobacter concisus

black pigmented not cultivated

others

A

Veillonella sp.
Veillonella parvula
Veillonella dispar
Tannerella orsythia
Streptococcus tigurinus
Streptococcus thermophilus
Streptococcus sanguinis
Streptococcus salivarius
Streptococcus pseudopneumoniae
Streptococcus parasanguinis
Streptococcus oralis
Streptococcus oligofermentans
Streptococcus mitis
Sstreptococcus lactarius
Streptococcus infantis
Streptococcus gordonii
Streptococcus cristatus
Streptococcus australis
Streptococcus anginosus
Rothia sp.
Rothia mucilaginosa
Prevotellaceae uncultured
Prevotella veronalis
Prevotella sp./*oral*
Prevotella salivae
Prevotella pallens
Prevotella histicola
Prevotella melaninogenica
Photobacterium sp.
Okadaella gastrococcus
Leptotrichia sp.
Haemophilus parainfluenza
Haemophilus influenza
Alloprevotella sp.

0% 20% 40% 60% 80% 100%

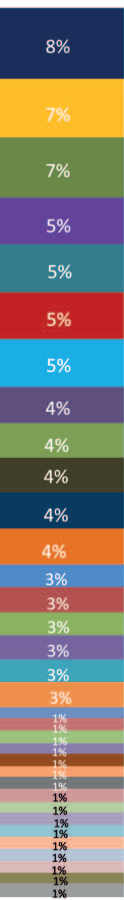
H1 H2 H3 H4 H5 H6

B

Veillonella sp.
Streptococcus tigurinus
Streptococcus thermophilus
Streptococcus salivarius
Streptococcus parasanguinis
Streptococcus oralis
Streptococcus mitis
Staphylococcus warneri
Selenomonas sp.
Selenomonas flueggei
Selenomonas dianae gene
Prevotella veronalis
Prevotella sp./*oral*
Prevotella pallens
Prevotella histicola
Porphyromonas sp.
Peptococcus sp.
Oribacterium parvum
Okadaella gastrococcus
Neisseria perflava
Neisseria flavescens
Leuconostoc mesenteroides
Lachnospiraceae oral clone
Lachnoanaerobaculum sp.
Lachnoanaerobaculum orale
Haemophilus parainfluenza
Granulicatella paraadiacens
Granulicatella adiacens
Gemella sanguinis
Gemella haemolysans
Fusobacterium periodonticum
Eubacterium sp.*oral*
Eubacterium infirmum
Centipeda periodontii
Alloprevotella sp.
Abiotrophia paraadiacens

0% 50% 100%

P1 P2 P3 P4 P5 P6


A
8%
7%
7%
5%
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4%
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1%
*Streptococcus mitis** (p<0.05)
Streptococcus oralis (p=0.8)
*Streptococcus pseudopneumoniae** (p<0.5)
Prevotella sp./*oral*
Streptococcus infantis (p=0.06)
Streptococcus salivarius
Streptococcus thermophilus
Okadaella gastrococcus
Streptococcus australis
Streptococcus parasanguinis
Streptococcus tigurinus
Veillonella sp.
Alloprevotella sp.
Prevotella melaninogenica
Prevotella pallens
Prevotellaceae uncultured
Streptococcus anginosus
Veillonella dispar
Haemophilus influenza
Haemophilus parainfluenza
Leptotrichia sp.
Photobacterium sp.
Prevotella histicola
Prevotella salivae
Prevotella veronalis
Rothia mucilaginosa
Rothia sp.
Streptococcus cristatus
Streptococcus gordonii
Sstreptococcus lactarius
Streptococcus oligofermentans
Streptococcus sanguinis
Tannerella forsythia
Veillonella parvula
B
8%
8%
6%
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6%
4%
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Streptococcus salivarius
Streptococcus thermophilus
Gemella sanguinis
Okadaella gastrococcus
Streptococcus parasanguinis
Prevotella histicola
Prevotella sp./*oral*
Abiotrophia paraadiacens
Alloprevotella sp.
Centipeda periodontii
Eubacterium infirmum
Eubacterium sp.*oral*
Fusobacterium periodonticum
Gemella haemolysans
Granulicatella adiacens
Granulicatella paraadiacens
Haemophilus parainfluenza
Lachnoanaerobaculum orale
Lachnoanaerobaculum saburreum
Lachnospiraceae oral clone
Leuconostoc mesenteroides
Neisseria flavescens
Neisseria perflava
Oribacterium parvum
Peptococcus sp.
Porphyromonas sp.
Prevotella pallens
Prevotella veronalis
Selenomonas dianae gene
Selenomonas flueggei
Selenomonas sp.
Staphylococcus warneri
Streptococcus mitis
Streptococcus oralis
Streptococcus tigurinus
Veillonella sp.