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ORIGINAL ARTICLE

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Volatilomic signatures of different strains of *Helicobacter pylori*

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Abstract

Background: *Helicobacter pylori* (*H. pylori*) infection is the most extensively studied risk factor for gastric cancer. As with any bacteria, *H. pylori* will release distinctive odors that result from an emission of volatile metabolic byproducts in unique combinations and proportions. Effectively capturing and identifying these volatiles can pave the way for the development of innovative and non-invasive diagnostic methods for determining infection. Here we characterize the *H. pylori* volatilomic signature, pinpoint potential biomarkers of its presence, and evaluate the variability of volatilomic signatures between different *H. pylori* isolates.

Materials and Methods: Using needle trap extraction, volatiles in the headspace above *H. pylori* cultures were collected and, following thermal desorption at 290°C in a splitless mode, were analyzed using gas chromatography–mass spectrometry. The resulting volatilomic signatures of *H. pylori* cultures were compared to those obtained from an analysis of the volatiles in the headspace above the cultivating medium only.

Results: Amongst the volatiles detected, 21 showed consistent differences between the bacteria cultures and the cultivation medium, with 11 compounds being elevated and 10 showing decreased levels in the culture's headspace. The 11 elevated volatiles are four ketones (2-pentanone, 5-methyl-3-heptanone, 2-heptanone, and 2-nonanone), three alcohols (2-methyl-1-propanol, 3-methyl-1-butanol, and 1 butanol), one aromatic (styrene), one aldehyde (2-ethyl-hexanal), one hydrocarbon (n-octane), and one sulfur compound (dimethyl disulfide). The 10 volatiles with lower levels in the headspace of the cultures are four aldehydes (2-methylpropanal, benzaldehyde, 3-methylbutanal, and butanal), two heterocyclic compounds (2-ethylfuran and 2-pentylfuran), one ketone (2-butanone), one aromatic (benzene), one alcohol (2-butanol) and bromodichloromethane. Of the volatile species showing increased levels, the highest emissions are found to be for 3-methyl-1-butanol, 1-butanol and dimethyl disulfide. Qualitative variations in their emissions from the different isolates was observed.

Conclusions: The volatiles emitted by *H. pylori* provide a characteristic volatilome signature that has the potential of being developed as a tool for monitoring

infections caused by this pathogen. Furthermore, using the volatilome signature, we are able to differentiate different isolates of *H. pylori*. However, the volatiles also represent potential confounders for the recognition of gastric cancer volatile markers.

KEYWORDS
biomarker, gastric cancer, GC–MS, *Helicobacter pylori*, volatile organic compounds, volatilome

1 | INTRODUCTION

Recent studies have documented those volatile organic compounds (VOCs), which form a "volatilome", play important roles in human organism.[1,2] For example, the biosignatures of human VOCs have the potential of providing information on the presence of disease or infection, and hence have the potential for use as diagnostic tools. This potential exists because the accompanying abnormal processes in the human body change the volatile signatures as a result of upregulation or downregulation of enzyme activities, oxidative stress, gene activation or repression, and microbiota alterations. Volatilomics tries to capture these alterations in the volatiles coming from different bodily excretions, such as breath, skin emanations, urine, saliva, or sweat. The collection of the volatiles is non-invasive, which causes no or little risk to patients, and forms the basis for development of novel, easy to use, and efficient diagnostic tests for clinical applications, especially as the VOC signatures can be readily mapped using portable and low-cost analyzers based on miniature chemical sensors.[3] For these reasons, volatilomic signatures have been a subject of intense research in recent years.[1,2]

With regards to volatiles associated with bacteria, a whole plethora of microbial microorganisms coexists in the human body, especially in the gut. Gut microbiota is a rich ecosystem, embracing bacteria, protozoa, archaea, viruses, and fungi, that plays a crucial role in the human organism via participation in digestion, immunomodulation, and cardiovascular system performance, but also in diseases and pathological disorders such as allergies, obesity, cardiovascular diseases, or colorectal cancer.[4,5] The microbiome species present in the gut emit a multitude of VOCs via aerobic and anaerobic fermentation processes, which enter the bloodstream via the gut-blood barrier. Once these volatiles have passed through this barrier, they are distributed throughout the body, thereby contributing to the human volatilome. Thus, profiling of human volatilome may be a good approach to find volatiles that have the potential for use as biomarkers for gastrointestinal diseases.[6,7] The identification of pathogen-specific compounds could lead the way for the rapid identification of pathogenic micro-habitants, which can form the basis for early diagnosis of acute and frequently lethal bacterial infections.

A common bacterial gut infection is *Helicobacter pylori* (*H. pylori*). *H. pylori* is a gram-negative bacterium that colonizes the human stomach and has been identified as a major risk factor for various gastrointestinal diseases, including chronic gastritis, peptic ulcers, and gastric cancer.[8] Chronic infection with *H. pylori* triggers an inflammatory response that can induce DNA damage, disrupt cell cycle regulation, and promote cancerous growth in the stomach lining.[8]

Gastric cancer is a globally important problem.[8] Mortality from gastric cancer is high and accounts for 22% of worldwide cancer related deaths. In its early stages, symptoms are not specific, resulting in an often late and incurable diagnosis and a 5-year-survival rate of 23%–36%.[8] Since detection at an early stage is critical for a patient's survival, non-invasive and low-cost screening methods are urgently needed. Several recent studies employed volatilomics for gastric cancer detection and classification. These studies, using sensor technologies to detect volatiles contained in the exhaled breath of humans, demonstrated that it is possible to discriminate gastric cancer patients from patients with other diseases, including pre-malignant conditions as well as controls, with a sensitivity and a specificity ranging from 0.67 to 1.0 and from 0.71 to 0.98, respectively.[9] However, the main limitation of this approach is the lack of knowledge on the identity of substances forming the disease-specific breathprints.

H. pylori infection is the most extensively studied risk factor for gastric cancer and accompanies the gastric cancer development.[9] Thus, the chemical signatures of *H. pylori* may overlap with the signatures related to gastric cancer, thereby complicating the identification of volatile biomarkers directly associated with the cancer. In other words, the *H. pylori*-related volatiles can be wrongly interpreted as gastric cancer biomarkers. In this context, *H. pylori* infection is a confounding factor in the use of volatile biomarker discovery phase for gastric cancer. Thus, the identification of VOCs released by *H. pylori* is of particular importance for the validation of potential respiratory markers of gastric cancer. Such knowledge may assist the discovery of biomarkers in breath or urine, and hence support studies that aim to investigate volatile signatures for use in the non-invasive detection of gastric cancer.

The key objective of the work presented in this paper is to characterize the *H. pylori* volatilomic signature and pinpoint useful biomarkers of its presence. A secondary objective is to evaluate the variability of volatilomic signatures between different *H. pylori* isolates, with the aim to investigate the potential differences between isolates that could then potentially be used to recognize different strains of *H. pylori*. Qualitative differences in the VOC patterns associated with the different isolates of *H. pylori* might help to identify a specific isolate and thereby optimize the treatment of *H. pylori* infection.

2 | MATERIALS AND METHODS

2.1 | Chemicals and standards

Calibration mixtures were produced using high-purity liquid substances. Reference chemicals, with stated purities of 95%–99.9%, were purchased from Merck (Austria) and Fluka (Switzerland). A calibration mixture was prepared by injecting and evaporating several μLs of a liquid compound into an evacuated and heated 1 L glass bulb (Supelco, Canada). Desired calibration concentrations were obtained by transferring appropriate volumes of a primary mixture into 3–25 L Tedlar bags (SKC Inc., USA) that were pre-filled with purified and humidified air (100% RH at 23°C). Ultimately, gas mixtures with VOC volume mixing ratios ranging from 0.07 to 60 parts per billion were used for calibration. The calibration curves relied on the use of seven distinct and independent concentration levels.

2.2 | Cultivation of *H. pylori*

H. pylori isolates used in this study were obtained from gastric mucosal biopsies and frozen at −80°C in a 20% glycerol solution containing glass beads (Viabank, Belgium). The intensities of the volatiles coming from *H. pylori* are relatively weak in comparison to those coming from the culturing media and any plastics in the system. Therefore, conventional gas generator bag-based culturing methods were not used, because they would greatly complicate the analysis to find definitive *H. pylori* VOC signatures. Furthermore, *H. pylori* cultures require a microaerophilic atmosphere. Hence, the cultivation environment had to be hermetically sealable, flushable with the required atmosphere, reusable and accessible for controlled headspace sampling. Taking all of this into account, a new approach to cultivate *H. pylori* was developed, using materials that contribute as little volatiles as possible.

For the bacterial cultivation, we used 500 mL rectangle shaped glass bottles (Duran, USA) fitted with GL45 thread polytetrafluoroethylene (PTFE) caps with two threaded ports and integrated, stopcocks. The peripheral connections, for example, the fittings and tubing, were also made from PTFE (Bola, Germany).

Each cultivation bottle contained a total of 50 mL Columbia agar medium (Oxoid, UK) supplemented with 10% defibrinated horse blood (E&O Laboratories, Scotland). The prepared PTFE bottle-cap assembly with agar medium was laid on their side horizontally. As the medium cooled, a large enough surface area of the agarized medium was formed for *H. pylori* culturing (see Figure 1). Prior to cultivation in the glass bottles and in order to obtain viable cultures, the frozen *H. pylori* isolates were thawed and cultured for 4–7 days at a temperature of 37°C on Columbia blood agar plates (10% horse blood) with atmospheric generator bags (Biomerieux, France). *H. pylori* suspensions were then prepared in sterile 0.9% sodium chloride for each isolate, equivalent to McFarland 3.0 turbidity standard. From this prepared *H. pylori* suspension, 100 μL were transferred to the prepared glass bottles and spread evenly over the entire surface

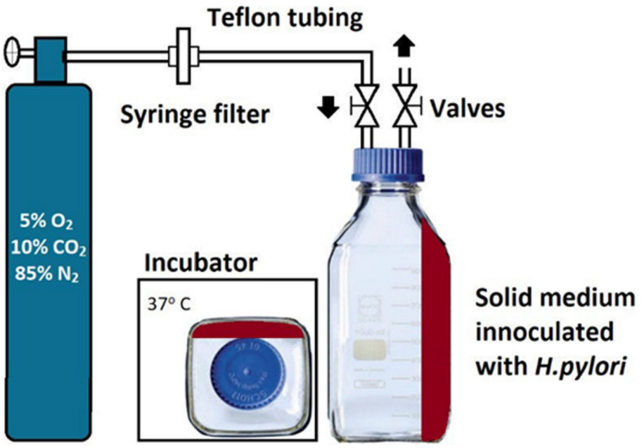

FIGURE 1 *Helicobacter pylori* cultivation setup.

area of the medium using a bacteriological loop. In parallel, 100 μL of sterile 0.9% sodium chloride were also similarly transferred to glass bottles and spread evenly over the entire surface area of the medium. This provided a negative control for any potential contamination and background VOCs. To ensure the necessary microaerophilic atmosphere, the glass cultivation bottle headspace was flushed through the cap ports for 3 min with a gas mixture containing 5% O_2, 10% CO_2, and 85% N_2. After this, the bottles were incubated at 37°C for 7 days. Additionally, each prepared *H. pylori* suspension was used to prepare serial dilutions in plates and cultivated in parallel to estimate viable cell count and to check for contamination. Petri plates containing the same medium as glass bottles were grown using a conventional *H. pylori* culturing method in a microaerobic environment for the same period. The scheme of the cultivation setup is presented in Figure 1.

Altogether nine isolates of *H. pylori* were cultivated. Each isolate was cultivated in triplicate together with triplicate medium controls. Each bottle was sampled only once. All cultures and controls were prepared and cultivated under identical conditions using the same protocols, materials, and reagents.

2.3 | Headspace needle trap extraction sampling protocol

The collection of the volatiles contained in a headspace relied on the use of needle trap extraction (NTE). The specific needle traps devices (NTDs) used were made from two-bed 23-gauge Silcosteel-treated stainless steel containing 2 cm of Carbopack X and 1 cm of Carboxen 1000, both with 60/80 mesh (PAS Technology, Germany). Prior to the extraction, the NTDs were pre-conditioned at 290°C under a flow of high purity helium (99.999%) for 20 min and the cultivation bottles were allowed to acclimate to room temperature for 20 min to reduce the water condensation in the needle trap. The NTE was performed by inserting the needle through a septa installed on a Luer adapter connected to one of the ports on the GL45 cap of the cultivation bottle. 70 mL of the headspace gas at a constant flow rate of 3 mL min^{-1} was

collected, while the other port was supplied with ultrapure nitrogen 6.0 at the same flow rate. After sampling the needle was plugged from both ends with Teflon caps and then stored at room temperature. The extracted sample was analyzed within 36 h. A 36-h window was a necessity owing to the time needed to transport the capped needle traps from the point of collection to the GC–MS facilities.

Trapped VOCs were thermally desorbed at 290°C in a splitless mode by inserting a needle into the inlet of the GC. For each replicate, one blank sample containing nitrogen was analyzed using the analogous protocol to identify possible contaminants stemming from sources other than bacterial metabolism, or the medium. If applicable, background concentration levels were subtracted from the corresponding values in the associated bacteria headspace samples. For the compounds reported here, this concerned only one volatile, namely 2 butanone. The scheme of the volatile extraction setup is presented in Figure 2.

2.4 | GC–MS analysis of *H. pylori* signatures

GC–MS analysis relied on an Agilent 8890/7079B GC–MS (Agilent, USA). The GC injector was equipped with an inert solid-phase microextraction liner (inner diameter 0.75 mm, Supelco, Canada) and operated in a splitless mode (0.75 min) followed by a split mode with a ratio 1:50. Extracted compounds were separated using a Rxi-624Sil MS column (30 m × 0.32 mm, layer thickness 1.8 μm, Restek, USA) operated at a constant helium flow of 1.4 mL min^{-1}. The oven temperature program was set as follows: 37°C for 12 min, followed by a rise of 5°C min^{-1} up to 150°C, then 10°C min^{-1} up to 290°C, and finally remaining at 290°C for 8 min. The untargeted VOC analysis relied on the mass spectrometer working in a SCAN mode with the associated *m/z* ranging from 20 to 250. The peak integration was based on extracted *m/z* ratio chromatograms. This approach allowed for a separation of most peaks of interest from their neighbors. The quadrupole rods, ion source, and transfer line were kept at 150°C, 230°C, and 280°C, respectively.

VOC identification was performed using a two-step process. First, the spectrum of a peak was checked against the NIST mass spectral library database. Second, the NIST identification was confirmed by comparing the temperature-programmed Kovats retention indices of peaks of the interest with retention indices obtained from the reference standards, which were prepared as outlined above. The retention indices were calculated using the homologous series of n-alkanes (C5-C15).

2.5 | Validation parameters

The validation parameters of the compounds of interest are provided in Table 1. Limits of detection (LOD) were calculated using the standard deviation of five consecutive blank signals,[10] and ranged from 0.01 to 0.22 ppb. The limit of quantification (LOQ) is defined as 3 × LOD. Relative standard deviations (RSDs) varied from 6% to 14%, which are considered adequate for the purposes of this study. The instrument response was found to be linear within the investigated concentration ranges, with coefficients of determination (R^2) ranging from 0.981 to 0.998.

3 | RESULTS & DISCUSSION

3.1 | *Helicobacter pylori* cultures

The estimated number of bacteria in the culturing flasks at the time of sampling was based on the colony counts observed in the serial dilution plates, which were cultured in parallel. The bacteria colonies were counted in the dilution plate, which contained between approximately 30–300 colonies and the cell count per mL was estimated using the following formula:

$$C = \frac{K_m}{V} N,$$

where, C is the cell count, K_m is the dilution factor, V is the inoculate volume in mL, N is the number of observed colonies in the petri plate.

The bacterial cell count obtained is provided in Table 2. The applied sampling procedure did not affect the bacteria's viability.

3.2 | General volatilomic signature of *H. pylori*

The characterization of the general signature of *H. pylori* comprised the identification of volatiles that are produced (released) and

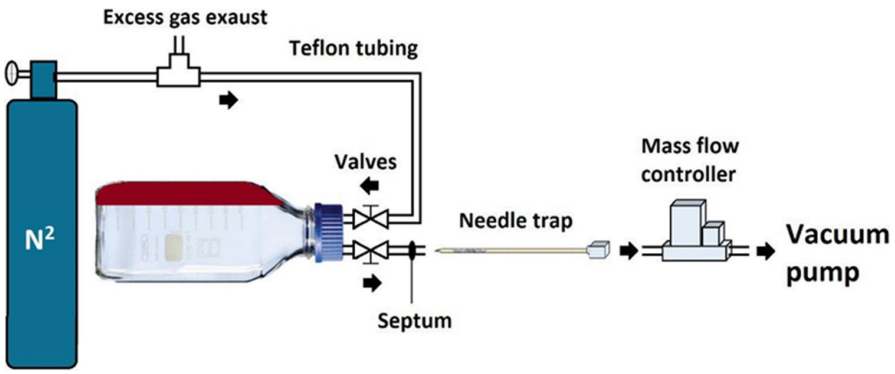

FIGURE 2 Volatile organic compounds (VOCs) extraction setup.

TABLE 1 Retention times (R_t) in min, quantifier ions, LODs [ppb], Relative Standard Deviations (RSDs) (%), coefficients of variation (R^2), and linear ranges [ppb] for volatile compounds of interest, which are ordered with respect to increasing retention time. RSDs were calculated for volatile organic compound (VOC) concentrations of 0.7–1.2 ppb.

VOCs name	CAS	Rt [min]	Quantifier ion	LOD [ppb]	RSD [%]	R^2	Linear range [ppb]
2-Butanone	78–93-3	6.41	72	0.22	10	0.98	0.73–60
1-Propanol, 2-methyl-	78–83-1	9.32	43	0.13	13	0.99	0.39–20
Butanal, 3-methyl-	590–86-3	9.82	57	0.06	9.0	0.981	0.17–50
Furan, 2-ethyl-	3208-16-0	11.97	81	0.04	10	0.990	0.12–6
1-Butanol	71–36-3	12.56	43	0.01	6	0.989	0.03–23
2-Pentanone	107–87-9	13.17	71	0.08	12	0.995	0.26–28
Dimethyl disulfide	624–92-0	16.30	94	0.05	11	0.988	0.15–30
1-Butanol, 3-methyl-	123–51–3	17.94	55	0.07	12	0.994	0.23–17
n-Octane	111–65-9	18.48	85	0.01	14	0.994	0.03–13
Styrene	100–42-5	24.64	78	0.02	7	0.992	0.06–22
2-Heptanone	105–42-0	25.09	58	0.04	13	0.989	0.12–11
2-Ethylhexanal	123–05-7	27.47	72	0.02	7	0.987	0.07–14
2-Pentylfuran	3777-69-3	28.10	81	0.02	7	0.989	0.08–3
2-Nonanone	821–55-6	32.63	58	0.03	14	0.994	0.08–9.5

TABLE 2 Total averaged number of bacteria across three replicate cultivation flasks at the start of cultivation.

	Total number of bacteria ($\times 10^4$)								
Isolate	IB0001909	IB0003214	IB0004118	IB0004960	IB0002577	IB0001982	IB0002104	IB0001352	IB0004718
N	16	2.1	0.3	0.2	0.55	9.5	15	1.6	0.05

metabolized (the uptake) by all isolates under study. The production and uptake of volatiles were evaluated using a Wilcoxon signed rank test, with a $p < 0.05$ being taken as significant. In other words, the levels of VOCs in the headspace above the bacteria cultures were compared to the respective levels in the medium only. For the Wilcoxon test, each *H. pylori* culture was randomly paired with one of the three control bottles containing medium only prepared within a particular experiment. All nine isolates were included for statistical analysis and only VOCs with an occurrence above 80% in bacteria cultures were used to represent the general signature of *H. pylori*. While all efforts were made to quantify the levels of the VOCs in the headspaces, this was not possible for a number of compounds owing either to the unavailability of pure substances or because of problems relating to the preparation of reliable reference mixtures. For these volatiles, a linear response of the MS detector over all the observed concentration levels was assumed. Thus, their levels could be compared using the peak areas alone. In such cases LOD was defined as three times the noise amplitude and only peaks with signal-to-noise ratio larger than nine (3 × LOD) were taken into account.

Amongst the volatiles detected, 21 fulfilled the aforementioned criteria by showing consistent differences between bacteria culture concentrations and those from the cultivation medium only. Of these 21 volatiles, 11 were found to have elevated headspace concentrations, whereas the other 10 exhibited decreased headspace levels. The detection incidences (occurrences) of the 21 VOCs of interest, their median concentrations (or peak areas in the cultivation flasks), concentration ranges, and the output of the Wilcoxon signed rank test are presented in Table 3. Comparisons of the headspace concentrations of released and consumed VOCs above the cultures of *H. pylori* isolates and medium only are presented in Figures 3 and 4.

The 10 VOCs found to have decreased levels are four aldehydes (2-methylpropanal, benzaldehyde, 3-methylbutanal, and butanal), two heterocyclic compounds (2-ethylfuran and 2- pentylfuran), one ketone (2-butanone), one aromatic VOC (benzene), one alcohol (2-butanol) and bromodichloromethane. Of the 11 volatile species with increased headspace levels, four are ketones (2- pentanone, 5-methyl-3-heptanone, 2-heptanone, and 2-nonanone), three are alcohols (2-methyl-1- propanol, 3-methyl-1-butanol and 1 butanol), one is an aromatic (styrene), one is an aldehyde (2- ethyl-hexanal), one is a hydrocarbon (n-octane) and one is a sulfur compound (dimethyl disulfide). Eight of these compounds were found in all *H. pylori* cultures and one in all samples but one. Whereas, all volatile species of interest but one (bromodichloromethane) were identified using the retention index library based on the reference standards, only 15 compounds were quantified. However, it must be stressed here that the set of calibrated compounds embraced all of the emitted VOCs but one. Of all of these, the highest levels of emissions were found to be 3-methyl-1-butanol, 1-butanol and dimethyl disulfide.

Of the VOCs that are released, ketones are the dominant chemical class with four representatives. All of the detected ketones are

TABLE 3 Occurrence, concentration medians and ranges of volatile organic compounds (VOCs) in the headspace of media and all bacteria cultures (nine isolates) and the outcome of a Wilcoxon signed rank test. Compounds are ordered with respect to decreasing occurrence. For the uncalibrated species (identified by italics) data of peak areas are only provided.

	VOCs name	CAS	Occurrence *H. pylori* [%]	Occurrence medium [%]	*H. pylori* median (range) [ppb]	Medium median (range) [ppb]	*p*-value
Release	2-Heptanone	110–43-0	100	72	4.2 (0.46–26.3)	0.56 (LOD–5.2)	3.0×10^{-8}
	1-Propanol, 2-methyl-	78–83-1	100	100	53 (4–182)	9.4 (2.6–149)	1.5×10^{-7}
	Disulfide, dimethyl	624–92-0	100	60	6.2 (0.74–206)	0.86 (LOD-5.1)	9.0×10^{-6}
	1-Butanol, 3-methyl-	123–51–3	100	100	82 (39–267)	25 (2–240)	1.9×10^{-5}
	2-Pentanone	107–87-9	100	68	8.8 (1.5–38)	2.5 (LOD-34)	8.1×10^{-5}
	Hexanal, 2-ethyl-	123–05-7	100	100	8.8 (2.2–232)	4.9 (1.1–19)	3.4×10^{-3}
	n-Octane	111–65-9	100	96	0.6 (0.3–1.7)	0.45 (0.3–1.2)	3.4×10^{-3}
	Styrene	100–42-5	100	100	2.2 (0.88–12)	1.4 (0.6–1.4)	7.4×10^{-3}
	1-Butanol	71–36-3	96	92	223 (LOD-3050)	148 (LOD-2700)	3.9×10^{-4}
	2-Nonanone	821–55-6	92	36	2.5 (LOD-12.5)	<LOD (LOD-3.9)	7.7×10^{-5}
	3-Heptanone, 5-methyl-	*541–85-5*	*85*	*54*	*8.4 (0–38)* $\times 10^6$	*5.5 (0–20)* $\times 10^6$	3.4×10^{-3}
	1-Butanol, 2-methyl-	*137–32-6*	*77*	*73*	*3.7 (0–96)* $\times 10^6$	*1.6(0–100)* $\times 10^6$	0.04
	Furan, 3-methyl-	*930–27-8*	*69*	*38*	*1.3 (0–8.8)* $\times 10^6$	*0 (0–5.8)* $\times 10^6$	1.1×10^{-4}
	1-Propanol	*71–23-8*	*62*	*31*	*0.4 (0–4.4)* $\times 10^6$	*0 (0–2.6)* $\times 10^6$	5.3×10^{-3}
	n-Pentane	109–66-0	58	44	4.0 (LOD-65)	LOD (LOD-44)	1.4×10^{-2}
	o-Xylene	95–47-6	58	46	*0.4 (0–3.1)* $\times 10^6$	*0 (0–2.8)* $\times 10^6$	0.03
	2-Octanone	111–13-7	50	35	*0.13 (0–11.2)* $\times 10^6$	*0 (0–10)* $\times 10^6$	0.02
Uptake	2-Butanone	78–93-3	100	96	50 (5.4–220)	86 (6–300)	5.2×10^{-5}
	Furan, 2-pentyl-	3777-69-3	92	96	0.9 (LOD-11)	1.5 (LOD-18)	6.7×10^{-3}
	Furan, 2-ethyl-	3208-16-0	88	88	0.5 (LOD-3.8)	1.0 (LOD-13)	4.4×10^{-4}
	Butanal, 3-methyl-	590–86-3	81	96	33 (LOD-960)	1200 (30–4000)	8.9×10^{-8}
	Methane, bromodichloro-	*75–27-4*	81	81	*1.2 (0–7.4)* $\times 10^6$	*2.0 (0–14.6)* $\times 10^6$	7.2×10^{-3}
	Benzene	*71–43-2*	81	100	*3.6 (0–4.6)* $\times 10^5$	*6.0 (2,6–100)* $\times 10^5$	0.03
	Butanal	*123–72-8*	77	92	*2.2 (0–16)* $\times 10^6$	*3.2 (0–19)* $\times 10^6$	3.7×10^{-3}
	Benzaldehyde	*100–52-7*	58	100	*3.0 (0–12)* $\times 10^5$	*15 (3.6–15.3)* $\times 10^5$	6.2×10^{-6}
	Propanal, 2-methyl-	78–84-2	58	96	1.9 (LOD-52)	73 (LOD-166)	2.1×10^{-5}
	2-Butanol	*78–92-2*	46	81	*0 (0–40)* $\times 10^6$	*2.9 (0–50)* $\times 10^5$	5.2×10^{-3}

reported to mirror microbiota activity. For examples, 2-pentanone, 2-heptanone and 2-nonanone are produced by *Pseudomonas aeruginosa*.[11] Moreover, 2-pentanone was reported to be released by *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Haemophilus influenza*,[12–15] whereas, 2-nonanone was reported to be released by *S. pneumoniae*.[12] The production of ketones by *H. pylori* can be ascribed to the activity of alcohol dehydrogenases (ADH) oxidizing secondary alcohols. The latter could be present in the applied medium or stem from the hydrocarbon's metabolism. Methyl ketones with an odd number of carbon atoms (from acetone to pentadecan-2-one) can also derive from even-numbered β-keto acids by decarboxylation in the β-oxidation pathway that occurs in many bacteria.[16]

Aerobic bacterial degradation of alkanes involves monooxygenases that activate these compounds by generating reactive oxygen species.[17] Several monooxygenases families can be involved in this step. These include for example, AlkB alkane hydroxylases.[17] A number of bacterial strains can degrade C5–C10 alkanes using alkane hydroxylases, which belong to a family of cytochrome P450 monooxygenases (e.g., CYP153A1 identified in *Acinetobacter* sp. *EB104*).[17] The conversion of alkanes usually starts with the oxidation of a terminal methyl group to render a primary alcohol that is further oxidized by

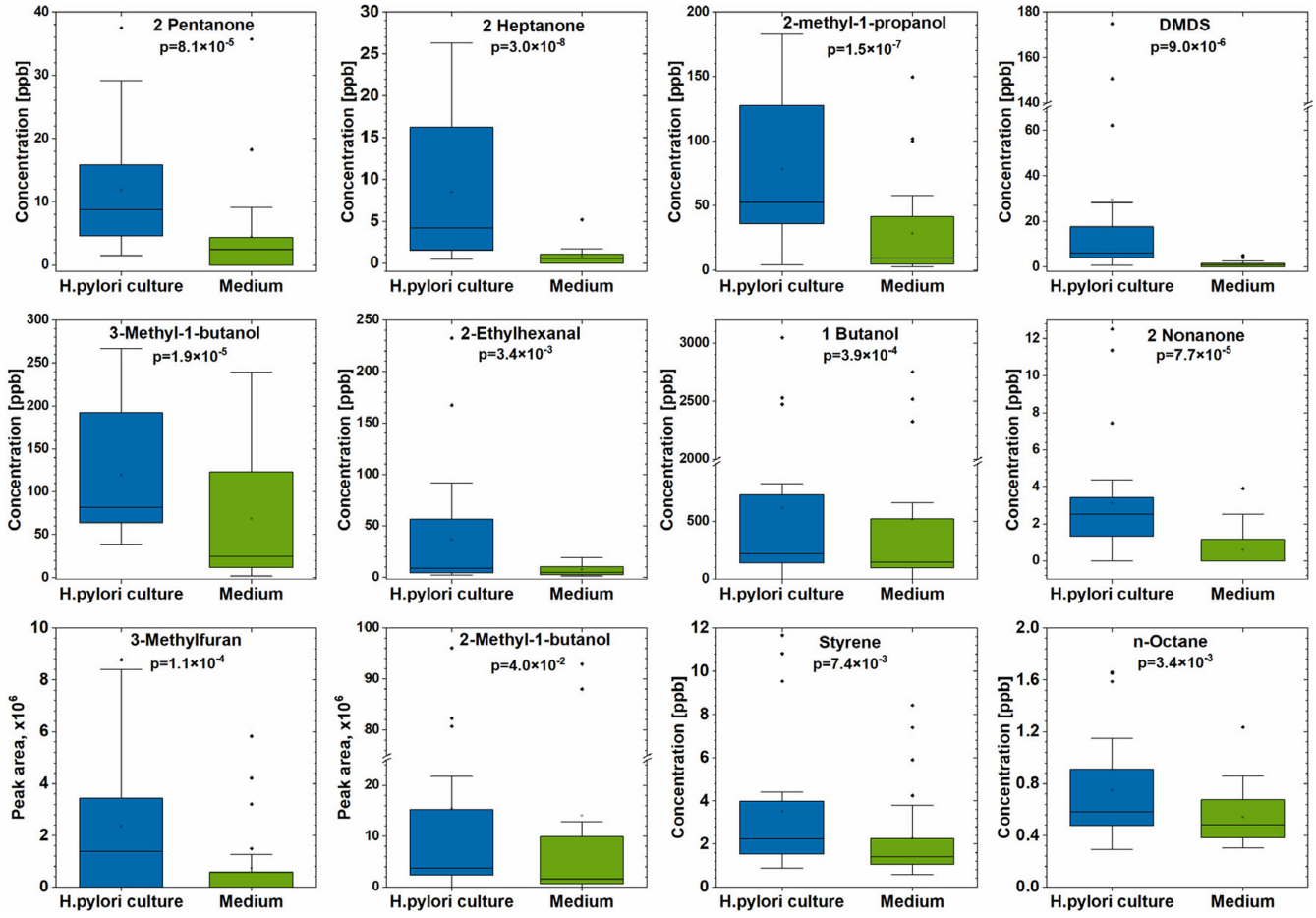

FIGURE 3 Comparison of the headspace concentrations of selected released volatile organic compounds (VOCs) above the cultures of *Helicobacter pylori* (*H. pylori*) isolates ($n = 26$) and medium only. Blue—bacteria, green—medium. DMDS, dimethyl disulfide.

ADH into the corresponding aldehyde and next converted into a fatty acid by aldehyde dehydrogenases (ALDH). Finally, fatty acids are conjugated to coenzyme A (CoA) and further metabolized by β-oxidation to generate acetyl-CoA. However, subterminal oxidation of n-alkanes is also possible. In such a case, a secondary alcohol is generated, which is next bioconverted into the corresponding ketone.[17] While *H. pylori* exhibits significant cytosolic alcohol dehydrogenase activity,[18,19] it is not clear if this species is able to activate the alkane molecules.

The activity of the aforementioned ADH reversibly reducing aldehydes to alcohols could be responsible for the generation of 2-methyl-1-propanol, 3-methyl-1-butanol and 1-butanol. Alternatively, the alcohols could be triggered by the degradation of alkanes (2-methyl-propane, n-butane and 3-methyl-butane).[17] However, the high occurrence of respective aldehydes, that is, 2-methylpropanal, 3-methylbutanal and butanal, in the applied medium and their decreased levels in the *H. pylori cultures* render the former pathway more plausible. Moreover, the degradation of branched-chain alkanes is more difficult than the linear ones.[17]

Interestingly, 2-ethyl-1-hexanal was the only aldehyde found to be emitted by the isolates of interest. The release of 2-ethyl-1-hexanal can be attributed to the oxidation of 2-ethyl-1-hexanol by ADH. Indeed, this alcohol was present in the medium at high abundances. Dimethyl disulfide can be derived from l-methionine catabolism producing methanethiol.[16] Dimethyl disulfide is postulated to arise from hydrogen sulfide and methanethiol by rapid autoxidation mediated by ascorbate and transition-metal ions. Interestingly, methyl mercaptan was reported to be produced by *H. pylori*.[20] Dimethyl disulfide may be synthesized by the methylation of hydrogen sulfide by thiol S-methyltransferase. An alternative mechanism for dimethyl disulfide formation involves the disproportionation of S-methyl methanethiosulfinate.[17]

The ability to synthesize hydrocarbons is widespread amongst bacteria.[21] Aliphatic hydrocarbons are considered to derive from fatty acids. This process involves decarboxylation of a respective acid with an aldehyde as the immediate precursor of a hydrocarbon. It is postulated, that fatty acids are first converted into aldehydes by fatty acyl-CoA reductase and next decarbonylated to hydrocarbons.[21] Only one alkane, n-octane, was found to be released by the isolates of interest. However, it is worthy of note, that the applied medium contained numerous hydrocarbons at high quantities. Perhaps, the production of other alkanes by *H. pylori* did occur, but was too low for their detection given their relatively high levels in the medium. Nevertheless, it is not clear if the aforementioned pathway occurs in *H. pylori*.

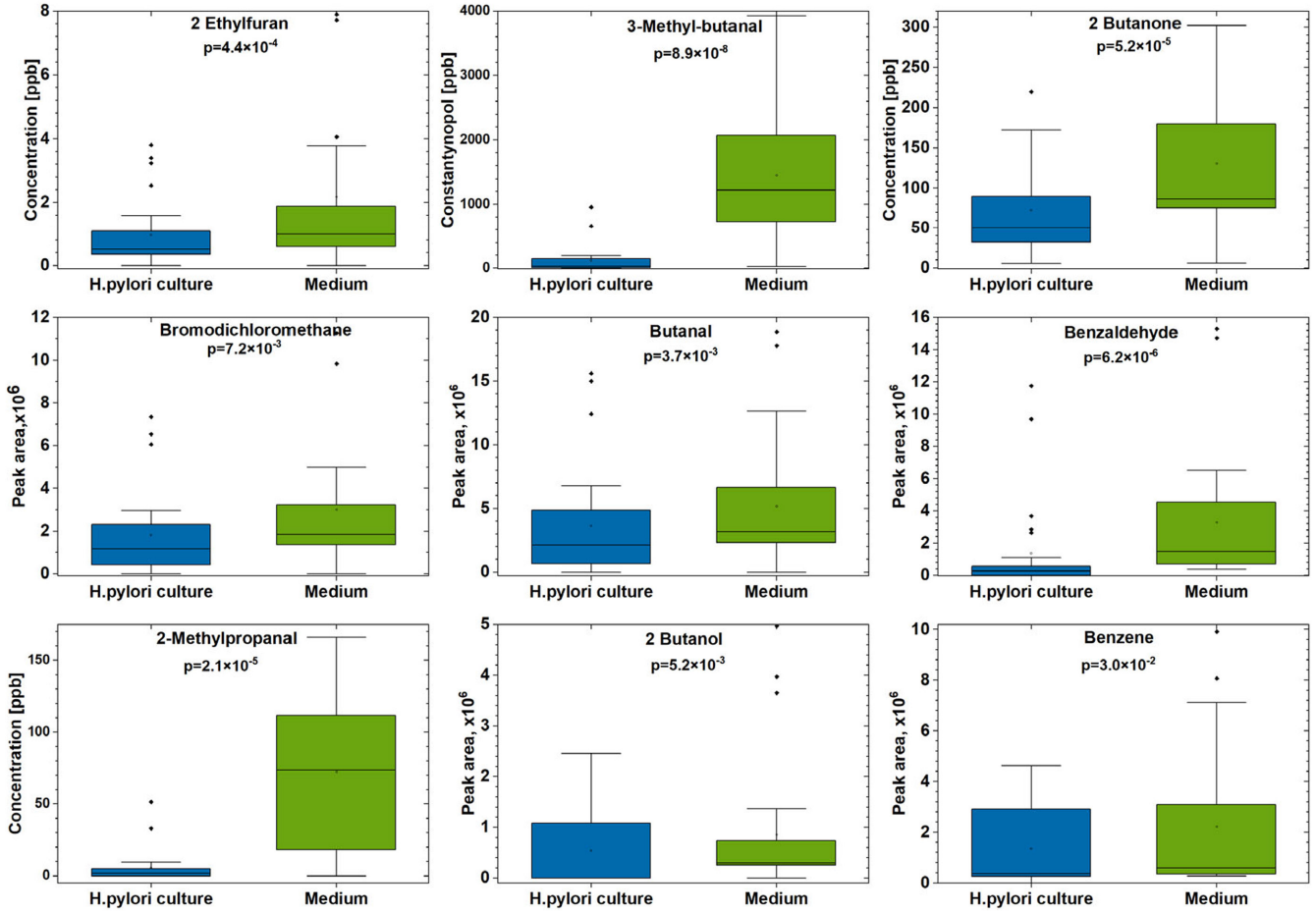

FIGURE 4 Comparison of the headspace concentrations of selected consumed volatile organic compounds (VOCs) above the cultures of *Helicobacter pylori* (*H. pylori*) isolates ($n = 26$) and medium only. Blue—bacteria, green—medium.

It is difficult to explain the release of styrene by isolates under study. Perhaps, this aromatic stems from the catabolism of aromatic amino acids, for example, L-phenylalanine or L-tyrosine, which are produced in the shikimate pathway.[16] Furthermore, heteroaromatic compounds can derive from L-tryptophan. It is possible that *H. pylori* is able to metabolize aromatic amino acids and, thereby, generate styrene.

Contrary to 2-pentanone, 5-methyl-3-heptanone, 2-heptanone, and 2-nonanone, it is intriguing that 2-butanone was found to be metabolized by all *H. pylori* isolates. Two potential pathways could lead to the consumption of 2 butanone: (i) its reduction into 2-butanol catalyzed by ADH and/or (ii) its oxidation by a Baeyer–Villiger monooxygenase to an ester.[22] Since, 2-butanol was also consumed by *H. pylori* the former route seems to be unlikely. On the other hand, no ester production from the products of 2-butanone oxidation were detected in the cultures of *H. pylori*. Thus, the optional pathway cannot be confirmed.

Interestingly, *H. pylori* metabolized benzene. Numerous bacteria can degrade benzene under aerobic and anaerobic conditions.[23] Under anoxic conditions, several bioconversion mechanisms of the benzene have been proposed. These include hydroxylation, methylation, or carboxylation.[23] Perhaps, one of these mechanisms occurs in *H. pylori*. Two heterocyclic compounds were metabolized by the bacteria of interest: 2-ethyl-furan and 2- pentyl-furan. However, the metabolic pathways leading to their consumption by *H. pylori* are not known.

The volatilomic studies on *H. pylori* are relatively sparse. Thus, it is difficult to relate the results obtained in this study to the data provided in other studies. For instance, Lee et al.[20] investigated volatile sulfur compounds (VSCs) produced by three strains of *H. pylori* in broth cultures mixed with sulfur-containing amino acids using gas chromatography. *H. pylori* was shown to produce hydrogen sulfide and methyl mercaptan; moreover, their production were shown to be also strain-specific. In conclusion, both sulfur-containing compounds were suggested to play a role in tissue destruction in the gastrointestinal tract. Buszewski et al.[24] employed GC–MS to determine the volatilomic signatures of a single *H. pylori* strain. The GC–MS study resulted in the recognition of 25 VOCs. However, no data on their occurrence and culture replicates were provided. In a follow-up study, Ulanowska et al.[25] investigated the volatilomic signatures of 11 *H. pylori* strains. They reported 48 volatiles; however, the incidence of the overwhelming majority of them was very low, that is, well below 50% ($n = 11$). Only two VOCs in their study exhibited an occurrence higher than 80%, namely n-butane and 2-butene. Moreover, in that study the differences between strains were not investigated.

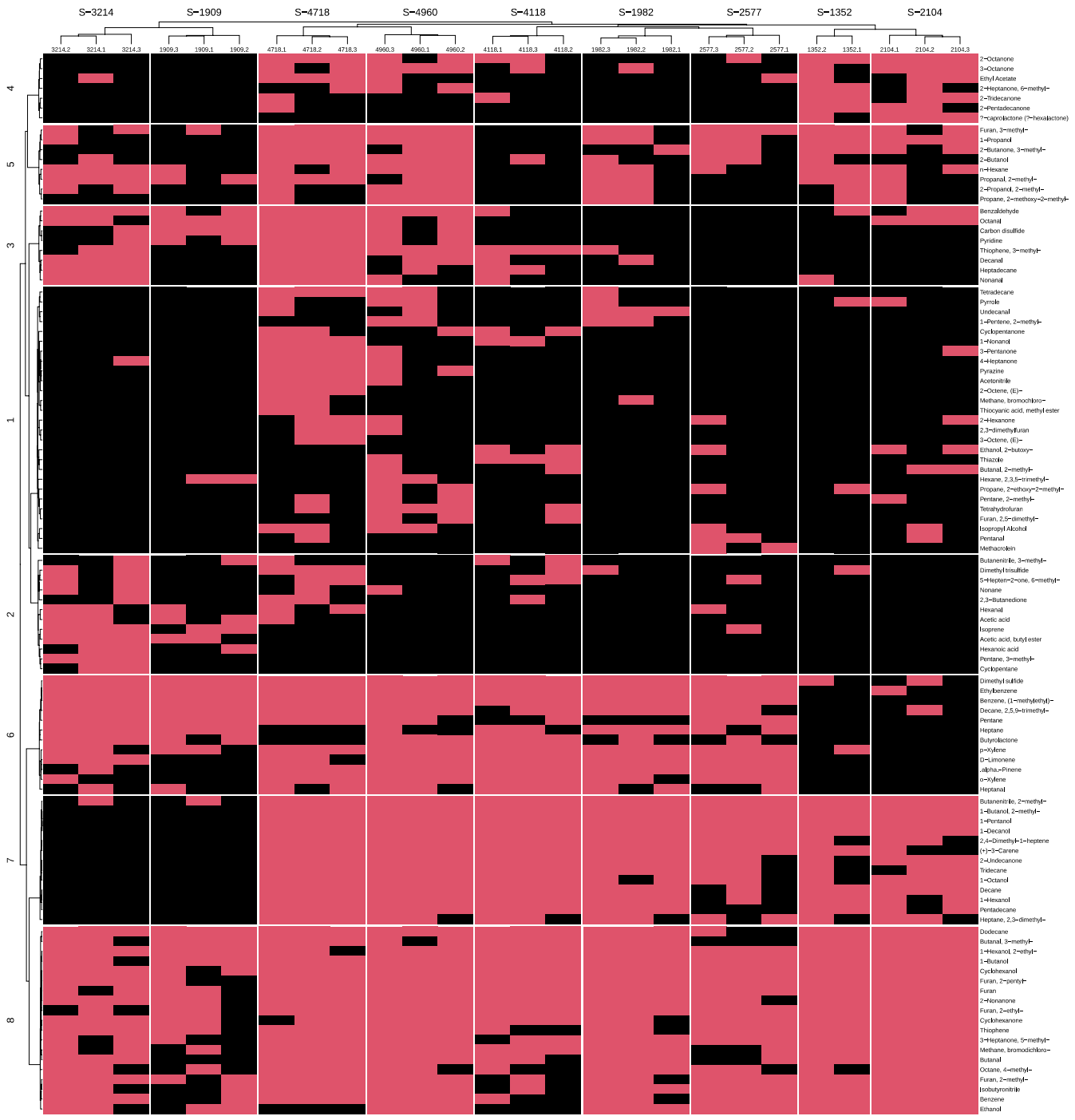

FIGURE 5 Heatmap illustrating the presence and absence patterns of compounds across various samples. The compounds under consideration were detected in at least one sample but not in all of them. The columns were grouped by samples and replicates are ordered based on their Jaccard distance similarity.

3.3 | Variability in volatilomic signatures of different *H. pylori* isolates

One of the aims of this study was to investigate the variability in the volatilomic signatures of different isolates of *H. pylori*, because any differences between these signatures could be potentially employed for their identification. The variability in volatilomic signatures was checked by comparing the signatures of isolates. Since the number of replicates was low, strict criteria were applied to identify VOCs: the levels of VOCs in the gaseous phase in the bacteria culture were binarized, if VOC >0 then 1 (on) or else 0 (off). Then, all those compounds that were present (on or 1) in all the samples were filtered out as well as those that were not present (off or 0) in at least two out of three. Figure 5 presents a heatmap of the remaining samples. The columns represent the sample replicates ordered by condition and the rows are ordered according to hierarchical clustering performed

on compounds. Volatilomic signatures of the isolates under study are compared in Figure 5.

Effectively, VOCs present in the headspace of the *H. pylori* culture have been grouped into eight clusters. Cluster 4 contains predominantly ketones (six out of eight members), whereas cluster 3 is rich in aldehydes (50%). Cluster 6, in turn, consists mainly of aromatic and aliphatic hydrocarbons. Cluster 7 embraces five primary alcohols. The remaining clusters (1, 2, 5, 8, and) consist of VOCs from very different chemical families. Within the limits of this approach, some valuable information can be extracted concerning the metabolic differences between the *H. pylori* isolates of interest. First, the isolates IB0003214, IB0001909, IB0001982, and IB0002577 exhibit significantly reduced emission of ketones as compared to the isolates IB0001352 and IB0002104 (see cluster 4). The majority of VOCs that are grouped within cluster 4 are methyl ketones. This difference might be caused by the differences in the ability of isolates under study to decarboxylate even-numbered β-keto acids. Second, signatures of the isolates IB0001352, IB0001982, and IB0002577 contain very few aldehydes (see e.g., cluster 3). This feature can stem from the elevated expression of ALDH in these isolates. Conversely, the occurrence of aldehydes in the fingerprints of isolates IB0004718 and IB0004960 was higher. Moreover, scrutiny of VOCs forming cluster 6 reveals some interesting information on the metabolism of isolates IB0001352 and IB0002104. The volatilomic signatures of these two isolates are characterized by the lowered occurrence of hydrocarbons, both aromatic and aliphatic, as compared to the remaining strains. Due to the shortage of information on the activity of the *H. pylori* enzymes, it is difficult to interpret this finding. Perhaps, the lowered level of hydrocarbons stems from an increased expression of alkane hydroxylases. Finally, signatures of isolates IB0003214 and IB0001909 exhibit reduced levels of primary alcohols (cluster 7) that can reflect upregulated expression of ADH and/or ALDH. With the aforementioned information in mind, it can be anticipated that it is feasible to differentiate different strains of *H. pylori* based only on biochemical volatile signatures. This knowledge could be used to improve the treatment of the infections caused by *H. pylori* through the use of non-invasive diagnostic methods to analyze exhaled breath volatiles.

4 | CONCLUSIONS

This study investigated the volatilomic signatures of nine isolates of *H. pylori* using GC–MS. Collectively, 21 volatiles were found to be omnipresent in the *H. pylori* volatilome that showed consistent differences in concentrations in the headspace above bacteria cultures compared to that above the cultivation medium. These volatiles are recognized as representing general volatilomic signatures of *H. pylori*. Of these VOCs, 11 were found to have elevated concentrations in the headspace above the bacterial cultures, whereas, the other 10 exhibited decreased levels in the bacterial headspace.

H. pylori infection is one of the best described risk factors for gastric cancer and the *H. pylori*-related compounds can thus be incorrectly correlated with this disease, that is, the VOCs commonly produced by *H. pylori* form a set of potential confounders for the recognition of gastric cancer volatile markers. On the other hand, these compounds are candidates for detecting *H. pylori* infection. Whether one VOC or a certain set of VOCs found within this study are of the value to diagnose *H. pylori* infection, via for example, breath and/or urine analyses, remains to be clarified in future studies.

Qualitative variations in the VOC emissions from the different isolates were observed. To the best of our knowledge, this phenomenon has not been reported previously for *H. pylori*. Our observations indicate that it might be possible to differentiate between different isolates of *H. pylori* using biochemical volatile signatures and that VOC analysis may provide a useful tool for monitoring infections caused by this pathogen. Further studies involving genotypic identification on the *H. pylori* isolates are needed to show whether this chemical information could be employed for the recognition of different *H. pylori* strains. It would be highly beneficial to collect the exact culture passage, used for headspace collection, for subsequent sequencing, based on species quality control and genotypic comparison.

The new knowledge gained within this project will support the discovery of volatile biomarkers in breath or contained in the headspace of urine, and hence support studies that are aiming to provide the volatile signatures for use in noninvasively detecting people with gastric cancer.

AUTHOR CONTRIBUTIONS

Conceptualization—R.V., P.M.; methodology—R.V., E.D.; formal analysis—C.A., E.A.F., D. Ś.W.; investigation—L.M., P.M., R.V. E.D.; data curation—M.L., R.V.; supervision—M.L., P.M., CAM; writing—original draft preparation—P.M., A.H.C., R.V., M.L.; writing—review and editing—L.M., M.L., CAM, E.A.F. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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