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Original article

Breath volatilome analysis reveals new gut microbiome-related metabolites that discriminate high versus low dietary fibre intake

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SUMMARY

Background & aims: Gut bacteria produce a wide variety of metabolites that are playing important roles in human health. Dietary fibres (DF) are beneficial nutrients that have been shown to modulate key intestinal functions when fermented by gut bacteria. Since most bacteria-derived metabolites are volatile, their presence in exhaled breath allows to propose new non-invasive methods to study DF-microbiome interactions in humans. We aimed to identify potential novel biomarkers of gut microbiota activity released in exhaled breath following the consumption of DF at breakfast, upon untargeted analysis in healthy volunteers.

Methods: 14 volunteers (7 women/7 men, 21 ± 2 years old) participated to two test days at a one-month interval, where they received either a low-(2.6 g) or high-(16.1 g) fibre breakfast. Before each test days, stools were collected to evaluate the microbiota composition using Illumina sequencing (V5–V6 region of 16S rRNA gene). Throughout the test days, breath samples were analysed using selected-ion flow-tube mass spectrometry (SIFT-MS). A sparse partial least squares-discriminant analysis (sPLS-DA) identified 30 signals that best discriminated between test days, corresponding to 173 candidate breath compounds.

Results: The gut microbiota of the volunteers remained stable one month apart. The composition of exhaled breath shifted starting from 5 h after the high-fibre breakfast ingestion. Ninety compounds were identified as potential metabolites of gut microbes, with 81 showing increased concentrations after the high-fibre breakfast. These included acrylic acid (positively correlated with *Faecalibacterium/Ruminococcaceae/Bacillota* and negatively correlated with *Bifidobacterium/Bifidobacteriaceae/Actinomycetota)*. The high-fibre breakfast also led to increases in limonene, ethylbenzene/xylene, p-cymene, and methionol that were positively correlated with the genus *Faecalibacterium*. Moreover, positive correlations were observed between cyclooctane/ethylcyclohexane, methanol and the phylum *Bacillota*. Dimethyl disulfide was strongly negatively correlated with the genus *Bacteroides* and its family *Bacteroidaceae*.

Conclusion: This study shows that DF consumption at breakfast stimulates the production of exhaled bacteria-derived metabolites reflecting profound changes in the metabolic activity of the gut microbiota. We also identified new potential biomarkers of DF intake, that are not directly linked to DF

Abbreviations: AUC, Area under the curve; BMI, Body mass index; CICN, Centre of Investigation in Clinical Nutrition; DF, Dietary fibres; FDR, False discovery rate; FOS, Fructo-oligosaccharides; FRS-FNRS, Fonds de la Recherche Scientifique; GFS, Gut fermentation syndrome; GOS, Galacto-oligosaccharides; HF, High-fibre; IBD, Inflammatory bowel disease; IBS, Irritable bowel syndrome; IBS-C, Irritable bowel syndrome with constipation; IBS-D, Irritable bowel syndrome with diarrhoea; IDF, Insoluble dietary fibre; LAB, Lactic acid bacteria; LF, Low-fibre; *m/z*, Mass-to-charge ratio; OTU, Operational taxonomic unit; PCA, Principal component analysis; SCFA, Short-chain fatty acids; SDF, Soluble dietary fibre; SIBO, Small intestinal bacterial overgrowth; SIFT-MS, Selected-ion flow-tube mass spectrometry; sPLS-DA, Sparse partial least squares-discriminant analysis; TDF, Total dietary fibre; VAS, Visual analog scale.

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fermentation. Specific bacteria known to play a role in gut barrier, immunity and host metabolism were associated with those new metabolites.
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1. Introduction

Dietary fibres (DF) are an important class of nutrients that contribute to health; the increase in DF intake being inversely correlated with all risks of mortality in humans [1,2]. Fermentable DF serve as a primary energy source for gut microbes, a process leading to the production of gases (such as hydrogen, methane, hydrogen sulfide) and of beneficial short-chain fatty acids (SCFA) like acetate, propionate, and butyrate. These SCFA support intestinal barrier function by enhancing mucus synthesis and quality, by promoting antimicrobial peptide production, and by reducing epithelial permeability and increasing tight junction complex integrity. In addition to their effect on the gut, SCFA support host health by preventing weight gain, regulating energy metabolism, and improving glucose homeostasis and insulin sensitivity [3].

Beyond SCFA, the gut microbiota produces various compounds that can play a role in host physiology, including vitamins, neuromediators, or bile acids [4,5]. Other microbial metabolites, such as alcohols and aromatic compounds, have unclear relevance for health. For instance, ethanol is produced from the fermentation of carbohydrates primarily by *Candida albicans* and *Saccharomyces cerevisiae* in gut fermentation syndrome (GFS); this rare condition causing symptoms such as slurred speech and disorientation [6]. More recent studies also propose that gut microbiota dysbiosis is associated with inflammation in the liver and that ethanol production by specific gut bacteria like *Enterobacteriaceae* can contribute to liver disease associated with obesity (for review, see Lanthier and Delzenne [7]). Phenolic and indolic compounds are derived from the degradation of aromatic amino acids (phenylalanine, tyrosine, tryptophane) by anaerobic bacteria belonging to *Bacteroides*, *Bifidobacterium*, *Clostridium, Lactobacillus* and *Peptostreptococcus,* and can exert potentially harmful or beneficial effect for health [8].

Interestingly, numerous volatile microbial products produced in the gut can enter the bloodstream and be exhaled in breath. Measurement of breath hydrogen and methane after ingestion of test-carbohydrates is routinely used for diagnostic purposes [9]. Beyond these gases, breath analysis appears promising to assess a wide range of gut microbial metabolites. As evidence, De Vincentis *et al.* [10] showed that breath analysis could detect changes in the gut microbiota after rifaximin – an antibiotic – treatment in volunteers with diverticular disease. Similarly, Shahi *et al.* [11] reported significant shifts in breath composition and microbial communities after ertapenem and ciprofloxacin therapy in a patient with a long-term biliary system infection. Previous work from our group showed that both acute ingestion and 3-week supplementation of chitin-glucan, a fermentable insoluble DF, modified breath composition compared with maltodextrin as a placebo [12,13].

This study explored the effects of breakfast fibre content on exhaled breath composition, with the aim of identifying potential novel biomarkers reflecting gut microbial activity.

2. Material and methods

2.1. Intervention study

This paper compares the first test day of two different interventions conducted in the same volunteers one month apart. Our objective was to reuse the dataset in order to identify breath volatile compounds associated with the intake of DF at breakfast (low-fibre on test day 1 and high-fibre on test day 2). The high-fibre breakfast contained 16.1 g of fibre, approaching the European daily reference value of 25 g per day for adults [14]. Although this recommendation applies to total daily intake, this amount within a single meal is high relative to the average Belgian intake of 16 g per day [15]. The low-fibre breakfast contained 2.6 g of fibre, consistently with previous definitions of low-fibre diets, which have been described as providing less than 10 g of fibre per day [16]. An overview of the study design is shown in Fig. 1.

2.2. Participant recruitment

Healthy volunteers were recruited by the Centre of Investigation in Clinical Nutrition (CICN, UCLouvain) through posters on the university campus, emails, social media, local newspapers, and flyers distributed in nearby shops and doctors' offices. Participants were pre-screened via phone or email using a questionnaire to determine eligibility based on inclusion and exclusion criteria, as previously described [12]. Briefly, inclusion criteria were the following: men or women aged 18–40 years old, body mass index (BMI) between 18 and 25 kg/m^2, Caucasian, non-smoker, in good general health (verified by medical history and physical examination), effective contraception for women, and hydrogen-producer status. Exclusion criteria were the following: gastrointestinal disorders, chronic or intestinal diseases (e.g., ulcers, diverticulitis, inflammatory bowel diseases (IBD)), smoking, recent or current use of antibiotics, pro/prebiotics (as dietary supplements), or gut transit-affecting products (e.g., laxatives) within 4 weeks prior to the study, use of drugs altering gut microbiota composition (e.g., antidiabetic drugs, cholesterol-lowering drugs, proton pump inhibitors), pregnancy or lactation, psychiatric issues (and/or use of antipsychotics), chronic drug use, specific diets (e.g., vegetarian, high-fibre, high-protein), food allergies or intolerances (e.g., lactose, gluten), allergy or intolerance to any component of the meals, excessive alcohol consumption (more than 3 units/day), intense physical activity (more than 10 h/week), and participation in another clinical trial within 1 month before screening.

2.3. Screening and pre-study procedures

At least 4 weeks before the first day of intervention, participants underwent a lactulose screening test to identify hydrogen-producers, based on hydrogen rather than methane exhalation during indigestible sugar fermentation. A high abundance of methanogens is associated with conditions like obesity and irritable bowel syndrome with constipation (IBS–C), while a low abundance is linked with IBD and irritable bowel syndrome with diarrhoea (IBS-D) [17]. Volunteers, who had fasted for at least 10 h, ingested 10 g of lactulose orally and exhaled hydrogen was measured every 30 min for 4 h using the Lactotest 202 (Medical Electronic Construction, MEC). A minimum increase of 10 ppm of hydrogen over 3 consecutive measurements was required for selection. No increase in the first 30 min was necessary to exclude volunteers with small intestinal bacterial overgrowth (SIBO). After selection, volunteers underwent a medical examination by the

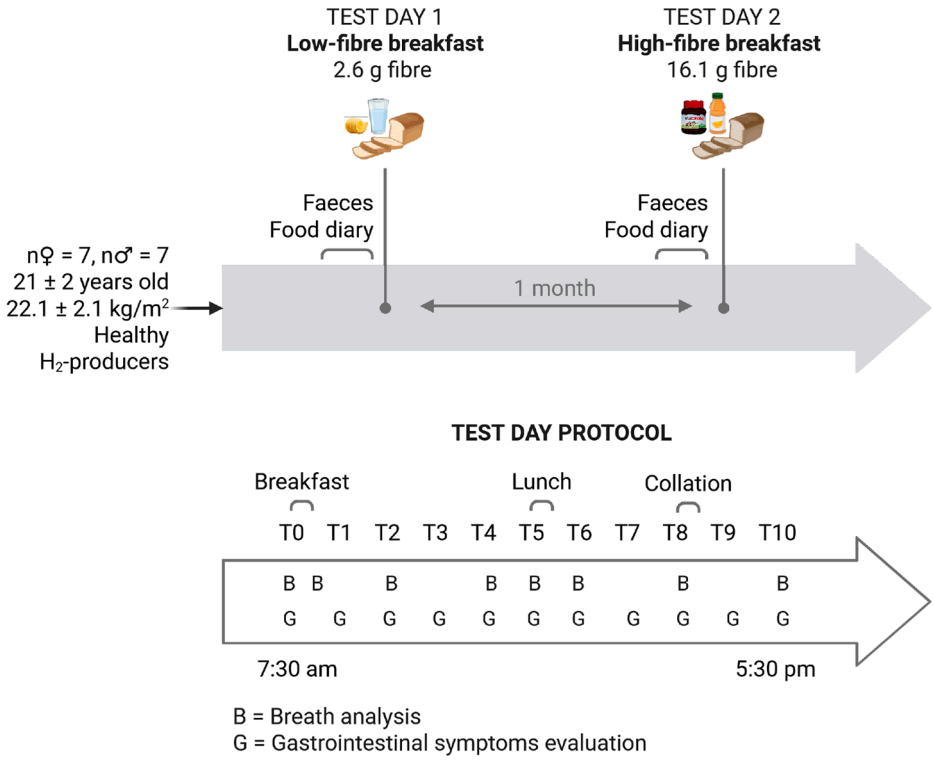


Fig. 1. Overview of the study design. This paper compares the first test day of two different interventions conducted in the same volunteers one month apart with the objective to identify breath volatile compounds following dietary fibre consumption. The 14 healthy volunteers consumed a low-fibre breakfast and a high-fibre breakfast, both test days being organised in the same way.

physician investigator to confirm their health status. Fourteen healthy volunteers (7 women and 7 men, 21 ± 2 years old) who fitted with all inclusion and exclusion criteria participated in the two test days. Baseline characteristics of the participants are detailed in Table S1. Of note, a post hoc statistical power analysis indicated that the sample size was sufficient to detect significant differences between test days (power higher than 90% for the compounds presented in the main figures at the 8-h time point; Wilcoxon tests, two-tailed, $\alpha = 0.05$, G*Power 3.1.9.7 [18,19]).

After the screening test, participants received dietary recommendations and were instructed to hold food diaries and collect faecal samples. A dietician inquired the volunteers to avoid consuming fibre-rich foods containing high amounts of prebiotic DF more than once a week at least 12 days before each test day. These foods included whole grains, artichokes, Jerusalem artichokes, salsifies, leeks, and onions. Prebiotic and probiotic supplements, as well as fibre-enriched foods, were prohibited. Food diaries were self-completed by the participants for three days (two weekdays and one weekend day) during the week prior to each test day, recording all food and beverages consumed. Macronutrient and total fibre intake were assessed using the Nubel Pro program and the Nubel 2010 composition table. Faecal samples were collected up to 4 days before each test day and stored at −20 °C by the volunteer. They were then transported to the CICN for storage at −80 °C until analyses.

During the intervention, participants were frequently contacted via email or phone to ensure they met the inclusion/exclusion criteria, adhered to dietary guidelines, and collected stool samples. Additionally, participants completed an electronic questionnaire using REDCap (Research Electronic Data Capture) on test days to monitor side effects and compliance.

2.4. Test day protocol

Both test days followed the same protocol. The evening before each intervention day, participants consumed rice and meat without vegetables and avoided alcohol. Volunteers who had fasted from 9 pm the previous night arrived at the CICN at 7 am. The study coordinator reviewed the inclusion and exclusion criteria and ensured compliance with instructions. After a mouthwash (Perio Aid Intensive Care), baseline breath samples were collected. Participants then completed a 100-mm visual analog scale (VAS) questionnaire on 8 gastrointestinal symptoms typically associated with fibre consumption (bloating, burps, cramps, discomfort, flatulence, nausea, reflux, and rumbling). Scores were determined by measuring the distance from 0 (in mm) and expressed as changes from baseline. On the first test day, participants received a low-fibre breakfast of white bread and butter with 240 ml of water, while on the second test day, they had a high-fibre breakfast of rye bread with 25 g of carob spread and 200 ml of orange juice (see Table S2 for breakfast composition). Breakfast was consumed within 15 min. Participants provided breath samples 30 min and every 2 h post-ingestion and completed the gastrointestinal tolerance questionnaire hourly, both for 10 h. Two hours post-breakfast, participants were allowed to drink water. Five hours after the start, they provided an additional breath sample before receiving a standardized lunch of white bread, ham, and cheese, to be eaten within 15 min. Eight hours after the start, they were given a sweet waffle snack. The test day concluded at 6 pm.

2.5. Gut microbiota analysis

Bacterial DNA was extracted using the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany). The complete methodology and

bioinformatic analysis were previously described [20]. α-Diversity indices were calculated with QIIME2. The raw sequencing data are available in the Sequence Read Archive (SRA) of NCBI (http://www.ncbi.nlm.nih.gov/sra) under BioProject PRJNA636138.

2.6. Breath volatile metabolites analysis

Breath samples were collected in bags (FMONP.SBAG1.01 from MEC R&D SPRL, Belgium) sent to Interscience (Louvain-La-Neuve, Belgium) for measurement within 2 h using selected-ion flow-tube mass spectrometry (SIFT-MS, Voice 200ultra, Syft Technologies, New Zealand). Reagent ions (H_3O^+, NO^+, and O_2^+) were generated by a microwave air discharge at 0.5 Torr, selected with a quadrupole mass filter, and injected into a helium carrier gas stream in the flow tube. Product ions of the chemical ionization and unreacted reagent ions were monitored by a quadrupole mass spectrometer in full scan mode in the mass-to-charge ratio (m/z) range of 15-150 (unit mass resolution) over 60 s. Helium-filled bags were analysed at each time point to serve as blanks. Breath data were of two types: untargeted (counts of product ions) and targeted (concentrations of targeted compounds calculated from the untargeted data). The LabSyft software (version 1.4.4, Syft Technologies) was used to export data acquired with the SIFT-MS device, as well as to identify and quantify compounds present in breath samples.

While SIFT-MS enables real-time and quantitative analysis of volatile compounds without sample preparation, structural isomers and compounds yielding similar product ion patterns can produce overlapping signals preventing unequivocal identification. For this reason, in the present study, certain compounds are reported as alternative possibilities (e.g., 1-pentanol/2-pentanol/3-pentanol) when the spectral data did not allow clear discrimination between them.

2.7. Statistical analysis

Data were assessed for normality using the D'Agostino and Pearson test ($p < 0.05$). Baseline characteristics followed a normal distribution and are therefore presented as mean ± standard deviation. Other variables did not follow a normal distribution and are presented as median [P25-P75], except for gastrointestinal symptoms data, which are shown as mean ± standard deviation because the median values were 0 for most time points.

The breath measurement of volunteer D 4 h after the high-fibre breakfast was not considered for the analysis due to analytical inaccuracy. The values measured in helium-filled bag were substracted to values measured in samples at each time point for both targeted and untargeted breath data analysis.

Novel compounds of interest for studying gut microbiota activity were identified from breath untargeted data using the "mixOmics" package in R (v4.2.2). To achieve this, a scaled principal component analysis (PCA) was applied to untargeted breath data to visualize the changes induced by the two different breakfasts on breath composition throughout the day. Then, a sparse partial least squares-discriminant analysis (sPLS-DA) was employed on untargeted breath data obtained at the most relevant time points to study gut microbial metabolites. This analysis identified the loadings – corresponding to ions produced by the reaction between compounds present in the exhaled breath and the reagent ions H_3O^+, NO^+, and O_2^+ in the SIFT-MS device – most noteworthy for explaining the difference between the low-fibre and high-fibre test days. For arbitrary reasons, the first 30 loadings were considered to extract compounds in breath samples that might have formed them in the device from the LabSyft library.

Test days were compared using GraphPad Prism (v10.2.3) and visualised using the "ggplot2" package in R (v4.2.2). Data measured once before each test day (i.e., dietary intake, relative microbial abundance, alpha-diversity index) were compared using a Wilcoxon test ($p < 0.05$). Data measured over time (i.e., gastrointestinal symptoms, breath targeted data) were compared using a Wilcoxon test on the net area under the curve (AUC) calculated from baseline to 10 h post-breakfast ($p < 0.05$), as well as a mixed model followed by Šídák test ($p < 0.05$). The Wilcoxon test on net AUC assessed overall changes, while Šídák test compared values at each time point.

Correlation analysis was performed between the concentration of compounds of interest in exhaled breath at 8 h post-ingestion of the high-fibre breakfast and gut microbiota composition (phylum, family, and genus taxonomic levels) before the high-fibre test day filtered with a mean relative abundance above 1%. To do this, Spearman test ($p < 0.05$) and the "corrplot" package in R (v4.2.2) were used. The 8-h post-ingestion time point was selected as it represented the peak concentration of the compounds in the exhaled breath following the high-fibre breakfast. For correlation analysis, false discovery rate (FDR) correction was applied to p-values using the Benjamini-Hochberg procedure ($q < 0.05$).

2.8. Ethics

The protocols were approved by the local ethical committee (Comité d'Ethique Hospitalo-Facultaire UCLouvain/Cliniques Universitaires Saint-Luc) and registered at ClinicalTrials.gov under identification numbers NCT03494491 and NCT03505177. Written informed consent was obtained from all volunteers. The trials were carried out in accordance with the Good Clinical Practice as required by the following regulations: the Belgian law of 7 May 2004 regarding experiments on human persons and the EU Directive 2001/20/EC.

3. Results

3.1. Characteristics of volunteers: dietary anamnesis and microbiota composition prior intervention test days

The dietary habits were evaluated using a food diary during the week prior to each test day. The usual intake of lipids, proteins and carbohydrates (including total dietary fibre (TDF), soluble dietary fibre (SDF), insoluble dietary fibre (IDF), galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS), inulin, and fructans) did not significantly differ before the two test days (Wilcoxon test, $p > 0.05$, data not shown). However, energy intake significantly decreased from the first to the second test day (Wilcoxon test, $p < 0.05$, data not shown).

The alpha diversity – an indicator of microbial diversity within an individual – did not change significantly from test days one month apart (Wilcoxon test, $p > 0.05$) (Fig. 2A). This was confirmed using the microbe count (richness), the Pielou index (evenness) and the Shannon index (richness and evenness).

The most prevalent phyla in the volunteers on both test days were *Bacillota* (previously known as *Firmicutes*), followed by *Bacteroidota* (previously known as *Bacteroidetes*) and *Actinomycetota* (previously known as *Actinobacteria*) (Fig. 2B). The most abundant families were *Lachnospiraceae*, followed by *Ruminococcaceae*. Volunteer I exhibited a different gut microbiota compared to the others, with a greater relative abundance of the genus *Agathobacter* and *Bifidobacterium* (Fig. 2C). None of the taxa that had a mean relative abundance above 1% reached a significant difference at the q value; only the genera *[Eubacterium] hallii* group (reclassified as *Anaerobutyricum hallii* group) and *Dorea* were more

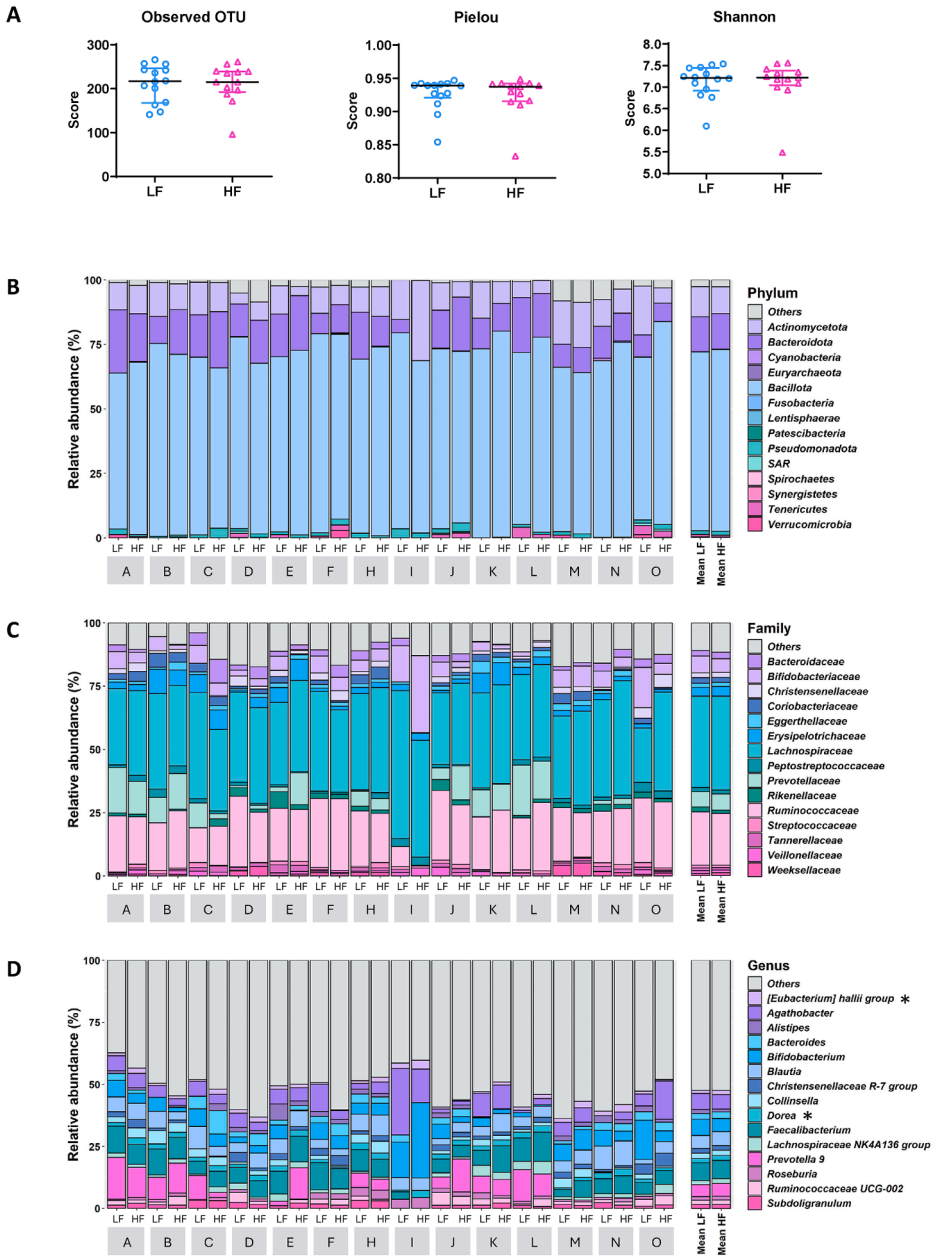


Fig. 2. Comparison of gut microbiota composition in faeces sampled in 14 healthy volunteers the week before low-fibre (LF) and high-fibre (HF) breakfast test days. (**A**) α-Diversity indexes related to bacterial richness (observed operational taxonomic unit, OTU), evenness (Pielou), or both (Shannon). Data are not normally distributed and expressed as median [P25-P75]. Wilcoxon test ($p > 0.05$). (**B-D**) Relative abundance at several taxonomic levels – phylum, family and genus – for each volunteer (unfiltered data, volunteer A to O, no volunteer G) and mean value. * Wilcoxon test ($p < 0.05$).

abundant in volunteers on the second test day (Wilcoxon test, $p < 0.05$, Fig. 2D).

The tolerance of meals provided during the test days was generally good. Indeed, the progression of 8 gastrointestinal tolerance scores on the test days did not significantly differ between the consumption of the low-fibre breakfast and the high-fibre breakfast neither globally nor at a specific time point (Wilcoxon test on net AUC and Šídák test, $p > 0.05$, respectively, Fig. S1).

3.2. *Identification and quantification of novel compounds in exhaled breath as potential microbial metabolites*

The PCA of the untargeted breath data revealed that fibre ingestion led to changes in breath composition from 5 h after the breakfast (Fig. 3A). Throughout the day, the breath composition remained relatively stable following the low-fibre breakfast, closely resembling the baseline and up to 4 h after the high-fibre breakfast ingestion. However, a distinct cluster was observed for all breath samples collected between 5 and 10 h after the high-fibre breakfast, indicating a shift in the composition.

The sPLS-DA of these same untargeted breath data allowed the identification of the product ions (loadings) most noteworthy for explaining the difference between the low-fibre and high-fibre groups between 5 and 10 h post-breakfast ingestion (Fig. 3B). For arbitrary reasons, the first 30 loadings were considered for further exploration (Fig. 3C). Twenty-five out of the 30 loadings were ions associated with the ingestion of the high-fibre breakfast. Conversely, the 5 other loadings corresponded to ions associated with the ingestion of the low-fibre breakfast.

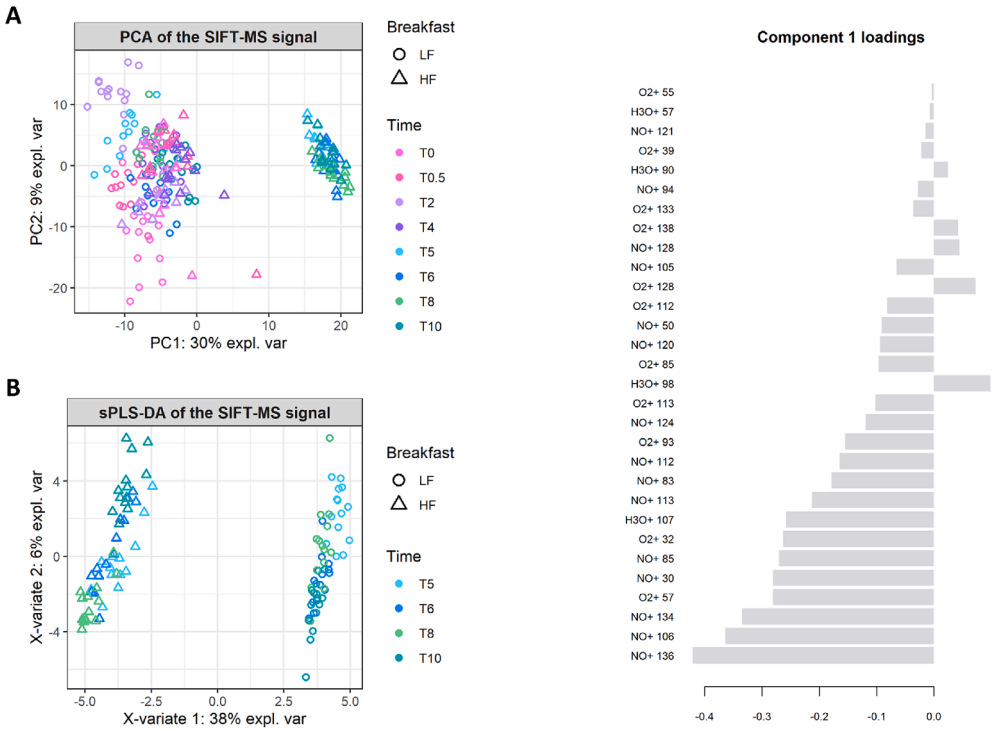


Fig. 3. Untargeted analysis (signals from the reactions of analytes with reagent ions H_3O^+, NO^+ and O_2^+ in the selected-ion flow-tube mass spectrometry (SIFT-MS) device) of breath samples from 14 healthy volunteers. (**A**) Principal component analysis (PCA) individual plot from 0 and up to 10 h after low-fibre (LF) and high-fibre (HF) breakfast ingestion. (**B**) Sparse partial least squares-discriminant analysis (sPLS-DA) individual plot from 5 h and up to 10 h after LF and HF breakfast ingestion. (**C**) Loading plot on component 1 corresponding to figure B.

The 173 compounds that might have formed these 30 product ions detected in the device were filtered and sorted to identify those most likely present in the samples following a decision tree and criteria described in the legend of Fig. S2. Based on this decision tree, 90 compounds were identified and classified according to their production by microbes and their previous detection in human samples (Fig. S3, Table S3). Of these, 81 showed increased concentrations from 5 to 10 h after the ingestion of the high-fibre breakfast compared to the low-fibre breakfast, 56 have already been identified in breath, blood, faeces, and/or saliva and 50 are described as produced by microbes.

The focus was placed on the 23 compounds most likely produced by gut microbes, identified through an intensive literature review assessing the capacity of the gut microbiota to produce them (Table S4). The evolution of the other remaining compounds is presented in Fig. S4 and Fig. S5. To avoid misidentification, compounds with similar spectra were merged. Cyclooctane, 2-pentanol and 3-pentanol were added as they could be confounded with certain of the 23 selected molecules. In total, 26 compounds were considered, accounting for potential spectral conflicts.

The evolution of the concentrations of the 26 selected compounds over time (Fig. 4A) revealed increased levels starting 5 h after the ingestion of the high-fibre breakfast, while concentrations remained stable following the low-fibre breakfast. Several patterns were observed depending on the compound, with some showing rapid maximum increases (e.g., acrylic acid) or continuing to rise until the end of the test (e.g., methanol). Additionally, certain compounds showed a slight increase in concentration immediately after breakfast (e.g., methionol), while others did not (e.g., acrylonitrile). Among the selected compounds, the most concentrated were methanol (median concentration up to 1000 ppb), dimethyl disulfide (median concentration up to 500 ppb), acrylic acid (median concentration up to 100 ppb), and limonene (median concentration up to 70 ppb).

Individual variations in the concentrations (Fig. 4B) indicated that all participants exhibited similar responses in both timing and concentration following breakfast ingestion.

3.3. Correlation between concentration of identified compounds in exhaled breath and relative abundance of gut microbes

The correlations performed between breath compounds likely produced by gut microbes and gut microbiota at phylum, family, and genus taxonomic levels are presented in Fig. 5 (Spearman test, $p < 0.05$; FDR correction, $q < 0.05$). A significant q value was obtained only for dimethyl disulfide that was strongly negatively correlated with the genus *Bacteroides* and its family *Bacteroidaceae* ($q < 0.05$). This exhaled metabolite was also negatively correlated to a lesser extent with the genus *Parabacteroides* and its family *Tannerellaceae* ($p < 0.05$).

Acrylic acid showed significant correlations at multiple taxonomic levels: it was positively correlated with the genus *Faecalibacterium*, and coherently with its family *Ruminococcaceae*, and phylum *Bacillota* ($p < 0.05$). In addition, acrylic acid was negatively correlated with the genus *Bifidobacterium*, its family *Bifidobacteriaceae*, and its phylum *Actinomycetota* ($p < 0.05$). Several other metabolites – i.e., p-cymene, ethylbenzene/xylene, limonene, and methionol – were also positively correlated with the genus *Faecalibacterium* whereas sec-butylamine, and limonene were

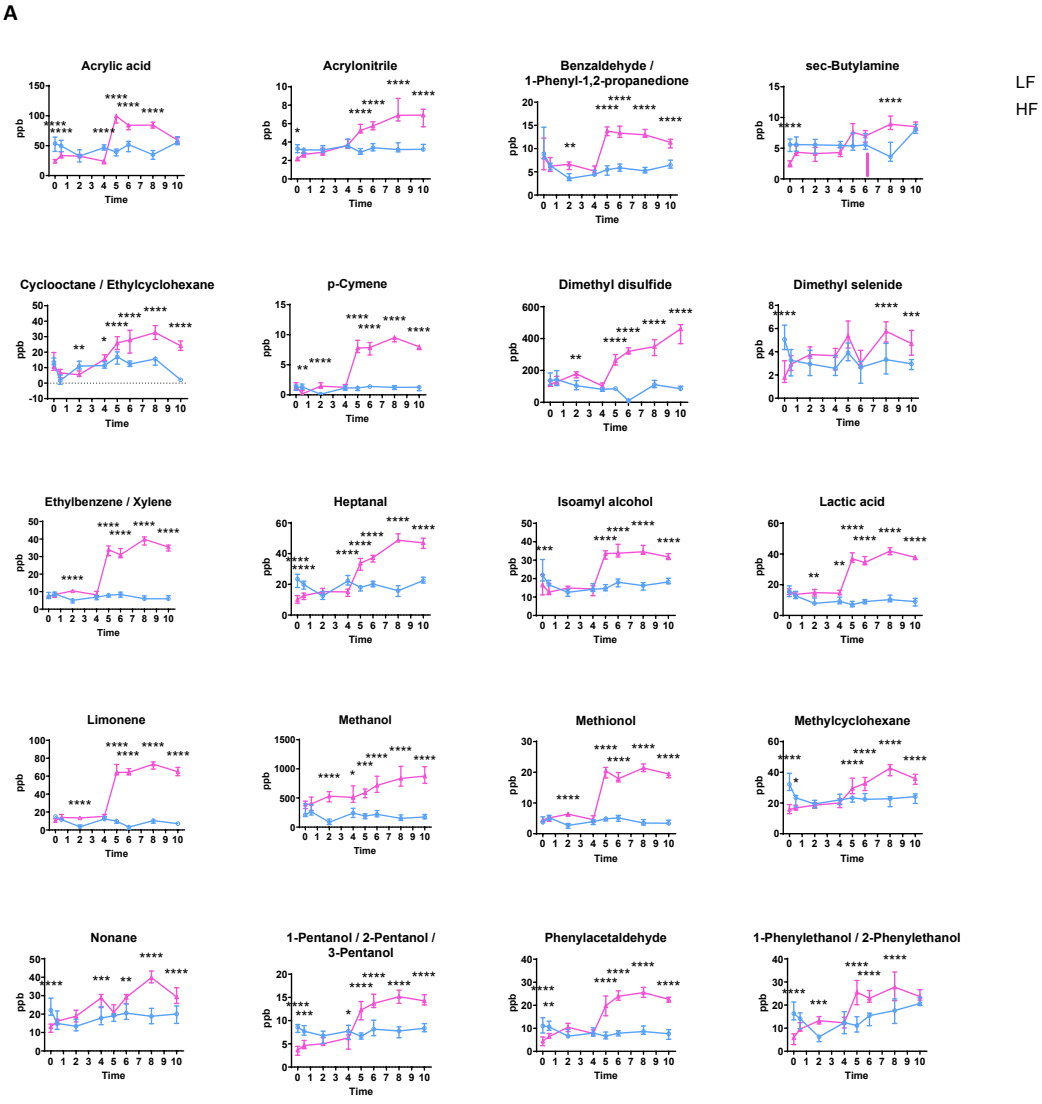


Fig. 4. Evolution of 26 selected compounds measured in breath samples from 14 healthy volunteers after low-fibre (LF) versus high-fibre (HF) breakfast ingestion. The compounds are sorted in alphabetical order. (**A**) Data expressed as median [P25-P75] as they are not normally distributed. Wilcoxon test on net area under the curve (AUC, $p < 0.05$). Šídák test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). (**B**) Data expressed as individual values.

negatively correlated with the genus *Bifidobacterium* and its family *Bifidobacteriaceae* ($p < 0.05$).

Positive correlations were also observed between cyclooctane/ethylcyclohexane, and methanol with the phylum *Bacillota* ($p < 0.05$). More specifically, cyclooctane/ethylcyclohexane was positively correlated with the genus *Ruminococcaceae UCG-002* and its family *Ruminococcaceae*, as well as the *[Eubacterium] coprostanoligenes group* ($p < 0.05$). Methanol also showed a positive correlation with the *[Eubacterium] coprostanoligenes group* ($p < 0.05$).

Benzaldehyde/1-phenyl-1,2-propanedione showed a negative correlation with the genus *Collinsella* and its family *Coriobacteriaceae* ($p < 0.05$). Dimethyl selenide was negatively correlated with the *Christensenellaceae R-7 group* and its family *Christensenellaceae* ($p < 0.05$). Lastly, sec-butylamine, p-cymene, limonene, and methanol were negatively correlated with the phylum *Pseudomonatota* ($p < 0.05$).

4. Discussion

In this study, we explored the impact of a low-fibre breakfast compared to a high-fibre breakfast on the composition of exhaled breath, to identify potential biomarkers indicative of gut microbiota activity. The study design ensured that the characteristics of volunteers, particularly their gut microbiota composition, remained stable over the one-month interval between test days, prior the intervention. Only two minor changes were observed between the two test days at the genus taxonomic level (*[Eubacterium] hallii group* and *Dorea*). Importantly, our findings demonstrated that the ingestion of a high-fibre breakfast significantly altered the breath profile; 25 out of 30 discriminating product ions being increased from 5 h post-DF ingestion. Based on these data, 81 out of 90 identified compounds increased from 5 to 10 h after the high-fibre breakfast compared to the low-fibre breakfast.

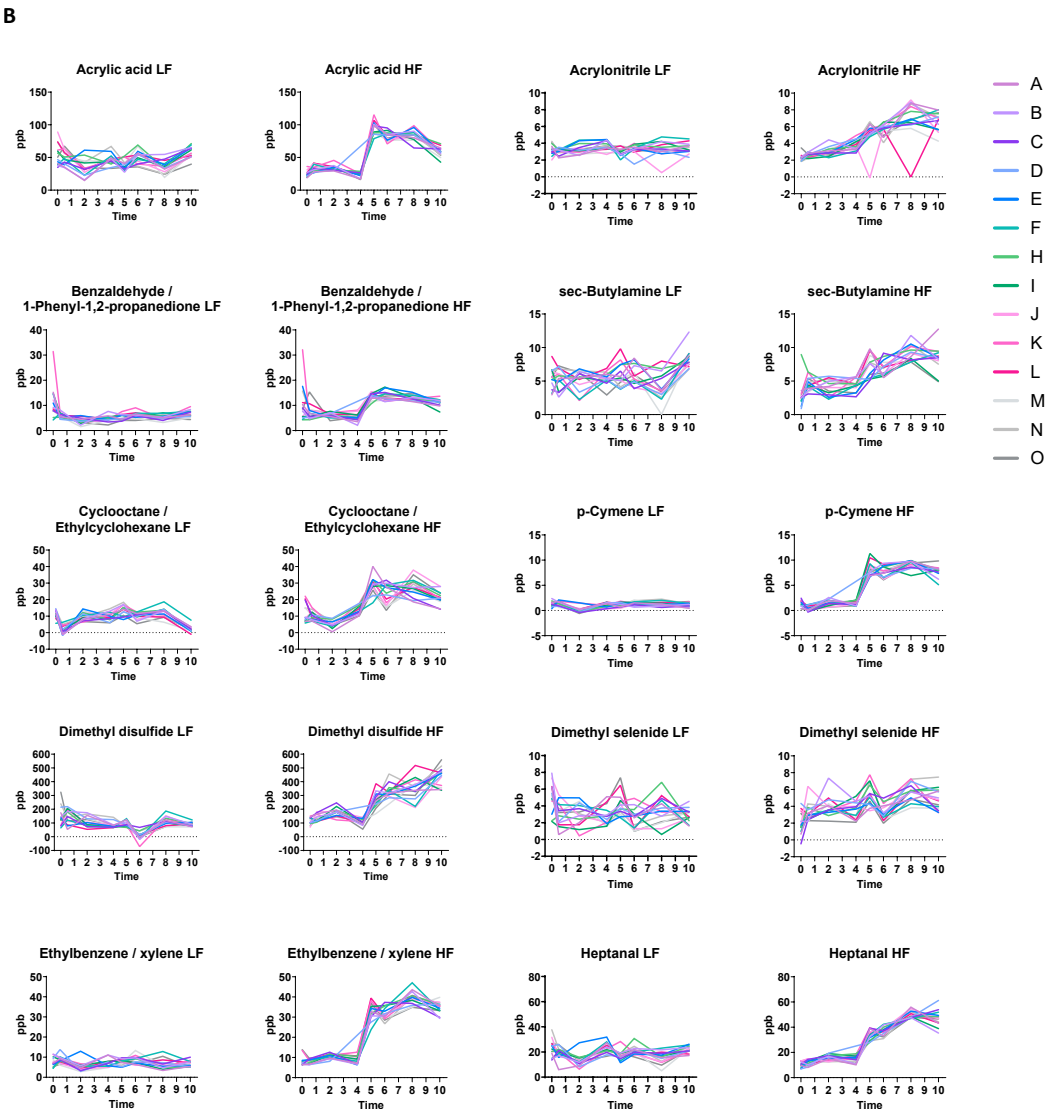


Fig. 4. (*continued*).

An exhaustive search was conducted to determine whether these metabolites had already been measured in human samples (breath, blood, faeces, and/or saliva) and whether they had been described as being produced by microbes (see Fig. S3, Table S3). Based on the outcome of this review, 23 out of the 90 compounds were highlighted as most likely to be produced by the gut microbiota. Among the compounds considered, all had previously been detected in breath except for acrylic acid, sec-butylamine, methionol, 3-pentanol, phenylacetaldehyde and 1-phenyl-1,2-propanedione [21]. The increase in breath concentration of several compounds from 5 h after the ingestion of the high-fibre breakfast could be explained by their production by gut microbes during fermentation of DF. This shift in breath composition appeared after the time required for gastric emptying (described to be lower than 5 h average after a typical breakfast [22]) and consequently with the arrival of DF in the gut. As example supporting this hypothesis, lactate production is possible from most monosaccharides (derived from the breakdown of DF) through glycolysis followed by the reduction of the subsequent pyruvate [23]. Also, methanol can be produced through the hydrolysis of pectin methyl esters by bacterial pectinesterase [24].

Since most identified compounds cannot be issued from DF fermentation, an explanation is that DF might have also influenced the availability of other nutrients for gut microbes. Surprisingly, most identified compounds are rather expected to be formed from lipid or amino acid metabolism even though the breakfasts differed primarily in their DF content. DF could have trapped nutrient and alter transit time, which would have modified the nutrient profile accessible to microbes and impacted the duration nutrients are available for microbial fermentation [25]. This hypothesis aligns with the increased concentrations of methionol and dimethyl disulfide after the high-fibre breakfast, which cannot be explained by a higher abundance of sulphated amino acids methionine (108.9 g in low-fibre versus 111.3 g in high-fibre) and cystine (127.0 g in low-fibre versus 141.2 g in high-fibre). Similarly, higher abundance of dimethyl selenide in exhaled breath was not explained by selenium content in the high-fibre breakfast (2.0 g in low-fibre versus 2.4 g in high-fibre).

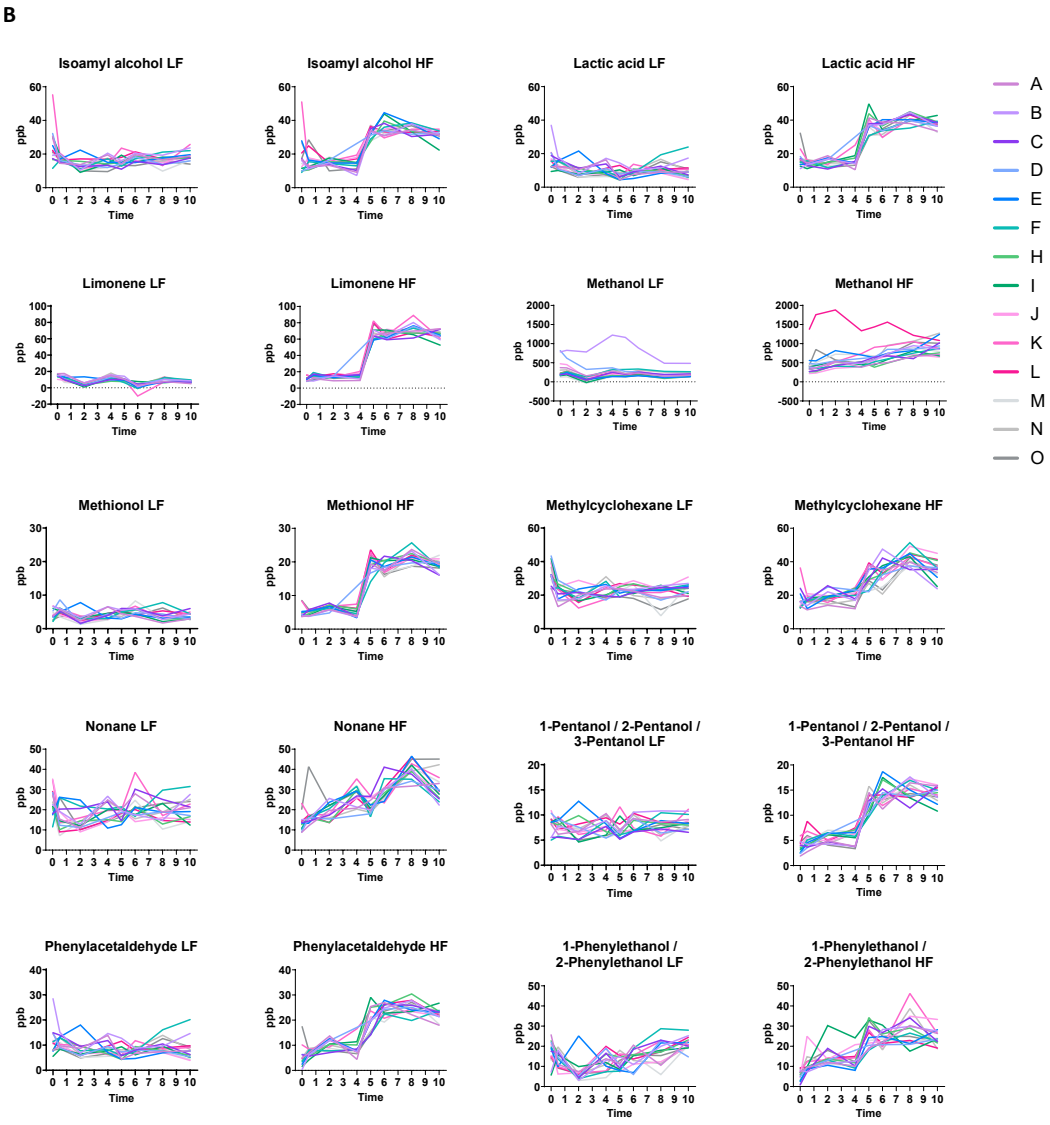


Fig. 4. (*continued*).

Certain compounds identified as potential breath biomarkers of gut microbiota activity in this study have already been shown to be produced by gut microbes in humans or other living organisms. Methanol formation by gut bacteria was shown in rats by Komarova *et al.* [26], who demonstrated a significantly decreased rate of methanol addition to the plasma after removing the intestine. Isoamyl alcohol and 2-phenylethanol were shown to be produced by gut bacteria belonging to two different species of insects. Axelsson *et al.* [27] identified isoamyl alcohol and 2-phenylethanol as main compounds released by *Roseateles aquatilis*, which was isolated from the gut of the *Hylobius abietis* (pine weevil). Hadapad *et al.* [28] found isoamyl alcohol and 2-phenylethanol to be part of the most abundant chemical constituents in the supernatants of *Klebsiella oxytoca* and *Citrobacter freundii*, which are midgut bacteria of *Bactrocera cucurbitae* (melon fly). *Klebsiella oxytoca* and *Citrobacter freundii* have already been detected in the human gut while *Roseateles aquatilis* is expected to be part of it [29].

Other compounds we highlighted as potential breath biomarkers are known to be produced by bacteria belonging to the human gut. Hernandez-Leyva *et al.* [30] showed that mono-colonised mice with *Akkermansia muciniphila*, *Bacteroides thetaiotaomicron*, *Escherichia coli* and *Ruminococcus torques* exhaled more xylene compared to germ-free controls. Similarly, mice mono-colonised with *Bacteroides thetaiotaomicron* and *Ruminococcus torques* had more ethylbenzene in their breath. Besides, lactic acid is formed by lactic acid bacteria (LAB) through lactic fermentation. LAB are most widely studied in food but are also members of the gut microbiome of human and animal hosts, where their origin, role and activity are still being explored [31]. We propose that measuring lactic acid in exhaled breath could be a valuable tool for exploring the role of LAB in the gut microbiome. Despite its potential, lactic acid has only been measured in a few studies focusing on breath and saliva [21].

To elucidate the microbial origins of the highlighted breath compounds, we performed correlation analyses with gut microbiota composition, identifying associations consistent with existing literature. In our cohort, limonene was significantly negatively correlated with the genus *Bifidobacterium* and its family *Bifidobacteriaceae*. Nissen *et al.* [32] provided data in this regard, showing that D-Limonene adsorbed on coca fibre induced increased abundance of *Bifidobacteriaceae* in the multi-unit *in vitro*

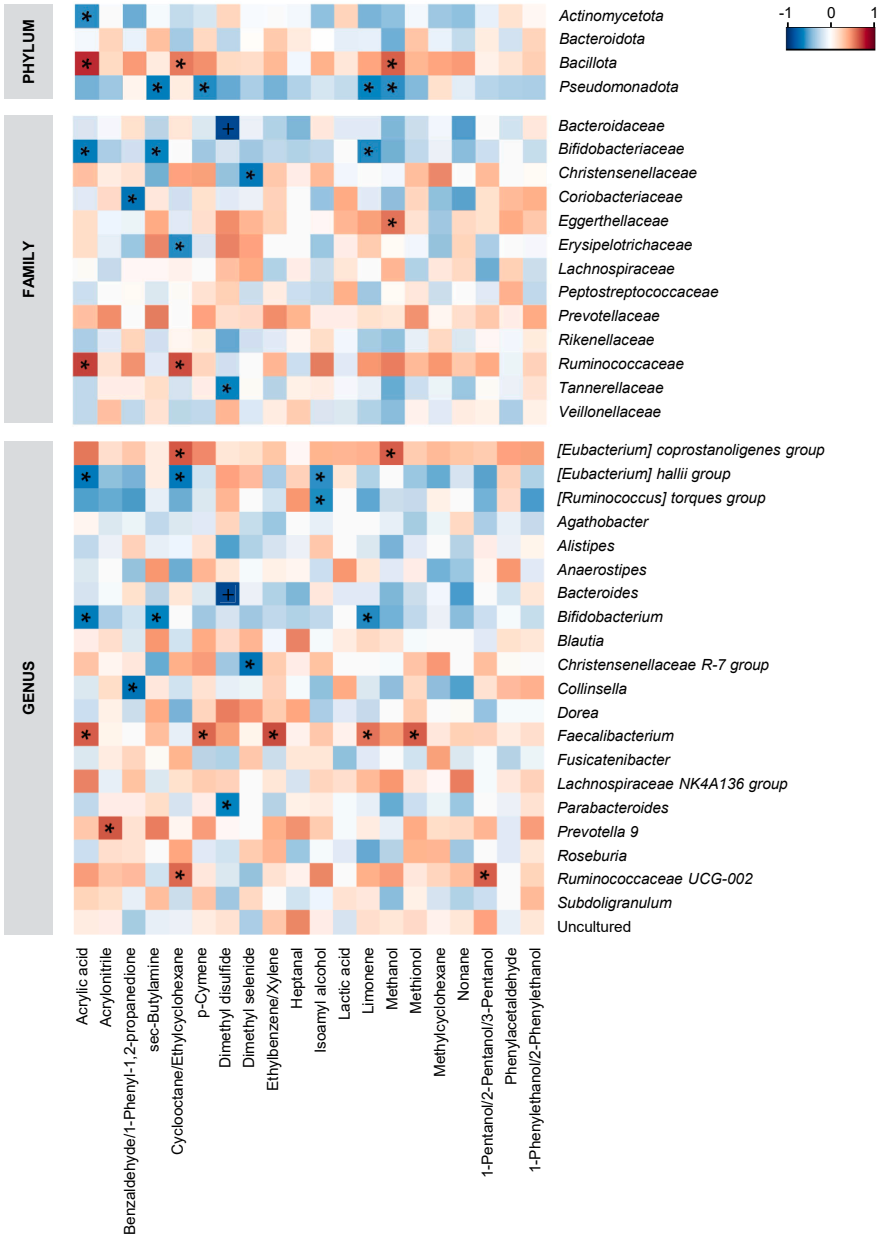


Fig. 5. Correlation plot between breath compounds (ppb, exhaled at time 8 h after the ingestion of a high-fibre breakfast) and gut microbiota composition at the phylum, family and genus taxonomic levels (mean relative abundance >1%, before high-fibre test day) for 14 healthy volunteers. * Spearman test ($p < 0.05$); + False discovery rate (FDR) correction ($q < 0.05$).

colon gut model MICODE. Members of the *Bifidobacterium* genus, often considered as beneficial gut microorganisms [33], might therefore be involved in the consumption of limonene. Our dataset also revealed a significant positive correlation between ethylbenzene/xylene, p-cymene and the genus *Faecalibacterium*. Similarly, Philips *et al.* [34] reported ethylbenzene degradation pathways in the microbiota of patients with acute alcoholic pancreatitis who are characterized by low levels of *Faecalibacterium* as compared to patients with alcoholic hepatitis. Furthermore, Watai *et al.* [35] described that xylene degradation pathway was significantly enriched in patients with multiple chemical sensitivity, while *Faecalibacterium prausnitzii* was significantly less abundant in these volunteers compared to healthy controls. Based on our data and those of others, we can propose *Faecalibacterium* are associated with higher concentrations of ethylbenzene and/or xylene but how DF intake influences the process remains unclear.

Among the significant correlations we identified, several were less evident in existing literature, indicating previously unrecognized interactions between breath compounds and gut microbes. We observed that dimethyl disulfide was significantly negatively correlated with *Bacteroides*, *Parabacteroides*, and their families *Bacteroidaceae* and *Tannerellaceae*. This contrasts with Zhang *et al.* [36], who found a positive correlation between dimethyl disulfide and *Bacteroides* in decomposing food waste – a genus also linked to sulfur-based halitosis [37]. These contrasting patterns suggest that the gut, as a different environment, may exhibit a different balance

between microbial producers and consumers. In our study, benzaldehyde/1-phenyl-1,2-propanedione was significantly negatively correlated with the genus *Collinsella* and its family *Coriobacteriaceae*, consistently with the role of this genus in the degradation of dietary polyphenols and other aromatic compounds [38]. We also revealed a significant negative correlation between dimethyl selenide, the genus *Christensenellaceae R-7 group* and its family *Christensenellaceae*. This suggests a role of this bacteria in the ecosystem related to selenium metabolism, that can either result in available selenium pool for the host or bacteria or to excretion in urine and breath [39].

Lastly, we discovered consistent and significant correlations across taxonomic levels between several breath compounds and gut microbes that had not been reported until now. Notably, acrylic acid was negatively correlated with the genus *Bifidobacterium*, its family *Bifidobacteriaceae*, and its phylum *Actinomycetota*, and positively correlated with the genus *Faecalibacterium*, its family *Ruminococcaceae* and its phylum *Bacillota*. Likewise, cyclooctane/ethylcyclohexane were positively correlated with the genus *Ruminococcaceae UCG-002,* its family *Ruminococcaceae* and its phylum *Bacillota*. Such cross-level consistency supports the robustness of these associations and point out their potential biological significance, warranting deeper exploration.

Although exploratory, our findings demonstrate the potential of breath analysis as a translational tool in clinical and nutritional practice. By identifying volatile compounds likely originating from gut microbial metabolism, we provide a basis for developing non-invasive, rapid, and repeatable assessments of gut microbiota activity. In a clinical context, such breath-based biomarkers could be applied to monitor individual responses to prebiotic or other dietary interventions, track restoration of microbiota balance after dysbiosis (e.g., following antibiotic therapy), or support the dietary management of conditions linked to gut microbial composition, such as irritable bowel syndrome (IBS), IBD, or metabolic disorders. Moreover, integrating breath analysis into personalised nutrition strategies could facilitate real-time feedback to patients and practitioners, improving adherence and enabling adaptive dietary adjustments.

This study has several limitations. First, the small number of volunteers, all of whom were healthy hydrogen-producers, limits the generalisability of the findings. Methane-producers, who harbour higher abundances of methanogenic microbes, may exhibit different fermentation patterns and breath metabolite profiles. Second, the energy and macronutrient composition of the two test meals was not perfectly matched. Although the primary focus was on the dietary fibre composition, the potential contribution of these differences to the gut microbiota response cannot be entirely excluded. Third, the menstrual day of female participants was not recorded and could have influenced gastrointestinal symptoms. However, no major symptoms were reported, and no significant differences were observed between test days, suggesting that this factor likely did not affect our conclusions. Fourth, although we prioritised evidence from human studies when exploring links between breath compounds and specific microbes, the literature in this area remains sparse. Consequently, some interpretations draw on findings from animal models, which should be viewed with caution until validated in human cohorts due to differences in gut physiology, microbial community structure, and host–microbe interactions. Fifth, establishing a direct causal link between specific microbiota and health outcomes remains challenging, requiring robust human intervention trials. To move from correlation to causation, gnotobiotic animal studies and experiments comparing microbiota-derived metabolites to control are essential. Sixth, the ability of SIFT-MS to confidently identify unknown compounds is limited, as the method is primarily designed for the quantification of predefined targets in samples with expected compositions. We notably pointed out the lack of clear distinction between isomers (ethylbenzene/xylene, 1-pentanol/2-pentanol/3-pentanol, 1-phenylethanol/2-phenylethanol) and other molecules with similar patterns of product ions (benzaldehyde/1-phenyl-1,2-propanedione, cyclooctane/ethylcyclohexane).

Future research should focus on validating these findings in larger and more diverse cohorts. Notably, investigating the differential impacts of hydrogen-producers versus methane-producers is warranted. The development of algorithms to suggest the presence of compounds in the spectra obtained by SIFT-MS, combined with other techniques more suitable for identification, is essential. The bacterial pathways and the roles of these volatiles in gut microbiota–host interactions, as well as their clinical relevance, need further investigation.

5. Conclusion

We identified 90 potential biomarkers, with 81 showing increased concentrations from 5 to 10 h after the ingestion of the high-fibre breakfast compared to the low-fibre breakfast. These compounds provide valuable insights into the metabolic processes occurring in the gut and their systemic effects. Some of these compounds are well-known in human samples and described as produced by gut microbes, while others are less clearly defined, highlighting the novelty of our findings.

Significant correlations between breath compounds and specific gut microbiota taxa further elucidate the relationships between microbial metabolism and the composition of exhaled breath. Notable patterns, such as the contrasting associations of *Bacillota* and *Pseudomonadota* with key compounds, suggest distinct metabolic roles within the gut microbiome. Further studies are needed to clarify the mechanistic underpinnings of these relationships and their implications for host health and disease.

This study demonstrates that gut microbiota activity can be effectively measured through breath analysis and provides a reliable methodology for this purpose. Breath analysis offers a promising approach for studying the gut microbiota, as it is non-invasive, fast, low-cost, sustainable and enables measurements across different time points. We propose new biomarkers for gut microbiota activity and present a flowchart for identifying additional biomarkers in future research. Overall, this study underscores the complex interactions between DF and the gut microbiota and highlights the potential of breath analysis in revealing microbial contributions to host health.

Authors contributions

Conceptualization: A.M.N., M.A., N.M.D.; Data curation: M.A., A.M.N., M.R.; Formal analysis: M.A., A.M.N., M.R., L.L., E.V.O, J.R.; Funding acquisition: N.M.D., J.W.; Investigation: A.M.N., J.R. Methodology: M.A., A.M.N., J.R., L.B.B.; Project administration: N.M.D., A.M.N.; Resources: P.D.C., N.M.D.; Supervision: N.M.D.; Validation with verification of the underlying data: N.M.D., M.A., A.M.N.; Visualization: M.A., A.M.N.; Writing—original draft: M.A.; Writing—review and editing: M.A., A.M.N., L.L., E.V.O., J.R., P.D.C., L.B.B., N.M.D. All authors read and approved the final version of the manuscript. No one eligible for authorship has been excluded from the list of authors.

Data sharing statement

The raw sequencing data are available in the Sequence Read Archive (SRA) of NCBI (http://www.ncbi.nlm.nih.gov/sra) under

BioProject PRJNA636138. Other data described in the manuscript will be made available upon reasonable request from the corresponding author.

Declaration of generative AI and AI-assisted technologies

No Generative AI and AI-assisted technologies were used in the writing process.

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Conflict of interest

PDC was co-founder of Enterosys. The other authors report no financial interests or potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2026.106662.

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