

**Faculté de pharmacie
et des sciences biomédicales**

Breath volatilome as a new tool to study nutrition-host-microbiota interactions: the example of kombucha intake and psychological stress level

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Année académique 2025-2026
Master [120] en sciences biomédicales, finalité nutrition humaine

The research leading to these results has been founded by the Public Service of Wallonia (Economy, Employment and Research), under the FoodWal agreement n°2210182 from the Win4Excellence project of the Wallonia Recovery Plan.



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Acknowledgements

I would like to express my sincere gratitude to my supervisor, Pr. Nathalie Delzenne, for her guidance, support, and valuable advice throughout the course of this master's thesis. Her expertise and availability were essential to the completion of this work.

I am also deeply grateful to my PhD student supervisor, Ir. Manon Autuori, for her constant support, patience, and willingness to share her knowledge. Her help, insightful discussions, and constructive feedback greatly contributed to my learning and to the quality of this work. I would like to thank as well the other members of my team for their collaboration, assistance, and for generously sharing their skills and expertise.

I would like to thank the University of Liège and the Smart Gastronomy Lab for their collaboration, in particular Dr. Dorothée Goffin and Sarah Suffys (PhD student, Faculty of Gembloux Agro-Bio Tech, University of Liege), for providing kombucha and for sharing their data with us.

I would also like to extend my sincere thanks to the Centre d'Investigation Clinique en Nutrition (CICN) for their collaboration and for providing access to the data that made this project possible. Working with the CICN team was both enriching and enjoyable, thanks to their remarkable organisation and professionalism. I am particularly grateful to Dr. Valérie Dormal for her availability to answer all my questions throughout the process.

More broadly, I wish to thank all members of the Metabolism and Nutrition (MNUT) laboratory for their help, kindness, and the stimulating working environment they provided. Their professionalism and friendliness made my time in the lab both enriching and enjoyable.

Finally, I would like to thank my parents and my sister for their unconditional support, encouragement, and understanding throughout my studies. Their trust and motivation were invaluable and helped me reach this important milestone.

List of abbreviations

AMPK: AMP-activated protein kinase	PC: principal component
ASA24: automated self-administered 24-hour dietary assessment	PCA: principal component analysis
BDI: beck depression inventory	PGC1- α : peroxisome proliferator-activated receptor gamma coactivator 1-alpha
BMI: body mass index	ppb: parts-per-billion by volume
BVC: breath volatile compounds	ppm: parts-per-million by volume
CI95: 95% confidence interval	ppt: parts-per-trillion by volume
CICN: Centre d'Investigation Clinique en Nutrition	PSS: perceived stress scale
CRH: corticotropin-releasing hormone	PTFE: polytetrafluoroethylene
eNose: electronic nose	PTR-MS: proton transfer reaction mass spectrometry
FAS: fatigue assessment scale	QMF: quadrupole mass filter
FS: full scan	R ⁺ or R ⁻ : reagent ions
GC-MS: gas chromatography - mass spectrometry	R ² : coefficient of determination
HPA: hypothalamic-pituitary-adrenal	RE: response element
IKB- α : inhibitor of NF κ B alpha	ROS: reactive oxygen species
IKK: I κ B kinase	SAM: sympathetic-adreno-medullar
IL-6: interleukin-6	SBSE: stir bar sorptive extraction
IMS: ion mobility spectrometry	sCD14: soluble cluster of differentiation 14
ISI: insomnia severity index	SCFA: short-chain fatty acid
<i>k</i> : reaction rate constants	SCOBY: symbiotic culture of bacteria and yeast
Keap1: Kelch-like ECH-associated protein-1	SERBP: sterol regulatory element-binding protein
LAS: laser absorption spectroscopy	SIFT-MS: selected-ion flow-tube mass spectrometry
LBP: LPS-binding protein	SIM: selected ion monitoring
LC: locus coeruleus	SIRT1: sirtuin 1
LOD: limit of detection	sPLS-DA: sparse partial least squares-discriminant analysis
LOQ: limit of quantification	STAI: state-trait anxiety inventory
LPS: lipopolysaccharide	TNF- α : tumor necrosis factor α
<i>m/z</i> : mass to charge ratio	TLR4: Toll-like receptor 4
M: analyte molecules	TSST: trier social stress test
NAC: "notional analyte concentrations"	VAS: visual analog scale
NF κ B: nuclear factor-kappa B	w/v: weight per volume
Nrf2: nuclear factor erythroid 2-related factor 2	
P ⁺ or P ⁻ : product ions	
PASAT: paced auditory serial addition test	

Résumé

Introduction : L'analyse des composés volatils expirés – le volatilome respiratoire – est une approche non invasive prometteuse pour étudier la physiologie humaine. Ces composés, provenant de l'environnement ou du métabolisme endogène, pourraient également refléter l'activité du microbiote intestinal. Cette thèse de master étudie in vivo l'impact sur le volatilome respiratoire (1) de l'ingestion, chez des volontaires sains, de kombucha – un thé fermenté à base de bouleau présentant des bienfaits potentiels pour la santé – et (2) du niveau de stress psychologique. L'objectif est de découvrir des marqueurs reflétant l'interaction entre le métabolisme humain et le microbiote intestinal à travers l'analyse de l'haleine.

Matériel and méthodes : Dans l'étude sur le kombucha, 5 volontaires à jeun en bonne santé (2 femmes, 3 hommes ; 20 [19 ; 21] ans) ont participé à deux journées de test afin de comparer la consommation de kombucha à celle d'eau (témoin). Plusieurs échantillons d'haleine ont été prélevés avant et jusqu'à 360 minutes après l'ingestion. Dans l'étude sur le stress, menée par le CICN, 70 volontaires (48 femmes, 22 hommes ; 31 [24 ; 46] ans) sélectionnés sur base d'un score supérieur à 14 sur l'échelle de stress perçu (PSS), ont fourni un seul échantillon d'haleine à jeun afin de comparer les niveaux de stress modéré et élevé. Les deux études se sont appuyées sur la spectrométrie de masse à tube à flux d'ions sélectionnés (SIFT-MS), permettant un profilage non ciblé du volatilome et une quantification ciblée de composés spécifiques.

Résultats : L'étude sur le kombucha a montré des changements dans la composition de l'haleine apparaissant 5 minutes et après 120 minutes suivant la consommation de kombucha. Le pic transitoire de plusieurs composés (comme l'éthanol et l'acide lactique) 5 minutes après l'ingestion serait dû aux résidus de kombucha dans le tractus gastro-intestinal supérieur. L'étude sur le stress n'a pas révélé de changement global dans la composition de l'haleine en fonction du niveau de stress, mais a montré une réduction des concentrations de métabolites spécifiques déjà observé dans le volatilome de volontaires plus stressés, comme l'acétate d'éthyle et des isomères de xylène, ainsi que de nouveaux composés comme l'acide butyrique, qui peut signer une diminution des bactéries productrices de butyrate associée.

Conclusion : Cette thèse de master a confirmé que l'analyse de l'air expiré permet de détecter les changements liés à la consommation de kombucha et au stress psychologique. À l'avenir, une telle approche pourrait permettre un suivi personnalisé en temps réel des réponses microbiennes et physiologiques liées à l'alimentation et au stress.

1. Introduction

1.1. Interest of breath volatile compounds analysis in health and nutrition

Breath analysis has been known for a long time and is a promising strategy to evaluate the physiological state of the human body. Unlike blood and urine samples, breath sampling is a non-invasive and rapid procedure, that can be adapted for continuous monitoring (Sharma et al., 2023). The first attempts to analyse breath for physiological insights date back to Hippocrates (460–370 BC) (Ajibola et al., 2013; Sharma et al., 2023). Indeed, ancient Greek physicians already knew that human breath could provide information about a person's state of health and even, in some cases, about a diagnosis (Ajibola et al., 2013). For example, a skilled clinician can easily recognise the typical fruity odour of acetone in diabetes, the musty and fishy smell of advanced liver disease or the putrid smell of a lung abscess (Sharma et al., 2023). In the 1970s, Pauling et al. (1971) investigated the relationship between breath composition and diet and recognised the potential impact of intestinal microbiota – an assemblage of living microorganisms (bacteria, archaea, fungi) which reside in the intestine (Delzenne & Rodriguez, 2022) – as a contributing factor to breath composition. However, despite these appealing approaches, breath analysis is still in its infancy. Many questions remain, making exhaled breath a highly promising emerging area of research.

Exhaled human breath is a complex mixture of volatile compounds influenced by intrinsic and external conditions. Exhaled human breath contains predominantly nitrogen (78.6% weight per volume – w/v), oxygen (16% w/v), carbon dioxide (4.5% w/v), and a small fraction (0.9% w/v) of other breath volatile compounds (BVC). The relative humidity levels range from 91% to 96% in oral exhalations, and from 82% to 85% in nasal exhalations (Hibbard & Killard, 2011). Exhaled breath compounds can originate from exogenous sources – environmental exposures, inhalation or ingestion – and from endogenous sources including human and microbial metabolism (Ajibola et al., 2013; Sharma et al., 2023). Volatile metabolites generated within the body travel around the body via the blood and then can cross the alveolar interface and appear in exhaled breath. Their concentration is measured at trace concentrations in parts-per-million by volume (ppm), parts-per-billion by volume (ppb) levels or even lower (Lourenco & Turner, 2014). Their concentration can be influenced by many factors such as age, gender, physiological status, genetic make-up and diet

(Ajibola et al., 2013; Sharma et al., 2023). This master's thesis will evaluate BVC under two conditions: acute kombucha ingestion and psychological stress condition.

1.2. Overview of breath analysis techniques

Due to their low concentration, BVC detection requires detectors with high sensitivity. Gas chromatography - mass spectrometry (GC-MS) was among the first methods used for BVC detection and remains one of the most widely used today. This technique offers an extremely low limit of detection (LOD), allowing the identification of trace compounds with remarkable accuracy and specificity (Lourenco & Turner, 2014). However, the duration of GC-MS analysis is relatively long – ranging from 10 to 60 minutes depending on the elution time of analytes, as well as the time required for column cleaning and oven cooling (Langford & Perkins, 2025). This extended analysis time represents a major drawback for kinetic studies or experiments involving multiple volunteers in parallel, where rapid analysis is essential. More recently, other approaches enabling real-time analysis of exhaled breath have been developed (**Table 1**). Among these techniques, selected-ion flow-tube mass spectrometry (SIFT-MS) offers the best compromise between specificity, sensitivity, LOD and time required for the analysis (Lourenco & Turner, 2014).

Table 1 | Comparison of different analytical method for exhaled breath analysis.
Abbreviation: eNose, electronic nose; GC-MS, gas chromatography - mass spectrometry; IMS, ion mobility spectrometry; LAS, laser absorption spectroscopy; ppb, parts-per-billion by volume; ppt, parts-per-trillion by volume; PTR-MS, proton transfer reaction mass spectrometry; SIFT-MS, selected-ion flow-tube mass spectrometry (Lourenco & Turner, 2014).

Analytical Method	Mode of operation	Limit of detection	Sensitivity	Specificity
eNose	Real time	ppb	Medium	Medium
GC-MS	Pre-concentration	ppt-ppb	Very-high	Very-high
IMS	Real time	ppb	Medium	Medium
LAS	Real time	ppb	High	High
PTR-MS	Real time	ppt	High	Medium-High
SIFT-MS	Real time	ppb	High	High

SIFT-MS technique relies on soft chemical ionisation of sample molecules by multiple reagent ions and detection of mass to charge ratio by mass spectroscopy. Three regions can be distinguished in the SIFT-MS device (**Figure 1**). In the first region, reagent ions are generated and selected. Reagent ions (R^+ or R^- ; e.g. H_3O^+ , NO^+ , O_2^+ , OH^- , O^- , O_2^- , NO_2^- and NO_3^-) are generated from wet air in plasma using a microwave discharge. These ions are safe, environmentally friendly (both in

terms of the abundance of their natural source and their sustainability) and can be produced continuously at very low cost. The plasma contains a mixture of reagent ions and is passed into a quadrupole mass filter (QMF) that, under automated software control, selects the appropriate, single reagent ion for introduction as a pure stream of reagent ions into the second region, the flow tube. In the flow tube, reagent ions first encounter carrier gas molecules (either helium or nitrogen), and react consecutively with analyte molecules (M) to form characteristic product ions. For trace analytes, the rate of conversion of reagent ions to product ions is thus directly proportional to the analyte concentration. All ions – both product ions and unreacted reagent ions – are directed into the final region of the system for mass spectrometric analysis. This detection zone includes an ion guide that enhances the transmission of heavier product ions, followed by a QMF that selectively transmits ions based on their mass to charge ratio (m/z), and concludes with a channel electron multiplier that counts the ions. The resulting ion count rates, expressed in counts per second ($\text{count} \times \text{s}^{-1}$), are directly proportional to the ion number densities at the end of the flow tube. Quantification is achieved by comparing the count rates of product ions (P^+ or P^-) to those of reagent ions, providing a reliable measure of analyte concentration that remains unaffected by fluctuations in reagent ion intensity. This process is supported by a built-in reaction kinetics library, which contains detailed information on target analytes, their corresponding reagent ions, specific product ions, and associated reaction rate constants (k) (Benchennouf et al., 2023; Langford & Perkins, 2025).

SIFT-MS data can be collected using two distinct operating modes: full scan (FS) and selected ion monitoring (SIM). FS mode provides qualitative insights by scanning across a broad m/z range without requiring prior knowledge of the compounds contributing to the detected signals. Multiple reagent ions can be used, and successive cycles enable untargeted data collection across all reagent ions and m/z ranges. FS mode is particularly advantageous for rapid acquisition and offers the flexibility to revisit and reanalyse datasets for different target compounds. In contrast, SIM mode is optimized for quantitative analysis of predefined target compounds. Data are repeatedly collected at the m/z values corresponding to the analytes of interest, as specified in the SIFT-MS library. Because acquisition is focused on targeted ions, SIM mode requires longer measurement times and lacks flexibility: once the target molecules are defined, the analysis cannot be redirected to other compounds.

Consequently, SIM mode is best applied for data extraction and quantitative follow-up after FS mode, rather than for direct acquisition (Langford & Perkins, 2025).

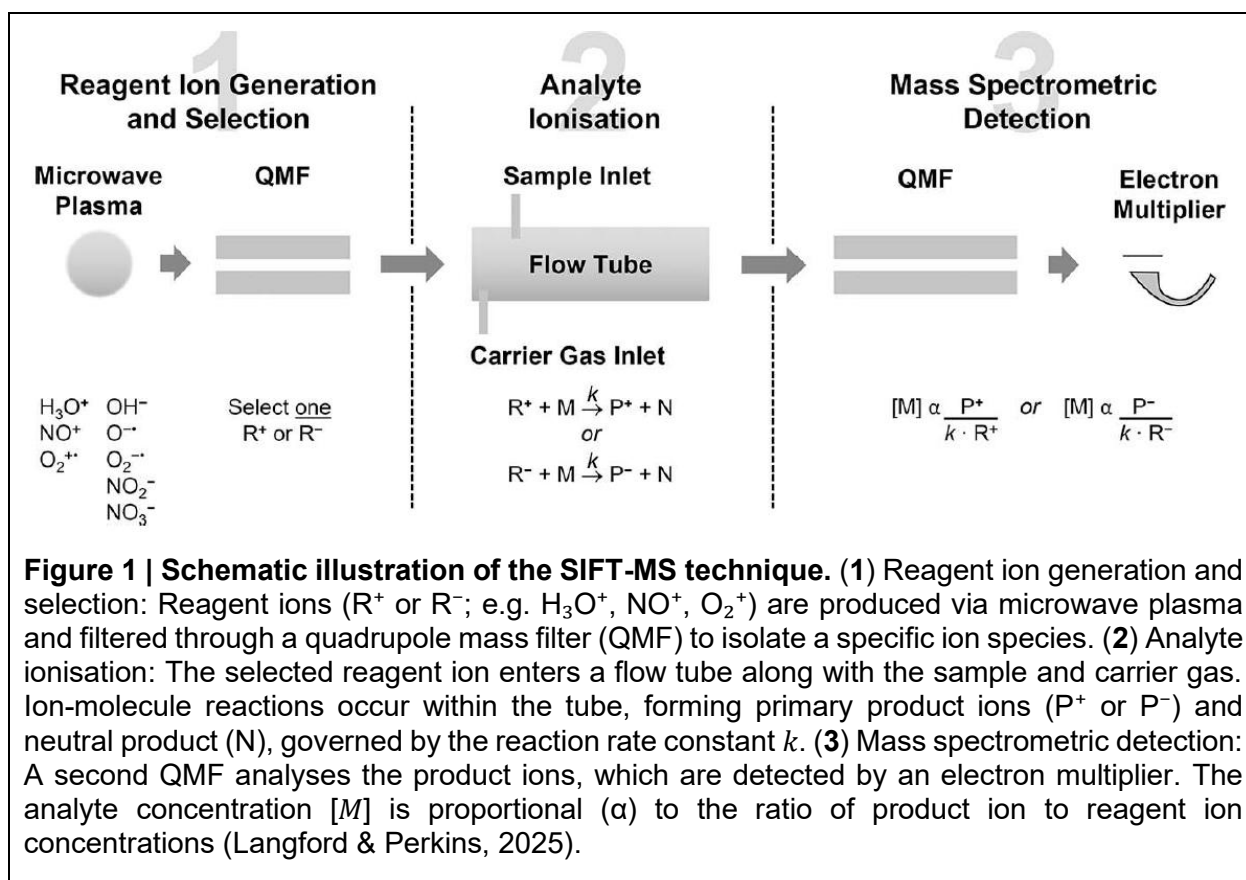


Figure 1 | Schematic illustration of the SIFT-MS technique. (1) Reagent ion generation and selection: Reagent ions (R⁺ or R⁻; e.g. H₃O⁺, NO⁺, O₂⁺) are produced via microwave plasma and filtered through a quadrupole mass filter (QMF) to isolate a specific ion species. (2) Analyte ionisation: The selected reagent ion enters a flow tube along with the sample and carrier gas. Ion-molecule reactions occur within the tube, forming primary product ions (P⁺ or P⁻) and neutral product (N), governed by the reaction rate constant *k*. (3) Mass spectrometric detection: A second QMF analyses the product ions, which are detected by an electron multiplier. The analyte concentration [M] is proportional (α) to the ratio of product ion to reagent ion concentrations (Langford & Perkins, 2025).

Correcting SIFT-MS data is essential to ensure reliable results, especially when measurements are collected over a long period of time. Like other mass spectrometry techniques, SIFT-MS is subject to systematic and random variations that can compromise statistical analysis. While normalisation strategies such as internal standards or quality control samples are commonly used in GC-MS, their application in SIFT-MS is limited due to the lack of chromatographic separation (Benchennouf et al., 2023). Targeted data acquired in SIM mode benefit from correction procedures that account for daily variation, but untargeted data obtained in FS mode remain unprocessed. Therefore, for statistical analysis, untargeted data can be normalised by dividing each *m/z* abundance either by the total count of all *m/z* for a given reagent ion (e.g., H₃O⁺ *m/z* 45 were normalised by the total signal of H₃O⁺ *m/z* 15 until H₃O⁺ *m/z* 250) or by the total reagent ion signal (e.g., H₃O⁺ *m/z* 45 were normalised by the total signal of H₃O⁺ *m/z* 19, H₃O⁺ *m/z* 37, H₃O⁺ *m/z* 55 and H₃O⁺ *m/z* 73) (Langford & Perkins, 2025). For longitudinal studies, it is recommended to convert *m/z* counts into “notional analyte concentrations” (NAC); a more robust approach mitigating temporal drift and improving data comparability (Benchennouf et al., 2023; Langford & Perkins,

2025). Concentration for each m/z is then calculated in SIM mode, regardless of which reagent ion it was produced from. Although this method sacrifices information about the specific reagent ion responsible for each m/z , it enables the application of the robust correction procedures available in SIM mode to untargeted data (Benchennouf et al., 2023).

1.3. Gut microbiome and health

Microbiome contains various microorganisms essential for physiological functions. The human microbiome refers to the vast community of microorganisms – bacteria, archaea, viruses, fungi, and unicellular eukaryotes – that inhabit the body, along with their “theatre of activity,” meaning their structural elements, metabolites, and surrounding environmental conditions. In the gut, where microbial density is highest, the dominant bacterial phyla include *Bacillota* (previously known as *Firmicutes*), *Bacteroidota* (previously known as *Bacteroidetes*), *Actinomycetota* (previously known as *Actinobacteria*), *Pseudomonadota* (previously known as *Proteobacteria*), and *Verrucomicrobia*. Each phylum contains key genera such as *Lactobacillus*, *Ruminococcus*, *Bacteroides*, *Prevotella*, *Bifidobacterium*, *Escherichia*, and *Akkermansia*, all contributing to essential physiological functions including immunity, metabolism, appetite regulation, and even behaviour (Delzenne & Rodriguez, 2022).

Microbial fermentation in gut produces a lot of BVC as short-chain fatty acids (SCFA). Intestinal microorganisms have the ability to ferment the substrates that are not digested or absorbed in the upper gastrointestinal tract. These include carbohydrates, proteins, fats, and other dietary components that escape digestion due to molecular or physical structural complexity (Delzenne & Rodriguez, 2022; Mutuyemungu et al., 2023). This fermentation produces a wide range of metabolites, the best-known being SCFA such as acetate, propionate and butyrate (**Figure 2**), which serve as energy sources for colonocytes, modulate inflammation, and regulate host metabolism (Delzenne & Rodriguez, 2022). Other well-known fermentation products include hydrogen (H_2), methane (CH_4), hydrogen sulfide (H_2S) (Mutuyemungu et al., 2023). While SCFA are beneficial products derived mainly from the fermentation of non-digestible carbohydrates (Delzenne & Rodriguez, 2022), H_2S is a potentially harmful byproduct of protein fermentation. Elevated H_2S can disrupt colonocyte metabolism and growth by impairing butyrate oxidation and protein synthesis (Mutuyemungu et al., 2023).

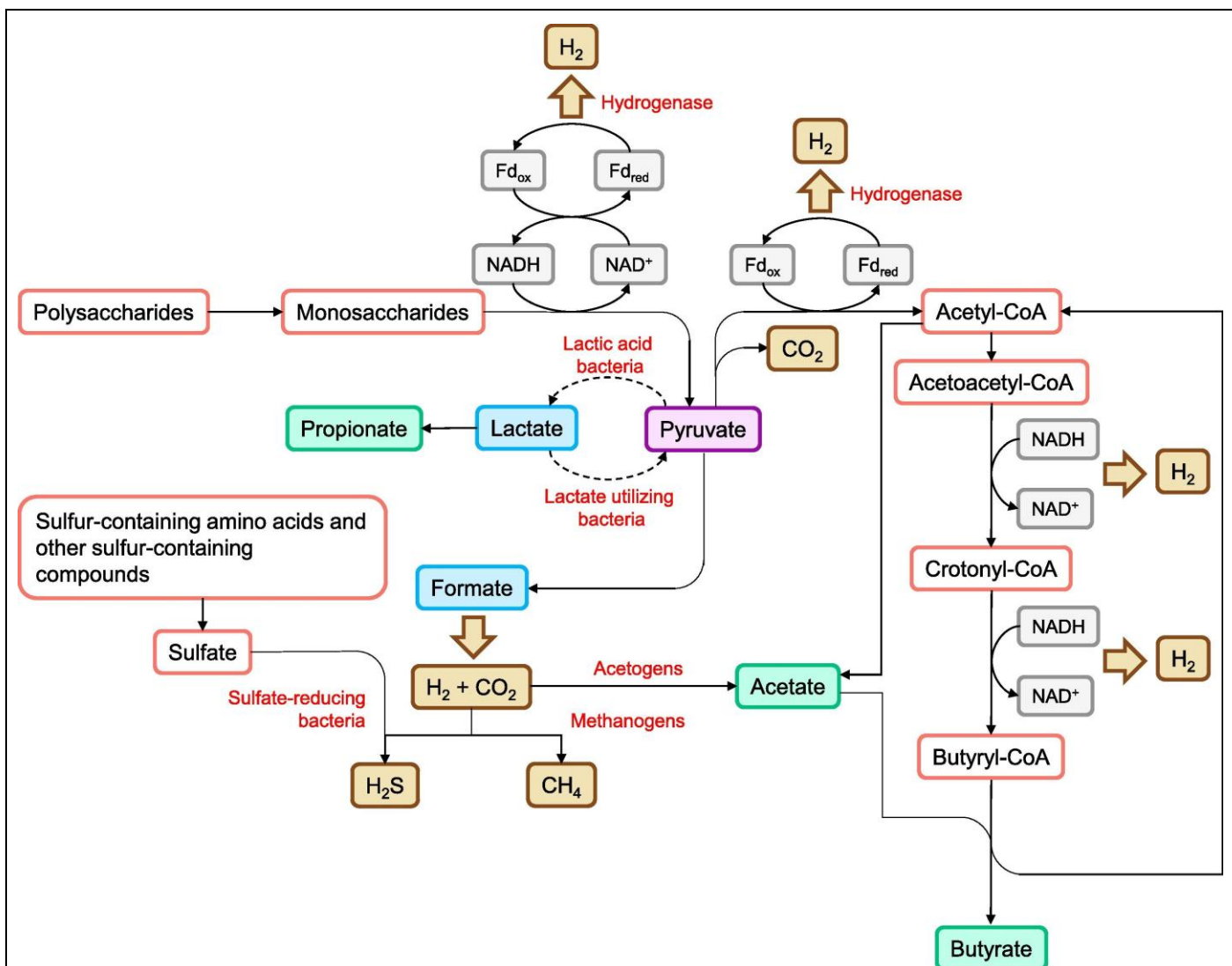


Figure 2 | Biochemical pathway of H_2 , H_2S and CH_4 production from bacterial fermentation. Gases are shown in brown boxes; intermediate products of bacterial fermentation are shown in blue boxes and primary products of bacterial fermentation (short chain fatty acids) are shown in green boxes; electron carriers are shown in grey boxes. **Abbreviation:** Fd_{ox} , oxidized flavin adenine dinucleotide; Fd_{red} , reduced flavin adenine dinucleotide; $NAD^+/NADH$, nicotinamide adenine dinucleotide (Mutuyemungu et al., 2023).

Multiple factors influence the composition and activity of the gut microbiome, and disturbances in this balance can lead to dysbiosis. These factors include initial colonisation after birth, which depends on the mode of delivery and infant feeding practices, as well as other factors throughout life, including environmental exposure, medication use, diet, physical activity and psychological stress. Among these, diet is considered one of the most powerful modulators. Fibre-rich diets promote the growth of SCFA-producing bacteria, whereas high-fat or low-carbohydrate diets may diminish beneficial taxa and disrupt fermentation processes. Such alterations can induce dysbiosis – defined as changes in both microbial composition and function – which is linked to metabolic disorders including

obesity, type 2 diabetes, and non-alcoholic fatty liver disease. A central mechanism involves lipopolysaccharides (LPS), structural components of Gram-negative bacteria. When dysbiosis compromises gut barrier integrity, LPS can translocate into the bloodstream, eliciting chronic low-grade inflammation, termed metabolic endotoxemia, that contributes to insulin resistance, hepatic steatosis, and systemic inflammation (Delzenne & Rodriguez, 2022).

1.4. Interest of fermented foodstuffs in nutrition: Health effects of kombucha

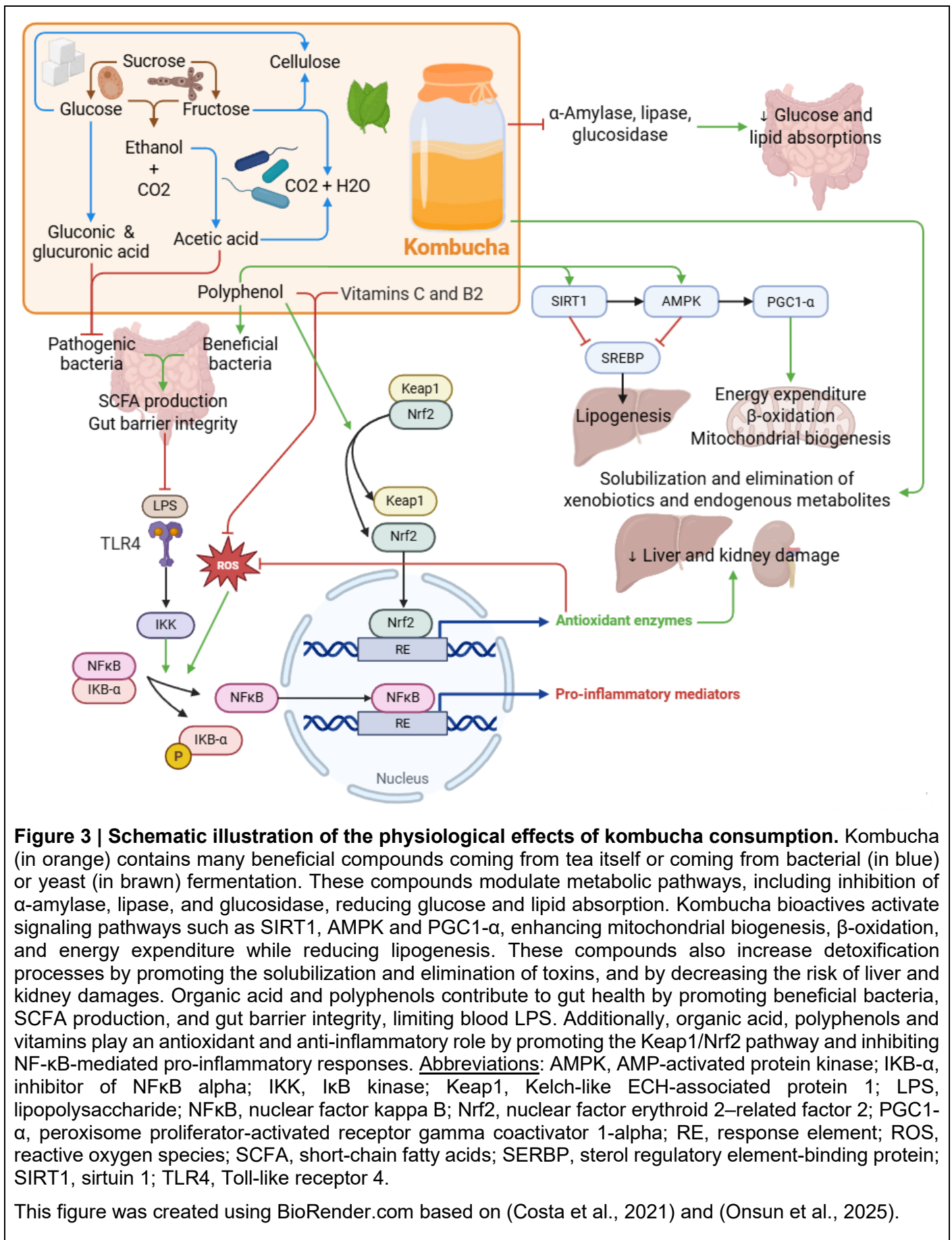
Fermented foods and drinks, which have been part of the human diet for nearly 10,000 years, have gained diversity and interest in the 21st century. While food fermentation was primarily intended to improve food safety and extend shelf life, fermented foods are also associated with health benefits. Proposed mechanisms to explain these health benefits include: **(1)** the direct nutritional value of fermented foods, including bioactive compounds produced as a result of the fermentation process; **(2)** the provision of nutrients that promote the growth of gut microbes; and **(3)** the ability of microbes present in fermented foods to survive gastric transit and either become a component of the gut microbiome or inhibit/compete with existing members of the gut microbiome. Each fermented food, which can come from a wide variety of sources (meat, milk, plants, etc.), generally harbours a distinct population of microorganisms (Leeuwendaal et al., 2022). In the first part of our work, we studied the potential effect of new sources of kombucha on the gut microbiota and health by analysing the breath volatilome.

Kombucha is an ancient fermented and sparkling tea-based drink that has recently gained popularity. Kombucha, also known as Haipao, Teakwass, Tea Fungus, Manchurian Mushroom and Kambotscha, is believed to have originated in the Manchurian region of north-eastern China around 220 BC, where it was prized for its detoxifying and energising properties (Chong et al., 2023). The word “kombucha” is a combination of “kombu”, which refers to the broad-leafed seaweed *Laminaria japonica*, and “cha”, which means tea. Around 400 BC, the drink was introduced in Japan to treat an emperor's digestive problems. Kombucha was then introduced in Russia and spread throughout Eastern Europe during the 20th century. Kombucha's popularity in Russia was attributed to its supposed beneficial effects on the treatment of metabolic diseases, haemorrhoids and rheumatism. During the Second World War, kombucha

was introduced to Western Europe and North Africa (Onsun et al., 2025). In recent years, kombucha has grown in popularity around the world because it has the characteristics of a “functional food”. “Functional food” is a term coined in the early 1990s to describe food that provides essential nutrients but also contains biologically active compounds that can promote health (Roberfroid, 2002).

Kombucha is produced by the aerobic fermentation of tea and white sugar by a symbiotic culture of bacteria and yeast (SCOBY) over a period of 7 to 10 days. The base usually consists of black, green, white or oolong tea combined with white sugar. Bacteria typically include acetic acid bacteria, such as *Komagataeibacter*, *Acetobacter*, *Gluconacetobacter*, and *Gluconobacter*, and lactic acid bacteria, such as *Lactobacillus* and *Lactococcus*. Yeast typically include *Schizosaccharomyces*, *Candida*, *Zygosaccharomyces*, *Saccharomyces*, and *Brettanomyces* (Chong et al., 2023; Dimidi et al., 2019). During fermentation, sucrose is hydrolysed to produce glucose and fructose by the action of yeast invertase. The yeasts then metabolise these monosaccharides to produce ethanol and CO₂, which are then oxidised by acetic acid bacteria to produce acetic acid. This interaction stimulates the mutual growth of yeasts and bacteria. Production of acetic acid contributes to decrease the pH, which is essential to prevent microbial contamination that could compromise the quality and safety of the final product. Simultaneously, acetic acid bacteria synthesise cellulose, forming the characteristic SCOBY layer that thickens on the surface of the liquid and that will be used for to produce another kombucha. Throughout fermentation, numerous bioactive compounds very interesting for human health are formed, including polyphenols, organic acids (such as acetic, gluconic and glucuronic acids), amino acids, vitamins, minerals and antimicrobial substances (Chong et al., 2023; Onsun et al., 2025).

Kombucha has shown several promising properties *in vitro* and in animal studies (Figure 3), but research on its relevance in human health remains limited. A PubMed search for “kombucha” under the “Clinical Trial” filter identified only five studies, with text available for four. The first, an eight-week randomized trial, evaluated a four-week kombucha supplementation in healthy adults following a Western diet (kombucha group, n=16; control, n=8). No significant changes were found in inflammatory markers. However, an increase in SCFA-producing bacteria and kombucha-associated microbes such as *Weizmannia coagulans* was observed in gut microbiome (Ecklu-Mensah et al., 2024). The second, a ten-week study involving



overweight volunteers under caloric restriction (kombucha group, n=30; control, n=29), showed no differences in weight loss but revealed a significant reduction in lipid accumulation product and a decrease in salivary pathogen species (Fraiz et al., 2024). The third, a ten-week trial with a similar design, reported that kombucha consumption significantly lowered hydrogen peroxide levels, a pro-oxidant marker. Finally, the fourth, a short-term ten-day pharmacokinetic study involving 7 volunteers, found that acetate levels remained stable after a control drink but increased significantly after 60 minutes after consuming kombucha, indicating enhanced acetate absorption and circulation in blood (Gill et al., 2021).

1.5. Psychological stress: a disease related to nutrition and gut microbiota

Stress is fundamentally a physiological response that enables the body to adapt to internal or external challenges. The physiology of stress response has 2 components: the sympathetic–adreno-medullary (SAM) axis and the hypothalamic–pituitary–adrenal (HPA) axis (**Figure 4**). The SAM axis initiates the immediate response by releasing catecholamines, which increase heart rate, blood pressure, cardiac output, and skeletal muscle perfusion, while also stimulating glycogenolysis, gluconeogenesis, and lipolysis to rapidly mobilize energy substrates. These effects are accompanied by reduced intestinal motility and bronchiolar dilation, optimizing the body for short-term survival demands. In parallel, the HPA axis governs the slower, sustained phase of the stress response through the secretion of glucocorticoids – primarily cortisol in humans – which further support energy availability by promoting glycogenolysis, gluconeogenesis, and lipolysis. Together, these systems allow the organism to respond effectively to acute stressors and maintain homeostasis (Chu et al., 2025).

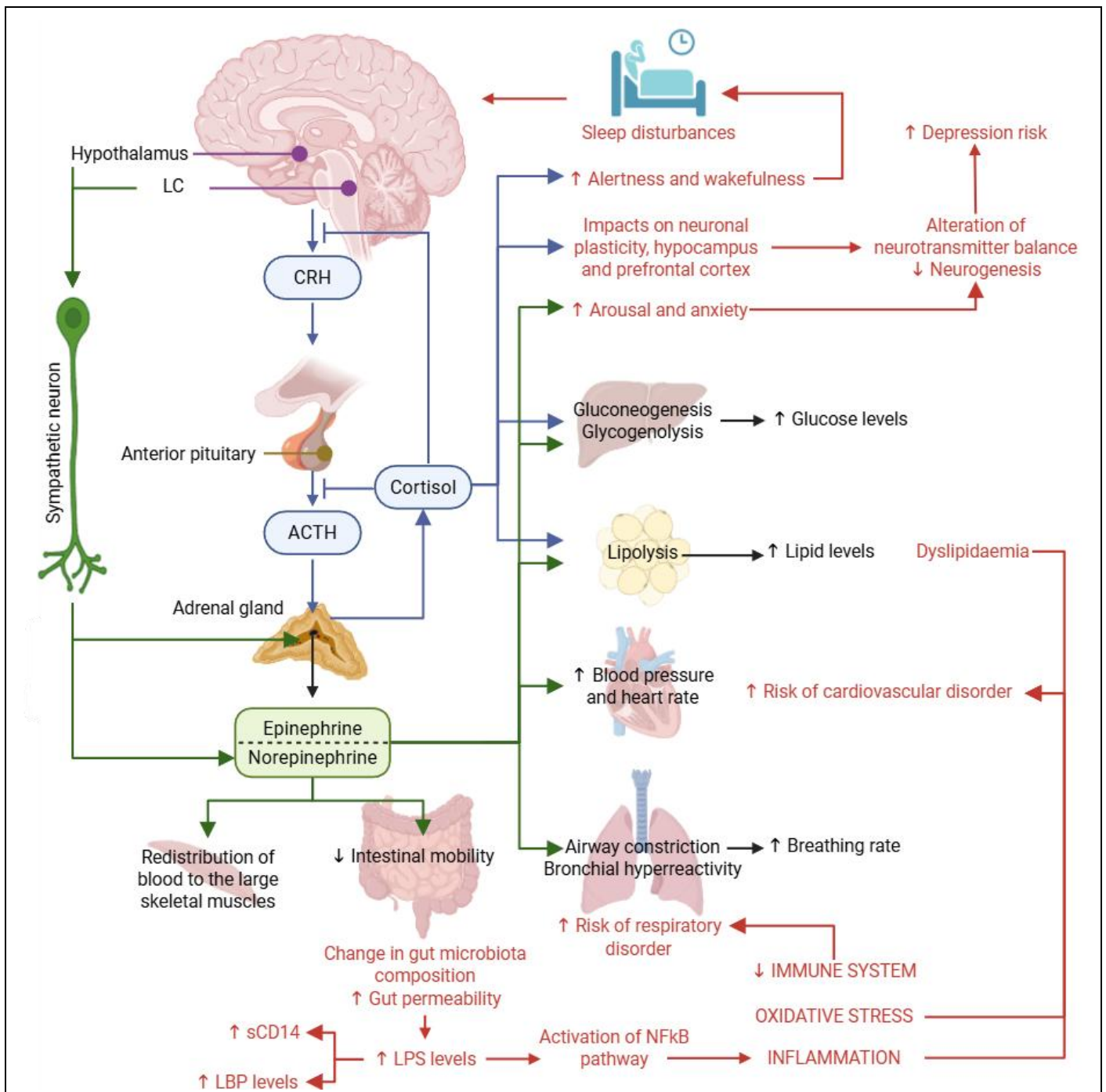


Figure 4 | Schematic illustration of the physiological and pathological effects of stress. The 2 main neuroendocrine pathways activated during stress – hypothalamic-pituitary-adrenal (HPA) axis (in blue) and sympatho-adreno-medullary (SAM) axis (in green) – coordinate adaptive responses that facilitate coping with acute stress. The SAM axis promotes catecholamine (epinephrine and norepinephrine) release via sympathetic neurons and the adrenal medulla. In parallel, the HPA axis regulates cortisol secretion through activation of the anterior pituitary by CRH and the adrenal cortex by ACTH. When stress becomes chronic, these adaptive mechanisms transition to maladaptive states (highlighted in red). Abbreviations: ACTH, adrenocorticotropic hormone; sCD14, soluble cluster of differentiation 14; CRH, corticotropin-releasing hormone; LBP, LPS-binding protein; LC, locus coeruleus; LPS, lipopolysaccharide; NFκB, nuclear factor-kappa B.

This figure was created using BioRender.com based on (Anand et al., 2022; Chu et al., 2025; Cristina Rabasa, 2016; Cryan & Dinan, 2012; Godoy et al., 2018; Wasiak & Gawlik-Kotelnicka, 2023; Xue et al., 2025).

While acute stress can be adaptive, chronic psychological stress becomes maladaptive and contributes to a wide range of health problems. Psychological stress originates from cognitive or emotional factors, including work pressure, academic demands, or negative thoughts, often manifesting as anxiety or rumination. When stress is intense, persistent, or frequently repeated, the physiological response shifts from eustress – “good” stress – to distress – “bad” stress – disrupting normal regulatory mechanisms and impairing both physical and mental well-being. Indeed, prolonged activation of the SAM and HPA axes leads to sustained elevations in catecholamines and cortisol, which can promote cardiovascular dysfunction, metabolic disturbances, sleep disruption, mood disorders, and gastrointestinal problems (Chu et al., 2025). The growing prevalence of stress highlights its societal impact: according to the World Mental Health Day report of Ipsos (2024), 62% of respondents reported stress severe enough to affect daily life, and 40% experienced stress so intense that they were unable to work for a period of time.

Chronic stress profoundly shapes dietary behaviours and gut microbial ecology, creating a bidirectional loop in which each component reinforces the others. Psychological stress often alters eating patterns – either driving increased intake of high-fat, high-sugar foods or reducing food consumption – both of which negatively affect gut microbiota composition and function (Schneider et al., 2024). At the same time, stress itself directly perturbs the gut ecosystem: chronic psychosocial stress has been shown to decrease *Bacteroides* spp. and increase *Clostridium* spp. in mice, alongside elevations in circulating interleukin-6 (IL-6) and C-C motif chemokine ligand 2 (CCL2) that correlate with shifts in *Coprococcus*, *Pseudobutyrvibrio* and *Dorea* abundance (Bailey et al., 2011). Stress also compromises intestinal barrier integrity, increasing permeability and allowing bacterial components such as LPS to translocate into the bloodstream (Cryan & Dinan, 2012). Once systemic, LPS triggers the production of LPS-binding protein (LBP) and macrophages to release soluble cluster of differentiation (sCD14) and activates TLR4–NF- κ B (Toll-like receptor 4 – nuclear factor-kappa B) signalling, driving the release of pro-inflammatory cytokines including tumor necrosis factor α (TNF- α), IL-6, IL-8 and IL-12 (Wasiak & Gawlik-Kotelnicka, 2023). Dysbiosis resulting from poor diet or chronic stress can therefore exacerbate stress reactivity by reducing beneficial metabolites such as SCFAs, promoting oxidative stress and systemic inflammation, and weakening gut–brain communication (Schneider et al., 2024).

2. Aims of the work

The aims of this master's thesis are to study nutrition-host-microbiota interactions using breath analysis, in two different contexts (Figure 5).

The first study, called MICROBOOST-1 Kombucha study, aims to study in vivo in healthy volunteers, the potential impact of acute kombucha intake on exhaled breath over time, in order to unravel its potential effect on microbial-derived metabolites present in kombucha versus those potentially issued from the interaction with the human gut microbiota or health. This small cohort served as a pilot, allowing me the familiarisation with the analytical methods and data processing approaches.

The second study, called MICROBIOSTRESS study, aims to study in vivo, using stressed volunteers recruited in the context of a study performed by the CICN, the impact of stress level on fasting exhaled breath, the main objective being to evaluate the potential link between exhaled metabolites, human versus microbiota-related metabolism and psychological stress. This cohort also enabled the assessment of fasting breath profiles in relation to age, sex, and body mass index (BMI).

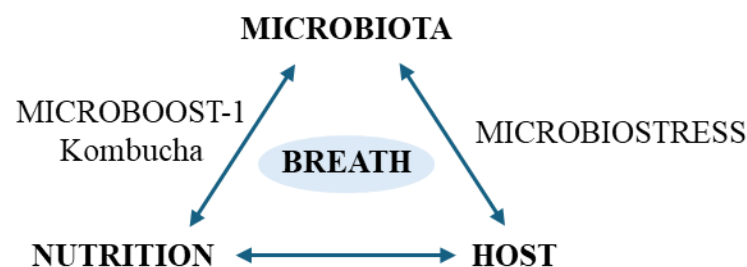


Figure 5 | Conceptual overview of the objectives of this master's thesis. Nutrition, host physiology, and the gut microbiota are interconnected systems that contribute to the production of volatile compounds detectable in exhaled breath. This master's thesis explores these interactions through two studies: the MICROBOOST-1 Kombucha study investigates the nutrition–microbiota axis using kombucha as a dietary intervention, while the MICROBIOSTRESS study examines the host–microbiota relationship in the context of psychological stress. Both studies employ breath analysis as a non-invasive tool.

3. Materials and methods

3.1. MICROBOOST-1 Kombucha study

Study overview

The MICROBOOST-1 Kombucha study is an interventional monocentric study including 2 test days that was approved by the local ethical committee (Comité d'Ethique Hospitalo-Facultaire UCLouvain/Cliniques Universitaires Saint-Luc; reference (2023/13AVR/183)) and carried out in accordance with the Good Clinical

Practice. The study aimed to characterise changes in the composition of the exhaled breath over time after drinking kombucha compared with water (control) (**Figure 6**). The recruitment was conducted from the 3rd to the 13th of February 2025, and the test days occurred on 20th and 27th February 2025. Written informed consent was obtained from all volunteers before inclusion in the study. A total of 5 healthy volunteers were included in the study based on the predefined inclusion and exclusion criteria.

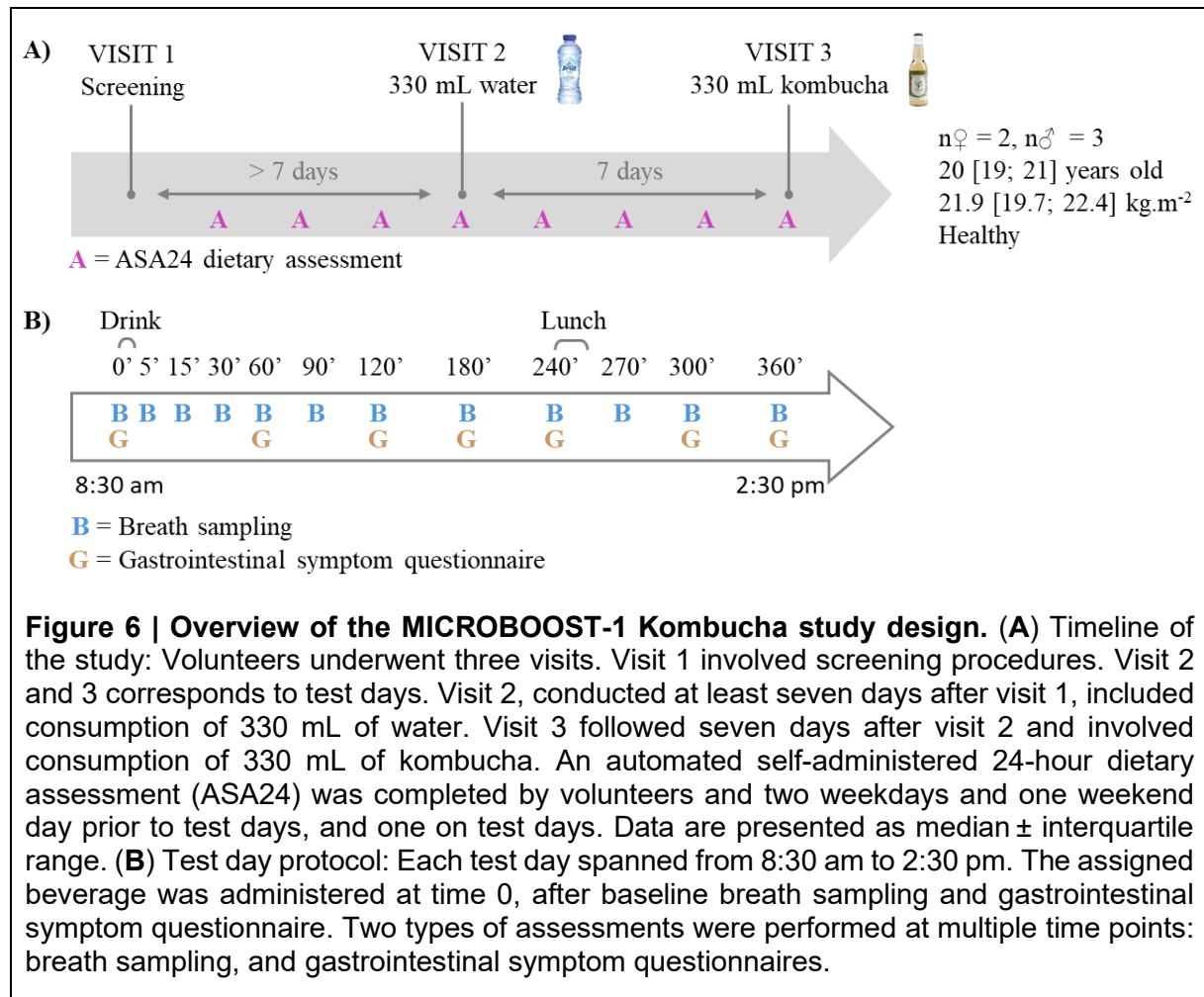


Figure 6 | Overview of the MICROBOOST-1 Kombucha study design. (A) Timeline of the study: Volunteers underwent three visits. Visit 1 involved screening procedures. Visit 2 and 3 corresponds to test days. Visit 2, conducted at least seven days after visit 1, included consumption of 330 mL of water. Visit 3 followed seven days after visit 2 and involved consumption of 330 mL of kombucha. An automated self-administered 24-hour dietary assessment (ASA24) was completed by volunteers and two weekdays and one weekend day prior to test days, and one on test days. Data are presented as median $\pm$ interquartile range. **(B)** Test day protocol: Each test day spanned from 8:30 am to 2:30 pm. The assigned beverage was administered at time 0, after baseline breath sampling and gastrointestinal symptom questionnaire. Two types of assessments were performed at multiple time points: breath sampling, and gastrointestinal symptom questionnaires.

Inclusion criteria required volunteers to be adults aged 18 to 65 years; with a BMI between 18 and 30 kg/m²; non-smokers; French-speaking; willing to comply with study procedures; and able to provide written informed consent.

Exclusion criteria comprised severe systemic diseases (e.g., renal or hepatic failure, cancer, chronic pancreatitis, asthma); chronic or severe gastrointestinal disorders (e.g., duodenal ulcer, inflammatory bowel disease, coeliac disease, irritable bowel syndrome); gastrointestinal surgery (except appendectomy) within the past two month; psychiatric disorders or use of antipsychotic medication; recent use of antibiotics; fibre supplements, or agents affecting intestinal transit or gut microbiota

within the past four weeks; chronic medication use (except contraceptives); medically supervised hypocaloric diets within the past six weeks; pregnancy, breastfeeding, or lack of contraception; excessive alcohol intake (three alcoholic drinks per day, > 30 g/day); participation in another clinical trial within the past two weeks; intense physical activity (> 10 hours/week); and any language, mental or legal barrier preventing understanding or compliance with the study.

Organisation of the visit 1

During the inclusion visit, volunteers received detailed information about the study procedures. The volunteers were tasked to continue to respect inclusion and exclusion criteria throughout the intervention period. They were therefore not allowed to smoke; to consume antibiotics, fibre supplements and/or any product that modulates intestinal transit (laxatives, anti-diarrheal agents, etc.) or the composition of the gut microbiota; to follow a low-calorie diet; to consume more than 3 glasses of alcohol per day (>30 g alcohol per day); to take part in any other clinical trial in parallel with the study; and to engage in more than 10 hours of intense sporting activity per week.

After providing informed consent and declaring willingness to comply with all study procedures and availability for the duration of the study, volunteers completed an electronic questionnaire (via REDCap, research electronic data capture) about their medical and medication history, general information and intestinal transit. Moreover, during each week preceding the 2-test days, volunteers completed 3 days-food diaries (two weekdays and one weekend day), called ASA24 (automated self-administered 24-hour dietary Assessment), to report all food and beverages consumed during the day (**Figure 6**).

To standardize conditions prior to each test day, volunteers received detailed instructions regarding diet and behaviour. On the day before each intervention, they were asked to abstain from alcohol and, after 4 pm, to consume only a prescribed meal consisting of cooked hot rice and chicken. The use of cooking fats, spices, and small amounts of ketchup or mayonnaise was permitted, whereas all other foods were prohibited. On the morning of each test day, volunteers were required to arrive in a

fasted state, with their teeth brushed, without having drunk anything (no water, tea or coffee), and without having engaged in any intense physical activity.

Organisation of visits 2 and 3

Both test days (water day and kombucha day) were organized in the same way (**Figure 6**). The study coordinator (Manon Autuori, PhD student under the supervision of Pr. Nathalie Delzenne) reviewed the inclusion and exclusion criteria, and verified if instructions were adequately followed. Any event that deviated from the usual recommendations during the study, or that seemed important to report during the test days, had to be reported by the volunteers using the form provided for this purpose. On the first test day (water day) only, the volunteers were weighed and measured.

After mouth wash with water (SPA® Reine, Liège), breath was sampled at baseline. At 8:30 am, volunteers received 330 mL of water very low in mineral salts (SPA® Reine, Liège) or kombucha (“So Wood Kombucha® - Bouleau”, batch no. 3.021, Gembloux), which they had to drink within 2 minutes. Ingredients of the kombucha beverage used in the study included Forest infusion (water, beet sugar, birch leaves 8%) and So Wood Kombucha (green tea, black tea, beet sugar, kombucha culture). Its nutritional composition is detailed in **Table 2**. Subsequent breath samples were collected 5, 15, 30, 60, 90, 120, 180, 240, 270, 300, and 360 minutes after ingestion, each time after rinsing the mouth with water. For each time point, breath samples were collected in 2 bags for different types of analysis: H₂, CH₄ and CO₂ analysis by Lactotest®, and other BVC analysis by SIFT-MS. Breath samples for the Lactotest® were collected 2 minutes after those for the SIFT-MS. Additionally, volunteers were asked to complete the ASA24 and to answer hourly a questionnaire about gastrointestinal symptoms.

Table 2 | Nutritional composition of the morning and midday foodstuffs consumed in the study. Two meals were provided to volunteers: a morning beverage (water or kombucha) and a midday lunch composed of white bread and Gouda cheese. The lunch values are shown both as a total (combined intake) and as individual components. Abbreviations: Glu, glucose; Lip; lipid; Pro, protein; SFA, saturated fatty acid.

	Quantity	Kcal	Glu (g)	Pro (g)	Lip (g)	SFA (g)	Fibre (g)	Sugar (g)	Salt (g)
Beverage									
a) Water	330 mL	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
b) Kombucha	330 mL	48.8	12.2	0.0	0.0	0.0	0.0	12.2	0.0
Lunch	192 g	601.0	68.6	24.3	24.9	12.2	3.2	1.6	2.5
→ White bread	132 g	393.4	68.6	9.9	8.1	0.8	3.2	1.6	1.5
→ Gouda cheese	60 g	207.6	0.0	14.4	16.8	1.4	0.0	0.0	1.1

Of note, volunteers were free to drink water 2 hours after consuming the morning beverage, and they received a standardized lunch composed of white bread and cheese 4 hours after consuming the morning beverage. Nutritional composition of lunch is detailed in **Table 2**. All volunteers consumed the lunch within 15 minutes, immediately after the 240' breath sampling.

Kombucha aroma composition

The aroma profile of So Wood Kombucha® was analysed by Sarah Suffys (PhD student, Faculty of Gembloux Agro-Bio Tech, University of Liege) using stir bar sorptive extraction (SBSE) coupled with GC-MS. For sample preparation, 5 mL of kombucha were incubated at 25 °C for 20 minutes, with three replicates performed. Following incubation, aliquots were transferred into vials, and NaCl was added to achieve a final concentration of 20% (w/v). A pre-conditioned SBSE was placed in each vial and subjected to magnetic stirring for two hours. After agitation, the stir bars were rinsed with distilled water and gently blotted with absorbent paper to remove residual moisture. Finally, 1 µL of heptan-1-ol (100 mg/L in ethanol) was added as an internal standard.

Dietary assessment

ASA24 is a validated, web-based dietary 24-hour recall tool developed by the National Cancer Institute. It uses the Automated Multiple-Pass Method and standardized food photographs to enhance accuracy and completeness (Blanton et al., 2006; Moshfegh et al., 2008). The Canadian 2018 version, developed by the National Cancer Institute (Bethesda, MD, USA), was customized to reflect volunteers' dietary habits. Nutrient intake was analysed by Laetitia Lengele (PhD working in the Pr. Nathalie Delzenne 's team).

Gastrointestinal symptoms evaluation

Gastrointestinal symptoms were assessed every hour using an online Visual Analog Scale (VAS) questionnaire implemented in REDCap (Harris et al., 2009). The 8 symptoms –bloating, burps, cramps, discomfort, flatulence, gastrointestinal reflux, nausea and rumbling – were scored on a scale from 0 (no symptom) to 10 (very severe symptom).

Breath sample collection and analysis

To analyse H₂, CH₄, and CO₂, exhaled breath was sampled using MEC bags (300 mL; ref. FMONP.SBAG1.AA, MEC R&D SPRL, Belgium). Volunteers took a deep inhalation, held their breath for 15 seconds, and then exhaled as long as possible into the bag. To ensure measurement accuracy, the bags were connected to the Lactotest® (Medical Electronic Construction, R&D, Belgium) within 2 hours of exhaled breath collection.

To analyse other BVC, exhaled breath samples were taken using Supelco bags (1 L; ref. 30272-U, Supelco, Merck, Belgium). Volunteers were instructed to inhale deeply before exhaling into the bag. The bags were subsequently connected using a polytetrafluoroethylene (PTFE) tube (4 × 6 mm; PTFE Tube Shop, Breda, Netherlands) to the SIFT-MS instrument (Voice200ultra, Syft Technologies, New Zealand) for analysis within 2 hours of the exhaled breath being collected to ensure measurement accuracy.

SIFT-MS measurements were performed using a Voice200ultra instrument equipped with H₃O⁺, NO⁺, and O₂⁺ reagent ions. BVC were carried into the flow tube via a helium stream, where they reacted with the reagent ions. The resulting product ions were detected by a quadrupole mass spectrometer, operated in FS mode over a *m/z* range of 15-250 amu, with unit mass resolution, 3 repetitions, and a 100 ms integration time per product ion.

To ensure that environmental conditions did not introduce background bias, ambient air samples were taken at different time points (0, 120, 240, and 360 minutes after beverage ingestion) during test days. Moreover, to account for potential background biases from our device, limit of quantification (LOQ) was calculated based on a helium sample analysed in FS mode over a *m/z* range of 15-250 amu, with unit mass resolution, 50 repetitions, and a 100 ms integration time per product ion. LOQ was defined as: LOQ = mean + 10 × SD, where the mean and standard deviation (SD) were derived from the helium sample measurements (U.S. Food and Drug Administration [FDA], 1996).

Breath data obtained with the SIFT-MS instrument were initially analysed using an untargeted approach (FS mode), which recorded ion signal intensities (count × s⁻¹) across the full *m/z* spectrum without focusing on specific compounds. This provided an overview of breath profile. Following this, a targeted approach (SIM mode) was

employed on the full m/z spectrum to quantify specific volatile compounds of interest. These included (1) 61 aroma compounds present in the kombucha used (see section 3.1; Kombucha aroma composition; full list not shown), (2) 13 compounds typically associated with microbial fermentation processes, and (3) 2 of the most abundant endogenous compounds in exhaled breath (acetone and isoprene) (**Table S1**). All stages of data acquisition, compound identification and quantification were performed using LabSyft software (version 1.4.4, Syft Technologies, New Zealand).

Of note, because structural isomers may generate overlapping signals in SIFT-MS, spectral information did sometimes not permit clear differentiation. Consequently, some compounds are presented collectively (e.g., propanol isomers for 1-propanol / 2-propanol / 3-propanol) in this master's thesis.

Statistical analysis

The distribution of variables – namely dietary intake, breath composition and gastrointestinal symptom – was considered to deviate from a normal distribution due to the small sample size. Results were therefore reported as median $\pm$ interquartile range, except for gastrointestinal symptoms, reported as mean $\pm$ standard deviation, due to the predominance of zero scores making median values uninformative.

Comparisons between test days were conducted using GraphPad Prism (version 9.5.1) (GraphPad Software, San Diego, CA, USA). Variables measured once per test day – namely dietary intake – were analysed using the Friedman test ($p < 0.05$) by Laetitia Lengele (PhD working in Nathalie Delzenne 's team). Time-resolved data – namely breath composition and gastrointestinal symptoms – were analysed using mixed models with Šídák's multiple comparisons test ($p < 0.05$).

Untargeted breath data were analysed by conducting statistical analyses performed in R (version 4.4.3) after normalization made to the total ion signal summed over all m/z for a given reagent ion (e.g., H_3O^+ m/z 45 were normalised by the sum of the signals of all m/z obtained by the ionisation by H_3O^+) as recommended by Langford and Perkins (2025). Signals corresponding to reagent ions and related secondary chemistry (H_3O^+ m/z 19, $\text{H}_3\text{O}^+ \cdot \text{H}_2\text{O}$ m/z 37, $\text{H}_3\text{O}^+ \cdot (\text{H}_2\text{O})_2$ m/z 55, $\text{H}_3\text{O}^+ \cdot (\text{H}_2\text{O})_3$ m/z 73, NO^+ m/z 30, NO^+ m/z 48, O_2^+ m/z 32, O_2^+ m/z 50) were excluded from the untargeted analysis, as these ions are present in large excess and could otherwise dominate apparent differences between test days. Principal component analysis (PCA) of the MS-signals obtained from the H_3O^+ , NO^+ , and O_2^+ ionisation modes was first

conducted to explore potential clusters between the two conditions (water day vs kombucha day). PCA was performed with scaling to prevent the clustering from being determined solely by the most abundant volatile compounds in the breath, as reflected by the number of their respective MS signals. Subsequently, sparse partial least squares-discriminant analysis (sPLS-DA) was applied to determine the MS-signals contributing most strongly to the separation of clusters.

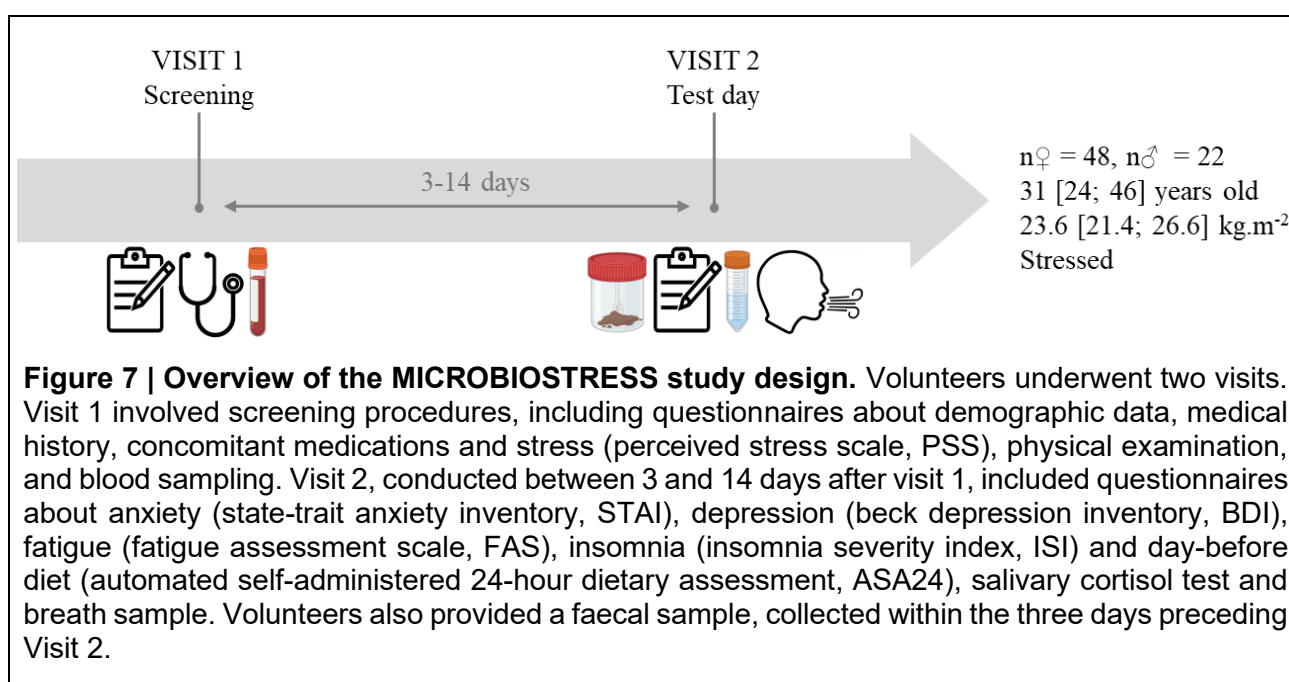
The identification of discriminant volatile compounds between the water and kombucha days from untargeted breath data was performed based on the most discriminant MS-signals – those with the highest loadings on the two first principal components. For each selected MS-signals, potential volatile compounds responsible for the detected signals were identified using the LabSyft library, restricting the selection to compounds with a boiling point below 250 °C at 760 mmHg to ensure adequate volatility. To ensure the presence of these potential volatile compounds, Spearman correlation between all MS-signals was performed in R (version 4.4.3).

3.2. MICROBIOSTRESS study

Study overview

The MICROBIOSTRESS study is an interventional monocentric study conducted by the Centre d'Investigation Clinique en Nutrition (CICN) of UCLouvain. This study, including 3 visits, was approved by the local ethical committee (Comité d'Ethique Hospitalo-Facultaire UCLouvain/Cliniques Universitaires Saint-Luc; reference (2025/23JUI/247)) and carried out in accordance with the Good Clinical Practice. Analyses in this master's thesis focused on 2 visits to explore the composition of the exhaled breath in stressed volunteers and compare their breath profile in fasting state with their level of stress. Both visits, separated between three to fourteen days, occurred between September and November 2025 (**Figure 7**). The questionnaires and blood and saliva analyses were performed by the CICN (Dr. Valérie Dormal) and provided to us in order to allow the statistical analysis of the correlation between all parameters and breath volatile compounds. The sampling of breath and analysis was performed by me in collaboration with my PhD student supervisor, Manon Autuori.

Written informed consent was obtained from all volunteers before inclusion in the study. A total of 71 stressed volunteers were included in the study based on the predefined inclusion and exclusion criteria, but one volunteer was excluded due to the presence of strange odours during breath analysis.



Inclusion criteria required volunteers to be adults aged 18 to 60 years old); with moderate-to-high level of perceived stress scale (PSS), i.e. scores ranging from 14 to 30 at the PSS and a score ≥ 3 for frequency of anxiety and nervousness (item 3 of PSS scale); French-speaking; willing to comply with study procedures; and able to provide written informed consent.

Exclusion criteria comprised a history or current serious medical or cognitive condition (especially gastrointestinal) that could affect study results or safety; medical treatments that could affect cognition, emotions, or gut microbiota; drug use or history of addiction in the past 5 years; use of food supplements or foods with active ingredients (e.g., probiotics, prebiotics); high coffee intake (> 5 cups/day); high alcohol consumption (> 30 g/day); type 1 or type 2 diabetes; vegetarian or vegan diet; known hypersensitivity to any study product ingredient; participation in another clinical trial; pregnancy, breastfeeding, and plans to become pregnant within 6 weeks or not using reliable contraception (e.g., pill, IUD, abstinence).

Organisation of the visit 1

During the first visit, volunteers received detailed information about the study procedures. The volunteers were tasked not to change current drinking, eating and exercise habits during the study. After providing informed consent and declaring willingness to comply with all study procedures and availability for the duration of the study, volunteers provided demographic data (age, gender, race), medical history, concomitant medication and daily caffeine consumption, and completed the PSS

(**Figure 7**). After checking eligibility against inclusion and exclusion criteria, physical examination (including measurement of weight, height, blood pressure and heart rate) and blood sampling were performed, and the instructions and the material to collect a stool sample within 3 days before next visit were provided. In case volunteers had taken medications or products that modify cognition and/or microbiota within 3 weeks before the screening visit, it was proposed to the volunteers to realize a washout to meet inclusion/exclusion criteria.

To standardize conditions prior to each test day, volunteers received detailed instructions regarding diet and behaviour. On the day before each intervention, they were asked to abstain from alcohol and, after 4 pm, to consume only a prescribed meal consisting of cooked hot rice and chicken. The use of cooking fats, spices, and small amounts of ketchup or mayonnaise was permitted, whereas all other foods were prohibited. On the morning of each test day, volunteers were required to arrive in a fasted state, with their teeth brushed, without having drunk anything (except water), and without having engaged in any intense physical activity.

Organisation of the visit 2

The second visit was scheduled between 3 and 14 days after the first one (**Figure 7**). Volunteers had to arrive fasted (no tea, water or coffee), with their teeth brushed and without having made any intense effort. Inclusion and exclusion criteria were reviewed, compliance with the instructions was verified, and volunteers brought back a stool sample for microbiota analysis and a salivary sample for cortisol analysis. Any event that deviated from the usual recommendations during the study, or that seemed important to report during the test days, had to be reported by the volunteers using the form provided for this purpose.

During this visit, evaluation of emotional processing was carried out. Volunteers completed online questionnaires assessing anxiety level (state-trait anxiety inventory, STAI), depression (beck depression inventory, BDI), fatigue (fatigue assessment scale, FAS), and insomnia (insomnia severity index, ISI). They also reported all food and beverages consumed during the previous day in a food diary called ASA24. A breath sampling was performed after mouth wash with water (SPA® Reine, Liège) for BVC analysis by SIFT-MS.

Emotional evaluation

PSS evaluates how individuals perceive stress based on their thoughts and feelings over the past month. Scores on the PSS range from 0 to 40, with higher scores indicating greater perceived stress. Scores ranging from 0 to 13 indicate low stress; scores ranging from 14 to 26 indicate moderate stress; and scores ranging from 27 to 40 indicate high stress.

STAI is a psychological inventory based on a 4-point Likert scale. The STAI measures state anxiety, or anxiety about an event, and consists of 20 different questions that are scored from 0 to 80, with higher scores being positively correlated with higher levels of anxiety. Scores ranging from 20 to 37 indicate low anxiety; scores ranging from 38 to 44 indicate moderate anxiety; and scores ranging from 45 to 80 indicate high anxiety.

BDI is a 21-question multiple-choice self-report inventory, one of the most widely used psychometric tests for measuring the severity of depression. Scores on the BDI range from 0 to 63, with higher scores indicating greater depression. Scores ranging from 0 to 9 indicate minor depression; scores ranging from 10 to 18 indicate mild depression; scores ranging from 19 to 29 indicate moderate depression; and scores ranging from 30 to 63 indicate severe depression.

FAS is a 10-item questionnaire designed to measure general fatigue. It includes 5 items related to physical fatigue and 5 items that assess mental fatigue. Scores on the FAS range from 10 to 50, with higher scores indicating greater fatigue. Scores of 22 or below indicate “normal” fatigue; scores ranging from 22 to 34 indicate mild-to-moderate fatigue; and scores of 35 or above indicate severe fatigue.

ISI is a brief 7-item scale that evaluates the nature of insomnia, satisfaction with sleep, daily functioning, and anxiety related to sleep disturbances. The usual recall period comprises the past month. Scores on the ISI range from 0 to 28, with higher scores indicating greater insomnia. Scores ranging from 0 to 7 indicate no insomnia; scores ranging from 8 to 14 indicate subclinical (mild) insomnia; scores ranging from 15 to 21 indicate clinical (moderate) insomnia; and scores ranging from 22 to 28 indicate clinical (severe) insomnia.

Dietary assessment

ASA24 is a validated, web-based dietary 24-hour recall tool developed by the National Cancer Institute. It uses the Automated Multiple-Pass Method and

standardized food photographs to enhance accuracy and completeness (Blanton et al., 2006; Moshfegh et al., 2008). The Canadian 2018 version, developed by the National Cancer Institute (Bethesda, MD, USA), was customized to reflect volunteers' dietary habits. Nutrient intake will be analysed by Laetitia Lengele (PhD working in the Pr. Nathalie Delzenne 's team).

Blood sample collection and analysis

A blood sampling has been performed at the first visit to evaluate gut permeability by LBP analysis. Samples will be immediately centrifuged at 4°C and stored at the CIGN until analysis by LIMS lab (Fond des Més, 5/3 (Tower F) 1348 Louvain-la-Neuve, Belgium).

Stool sample collection and analysis

A stool sample was collected by each volunteer within 3 days before visit 2 to analyse (1) gut microbiota composition and (2) inflammation. Those analyses have not been assessed yet, and therefore, will not be presented in the thesis.

Saliva sample collection and analysis

A single saliva sample was collected on the morning of the second visit using a Salivette® Cortisol device (ref.51.1534.500, Sarstedt AG & Co., Germany) to evaluate the physiological stress response. Volunteers collected the sample immediately upon waking, before eating, drinking, brushing their teeth, or smoking. After washing their hands, volunteers placed the swab in the mouth for 2 minutes without chewing. After sampling, tubes were stored in the refrigerator until the volunteers arrived at the CIGN. Salivary cortisol quantification was performed by the LIMS laboratory (Louvain-la-Neuve) for cortisol analysis. At the time of writing this master's thesis, only 59 of the 70 volunteers included had been analysed.

Breath sample collection and analysis

Exhaled breath was sampled and analysed in the same way that for the MICROBOOST-1 Kombucha study (see section 3.1; Breath sample collection and analysis). BVC were analysed by the SIFT-MS instrument (Voice200ultra, Syft Technologies, New Zealand). To ensure measurement accuracy, all bags were analysed within 2 hours of exhaled breath collection. ambient air samples were collected prior to analysis to ensure that environmental conditions did not introduce

background bias, and the LOQ was calculated from a helium sample to account for potential background bias in our instrument.

Breath data obtained with the SIFT-MS instrument were initially analysed using an untargeted approach (FS mode), like for MICROBOOST-1 Kombucha study (see section 3.1; Breath sample collection and analysis). Because the study extended over multiple days, untargeted breath data were extracted using NAC approach to benefit of robust daily correction of SIM mode and decrease the differences between test days. NAC were incorporated into the statistical analyses (see Section 3.2; Statistical analysis) to investigate volatile breath profiles across varying levels of stress, anxiety, depression, fatigue and insomnia. Subsequently, a targeted approach (SIM mode) was employed to focus on quantifying specific volatile compounds of interest. The compounds of interest included (1) 18 compounds that are associated with stress based on the literature (2) 13 compounds typically associated with microbial fermentation processes, and (3) 2 of the most abundant endogenous compounds in exhaled breath (acetone and isoprene) (Table S2). Initially, measuring BVC in relation to fermentation allowed us to indirectly study the impact of stress levels on the gut microbiota. Subsequently, this will enable us to link our results with those of the stool analysis. All stages of breath data acquisition were performed using LabSyft software (version 1.4.4, Syft Technologies, New Zealand).

Statistical analysis

The distribution of all variables – namely questionnaire scores, BVC concentration composition, cortisol concentration, LBP concentration, age, sex and BMI – was assessed using the D'Agostino test and found to deviate from normality, reflecting predominantly non-parametric data. Consequently, results were presented as median $\pm$ interquartile range.

Comparisons between different parameters were conducted using GraphPad Prism (version 9.5.1) (GraphPad Software, San Diego, CA, USA). Comparison of the different parameters between moderate and high stress were analysed using Mann-Whitney test ($p < 0.05$) excepted for the number of men and women across these 2 stress categories where Fisher's exact test ($p < 0.05$) was used. Comparison of BVC concentration between STAI, BDI, FAS or ISI scores were analysed using Kruskal-

Wallis test ($p < 0.05$). Evolution of BVC concentrations across cortisol concentration, LBP concentration, age or BMI were analysed using Spearman correlation ($p < 0.05$).

Untargeted breath data were analysed by conducting statistical analyses performed in R (version 4.4.3). Because the study extended over multiple days, untargeted breath data were extracted using NAC approach to benefit of robust daily correction of SIM mode and decrease the differences between test days. PCA of NAC was conducted to explore potential clusters between the questionnaire scores. PCA was performed with scaling to prevent the clustering from being determined solely by the most abundant volatile compounds in the breath, as reflected by the number of their respective MS signals.

4. Results – MICROBOOST-1 Kombucha study

4.1. Study population

The five volunteers included in the study had minor gastro-intestinal symptoms after kombucha ingestion and maintained consistent dietary habits throughout the study. Among the 5 healthy included volunteers – 2 women and 3 men (20 [19; 21] years old, 21.9 [19.7; 22.4] kg/m²), only minor differences in gastrointestinal symptom scores reached statistical significance prior to ingestion, with higher flatulence scores on the water day ($p < 0.01$, Šídák's test) and higher nausea on the kombucha day ($p < 0.05$, Šídák's test), reflecting baseline variations unrelated to the beverages tested (**Figure S1**). A slight but significant increase in rumbling was also observed three hours after kombucha ingestion compared to water ($p < 0.05$, Šídák's test). Dietary habits, evaluated by postdoctoral researcher Laetitia Lengele based on three non-consecutive 24-hour recalls (ASA24) during the week preceding the control and kombucha days, showed no significant differences in energy, macronutrient, sugar, or fibre intake between the control and kombucha days ($p > 0.05$, Friedman test; data not shown).

4.2. Untargeted analysis of the SIFT-MS signals

PCA on untargeted breath data revealed 3 clusters on the kombucha day. To explore overall changes in breath composition following kombucha ingestion compared to water, we applied PCA; an unsupervised multivariate method summarising complex data into key axes capturing the main sources of variation. The PCA score plot of the normalised MS-signal (from H₃O⁺, NO⁺, and O₂⁺ ionisations)

revealed two distinct shifts in breath composition – one at 5 minutes and another after 90 minutes post-ingestion – while samples from other time points and the water day remained relatively stable (**Figure 8A**). The first principal component (PC1), distinguishing the >90-minute (120', 180', 240', 270', 300', 360') samples from the rest, accounted for 21% of the total variance. The extent of the >90-minute shift did not vary considerably among volunteers. The second principal component (PC2), distinguishing the 5-minute samples from the others, explained 8% of the variance. The extent of the 5-minute cluster showed great variation among volunteers, with volunteer 4 showing the smallest change – suggesting a weaker response – and volunteer 5 showing the greatest change – suggesting a stronger response.

The sPLS-DA on breath data revealed 10 key MS-signals explaining the 3 clusters identified based on the PCA. To identify the signals responsible for the observed shifts in breath composition at 5 minutes and after 90 minutes on the kombucha day, we applied a sPLS-DA, a supervised multivariate method that highlights variables contributing most to the separation between predefined groups. Focusing on the kombucha day, we compared the 5-minute samples, the >90-minute (120', 180', 240', 270', 300', 360') samples, and <90-minute (0', 15', 30', 60') samples (**Figure 8B**). Using the R function “tune.splsda,” we determined that the top five MS-signals from each principal component were sufficient to distinguish the three clusters. The five MS-signals contributing most to the separation of the >90-minute samples were, in descending order of importance: m/z 169 generated by NO^+ , m/z 120 generated by O_2^+ , m/z 120 generated by NO^+ , m/z 155 generated by NO^+ , and m/z 179 generated by NO^+ (**Figure 8C**). These MS-signals were negatively associated with breath composition after 90 minutes post-ingestion, as indicated by their positive loadings and the leftward positioning of the samples on the sPLS-DA plot. In contrast, the five MS-signals most strongly associated with the 5-minute samples were: m/z 45 generated by H_3O^+ , m/z 46 generated by NO^+ , m/z 76 generated by NO^+ , m/z 31 generated by O_2^+ , and m/z 66 generated by H_3O^+ . These MS-signals were positively associated with breath composition at 5 minutes post-ingestion, as indicated by their negative loadings and the downward positioning of the samples on the sPLS-DA plot.

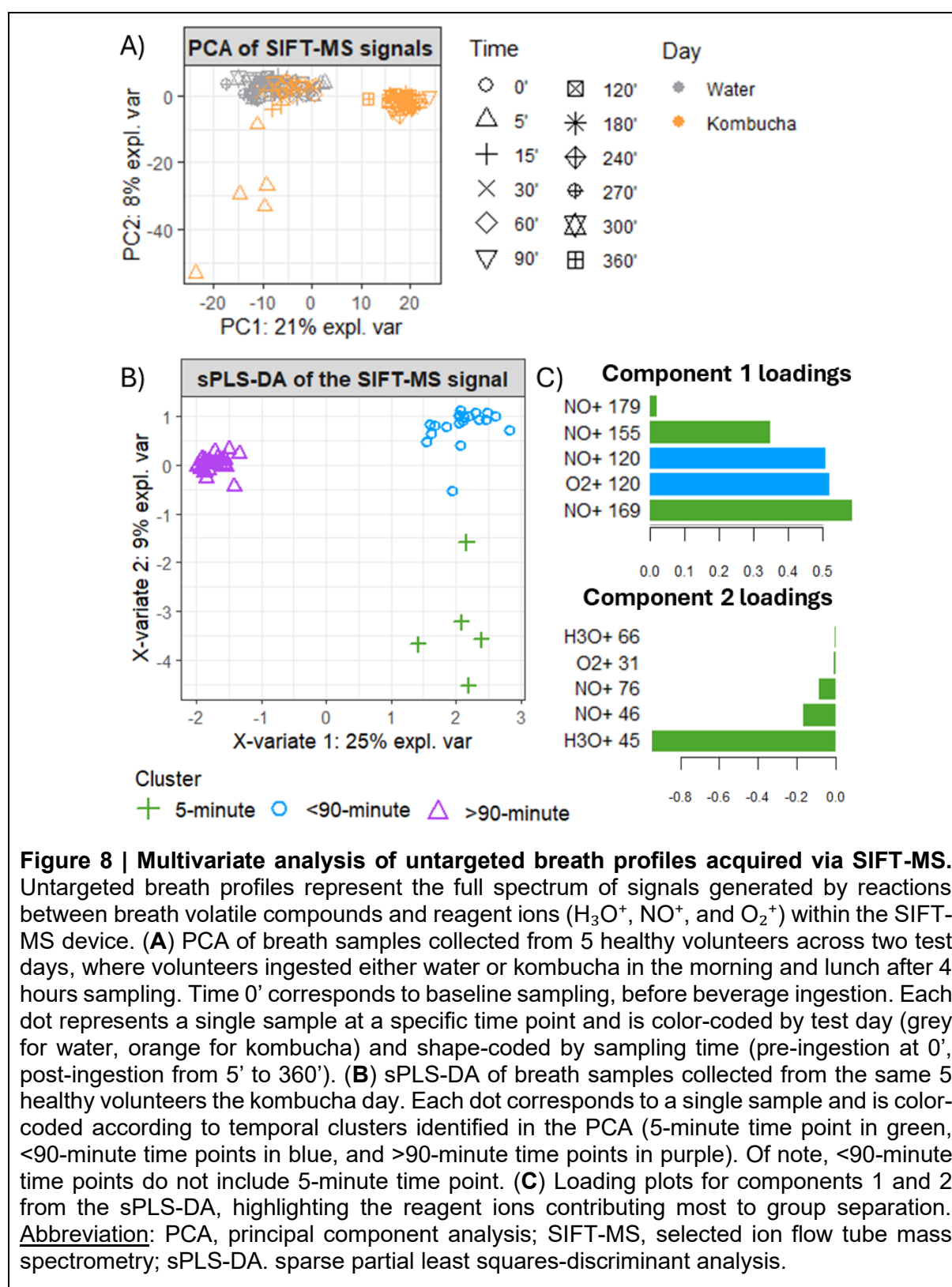


Figure 8 | Multivariate analysis of untargeted breath profiles acquired via SIFT-MS. Untargeted breath profiles represent the full spectrum of signals generated by reactions between breath volatile compounds and reagent ions (H_3O^+ , NO^+ , and O_2^+) within the SIFT-MS device. **(A)** PCA of breath samples collected from 5 healthy volunteers across two test days, where volunteers ingested either water or kombucha in the morning and lunch after 4 hours sampling. Time 0' corresponds to baseline sampling, before beverage ingestion. Each dot represents a single sample at a specific time point and is color-coded by test day (grey for water, orange for kombucha) and shape-coded by sampling time (pre-ingestion at 0', post-ingestion from 5' to 360'). **(B)** sPLS-DA of breath samples collected from the same 5 healthy volunteers the kombucha day. Each dot corresponds to a single sample and is color-coded according to temporal clusters identified in the PCA (5-minute time point in green, <90-minute time points in blue, and >90-minute time points in purple). Of note, <90-minute time points do not include 5-minute time point. **(C)** Loading plots for components 1 and 2 from the sPLS-DA, highlighting the reagent ions contributing most to group separation. Abbreviation: PCA, principal component analysis; SIFT-MS, selected ion flow tube mass spectrometry; sPLS-DA, sparse partial least squares-discriminant analysis.

4.3. Identification of potential BVC discriminating both tested conditions based on the sPLS-DA

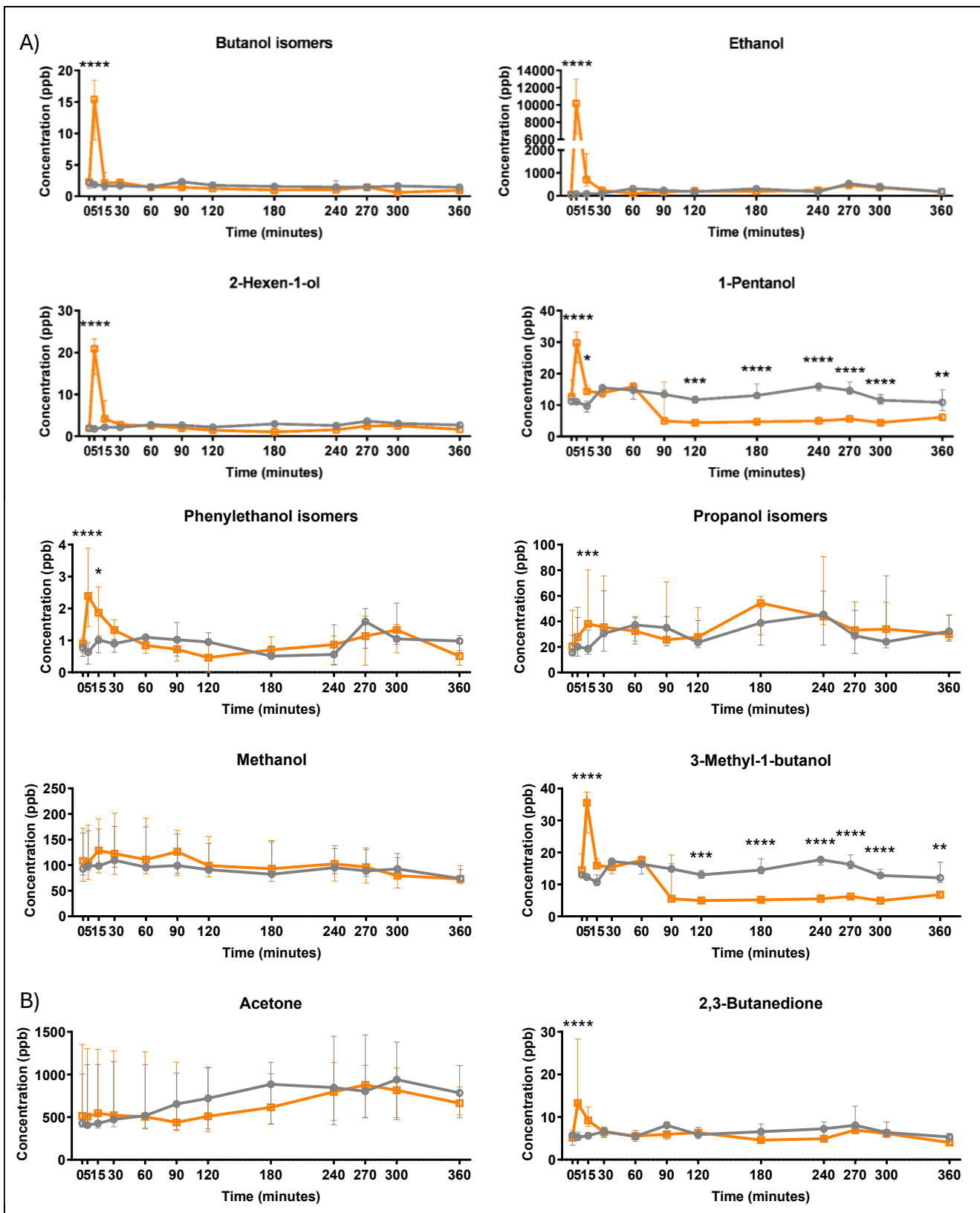
No compounds could be clearly identified based on the MS-signals from the sPLS-DA. The ten key MS-signals identified as major contributors to the changes

in breath composition following kombucha ingestion were used to search for potential exhaled volatile compounds responsible for their formation in the SIFT-MS device. All candidate matches from the LabSyft library were screened, and only those meeting two criteria were retained: (1) a significantly different concentration between test days either 5 minutes after ingestion or consistently after 90 minutes (Šídák's test, $p < 0.0001$), supporting the PCA findings, and (2) a boiling point below 250 °C at 760 mmHg, ensuring sufficient volatility for SIFT-MS detection. This selection yielded 18 compounds (data not shown): 10 that only increased at 5 minutes (e.g., diethyl ether, methylamine, phenylacetaldehyde, methylhydrazine), 4 that increased at 5 minutes and decreased after 90 minutes (e.g., lactic acid, styrene oxide), and 4 that only decreased after 90 minutes (e.g., menthol, 2-ethyltoluene). To confirm the presence of these compounds, we performed correlation analyses across all MS-signals coming from FS analysis and examined whether signals associated with each candidate compound were significantly correlated ($p < 0.05$, Spearman test). As no candidate compound showed consistent correlation across all its associated signals, we refrained from proposing specific compound identities based solely on untargeted data.

4.4. Targeted analysis of compound of interest

The concentrations of several compounds changed following kombucha ingestion, most notably showing an increase five minutes after consumption. A set of targeted analytes was selected to characterize the temporal evolution of specific compounds after kombucha intake. We targeted 61 specific compounds known to be present in the kombucha used in the study. These were identified through GC-MS analysis performed by Sarah Suffys (PhD student, Faculty of Gembloux Agro-Bio Tech, University of Liege) to characterize its aroma profile (list not shown). The 6 compounds showing a significant increase ($p < 0.05$, Šídák's test) at the 5-minute time point were selected (**Figure 9**). Additionally, we targeted 13 compounds originating from fermentation processes and 2 endogenous compounds commonly found at high concentrations in exhaled breath. In total, 19 compounds were considered (**Table S1**), of which 11 showed significantly ($p < 0.05$, Šídák's test) increased levels at 5 minutes post-kombucha ingestion and 2 appeared to decrease in concentration after 90 minutes post-kombucha ingestion. **Alcohols** were among the most responsive at 5 minutes, with ethanol showing the highest concentration (median peak ~ 10,000 ppb), followed by significant increase in concentrations ($p < 0.0001$, Šídák's test) in 3-methyl-

1-butanol, 1-pentanol, 2-hexen-1-ol, butanol isomers and phenylethanol isomers (**Figure 9A**). Methanol concentration remained stable over time. Propanol isomers concentration showed significant increased at 15 minutes post-kombucha ingestion. 3-Methyl-1-butanol and 1-pentanol were the 2 compounds whose concentration decreases significantly ($p < 0.05$, Šídák's test) 90 minutes after kombucha ingestion. **Ketones** included 2,3-butanedione, which concentration significantly increased at 5 minutes ($p < 0.0001$, Šídák's test), while acetone only showed a slight rise until lunch, probably reflecting fasting status (**Figure 9B**). **Acids** such as butyric, lactic and octanoic acids also increased significantly in concentration at this time point ($p < 0.0001$, Šídák's test), whereas acetic and propanoic acids concentrations remained relatively stable over time (**Figure 9C**). **Other chemicals** such as acetaldehyde and myrcene peaked at 5 minutes ($p < 0.001$, Šídák's test), while isoprene and hydrogen sulfide concentrations remained relatively stable over time (**Figure 9D**). The individual breath profiles of the metabolites were broadly similar across volunteers (**Figure S2**). However, volunteers 1 and 2 exhibited a delayed decline in 3-methyl-1-butanol and 1-pentanol concentrations compared to the rest of the cohort. Notably, these two volunteers showed a higher level of hydrogen on the kombucha day (**Figure S3**). Besides, volunteer 4 showed lower peak concentrations 5 minutes after ingesting kombucha for most compounds, including ethanol, which explains the less pronounced change in exhaled air composition observed in the PCA. This volunteer also differed from the others by exhibiting higher baseline levels of hydrogen sulfide, which interestingly, appeared to homogenise across all volunteers after lunch.



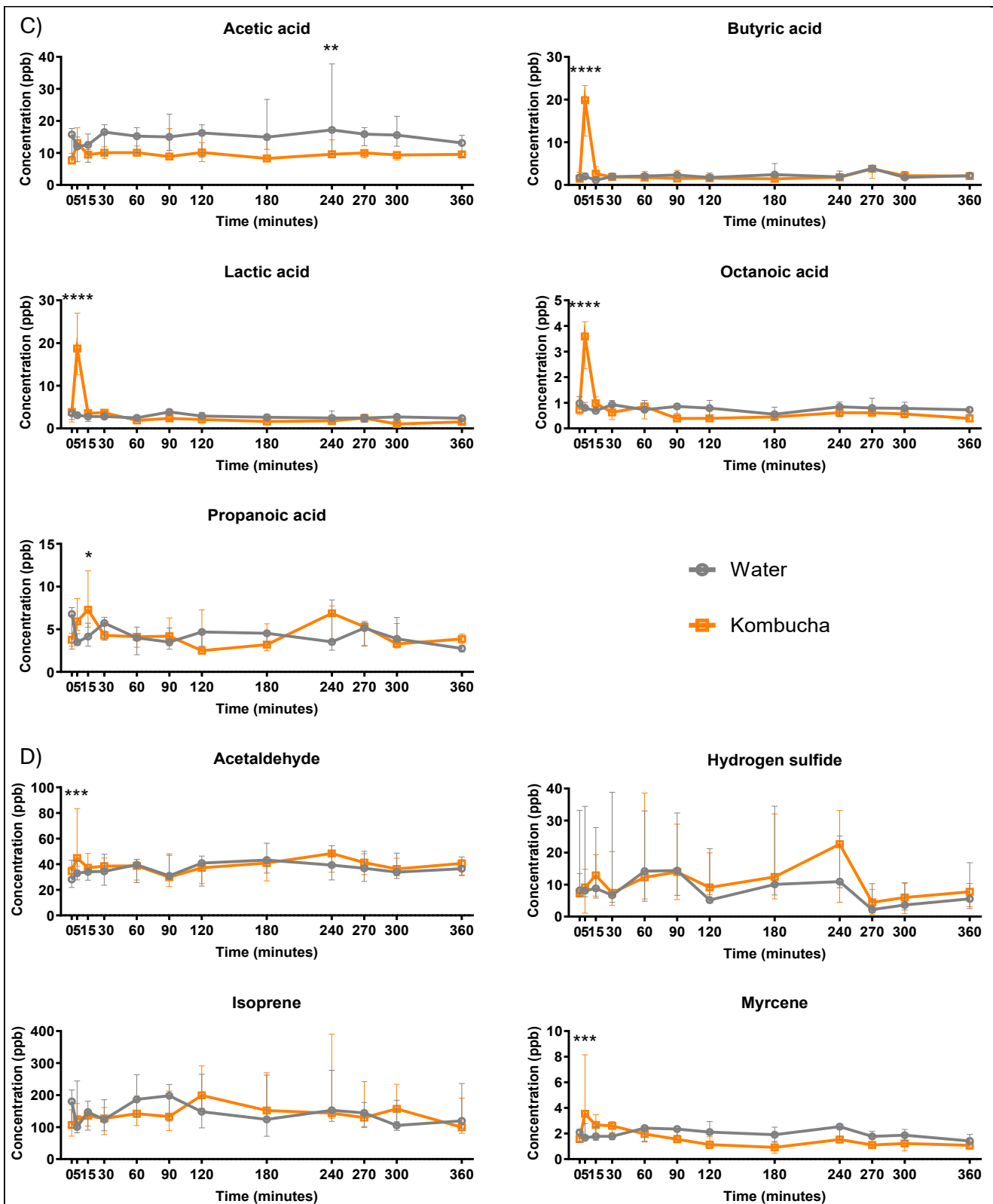


Figure 9 | Evolution of the concentration of targeted breath volatile compounds measured with SIFT-MS. Concentration were measured in exhaled breath of 5 healthy volunteers across two test days, where they ingested either water (in grey) or kombucha (in orange) in the morning, and lunch after 4 hours sampling. Time 0' corresponds to baseline sampling, before beverage ingestion. Compounds are grouped into four chemical classes: (A) alcohols, (B) ketones, (C) acids, and (D) other compounds. Data did not follow a normal distribution and are consequently presented as median $\pm$ interquartile range. Statistical significance was assessed using Šídák's multiple comparisons test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

5. Discussion – MICROBOOST-1 Kombucha study

In this study, we investigated the impact of kombucha ingestion on gut microbiota and host-related metabolism using breath volatilome analysis. The PCA of untargeted breath profile revealed 2 main trends post-kombucha ingestion: a cluster at 5 minutes, explainable by increased concentrations of BVC (e.g., ethanol, butyric acid), and a cluster after 90 minutes, explainable by decreased concentrations of unidentified BVC.

The extended 5-minute cluster observed in the PCA likely reflected residual traces of kombucha remaining in the upper gastrointestinal tract. A number of volatile compounds present in food can rapidly appear in the breath following consumption (Ajibola et al., 2013). The transient peak concentration at 5 minutes of BVC already detected in the aromatic profile of the liquid fraction of kombucha (e.g. 2-hexen-1-ol, octanoic acid; analysed by Sarah Suffys) or linked to fermentation (e.g. butyric acid, ethanol) supported this hypothesis. Therefore, the extent of the 5-minute cluster, which was consistent with inter-individual variation in the concentrations of the target compounds at this time point, may be explained by differences in the effectiveness of mouth rinsing. Nevertheless, not all compounds identified in the liquid fraction (by Sarah Suffys; data not shown) of kombucha showed a peak at 5 minutes, which may be explained by the non-volatility of compounds present in the kombucha or a rapid interaction of kombucha with saliva or oral microbiota, altering the chemical properties of certain compounds such as their volatility. Indeed, saliva contains numerous enzymes capable of rapidly reacting with food (Uchida & Ovitt, 2022).

The >90-minute cluster observed in the PCA of untargeted breath profile suggests a global decline in several unidentified BVC concentrations. The underlying cause of this observation remains unclear. Some targeted compounds originating from the kombucha beverage (as analysed by Sarah Suffys) or associated with fermentation processes (e.g. 1-pentanol, 3-methyl-1-butanol) showed reduced concentrations after 90 minutes post-kombucha ingestion. However, their concentration consistently fell below the LOQ (Table S1), making meaningful interpretation difficult. More broadly, the observed decrease in a wide range of BVC after kombucha intake is difficult to explain, as the literature provides limited evidence for such a phenomenon. A possible explanation is that components of kombucha may

influence host or microbial metabolic pathways, for example by altering the activity of transporters or enzymes (Costa et al., 2021), thereby promoting the conversion of volatile compounds into non-volatile compounds that are not detectable in exhaled breath. The delayed decrease in BVC concentrations (e.g., 1-pentanol, 3-methyl-1-butanol) observed in volunteers 1 and 2, together with their elevated hydrogen levels, further supports a potential influence of kombucha on gut microbial activity, given that hydrogen is a well-established fermentation product (Mutuyemungu et al., 2023).

This study underscored the need for continued methodological advances to strengthen compound identification from SIFT-MS signals, particularly in complex matrices such as exhaled breath. While some identifications – such as methylhydrazine, which appeared as a candidate driver of the 5-minute shift in the sPLS-DA – were unlikely to reflect true biological presence given its toxicological profile and implausibility in a nutritional study (Kim, 2025), other more plausible candidates also emerged from the analysis. Indeed, lactic acid, diethyl ether, and methylamine, for example, could reasonably originate from fermentation processes or tea-derived constituents of kombucha (*The Good Scents Company - Flavor, Fragrance, Food and Cosmetics Ingredients Information*, n.d.) and may explain the transient peak observed at 5 minutes. These observations suggested that our filtering criteria may have been overly restrictive and highlighted the importance of complementary analytical approaches – such as GC-MS – to confidently validate compound identity. Overall, the findings illustrated both the potential and the current limitations of untargeted SIFT-MS for breath metabolomics and point toward clear paths for methodological improvement.

This study presented several limitations that should be considered when interpreting the results. **First**, the small sample size and limited demographic diversity restricted the generalizability of the results. All volunteers were young adults, which limited the extent to which the outcomes can be extrapolated to other age groups or physiological conditions. Future studies involving larger and more heterogeneous cohorts will therefore be essential to capture the natural variability in breath composition and metabolic responses associated with age, lifestyle, and underlying physiology. **Second**, stool samples were not collected, which prevents direct comparison of breath-derived metabolites with gut microbiota composition. Integrating microbiome data would provide valuable insights into the microbial origins of specific BVC. **Third**, analytical constraints inherent to SIFT-MS may also influence the

interpretation of the data. Certain compounds can produce identical or near-identical ion-molecule reaction signatures, particularly structural or positional isomers, which often share similar reaction behaviours and thus generate indistinguishable MS fingerprints. As a result, SIFT-MS cannot reliably differentiate between such isomers, which is why multiple candidate identities were sometimes reported for a given signal.

This work, which is the subject of an article currently in preparation, opens several avenues for future research. First, the use of complementary analytical techniques such as GC-MS could enable the precise identification of specific BVC whose concentration was affected after kombucha ingestion, thereby enhancing compound annotation beyond what is possible with SIFT-MS alone. **Second**, extending the breath sampling window would provide a more complete picture of the temporal dynamics of BVC. This would help determine how long compounds belonging to the >90-minute group remain reduced and whether their concentrations return to baseline or follow a different trajectory. **Third**, a longitudinal study evaluating chronic kombucha consumption could clarify whether BVC concentration that decrease after 90 minutes also show sustained reductions at baseline when assessing fasting breath profiles. This information would help determine whether acute effects translate into longer-term metabolic adaptations. **Fourth**, future studies could benefit from the inclusion of stool samples, which would enable direct comparison between breath-derived metabolites and gut microbiota composition. Integrating microbiome data would offer valuable insights into the microbial origins of specific BVC and strengthen the interpretation of host–microbiota metabolic interactions.

Furthermore, future research could explore the influence of other dietary components known to modulate the gut microbiota and assess their impact on breath composition. In particular, our laboratory, in collaboration with the University of Mons and the CICN, will study for interventions involving yoghurts enriched with purple bacteria, as a prebiotic or probiotic strategy, to assess potential changes in the breath volatilome.

6. Results – MICROBIOSTRESS study

6.1. Study population

The 70 volunteers included in the study were classified in moderate-stress group and high-stress group based on PSS score. Although the 70 fasted stressed volunteers – 48 women and 22 men (31 [24; 46] years old, 23.6 [21.4; 26.6] kg/m²) – exhibited wide variability in biological characteristics, their repartition into the

moderate-stress and the high-stress groups was dependant of sex ($p > 0.05$, Fisher's exact test), age or BMI on stress level ($p > 0.05$, Mann-Whitney test) (**Table 3**). Volunteers with high stress showed significantly ($p < 0.05$, Mann-Whitney test) higher scores on STAI, FAS and ISI, suggesting that high stress is strongly associated with more anxiety, more fatigue, and more insomnia. No significant differences ($p > 0.05$, Mann-Whitney test) were observed between moderate and high stress groups for BDI scores, blood LBP concentrations, or salivary cortisol levels. Repartition of the number of volunteers with moderate-stress and the high-stress in each questionnaire scores was presented in supplemental data (**Table S3**).

Table 3 | Baseline characteristics of 70 fasted stressed volunteers classified into moderate stress and high stress groups according to the Perceived Stress Scale (PSS).

Data did not follow a normal distribution and are consequently presented as median $\pm$ interquartile range. P-values were obtained using Fisher's exact test (a) for categorical variables and the Mann-Whitney test (b) for continuous variables. Abbreviations: BDI, beck depression inventory; BMI, body mass index; FAS, fatigue assessment scale; ISI, insomnia severity index; LBP, LPS-binding protein; ns, not significant; STAI, state-trait anxiety inventory.

Characteristic	Total (n=70)	Moderate stress (n=44)	High stress (n=26)	P-value
Sex (♀/♂)	48/22	31/13	17/9	ns ^a
Age (years)	31 [24; 46]	31 [25; 49]	30 [24; 42]	ns ^b
BMI (kg/m ²)	23.6 [21.4; 26.6]	24.1 [22.0; 27.1]	22.5 [21.3; 25.0]	ns ^b
STAI score	52.5 [46.0; 61.5]	50.0 [42.5; 55.0]	60.0 [52.0; 64.0]	0.0003 ^b
BDI score	19.5 [12.5; 24.0]	15.5 [11.0; 20.3]	24.0 [20.0; 30.8]	ns ^b
FAS score	33.0 [27.0; 37.0]	31.0 [25.8; 36.0]	35.5 [30.0; 39.5]	0.02 ^b
ISI score	13.0 [8.0; 17.0]	12.5 [9.0; 17.0]	14.5 [7.3; 18.8]	0.0001 ^b
LBP (µg/mL)	10.3 [7.5; 13.5]	11.0 [7.8; 13.4]	9.8 [7.0; 13.4]	ns ^b

Characteristic	Total (n=59)	Moderate stress (n=39)	High stress (n=20)	P-value
Cortisol (nmol/L)	8.6 [6.1; 12.1]	8.6 [6.1; 12.1]	8.7 [7.1; 12.3]	ns ^b

6.2. Untargeted analysis of the SIFT-MS signals

PCA of untargeted breath profiles revealed no clear clustering based on questionnaire levels. To investigate whether overall breath composition varied with stress level (PSS), we applied PCA. The PCA score plot of the NAC based on MS-signal (from H₃O⁺, NO⁺, and O₂⁺ ionisations) revealed no distinct shifts in breath composition between moderate- and high-stressed groups (**Figure 10**). Some volunteers (222, 203, 253, 211) appeared slightly distinct. The first two components explained only a small proportion of variance (PC1: 9%, PC2: 5%). Similar analyses for anxiety (STAI), depression (BDI), fatigue (FAS), and insomnia (ISI) yielded comparable results, with no evident clustering in breath profiles according to questionnaire scores.

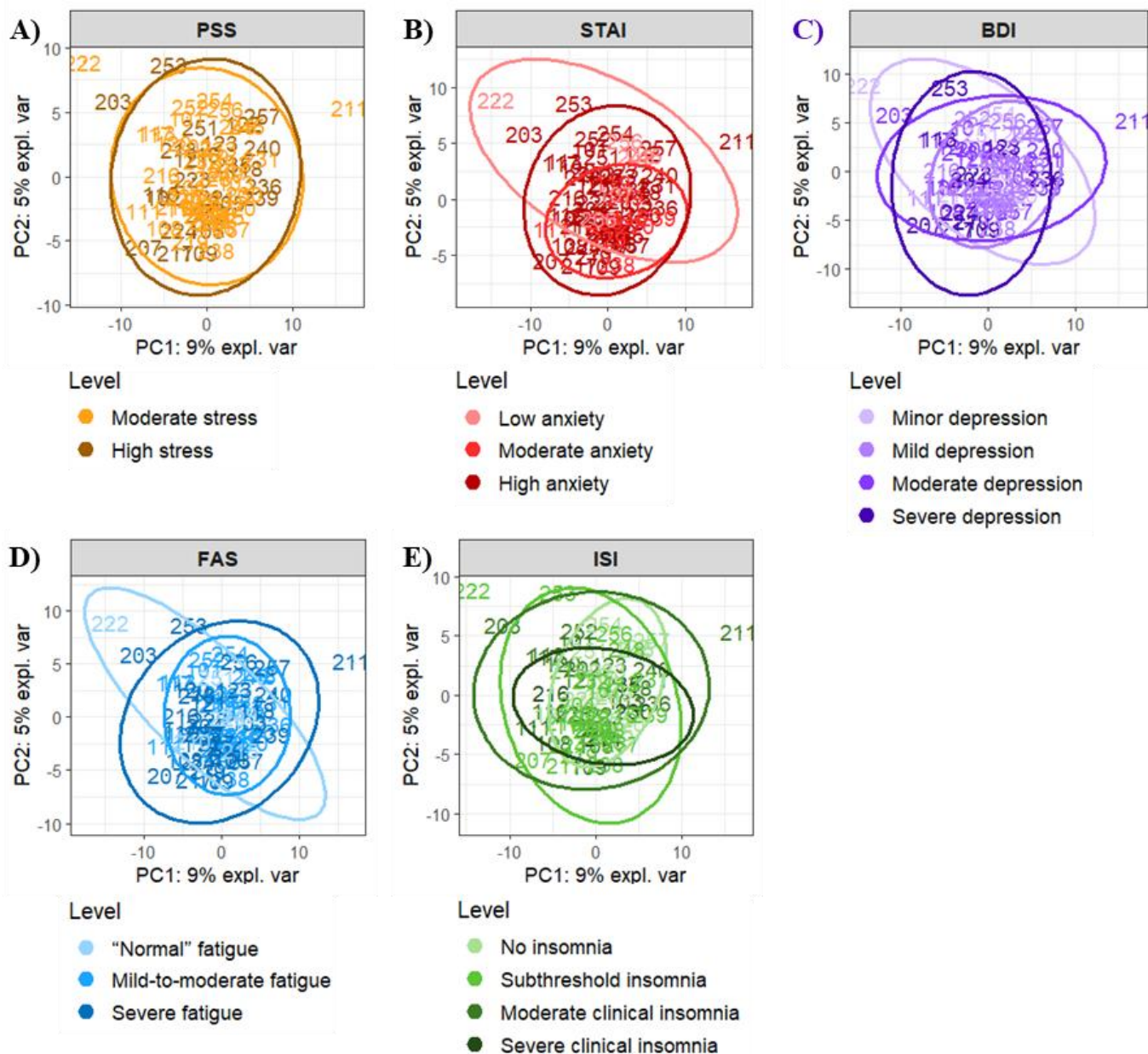


Figure 10 | PCA of untargeted breath profiles obtained using SIFT-MS. These untargeted breath profiles reflect the concentration of all signals generated from reactions of breath volatile compounds with reagent ions (H_3O^+ , NO^+ , and O_2^+) within the SIFT-MS device. The PCA plots represent breath samples collected from 70 fasted stressed volunteers. Each dot corresponds to a single breath sample from one volunteer and is color-coded, with darker colours corresponding to a higher score, according to questionnaire scores: (A) stress, (B) anxiety, (C) depression, (D) fatigue, and (E) insomnia. Abbreviation: BDI, beck depression inventory; FAS, fatigue assessment scale; ISI, insomnia severity index; PCA, principal component analysis; PSS, perceived stress scale; SIFT-MS, selected ion flow tube mass spectrometry; STAI, state-trait anxiety inventory.

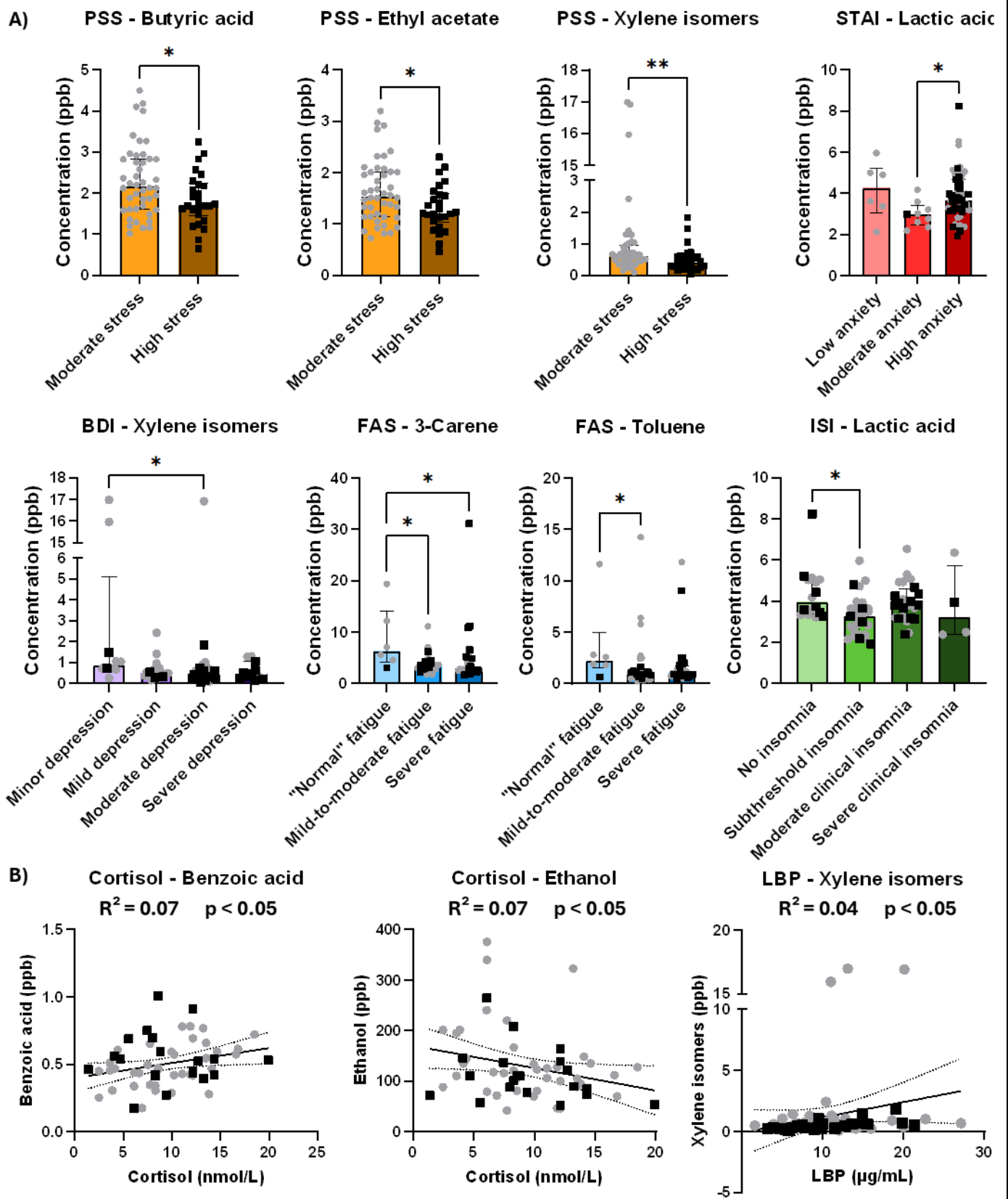
6.3. Targeted analysis of compound of interest

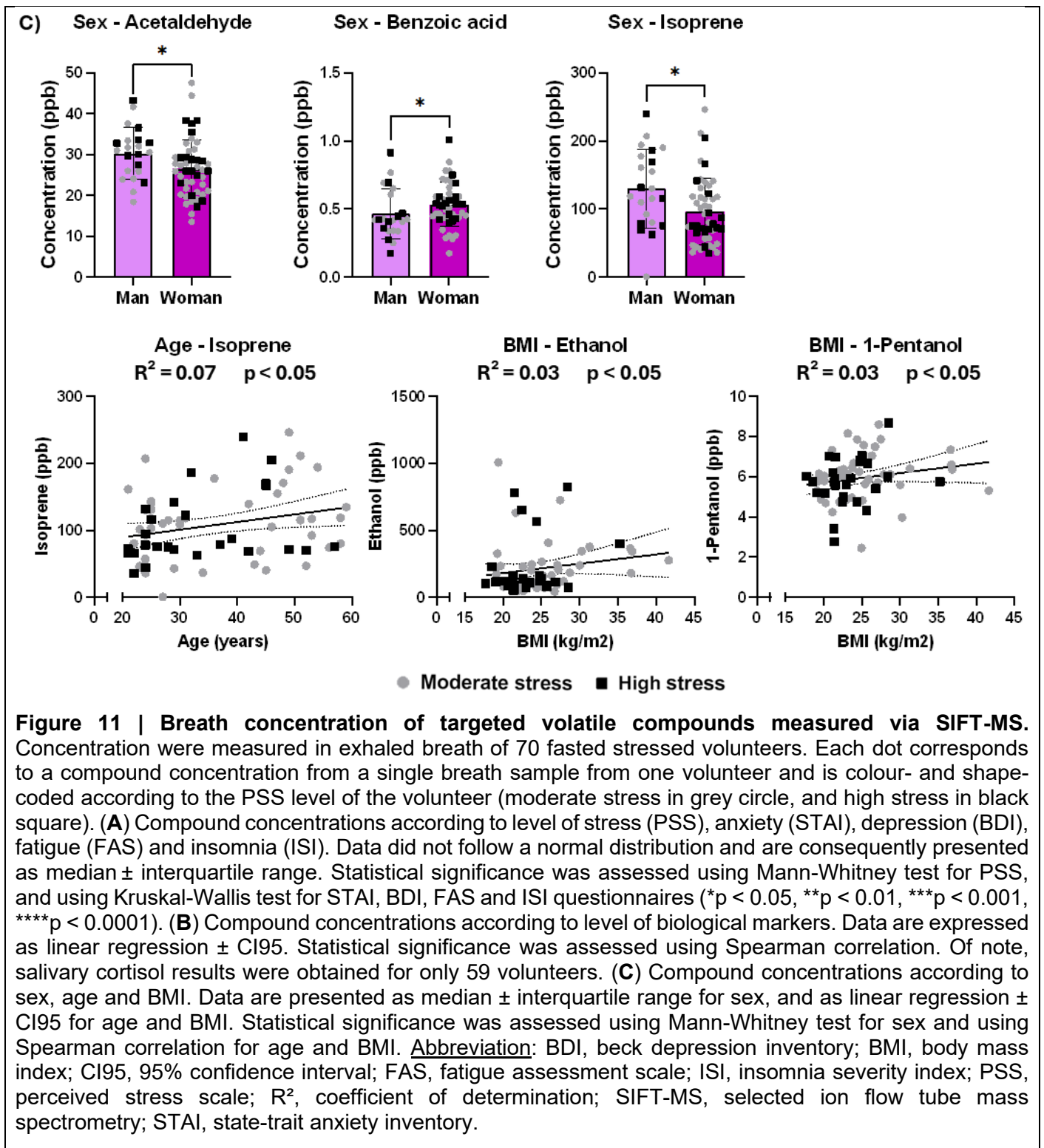
Targeting some compounds highlighted differences in breath composition related to questionnaire scores. While no global change in breath profile was observed in the context of stress, anxiety, depression, fatigue and

insomnia, specific concentration changes were observed for some targeted BVC that had already been discussed in the context of stress and in the context of microbial fermentation in the literature (**Figure 11A**). A significant decrease ($p < 0.05$, Mann-Whitney test) in the concentration of butyric acid, ethyl acetate, xylene isomers was observed with high stress assessed by PSS. Lactic acid concentration increased ($p < 0.05$, Kruskal-Wallis test) in high anxiety compared to moderate anxiety (STAI). For BDI, xylene isomer levels dropped ($p < 0.05$, Kruskal-Wallis test) in moderate depression compared to minor depression. 3-Carene levels decreased ($p < 0.05$, Kruskal-Wallis test) in mild-to-moderate and severe fatigue (FAS) compared to normal fatigue. A significant diminution ($p < 0.05$, Kruskal-Wallis test) of concentration was also observed in toluene in mild-to-moderate fatigue compared to normal fatigue. Lactic acid concentration also dropped ($p < 0.05$, Kruskal-Wallis test) in subthreshold insomnia compared to no insomnia (ISI).

Some breath compounds concentration was related to the level of biological markers of stress and inflammation. The concentration of benzoic acid increased while the concentration of ethanol decreased ($p < 0.05$, Spearman correlation) with the concentration of salivary cortisol, a stress hormone (**Figure 11B**). The concentration of xylene isomers increased ($p < 0.05$, Spearman correlation) with the concentration of LBP, an inflammatory blood marker reflecting the body's exposure to bacterial components from the gut.

Host factors had an influence on specific compounds levels in exhaled breath. Concentration of acetaldehyde and isoprene were lower in women than in men while benzoic acid was higher (**Figure 11C**). Concentration of isoprene increased with age. Concentration of ethanol and 1-pentanol increased with BMI. All these correlations were significative ($p < 0.05$, Spearman correlation), but coefficients of determination (R^2) were close to zero.





7. Discussion – MICROBIOSTRESS study

In this study, we investigated the impact of the level of stress on gut microbiota and host-related metabolism using breath volatilome analysis. While no global change in breath profile was observed in the PCA in the context of stress, anxiety, depression, fatigue, and insomnia, the targeted analysis revealed decreased concentrations of

butyric acid, ethyl acetate and xylenes in volunteers with higher stress levels. We also explored associations between the breath volatilome and biological markers including cortisol and LBP, as well as host factors including age, sex, and BMI.

Stress levels, as reflected in higher PSS scores, appear closely linked to elevations in anxiety (STAI), fatigue (FAS), and insomnia (ISI) severity. Chronic activation of the HPA axis leads to persistent glucocorticoid release, while heightened sympathetic activity and increased catecholamine levels intensify arousal and anxiety, potentially contributing to depressive symptoms (Godoy et al., 2018). Sustained HPA axis activation under prolonged stress also keeps evening cortisol abnormally high, disrupting sleep onset, fragmenting deep sleep, and increasing nighttime awakenings (Xue et al., 2025). Although our findings confirm a clear association between stress levels and anxiety, fatigue, and insomnia, no significant link with depression was observed in our data.

PSS levels did not significantly influence salivary cortisol concentration. Saliva is increasingly recognized as a valuable biofluid for non-invasive cortisol monitoring, providing a convenient and stress-free alternative to blood or serum sampling. Its simplicity, painlessness, and independence from trained personnel make it particularly suitable for repeated or ambulatory testing, with strong relevance for stress research, occupational health, and clinical diagnostics. Importantly, salivary cortisol reflects the free, biologically active fraction of circulating hormone. However, salivary analysis is subject to several methodological challenges. Saliva is a complex and variable matrix, and factors such as flow rate, circadian fluctuations, pH changes, and contamination from food or oral hygiene products can introduce noise and reduce reproducibility (Messina et al., 2025).

In the PCA of untargeted breath data, four volunteers (ID 222, 203, 253, and 211) were separated from the main cluster of volunteers without an apparent explanation. Volunteers 203 and 253 had medical histories (a caesarean section and a surgically corrected congenital colon malformation, respectively), but these conditions are not known to influence breath composition. Both volunteers also reported the use of medications (contraception and cold medication, respectively) and experienced minor health symptoms such as headache or cold symptoms during the study; however, similar reports were common among other volunteers. No particular characteristics or events were reported for volunteers 211 and 222.

The lower concentrations of butyric acid, ethyl acetate, and xylene isomers observed in volunteers with higher stress levels are consistent with previous data. Reduced concentrations in butyric acid has been repeatedly reported in patients with major depressive disorder across stool and blood matrices, likely reflecting a diminished abundance of butyrate-producing genera (Cheng et al., 2024; Yu et al., 2023). Interestingly, in this master's thesis, these associations did not appear when using BDI scores. Decreased concentrations in ethyl acetate and xylene isomers have similarly been documented in the breath volatilome of healthy adults undergoing acute stress induced by the Paced Auditory Serial Addition Test (PASAT) (Martin et al., 2016; Santos et al., 2020). Both compounds are considered to be of exogenous origin: ethyl acetate through environmental or dietary exposure (Kim, 2025), and xylenes through coating and paints, vehicle exhaust, or cigarette smoke (Tang et al., 2024). Their reduced levels under stress have been attributed to physiological responses, including increased ventilation, respiratory rate, and heart rate (Martin et al., 2016; Santos et al., 2020). Although ethyl acetate has previously been detected in exhaled breath (Martinez-Lozano Sinues et al., 2013), its identification by Santos et al. (2020) remained uncertain. Beyond acute stress, evidence from another study suggests that more chronic states, such as depressive symptoms, may also be associated with increased levels of xylenes (Tang et al., 2024). However, this finding is inconsistent with the decreased concentrations in xylenes observed with moderate depression compared to minor depression.

The changes observed in 3-carene, toluene and lactic acid concentrations in relation to potential stress consequences are more difficult to interpret considering existing literature. Decreased concentrations in 3-carene and toluene have previously been reported following acute stressors such as PASAT or Trier social stress test (TSST) (Mansour et al., 2023; Martin et al., 2016). While no consistent evidence links 3-carene to fatigue, toluene – a component of gasoline, paints and cleaning agents (Kim, 2025) – is known to cause symptoms such as fatigue and headache (Baelum et al., 1985). However, these findings do not allow us to explain the significant diminution in 3-carene with mild-to-moderate and severe fatigue compared to normal fatigue, and the significant diminution in toluene with mild-to-moderate compared to normal fatigue. Decreased lactic acid levels have also previously been reported, in both anxiety and insomnia. For example, athletes with moderate to severe anxiety exhibited lower faecal L-lactic acid levels, and experimental work suggests that

lactate in combination with *Akkermansia muciniphila*, may decrease anxiety-related metabolic pathways (Pan et al., 2025). Similarly, insomnia has been linked to reductions in circulating D-lactic acid and related gut-derived markers, likely reflecting disruptions in gut barrier and blood–brain barrier integrity (Cai et al., 2024). While our observation of elevated lactic acid in high versus moderate anxiety does not align with these findings, the decrease in lactic acid with subthreshold insomnia compared to no insomnia is directionally consistent with the literature. Nonetheless, in both anxiety and insomnia, our results did not reveal a coherent pattern across severity categories, limiting firm interpretation.

We report, as previously suggested in animals, an increased concentration in benzoic acid correlated with increased concentration of cortisol. In weaned piglets, stress-induced cortisol elevation was shown to modify the gut microbiome, favouring *Prevotellaceae* dominance. This microbial shift was positively correlated with metabolites such as benzoic acid, acetic acid, cholesterol, and glycerol. Importantly, the rise in benzoic acid appeared not to result from a direct metabolic action of cortisol, but rather from cortisol-driven alterations in microbial composition (Jiang et al., 2020).

The increased concentration of xylenes with higher LBP levels was consistent with previous data. Indeed, recent research shows that air pollutants like PM2.5 often carry LPS and other pro-inflammatory components capable of activating TLR4 signalling, disrupting gut homeostasis, and promoting both systemic and neuroinflammation (Campolim et al., 2024).

Variations in exhaled breath composition attributable to host factors (sex, age, BMI) have been previously reported, independently of stress. The lower acetaldehyde concentrations observed in women compared with men are consistent with earlier findings (Jia et al., 2024), which is consistent with our results. Isoprene, a breath volatile linked to cholesterol metabolism and muscle activity (Sukul et al., 2023), has also been shown to vary with host factors. Several studies have reported higher isoprene levels in men and with age (Jia et al., 2024; Sukul et al., 2023), in agreement with our results. However, results across the literature remain inconsistent: large cohort analyses found no significant sex differences and even reported age-related declines in men only (Kushch et al., 2008), while other longitudinal SIFT-MS studies detected no clear associations with sex, age, BMI, or cholesterol status (Turner et al., 2006). Collectively, these findings highlight that while isoprene is a robust breath volatile, its

dependence on sex and age remains inconsistent, likely reflecting methodological differences and population heterogeneity. In contrast, several other differences in host factors identified in our study have not yet been documented. For example, no published evidence was found establishing a link between 1-pentanol or ethanol and BMI, or between benzoic acid and sex.

This study presents several limitations that should be considered when interpreting the results. First, volunteers' dietary habits and smoking status missed. Dietary intake was collected using the ASA24 questionnaire but has not yet been analysed. Given that previous work has shown that BVC can reflect gut microbiota activity (Neyrinck et al., 2022; Neyrinck et al., 2021), incorporating habitual dietary data would help determine whether systematic differences in diet confound the observed metabolic patterns and would strengthen the robustness of the conclusions. Besides, smoking status was not recorded, despite smoking being a well-established confounder known to increase the exhalation of several volatile organic compounds – including toluene, benzene, xylenes, and acetaldehyde (Filipiak et al., 2012). **Second,** the statistical interpretation of BVC associations with stress and biological is constrained by multiple testing, low analyte concentrations, and weak correlations. Several associations, appearing significant at $p < 0.05$ in **Figure 11**, did not remain significant after false discovery rate correction ($q < 0.1$). Moreover, many compounds – butyric acid, ethyl acetate, xylene isomers, lactic acid, benzoic acid and 1-pentanol – initially linked to emotional scores, salivary cortisol, LBP, or host factors were present at very low levels – often below the LOQ. However, the LOQ used may have been too strict. Finally, despite some significant Spearman correlations, coefficients of determination were near zero and scatter plots showed no clear trends, suggesting that these findings may reflect random variation rather than true biological effects. Nevertheless, some of the trends observed were consistent with the previous studies presented above.

The next steps in this work include several planned analyses and follow-up measures. First, ASA24 dietary assessment and stool samples still need to be analysed. The ASA24 data will provide valuable insights into habitual eating patterns and may help clarify how diet contributes to variability in breath metabolites, ultimately reinforcing the interpretation of stress-related effects. Stool sample analysis will provide gut microbiota composition, creating an important opportunity to link gut microbial activity with exhaled BVC patterns in stressed volunteers. **Second,** as the

study progresses toward its target of 150 volunteers, the larger sample size will enhance cohort representativeness and allow more refined subgroup analyses across demographic and lifestyle variables, ultimately providing a more robust foundation for assessing variability in BVC profiles and their potential clinical relevance. **Third**, statistical mediation analysis could be conducted to investigate the relationships between microbiota, stress and exhaled breath. **Fourth**, a subset of volunteers will provide a second breath sample after treatment (tablet with plant combination vs plant combination plus probiotics vs control), offering the opportunity to assess the impact of plants and probiotics on stress level, gut microbiome and breath composition.

8. Conclusion and perspectives

This master's thesis demonstrates that exhaled breath analysis represents an innovative, non-invasive approach to study human metabolism and physiological responses to nutrition and stress, and reveals the potential impact on breath composition of compounds issued from microbial fermentation.

The MICROBOOST-1 Kombucha study highlighted time-dependent changes in breath composition upon ingestion of a fermented drink, namely kombucha. Five minutes post-ingestion, several compounds (e.g., ethanol, lactic acid) showed a transient peak, probably explained by residuals from the kombucha in the upper gastrointestinal tract. After 90 minutes, the concentration of unidentified compounds decreased, this event being hardly explained.

The MICROBIOSTRESS study revealed no major changes in breath composition among stress levels but highlighted reduced concentrations of specific compounds in highly stressed volunteers. Indeed, butyric acid levels decreased, which reflects a depletion of butyrate-producing bacteria in the gut microbiota associated with changes of mood and psychotic disorders. Moreover, we confirmed ethyl acetate and xylenes depletion in the context of stress. We also related breath BVC profiles to questionnaires assessing psychological stress parameters (anxiety, depression, fatigue and insomnia), to biological stress/inflammation markers (cortisol, LBP) as well as to other host factors (sex, age, BMI). We could point out changes in the concentration in targeted compounds linked to stress and that could be linked to gut microbial fermentation and/or to changes in metabolism (e.g. lactic acid, benzoic acid, ethanol, isoprene).

In conclusion, breath analysis could evolve into a valuable tool for personalized, real-time monitoring of diet and stress-related physiological responses. The next step will be to analyse the faecal microbiome profile in order to establish direct links between the intestinal microbial composition and metabolites derived from breath.

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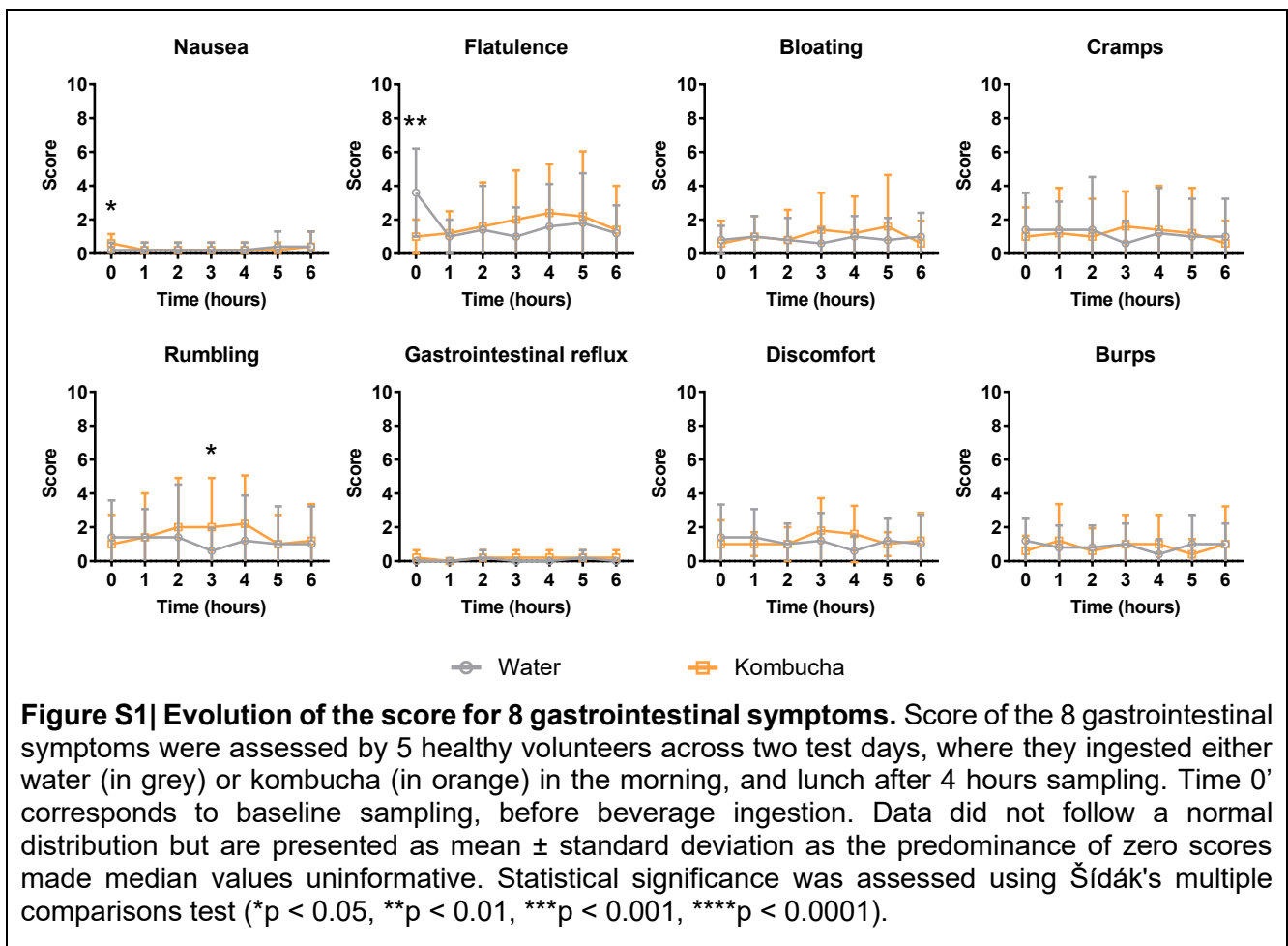
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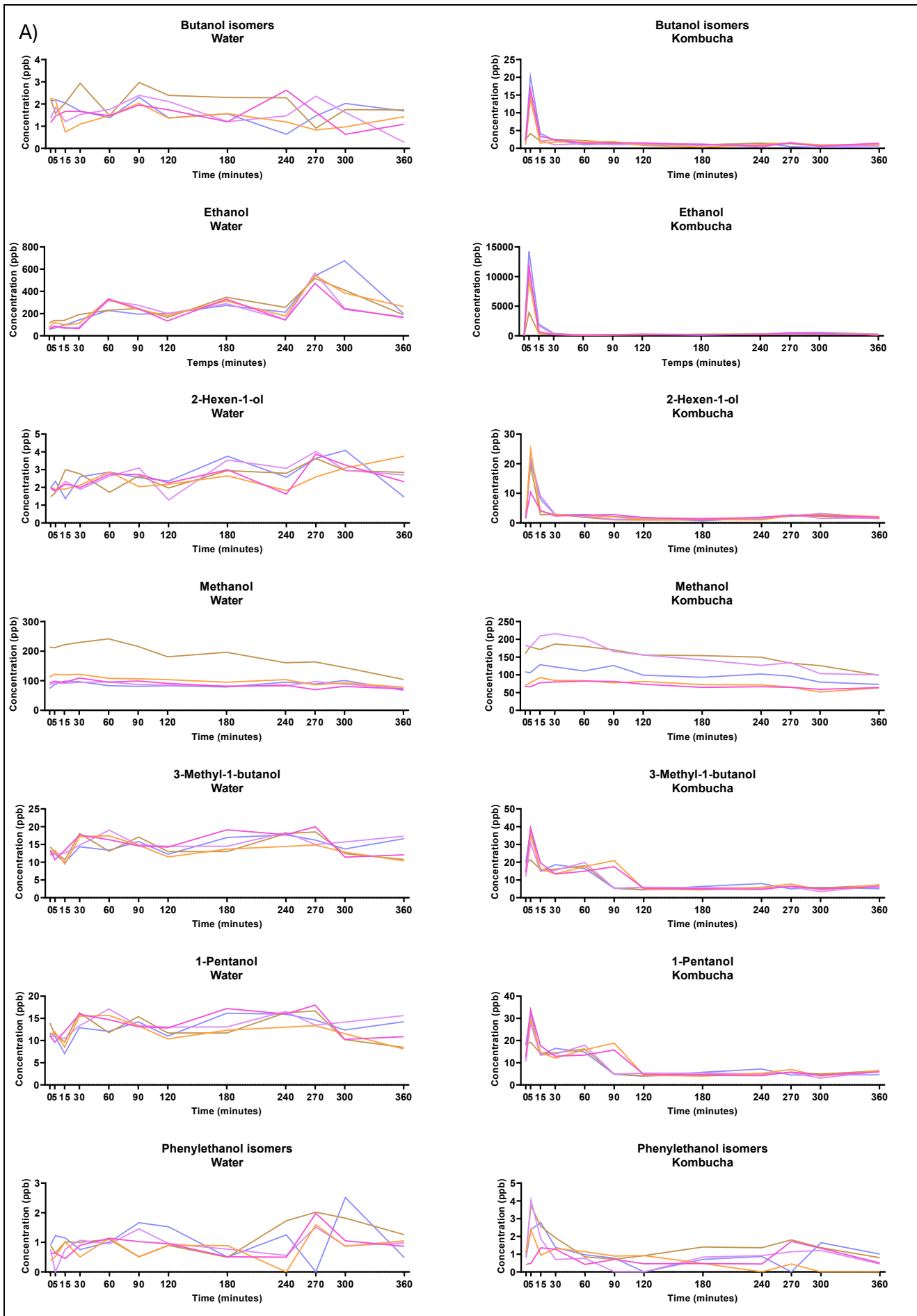
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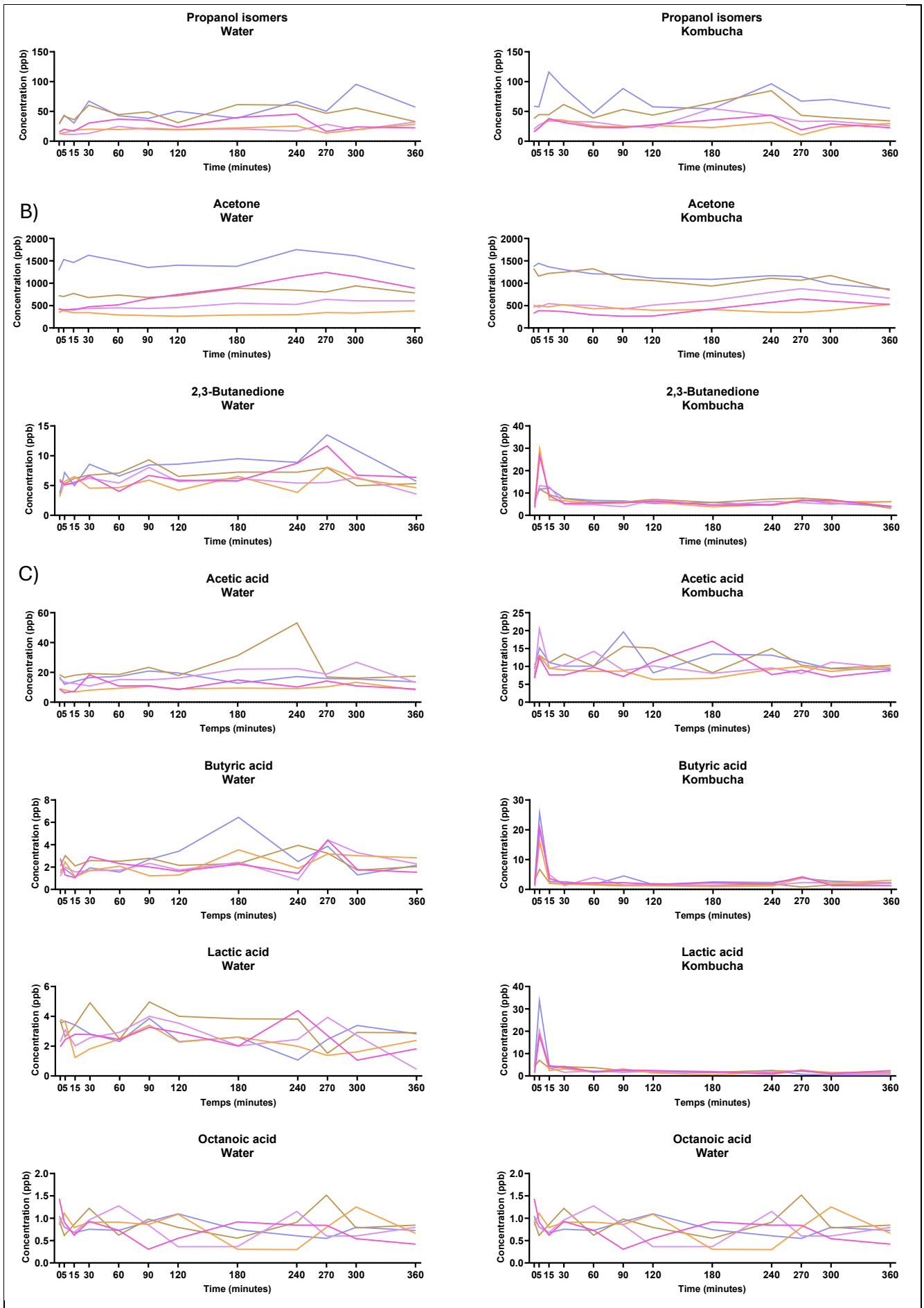
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Supplementary data

1. Supplementary figures







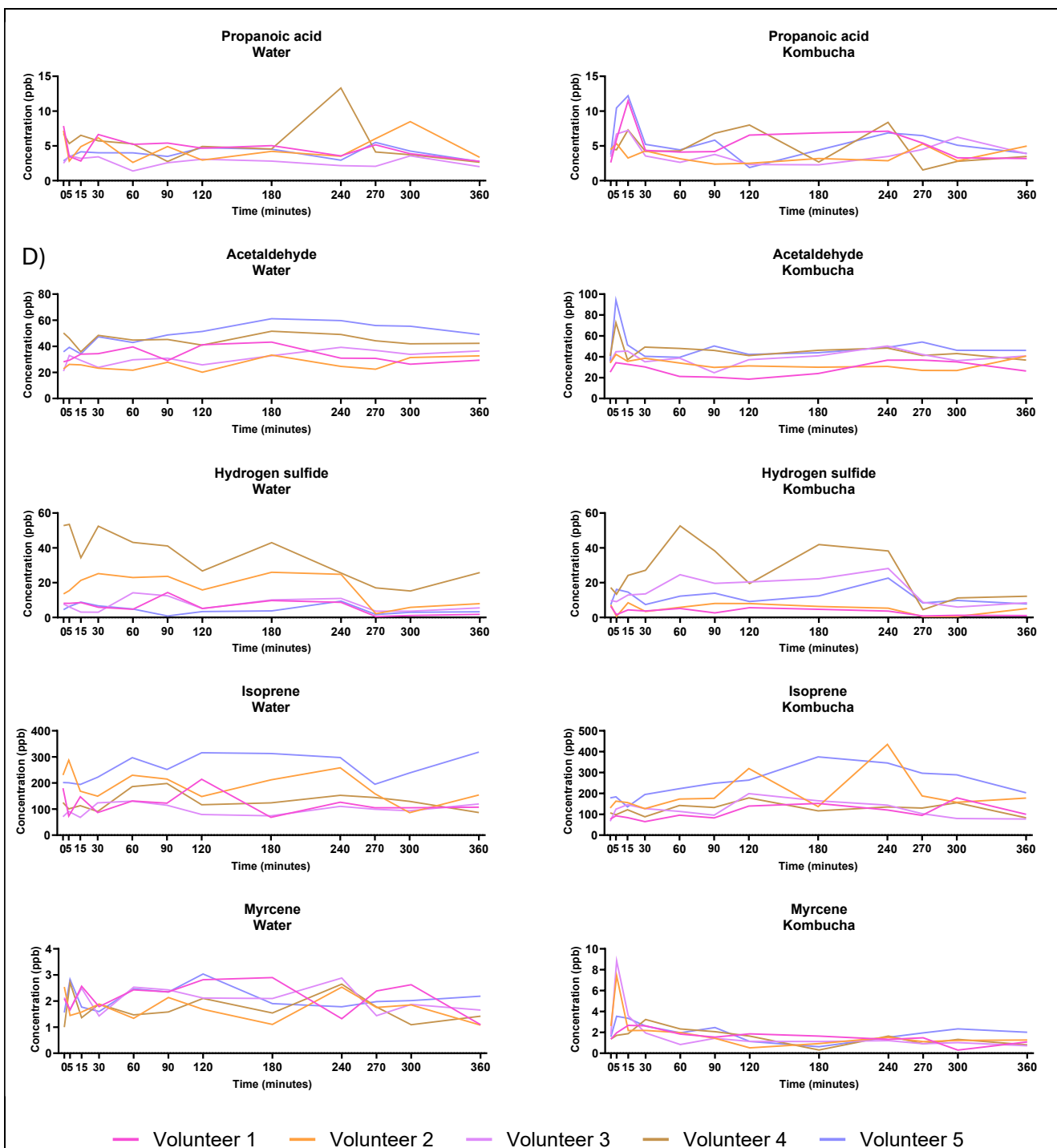


Figure S2 | Evolution of the concentration of targeted breath volatile compounds calculated via SIFT-MS. Concentrations were measured in exhaled breath of 5 healthy volunteers across two test days, where they ingested either water (on the right) or kombucha (on the left) in the morning, and lunch after 4 hours sampling. Time 0' corresponds to baseline sampling, before beverage ingestion. Compounds are grouped into four chemical classes: (A) alcohols, (B) ketones, (C) acids, and (D) other compounds. Data are expressed as individual values.

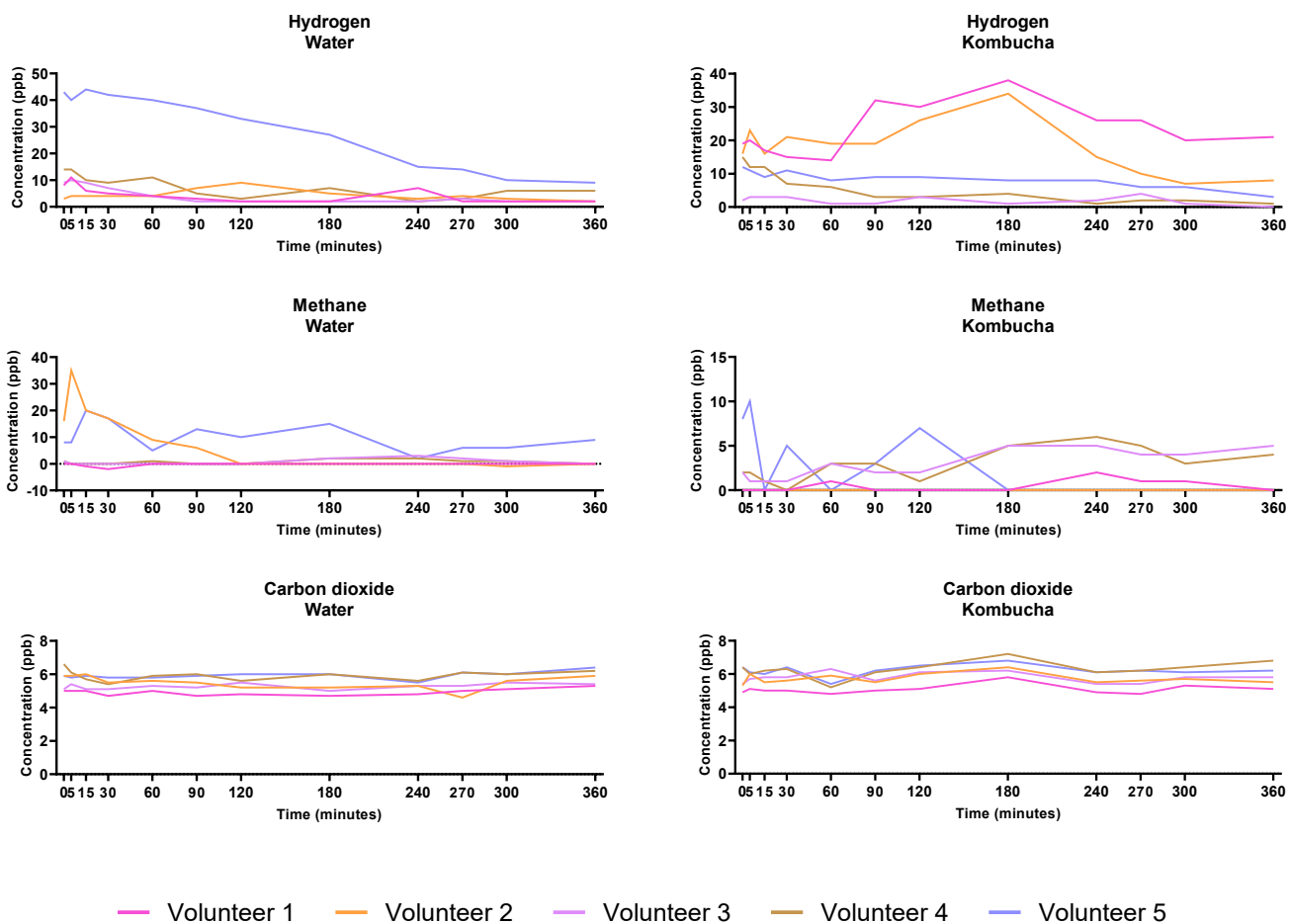


Figure S3 | Evolution of the concentration of hydrogen, methane and carbon dioxide measured with Lactotest®. Concentrations were measured in exhaled breath of 5 healthy volunteers across two test days, where they ingested either water (on the right) or kombucha (on the left) in the morning, and lunch after 4 hours sampling. Time 0' corresponds to baseline sampling, before beverage ingestion. Data are expressed as individual values.

2. Supplementary tables

Table S1 | Summary list of breath volatile compounds targeted in the context of kombucha intake. Volatile compounds were selected based on (1) their presence in the kombucha used (So Wood Kombucha®), as determined by aroma profiling using gas chromatography - mass spectrometry, and (2) previously reported associations with microbial fermentation processes in the gut. Isoprene and acetone – two of the most abundant endogenous compounds in exhaled breath – were also included. For each compound, the table indicates its relevance to kombucha, fermentation, or endogenous abundance, as well as the analytical limit of quantification (LOQ), expressed in parts-per-billion (ppb).

Name	Compounds detected in So Wood Kombucha	Compounds associated with fermentation	Most abundant endogenous compounds	LOQ (ppb)
Acetaldehyde		(Dinis-Oliveira, 2021)		11.6
Acetic acid	Yes	(Mutuyemungu et al., 2023; Oliphant & Allen-Vercoe, 2019)		6.5
Acetone			(Fenske & Paulson, 1999)	6.0
2,3-Butanedione		(Li et al., 2023)		9.6
Butanol isomers	Yes	(Vernocchi et al., 2023)		3.3
Butyric acid		(Mutuyemungu et al., 2023; Oliphant & Allen-Vercoe, 2019)		8.2
Ethanol		(Dinis-Oliveira, 2021; Oliphant & Allen-Vercoe, 2019)		26.8
2-Hexen-1-ol	Yes			3.4
Hydrogen sulfide		(Barton et al., 2017; Oliphant & Allen-Vercoe, 2019)		3.2
Isoprene			(Fenske & Paulson, 1999)	1.8
Lactic acid		(De Filippis et al., 2020; Mutuyemungu et al., 2023; Oliphant & Allen-Vercoe, 2019)		5.4
Methanol		(Oliphant & Allen-Vercoe, 2019)		85.6
3-Methyl-1-butanol	Yes			12.1
Myrcene	Yes			2.0
Octanoic acid	Yes			1.8
1-Pentanol		(Vernocchi et al., 2023)		11.6
Phenylethanol isomers	Yes	(Kanehisa et al., 2023; Wishart et al., 2023)		1.6
Propanoic acid		(Louis & Flint, 2017; Mutuyemungu et al., 2023; Oliphant & Allen-Vercoe, 2019)		4.9
Propanol isomers		(Louis & Flint, 2017; Oliphant & Allen-Vercoe, 2019)		5.1

Table S2 | Summary list of breath volatile compounds targeted in the context of psychological stress level. Volatile compounds were selected based on previously reported associations with psychological stress and/or microbial fermentation processes in the gut. Isoprene and acetone – two of the most abundant endogenous compounds in exhaled breath – were also included. For each compound, the table indicates its relevance to psychological stress, fermentation, or endogenous abundance, as well as the analytical limit of quantification (LOQ), expressed in parts per billion (ppb).

Name	Compounds associated with stress	Compounds associated with fermentation	Most abundant endogenous compounds	LOQ (ppb)
Acetaldehyde		(Dinis-Oliveira, 2021)		11.6
Acetic acid	(Mansour et al., 2023)	(Mutuyemungu et al., 2023; Oliphant & Allen-Vercoe, 2019)		6.5
Acetone			(Fenske & Paulson, 1999)	6.0
Benzaldehyde	(Turner et al., 2013) (Santos et al., 2020)			2.0
Benzoic acid	(Mansour et al., 2023; Martin et al., 2016)			1.8
2,3-Butanedione		(Li et al., 2023)		9.6
Butanol isomers		(Vernocchi et al., 2023)		3.3
Butanone	(Mansour et al., 2023)			1.3
Butyric acid		(Mutuyemungu et al., 2023; Oliphant & Allen-Vercoe, 2019)		8.2
3-Carene	(Martin et al., 2016)			2.9
Dimethyl sulfone	(Mansour et al., 2023)			3.4
Ethanol	(Santos et al., 2020)	(Dinis-Oliveira, 2021; Oliphant & Allen-Vercoe, 2019)		26.8
Ethyl acetate	(Santos et al., 2020)			5.1
Hydrogen sulfide	(De Lima et al., 2020; Lima et al., 2013)	(Barton et al., 2017; Oliphant & Allen-Vercoe, 2019)		3.2
2-Hydroxy acetophenone	(Lucchi et al., 2024)			1.8
Indole	(Turner et al., 2013)			1.0
Isoprene			Yes (Fenske & Paulson, 1999)	1.8
Lactic acid		(De Filippis et al., 2020; Mutuyemungu et al., 2023; Oliphant & Allen-Vercoe, 2019)		5.4
Methanol		(Oliphant & Allen-Vercoe, 2019)		85.6
Xylene isomers	(Martin et al., 2016)			2.1
2,3-Pentanedione	(Santos et al., 2020)			3.8
1-Pentanol		(Vernocchi et al., 2023)		11.6
1-Pentene	(Mansour et al., 2023)			22.9
Phenylethanol isomers		(Kanehisa et al., 2023; Wishart et al., 2023)		1.6
Propanoic acid	(Lucchi et al., 2024; Mansour et al., 2023)	(Louis & Flint, 2017; Mutuyemungu et al., 2023; Oliphant & Allen-Vercoe, 2019)		4.9

Propanol isomers	(Santos et al., 2020)	(Louis & Flint, 2017; Oliphant & Allen- Vercoe, 2019)	5.1
Styrene	(Santos et al., 2020)		1.4
Toluene	(Mansour et al., 2023)		2.0

Table S3 | Distribution of volunteers across emotional questionnaires according to perceived stress levels. The 70 fasted stressed volunteers included in the study were classified into moderate stress and high stress groups according to the Perceived Stress Scale (PSS). Volunteers completed questionnaires about anxiety (State-Trait Anxiety Inventory, STAI), depression (Beck Depression Inventory, BDI), fatigue (Fatigue Assessment Scale, FAS) and insomnia (Insomnia Severity Index, ISI). For each scale, the number of volunteers belonging to defined severity categories is indicated for the total sample and each stress subgroup.

		Total (n=70)	Moderate stress (n=44)	High stress (n=26)
STAI	Low anxiety	6	6	0
	Moderate anxiety	9	8	1
	High anxiety	55	30	25
BDI	Minor depression	10	8	2
	Mild depression	22	19	3
	Moderate depression	27	14	13
	Severe depression	11	3	8
FAS	“Normal” levels of fatigue	6	5	1
	Mild-to-moderate fatigue	34	24	10
	Severe fatigue	30	15	15
ISI	No insomnia	16	9	7
	Subthreshold insomnia	26	20	6
	Clinical insomnia (moderate severity)	24	12	12
	Clinical insomnia (severe)	4	3	1

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Introduction: Analysis of exhaled volatile compounds – the breath volatilome – is a promising non-invasive approach for studying human physiology. These compounds, which originate from the environment or endogenous metabolism, may also reflect the activity of the gut microbiota. This master's thesis studies in vivo the impact on the breath volatilome (1) of the ingestion of kombucha – a fermented birch-based tea with potential health benefits – in healthy volunteers and (2) of psychological stress levels. The aim is to discover markers reflecting the interaction between human metabolism and the gut microbiota through breath analysis.

Materials and methods: In the kombucha study, five healthy fasting volunteers (two women, three men; 20 [19; 21] years old) participated in two days of testing to compare kombucha consumption with water consumption (control). Several breath samples were collected before and up to 360 minutes after ingestion. In the stress study, conducted by the CICN, 70 volunteers (48 women, 22 men; 31 [24; 46] years old) selected on the basis of a score above 14 on the Perceived Stress Scale (PSS) provided a single fasting breath sample to compare moderate and high stress levels. Both studies used selected ion flow tube mass spectrometry (SIFT-MS), enabling untargeted volatilome profiling and targeted quantification of specific compounds.

Results: The kombucha study showed changes in breath composition appearing 5 minutes and 120 minutes after kombucha consumption. The transient peak of several compounds (such as ethanol and lactic acid) 5 minutes after ingestion was thought to be due to kombucha residues in the upper gastrointestinal tract. The stress study did not reveal any overall change in breath composition based on stress level, but showed a reduction in the concentrations of specific metabolites already observed in the volatilome of more stressed volunteers, such as ethyl acetate and xylene isomers, as well as new compounds such as butyric acid, which may indicate a decrease in associated butyrate-producing bacteria.

Conclusion: This master's thesis confirmed that breath analysis can detect changes related to kombucha consumption and psychological stress. In the future, such an approach could enable personalised, real-time monitoring of diet and its impact on stress-related physiological responses.