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The tongue microbiome in healthy subjects and patients with intra-oral halitosis

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Abstract

Intra-oral halitosis (IOH) is an unpleasant odor emanating from the oral cavity. It is thought that the microbiota of the dorsal tongue coating plays a crucial role in this condition. The aim of the study was to investigate the composition of the tongue microbiome in subjects with and without IOH. A total of 26 subjects, 16 IOH patients and 10 healthy subjects were recruited based on their organoleptic score and volatile sulfur compound (VSC) measurements. The composition of the tongue microbiome was studied using the 16s amplicon sequencing of the V3-V4 hyper variable region with an Illumina MiSeq. The sequenced data were analyzed using QIIME, and the sequences obtained were distributed across 7 phyla, 27 genera and 825 operational taxonomic units (OTUs). At a higher taxon level, *TM7* was associated with IOH patients whereas *Gemellaceae* was significantly abundant in the healthy subjects. At OTU level, we found several significant OTUs that differentiated the IOH patients from the controls. These included *Aggregatibacter* (OTU id 4335776), *Aggregatibacter segnis* (*A. segnis*), *Campylobacter, Capnocytophaga*, *Clostridiales, Dialister*, *Leptotrichia*, *Parvimonas*, *Peptostreptococcus*, *Peptococcus*, *Prevotella*, *Selenomonas*, *SR*1, *Tannerella*, *TM*7-3 and *Treponema* in the IOH group. In the control group, *Aggregatibacter* (OTU id 4363066), *Haemophilus*, *Haemophilus parainfluenza* (*H. parainfluenza*), *Moryella*, *Oribacterium*, *Prevotella*, several *Streptococcus*, *Rothia dentocariosa* (*R. dentocariosa*) and OTU from *Gemellaceae* were significantly abundant. Based on our observation, it was concluded that the bacterial qualitative composition of the IOH and the control group was almost the same, except for the few above-mentioned bacterial species and genera.

Nomenclature

IOH	Intra-oral halitosis
DPSI	Dutch periodontal screening index
EOH	Extra-oral halitosis
EDTA	Ethylenediaminetetraacetic acid
HCl	Hydrochloric acid
H_2S	Hydrogen sulfide
CH_3SH	Methyl mercaptan
$(CH_3)_2S$	Dimethyl sulfide
OLS	Organoleptic score
OTU	Operational taxonomic unit
PCoA	Principal component analysis
PCR	Polymerase chain reaction
VSC	Volatile sulfur compound
TE	Tris-EDTA
WTCI	Winkel tongue coating index

Introduction

Halitosis or oral malodor is defined as an unpleasant odor in expired air [1]. Halitosis has no gender-specific differences and patients with this condition can face significant emotional and psychological stress [2]. Halitosis can be subdivided into extra- and intra-oral halitosis. Blood-borne causes of extra-oral halitosis (EOH) involve liver and kidney diseases, diabetes, metabolic disorders, certain drugs (disulfiram, dimethyl sulfoxide, cysteamine) and food (onion, garlic) [3]. Non-blood-borne causes of extra-oral halitosis include respiratory diseases and certain stomach conditions [3]. Intra-oral halitosis (IOH) is caused by conditions in the oral cavity and accounts for 80%–90% of halitosis [1]. Bacteria in the coating of the tongue dorsum are believed to be the main cause of physiological IOH [4] and oral conditions such as gingivitis, periodontitis and xerostomia may also contribute to it [5]. The major IOH imputes are volatile sulfur compounds (VSCs) including hydrogen sulfide and methyl mercaptan [1]. Other compounds that have been linked to IOH are indole, short chain fatty acids and polyamines, such as putrescine and cadaverine [6], although this is questioned by Tangerman and Winkel (2007) [7] and Van den Velde *et al* (2009) [8]. VSCs result from the bacterial degradation of the sulfur-containing amino acids cysteine and methionine [9]. Gram-negative as well as Gram-positive anaerobic bacteria produce VSCs and therefore are thought to be involved in IOH [9, 10]. The topography of the tongue (roughness, papillae, fissures, crypts) favors the development of the tongue coating and growth of anaerobic bacteria [11]. IOH tongue microbes were first studied with aerobic and anaerobic culture techniques and reported in the involvement of *Porhyromonas, Prevotella,* fusiforms, *Peptostreptococcus, Eubacterium, Selenomonas,* and *Centipeda* species [12, 13]. However, due to limitations in culture techniques, the tongue microbiota has not been fully characterized. Culture- independent molecular techniques, such as 16s rRNA sequencing on cloned genes, have identified different species in IOH such as *Atopobium parvulum* (*A. parvulum*), *Dialister* spp., *Eubacterium sulci,* a phylotype of *TM7*, *Streptococcus* and *Prevotella* [14, 15]. The direct amplification of 16s rRNA using a broad range polymerase chain reactions (PCRs) identified *Solobacterium moorei* (*S. moorei)* in the IOH patient group but not in the controls [16]. More recently, next generation sequencing has revealed the positive correlation of *Leptotrichia* spp. and *Prevotella* spp. to oral malodor severity, whereas *Haemophilus* spp. and *Gemella* spp. showed a negative correlation [17]. Overall, the findings from these studies showed significant differences in the microbial composition of the tongue microbiota between patients with IOH and the healthy controls.

Previous studies have provided new insights into the tongue microbiome; however, an in-depth analysis at the species level has not yet been provided. Understanding the composition and function of microbial communities may lead to the development of diagnostic and therapeutic tools. Moreover, recent research posits 'integrated bacterial communities' as being responsible for the development of microbial disease [18]. This concept came to light in IOH studies, but the cause of it remains unclear. To investigate this, we studied the microbial composition of the tongue microbiota with a focus on the taxon abundance from the phylum to species-level operational taxonomic units (OTU) by 16s amplicon sequencing.

Materials and methods

Ethics statement

The study was conducted in accordance with Dutch laws on ethical rules and principles for human research and with the approval of the medical ethical committee of the University Medical Center Groningen (METC 2015/458) in accordance with the Helsinki Declaration 2013. Written informed consent was obtained from all subjects participating in the study.

Study population and design

Patients and controls were recruited from the Clinic for Periodontology Amsterdam, Amsterdam, The Netherlands. The total number of participants in the study was 26. The subjects who reported to the clinic complaining of halitosis were consecutively screened. Prior to their visit, subjects were instructed to: (1) avoid onion, garlic and hot spices in their diet for 48 h before their appointment, (2) refrain from alcohol intake and smoking 12 h prior to the halitosis examination, (3) abstain from normal oral hygiene procedures and (4) avoid mint containing products, aftershave lotions and highly scented cosmetics on the day of the examination. The subjects were allowed to eat and drink up to 8 h before the examination. Water drinking was allowed up to 3 h before the examination. Subjects filled in the detailed halitosis and medical questionnaire (CAI, www.healthquestionnaires.eu) and a thorough periodontal and halitosis examination was performed to determine whether the patient fulfilled the entrance criteria.

Exclusion criteria

Subjects with the presence of systemic diseases and on systemic medication related to oral dryness, pregnancy, those using antimicrobial therapy and mouth rinses during the three months prior to the start of the study, those with a history of fever and cold, as well as patients who had not followed the proper instructions for halitosis assessment, were excluded from the study. The periodontal condition of the subjects was investigated using the Dutch periodontal screening index (DPSI). Subjects with a DPSI score of $\geqslant 3$ were excluded from participation in the study.

Inclusion criteria

After the screening session the following parameters were established:

1. Organoleptic score (OLS): (0 = no halitosis to 5 = presence of extreme halitosis) from nose and mouth [19, 20]
2. Volatile sulfur compound (VSC) level measured with (Halimeter®, Interscan corporation, California, United States)
3. VSC gases namely hydrogen sulfide (H_2S) and methyl mercaptan (CH_3SH) from OralChroma™ (Abilit Corporation, Japan)
4. Dutch periodontal screening index (DPSI) [21]
5. Winkel tongue coating index (WTCI) [22]

For organoleptic testing, patients were asked to close their mouth for 1 min, and then slowly exhale air from the nose and mouth at a distance of approximately 10 cm from the nose of an experienced examiner (EGW). Halimeter® and OralChroma VSC measurements were performed according to the manufacturer's instructions. The IOH patient group was selected based on an organoleptic score of $\geqslant 2$ from the mouth and nose $\leqslant 1$, having a VSC level >160 ppb, and $H_2S > 4\ \text{nmol}\,\text{l}^{-1}$ (96 ppb) and $CH_3SH > 0.5\ \text{nmol}\,\text{l}^{-1}$ (12 ppb) [7] and a DPSI of $\leqslant 2$. Patients with non-halitosis presenting an organoleptic score of 0 from the mouth and nose air, with a VSC level of <110 ppb (Halimeter®), $H_2S < 4\ \text{nmol}\,\text{l}^{-1}$ (96 ppb), $CH_3SH < 0.5\ \text{nmol}\,\text{l}^{-1}$ (12 ppb) (OralChroma) and a DPSI of $\leqslant 2$, were included in the control group.

Sample collection and DNA extraction

Tongue samples were collected in the morning. A tongue cleaning device (Scrapy™, Clevercool, Amsterdam, The Netherlands) [23] was used to dislodge the tongue coating by scraping from dorsal to ventral. The sample was collected in a Petri dish and the weight of the tongue coating was measured using an electronic pocket balance (Best home, Kijkshop, The Netherlands). Then, the sample was transferred to a 1 ml Tris-EDTA (Sigma-Aldrich Chemie N V, Zwijndrecht, The Netherlands) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) in an Eppendorf vial and stored at -20 °C until DNA extraction. The DNA was extracted using a PowerSoil® DNA isolation kit (MO BIO Laboratories, Qiagen company) according to the manufacturer's instructions. The DNA concentration was quantified with a Qubit® 2.0 fluorometer and the quantity was normalized to $5\ \text{ng}\,\mu\text{l}^{-1}$ for library preparation.

Library preparation and 16s rRNA sequencing

The 16s V3-V4 region of the 16s rDNA was amplified using a forward primer (**TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG** CCT ACG GGN GGC WGC AG) and a reverse primer (**GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G**GA CTA CHV GGG TAT CTA ATC C). Bold letters represent the adapter sequences. The PCR reaction was performed in a total volume of 25 μl containing 5 μl from the forward and reverse primer (1 μm), 2.5 μl ($5\ \text{ng}\,\mu\text{l}^{-1}$) microbial DNA and 12.5 μl 2× KAPA Hifi HotStart Ready Mix (KAPA Biosystems). The conditions of the reaction were as follows: denaturation at 95 °C for 3 min followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and subsequently an elongation step at 72 °C for 5 min. After the PCR clean up using AMPure XP beads (Beckman Coulter), dual barcoding PCR was performed in a total volume of 50 μl according to the manufacturer's instructions with 5 μl of the PCR product, 5 μl indexes and 5 μl adapters (Illumina Nextera index kit), 25 μl 2× KAPA Hifi HotStart Ready Mix (KAPA Biosystems) and 10 μl PCR grade water to obtain the DNA library. The library was cleaned up using AMPure XP beads (Beckman Coulter) and quantified using a fluorometric quantification method. Separate libraries obtained from the different samples were pooled in an equimolar amount. Subsequently, the libraries were sequenced on a MiSeq sequencer, using the MiSeq reagent kit v3, and sequencing aimed for at least 60-fold coverage. The MiSeq data was processed with MiSeq control software v2.4.0.4.

Bioinformatics and statistical analysis

The sequenced demultiplexed FASTQ raw data from the Miseq was analyzed with QIIME (quantitative insight into microbial ecology) version 1.8.1 according to the Caparso 1.1.0 workflow [24]. The forward and reversed paired end reads obtained from MiSeq were assembled into sequences and quality filtered at a phred quality score threshold of Q20. The sequences were clustered and aligned at 97% identity with the UCLUST algorithm [25] and assigned taxonomy using the Greengenes database 13–8 [26]. The representative sequences of the individual OTUs were aligned with pyNAST and a phylogenetic tree was generated with FastTree. To test the diversity within the samples (alpha diversity), the samples were rarefied at 11 000 reads per sample. Bacterial richness was estimated with ChaO1 and the evenness was estimated with the Shannon index. The overall community composition relationship (beta diversity) was analyzed with weighted unifrac and visualized with a principal coordinate analysis [27]. The phylum and the genus-level comparison between the healthy and the IOH samples was done with the Wilcox rank sum test. The student's t-test and Adonis were used to test the alpha and beta diversity respectively. For species-level OTUs,

Table 1. Demographic and clinical characteristics of intra-oral halitosis patients and controls.

Clinical parameters		Intra-oral halitosis ($n = 16$)	Controls ($n = 10$)	p value
Age (year)		36 ± 13	34 ± 6	0.66*
Gender	Female	10 (62%)	8 (80%)	0.41$
	Male	6 (37%)	2 (20%)	
Organoleptic score		Range (2–4)	Range 0	0.0001*
Winkel tongue coating index		6.00 ± 2.44	3.00 ± 2.44	0.02*
Plaque weight (milligrams)		357.00 ± 315.64	143.00 ± 92.38	0.01*
H_2S[a]		390.93 ± 293.95	9.50 ± 13.01	0.0003*
CH_3SH[a]		254.75 ± 261.11	5.70 ± 9.22	0.005*
$(CH_3)_2S$[a]		41.81 ± 46.60	7.10 ± 7.59	0.02*

[a] H_2S, CH_3SH and $(CH_3)_2S$ were measured in parts per billion (ppb). The continuous variables were represented as mean ± standard deviation.

*Two sample t-test, $ Pearson Chi-square test or Fisher exact test.

DESeq2, a negative binomial test, was used to test the differentially abundant OTUs based on log2 fold changes between the IOH and control group [28, 29]. In order to minimize the library size variation, we selected fifteen samples with the maximum number of reads ranging from (72 087–198 212) and the mean library size of the IOH (132 678 reads) and control group (161 334 reads) was matched. Fifteen samples were included in total—10 with IOH and 5 control samples; the samples included were (16A, 20A, 26A, 28A, 38A, 7A, 12A, 14A, 18A, 27A, 29A, 33A, 35A, 36A, 37A). Further, the sequences of significantly abundant OTUs were compared with BlastN in order to assign the closest species taxonomic level [30]. The statistics were done using the R statistical package (3.3.0) and QIIME version 1.9.1.

Results

Of the 26 participants, 16 subjects (10 females and 6 males) complied with the criteria of IOH, whereas 10 subjects (8 females and 2 males) complied with the criteria of controls. The mean age of the IOH patients and controls was 36 (±12.96, range 20–67 years) and 34 (±5.53, range 23–43 years), respectively. There was no significant difference in age ($p = 0.66$) or sex distribution ($p = 0.41$) between the patients and controls. The demographic and clinical characteristics of the study population are shown in table 1.

The mean OLS, WTCI, plaque weight and mean concentrations of H_2S, CH_3SH and $(CH_3)_2S$ were statistically higher in the patients compared to the controls (table 1). In the patient group, Pearson's correlation coefficient showed a positive correlation between $(CH_3)_2S$ and CH_3SH level ($r = 0.6, p = 0.01$) but no correlation between the $(CH_3)_2S$ and H_2S level ($r = 0.1, p = 0.65$) was observed.

Overall sequencing data and microbial profile

All 26 samples were sequenced, resulting in a total of 3419 715 reads after quality filtering, with an average of 131 528 (median of 117 548) reads per sample. We detected 7 phyla, 27 genera and 825 OTUs with singletons. The average length of the reads was approximately 460 base pairs (bp) (excluding the PCR primer and barcode sequences).

Bacterial diversity

Bacterial diversity such as alpha diversity (Shannon, Chao1) was measured at a rarefaction level of 11 000 reads per sample. Figure 1(a) shows the rarefaction curves determined by the Shannon index, and figure 1(b) shows the rarefaction measure of ChaO1. The species richness and evenness estimated with the ChaO1, and the species richness measured with Shannon in the individual samples, were consistent between the two groups ($p = 0.15$, $p = 0.45$) respectively. The bacterial community composition based on the phylogenetic relationship was determined with weighted unifrac, a distant matrix, and the result was depicted in a principal coordinate analysis (PCoA). Figure 1(c) shows the PCoA analysis of the IOH and control group based on weighted unifrac analysis. No statistically significant difference was found between the groups ($p = 0.30$). Therefore, the weighted unifrac represented a similar community composition between the IOH and control group.

Microbial profiles related to halitosis and oral health

All sequences obtained were clustered into the OTUs based on 97% identity. OTUs with >1% relative abundance were represented in the phyla: *Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria* and *TM7*. Figure 2 shows the mean relative abundance of individual samples of IOH patients and controls. The mean relative abundance of each phyla in the IOH and control group were: *Actinobacteria* (4.89% versus 4.38%), *Bacteroidetes* (37.53% versus 33.66%), *Firmicutes* (30.13% versus 38.86%),

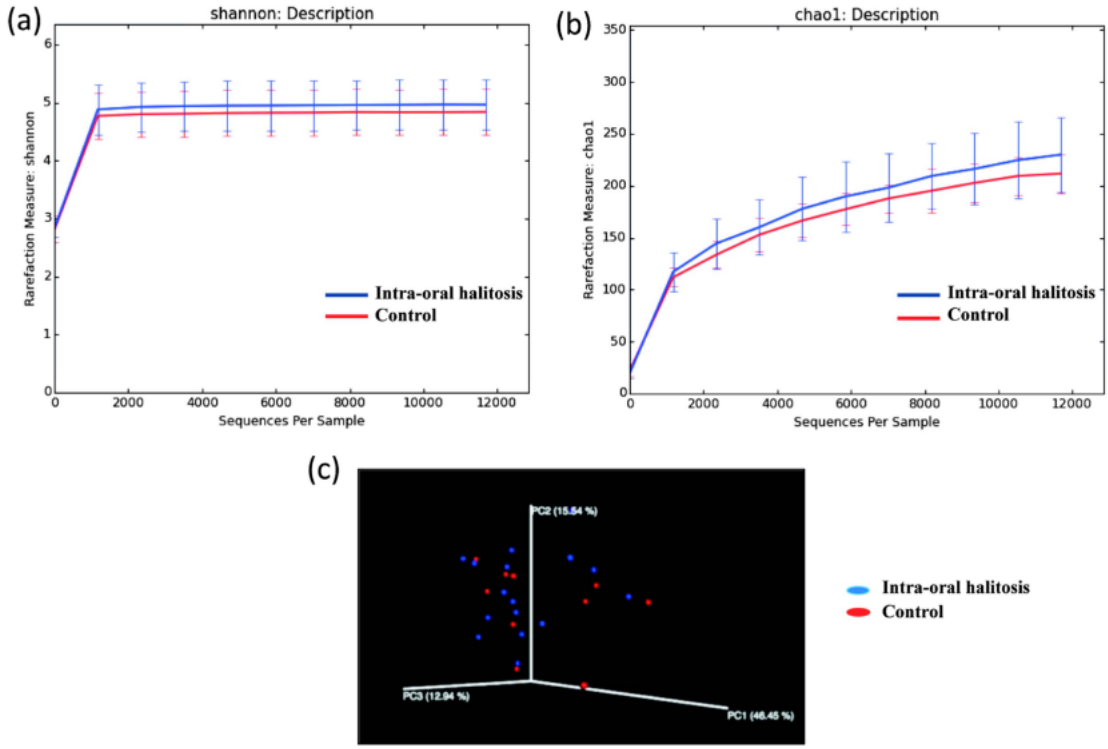

Figure 1. Alpha diversity analysis (Shannon and ChaO1 index) comparing intra-oral halitosis and the control group and a principal coordinate analysis of weighted unifrac at a sequencing depth of 11 000 sequences/sample for intra-oral halitosis and the control samples. (a) Rarefaction curves (Shannon index on *Y*-axis) and (b) rarefaction curve (ChaO1 on *Y*-axis) for intra-oral halitosis (blue curves) versus control (red curves). (c) Principal coordinate analysis of 16 intra-oral halitosis patients and 10 controls based on weighted unifrac analysis.

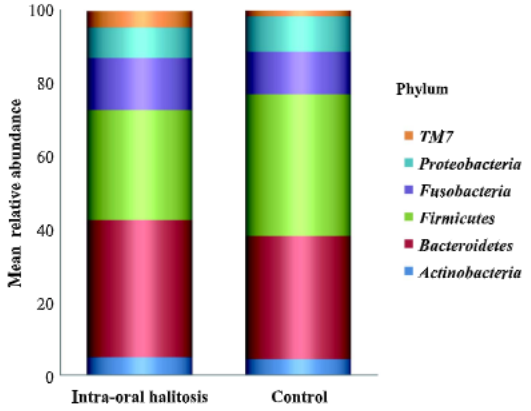

Figure 2. A comparison of the phyla (mean relative abundance) of intra-oral halitosis and the control group.

Fusobacteria (14.32% versus 11.67%), *Proteobacteria* (8.35% versus 9.73%) and *TM7* (4.51% versus 1.55%). The Wilcoxon rank sum statistical comparison at the phylum level showed a significantly higher proportion of *TM7* in the IOH group ($p = 0.009$). No significant difference in the relative abundance was found in *Actinobacteria* ($p = 0.38$), *Bacteroidetes* ($p = 0.41$), *Firmicutes* ($p = 0.09$), *Fusobacteria* ($p = 0.31$) or *Proteobacteria* ($p = 0.95$). At the family level, *Gemellaceae* was significantly associated with the controls ($p = 0.03$). Genera with a mean relative abundance $\geqslant$1% were taken into account and 17 genera including *Actinomyces*, *Rothia*, *Atopobium*, *Porphyromonas*, *Prevotella*, an uncharacterized genus of *Gemellaceae*, *Granulicatella*, *Streptococcus*, an uncharacterized genus of *Lachnospiraceae*, *Selenomonas*, *Veillonella*, *Fusobacterium*, *Leptotrichia*, *Neisseria*, *Campylobacter*, Haemophilus and *TM7* were detected in the patients and controls. The remaining genera with <1% mean relative abundance were excluded for genus-level comparisons between the groups. Table 2 represents the statistical analysis of each genus of the IOH and healthy group. Figure 3 represents the relative abundance of the genera in individual samples of the IOH patients and healthy controls.

Species-level OTUs

The samples with the maximum number of reads were selected and the mean library size was matched between the groups. OTUs present in fewer than two samples were removed. After filtering the singletons, the total number of OTUs obtained was 695 and these were further analyzed with DESeq2. Based on the log2 fold changes in an abundance of OTUs between patients and controls, a total of 37 OTUs were found that discriminated between the two groups. The OTUs that were significantly associated with IOH include *Aggregatibacter* (OTU id 4335776), *A. segnis*, *Campylobacter*, *Capnocytophaga*, *Clostridiales*, *Dialister*, *Leptotrichia* (four OTUs).

Table 2. A list of the genus with mean abundance and standard deviation in the intra-oral halitosis and control group.

Genera	Intra-oral halitosis (mean ± SD*)	Control (mean ± SD*)	*p* value
Actinomyces	2.80 ± 1.84	1.82 ± 1.69	0.08
Atopobium	1.06 ± 0.79	0.74 ± 0.66	0.24
Campylobacter	1.70 ± 1.20	1.48 ± 1.12	0.61
Fusobacterium	4.61 ± 3.77	3.49 ± 2.32	0.97
Granulicatella	1.33 ± 2.28	1.81 ± 2.09	0.54
Haemophilus	3.89 ± 5.02	4.30 ± 5.51	1.00
Leptotrichia	9.70 ± 3.30	8.16 ± 7.98	0.57
Neisseria	2.65 ± 4.20	3.88 ± 5.92	0.97
Porphyromonas	2.51 ± 3.92	1.15 ± 1.13	0.73
Prevotella (family *Prevotellaceae*)	31.52 ± 11.70	29.49 ± 12.96	1.00
(*Prevotella*) (family *Paraprevotellaceae*)	2.53 ± 3.01	2.53 ± 2.97	1.00
Rothia	0.98 ± 1.18	1.73 ± 1.76	0.24
Selenomonas	1.96 ± 1.97	1.29 ± 1.14	0.50
Streptococcus	7.14 ± 5.05	11.21 ± 8.83	0.27
Veillonella	14.95 ± 5.11	18.26 ± 6.60	0.17

SD* standard deviation.

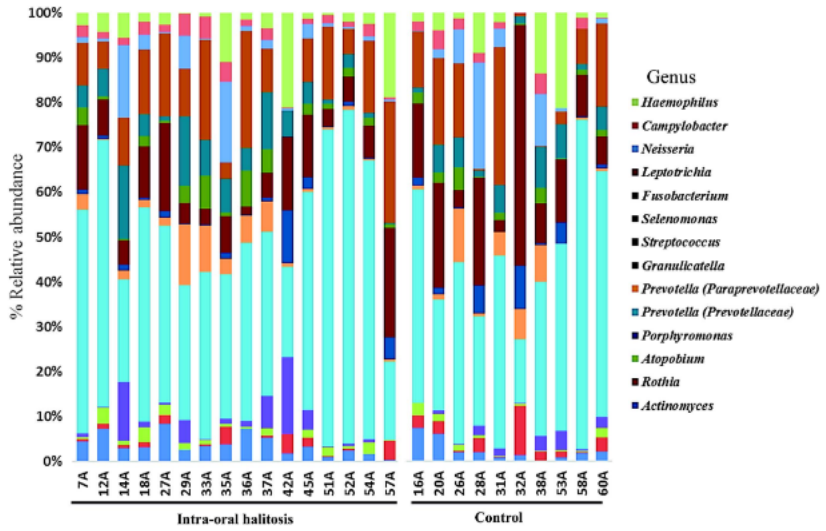

Figure 3. Relative abundance of genera present in individual samples of the intra-oral halitosis and control group.

Parvimonas, *Peptostreptococcus*, *Peptococcus*, *Prevotella*, *Selenomonas* (three OTUs), *Tannerella*, *SR*1 (three OTUs), *Treponema* and *TM*7-3. The OTUs that were significantly associated with the control group included *Aggregatibacter* (OTU id 4363066), *Haemophilus*, *H. parainfluenza* (2 OTUs), several *Streptococcus* (five OTUs), *Moryella*, *Oribacterium*, *Prevotella*, *R. dentocariosa* and an OTU from *Gemellaceae*. Table 3 represents the differentially abundant significant OTUs of the IOH and control group (adjusted *p* value < 0.05) and their BlastN results. Figure 4 presents the differentially abundant significant OTUs of the IOH and control group.

Discussion

It is generally thought that intra-oral halitosis is a bacteria-driven disorder. The hypothesis, therefore, was that the composition of the tongue microflora in patients with this condition would be different compared to subjects without oral halitosis. The microbiological analysis involved sequencing of the 16s rRNA gene, which is a sensitive technique for studying the composition of complex microflora such as the tongue biofilm. Participants were selected on the basis of critical objective and subjective parameters. The number of subjects included in this study was limited. However, the microbiological data obtained from individuals was similar, and therefore, the variation in the composition of the tongue biofilm between the healthy group and IOH was rather limited. All clinical parameters, as well as the level of relevant VSCs between the groups, were statistically different. Statistically higher levels of H_2S and CH_3SH, and to a lesser extent $(CH_3)_2S$ from IOH patients, were observed, which is in agreement with previous studies on IOH [7]. Moreover, a significant correlation was found between CH_3SH and $(CH3)_2S$, and therefore this

Table 3. Differentially abundant operational taxonomic units of the intra-oral halitosis and control group that are significantly different (p adjusted <0.05) and BlastN result.

OTU* id	OTU* taxonomy (97% identity) Greengenes database	Sequence identity	Closely matched species (BlastN)	Sequence ID	p value adjusted (<0.05)
IOH					
4335 776	*Aggregatibacter*	99%	*A. segnis*	JN713257.1	0.03
4294 655	*A. segnis*	98%	*A. segnis*	GU727818.1	0.04
32 546	*Campylobacter*	98%	*Campylobacter gracilis*	CP012196.1	0.0001
664 697	*Capnocytophaga*	99%	*Capnocytophaga gingivalis*	NR113368.1	0.03
4432 435	*Clostridiales*	100%	*Clostridiales bacterium* oral taxon	GU400575.1	0.01
174 016	*Dialister*	99%	*Dialister invisus*	LT223661.1	0.0002
4441 038	*Leptotrichia*	99%	*Leptotrichia buccalis*	CP001685.1	0.007
923 032	*Leptotrichia*	96%	*Leptotrichia hofstadii*	NR113161.1	0.003
4400 260	*Leptotrichia*	97%	*L. buccalis*	KX286353.1	0.03
4419 634	*Leptotrichia*	98%	*Leptotrichia* sp. oral taxon	GU408396.1	0.03
42 091	*Peptococcus*	99%	*Peptococcus* sp. oral taxon	GU407070.1	0.006
527 630	*Peptostreptococcus*	100%	*Peptostreptococcus stomatitis*	KF933775.1	0.0001
557 665	*Prevotella*	99%	*Prevotella shahii*	NR024815.1	0.0002
4377 418	*Parvimonas*	99%	*Parvimonas* sp. oral taxon	HM596290.1	0.01
3581 175	*Selenomonas*	94%	*Selenomonas* sp. oral taxon	CP012071.1	0.008
4455 183	*Selenomonas*	99%	*Selenomonas infelix*	NR028797.1	0.04
4432 347	*Selenomonas*	99%	*Selenomonas* sp. oral taxon	CP017042.1	0.04
4213 913	*SR*1	100%	*SR*1 bacterium oral taxon	KM018314.1	0.03
4330 849	*SRI*	97%	*Candidate* division *SR*1 bacterium	KM462162.1	0.0002
4400 869	*SRI*	99%	*SR*1 bacterium oral taxon	KM018323.1	0.03
4443 201	*Tannerella*	95%	*Tannerella forsythia*	AP013045.1	0.0009
799 024	*TM7*-3	99%	*Candidatus Saccharibacteria* oral taxon	CP007496.1	0.04
73 875	*Treponema*	98%	*Treponema refringens*	AF426101.1	0.02
CONTROL					
4363 066	*Aggregatibacter*	99%	*H. parainfluenza*	KC632194.1	0.02
4446 902	*Gemellaceae*	100%	*Gemella haemolysans*	KP192305.1	0.03
3462 224	*H. parainfluenza*	99%	*H. parainfluenza*	JF506652.1	0.02
4375 080	*H. parainfluenza*	99%	*H. parainfluenza*	JF506652.1	0.03
4318 872	*Haemophilus*	100%	*Haemophilus influenzae*	AF224308.1	0.04
714 766	*Moryella*	100%	*Stomatatobaculum longum*	NR117792.1	0.04
749 837	*Oribacterium*	99%	*Oribacterium parvum*	HM120212.1	0.03
4315 804	*Prevotella*	99%	*Prevotella oris*	JF803574.1	0.03
4311 939	*R. dentocariosa*	99%	*R. dentocariosa*	KM225760.1	0.04
1010 458	*Streptococcus*	99%	*Streptococcus mitis*	KX661103.1	0.003
525 391	*Streptococcus*	98%	*Granulicatella adiacens*	LC125191.1	0.01
528 357	*Streptococcus*	98%	*Streptococcus parasanguinis*	KJ566187.1	0.03
2819 725	*Streptococcus*	99%	*Streptococcus mitis*	CP014326.1	0.03
4402 254	*Streptococcus*	100%	*S. parasanguinis*	HM560705.1	0.04

*OTU: Operational taxonomic unit.

elevated amount of $(CH_3)_2S$ from the oral cavity might be due to the methylation of CH_3SH [31]. However, our metagenomic sequencing data revealed that there was no difference in the bacterial diversity of the groups (alpha and beta) based on the phylogenetic relationship. This confirms previous studies showing a similar bacterial species richness as well as community structure between the healthy and IOH groups [17]. At the phylum level, the differences between the patients and controls in our study were minimal with only a higher abundance of *TM7*, which is in agreement with a previous observation [14]. At the family level, *Gemellaceae* was the only significant group that was more abundant in the control subjects.

At OTU level, several *Streptococcus* OTUs were significantly abundant in the control group, and this finding is also in line with previous studies [14, 32]. We also found a significant abundance of *H. parainfluenza*, OTUs from *Haemophilus* (two) and *Prevotella*, which have been reported in the healthy tongue [15]. OTUs from *Aggregatibacter*, *Gemellaceae*, *Moryella*, *Oribacterium* and *R. dentocariosa* were significantly abundant in our control group, which has not been reported in earlier studies. Other species associated with the absence of IOH include *Rothia mucilaginosa*,

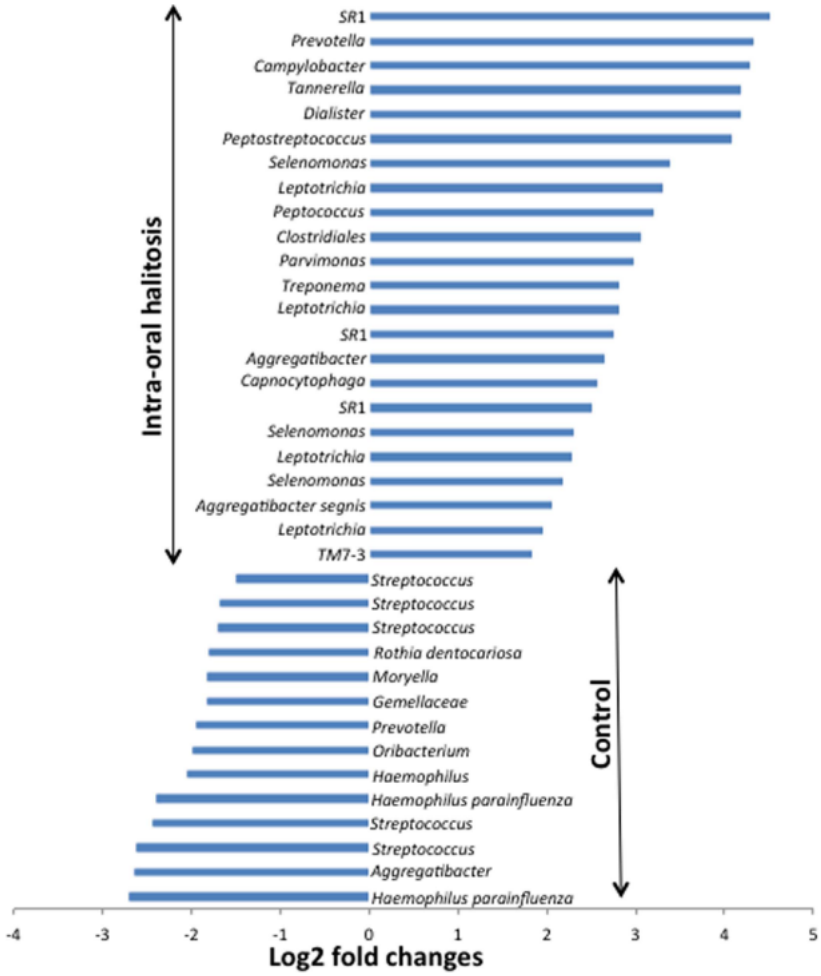

Figure 4. Differentially abundant operational taxonomic units of intra-oral halitosis and the control group using DESeq2. Positive log2 fold change values represent the abundant operational taxonomic units of the intra-oral halitosis group and the negative log2 fold change values represent the abundant operational taxonomic units of the control group. The figure presents the significantly different operational taxonomic units (p adjusted < 0.05).

Granulicatella adiacens and *Veillonella* species [14], which is in line with our observation.

Based on the log2 fold changes, we found some dissimilarity in the composition of the tongue microflora between the IOH patients and the controls. The IOH group in our study revealed several OTUs that were significantly more abundant in comparison to the controls, including *Peptostreptococcus* [33], *Peptococcus*, *Dialister* [14] and OTUs that belong to *Clostridiales*, *Capnocytophaga* [11], *Prevotella* [15], *Parvimonas*, *Tannerella* [34], *TM7* [14], *Treponema*, *Leptotrichia* [17], *Campylobacter* [13], *Aggregatibacter* and *SR*1. Our observations differ from the findings of Kazor *et al* (2003) [12], who found *A. parvulum*, *Fusobacterium periodonticum*, *Eubacterium sulci*, *Dialister* spp., *S. moorei*, and a phylotype of *Streptococcus* associated with IOH. This study has identified the above species in both IOH and the control group and found no differences in OTU abundance except for *Dialister*. *S. moorei*, which has previously been described as a marker species for IOH [16]. However, we found no significant difference in the presence and abundance between the IOH patients and control subjects. The differences in the microbiological outcome of the studies may be attributed to population differences, differences in the selection criteria of the study subjects, and the use of different molecular techniques.

Based on the small differences in the microbial composition of the IOH and healthy controls, we conclude that quantitative rather than qualitative parameters are important in oral malodor, which is in agreement with the findings of Riggio *et al* (2008) [15]. Based on these observations, we hypothesize that alterations in the metabolism of the tongue bacteria are determining factors in the onset of IOH rather than the qualitative composition of the tongue microflora. For instance, oral microbiota get their nutrients from the complex glycoproteins of saliva. These glycoproteins can be degraded by the microbial consortia of that particular ecosystem rather than the single bacteria [35]. Moreover, survival of the species in a particular ecosystem mainly depends on the metabolic activity of the bacteria, which results in the formation of a metabolic network based on the symbiotic relationship of the species [36]. An *in vitro* study on salivary malodor production strongly emphasized that

the metabolic activity of microbes plays a role in malodor production, and is influenced by the environmental conditions, such as reduced carbohydrate levels, a rise in pH and stagnant salivary flow [37]. Further, no single organism has been demonstrated to produce malodor *in vivo*. We speculate that a multi-species metabolic network might play a key role in IOH. Therefore, metabolic profiling (metabolomics) might provide some clues, which could help in the diagnosis and development of therapeutics.

Conclusion

Based on our observation, it was concluded that the qualitative bacterial composition was almost the same in the IOH and control group, and the quantitative increase in microbes may play a role in IOH. We hypothesize that a multi-species bacterial network might play a strong role in IOH. Metabolomics combined with metatranscriptome analysis may provide clues as to the cause of IOH.

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Authors' contributions

KS, EGW and AJvW designed the study, KS and EGW collected the samples, KS participated in the laboratory experiments and performed the bioinformatics analyses in collaboration with HH and JWAR. KS and AJvW drafted the manuscript. All authors read and approved the final version of the manuscript.

Conflict of interest

EGW is co-owner of CleverCool BV. EGW is working at the Clinic for Periodontology Amsterdam treating halitosis patients. All other authors declare no conflict of interests.

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