

University of Groningen

## Untargeted metabolomics of the bacterial tongue coating of intra-oral halitosis patients

Seerangaiyan, Kavitha; Maruthamuthu, Mukil; van Winkelhoff, Arie Jan; Winkel, Edwin G.

*Published in:*  
Journal of breath research

*DOI:*  
[10.1088/1752-7163/ab334e](https://doi.org/10.1088/1752-7163/ab334e)

**IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.**

*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
2019

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Seerangaiyan, K., Maruthamuthu, M., van Winkelhoff, A. J., & Winkel, E. G. (2019). Untargeted metabolomics of the bacterial tongue coating of intra-oral halitosis patients. *Journal of breath research*, 13(4), [046010]. <https://doi.org/10.1088/1752-7163/ab334e>

### Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

### Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

*Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.*

PAPER

# Untargeted metabolomics of the bacterial tongue coating of intra-oral halitosis patients

To cite this article: Kavitha Seerangaiyan *et al* 2019 *J. Breath Res.* **13** 046010

View the [article online](#) for updates and enhancements.



**NEW BREATH BIOPSY PRODUCTS**  
NEW FEATURES | NEW LOOK

SAME WORLD-LEADING  
BREATH RESEARCH PLATFORM

[VIEW OUR NEW RANGE](#)


[owlstonemedical.com](http://owlstonemedical.com)





## PAPER

## Untargeted metabolomics of the bacterial tongue coating of intra-oral halitosis patients

RECEIVED  
24 March 2019REVISED  
10 July 2019ACCEPTED FOR PUBLICATION  
18 July 2019PUBLISHED  
20 August 2019Kavitha Seerangaiyan<sup>1,4</sup> , Mukil Maruthamuthu<sup>2,4</sup>, Arie Jan van Winkelhoff<sup>1</sup> and Edwin G Winkel<sup>1,3,5</sup><sup>1</sup> Center for Dentistry and Oral Hygiene, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands<sup>2</sup> Department of Medicine, University of Miami Miller school of Medicine, Miami, Florida, United States of America<sup>3</sup> Clinic for Periodontology Amsterdam, Amsterdam, The Netherlands<sup>4</sup> Equal contribution.<sup>5</sup> Author to whom any correspondence should be addressed.E-mail: [dr.kavithashree@gmail.com](mailto:dr.kavithashree@gmail.com) and [edwinwinkel@mac.com](mailto:edwinwinkel@mac.com)**Keywords:** intra-oral halitosis, tongue coating, microbiome, metabolites, hydrogen sulfide (H<sub>2</sub>S)Supplementary material for this article is available [online](#)**Abstract**

Intra-oral halitosis (IOH) refers to an unpleasant odor from the oral cavity that is mainly caused by the tongue coating. Although the tongue coating microbiome is thought to play an essential role in IOH, the exact aetiology of IOH remains unclear. Here we investigated and compared the metabolic profiles of the tongue coating microbiomes of patients with IOH versus healthy control. The metabolic profiles were significantly different in IOH patients than in healthy controls. Healthy controls showed higher selenoamino acid and nicotinamide metabolism; these metabolic pathways are mainly involved in maintaining the oxidation-reduction potential and redox state. A total of 39 putative metabolites were associated with IOH. Remarkably, 3 of the metabolites, branched-chain fatty acids (BCFA), 3-fumaryl pyruvate, and acetyl phosphate, are potential key players in IOH. Interestingly, the predominant metabolite in IOH is BCFAs, which might underlie tongue coat formation. In addition, the key metabolite acetyl phosphate has a clear association with the hydrogen sulfide- (H<sub>2</sub>S-) producing metabolic pathway and anaerobic fermentation. These novel metabolomic findings provide insights into the formation of the tongue coating and the production of H<sub>2</sub>S, which causes bad breath.

**List of abbreviations**

|                                   |                                    |
|-----------------------------------|------------------------------------|
| CH <sub>3</sub> SH                | methyl mercaptan;                  |
| (CH <sub>3</sub> ) <sub>2</sub> S | dimethyl sulfide;                  |
| DPSI                              | Dutch periodontal screening index; |
| EOH                               | extra-oral halitosis;              |
| H <sub>2</sub> S                  | hydrogen sulfide;                  |
| IOH                               | intra-oral halitosis;              |
| OLS                               | organoleptic score;                |
| OTU                               | operational taxonomic unit;        |
| PCA                               | principal component analysis;      |
| PCR                               | polymerase chain reaction;         |
| TE                                | tris-EDTA;                         |

|      |                              |
|------|------------------------------|
| VSC  | volatile sulfur compound;    |
| WTCI | Winkel tongue coating index. |

**Introduction**

Halitosis or bad breath is commonly classified into three categories: intra-oral halitosis (IOH), extra-oral halitosis (EOH), and transient halitosis [1]. The source of IOH is the oral cavity, whereas in EOH, malodorous compounds are produced in the human body but not in the oral cavity [1]. EOH can itself be divided into blood-borne and non-blood-borne halitosis. The sources of blood-borne halitosis include systemic diseases, metabolic disorders, medications, and certain foods. Non-blood-borne halitosis can be caused by disorders that affect the nose and the upper and

lower respiratory tracts [2]. Recently, a genetic disorder was shown to contribute to IOH [3]. The tongue coating, which is a major causative factor in IOH [4], consists of large bacterial deposits, food debris, and desquamated epithelial cells. Gingivitis and periodontitis are oral pathological conditions caused by anaerobic bacteria that are also associated with IOH. The known risk factors for IOH include stress and xerostomia [1].

About 90% of halitosis cases are due to IOH; notably, halitosis can negatively impact an individual's social life and psychological well-being. IOH is a widespread condition that affects 22%–50% of the population worldwide [5]. Subjects with IOH typically have more or a thicker tongue coating than subjects without IOH [6]. Volatile sulfur compounds (VSCs) are largely responsible for IOH, including hydrogen sulfide, methyl mercaptan, and, to a lesser extent, dimethylsulfide [7]. Other volatile compounds, such as putrescine, cadaverine, indole, and skatole, are also putative causes of bad breath [8], but this is somewhat controversial [9]. Bacterial putrefaction that produces unpleasant volatile compounds is thought to be involved in oral malodor production [10]. Bacteria that are present on the dorsum of the tongue degrade the sulfur-containing amino acids (cysteine, cysteine, homocysteine, and methionine) to produce VSCs and thereby cause IOH [11, 12].

Several techniques have been used to study the composition of bacteria on the tongues of subjects with IOH [13–15]. Studies of anaerobic cultures of tongue samples implicate *Peptostreptococcus anaerobius*, *Collinsella aerofaciens*, *Eubacterium* group, *Actinomyces* spp., *Eikenella corrodens*, *Veillonella* spp., *Fusobacterium nucleatum*, pigmented *Prevotella* spp, and *Selenomonas* spp. In IOH, culture-independent molecular methods revealed additional species associated with IOH, including *Atopobium parvulum*, *Dialister* spp., *Eubacterium sulci*, a phylotype of the uncultivated phylum TM7, *Solobacterium moorei*, and a phylotype of *Streptococcus* [14]. Studies that used PCR amplification, gene cloning, and 16S rRNA sequencing describe increased species diversity in IOH and report that *Lysobacter*-type species, *Streptococcus salivarius*, *Prevotella melaninogenica*, *Prevotella veroralis*, and *Prevotella pallens* are commonly found in the tongue biofilms of people with IOH [15]. However, a recent study showed a high degree of similarity between the bacterial composition of the tongue coatings of IOH patients and subjects without halitosis [6]. Based on this observation, it was hypothesized that bacterial metabolism plays a major role in IOH.

In this context, knowing more about the function of bacterial communities [16] may help clarify the associations between the microbiome and diseases. Microbial omics-based approaches, including metagenomics, transcriptomics, and metabolomics, may provide information that will help us understand the role

of the microbiome in condition like halitosis. Bacteria produce various metabolites, but their possible roles in oral diseases have not been extensively investigated [17]. In addition, integrating microbiome and metabolome data may provide insights into healthy and disease states, giving us a clearer picture of dynamic changes in cells that can be quantified by analyzing small molecules, such as lipids and amino acids [16]. Metabolomics has been used to study several oral diseases, such as dental caries and periodontitis. Microbial metabolites can alter conditions in the oral environment, thereby increasing bacterial pathogenicity and enriching the ecosystem to create a potentially more pathogenic environment [17]. In order to investigate this in IOH, we used an untargeted metabolomic approach based on LC-MS/MS to analyze and compare the bacterial metabolome of the tongue biofilms of subjects with and without IOH (figure 1). This approach may provide insights into the mechanisms responsible for bad breath. In addition, this approach may help establish well-defined diagnostic markers for IOH and suggest therapeutic strategies.

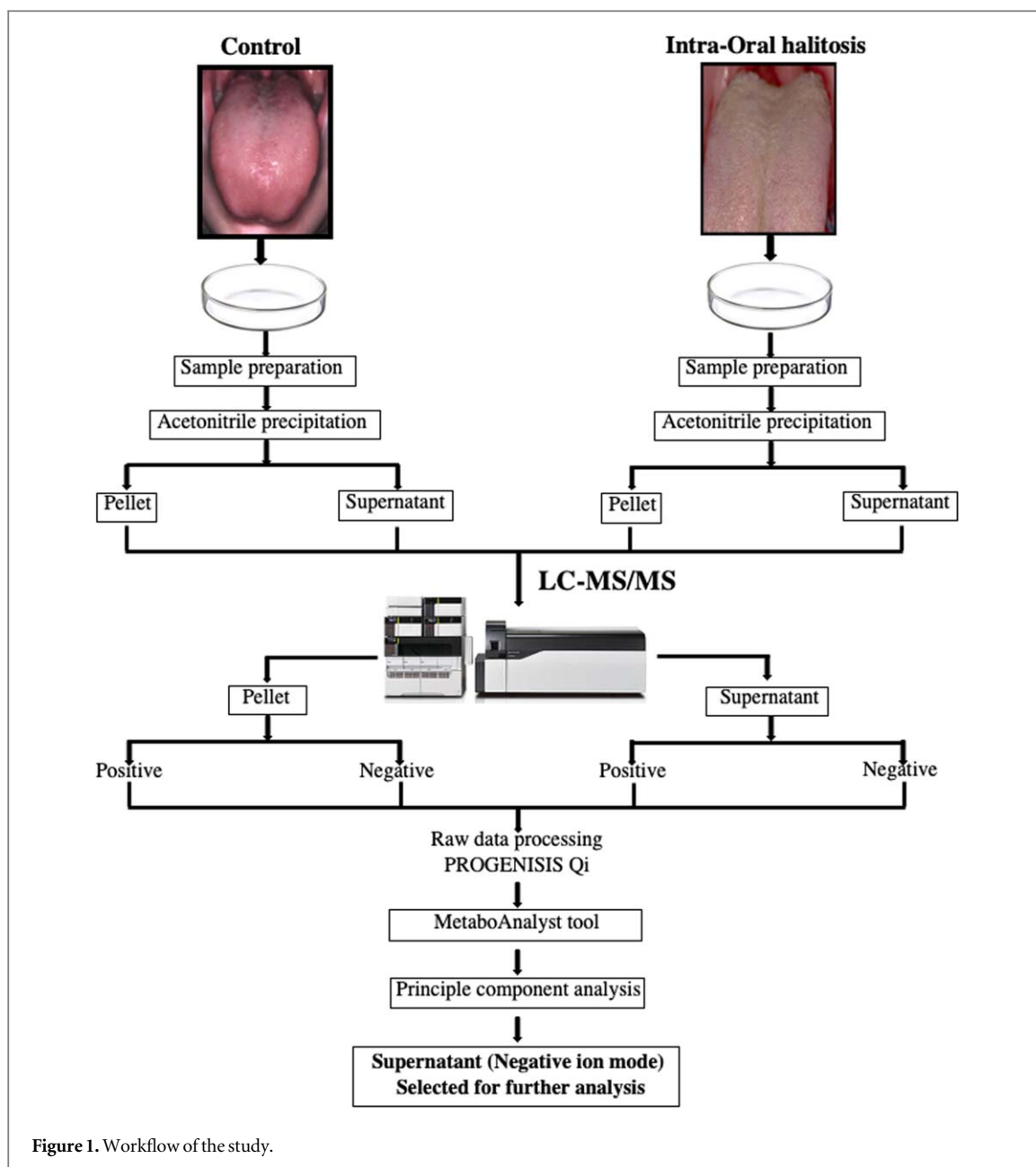
## Methods

### Ethics statement

The study was conducted in accordance with Dutch ethics laws and with the principles for human research. All participants provided informed written consent. The medical ethics committee at the University Medical Center Groningen (METC 2015/458) approved the study protocol, and the study was conducted in accordance with the tenets of the Declaration of Helsinki (2013).

### Halitosis assessment and tongue sample collection

A total of 24 subjects participated in this study, 14 patients with IOH and 10 controls without IOH. Patients were recruited at the Clinic for Periodontology Amsterdam, The Netherlands. Subjects without halitosis were volunteers from the Center for Dentistry and Oral Hygiene, University Medical Center Groningen, Groningen, The Netherlands. All subjects were included in the study based on careful periodontal and halitosis examinations. Prior to their visit, the subjects were instructed to do the following: (1) avoid consuming onions, garlic, and hot spices in the 48 h before the 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, after-shave 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 and were allowed to drink water up to 3 h before the examination. The inclusion and the exclusion criteria were the



same as in our previous study [6] and are described below.

#### Exclusion criteria

We excluded subjects with periodontitis or systemic diseases; those who smoked, were pregnant, or used antimicrobial therapy and mouth rinses in the three months prior to the start of the study; subjects with a history of fever or cold in previous four weeks; and patients who failed to follow the instructions for the halitosis assessment.

#### Inclusion criteria

For inclusion, we first determined the following in the 24 subjects who participated in the study:

1. Organoleptic score (OLS): (0 = no halitosis to 5 = the presence of extreme halitosis) from the nose and mouth [18, 19].
2. VSC gases, namely hydrogen sulfide ( $H_2S$ ) and methyl mercaptan ( $CH_3SH$ ), using OralChroma™ (Abilit Corporation, Japan).
3. Dutch periodontal screening index (DPSI) [20].
4. Winkel tongue coating index (WTCl) [21].

The Winkel tongue coating index is a visual index with which the dorsum of the tongue is divided into six areas, three in the posterior and three in the anterior region of the tongue. The tongue coating in each area is scored as 0=no coating, 1=light coating and 2=severe coating. The tongue coating value is

obtained by addition of the scores from all six areas, ranging from 0 to 12 [21].

IOH patients presented with an organoleptic score  $\geq 2$  from the mouth and  $\leq 1$  from the nose, had  $\text{H}_2\text{S} > 4 \text{ nmol l}^{-1}$  (96 ppb) and  $\text{CH}_3\text{SH} > 0.5 \text{ nmol l}^{-1}$  (12 ppb) [9], and had a DPSI score  $\leq 2$ . Control subjects presented with an organoleptic score of 0 from the mouth and nose and had  $\text{H}_2\text{S} = 0$ ,  $\text{CH}_3\text{SH} = 0$  (Oral-Chroma), and a DPSI score  $\leq 2$ .

### Tongue samples

Tongue samples were collected in the morning on the day of the examination. A tongue-cleaning device was used (Scrapy, CleverCool, Amsterdam, The Netherlands) to dislodge the tongue coating from the posterior to the anterior side of the tongue. The sample was weighed and 100 mg of the sample was transferred to a petri dish containing 1 ml of sterile phosphate-buffered saline (1X) pH 7.5. The sample was then collected in an Eppendorf tube, incubated at room temperature for two hours, and centrifuged for 10 min at  $1750 \times g$  at  $4^\circ\text{C}$  to collect the extracellular metabolites [22]. Following centrifugation, the supernatant and the pellet were both carefully collected and stored at  $-80^\circ\text{C}$  until the analysis.

### LC-MS/MS analysis

The metabolites were extracted from the pellet and supernatant samples using acetonitrile. Specifically, 200  $\mu\text{l}$  of supernatant was mixed with 200  $\mu\text{l}$  of acetonitrile, vortexed for 30 s, and centrifuged for 3 min at  $12000 \times g$  at  $4^\circ\text{C}$ . The supernatant was collected, freeze-dried, and suspended in 200  $\mu\text{l}$  of 80% acetonitrile. The same procedure was followed for the pellet. Each extract was injected into an Ultimate 3000-UPLC system (Dionex, Amsterdam, The Netherlands) connected to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) and separated on a Kinetex 2.6u EVO C18 100A column (Phenomenex, Utrecht, The Netherlands). The following mobile phase gradient was delivered at a flow rate of  $0.4 \text{ ml min}^{-1}$ : start 1% B, 1 min hold; linear gradient 1%–94% B in 11 min; hold 94% B, 8 min. Solvent A was  $\text{H}_2\text{O}$  with 0.1% formic acid, and solvent B was acetonitrile with 0.1% formic acid. The column temperature was kept constant at  $40^\circ\text{C}$ . The mass spectrometer was operated in positive or negative ionization mode. Full scan MS spectra were acquired from  $m/z$  120 to 1500 at a target value of  $1\text{E}6$  and a max IT of 50 ms with a resolution of 70 000 at  $m/z$  200.

### MS analysis

Raw MS data files were analyzed using Progenesis QI software (Waters Corporation, Milford, MA) for peak alignment, peak picking, and normalization of the LC-MS/MS data. Peak alignment was performed to correct drifts in retention times. A reference LC-MS/

MS run that was the most representative of the whole data set was automatically selected. All other runs were then aligned to this reference. The following adduct forms were used for the peak picking and feature selection: [M + H], [M +  $\text{NH}_4$ ], [M + Na], [M + K], [M + H- $\text{H}_2\text{O}$ ], [M +  $\text{CH}_3\text{OH}$  + H], [2M + H], [2M +  $\text{NH}_4$ ], and [2M + Na] in positive mode; and [M-H], [M + FA-H], [M + Cl], [2M + FA-H], and [M- $\text{H}_2\text{O}$ -H] in negative mode. The peak picking limits were set to a minimum absolute intensity of 50 000, and the default automatic normalization method was used.

### Statistical analysis

Subject age and clinical parameters, including the organoleptic score, the WTCl, and VSC concentrations, were compared using unpaired t-tests in the R statistical package. Values of  $p < 0.05$  were considered statistically significant. The multivariate analyses, including principal component analysis (PCA) and partial linear square discriminant analysis (PLS-DA), were performed using MetaboAnalyst software version 3.0 ([www.metaboanalyst.ca](http://www.metaboanalyst.ca)). For multivariate analysis, three -step normalization procedure was followed. (i) Quantile sample normalization was performed to adjust the differences among samples, then (ii) log data transformation and (iii) pareto scaling were performed to make individual features more comparable (supplementary figure 1 is available online at [stacks.iop.org/JBR/13/046010/mmedia](http://stacks.iop.org/JBR/13/046010/mmedia)).

### Identification and evaluation of the biomarker compounds and metabolic pathways

PLS-DA was used to identify putative biomarkers for metabolomics studies, and the VIP (variable importance in projection) scores were used for metabolite selection. A VIP score  $> 1$  is typically used to identify compounds that are the most important in the projection. Since this was an untargeted metabolomics approach, there were a considerable number of metabolites with  $\text{VIP} \geq 1$ . Accordingly, we used VIP scores  $\geq 1.8$  (table S1) to differentiate groups. After the selection of a metabolite, the fold-change of that particular metabolite in the IOH patient group versus the control group was determined; T-test was used for the comparisons and  $p$ -value adjustments for multiple metabolites were carried out by false discovery rate adjustment (FDR) using a cut-off of 0.05. The list of selected compounds with the corresponding molecular weights ( $m/z$ ) and a mass error of  $\pm 5$  ppm was further checked against the Human Metabolome Database (HMDB) [23] to identify each compound by name. The compounds that could not be identified in the HMDB were screened further using the LIPID MAPS database [24] using the mass ( $m/z$ ) and mass error  $\pm 5$  daltons. The enrichment analysis and the metabolic pathway analysis were performed using HMDB ID in MetaboAnalyst [25]. The metabolite model was evaluated using receiver operating

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

| Clinical parameters                            | Intra-oral halitosis ( <i>n</i> = 16) |         | Controls ( <i>n</i> = 10) | <i>p</i> value      |
|--|---------------------------------------|---------|---------------------------|---------------------|
|  |                                       |         |                           |                     |
| Age (years)                                    | 44 ± 15                               |         | 47 ± 14                   | 0.58 <sup>b</sup>   |
| Gender   | Female                                | 8 (57%) | 5 (50%)                   | 0.51 <sup>c</sup>   |
|  | Male                                  | 6 (43%) | 5 (50%)                   |                     |
| Organoleptic score range                       | 2–4                                   |         | 0                         | 0.0001              |
| Winkel tongue coating index                    | 6.21 ± 1.96                           |         | 0.5 ± 0.7                 | 0.0001 <sup>b</sup> |
| H <sub>2</sub> S <sup>a</sup>                  | 394.83 ± 272.48                       |         | 8.30 ± 9.15               | 0.0002 <sup>b</sup> |
| CH <sub>3</sub> SH <sup>a</sup>                | 248.87 ± 360.70                       |         | 5.45 ± 4.82               | 0.04 <sup>b</sup>   |
| (CH <sub>3</sub> ) <sub>2</sub> S <sup>a</sup> | 16.71 ± 13.91                         |         | 2.50 ± 3.17               | 0.003 <sup>b</sup>  |

<sup>a</sup> H<sub>2</sub>S, CH<sub>3</sub>SH, and (CH<sub>3</sub>)<sub>2</sub>S were measured in parts per billion (ppb). The continuous variables are reported as means ± standard deviations.

<sup>b</sup> Two-sample t-test,

<sup>c</sup> Pearson's chi-square test or Fisher's exact test.

**Table 2.** The total number of metabolites (peaks) detected in LC-MS/MS analysis (all modes).

| No. | Sample          | Identification (LC-MS/MS) | Based on ≥2-fold difference |     |
|-----|-----------------|---------------------------|-----------------------------|-----|
|     |                 |                           | Control                     | IOH |
| 1   | Supernatant (–) | 1903                      | 1015                        | 888 |
| 2   | Supernatant (+) | 1623                      | 865                         | 758 |
| 3   | Pellet (–)      | 521                       | 166                         | 355 |
| 4   | Pellet (+)      | 834                       | 465                         | 366 |

characteristic (ROC) curves, which is considered an effective way to determine the clinical utility of a biomarker in metabolomic studies. The area under the curve (AUC) of the ROC curve allows the identification of sensitive and specific biological markers [26].

## Results

### Demographic and clinical characteristics of the study subjects

The study included 14 patients with IOH (8 men and 6 women) and 10 control subjects (5 men and 5 women). The mean age of the IOH patients was 44 ± 15.34 years (range 18–64 years), and the mean age of the control subjects was 47 ± 13.94 years (range 29–68 years) (*p* = 0.58). The mean OLS and WTCI and the mean H<sub>2</sub>S and CH<sub>3</sub>SH concentrations were all higher in patients compared to controls (table 1).

### Metabolomic profiling

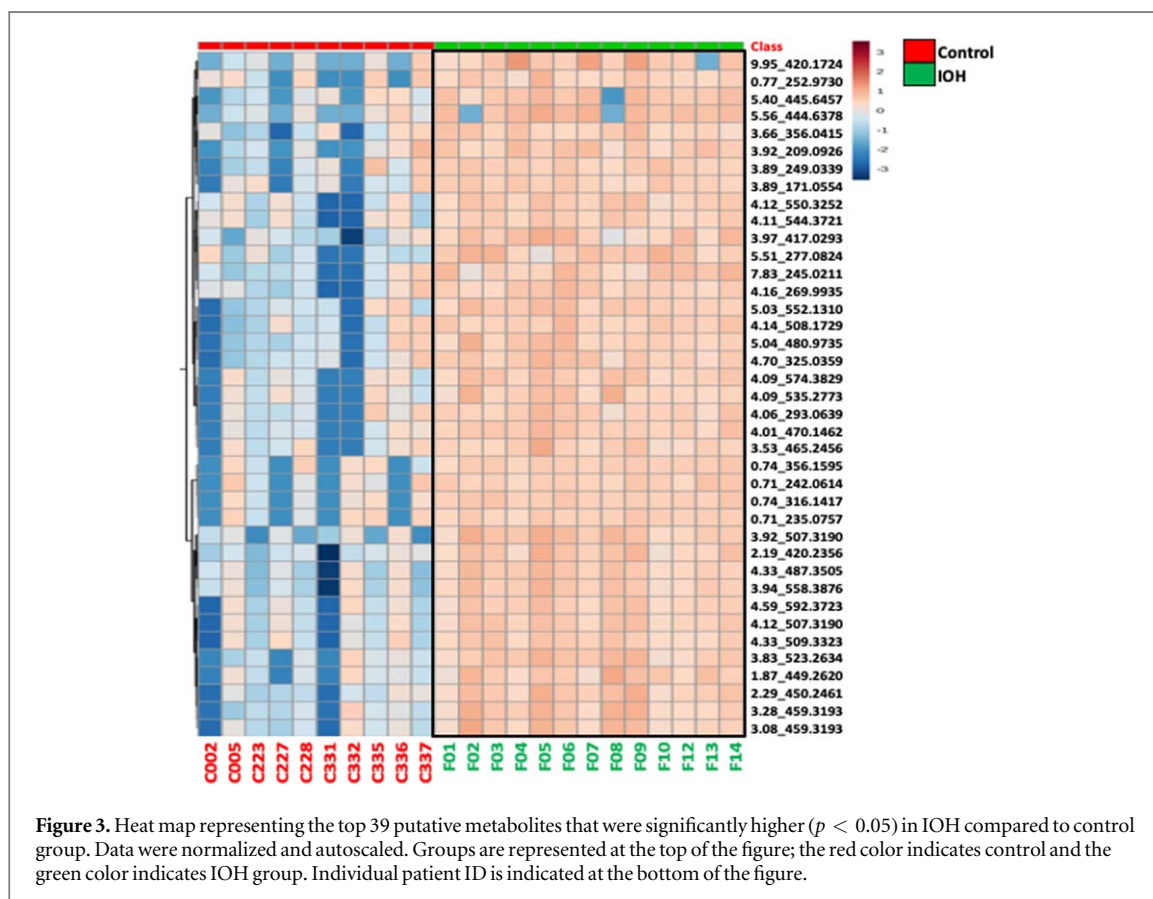
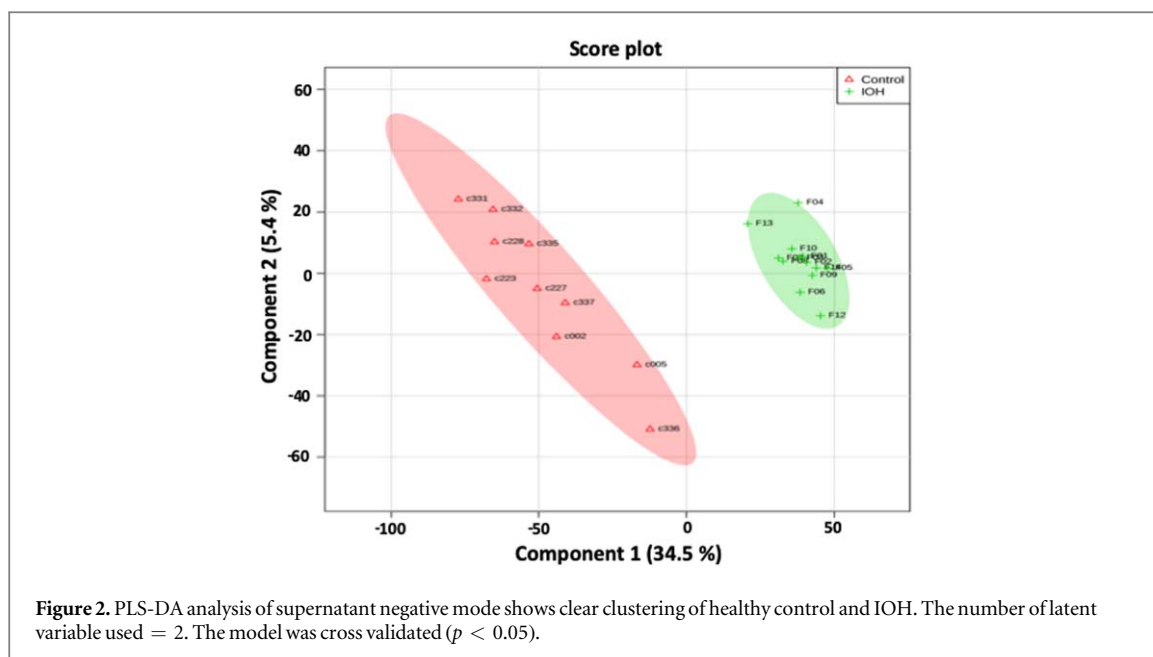
Table 2 shows the total number of peaks detected in the supernatants and in the pellets in LC-MS positive and negative modes. The unsupervised PCA of the supernatant and the pellet samples for the positive and negative modes was performed. In the negative mode,

the supernatant samples showed a clear clustering pattern for the patient and control groups with an outlier (F11). After removing the outlier, the supernatant in the negative mode was subjected to further analysis. The other three modes did not show clear clustering pattern of groups and were excluded from the analysis (data not shown). In order to model the differences between the results of the supernatant analysis in patients and controls in negative mode, supervised PLS-DA was performed with two latent variables, and the model was cross-validated using the leave-out-one method (figure 2). The *R*<sup>2</sup> (explained variance) and the *Q*<sup>2</sup> (predicted variance) of the cross validation were 0.9 and 0.8, respectively. The difference between *R*<sup>2</sup> and *Q*<sup>2</sup> was 0.1; this is very small and this model was therefore considered to be the best model for PLS-DA analysis. The model was validated using the permutation test and the observed statistic was found significant (*p* < 0.05).

### Identification of the biomarkers

A total of 39 putative markers were identified that had VIP scores ≥1.8 and that showed a ≥2-fold significant difference between the patient and control groups. The metabolites that were found at significantly higher levels in the IOH group were mostly branched chain fatty acids (BCFAs) that were present at 3- to 3601-fold higher levels in patients with IOH (supplementary table 1). The amino acid metabolites included leucine, isoleucine, and valine, aspartyl-tyrosine, methionyl-serine, isoleucyl-methionine, and cysteinyl-arginine. Other metabolites included 10-formyl dihydrofolate, homocysteine thio-lactone, acetyl phosphate, 3-fumarylpyruvate, acetyl phosphate, and indole derivatives. Metabolites that were found at significantly higher levels in the control group included fatty acyl glycosides of mono- and disaccharides, diacylglycerophospho-ethanolamines, gamma-glutamyl-Se-methylselenocysteine, threonyl-glutamate, and S-formylglutathione (supplementary table 2).

A heat map was constructed (figure 3) for the top 100 putative metabolites that showed significant differences between healthy controls and IOH patients; the mass (*m/z*) of these metabolites was used in HMDB database to retrieve the known putative metabolites. Metabolites that were not identified in HMDB were subject for analyses using the lipidmaps database. The known metabolites identified were categorized by possible metabolomic pathway/enrichment analysis using KEGG database [27]. The enrichment analysis shows the functionally related metabolites (or gene sets) rather than individual metabolites. Figure 4(a) shows the enrichment of nicotinate and nicotinamide metabolism and selenoamino acid metabolism in the control group. Figure 4(b) shows the detection of oxidation of BCFAs and long chain fatty acids based on the presence of L-acetyl carnitine. L-acetyl carnitine is the carrier of acetyl CoA during fatty acid metabolism. Figures 4(c) and (d) shows the

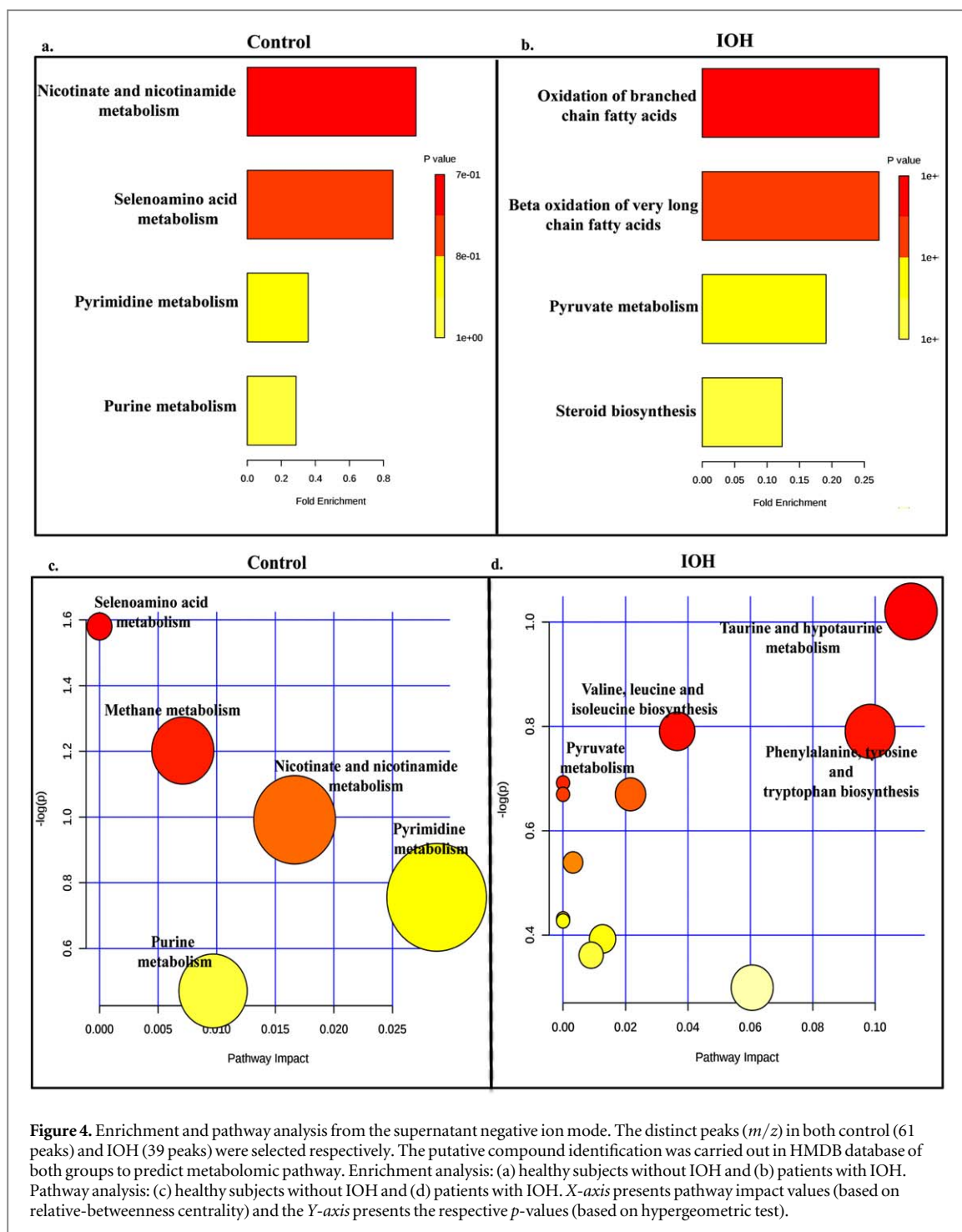


metabolic pathways which is markedly influenced by big pathway impact and small  $p$  value. Figure 4(c) shows that the metabolic pathways in the healthy subjects involved selenoamino acid, nicotinate and nicotinamide, and methane. Figure 4(d) shows that the metabolic pathways in the IOH patients involved taurine and hypotaurine, phenylalanine, tyrosine and tryptophan biosynthesis, valine, leucine and isoleucine biosynthesis, and pyruvate.

### Biomarker evaluation

The classification (probability view) of the IOH and the control groups are shown in supplementary figure 2. In order to determine the sensitivity and specificity of a marker, multivariate exploratory ROC analysis was performed to evaluate the effectiveness of all biomarkers of IOH. The classification method and feature ranking methods were PLS-DA and PLS-DA built-ins respectively with 2 latent variables. An AUC





**Figure 4.** Enrichment and pathway analysis from the supernatant negative ion mode. The distinct peaks ( $m/z$ ) in both control (61 peaks) and IOH (39 peaks) were selected respectively. The putative compound identification was carried out in HMDB database of both groups to predict metabolomic pathway. Enrichment analysis: (a) healthy subjects without IOH and (b) patients with IOH. Pathway analysis: (c) healthy subjects without IOH and (d) patients with IOH.  $X$ -axis presents pathway impact values (based on relative-betweenness centrality) and the  $Y$ -axis presents the respective  $p$ -values (based on hypergeometric test).

value  $>0.7$  indicates that the marker is a more sensitive and specific marker. As shown in supplementary figure 2 the values obtained in the AUC analysis were  $\geq 0.8$  within the 95% confidence intervals.

## Discussion

The microbiome of the tongue coating plays a vital role in IOH [14, 15]. Our previously published 16S rRNA sequencing study on tongue coating showed that the microbial community composition in IOH is highly similar compared to healthy controls. However, minor

differences were observed in the quantitative abundance of few OTUs including *Clostridiales*, *SRI*, *TM7*, *Campylobacter*, *Dialister*, *Leptotrichia*, *Peptostreptococcus*, *Prevotella*, *Selenomonas*, *Peptococcus*, *Aggregatibacter*, *Capnocytophaga*, *Parvimonas*, *Treponema* and *Tannerella* [6]. Based on this finding, we speculated that the changes in the tongue microbiome derived metabolites are responsible for the symptoms of IOH. Yet the tongue microbiome makes countless/numerous metabolites whose function is unknown and may have profound effects on the tongue coating. Therefore, we investigated the metabolite (bioactive compounds) signatures of IOH using an untargeted

LC-MS/MS metabolomics approach (figure 1). The PLS-DA analysis showed distinct clustering of patients and healthy controls. A heat map based on the VIP score of PLS-DA indicates that the 39 metabolite markers were significantly higher in the tongue coating of IOH patients whereas 61 metabolite markers were associated with healthy subjects. Further, the HMDB and lipid maps database screening revealed the putative compound identity that belongs to carbohydrate, lipid, and amino acid group. Identified putative metabolites were used for the tentative metabolomic pathway prediction. In the healthy group, Selenoamino acid metabolism (figures 4(a) and (c)) was the most pronounced pathway and the hit metabolite is Gamma-glutamyl-se-methylselenocysteine; this type of amino acid is present mainly in human tissues and is an essential constituent of glutathione peroxidase, which prevents lipid peroxidation, and of phospholipid hydroperoxide enzymes, which are involved in the reduction of phospholipid hydroperoxide in cell membranes. Hence, selenium is an important factor in protecting the body from oxidative stress. However, selenoamino acid metabolism was not observed in IOH. This might result in an increased concentration of free oxygen radicals and peroxides, which could lead to increased oxidative stress, lipid peroxidation, and metabolic dysfunction [28].

This hypothesis was supported by our enrichment analysis, which showed the oxidation of BCFAs and long chain fatty acids (figures 4(b) and (d)). On the other hand, BCFAs are also produced during degradation of branched chain amino acids (BCAA) such as valine, leucine and isoleucine in an anaerobic environment. To support this finding, we observed valine, leucine and isoleucine (BCAA) as top metabolites in IOH samples (figure 4(d)).

The lipid profile of IOH was predominantly comprised of BCFAs, which are primarily saturated fatty acids (SFAs) with one or more methyl branches on the carbon chain that are categorized as mono-, di-, or multi-methyl BCFAs [29]. Interestingly, BCFAs are key components of several commensal bacterial membranes but are limited in human tissues [29, 30]. BCFAs can also be produced by proteolytic *Clostridiales* via the Stickland reaction; in this reaction, one amino acid is oxidized, and this is coupled to the deamination of a second amino acid [31]. Interestingly, in our microbiome study we found a significant quantitative increase of 23 OTUs in IOH compared to healthy controls and one among them is *Clostridiales* [6].

BCFAs can form a wax-like coating that is observed in vernix caseosa, a white cheesy substance found on the skin of newborn babies [29]. We hypothesize that bacterial BCFAs are essential for the formation of the tongue coating in IOH. Recently, Al-Beloshei et al (2015) showed that the bacterial BCFA levels increase during biofilm formation at neutral and alkaline pHs [32]. This observation is consistent with

our current findings. Also, BCFAs can induce the fermentation of food products like natto and dairy products and significantly increase bad odor [29], and we speculate that the increased amounts of BCFAs in IOH might induce the bacterial fermentation of tongue food particles and/or cell debris in IOH. Moreover, the BCFA level reflects the amount of protein breakdown and amino acid fermentation [33], which could explain the variations in the amount of volatile sulfur gases (which contribute to bad breath) in IOH of varying severity. Targeted lipidomics analysis is needed to quantify the BCFAs and to elucidate the exact mechanisms underlying tongue coating formation. The majority of the 39 biomarkers in IOH, were lipid metabolites (supplementary table 1). The fatty acid oxidation is a multistep process, requires acetyl CoA and we found higher levels of L-acetyl carnitine, the carrier of acetyl CoA, in the IOH group. L-acetyl carnitine stimulates the production of acetylcholine and enhances protein and membrane phospholipid synthesis [34].

Interestingly, we found 3-fumarylpyruvate and acetyl phosphate were linked to IOH (figure 4(d)); notably, 3-fumarylpyruvate is formed during the bacterial degradation of aromatic compounds in the gentisate pathway (figure 5). In this pathway, aromatic compounds are converted to 3-maleylpyruvate or isomerized to 3-fumarylpyruvate, which is further hydrolyzed to fumarate and pyruvate that enter into the prokaryotic tricarboxylic acid (TCA) cycle [35]. When this metabolic pathway is active, leucine, isoleucine, and valine are biosynthesised, and indeed, we found these metabolites among the selected 39 metabolites that were elevated in the IOH group (supplementary table 1). In the context of oral disease, an increase in this aromatic compound degradation pathway has been reported in periodontal disease [36]. Mostly gram-negative bacteria are involved in IOH, and these gram-negative bacteria predominantly use this gentisate pathway [37].

#### Acetyl phosphate: a key marker metabolite

Acetyl phosphate was one of the 39 metabolites found to be associated with IOH. This compound can be a product of pyruvate and taurine metabolism (figure 5). Acetyl phosphate is an energy phosphate and a precursor for acetic acid during fermentation [38]. H<sub>2</sub>S is an end product of the taurine pathway and has been linked to several diseases. Sulfacetaldehyde is an intermediate product in taurine metabolism and was a screening product in IOH secondary to H<sub>2</sub>S. We speculate that acetyl phosphate, which is produced by the taurine pathway, is linked to H<sub>2</sub>S production in IOH. In the taurine pathway, H<sub>2</sub>S is produced by prokaryotes such as *Firmicutes* and *Proteobacteria* [35]. In our previous microbiome study we showed a quantitative increase of *Clostridiales*, *Dialister*, *Peptostreptococcus*, *Peptococcus*, *Parvimonas* and *Selenomonas*

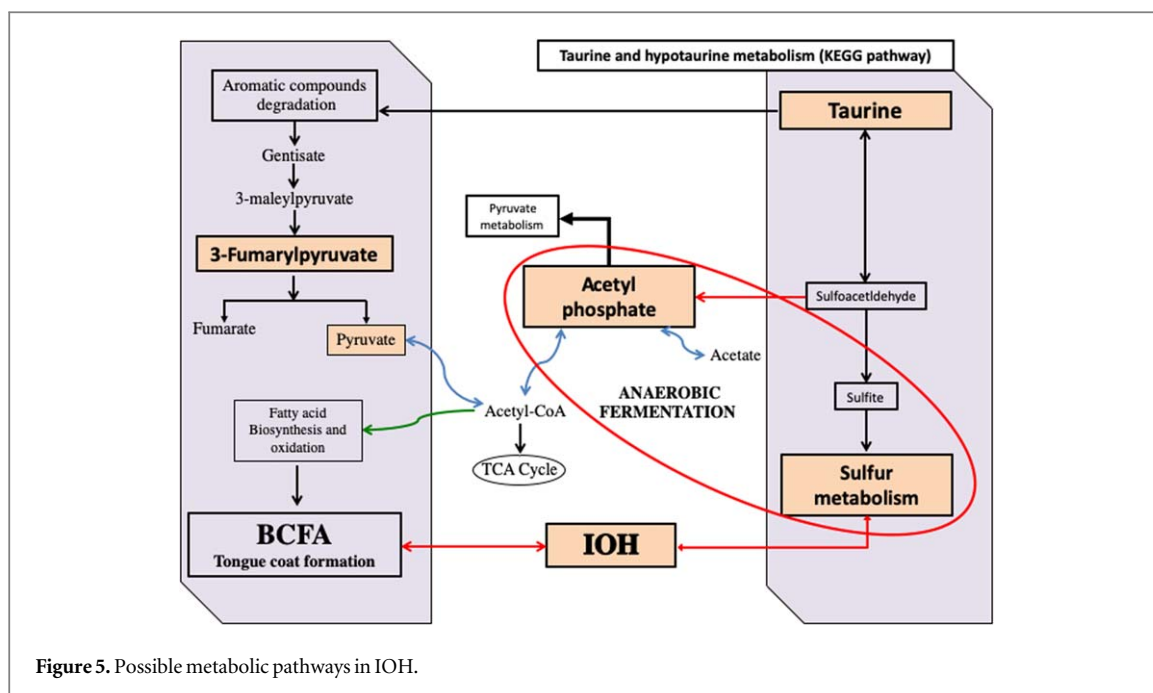


Figure 5. Possible metabolic pathways in IOH.

from *Firmicutes* and *Campylobacter* and *Aggregatibacter* from *Proteobacteria* in IOH. These bacteria might involve in the production of acetyl phosphate and  $H_2S$  through taurine pathway. These finding indicates that IOH is the result of both bacterial and metabolite-dependent reactions.

## Conclusion

We profiled the tongue coating metabolites in IOH and healthy control using LC-MS/MS approach. As far as we are aware, our study is the first to investigate the microbial metabolic profile of tongue coating. A total of 39 metabolites were associated with IOH, 3 of which, BCFA, 3-fumaryl pyruvate, and acetylphosphate, are potential key players in IOH. We conclude that the physiological changes of tongue bacterial may induce the production of different IOH -related metabolites such as BCFA that forms a white coating on the tongue. Tongue coating can serve as trap and a reservoir for the food particles and cell debris. BCFAs have link with the fermentation of the tongue coating debris (most likely food debris) and thus producing bad breath. This study reports novel metabolomic findings regarding the formation of a tongue coating and the production of bad breath  $H_2S$ . To conclude IOH is caused by changes at the functional (metabolite) level of the microbiome.

## Acknowledgments

The authors thank H J Permentier from the department of Mass Spectrometry, Eriba, University of

Groningen for performing the LC-MS/MS analyses and processing the raw data. Also, we acknowledge Dr P H M Savelkoul, Microbiome, Amsterdam, The Netherlands for the support in sample processing and storage.

## Funding

This study was supported by the Center for Dentistry and Oral hygiene, University Medical Center Groningen, Groningen, The Netherlands.

## Conflict of interest

Author KS, MMM, AJvW declared that they have no competing interests. Author EW is the co-owner of CleverCool BV. EW works at the Clinic for Periodontology Amsterdam, Amsterdam, The Netherlands, where he treats halitosis patients.

## Author's contributions

KS, MMM, EW, and AJvW designed the study, KS and EW collected the samples, MMM and KS performed the laboratory experiments and performed the bioinformatics analyses and drafted the manuscript with AJvW. All authors read and approved the final version of the manuscript.

## ORCID iDs

Kavitha Seerangaiyan  <https://orcid.org/0000-0002-8297-2430>

## References

- [1] Seemann R et al 2014 Halitosis management by the general dental practitioner—results of an international consensus workshop *J. Breath Res.* **8** 017101
- [2] Tangerman A 2002 Halitosis in medicine: a review *Int. Dental J.* **52** 201–6
- [3] Pol A et al 2018 Mutations in SELENBP1, encoding a novel human methanethiol oxidase, cause extraoral halitosis *Nat. Genet.* **50** 120–9
- [4] Quirynen M et al 2009 Characteristics of 2000 patients who visited a halitosis clinic *J. Clin. Periodontol.* **36** 970–5
- [5] Akaji E A, Folaranmi N and Ashiwaju O 2014 Halitosis: a review of the literature on its prevalence, impact and control *Oral Health Prev. Dental* **12** 297–304
- [6] Seerangaiyan K, van Winkelhoff A J, Harmsen H J M, Rossen J W A and Winkel E G 2017 The tongue microbiome in healthy subjects and patients with intra-oral halitosis *J. Breath Res.* **11** 036010
- [7] Tonzetich J 1977 Production and origin of oral malodor: a review of mechanisms and methods of analysis *J. Periodontol.* **48** 13–20
- [8] Scully C and Greenman J 2012 Halitology (breath odour: aetiopathogenesis and management) *Oral Diseases* **18** 333–45
- [9] Tangerman A and Winkel E G 2007 Intra- and extra-oral halitosis: finding of a new form of extra-oral blood-borne halitosis caused by dimethyl sulphide *J. Clin. Periodontol.* **34** 748–55
- [10] Kleinberg I and Westbay G 1992 Salivary and metabolic factors involved in oral malodor formation *J. Periodontol.* **63** 768–75
- [11] Persson S, Claesson R and Carlsson J 1989 The capacity of subgingival microbiotas to produce volatile sulfur compounds in human serum *Oral Microbiol. Immunol.* **4** 169–72
- [12] Sterer N, Shaharabany M and Rosenberg M 2009  $\beta$ -galactosidase activity and H<sub>2</sub>S production in an experimental oral biofilm *J. Breath Res.* **3** 016006
- [13] Tyrrell K L, Citron D M, Warren Y A, Nachnani S and Goldstein E J 2003 Anaerobic bacteria cultured from the tongue dorsum of subjects with oral malodor *Anaerobe* **9** 243–6
- [14] Kazor C E et al 2003 Diversity of bacterial populations on the tongue dorsa of patients with halitosis and healthy patients *J. Clin. Microbiol.* **41** 558–63
- [15] Riggio M P et al 2008 Molecular identification of bacteria on the tongue dorsum of subjects with and without halitosis *Oral Dis.* **14** 251–8
- [16] Shaffer M, Armstrong A J S, Phelan V V, Reisdorph N and Lozupone C A 2017 Microbiome and metabolome data integration provides insight into health and disease *Transl. Res.* **189** 51–64
- [17] Takahashi N 2015 Oral microbiome metabolism: from ‘who are they?’ to ‘what are they doing?’ *J. Dental Res.* **94** 1628–37
- [18] Rosenberg M, Kulkarni G V, Bosy A and McCulloch C A 1991 Reproducibility and sensitivity of oral malodor measurements with a portable sulphide monitor *J. Dental Res.* **70** 1436–40
- [19] Rosenberg M et al 1991 Halitosis measurement by an industrial sulphide monitor *J. Periodontol.* **62** 487–9
- [20] Van Der Velden U 2009 The Dutch periodontal screening index validation and its application in the Netherlands *J. Clin. Periodontol.* **36** 1018–24
- [21] Winkel E G, Roldán S, Van Winkelhoff A J, Herrera D and Sanz M 2003 Clinical effects of a new mouthrinse containing chlorhexidine, cetylpyridinium chloride and zinc-lactate on oral halitosis *J. Clin. Periodontol.* **30** 300–6
- [22] Sun Z-M et al 2013 Metabolic markers and microecological characteristics of tongue coating in patients with chronic gastritis *BMC Complementary Altern. Med.* **13** 227
- [23] Wishart D S et al 2013 HMDB 3.0—the human metabolome database in 2013 *Nucleic Acids Res.* **41** D801–7
- [24] Fahy E, Sud M, Cotter D and Subramaniam S 2007 Lipid maps online tools for lipid research *Nucleic Acids Res.* **35** W606–12
- [25] Xia J and Wishart D S 2011 Web-based inference of biological patterns, functions and pathways from metabolomic data using MetaboAnalyst *Nat. Protocols* **6** 743–60
- [26] Li Y et al 2016 A systematic strategy for screening and application of specific biomarkers in hepatotoxicity using metabolomics combined With ROC Curves and SVMs *Toxicol. Sci.* **150** 390–9
- [27] Kanehisa M, Goto S, Furumichi M, Tanabe M and Hirakawa M 2009 KEGG for representation and analysis of molecular networks involving diseases and drugs *Nucleic Acids Res.* **38** D355–60
- [28] Patching S G and Gardiner P H E 1999 Recent developments in selenium metabolism and chemical speciation: a review *J. Trace Elem. Med. Biol.* **13** 193–214
- [29] Ran-Ressler R R, Devapatla S, Lawrence P and Brenna J T 2008 Branched chain fatty acids are constituents of the normal healthy newborn gastrointestinal tract *Pediatric Res.* **64** 605–9
- [30] Kaneda T 1991 Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance *Microbiol. Rev.* **55** 288–302
- [31] Elsdén S R and Hilton M G 1978 Volatile acid production from threonine, valine, leucine and isoleucine by clostridia *Arch. Microbiol.* **117** 165–72
- [32] Al-Beloshei N E, Al-Awadhi H, Al-Khalaf R A and Afzal M A 2015 Comparative study of fatty acid profile and formation of biofilm in *Geobacillus gargensis* exposed to variable abiotic stress *Can. J. Microbiol.* **61** 48–59
- [33] Macfarlane G T, Gibson G R, Beatty E and Cummings J H 1992 Estimation of short-chain fatty acid production from protein by human intestinal bacteria based on branched-chain fatty acid measurements *FEMS Microbiol. Lett.* **101** 81–8
- [34] Calabrese V, Stella A M G, Calvani M and Butterfield D A 2006 Acetylcarnitine and cellular stress response: roles in nutritional redox homeostasis and regulation of longevity genes *J. Nutr. Biochem.* **17** 73–88
- [35] Caspi R et al 2016 The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases *Nucleic Acids Res.* **44**(D1) D471–80
- [36] Barnes V M et al 2011 Metabolomics reveals elevated macromolecular degradation in periodontal disease *J. Dental Res.* **90** 1293–7
- [37] Crawford R L and Frick T D 1977 Rapid spectrophotometric differentiation between glutathione-dependent and glutathione-independent gentisate and homogentisate pathways *Appl. Environ. Microbiol.* **34** 170–4
- [38] Brown T D, Jones-Mortimer M C and Kornberg H L 1977 The enzymic interconversion of acetate and acetyl-coenzyme a in *Escherichia coli* *J. Gen. Microbiol.* **102** 327–36