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The Use of Proton-Transfer-Reaction Time-of-Flight Mass Spectrometry (PTR-ToF-MS) to Determine the Volatile Organic Compounds (VOCs) Produced by Different Lactic Acid Bacterial Strains Growing in Defined Media

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Abstract: Lactic acid bacteria (LAB) fermentation has been claimed as an effective way of modifying the sensory properties of plant-based foods. However, not much has been published on the influence of different LAB strains on the flavour of the volatile organic compounds (VOCs) produced. Using a defined medium (DM) and proton-transfer-reaction time-of-flight mass spectrometry (PTR-ToF-MS), we assessed the VOCs produced by seven LAB strains, *Levilactobacillus brevis* WLP672 (LB672), *Lactobacillus delbrueckii* WLP677 (LD677), *Pediococcus damnosus* WLP661 (PD661), *Lactiplantibacillus plantarum* LP100 (LP100), *Pediococcus pentosaceus* PP100 (PP100), *Pediococcus damnosus* 5733 (PD5733), and *Lentilactobacillus buchneri* 5335 (LU5335), at three time points during fermentation (0, 7, and 14 days) at either 25 or 35 °C. Significant variations in VOC production were observed among LAB strains, growing in the same DM composition at either 25 °C or 35 °C. Specifically, the concentration of m/z 87.043 (t.i. diacetyl) was significantly ($p < 0.05$) higher at 7 days of fermentation at 35 °C by LP100, followed by PP100 at 35 °C and PD661 at 25 °C compared to the other strains at either 25 or 35 °C. The concentration of m/z 115.112 (t.i. 2-heptanone) was significantly ($p < 0.05$) higher at 7 days of fermentation at either 25 or 35 °C by LP100 compared to the other strains at all temperature and time points. The concentration of m/z 49.011 (t.i. methanethiol) was significantly ($p < 0.05$) higher after 7 days of fermentation at 35 °C by LB672 compared to the other strains at either 25 or 35 °C. The concentration of m/z 71.085 (t.i. 3-methyl butanol) was significantly ($p < 0.05$) higher after 7 days of fermentation at either 25 or 35 °C by PD661, LU5335, or PD5733 compared to the other strains studied. A notable increase in specific VOC concentrations was observed at 35 °C compared to 25 °C. This research demonstrates that LAB strains generate distinct VOC profiles in a DM based on strains and fermentation conditions. Therefore, this knowledge provides a basis for controlling and enhancing flavour in plant-based fermentations.

Keywords: defined medium; volatile organic compounds; lactic acid bacteria fermentation; proton-transfer-reaction time-of-flight mass spectrometry

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1. Introduction

Consumers are seeking to decrease their consumption of animal-based foods due to concerns about the impact of its production on the environment, the reported adverse health effects of high meat diets, and animal welfare implications, which in turn is driving demand for plant-based meat and dairy analogues [1–3]. However, the overall flavour of these analogues still falls short of their traditional meat or dairy counterparts [4–7]. Flavour perception is complex, with volatile organic compounds (VOCs) present in the food either singularly or together playing a significant role in how consumers perceive the flavour associated with food [8–11].

Plant-based substrates may contain VOCs associated with meat or dairy flavours; however, directly obtaining these VOC is challenging as their concentration and recovery rate is low [12]. Fermenting plant-based substrates using lactic acid bacteria (LAB) has been proposed to be an efficient way to produce higher concentrations of the desired VOCs [13–15]. During growth on plant substrates, LAB produce VOCs as secondary metabolites [16]. However, due to the complexity of the compounds that are present within plant-based systems, it is challenging to relate the impact of substrate, fermentation conditions or LAB strain to the production of specific VOCs.

LAB require a relatively rich cultivation media for growth, as they are auxotrophic to many vitamins and amino acids [17,18]. However, the use of poorly defined cultivation medium means that it can be difficult to identify which VOCs are being produced in response to substrates being metabolised [19]. To overcome this challenge, a well-defined minimal medium (DM) can be used. While many researchers have investigated the growth of LAB in a DM [20–24], only a few studies have examined the VOCs produced by LAB in DM [25–27]. Further, to maximise the formation of desirable VOCs in a natural or DM, a better understanding of the metabolic pathways present in different LAB strains and the VOC they produce is required [28,29].

While gas chromatography–mass spectrometry (GC-MS) remains a gold standard for VOC analysis, it is limited by its inability to perform real-time VOC analysis. As an alternative, proton-transfer-reaction time-of-flight mass spectrometry (PTR-ToF-MS) offers highly sensitive, real-time VOC detection in a direct and non-invasive manner [30,31]. However, as PTR-ToF-MS can only detect exact protonated masses, GC-MS and fastGC-PTR-MS can be used in conjunction to support VOC identification [32–34].

Therefore, the current study determined the VOCs associated with seven LAB strains growing in a DM, using PTR-ToF-MS, HS-SPME-GC-MS, and fastGC-PTR-ToF-MS.

2. Materials and Methods

2.1. LAB Strains

Seven commercially available LAB strains, namely *Levilactobacillus brevis* WLP672 (thereafter referred to as LB672), *Lactobacillus delbrueckii* WLP677 (LD677), *Pediococcus damnosus* WLP661 (PD661), *Lactiplantibacillus plantarum* LP100 (LP100), *Pediococcus pentosaceus* PP100 (PP 100), *Pediococcus damnosus* 5733 (PD5733), and *Lentilactobacillus buchneri* 5335 (LU5335), were used (Table 1). These strains were deemed appropriate for this study and for the commercial production of VOCs as they are all readily available, of food-grade standard, grown in the temperature range of 20 to 35 °C [35], and relatively nutritionally robust. The inoculums used for the fermentation trials were prepared following the procedure described in our previous experiments [27,36].

Table 1. LAB strains used in the study.

Abbreviation	LAB Strains	Culture Form	Source
LB672	*Lev. brevis* WLP672	Liquid	White labs, USA
LD677	*Lb. delbrueckii* WLP677	Liquid	White labs, USA
PD661	*P. damnosus* WLP661	Liquid	White labs, USA
LP100	*Lpb. plantarum* LP100	Lyophilised strain powder	Bioagro, Italy
PP100	*P. pentosaceus* PP100	Lyophilised strain powder	Bioagro, Italy
PD5733	*P. damnosus* 5733	Liquid	Wyeast, USA
LU5335	*Len. buchneri* 5335	Liquid	Wyeast, USA

2.2. Medium Composition

The DM was developed based on the findings of our previous research [27,37]. The DM contained D-glucose (20 g/L), peptone (enzymatic protein digest) (5 g/L), sodium acetate (12 g/L), mineral salts ($MgSO_4 \cdot 7H_2O$ (0.2 g/L), NaCl (0.01 g/L), $FeSO_4 \cdot 7H_2O$ (0.01 g/L), $MnSO_4 \cdot 5H_2O$ (0.04 g/L)), vitamins (calcium pantothenate (B5) (0.4 mg/L), nicotinic acid (B3) (0.2 mg/L), riboflavin (B2) (0.4 mg/L), thiamine HCl (B1) (0.2 mg/L)), and an amino acid mixture (0.4 g/L of each amino acid: L-leucine, L-isoleucine, L-phenylalanine, L-glutamic acid, L-aspartic acid, L-threonine, or L-methionine).

2.3. Fermentation

Before use, the DM was incubated at 25 °C for at least 3 days to ensure no bacterial growth (turbidity). Following the confirmation of the absence of contamination, 4 mL aliquots of DM were transferred into sterile headspace vials (20 mL) capped with PTFE/silicone septa (Agilent, Cernusco sul Naviglio, Italy). A 0.05 mL aliquot of each LAB cell suspension (1×10^9 CFU/mL) was subsequently inoculated into each headspace vial, which were flushed with N_2 at a rate of 10 mL/min for 20 min to establish an anaerobic environment. The inoculated vials were placed in sample trays in a randomised order in an autosampler (MPS Multi-Purpose Sampler, Gerstel, Germany) and held at either 25 or 35 °C for 14 days. Eight analytical replicates were prepared from each sample, four of which were kept at either 25 °C or 35 °C. The controls included uninoculated DM. After 14 days of fermentation, growth was confirmed by measuring pH (inoLab Level 1/WTW, Weilheim, Germany) and optical density (BioPhotometer/Eppendorf, Hamburg, Germany) in a sub-sample of the fermented culture.

2.4. Determination of Volatile Organic Compounds (VOCs)

2.4.1. PTR-ToF-MS

The VOCs produced during fermentation were measured at three time points (0, 7, and 14 days of fermentation) using a PTR-ToF-MS 8000 (Ionicon Analytik GmbH, Innsbruck, Austria). PTR-ToF-MS analysis was performed as previously described by Di Pierro, Franceschi [38], with some modifications [39–41].

2.4.2. HS-SPME-GC-MS

HS-SPME-GC-MS measurements were included to support with the identification of compounds detected by PTR-ToF-MS. At the end of fermentation (after 14 days), the samples were removed from the PTR-ToF-MS autosampler sample tray and transferred to a GC-MS autosampler sample tray held at either 25 or 35 °C. HS-SPME-GC-MS analysis was performed using methods described in our previous publications [36,37].

2.4.3. FastGC-PTR-ToF-MS

To assist with attributing each m/z to the correct compound and determining the number of isomeric compounds contributing to each m/z, fastGC-PTR-ToF-MS was carried

out on all samples at each time point after performing SHS-PTR-ToF-MS measurements using methods described in previous publications [36,37,42].

2.5. Statistical Analysis

ANOVA was used to determine which sample m/z values were significantly ($p < 0.05$) higher than those in blanks.

A three-way ANOVA using a general linear model was performed to identify m/z values that significantly discriminated between treatments. The main effects considered were LAB strain, fermentation temperature, and fermentation time, and all possible interactions were examined. Tukey's HSD test ($p < 0.05$) was used for post hoc mean separation. All statistical analyses were conducted using SPSS (IBM SPSS Statistics, v. 29.0.0.0, Armonk NY, USA).

To encompass variations between LAB strains and fermentation VOCs, a non-targeted approach was employed, generating heatmaps of significant m/z values. These heatmaps were created using R (version 4.2.1, R Foundation for Statistical Computing, Vienna, Austria). Log-transformed average VOC concentrations (ppbV) were used to create the heatmaps.

Selected VOCs (m/z) were plotted using two-way ANOVA for the main factors of the LAB strains, their fermentation temperature, and their interaction at 7 days of fermentation using the "ggplot2", "dplyr", "ggpubr", "reshape", "ggthemes", "multcompView", "readr", and "scales" packages in R.

3. Results

3.1. Physicochemical Properties After Fermentation

All LAB strains except LD677 exhibited good growth in the DM, as shown by decreased pH (due to acid production) [43] and increased OD_{600} (indicating cell growth) (Table 2). Significantly, the greatest pH reduction was observed in the DM inoculated with LP100, followed by LB672 and PP100 at either 25 or 35 °C. The highest OD_{600} values were observed in the DM inoculated with LP100, followed by LB672 and PP100 at 25 °C and LP100, followed by LB672 at 35 °C.

Table 2. The mean pH and OD_{600} of samples after 14 days of fermentation by different LAB strains in the DM.

DM Inoculated with Differ LAB Strains	Initial pH	at 25 °C		at 35 °C	
		pH	OD_{600}	pH	OD_{600}
LB672	5.67 ± 0.009^{Aa}	4.47 ± 0.03^{Cb}	1.40 ± 0.005^{Ba}	4.44 ± 0.018^{Db}	1.20 ± 0.04^{Bb}
LD677	5.66 ± 0.002^{Aa}	5.62 ± 0.003^{Aa}	0.05 ± 0.003^{Ea}	5.63 ± 0.003^{Aa}	0.04 ± 0.005^{Ea}
PD661	5.65 ± 0.005^{Aa}	5.22 ± 0.035^{Bb}	0.70 ± 0.01^{Da}	5.69 ± 0.06^{Aa}	0.70 ± 0.008^{Da}
LP100	5.65 ± 0.01^{Aa}	3.95 ± 0.1^{Db}	2.40 ± 0.05^{Aa}	4.02 ± 0.008^{Eb}	2.32 ± 0.005^{Aa}
PP100	5.66 ± 0.005^{Aa}	4.47 ± 0.001^{Cc}	1.31 ± 0.015^{Ba}	4.86 ± 0.055^{Cb}	0.75 ± 0.02^{Db}
PD5733	5.67 ± 0.003^{Aa}	5.09 ± 0.065^{Bb}	1.15 ± 0.015^{Ca}	4.94 ± 0.06^{Cb}	0.98 ± 0.008^{Cb}
LU5335	5.68 ± 0.006^{Aa}	5.08 ± 0.03^{Bc}	0.74 ± 0.005^{Da}	5.32 ± 0.005^{Bb}	0.70 ± 0.045^{Da}

Values are the means ± standard error of 2 replicates. Values with different superscript uppercase letters ($^{A–E}$) in the column (either pH or OD_{600}) are significantly different according to Tukey's test at $p< 0.05$. Values with different superscript lowercase letters ($^{a–c}$) in the row (either pH or OD_{600}) are significantly different according to Tukey's test at $p< 0.05$.

As LD677 did not grow sufficiently in the DM, the detected VOCs (all replicates) were excluded in the subsequent analysis.

It should be noted that there were no changes in pH and OD_{600} values in all uninoculated controls after fermentation, suggest that all uninoculated controls remained sterile throughout the experiment.

3.2. VOCs Produced During Fermentation

The fermentation of the DM at either 25 or 35 °C by the six (LD677 removed as no growth) LAB strains resulted in 104 m/z that were significantly ($p < 0.05$) higher than the baseline, after the removal of isotopologues (Table S1). The tentative identification (t.i.) of each m/z was based on its exact mass, supported by HS-SPME-GC-MS identification for 32 of the 104 (Tables 3, 4 and S1), fastGC-PTR-ToF-MS identification, and/or literature data.

Table 3. VOCs detected after 14 days of fermentation by different LAB strains (either LB672, LP100, PP100, PD661, PD5733 or LU5335) in the DM using HS-SPME-GC-MS at 25 and 35 °C.

No	VOCs	Formula	RT	RI. Cal	RI. Lit	at 25 °C	at 35 °C
	Acids						
1	Acetic acid	$C_2H_4O_2$	15.29	1467	1449	✓	✓
2	Butyric acid	$C_4H_8O_2$	19.63	1646	1625	✓	✓
3	Hexanoic acid	$C_6H_{12}O_2$	24.44	1862	1846	✓	✓
4	Octanoic acid	$C_8H_{16}O_2$	28.76	2035	2060	✓	✓
5	Decanoic acid	$C_{10}H_{20}O_2$	32.70	2154	2276	✓	✓
	Alcohols						
6	2-Propanol	C_3H_8O	3.07	934	927	✓	✓
7	Ethanol	C_2H_6O	3.16	941	932	✓	✓
8	2-Pentanol	$C_5H_{12}O$	6.69	1134	1119	✓	✓
9	1-Butanol	$C_4H_{10}O$	7.27	1158	1142	✓	✓
10	2/3-Methyl-1-butanol	$C_5H_{12}O$	8.86	1220	1208/1209	✓	✓
11	3-Methyl-3-buten-1-ol	$C_5H_{10}O$	9.99	1263	1248	✓	✓
12	2-Heptanol	$C_7H_{16}O$	11.78	1332	1320	✓	✓
13	Hexanol	$C_6H_{14}O$	12.67	1365	1355	✓	✓
14	2,3-Butanediol	$C_4H_{10}O_2$	17.44	1554	1543	✓	✓
15	1-Octanol	$C_8H_{18}O$	17.86	1571	1557	✓	✓
16	Menthol	$C_{10}H_{20}O$	19.81	1653	1637	✓	✓
17	2-Undecanol	$C_{11}H_{24}O$	21.59	1731	1717	✓	×
18	Benzyl alcohol	C_7H_8O	25.14	1895	1870	✓	✓
19	Phenylethyl alcohol	$C_8H_{10}O$	25.85	1930	1906	✓	✓
20	2-Tridecanol	$C_{13}H_{28}O$	25.90	1933	1903	✓	✓
21	P-cresol	C_7H_8O	29.45	2051	2080	✓	✓
	Aldehydes						
22	Butanal	C_4H_8O	2.75	911	877	✓	✓
23	2-Methyl butanal	$C_5H_{10}O$	2.90	922	914	✓	✓
24	3-Methyl butanal	$C_5H_{10}O$	2.96	926	918	✓	✓
25	2-Methyl-2-butenal	C_5H_8O	6.17	1114	1095	✓	✓
26	3-Methyl-2-butenal	C_5H_8O	8.77	1216	1215	✓	✓
27	2-Methyl pentanal	$C_6H_{12}O$	13.66	1403	-	✓	✓
28	Benzaldehyde	C_7H_6O	17.15	1542	1520	✓	✓
29	Benzeneacetaldehyde	C_8H_8O	20.03	1663	1640	✓	✓
	Esters						
30	Ethyl acetate	$C_4H_8O_2$	2.61	901	888	✓	✓
31	Isoamyl acetate	$C_7H_{14}O_2$	6.81	1139	1122	✓	✓
32	Octanoic acid ethyl ester	$C_{10}H_{20}O_2$	14.81	1448	1435	✓	✓
33	Decanoic acid ethyl ester	$C_{12}H_{24}O_2$	19.77	1652	1638	✓	✓
34	2-Phenylethyl acetate	$C_{10}H_{12}O_2$	23.89	1836	1813	✓	✓
35	Dodecanoic acid ethyl ester	$C_{14}H_{28}O_2$	24.30	1856	1841	✓	✓
	Furans						
36	Furfural	$C_5H_4O_2$	15.72	1484	1461	×	✓
37	2-Furanmethanol	$C_5H_6O_2$	20.40	1679	1660	✓	✓
	Ketones						
38	Acetone	C_3H_6O	1.97	823	819	✓	✓
39	Diacetyl	$C_4H_6O_2$	3.84	989	979	✓	✓
40	2-Heptanone	$C_7H_{14}O$	8.29	1198	1182	✓	✓
41	Acetoin	$C_4H_8O_2$	11.00	1302	1284	✓	✓
	Sulphur compounds						
42	Dimethyl disulphide	$C_2H_6S_2$	5.73	1095	1077	×	✓
43	Methional	C_4H_8OS	15.47	1474	1454	✓	✓
44	Cyclohexyl isothiocyanate	$C_7H_{11}NS$	20.61	1687	1667	✓	✓
45	3-(methylthio)-1-propanol (methionol)	$C_4H_{10}OS$	21.64	1734	1719	✓	✓
	Pyrazine						
46	Pyrazine	$C_4H_4N_2$	9.08	1228	1212	✓	✓
	Unknown compounds						
47	Unknown 1		4.92			✓	✓
48	Unknown 2		5.05			×	✓
49	Unknown 3		6.04			✓	✓
50	Unknown 4		12.53			✓	✓

✓: VOCs detected at given temperature. ×: VOCs not detected at given temperature.

Table 4. The VOCS (m/z) detected by PTR-ToF-MS during LAB fermentation in the DM that ANOVA analysis determined significantly ($p < 0.05$) distinguished between different LAB strains (S), fermentation time (0 and 7 days) (T), temperature (either at 25 or 35 °C) (Temp), and their interaction effects.

No	*m/z*	Sum Formula	Identification	*p* Value						
				S	T	Temp	S×T	S×Temp	T×Temp	S×T×Temp
1	26.016	$C_2H_2^+$	Common fragment	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
2	27.025	$C_2H_3^+$		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
3	28.031	$C_2H_4^+$		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
4	31.018	CH_2OH^+	Formaldehyde fragment	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
5	34.996	H_2SH^+	Hydrogen sulphide	<0.0001	<0.0001	0.010	<0.0001	<0.0001	0.017	<0.0001
6	41.039	$C_3H_5^+$		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.073	0.209
7	42.010			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
8	43.018	$C_2H_3O^+$	Common fragment	0.005	<0.0001	0.389	0.468	0.393	0.481	0.055
9	43.054	$C_3H_7^+$	Propanol fragment [1]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
10	47.049	$C_2H_6OH^+$	Ethanol [1]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.001
11	49.011	CH_4SH^+	Methanethiol	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.901	<0.0001
12	53.006			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.002
13	57.036	$C_3H_4OH^+$		<0.0001	<0.0001	0.050	<0.0001	0.602	0.412	0.806
14	57.070	$C_4H_9^+$	1-Butanol fragment [1]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
15	59.049	$C_3H_6OH^+$	Acetone[1]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.858	<0.0001
16	63.009	$CO_2H_3O^+$		<0.0001	<0.0001	0.166	<0.0001	0.407	0.975	0.059
17	64.005			<0.0001	<0.0001	<0.0001	<0.0001	0.008	0.009	<0.0001
18	71.085	$C_5H_{11}^+$	3-Methyl-butanol fragment [1,2]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.005	<0.0001
19	78.967	$CH_2S_2H^+$		<0.0001	0.008	<0.0001	<0.0001	<0.0001	0.002	0.001
20	81.016			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.001	<0.0001
21	85.066	$C_5H_8OH^+$	2-Methyl-2-butenal [1] and 3-Methyl-2-butenal [1]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.001	<0.0001
22	87.043	$C_4H_6O_2H^+$	Diacetyl [1,2]	<0.0001	<0.0001	0.004	<0.0001	<0.0001	0.282	<0.0001
23	89.060	$C_4H_8O_2H^+$	Ethyl acetate [1,2,3] and Acetoin [1,2,3]	<0.0001	<0.0001	0.682	0.001	0.239	0.918	0.161
24	91.027	$C_3H_6OSH^+$	Methyl thiolacetate/Mercaptoacetone	<0.0001	<0.0001	0.009	<0.0001	<0.0001	0.007	<0.0001
25	91.072	$C_4H_{10}O_2H^+$	2,3-Butanediol [1]	0.001	0.006	0.078	0.056	0.139	0.345	0.130
26	95.004	$C_2H_6S_2H^+$	Dimethyl disulphide[1]	<0.0001	0.052	<0.0001	<0.0001	<0.0001	0.001	<0.0001
27	95.093			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.001
28	97.063	$C_6H_8OH^+$	2,5-Dimethylfuran/Cyclohexen-2-one	<0.0001	<0.0001	<0.0001	0.002	<0.0001	<0.0001	<0.0001
29	97.106	$C_7H_{13}^+$		<0.0001	<0.0001	<0.0001	<0.0001	0.005	0.415	0.029
30	99.119	$C_7H_{15}^+$	2-Heptanol fragment [1]	<0.0001	<0.0001	<0.0001	<0.0001	0.001	0.013	<0.0001
31	103.074	$C_5H_{10}O_2H^+$	C5 esters and acids (i.e., pentanoic acid/3-methyl-butanoic acid)	<0.0001	<0.0001	0.037	<0.0001	0.204	0.761	0.207
32	105.046	$C_4H_8OSH^+$	Methional [1]	<0.0001	<0.0001	0.185	0.459	0.267	0.182	<0.0001
33	107.066	$C_4H_{10}OSH^+$	Methionol [1]	0.013	<0.0001	0.469	0.025	0.074	0.705	0.061
34	107.107			<0.0001	<0.0001	<0.0001	<0.0001	0.001	<0.0001	0.006
35	109.059	$C_7H_8OH^+$	Benzyl alcohol [1]	<0.0001	<0.0001	0.074	<0.0001	0.136	0.138	0.105
36	111.099			0.001	<0.0001	0.222	0.002	0.389	0.306	0.399
37	115.112	$C_7H_{14}OH^+$	2-Heptanone [1,2]	<0.0001	<0.0001	0.025	<0.0001	0.796	0.147	0.977
38	117.091	$C_6H_{12}O_2H^+$	Hexanoic acid [1]	<0.0001	<0.0001	0.562	0.012	0.125	0.606	0.036
39	119.093	$C_6H_{14}SH^+$		<0.0001	0.002	0.011	0.062	0.167	0.963	0.022
40	121.119			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
41	123.117	$C_9H_{15}^+$		<0.0001	0.015	0.434	0.005	0.241	0.840	0.432
42	126.967			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.111	<0.0001
43	127.050			0.007	<0.0001	0.098	0.089	0.436	0.608	0.831
44	131.105	$C_7H_{14}O_2H^+$	Isoamyl acetate [1]	<0.0001	<0.0001	0.137	<0.0001	0.645	0.848	0.415
45	133.117	$C_7H_{16}O_2H^+$		<0.0001	0.018	0.010	0.076	0.020	0.669	0.027
46	135.100	$C_6H_{14}O_3H^+$		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
47	135.134			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
48	139.137			<0.0001	<0.0001	<0.0001	<0.0001	0.001	0.002	0.001
49	143.137	$C_9H_{18}OH^+$	Nonanal/Nonanone	<0.0001	<0.0001	0.019	<0.0001	0.001	0.612	<0.0001
50	145.123	$C_8H_{16}O_2H^+$	Octanoic acid [1]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.006	<0.0001
51	163.077	$C_{10}H_{10}O_2H^+$		0.001	0.002	0.824	0.001	0.068	0.593	0.122
52	173.154	$C_{10}H_{20}O_2H^+$	Decanoic acid [1]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
53	201.185	$C_{12}H_{24}O_2H^+$	Decanoic acid ethyl ester [1]	<0.0001	<0.0001	<0.0001	<0.0001	0.005	<0.0001	0.020

[1]: m/z that HS-SPME-GC-MS identified. [2]: m/z identified by fastGC-PTR-ToF-MS and/or injection of pure standard. [3]: Ethyl acetate signal dominant in LB672 fermented samples, acetoin signal dominant in LP100 and PP100 fermented samples, ethyl acetate and acetoin (small) signals dominant in LU5335, and PD661 and PD5733 fermented samples based on fastGC-PTR-ToF-MS.

The concentrations of all the 104 m/z compounds' peaks were higher after 7 days compared to 14 days of fermentation. The reduction in VOC production after 7 days is likely a result of substrate depletion, leading to reduced microbial growth and metabolic activity. Additionally, the gas flushing of the headspace would have removed any remaining VOCs from day 7. Therefore, to more accurately assess the effects of the different LAB strains, only VOC data obtained from day 0 and day 7 were compared.

Three-way ANOVA was used to determine that of the 104 sample-related m/z; a total number of 81, 87, 61, 64, 52, 42, and 56 m/z were significantly ($p < 0.05$) differentiated based upon LAB strains, time (at 0 and 7 days), temperature (25 and 35 °C),

strain × time interactions, strain × temperature interactions, time × temperature interactions, and strain × time × temperature interactions, respectively (Table S1). Finally, 53 m/z were selected for which there was a significant ($p < 0.05$) increase (production) in their concentration during fermentation as opposed to a decrease (utilisation) and significant ($p < 0.05$) differences in either the main effects (LAB strains, time, or temperature) or their interaction effects (Tables 4 and 5).

Hierarchical clustering analysis and the heatmap visualisation of the selected m/z showed the VOCs (m/z) produced by the six differing LAB strains growing at either 25 °C (Figure 1) or 35 °C (Figure 2). In heatmap 1 (Figure 1), the VOCs, after 7 days of fermentation at 25 °C, were primarily grouped (column-wise) into two clusters based on the LAB strains used; cluster 1—LP100, PP100, and LB672 (subcluster-LP100 and PP100)—and cluster 2—LU5335, PD5733, and PD661 (subcluster-LU5335, and PD5733). The ketones (m/z 59.049 (t.i. acetone), m/z 87.043 (t.i. diacetyl)), and aldehydes (m/z 85.066 (t.i. 2-methyl-2-butenal and 3-methyl-2-butenal)) were present in higher proportions in the subcluster of cluster 1 LP100 and PP100, and the sulphur compounds (m/z 49.011 (t.i. methanethiol) and m/z 95.004 (t.i. dimethyl disulphide)) were present in higher proportions in cluster 1 LP100, PP100, and LB672. However, alcohols (m/z 43.054 (t.i. propanol fragment), m/z 57.070 (t.i. butanol fragment), m/z 71.085 (t.i. 3-methyl butanol fragment), m/z 91.072 (t.i. 2,3-butanediol), and m/z 109.059 (t.i. benzyl alcohol)) were present in a lower proportion in cluster 1 LP100, PP100, and LB672. In contrast, the alcohols (m/z 47.049 (t.i. ethanol), butanol, propanol, 3-methyl butanol, 2,3-butanediol, m/z 107.066 (t.i. methionol), and benzyl alcohol) were present in a higher proportion in cluster 2 LU5335, PD5733, and PD661, and the ketones (acetone and m/z 115.112 (t.i. 2-heptanone)), aldehydes (2-methyl-2-butenal and 3-methyl-2-butenal), and sulphur compounds (methanethiol and dimethyl disulphide) were present in a lower proportion. Further, the VOCs were row-wise clustered, mainly into two clusters (Figure 1 and Table S2); cluster 1 (orange) was characterised by a higher proportion of alcohols, while cluster 2 (pink) was characterised by sulphur compounds and ketones.

At 35 °C, the VOCs were also mainly clustered into two groups (column-wise) after LAB fermentation, as shown in heatmap 2 (Figure 2); cluster 1—LP100 and PP100—and cluster 2—LU5335, PD5733, PD661, and LB672 (subclusters—LU5335 and PD661; LU5335, PD661, and PD5733). The ketones (acetone, diacetyl, and 2-heptanone), aldehydes (2 and 3-methyl-2-butenal), and sulphur compounds (m/z 105.046 (t.i. methional) and dimethyl disulphide) were present in a higher proportion in the cluster 1 (LP100, and PP100), where alcohols (propanol, ethanol, butanol, 3-methyl butanol, 2,3-butanediol, methionol, and benzyl alcohol) were present in a lower proportion. In contrast, the ketones (acetone, 2-heptanone, and diacetyl) and aldehydes (2 and 3-methyl-2-butenal) were present in a lower proportion in cluster 2 (LU5335, PD5733, PD661 and LB672), and sulphur compounds (methional, methanethiol, and dimethyl disulphide) were present in a lower proportion in subcluster 2 LU5335, PD661, and PD5733. However, alcohols (propanol, butanol, 3-methyl butanol, and 2,3 butanediol) were present in a higher proportion in subcluster 2 LU5335, PD661, and PD5733. Further, VOCs were row-wise clustered into two groups (Figure 2 and Table S3); cluster 1 (orange) was characterised by having a higher proportion of alcohols, while cluster 2 (Pink) was characterised by having a higher proportion of ketones and sulphur compounds. The specific VOCs produced by the six differing LAB strains are discussed separately in the following sections.

Table 5. The mean concentration (ppbV) of selected VOCs (m/z) after 7 days of fermentation by different LAB strains in the DM at either 25 or 35 °C. Values are presented as mean ± standard error (n = 4).

No	*m/z*	LB672		PD661		LP100		PP100		PD5733		LU5335	
		at 25 °C	at 35 °C	at 25 °C	at 35 °C	at 25 °C	at 35 °C	at 25 °C	at 35 °C	at 25 °C	at 35 °C	at 25 °C	at 35 °C
1	26.016	167.048 ± 1.87	441.51 ± 7.46	521.94 ± 14.33	809.748 ± 37.0	26.42 ± 8.04	32.447 ± 4.88	14.709 ± 5.07	64.365 ± 38.27	432.918 ± 3.19	637.032 ± 6.32	408.468 ± 8.04	847.47 ± 72.77
2	27.025	80.096 ± 1.17	191.378 ± 4.39	270.533 ± 23.04	376.513 ± 23.34	14.404 ± 4.68	14.874 ± 2.25	7.521 ± 2.22	30.175 ± 17.55	205.566 ± 2.94	287.241 ± 4.45	195.772 ± 3.35	387.008 ± 31.54
3	28.031	8.817 ± 0.16	21.155 ± 0.51	27.7 ± 3.02	35.906 ± 1.64	1.554 ± 0.27	2.562 ± 0.13	1.156 ± 0.17	3.816 ± 1.49	21.056 ± 0.32	29.6 ± 0.16	19.782 ± 0.27	36.692 ± 2.36
4	31.018	928.421 ± 8.45	2132.831 ± 30.71	2193.987 ± 21.77	3378.789 ± 78.38	113.72 ± 2.24	132.123 ± 3.16	62.939 ± 15.28	165.598 ± 93.39	2105.244 ± 12.26	2959.667 ± 32.48	2006.605 ± 33.32	3231.393 ± 83.87
5	34.996	7.906 ± 0.25	14.439 ± 1.06	7.596 ± 1.83	14.935 ± 2.50	12.348 ± 0.62	0.692 ± 0.08	2.408 ± 0.23	0.758 ± 0.03	2.71 ± 0.19	3.876 ± 0.72	6.139 ± 0.34	4.801 ± 1.06
6	41.039	21.498 ± 0.42	53.09 ± 1.58	601.747 ± 118.91	822.194 ± 66.50	31.464 ± 0.46	56.122 ± 2.82	16.502 ± 2.74	73.972 ± 4.08	238.258 ± 6.52	236.99 ± 5.23	293.942 ± 9.25	516.946 ± 32.39
7	42.01	205.561 ± 2.28	547.972 ± 10.35	750.214 ± 51.63	1102.089 ± 76.24	48.238 ± 13.68	54.497 ± 7.61	25.718 ± 7.45	103.207 ± 57.47	541.102 ± 4.52	827.993 ± 8.69	512.99 ± 11.15	1178.195 ± 131.83
8	43.018	4158.139 ± 62.52	9122.072 ± 343.96	20261.619 ± 12219.10	8616.588 ± 327.42	6452.574 ± 201.49	6037.236 ± 340.36	3937.451 ± 294.38	3808.769 ± 771.34	6128.931 ± 68.91	8725.072 ± 116.73	6255.702 ± 108.34	11864.271 ± 1221.21
9	43.054	5.054 ± 1.14	18.602 ± 1.92	289.246 ± 26.12	519.05 ± 10.56	0 ± 0	0 ± 0	0 ± 0	2.77 ± 1.62	165.123 ± 5.13	145.644 ± 5.6	212.367 ± 6.92	327.327 ± 14.86
10	47.049	17,447.171 ± 176.66	44,863.183 ± 1176.33	51,191.95 ± 3319.03	82,907.442 ± 4413.17	286.84 ± 33.86	935.419 ± 85.42	498.008 ± 296.58	2407.504 ± 2051.69	44,565.668 ± 369.82	66,142.439 ± 1101.32	41,885.022 ± 781.09	88,784.653 ± 4937.75
11	49.011	182.917 ± 13.60	459.487 ± 31.33	49.417 ± 5.9	48.268 ± 3.96	210.152 ± 16.33	171.222 ± 41.01	54.535 ± 2.36	29.212 ± 3.78	51.715 ± 1.3	115.122 ± 16.4	58.243 ± 3.53	63.686 ± 6.42
12	53.006	9.648 ± 0.13	26.73 ± 0.48	104.944 ± 11.88	164.801 ± 12.26	8.502 ± 0.51	12.591 ± 0.41	3.941 ± 0.65	16.672 ± 2.26	52.793 ± 1.16	66.852 ± 1.10	59.265 ± 1.90	123.647 ± 11.11
13	57.036	5.489 ± 0.10	11.73 ± 0.39	138.128 ± 70.24	145.162 ± 12.15	6.711 ± 0.07	14.952 ± 0.76	5.347 ± 0.29	14.073 ± 0.33	35.028 ± 1.07	44.354 ± 1.26	40.768 ± 1.56	102.011 ± 5.78
14	57.07	13.976 ± 0.56	33.623 ± 1.16	558.761 ± 11.31	1080.518 ± 32.65	10.042 ± 0.65	30.006 ± 2.25	9.744 ± 0.16	20.545 ± 0.7	177.261 ± 3.79	236.018 ± 5.82	200.739 ± 7.39	527.166 ± 34.32
15	59.049	24.784 ± 3.49	32.257 ± 4.61	421.171 ± 68.79	534.178 ± 27.50	2206.881 ± 16.40	1745.93 ± 45.96	332.084 ± 15.63	847.678 ± 23.83	159.598 ± 3.26	388.981 ± 2.71	49.138 ± 6.58	382.086 ± 164.52
16	63.009	82.849 ± 1.97	127.534 ± 30.44	107.423 ± 4.06	111.562 ± 34.95	15.177 ± 0.47	17.316 ± 0.51	13.164 ± 1.42	12.978 ± 4.18	128.245 ± 6.27	97.983 ± 6.32	106.979 ± 5.34	121.257 ± 7.12
17	64.005	1.989 ± 0.12	3.025 ± 0.2	1.245 ± 0.17	1.453 ± 0.44	1.212 ± 0.05	0.969 ± 0.1	0.615 ± 0.09	0.383 ± 0.03	1.829 ± 0.06	1.726 ± 0.13	1.516 ± 0.08	1.502 ± 0.23
18	71.085	10.255 ± 0.53	21.316 ± 0.63	570.456 ± 11.35	1106.131 ± 32.59	4.368 ± 0.24	9.914 ± 0.63	5.146 ± 0.16	10.824 ± 0.17	370.814 ± 10.82	332.782 ± 8.55	475.144 ± 17.27	819.329 ± 47.8
19	78.967	5.29 ± 0.53	20.076 ± 2.17	1.37 ± 0.5	2.901 ± 0.78	6.06 ± 0.52	21.371 ± 6.39	2.336 ± 0.21	3.435 ± 0.33	1.372 ± 0.12	6.38 ± 0.84	1.961 ± 0.16	2.234 ± 0.45
20	81.016	3.575 ± 0.17	11.114 ± 0.18	7.418 ± 0.81	9.752 ± 2.77	0.208 ± 0.03	0.309 ± 0.07	0.357 ± 0.03	0.521 ± 0.17	8.684 ± 0.39	8.228 ± 0.53	7.232 ± 0.43	11.237 ± 0.71
21	85.066	0.667 ± 0.04	1.813 ± 0.18	1.760 ± 0.20	2.977 ± 0.40	4.007 ± 0.08	7.929 ± 0.28	2.836 ± 0.13	12.255 ± 0.39	0.836 ± 0.02	1.861 ± 0.08	0.956 ± 0.04	2.4 ± 0.18
22	87.043	2.494 ± 0.11	2.522 ± 0.07	19.148 ± 3.18	4.047 ± 0.69	10.271 ± 0.37	30.118 ± 0.89	4.348 ± 0.21	22.055 ± 2.13	2.624 ± 0.09	2.446 ± 0.09	2.821 ± 0.08	2.657 ± 0.07
23	89.06	107.588 ± 1.68	583.1 ± 11.55	513.225 ± 74.50	662.711 ± 77.45	21.932 ± 1.63	36.727 ± 2.05	6.935 ± 0.80	15.053 ± 1.39	768.849 ± 27.8	834.338 ± 15.05	688.761 ± 13.90	1323.605 ± 88.72
24	91.027	3.1 ± 0.16	14.864 ± 0.69	4.058 ± 2.11	3.108 ± 1.06	1.036 ± 0.03	1.64 ± 0.07	0.723 ± 0.03	1.06 ± 0.08	4.727 ± 0.2	4.062 ± 0.35	5.73 ± 0.32	3.626 ± 2.13
25	91.072	4.743 ± 0.19	27.938 ± 1.74	288.497 ± 51.59	137.918 ± 10.33	1.117 ± 0.18	3.118 ± 0.4	0.907 ± 0.07	16.874 ± 14.34	25.936 ± 0.85	76.375 ± 7.42	24.001 ± 0.96	79.585 ± 1.29
26	95.004	10.25 ± 0.81	37.968 ± 4.04	7.437 ± 1.90	6.718 ± 1.37	10.963 ± 1.47	32.705 ± 7.26	5.798 ± 0.6	7.747 ± 0.47	3.966 ± 0.31	13.75 ± 1.52	5.264 ± 0.45	5.799 ± 0.89
27	95.093	1.81 ± 0.07	12.486 ± 0.43	23.106 ± 10.48	35.172 ± 1.93	0.244 ± 0.04	0.893 ± 0.31	0.248 ± 0.06	0.27 ± 0.08	10.958 ± 0.28	24.895 ± 0.53	9.809 ± 0.53	37.52 ± 1.52
28	97.063	0.963 ± 0.09	10.732 ± 0.68	2.777 ± 2.34	0.478 ± 0.06	0.732 ± 0.04	1.093 ± 0.15	0.444 ± 0.02	0.424 ± 0.05	0.484 ± 0.04	2.646 ± 0.43	0.66 ± 0.03	1.806 ± 0.79
29	97.106	0.044 ± 0.02	0 ± 0	0.241 ± 0.05	0.364 ± 0.09	0.294 ± 0.04	0.31 ± 0.06	0.055 ± 0.01	0.27 ± 0.04	0.1 ± 0.01	0.052 ± 0.02	0.092 ± 0.02	0.204 ± 0.05
30	99.119	0.059 ± 0.03	0.239 ± 0.01	0.025 ± 0.02	0.01 ± 0.01	0.027 ± 0.01	0.08 ± 0.02	0.041 ± 0.00	0.016 ± 0.01	0.012 ± 0.01	0.038 ± 0.019	0.093 ± 0.02	0.135 ± 0.05
31	103.074	0.633 ± 0.07	0.997 ± 0.05	4.497 ± 2.24	3.697 ± 0.18	0.713 ± 0.07	0.747 ± 0.05	0.48 ± 0.02	0.675 ± 0.05	1.644 ± 0.06	2.076 ± 0.1	1.518 ± 0.05	4.072 ± 0.33
32	105.046	1.026 ± 0.06	1.22 ± 0.04	9.568 ± 4.36	2.927 ± 0.35	3.033 ± 0.68	4.07 ± 0.38	1.69 ± 0.3	4.448 ± 0.71	1.546 ± 0.1	1.62 ± 0.08	2.044 ± 0.10	4.597 ± 1.15
33	107.066	27.644 ± 1.29	143.431 ± 10.15	217.522 ± 151.90	61.948 ± 6.59	1.192 ± 0.08	8.355 ± 1.11	1.498 ± 0.64	5.285 ± 2.49	58 ± 0.56	113.907 ± 2.94	62.916 ± 2.64	121.476 ± 25.12
34	107.107	2.129 ± 0.12	7.99 ± 0.51	11.654 ± 3.37	14.393 ± 0.67	0.222 ± 0.04	0.512 ± 0.06	0.188 ± 0.05	0.483 ± 0.17	5.652 ± 0.17	8.888 ± 0.3	6.233 ± 0.20	13.897 ± 0.98
35	109.059	0.336 ± 0.02	2.186 ± 0.11	3.468 ± 2.03	2.352 ± 0.61	0.035 ± 0.02	0.136 ± 0.04	0.008 ± 0.01	0.08 ± 0.02	1.373 ± 0.05	2.024 ± 0.19	1.179 ± 0.1	3.182 ± 0.24

Table 5. *Cont.*

No	*m/z*	LB672		PD661		LP100		PP100		PD5733		LU5335	
		at 25 °C	at 35 °C	at 25 °C	at 35 °C	at 25 °C	at 35 °C	at 25 °C	at 35 °C	at 25 °C	at 35 °C	at 25 °C	at 35 °C
36	111.099	0.46 ± 0.02	2.347 ± 0.05	8.428 ± 6.18	5.95 ± 0.37	0.176 ± 0.02	0.187 ± 0.01	0.176 ± 0.03	0.217 ± 0.02	1.838 ± 0.05	4.268 ± 0.08	1.64 ± 0.09	6.77 ± 0.36
37	115.112	0.121 ± 0.03	0.204 ± 0.06	0.648 ± 0.02	0.938 ± 0.08	4.814 ± 0.16	3.831 ± 0.11	0.153 ± 0.03	0.277 ± 0.04	0.41 ± 0.04	0.676 ± 0.06	0.284 ± 0.01	0.77 ± 0.1
38	117.091	0.488 ± 0.03	1.118 ± 0.05	4.582 ± 2.78	1.807 ± 0.08	0.574 ± 0.03	0.513 ± 0.02	0.406 ± 0.02	0.403 ± 0.06	1.854 ± 0.15	1.738 ± 0.17	1.384 ± 0.11	3.8 ± 0.28
39	119.093	0.204 ± 0.01	0.368 ± 0.05	1.974 ± 0.81	1.592 ± 0.64	0.293 ± 0.01	0.248 ± 0.01	0.136 ± 0.02	0.258 ± 0.08	0.282 ± 0.02	0.608 ± 0.06	0.294 ± 0.04	1.642 ± 0.59
40	121.119	0.076 ± 0.01	0.448 ± 0.03	5.147 ± 1.81	25.03 ± 3.21	0 ± 0	0 ± 0	0 ± 0	0.016 ± 0.01	2.278 ± 0.06	5.06 ± 0.17	2.35 ± 0.14	11.913 ± 1.15
41	123.117	0.016 ± 0.01	0.067 ± 0.02	0.341 ± 0.2	0.183 ± 0.02	0.004 ± 0.00	0.049 ± 0.01	0.011 ± 0.01	0.037 ± 0.01	0.049 ± 0.01	0.079 ± 0.01	0.053 ± 0.01	0.162 ± 0.02
42	126.967	0.708 ± 0.05	1.55 ± 0.11	0.422 ± 0.02	0.449 ± 0.11	0.87 ± 0.07	1.09 ± 0.25	0.337 ± 0.01	0.428 ± 0.02	0.244 ± 0.01	0.549 ± 0.06	0.342 ± 0.02	0.368 ± 0.06
43	127.05	0.126 ± 0.02	0.125 ± 0.02	0.21 ± 0.08	0.214 ± 0.03	0.137 ± 0.02	0.15 ± 0.01	0.098 ± 0.01	0.119 ± 0.02	0.153 ± 0.02	0.157 ± 0.01	0.14 ± 0.01	0.224 ± 0.02
44	131.105	0.344 ± 0.02	0.67 ± 0.03	0.855 ± 0.03	1.078 ± 0.11	0.206 ± 0.01	0.219 ± 0.02	0.213 ± 0.01	0.184 ± 0.06	2.190 ± 0.18	3.322 ± 0.61	2.756 ± 0.12	6.385 ± 1.02
45	133.117	0.025 ± 0.02	0.036 ± 0.02	2.152 ± 0.66	1.873 ± 0.96	0.046 ± 0.02	0.094 ± 0.01	0.034 ± 0.02	0.1 ± 0.07	0.142 ± 0.01	0.344 ± 0.02	0.152 ± 0.02	3.062 ± 1.52
46	135.1	0.265 ± 0.02	2.334 ± 0.03	5.114 ± 2.89	5.545 ± 0.57	0.132 ± 0.01	0.12 ± 0.02	0.092 ± 0.01	0.139 ± 0.02	3.261 ± 0.11	5.336 ± 0.17	2.618 ± 0.07	10.64 ± 0.58
47	135.134	0.017 ± 0.01	0.126 ± 0.04	9.817 ± 1.56	28.434 ± 2.93	0 ± 0	0.011 ± 0.01	0.008 ± 0.01	0.045 ± 0.03	5.025 ± 0.19	6.919 ± 0.11	6.173 ± 0.46	20.47 ± 1.62
48	139.137	0.231 ± 0.01	1.246 ± 0.04	2.396 ± 1.4	4.003 ± 0.23	0.192 ± 0.02	0.389 ± 0.06	0.233 ± 0.03	0.333 ± 0.03	0.834 ± 0.03	2.522 ± 0.12	0.684 ± 0.06	4.342 ± 0.22
49	143.137	0.217 ± 0.03	0.161 ± 0.04	0.574 ± 0.24	0.411 ± 0.16	0.883 ± 0.03	0.778 ± 0.40	0.256 ± 0.02	0.198 ± 0.03	0.225 ± 0.04	1.254 ± 0.04	0.204 ± 0.02	0.296 ± 0.05
50	145.123	0.218 ± 0.03	0.43 ± 0.05	0.69 ± 0.24	0.617 ± 0.08	0.264 ± 0.01	0.202 ± 0.02	0.176 ± 0.02	0.209 ± 0.03	0.634 ± 0.1	0.883 ± 0.10	0.574 ± 0.05	1.592 ± 0.14
51	163.077	0.113 ± 0.02	0.118 ± 0.02	0.324 ± 0.12	0.194 ± 0.02	0.09 ± 0.01	0.1 ± 0.01	0.131 ± 0.02	0.112 ± 0.02	0.17 ± 0.01	0.131 ± 0.02	0.155 ± 0.00	0.274 ± 0.04
52	173.154	0.189 ± 0.01	0.629 ± 0.11	0.667 ± 0.15	1.103 ± 0.08	0.072 ± 0.03	0.116 ± 0.01	0.059 ± 0.02	0.066 ± 0.013	0.958 ± 0.18	1.692 ± 0.25	0.706 ± 0.016	2.857 ± 0.27
53	201.185	0.046 ± 0.01	0.177 ± 0.02	0.145 ± 0.022	0.195 ± 0.01	0.027 ± 0.01	0.034 ± 0.02	0.012 ± 0.01	0.026 ± 0.014	0.15 ± 0.04	0.241 ± 0.02	0.15 ± 0.014	0.186 ± 0.02

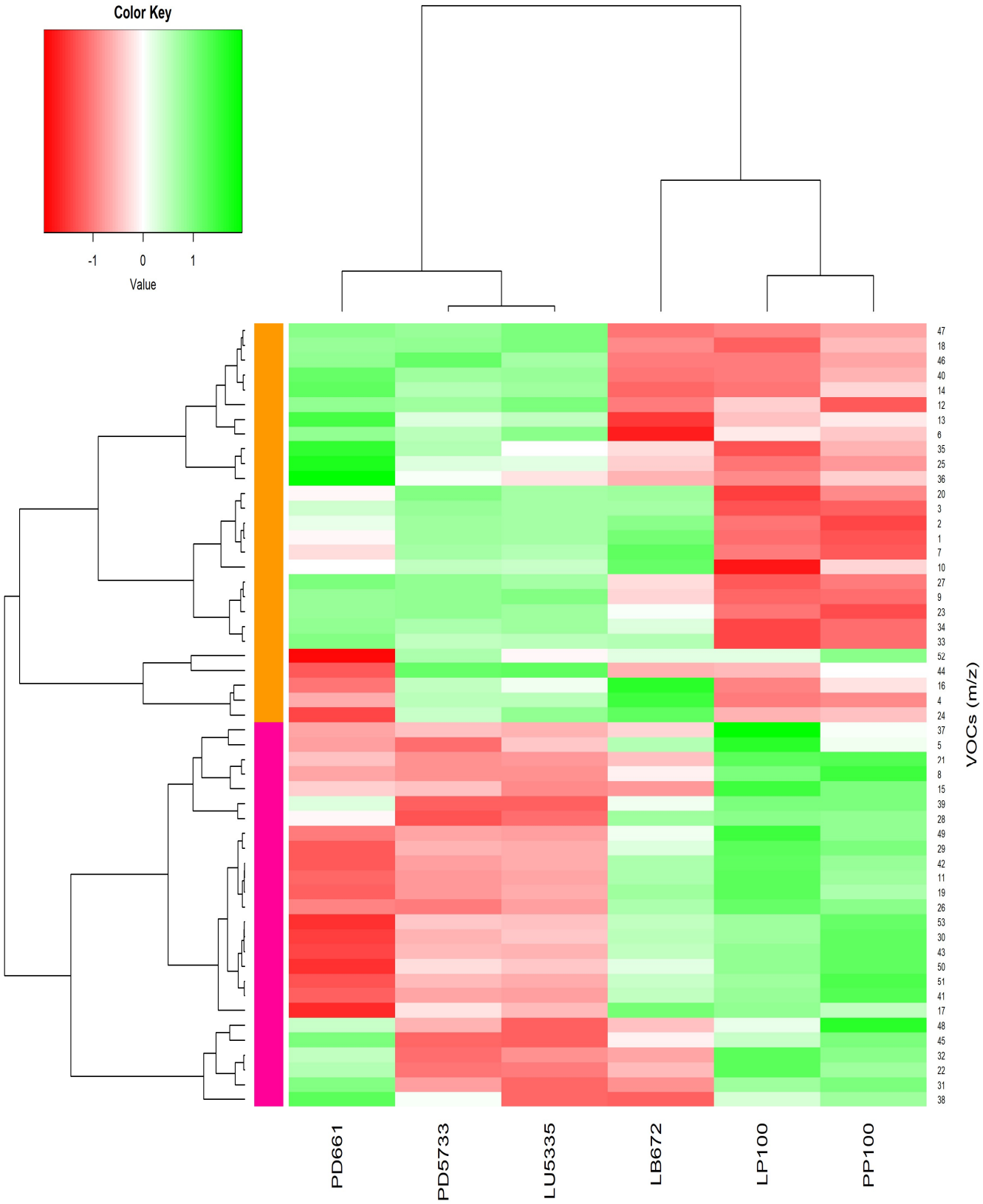

Figure 1. Heatmap visualisation and hierarchical clustering analysis of m/z (VOCs) produced by different LAB strains (Table 1) based on the log 2-transformed average concentration (ppbV) of each m/z. Fermentation was carried out in the DM for 7 days at 25 °C. The green colour represents a higher abundance, while the red colour indicates a lower abundance. The flavour VOCs represented in the heatmap are numbered according to the numbers in Table S2.

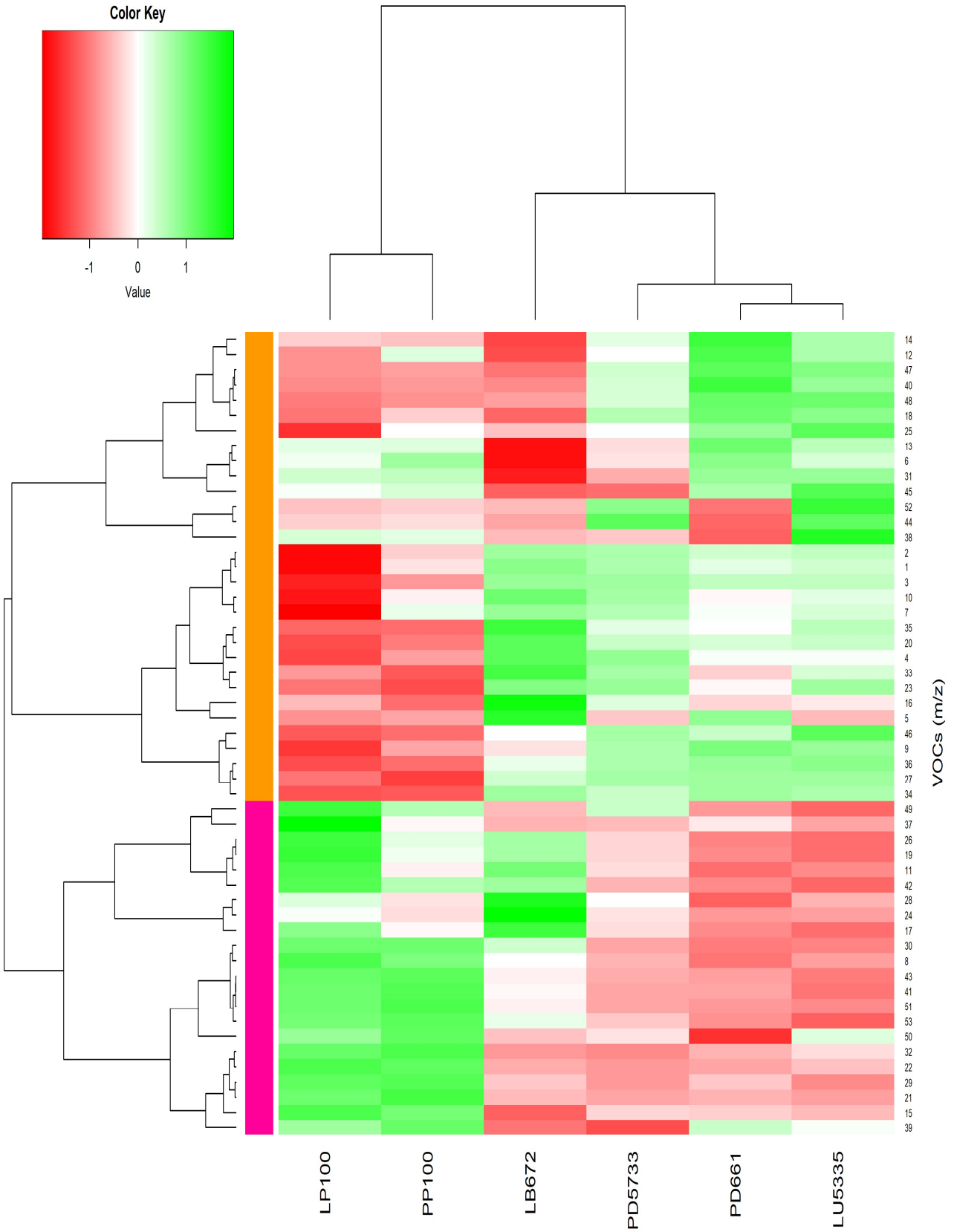

Figure 2. Heatmap visualisation and hierarchical clustering analysis of m/z (VOCs) produced by different LAB strains (Table 1) based on the log 2-transformed average concentration (ppbV) of each m/z. Fermentation was carried out in the DM for 7 days at 35 °C. The green colour represents a higher abundance, while the red colour indicates a lower abundance. The VOCs represented in the heatmap are numbered according to the numbers in Table S3.

3.2.1. Main Alcohols

Ethanol, which can be a key marker compound in fermentation studies, is produced by some LAB during sugar fermentation. In the present study, the concentration of m/z 47.049 (t.i. ethanol) was significantly ($p < 0.05$) higher after 7 days of fermentation at either 25 or 35 °C by PD661, LU5335, PD5733, and LB672 compared to the other two strains studied (Figure 3a). LU5335 and LB672 are heterofermentative LAB and produce ethanol via the phosphoketolase (PK) pathway. However, it seems that even though PD661 and PD5733 are classified as homofermentative LAB, the detection of ethanol suggests that under the fermentation conditions used in this study, glucose was fermented heterofermentatively [44]. Further, ethanol was not detected or was detected in very low concentrations after 7 days of fermentation by either LP100 or PP100, which are classified as being facultative heterofermentative or homofermentative LAB, respectively. Since acetaldehyde, an intermediate in the ethanol production pathway, was detected in DM after fermentation by LP100 and PP100 strains, these data suggest that these strains did not contain the alcohol dehydrogenase (AlcDH) enzyme. Besides sugar fermentation, ethanol can be produced from threonine amino acid [45–47]. Further, given the differences in the concentration of sugar (glucose) and threonine in the DM, it is speculated that the ethanol produced by PD661, LU5335, PD5733, and LB672 strains in this study was from mainly sugar metabolism.

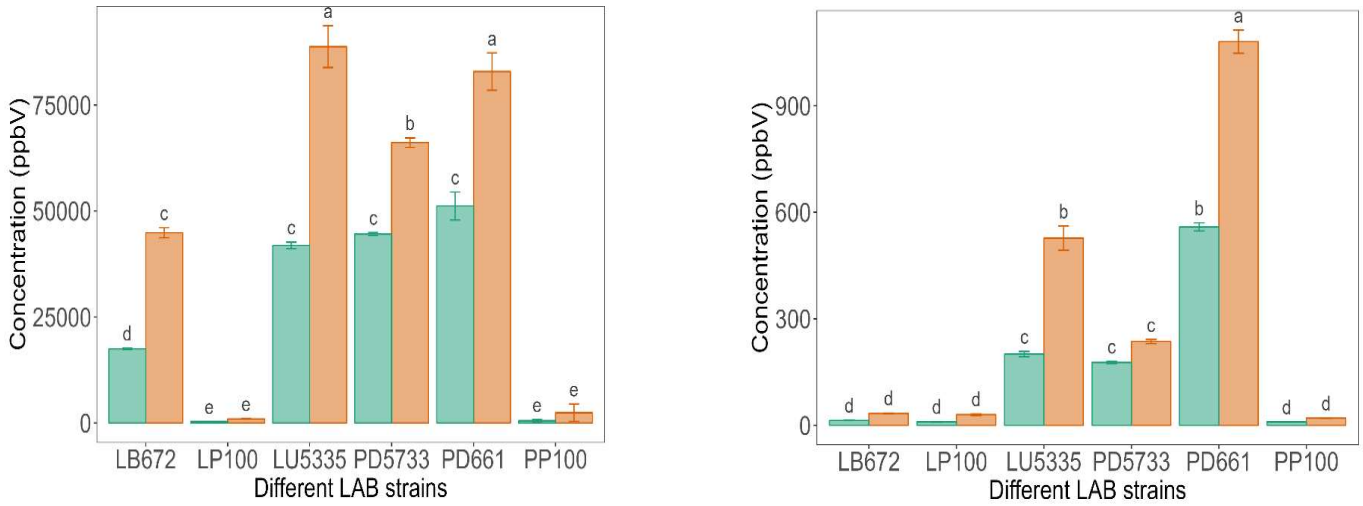

Figure 3. Mean concentrations (ppbV) of m/z 47.049 (t.i. ethanol) (**a**) and m/z 57.070 (t.i. butanol fragment) (**b**) across the DM fermented by different LAB strains at either 25 (■) or 35 (■) °C after 7 days. Values are presented as mean ± standard error (n = 4). Different superscript lowercase letters represent significant differences between the DM fermented by different LAB strains according to Tukey's test at $p < 0.05$.

The concentration of m/z 57.070 (t.i. butanol fragment), which is produced from sugars through the fatty acid biosynthesis pathway [48], was significantly ($p < 0.05$) higher after 7 days of fermentation at 35 °C by PD661, followed by LU5335 at 35 °C and PD661 at 25 °C compared to the other four strains studied, with PD5733 being significantly ($p < 0.05$) higher than the other three strains (Figure 3b).

3.2.2. VOCs Produced from Glucose and/or Aspartic Acid

Diacetyl, also known as 2,3-butanedione, is an important flavour compound across a range of dairy products, including in yoghurt [49]. It is generated from the metabolism of either sugar, citrate or aspartic acid, forming pyruvate [50]. Pyruvate is subsequently converted to acetaldehyde-TPP through a decarboxylation process, and then to α-acetolactate,

facilitated by α-acetolactate synthase (ALS). ALS has a low affinity to pyruvate; therefore, α-acetolactate synthesis is favoured in the presence of excess pyruvate. α-acetolactate is an unstable intermediate, which, in the presence of molecular oxygen, can be converted to diacetyl through nonenzymatic oxidative carboxylation [51–54]. In the present study, the concentration of m/z 87.043 (t.i. diacetyl) was significantly ($p < 0.05$) higher after 7 days of fermentation at 35 °C for LP100, followed by PD661 at 25 °C and PP100 at 35 °C compared to the other three strains studied (Figure 4a). The production of diacetyl by LAB has been reported to be strain-dependent, as the presence of enzymes varies between species and strains [55]. *Lpb. plantarum* fermented fruit juices have previously been reported to produce higher concentrations of diacetyl compared to unfermented juices [56,57]. Further, *Lpb. plantarum* strains have been reported to produce diacetyl in a complex medium containing glucose as a carbon source, where *Lev. brevis* strains did not produce diacetyl and *Lpb. plantarum* strains produced higher concentrations of diacetyl when the complex medium was supplemented with citrate [58]. Since citrate and glucose co-metabolism was not investigated in the current study, diacetyl was assumed to be produced either from glucose or glucose and aspartic acid. The presence of aspartic acid catabolic enzymes has been reported in *Lpb. plantarum* and *P. pentosaceus* strains by a genomic study [59]. Therefore, in the current study, aspartic acid catabolism may also lead to pyruvate accumulation in LP100 and PP100 strains, in addition to glucose metabolism, explaining the higher concentration of diacetyl in these strains at 35 °C. Interestingly, PD661 produced higher concentration of diacetyl at 25 °C compared to PD5733 at either 25 or 35 °C. It is obvious that the activity of enzymes can differ between various strains of the same LAB species [28,59].

The concentration of m/z 91.072 (t.i. 2,3-butanediol) was significantly ($p < 0.05$) higher after 7 days of fermentation at 25 °C by PD661 compared to the other five strains studied (Figure 4b). 2,3-butanediol can be produced from acetoin using the enzyme diacetyl acetoin reductase (DAR). Acetoin is synthesised from α-acetolactate, an intermediate in diacetyl synthesis. α-acetolactate is an unstable intermediate that is decarboxylated enzymatically to yield acetoin. Acetoin can also be synthesised from diacetyl using the enzyme DAR. The 2,3-butanediol can subsequently be reoxidised into acetoin by enzyme 2,3-butanediol dehydrogenase (BDH) [52,53].

The higher production of diacetyl and 2,3-butanediol after fermentation by PD661 is explained by higher DAR enzyme activity and lower BDH enzyme activity. However, an in-depth genomic study in relation to flavour-forming pathways is required to confirm the presence and the activity of DAR and BDH enzymes in all strains studied.

The concentration of m/z 89.060 (t.i. ethyl acetate and acetoin) was significantly ($p < 0.05$) higher after 7 days of fermentation at 35 °C by LU5335 compared to the other five strains studied at both temperatures (Figure 4c). Based on the RT of the main and fragment ions checked in the fastGC-PTR-ToF-MS for standards and samples (Table 6), the m/z 89.060 detected by PTR-ToF-MS was likely to be mainly ethyl acetate and acetoin (a small signal was observed for ethyl butanoate). However, there were differences between LAB strains in the contribution of ethyl acetate and acetoin for m/z 89.060; the ethyl acetate signal was dominant in LB672 fermentation, the acetoin signal was dominant in the fermentations carried out by LP100 and PP100, and for the LU5335, PD661, and PD5733 fermentations, ethyl acetate and acetoin (small signal) signals were dominant.

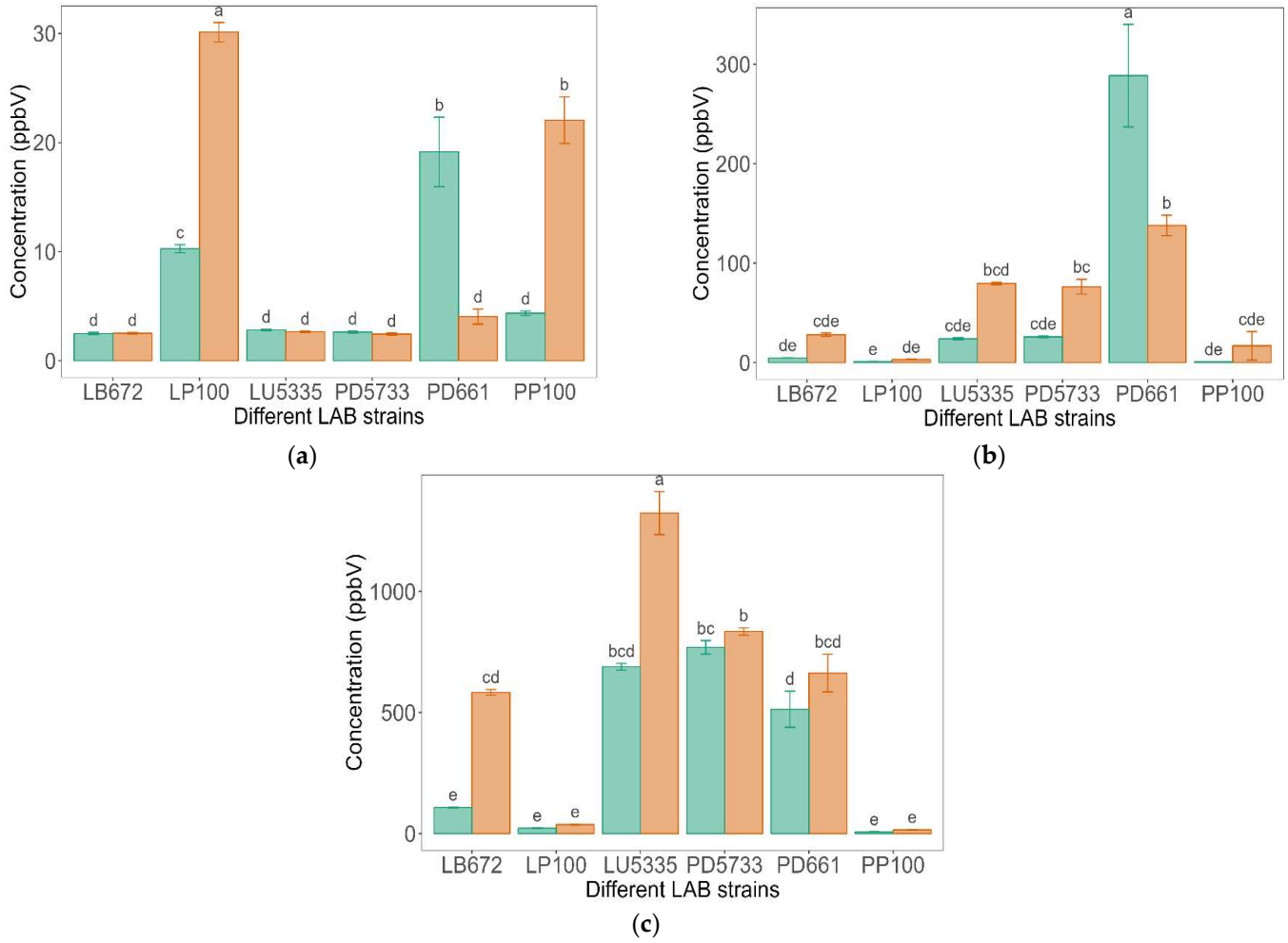

Figure 4. Mean concentrations (ppbV) of *m*/*z* 87.043 (t.i. diacetyl) (**a**), *m*/*z* 91.072 (t.i. 2,3-butanediol) (**b**), and *m*/*z* 89.060 (t.i. ethyl acetate and acetoin) (**c**) across the DM fermented by different LAB strains at either 25 (□) or 35 (□) °C after 7 days. Values are presented as mean ± standard error (n = 4). Different superscript lowercase letters represent significant differences between the DM fermented by different LAB strains according to Tukey's test at $p < 0.05$.

Table 6. Main and fragment ions checked for *m*/*z* 89.060 in fastGC-PTR-ToF-MS.

Compound Name	Molecular Formula	Main/Fragment Ions Checked			
		m/z	*m/z*	*m/z*	*m/z*
Ethyl acetate	$C_4H_8O_2$	89.06 $(C_4H_8O_2)H^+$	61.028 $(C_2H_4O_2)H^+$	43.018 $(C_2H_3O)H^+$	
Butyric acid	$C_4H_8O_2$	89.06 $(C_4H_8O_2)H^+$	71.049 $(C_4H_6O)H^+$	43.054 $(C_3H_7)H^+$	29.039 $(C_2H_5)H^+$
Acetoin	$C_4H_8O_2$	89.06 $(C_4H_8O_2)H^+$			

3.2.3. Other Specific VOCs

The concentration of *m*/*z* 49.011 (t.i. methanethiol) was significantly ($p < 0.05$) higher after 7 days of fermentation at 35 °C by LB672 compared to the other five strains studied (Figure 5a). Methanethiol, which contributes to the flavour of meat [60] and cheese [61,62], can be produced by LAB from methionine via transamination reactions or demethiolation, or from the enzymatic breakdown of methional [45,63–65].

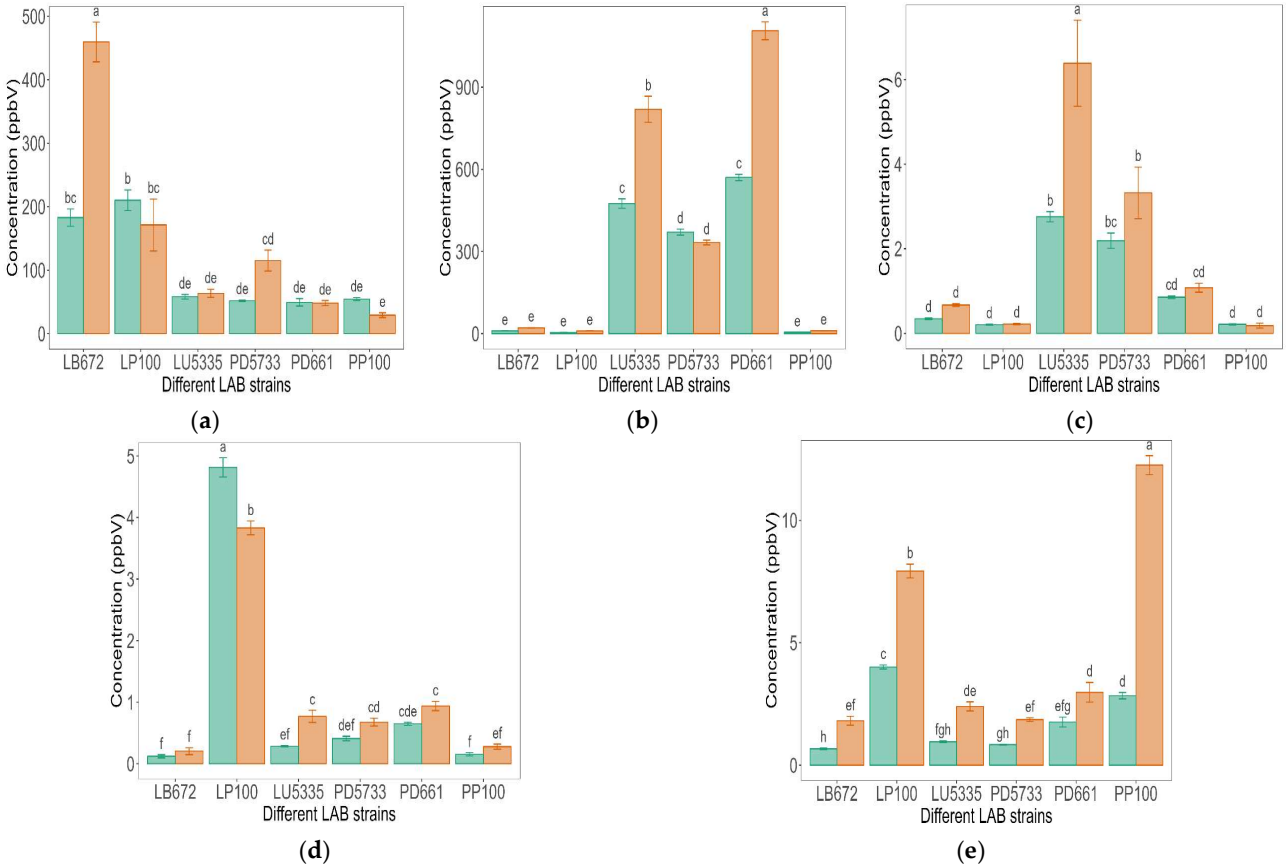

Figure 5. Mean concentrations (ppbV) of m/z 49.011 (t.i. methanethiol) (**a**), m/z 71.085 (t.i. 3-methyl butanol) (**b**), m/z 131.105 (t.i. isoamyl acetate) (**c**), m/z 115.112 (t.i. 2-heptanone) (**d**), and m/z 85.070 (t.i. 2-methyl-2-butenal and 3-methyl-2-butenal) (**e**) across the DM fermented by different LAB strains at either 25 (■) or 35 (■) °C after 7 days. Values are presented as mean ± standard error (n = 4). Different superscript lowercase letters represent significant differences between the defined medium fermented by different LAB strains according to Tukey's test at $p < 0.05$.

The concentration of m/z 71.085 (t.i. 3-methyl butanol fragment) was significantly ($p < 0.05$) higher after 7 days of fermentation at either 25 or 35 °C by PD661, LU5335, or PD5733 compared to the other three strains studied (Figure 5b). 3-Methyl butanol, which is an important flavour compounds in cheese [61,62], can be produced via the enzyme-mediated transamination of leucine [45,46].

The concentration of m/z 131.105 (t.i. 3-methyl butyl acetate), which is formed by a reaction between 3-methyl-butanol and acetyl CoA, was significantly ($p < 0.05$) higher after 7 days of fermentation at 35 °C by LU5335 compared to the other five strains studied at both temperatures (Figure 5c).

The concentration of m/z 115.112 (t.i. 2-heptanone), which is another important ketone in cheese [66], was significantly ($p < 0.05$) higher at 7 days of fermentation at either 25 or 35 °C by LP100 compared to the other strains at all temperature and time points (Figure 5d). 2-Heptanone is synthesised from octanoic acid. Octanoic acid is first oxidised into β-ketoacid via several β-oxidation steps using a range of enzymes, which is then decarboxylated into 2-heptanone with one less carbon atom [63,67].

The concentration of m/z 85.070 (t.i. 2-methyl-2-butenal and 3-methyl-2-butenal) was significantly ($p < 0.05$) higher after 7 days of fermentation at 35 °C for PP100, followed by LP100 at 35 °C compared to the other four strains studied (Figure 5e). 2-methyl-2-butenal and 3-methyl-2-butenal can be produced by LAB from isoleucine and leucine, respectively [68].

4. Discussion

The DM used was developed based on published studies and refined through several LAB growth trials. It contained glucose, peptone, and an amino acid mixture (leucine, isoleucine, phenylalanine, glutamic acid, aspartic acid, threonine, and methionine), sodium acetate, vitamins, and minerals at minimal concentrations needed for the optimum growth of LAB. The influence of seven LAB strains (LB672, LD677, PD661, LP100, PP100, PD5733, and LU5335) on the relative concentrations of VOCs in the DM was investigated in this study, as VOC production by LAB is strain-dependent [28,69].

All LAB strains except LD677 grew well in the developed DM, based on pH, OD_{600} values, and the visual inspection of the turbidity of the culture medium. As LAB's nutritional needs are strain-dependent, the better overall growth of all strains may have been obtained if the DM was optimised for each LAB strain separately. This could have provided further information on the generation of VOCs for each LAB strain in relation to the composition of the DM.

Fermentation reactions happen in real-time, where compounds can be produced and then consumed during fermentation, which means that during fermentation, compounds that could have been produced are subsequently converted into other compounds and hence are not present at the end of fermentation. To address this challenge, real-time PTR-ToF-MS was used to determine the VOCs produced by LAB strains in the DM overtime. A disadvantage of PTR-ToF-MS is that it is a one-dimensional analytical technique (i.e., no separation of VOCs occurs), meaning that unambiguous identification is not always possible. This limitation in part can be overcome by coupling PTR-ToF-MS with fastGC to improve compound identification and the use of complimentary analysis by GC-MS to support VOC identification [32–34]. Therefore, to support the identification of VOCs detected by PTR-ToF-MS, this study used fastGC-PTR-ToF-MS and HS-SPME-GC-MS (Tables 3–5 and S1).

The heatmap analysis highlighted differences between the VOCs produced by different LAB strains after 7 days of fermentation at either 25 or 35 °C (Figures 1 and 2, respectively). The DM used in this study had the same composition for all the LAB strains fermentation, but the VOCs produced varied among LAB strains incubated at either 25 or 35 °C. This result demonstrates that the presence of enzymes and/or enzyme activity varied among these strains.

As well as supporting growth, amino acids serve as the building blocks for important flavour compounds [64]. Though the amino acids methionine, leucine, and isoleucine were present in the DM in the same concentration, the concentrations of amino acid-derived VOCs such as methanethiol, 3-methyl butanol, 3-methyl-2-butenal, and 2-methyl-2-butenal differed based on the LAB strain, suggesting that the activity of methionine, leucine, and isoleucine catabolic enzymes varied between the LAB strains used in this study.

It is clear that at 35 °C as opposed to 25 °C, there was a significant increase in the concentration of specific VOCs (Figures 4 and 5). For example, the concentration of diacetyl was higher in LP100 fermentation at 35 °C; esters (isoamyl acetate and ethyl acetate) were higher in LU5335 ferment at 35 °C; the concentration of higher alcohol, 3-methyl butanol, was higher in PD661 and LU5335 ferments at 35 °C; and the sulphur VOC, methanethiol, was higher in LB672 ferment at 35 °C. This demonstrates that temperature influenced the VOC generation by these LAB strains in the DM.

In addition to the seven LAB strains used in the current study, strains such as *Lacticaseibacillus casei*, *Lacticaseibacillus paracasei*, *Lactobacillus acidophilus*, *Lacticaseibacillus rhamnosus*, *Lactobacillus helveticus*, and *Bifidobacterium lactis*, have been reported in plant-based fermentation studies [70]; therefore, further study using different commercial LAB strains is required.

5. Conclusions

The generation of fermentation VOCs by six (seven) commercial LAB strains growing in a DM was analysed using PTR-ToF-MS, HS-SPME-GC-MS, and fastGC-ToF-MS. The use of PTR–ToF–MS enabled the discovery of differences in VOCs produced between LAB strains (either homo, hetero, or facultative heterofermentative) and fermentation temperature (at either 25 or 35 °C). GC-MS-based techniques such as HS-SPME-GC-MS and fastGC-PTR-ToF-MS supported the identification of compounds detected by direct PTR-ToF-MS. Overall, differences between the relative concentrations of VOCs produced by LAB strains in the DM suggest the presence or differing activity of various enzymes.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/applmicrobiol5010033/s1: Table S1: The VOCS (m/z) detected by PTR-ToF-MS during LAB fermentation in defined medium that significantly ($p < 0.05$) distinguished between different LAB strains (S), fermentation time (0 and 7 days) (T), and temperature (either at 25 or 35 °C) (Temp) and their interaction effects; Table S2: Heatmap compounds (m/z) at 25 °C; Table S3: Heatmap compounds (m/z) at 35 °C.

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Conflicts of Interest: The authors declare no conflicts of interest.

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