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The Effect of Different Medium Compositions and LAB Strains on Fermentation Volatile Organic Compounds (VOCs) Analysed by Proton Transfer Reaction-Time of Flight-Mass Spectrometry (PTR-ToF-MS)

Sarathadevi Rajendran [1,2,3], Iuliia Khomenko [2], Patrick Silcock [1], Emanuela Betta [2], Michele Pedrotti [2], Franco Biasioli [2] and Phil Bremer [1,*]

[1] Department of Food Science, University of Otago, P.O. Box 56, Dunedin 9054, New Zealand; sarathadevi.rajendran@postgrad.otago.ac.nz (S.R.); pat.silcock@otago.ac.nz (P.S.)
[2] Sensory Quality Unit, Research and Innovation Centre, Edmund Mach Foundation, 38098 San Michele all'Adige, Italy; iuliia.khomenko@fmach.it (I.K.); emanuela.betta@fmach.it (E.B.); michele.pedrotti@fmach.it (M.P.); franco.biasioli@fmach.it (F.B.)
[3] Department of Agricultural Chemistry, Faculty of Agriculture, University of Jaffna, Kilinochchi 44000, Sri Lanka
* Correspondence: phil.bremer@otago.ac.nz

Abstract: Lactic acid bacteria (LAB) fermentation is a viable approach for producing plant-based flavour compounds; however, little is understood about the impact of different LAB strains and medium compositions on the production of volatile organic compounds (VOCs). This study investigated the impact of the addition of individual amino acids (AAs) (L-leucine, L-isoleucine, L-phenylalanine, L-glutamic acid, L-aspartic acid, L-threonine, or L-methionine) to a defined medium (DM) on the generation of VOCs (after 0, 7, and 14 days) by one of three LAB strains (*Levilactobacillus brevis* WLP672 (LB672), *Lactiplantibacillus plantarum* LP100 (LP100), and *Pediococcus pentosaceus* PP100 (PP100)), using proton transfer reaction-time of flight-mass spectrometry (PTR-ToF-MS). The concentration of m/z 45.031 (t.i. acetaldehyde) was significantly ($p < 0.05$) higher after 7 days of fermentation by LP100 in the DM supplemented with threonine compared to all other media fermented by all three strains. The concentrations of m/z 49.012 (t.i. methanethiol) and m/z 95.000 (t.i. dimethyl disulfide) were significantly ($p < 0.05$) higher after 7 days of fermentation by either LP100, PP100, or LB672 in the DM supplemented with methionine compared to all other media. Information on the role of individual AAs on VOCs generation by different LAB strains will help to guide flavour development from the fermentation of plant-based substrates.

Keywords: amino acids; defined medium; lactic acid bacteria; volatile organic compounds

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1. Introduction

Plant-based diets have become popular as a means of reducing the environmental impact associated with animal-based diets while also improving human health and animal welfare [1,2]. There is a wide range of meat and dairy analogues available in the market [3]; however, such products frequently lack the overall flavour of their traditional counterparts [4–7].

Flavour is a multifaceted sensory perception encompassing volatile organic compounds (VOCs) (aroma/odour) sensed at olfactory receptors in the nose, non-volatile organic compounds (taste) sensed at gustatory receptors on the tongue and influenced by chemesthetic responses [8–11]. VOCs have a significant impact on the food's overall flavour [8,11].

Fermentation via lactic acid bacteria (LAB) is a promising approach to generate specific meat and dairy VOCs from plant substrates [12–16]. However, it is difficult to relate the

influence of plant substrate, fermentation conditions, and LAB strain on VOCs produced due to the complexity of the compounds present in plant-based systems. In addition, LAB are a fastidious group of bacteria, and the majority of them are auxotrophic to vitamins and amino acids, requiring rich, complex cultivation media for normal growth [17,18]. However, using a rich, poorly defined cultivation medium makes it difficult to determine how changes in medium composition impact the production of specific VOC. To better understand the production of VOCs in accordance with the compositions of each medium, a defined medium (DM) is therefore required [19]. The growth of LAB in DM has been thoroughly investigated over many years [20–24]. However, only a few studies have examined the impact of LAB on the VOCs produced during growth in DM [25–27], and no studies have reported on the VOC production by different LAB strains in response to changes in DM composition.

LAB cultivation media commonly contain carbohydrates (simple sugars), proteins/amino acids, minerals, vitamins, and buffering agents [17]. Amino acids (AAs) serve as the building blocks for important flavour compounds in addition to being a substrate for growth. The first stage of AA catabolism is transamination, where LAB uses the aminotransferase enzyme to convert AAs into α-ketoacids with the presence of α-ketoglutarate. Produced α-ketoacids can then be decarboxylated into aldehydes, which can then be dehydrogenated into alcohols or carboxylic acids by alcohol dehydrogenase and aldehyde dehydrogenase, respectively [28–30]. The current study focuses on the impact of adding individual AA to a DM on the generation of specific fermentation VOCs.

The generation of specific VOCs by LAB fermentation is highly strain-dependent as it is dependent on different metabolic pathways carried out by LAB strains using various enzymes [31,32]. Thus, this study also aims to investigate how commercial LAB strains grown in a DM individually produce specific VOCs.

Proton transfer reaction-mass spectrometry (PTR-MS) enables the rapid, direct, and non-invasive real-time monitoring of VOC with extremely high sensitivity (parts per trillion (ppt) by volume) [33]. The fundamental principles of PTR-MS have been well covered in past reviews [34,35]. The main challenge with PTR-MS applications is that identification is based on the molecular formula without the capability to separate isomers. Thus, the parallel use of GC-MS and/or fastGC-PTR-MS analysis is usually required to support compound identification [36–38].

Therefore, the objective of this study was to use PTR-ToF (time of flight analyser)-MS, HS-SPME-GC-MS, and fastGC-PTR-ToF-MS to determine the VOCs produced by three commercial LAB strains (either *Levilactobacillus brevis* WLP672, *Lactiplantibacillus plantarum* LP100, or *Pediococcus pentosaceus* PP100) growing in DM supplemented with individual AAs (either L-leucine, L-isoleucine, L-phenylalanine, L-glutamic acid, L-aspartic acid, L-threonine, or L-methionine).

2. Materials and Methods

2.1. LAB Strains

Three commercial LAB strains were used in this study. The *Levilactobacillus brevis* WLP672 (hereafter referred to as LB672) was obtained from White labs, San Diego, CA, USA. *Lactiplantibacillus plantarum* LP100 (LP100) and *Pediococcus pentosaceus* PP100 (PP100) were obtained from BIOAGRO, Thiene, Italy. The strains were maintained at 4 °C prior to use. For activation, 1 mL of either a liquid stock culture or 1 g of lyophilized powder was added to 10 mL of de Man, Rogosa, and Sharpe (MRS) broth, which was incubated at 25 °C for 3 days under anaerobic conditions (Anaerobic packs, Mitsubishi Gas Chemical (MGC) Company, Tokyo, Japan). An aliquot of the resulting culture was inoculated onto MRS agar medium using the streak plate method to obtain single colonies and incubated at 25 °C for 3 days using MGC anaerobic packs. An inoculating suspension was prepared by adding three individual colonies from the streak plate to 10 mL of the MRS broth, which was incubated at 25 °C for 3 days using the MGC anaerobic packs. The cells were pelleted by centrifugation (5000× *g* for 5 min at 20 °C) (PK 121R/ALC International, Cologno Monzese,

Italy) and washed twice with sterilized phosphate-buffered saline (PBS) (100 mL; 0.8 g NaCl, 0.02 g KCl, 0.144 g Na_2HPO_4, and 0.0245 g KH_2PO_4, pH of 7.4) and then resuspended to a final concentration of 1×10^9 CFU/mL. The resulting suspension was used as the inoculum in the fermentation trials.

2.2. Medium Compositions

The DM was developed based on earlier research [20,23,24,39–42] and refined through a number of growth trials. The DM contained D-glucose (20 g/L), peptone (enzymatic protein digest) (5 g/L), sodium acetate (12 g/L), mineral salts ($MgSO_4.7H_2O$ (0.2 g/L), NaCl (0.01 g/L), $FeSO_4.7H_2O$ (0.01 g/L), and $MnSO_4.5H_2O$ (0.04 g/L)), and vitamins (calcium pantothenate (B5) (0.4 mg/L), nicotinic acid (B3) (0.2 mg/L), riboflavin (B2) (0.4 mg/L), and thiamine HCl (B1) (0.2 mg/L)), and an amino acid (AA) mixture (0.4 g/L of each AAs; L-leucine (Leu), L-isoleucine (Ile), L-phenylalanine (Phe), L-glutamic acid (Glu), L-aspartic acid (Asp), L-threonine (Thr), and L-methionine (Met)). Further, individual AAs (2 g/L) were added into DM. Therefore, eight types of media were prepared as shown in Table 1. The AAs were dissolved in an HCl solution (50 mM). All stock solutions were prepared using deionized water unless otherwise stated. The glucose and vitamin solutions were filter-sterilized using a 0.22 µm syringe filter (Nylon membrane; BIOFIL, Kowloon, Hong Kong), while all the other components were sterilized by autoclaving at 121 °C for 15 min. Unless otherwise specified, all of the chemicals used were of analytical grade. All procedures were carried out in a class II biological safety cabinet.

Table 1. Overview of medium compositions used.

Media	Glucose	Peptone	Vitamins	Mineral Salts	Sodium Acetate	AA Mixture	Glu	Asp	Leu	Ile	Thr	Phe	Met
DM	2%	0.5%	√	√	1.2%	0.04%	-	-	-	-	-	-	-
DMG	2%	0.5%	√	√	1.2%	0.04%	0.2%	-	-	-	-	-	-
DMA	2%	0.5%	√	√	1.2%	0.04%	-	0.2%	-	-	-	-	-
DML	2%	0.5%	√	√	1.2%	0.04%	-	-	0.2%	-	-	-	-
DMI	2%	0.5%	√	√	1.2%	0.04%	-	-	-	0.2%	-	-	-
DMT	2%	0.5%	√	√	1.2%	0.04%	-	-	-	-	0.2%	-	-
DMP	2%	0.5%	√	√	1.2%	0.04%	-	-	-	-	-	0.2%	-
DMM	2%	0.5%	√	√	1.2%	0.04%	-	-	-	-	-	-	0.2%

DM: original defined medium; DMG: DM added with glutamic acid (Glu); DMA: DM added with aspartic acid (Asp); DML: DM added with leucine (Leu); DMI: DM added with isoleucine (Ile); DMT: DM added with threonine (Thr); DMP: DM added with phenylalanine (Phe); DMM: DM added with methionine (Met).

2.3. Fermentation

Prior to fermentation, the prepared media were held for 3 days at 25 °C to ensure sterility. Then, 4 mL aliquots of the media 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 inoculated to each headspace vial, which was flushed with N_2 at a rate of 10 mL/min for 20 min to establish an anaerobic environment. The vials were placed in sample trays in a randomized order in an autosampler (MPS Multi-Purpose Sampler, Gerstel, Mülheim an der Ruhr, Germany) and held at 25 °C for 14 days. Three replicates were prepared from each sample. Controls were the uninoculated media. At the end of the fermentation (after 14 days), growth was confirmed by measuring the pH (inoLab Level 1/WTW, Weilheim, Germany) and optical density (BioPhotometer/Eppendorf, Hamburg, Germany) of a sub-sample of the fermented culture.

2.4. Determination of 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). The drift tube conditions were as follows: 110 °C drift tube temperature, 2.8 mbar drift pressure, and 628 V drift voltage. This led to an E/N ratio of about 140 Townsend (Td), with E corresponding to the electric field strength and N to the gas number density (1 Td = 10^{-17} V cm^2). The sampling time per channel of ToF acquisition was 0.1 ns, amounting to 350,000 channels for a mass spectrum ranging up to mass peak (m/z) = 340, which resulted in the acquisition rate of 1 spectrum/s. Each measurement was conducted automatically using an autosampler with 60 s between each measurement to prevent any memory effects/carry over. The sample headspace was withdrawn with a 2.5 mL syringe (CTC Analytics AG, Zwingen, Switzerland) and injected into the static headspace (SHS) module (Ionicon Analytik GmbH, Innsbruck, Austria). The flow of zero air inside the static headspace module was 90 sccm, and the syringe injection time was 25 s/sample at a rate of 100 µL/s, resulting in a 16-fold dilution of the sample [43]. Pure N_2 was flushed through the syringe immediately before withdrawal to prevent measurement contamination. PTR-MS performance was verified with certified calibration mixtures. Sensitivity was better than 10 cps/ppbv, and the limit of detection (LOD) was lower than 100 pptv at an acquisition rate of 1 spectrum/s. The mass resolution was at least 4000 M/ΔM. Deadtime correction, internal calibration of mass spectral data, and peak extraction were performed according to previously described procedures [44,45]. The peak intensity in ppb/v (parts per billion per volume) was estimated using the formula described in the literature. The formula uses a constant value for the reaction rate coefficient (k = $2 \cdot 10^{-9}$ cm^3 s^{-1}) [46].

2.4.2. HS-SPME-GC-MS

HS-SPME-GC-MS measurements were included to support the identification of compounds detected by PTR-ToF-MS. At the end of the 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 25 °C. An SPME fibre (DVB/CAR/PDMS, 2 cm, 50/30 µm thickness) was exposed to the headspace of the samples for 40 min at 25 °C. VOCs were desorbed from the SPME fibre at 250 °C for 5 min in the injector of the GC in splitless mode, and helium was used as the carrier gas at a flow rate of 2 mL/min for 5 min. The VOCs were separated using a capillary column (InnoWax 30 m/0.32 mm/0.5 µm). The oven temperature program was set at 40 °C held for 1 min, and then increased to 250 °C at 5 °C/min and held for 2 min. MS was performed with an ion source temperature of 200 °C and an electron ionization energy of 70 eV over the mass range of m/z 33–350.

Retention indices (RI) for each VOC were calculated from the retention time (RT) of an n-alkane series (C6-C30) obtained under the same HS-SPME-GC-MS conditions. By comparing the calculated RI and from the NIST library (NIST14, version 2.2, National Institute of Standards and Technology), the VOCs were identified.

2.4.3. FastGC-PTR-ToF-MS

To assist with attributing each signal (m/z) to the correct compound and determining the number of compounds contributing to each m/z (isomers), fastGC-PTR-ToF-MS was carried out on all the samples at each time point after performing SHS-PTR-ToF-MS measurements. The drift tube conditions were the same as described in Section 2.4.1. The polar capillary column (MXT®-WAX (Siltek®—treated stainless steel), 6 m) was maintained under pure helium with a constant flow rate of 2.5 sccm. Pure N_2 was used as a make-up gas with a flow of 50 sccm. Sample headspace air was injected through the purge tool (Gerstel, Germany) into a fastGC sampling loop for 15 s, guaranteeing its total filling. The chromatographic measurement was registered for 250 s with the thermal ramp from 40 to 200 °C and data acquisition was set to 5 spectra/s [47]. The following pure standards, ethyl acetate, ethyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl

decanoate, ethanol, 2-methyl propanol, 3-methyl butanol, phenylethyl alcohol, 2-butanone, 2-hexanone, 2-heptanone, 2-nonanone, and benzaldehyde were prepared individually and diluted to a final concentration of 10 ppm through serial dilutions. Acetic acid was diluted to a final concentration of 50 ppm through serial dilutions. The 15 pure standards were analysed as well in fastGC-PTR-ToF-MS to assist the identification of m/z. TofDAQViewer was used to visually inspect fastGC-PTR-ToF-MS data of the standards and samples after they were saved as h5-files. From the 15 standards, a table was prepared consisting of RT, the literature RI, and fragmentation pattern, which in combination with the literature fragmentation patterns and GC-MS results, was used to assist in assigning compound identities to each m/z (Table 2).

Table 2. Flavour standards checked in fastGC-PTR-ToF-MS.

No.	Flavour Standards	Molecular Formula	Molecular Weight	RI	RT (s)	Main/Fragment Ions Checked
1	Ethyl acetate	$C_4H_8O_2$	88	888	58	89.060, 61.028, 43.018
2	2-Butanone	C_4H_8O	72	918	60	73.065
3	Ethanol	C_2H_6O	46	932	59	47.049
4	Ethyl butanoate	$C_6H_{12}O_2$	116	1023	68	117.091, 89.060, 43.054
5	2-Methyl propanol	$C_4H_{10}O$	74	1092	69	57.07
6	2-Hexanone	$C_6H_{12}O$	100	1100	78	101.096
7	2-Heptanone	$C_7H_{14}O$	114	1182	84.5	115.112
8	3-Methyl butanol	$C_5H_{12}O$	88	1209	82.2	71.086
9	Ethyl hexanoate	$C_8H_{16}O_2$	144	1233	89	145.122, 117.091
10	2-Nonanone	$C_9H_{18}O$	142	1390	109	143.143
11	Ethyl octanoate	$C_{10}H_{20}O_2$	172	1435	111.5	127.112, 145.122
12	Acetic acid	$C_2H_4O_2$	60	1449	112.5	61.028, 43.018
13	Benzaldehyde	C_7H_6O	106	1520	115.5	107.049
14	Ethyl decanoate	$C_{12}H_{24}O_2$	200	1638	146	201.233, 155.107
15	Phenylethyl alcohol	$C_8H_{10}O$	122	1906	218	105.070

2.5. Statistical Analysis

To determine which m/z were significantly ($p < 0.05$) higher in the samples than in the blanks, an analysis of variance (ANOVA) was run between each sample type and the corresponding blank.

Principal component analysis (PCA) was performed on all samples using related m/z concentrations and coded to highlight the sample differentiation based on DM compositions (DM and DM supplemented with individual AAs), LAB strains, and fermentation time. To ensure that the PCA was focusing on variation in the data from different DM compositions, LAB strains, and fermentation time, data from the control (uninoculated) treatments were excluded from the PCA. The analysis was performed in R (version 4.2.1, R Foundation for Statistical Computing, Vienna, Austria) by using "factoextra", "ggplot2", "reshape", "ggpubr", and "dplyr" packages [48]. Data were normalized by autoscaling (mean-centred and divided by the standard deviation) using the "prcomp" function of the "factoextra" package.

To identify m/z that were significantly influenced by experimental conditions, all sample-related m/z were subjected to three-way ANOVA using a general linear model (significance level at $p < 0.05$), where the main effects were DM compositions, LAB strains, and fermentation time, and all interactions were investigated. The mean separations for each m/z were calculated using Tukey's HSD test at $p < 0.05$. Analysis was carried out using SPSS (IBM SPSS statistics, version 29.0.0.0 (241), Chicago, IL, USA).

The selected VOCs (m/z) were plotted in bar graphs separating data based upon main experimental factors of DM compositions, and LAB strains as well as the interactions between DM compositions and LAB strains at 7 days of fermentation using "ggplot2", "dplyr", "ggpubr", "reshape", "ggthemes", "multcompView", "readr", and "scales" pack-

ages in R. The mean separations for each m/z were calculated using Tukey's HSD test at $p < 0.05$.

3. Results and Discussion

3.1. Physicochemical Properties after Fermentation

All the LAB strains grew well either in the DM or in the DM supplemented with individual AAs, as indicated by a decrease in pH (due to acid production) [49], and an increase in optical density (OD_{600}) values (resulting from cell growth) (Table 3). The OD_{600} values of the inoculated samples at day 0 ranged between 0.01 and 0.03. There were significant ($p < 0.05$) differences in the pH and OD_{600} values between LAB strains across different medium compositions after 14 days of fermentation. In general, the pH of medium compositions fermented by LB672 was highest except for DMA, DML, and DMI, where there were no significant ($p > 0.05$) differences in the pH between LAB strains.

Table 3. The mean pH and OD_{600} of the samples after 14 days of fermentation by three LAB strains across different medium compositions at 25 °C. Values are the means ± standard error of 2 replicates. Values with different superscript lowercase letters ($^{a–c}$) in each row (either pH or OD_{600} column wise) are significantly different according to Tukey's test at $p < 0.05$.

No.	Medium	Initial pH	LAB Fermentation	pH after 14 Days	OD_{600} after 14 Days
1	DM	5.70	LB672	4.30 ± 0.05 ^{a}	0.95 ± 0.033 ^{c}
			LP100	4.12 ± 0.008 ^{b}	1.26 ± 0.01 ^{b}
			PP100	4.05 ± 0.01 ^{b}	1.48 ± 0.005 ^{a}
2	DMG	5.16	LB672	4.15 ± 0.05 ^{a}	1.15 ± 0.05 ^{a}
			LP100	3.94 ± 0.008 ^{b}	0.61 ± 0.025 ^{b}
			PP100	4.02 ± 0.02 ^{ab}	0.63 ± 0.02 ^{b}
3	DMA	5.34	LB672	4.22 ± 0.005 ^{a}	0.84 ± 0.013 ^{c}
			LP100	4.06 ± 0.06 ^{a}	1.86 ± 0.015 ^{a}
			PP100	4.17 ± 0.005 ^{a}	1.00 ± 0.005 ^{b}
4	DML	5.49	LB672	4.09 ± 0.005 ^{a}	1.64 ± 0.04 ^{a}
			LP100	4.05 ± 0.005 ^{a}	1.40 ± 0.013 ^{b}
			PP100	4.07 ± 0.02 ^{a}	1.38 ± 0.015 ^{b}
5	DMI	5.52	LB672	4.23 ± 0.005 ^{a}	1.48 ± 0.003 ^{c}
			LP100	4.18 ± 0.002 ^{a}	2.21 ± 0.01 ^{a}
			PP100	4.24 ± 0.025 ^{a}	1.65 ± 0.025 ^{b}
6	DMT	5.60	LB672	4.20 ± 0.003 ^{a}	1.80 ± 0.045 ^{a}
			LP100	4.08 ± 0.005 ^{b}	1.26 ± 0.015 ^{b}
			PP100	4.08 ± 0.002 ^{b}	0.96 ± 0.03 ^{c}
7	DMP	5.53	LB672	4.22 ± 0.02 ^{a}	1.28 ± 0.005 ^{b}
			LP100	4.08 ± 0.002 ^{b}	2.11 ± 0.006 ^{a}
			PP100	4.17 ± 0.01 ^{a}	1.07 ± 0.001 ^{c}
8	DMM	5.53	LB672	4.26 ± 0.005 ^{a}	0.86 ± 0.04 ^{b}
			LP100	4.11 ± 0.005 ^{b}	2.13 ± 0.08 ^{a}
			PP100	4.14 ± 0.003 ^{b}	0.65 ± 0.005 ^{c}

DM: original defined medium; DMG: DM added with glutamic acid (Glu); DMA: DM added with aspartic acid (Asp); DML: DM added with leucine (Leu); DMI: DM added with isoleucine (Ile); DMT: DM added with threonine (Thr); DMP: DM added with phenylalanine (Phe); DMM: DM added with methionine (Met).

3.2. VOCs Produced during Fermentation

Fermentation by three LAB strains either in the DM or in the DM supplemented with different AAs, resulted in a total of 184 m/z being measured in the raw PTR-ToF-MS data. After the removal of isotopologues and m/z that were not significantly ($p > 0.05$) different from the baseline, this number decreased to 88 (Table S1). m/z were tentatively identified (t.i.) based on their exact mass, supported by HS-SPME-GC-MS (22 out of 88 m/z) (Tables 4, 5 and S1), fastGC-PTR-ToF-MS identification (Tables 2 and 5), and/or literature data.

Table 4. VOCs detected after fermentation by three LAB strains in either DM or DM supplemented with individual AAs using HS-SPME-GC-MS at 25 °C.

No.	Name	RT (min)	RI. Cal	RI. Lit	LAB Strains Used		
					LB672	LP 100	PP100
	Acids						
1	Acetic acid	15.29	1467	1449	√	√	√
2	Butyric acid	19.63	1646	1625	√	√	√
3	Hexanoic acid	24.44	1862	1846	√	√	√
4	Octanoic acid	28.76	2035	2060	√	√	√
5	Decanoic acid	32.70	2154	2276	√	√	√
	Alcohols						
6	2-Propanol	3.07	934	927	√	×	×
7	Ethanol	3.16	941	932	√	√	√
8	2-Pentanol	6.69	1134	1119	√	×	×
9	1-Butanol	7.27	1158	1142	√	√	√
10	2/3-Methyl-1-butanol	8.86	1220	1208/1209	√	√	√
11	3-Methyl-3-buten-1-ol	9.99	1263	1248	√	√	√
12	2-Heptanol	11.78	1332	1320	√	√	√
13	1,6-Heptadien-4-ol	11.89	1335	1330	√	√	√
14	Hexanol	12.67	1365	1355	√	√	√
15	2-Ethyl-1-hexanol	16.16	1501	1491	√	√	√
16	2,3-Butanediol	17.44	1554	1543	√	×	×
17	Menthol	19.81	1653	1637	√	√	√
18	2-Undecanol	21.59	1731	1717	√	√	√
19	Benzyl alcohol	25.14	1895	1870	√	√	√
20	Phenylethyl alcohol	25.85	1930	1906	√	√	√
21	2-Tridecanol	25.90	1933	1903	√	√	√
22	P-cresol	29.45	2051	2080	√	√	√
23	2-Tetradecanol	29.88	2062	2013	√	√	√
	Aldehydes						
24	Butanal	2.75	911	877	√	√	√
25	2-Methyl butanal	2.90	922	914	√	√	√
26	3-Methyl butanal	2.96	926	918	√	√	√
27	2-Methyl-2-butenal	6.17	1114	1095	√	√	√
28	3-Methyl-2-butenal	8.77	1216	1215	√	√	√
29	2-Methyl pentanal	13.66	1403		√	√	√
30	Benzaldehyde	17.15	1542	1520	√	√	√
31	Benzeneacetaldehyde	20.03	1663	1640	√	√	√
	Esters						
32	Ethyl acetate	2.61	901	888	√	√	√
33	Isoamyl acetate	6.81	1139	1122	√	×	×
	Furans						
34	Furfural	15.72	1484	1461	×	√	×
35	2-Furanmethanol	20.40	1679	1660	√	√	√
	Ketones						
36	Acetone	1.97	823	819	√	√	√
37	2,3-Butanedione (Diacetyl)	3.84	989	979	√	√	√
38	2-Pentanone	3.91	994	981	√	√	√
39	3-Penten-2-one	6.92	1144	1128	√	√	√
40	2-Heptanone	8.29	1198	1182	√	√	√

Table 4. *Cont.*

No.	Name	RT (min)	RI. Cal	RI. Lit	LAB Strains Used		
					LB672	LP 100	PP100
41	Acetoin	11.00	1302	1284	√	√	√
42	2-Tridecanone	23.60	1822	1809	√	√	√
	Pyrazines						
43	Pyrazine	9.08	1228	1212	√	√	√
	Sulphur compounds						
44	Dimethyl disulfide	5.73	1095	1077	√	√	√
45	Methional	15.47	1474	1454	√	√	√
46	Cyclohexyl isothiocyanate	20.61	1687	1667	√	√	√
47	3-(methylthio)-1-propanol (methionol)	21.64	1734	1719	√	√	√
	Unknown compounds						
48	Unknown 1	11.89			×	√	√
49	Unknown 2	12.53			√	√	×
50	Unknown 3	26.61			√	√	√

√: VOCs detected at given LAB-fermented medium. ×: VOCs not detected at given LAB-fermented medium.

Table 5. VOCs (m/z) detected by PTR-ToF-MS after fermentation by different LAB strains in DM and DM supplemented with individual AAs at 25 °C that significantly ($p < 0.05$) distinguished between different medium compositions (M), LAB strains (S), and fermentation time (0 and 7 days) (T) and their interaction effects.

No.	*m/z*	Sum Formula	Identification	Media	Strain	T	M × S	M × T	S × T	M × S × T
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	29.04	$C_2H_5^+$	Ethanol fragment	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
4	29.145			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
5	31.018	CH_2OH^+	Formaldehyde	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
6	34.997	H_2SH^+	Hydrogen sulfide	0.843	0.263	<0.0001	0.033	0.828	0.256	0.031
7	40.028			0.039	<0.0001	<0.0001	<0.0001	0.004	<0.0001	0.003
8	41.039	$C_3H_5^+$	Common fragment	0.003	<0.0001	<0.0001	<0.0001	0.001	<0.0001	<0.0001
9	42.011	C_2HOH^+		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
10	43.017	$C_2H_3O^+$	Common fragment	0.015	0.158	<0.0001	0.238	0.152	0.221	0.023
11	44.022			0.007	0.169	<0.0001	0.656	0.346	0.111	0.933
12	44.993	CO_2H^+	Carbon dioxide	<0.0001	0.034	<0.0001	<0.0001	<0.0001	0.100	<0.0001
13	45.031	$C_2H_4OH^+$	Acetaldehyde	<0.0001	<0.0001	<0.0001	0.002	<0.0001	0.015	<0.0001
14	47.045	$C_2H_6OH^+$	Ethanol [1]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
15	48.008			<0.0001	<0.0001	<0.0001	0.208	<0.0001	0.016	0.167
16	49.012	CH_4SH^+	Methanethiol	<0.0001	0.092	<0.0001	0.785	<0.0001	0.092	0.786
17	51.009			<0.0001	0.136	<0.0001	0.811	<0.0001	0.095	0.809
18	53.005			0.043	<0.0001	<0.0001	0.099	0.021	<0.0001	0.456
19	54.006			0.001	<0.0001	<0.0001	0.860	0.002	<0.0001	0.910
20	57.039	$C_3H_4OH^+$		0.193	<0.0001	<0.0001	<0.0001	0.023	<0.0001	0.002
21	59.049	$C_3H_6OH^+$	Acetone [1]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
22	60.021			<0.0001	0.014	<0.0001	0.921	0.112	0.006	0.663
23	63.01	$CO_2{*}H_3O$	Carbon dioxide-water cluster	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
24	63.04	$C_2H_6O_2H^+$	Acetaldehyde hydrate cluster	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
25	65.057		Ethanol hydrate cluster	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
26	67.06	$C_5H_7^+$		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
27	71.05			<0.0001	<0.0001	<0.0001	0.412	<0.0001	<0.0001	0.178
28	75.08	$C_2H_5^+[C_2H_5OH]$	Ethanol cluster	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
29	77.016			0.058	<0.0001	<0.0001	0.058	0.030	0.055	0.512
30	77.057			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
31	83.066			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
32	85.064	$C_5H_8OH^+$	2-Methyl-2-butenal [1,2,3] and 3-Methyl-2-butenal [1,2,3]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
33	89.058	$C_4H_8O_2H^+$	Ethyl acetate [1,2,4], Acetoin [1,2,4] and Butyric acid [1,2,4]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
34	91.058	$C_4H_{10}SH^+$		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
35	93.087	$C_7H_8H^+$	Toluene	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
36	95.000	$C_2H_6S_2H^+$	Dimethyl disulfide [1]	0.008	0.790	0.007	0.899	0.018	0.064	0.886
37	97.061	$C_6H_8OH^+$	2,5-Dimethylfuran/Cyclohexen-2-one	<0.0001	<0.0001	<0.0001	0.076	<0.0001	<0.0001	0.018
38	107.063	$C_4H_{10}OSH^+$	Methionol [1]	0.299	<0.0001	<0.0001	0.161	0.357	<0.0001	0.387
39	115.108	$C_7H_{14}OH^+$	2-Heptanone [1,2]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
40	117.078			0.031	0.032	<0.0001	0.328	0.021	0.001	0.151

Table 5. *Cont.*

No.	*m/z*	Sum Formula	Identification	Media	Strain	T	M × S	M × T	S × T	M × S × T
41	119.075			0.016	<0.0001	<0.0001	0.244	0.014	0.001	0.089
42	143.14	$C_9H_{18}OH^+$	Nonanal/Nonanone	0.002	<0.0001	<0.0001	<0.0001	0.028	<0.0001	<0.0001
43	171.17	$C_{11}H_{22}OH^+$	Undecanal/Undecanone	<0.0001	<0.0001	<0.0001	<0.0001	0.002	<0.0001	<0.0001

[1]: m/z that HS-SPME-GC-MS identified. [2]: m/z identified by fastGC-PTR-ToF-MS and/or the injection of pure standard. [3]: 2-methyl-2-butenal and 3-methyl-2-butenal both present, but mainly 2-methyl-2-butenal due to elevated treatment effect based on HS-SPME-GC-MS. [4]: Ethyl acetate + butyric acid dominant in LB672 fermented samples, and acetoin + butyric acid dominant in LP100 and PP100 fermented samples based on fastGC-PTR-ToF-MS.

The concentrations of the 88 m/z were higher at 7 days. At 14 days, the concentrations were lower, which was assumed to be due to a decrease in LAB growth/metabolism as substrates became depleted, which resulted in a decrease in the concentration of the volatiles in the headspace as those present at day 7 were removed during the flushing of the headspace. Hence, to better determine the effects of different LAB strains on the VOCs produced across different medium compositions, only the data on day 0 and after 7 days of fermentation were considered.

To understand the differences between m/z produced by three LAB strains across different medium compositions, PCA was performed for the 88 m/z detected by PTR-ToF-MS. As shown in Figure 1a, the PCA score plot of LB672-, LP100-, and PP100-fermented DM and DM supplemented with individual AAs samples explained 52.7% of the total variance, comprising 38.4% from the first principal component (Dim1) and 14.3% from the second principal component (Dim2). The explained variance was mainly attributed to the separation of LB672-, LP100-, and PP100-fermented DM and DM supplemented with individual AA samples at 7 days from the 0 days samples and LB672-fermented DM samples from LP100- and PP100-fermented DM and DM supplemented with AA samples, and LB672-fermented DM supplemented with AA samples at 7 days (Figure 1a). The separation along Dim 1 was attributed to the presence of higher concentrations of m/z 60.021, 126.968, 128.058, 89.058 (t.i. ethyl acetate, acetoin, and butyric acid), 117.078, 54.006, 57.039, 43.017 (t.i. common fragment), 61.028 (t.i. acetic acid), 49.012 (t.i. methanethiol), and 44.022 associated with mainly LB672-fermented DM supplemented with AA samples at 7 days from all samples at 0 days (Figure 1b). Variation along Dim 2 was attributed to the separation of 7 days of LB672-fermented DM samples, in particular DMA (negative Dim 2), from LP100- and PP100-fermented DM and DM supplemented with AA samples at 7 days. The separation along Dim 2 was mainly attributed to ethanol-related m/z (47.045, 29.04, 65.057, and 75.08) along with m/z 26.016 (common fragment), 27.025, 31.018 (t.i. formaldehyde), 63.01 (t.i. carbon dioxide/water cluster), 83.066, and 93.087 (t.i. toluene) which had negative loadings, and 71.050 and 75.036 with positive loadings (Figure 1b).

Three-way ANOVA was used to further investigate differences in VOC emissions from the different media and LAB strain combinations. The analysis found that of the 88 m/z signals attributed to the samples, 46, 55, 81, 32, 44, 48, and 33 m/z were significantly ($p < 0.05$) differentiated based upon LAB strains, DM compositions, time (at 0 and 7 days), DM compositions*LAB strains interactions, time*LAB strains interactions, DM compositions*time interactions, and DM compositions*time*LAB strains interactions, respectively (Table S1). Finally, 43 m/z were identified (Table 5) that demonstrated a significant ($p < 0.05$) increase in their concentration during fermentation (time) and significant ($p < 0.05$) differences in either DM compositions, or LAB strains or interaction effects.

The specific fermentation VOCs (m/z) produced by different LAB strains in DM supplemented with different AAs are discussed in the following sections.

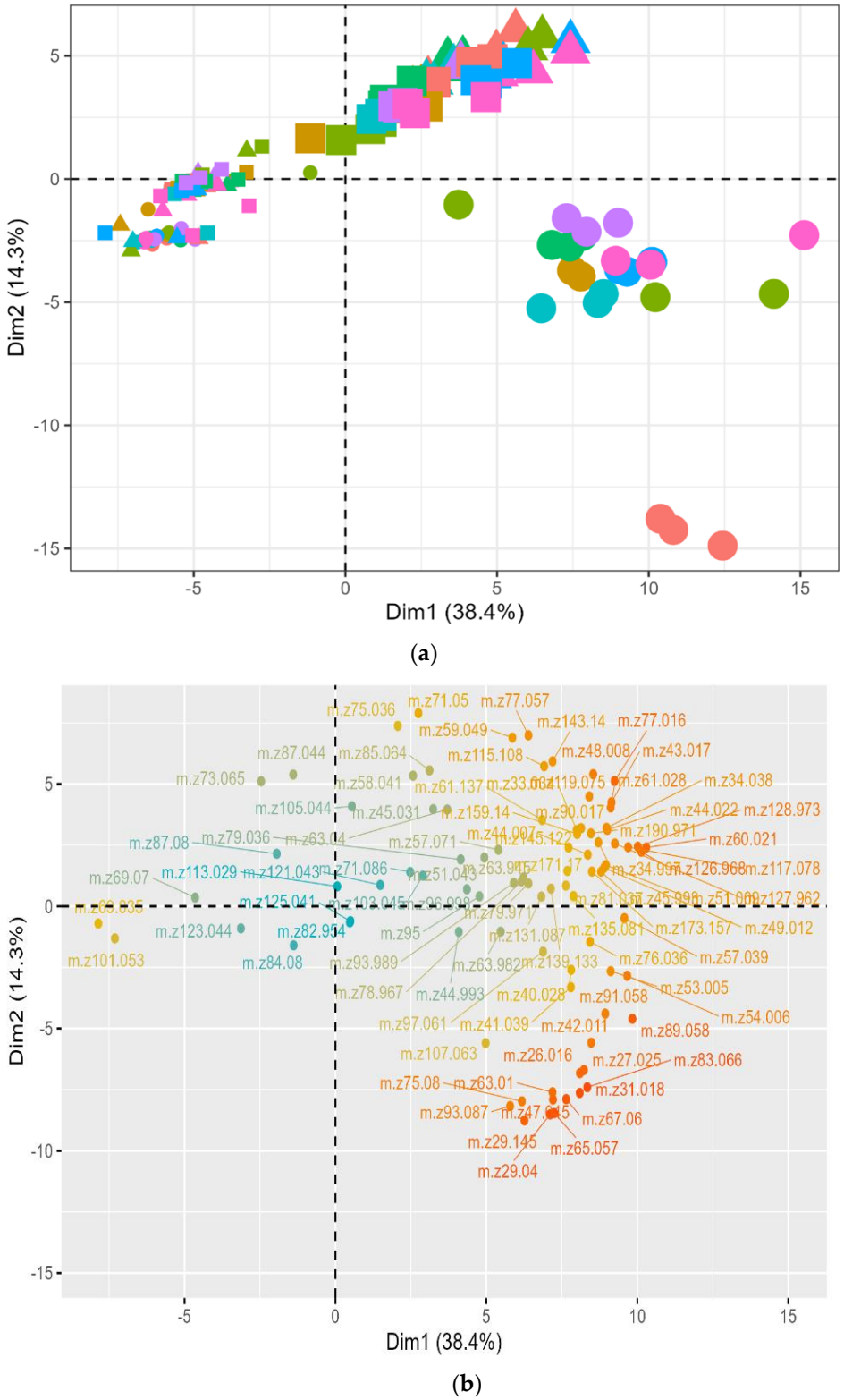

Figure 1. Score plot (**a**) and loadings plot (**b**) of the principal components (PC) of VOCs produced by three LAB strains (LB672 (●), LP100 (▲), and PP100 (■)) across different medium compositions (DM ●, DMA ●, DMG ●, DMI ●, DML ●, DMM ●, DMP ●, and DMT ●) at 0 (●) and 7 (●) days of fermentation at 25 °C based on the concentrations (ppbV) of finalized 88 m/z from PTR-ToF-MS. The first and second components represented for 38.4% and 14.3% of total variance.

3.2.1. Ethanol

Ethanol is an end product of sugar fermentation by LAB. Heterofermentative LAB (*Lev. brevis*) utilise hexose sugars (glucose) via the phosphoketolase (PK) pathway and produces ethanol as the end product via an intermediate of acetaldehyde using an alcohol dehydrogenase (AlcDH) enzyme. In contrast, homofermentative LAB (*P. pentosaceus*) ferment hexose sugars solely into lactic acid through the Embden/Meyerhof/Parnas (EMP) pathway [16]. However, at slow growth and low glycolytic flux rates, homofermentative LAB can shift to a mixed acid fermentation with ethanol as one of the end products [50]. Notably, facultative heterofermentative LAB (*Lpb. plantarum*) utilise hexose sugars to produce lactic acid through the EMP pathway and pentose sugars by the PK pathway [51] and under certain conditions, these LAB have been reported to ferment hexoses through the PK pathway [52]. Furthermore, ethanol can also be produced by LAB through the degradation of the AA Thr [29,30,53].

In the present study, the concentration of m/z 47.045 (t.i. ethanol) was significantly ($p < 0.05$) higher after 7 days of fermentation by LB672 (Figure 2) across the different medium compositions, where ethanol was detected at trace amounts after either LP100 or PP100 fermentation. In the LB672 fermentation, ethanol was higher in the DM compared to DM supplemented with individual AAs. Given the similarities in pH after LB672 fermentation across different medium compositions (Table 3) after 14 days, differences in ethanol in LB672 after 7 days are likely due to differences in fermentation rate.

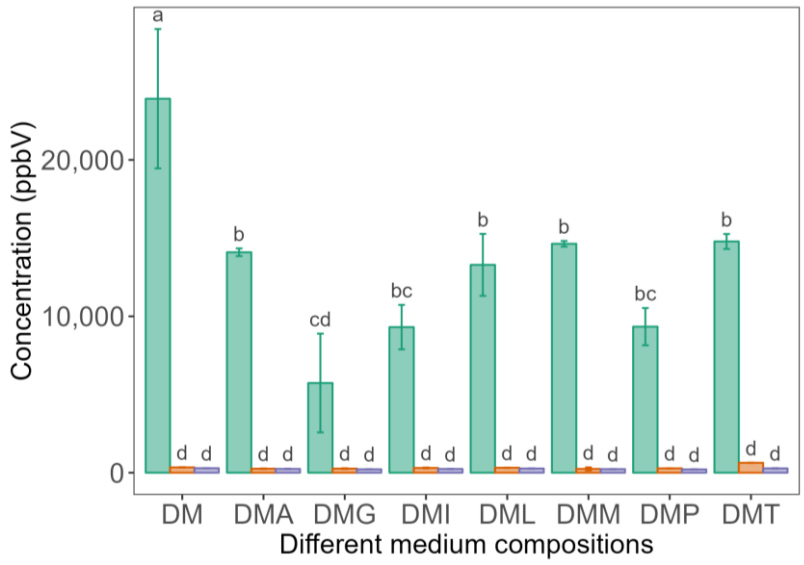

Figure 2. Mean concentration (ppbV) of m/z 47.045 (t.i. ethanol) across different medium compositions after 7 days of fermentation by LB672 (■), LP100 (■), and PP100 (■) at 25 °C. Values are presented as mean ± standard error (n = 3). Different superscript lowercase letters represent significant differences between different medium compositions fermented by different LAB strains according to Tukey's test at $p < 0.05$.

3.2.2. Thr-Derived VOCs

Acetaldehyde, which is a main flavour compound in yogurt [54], is produced by LAB either from the catabolism of Thr using either threonine aldolase (TA) or serine hydroxymethyltransferase (SHMT) enzymes [29,30,53] or from sugars via the PK pathway (intermediate in the ethanol production pathway) [16]. In the current study, the concentration of m/z 45.031 (t.i. acetaldehyde) was significantly ($p < 0.05$) higher after 7 days of fermentation by LP100 in the DM with added Thr (DMT), followed by PP100 fermentation in the medium DMT compared to all other media fermented by either LP100, PP100, or LB672 (Figure 3). This suggests that the presence/activity of TA/SHMT enzymes is more likely/higher in LP100 and PP100 strains. However, it is not possible to confirm

the presence or absence of TA/SHMT enzymes in LB672 as the highest concentration of ethanol was detected in LB672-fermented media (Figure 2), which suggests that produced acetaldehyde was converted into ethanol by the AlcDH enzyme. The facultative heterofermentative and homofermentative LAB strains, LP100 and PP100, respectively, did produce acetaldehyde, but not ethanol after fermentation in all media studied, which suggests that the AlcDH enzyme is most likely absent/not active in these strains. Notably, acetaldehyde was detected in all media possibly due to the presence of glucose, peptone, and an AA mixture in all DM compositions.

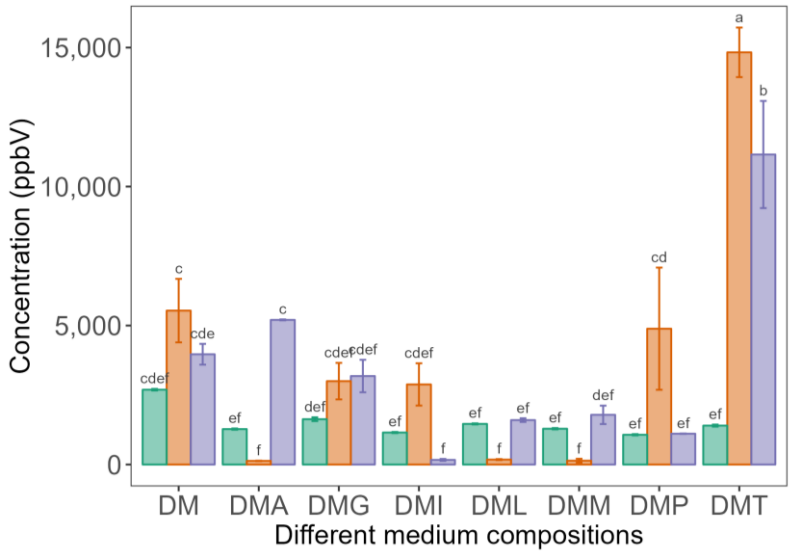

Figure 3. Mean concentration (ppbV) of m/z 45.031 (t.i. acetaldehyde) across different medium compositions after 7 days of fermentation by LB672 (■), LP100 (■), and PP100 (■) at 25 °C. Values are presented as mean ± standard error (n = 3). Different superscript lowercase letters represent significant differences between different medium compositions fermented by different LAB strains according to Tukey's test at $p < 0.05$.

3.2.3. Met-Derived VOCs

Met is a sulphur-containing AA whose catabolism is initiated by a transamination step, in which the aromatic aminotransferase (ArAT,) or the branched-chain aminotransferase (BcAT) is involved, in the presence of α-ketoglutarate, yielding 4-methylthio-2-ketobutyric acid (KMBA). The KMBA produced can subsequently be chemically converted into methanethiol. KMBA can also be converted into methional via a decarboxylation reaction, and the resulting methional is converted into methanethiol and α-ketobutyrate by an unknown pathway. In addition, KMBA can also be enzymatically converted into 2-hydroxyl-4-methylthiobutyric acid and methanethiol. Further, the demethiolation of Met produces methanethiol, α-ketobutyrate, and ammonia via two pyridoxal phosphate-dependent lyases (cystathionine β-lyase (CBL) and cystathionine γ -lyase (CGL)). Methanethiol produced from transamination, demethiolation, and through methional, can be further converted into dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide by auto-oxidation [28,30,55,56].

In the current study, the concentration of m/z 49.012 (t.i. methanethiol) was significantly ($p < 0.05$) higher after 7 days of fermentation by either LP100, PP100, or LB672 in the DM supplemented with Met (DMM) compared to all other media fermented by all three strains (Figure 4a). Methanethiol is a characteristic flavour compound in meat [57] and cheese [58,59]. Similarly, the concentration of m/z 95.000 (t.i. dimethyl disulfide) was significantly ($p < 0.05$) higher after 7 days of fermentation by either LP100, PP100, or LB672 in the medium DMM, compared to all other media fermented by all of these strains (Figure 4b). Notably, methanethiol and dimethyl disulfide were detected in all media possibly due to the presence of peptone and an AA mixture in all DM compositions, which contained Met.

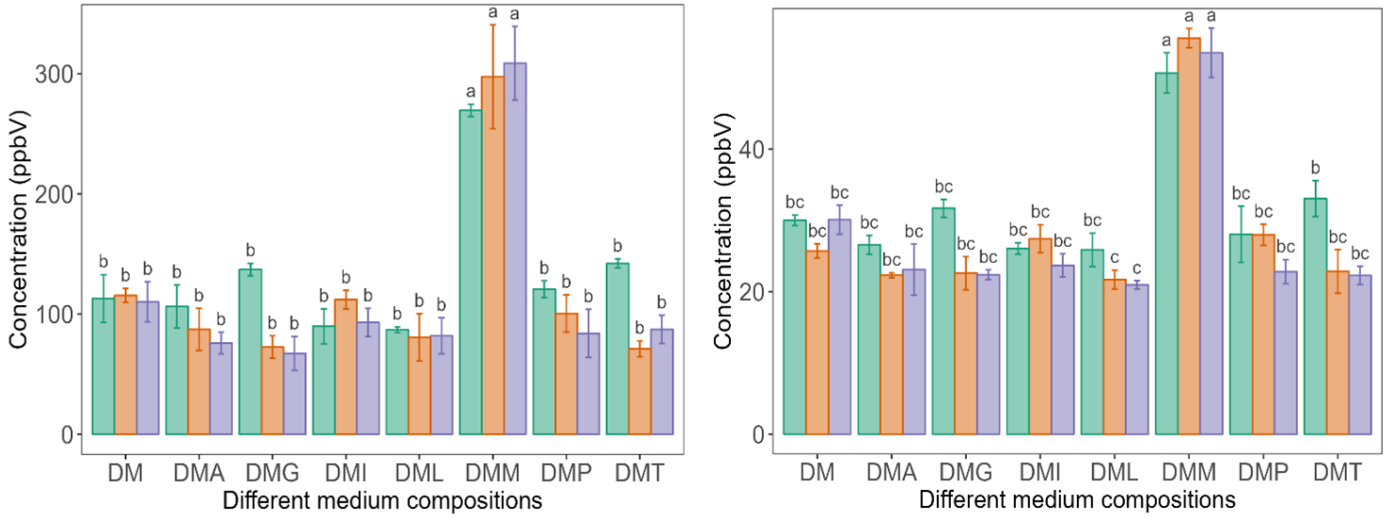

Figure 4. Mean concentrations (ppbV) of m/z 49.012 (t.i. methanethiol) (**a**) and m/z 95.000 (t.i. dimethyl disulfide) (**b**) across different medium compositions after 7 days of fermentation by LB672 (■), LP100 (■), and PP100 (■) at 25 °C. Values are presented as mean ± standard error (n = 3). Different superscript lowercase letters represent significant differences between different medium compositions fermented by different LAB strains according to Tukey's test at $p < 0.05$.

According to a study by Liu et al. [60], the genes *cblA* and *cglA* encoding CBL and CGL lyases are present in *Lev. brevis* ATCC 367 and *Lpb. plantarum* WCFS1, which catalyses the demethiolation reaction of Met. Whereas aminotransferases such as ArAT are present in *Lpb. plantarum* WCFS1 and *P. pentosaceus* ATCC 25745 and BcAT is only present in *Lpb. plantarum* WCFS1 and is involved in the transamination reaction. It is obvious that without these enzymes, methanethiol production is not possible. Therefore, the LAB strains used in the current study LP100, PP100, or LB672 likely contain either CBL and CGL lyases and/or ArAT/BcAT transferases. A genomic study is therefore required to confirm the presence of CGL, CBL, ArAT, and BcAT enzymes in these strains.

3.2.4. Ile-Derived VOCs

The concentration of m/z 85.064 (t.i. 2-methyl-2-butenal and 3-methyl-2-butenal) was significantly ($p < 0.05$) higher after 7 days of fermentation by either LP100, or PP100 in the DM supplemented with Ile (DMI) compared to all other media fermented by either LB672, LP100, or PP100 (Figure 5). As 2-methyl-2-butenal is an Ile-derived compound [61], m/z 85.064 was most likely contributed by 2-methyl-2-butenal, which was confirmed with HS-SPME-GC-MS data.

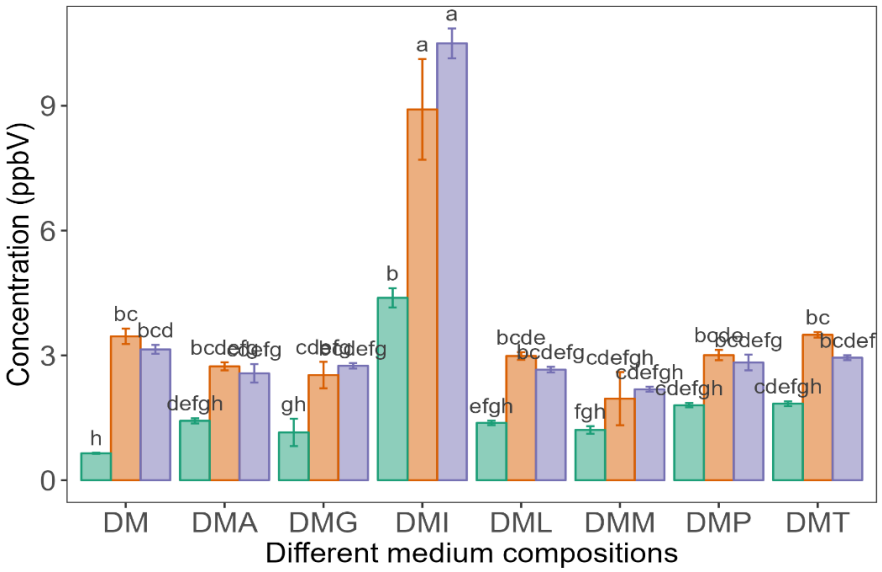

Figure 5. Mean concentration (ppbV) of m/z 85.064 (t.i. 2-methyl-2-butenal and 3-methyl-2-butenal) across different medium compositions after 7 days of fermentation by LB672 (■), LP100 (■), and PP100 (■) at 25 °C. Values are presented as mean ± standard error (n = 3). Different l superscript lowercase letters represent significant differences between different medium compositions fermented by different LAB strains according to Tukey's test at $p < 0.05$.

3.2.5. Other VOCs

The concentration of m/z 89.058 (t.i. acetoin, ethyl acetate, and butyric acid) was significantly ($p < 0.05$) higher after 7 days of fermentation by LB672 across different medium compositions compared to either LP100 or PP100 fermentation across all media studied (Figure 6). Based on the fastGC-PTR-ToF-MS results, the m/z 89.058 detected by PTR-ToF-MS was considered to be comprised of contributions from ethyl acetate, acetoin, and butyric acid (and a small signal was observed for ethyl butanoate). However, there were differences between LAB strains in the contribution of ethyl acetate, acetoin, and butyric acid to this m/z: acetoin and butyric acid were dominant in LP100 and PP100 ferments, whereas ethyl acetate and butyric acid were dominant in LB672 ferments.

Based on previous experiments, to obtain good growth of LAB in the DM, the DM was supplemented with an AA mixture in addition to peptone. While the AA mixture addition made determining the impact of individual AAs on VOC production a little more challenging, the obvious advantage was that the cultures all grew. It is important to note that the concentration of each individual AA added to the DM was at least five times higher than its concentration in the DM from the AA mixture. A closer look at the VOCs derived from individual AAs reveals that Thr, Met, and Ile had the highest impact on specific VOCs production. In contrast, the addition of either Leu, Glu, Asp, or Phe had no significant effect on the production of specific VOCs. This might be because the addition of a higher concentration of a single AA may result in an increased requirement for other AA, which will influence the VOCs produced [62].

This study investigated how the relative concentrations of VOCs in the DM and DM supplemented with AAs were influenced by only three commercial LAB strains. To better understand the impact of strain on particular VOCs, further study using different commercial LAB strains is required. Further, temperature controls the growth rate of LAB, resulting in impact on VOC generation. As only 25 °C was used in the present study, different fermentation temperatures need to be investigated to determine their effect on the VOCs production.

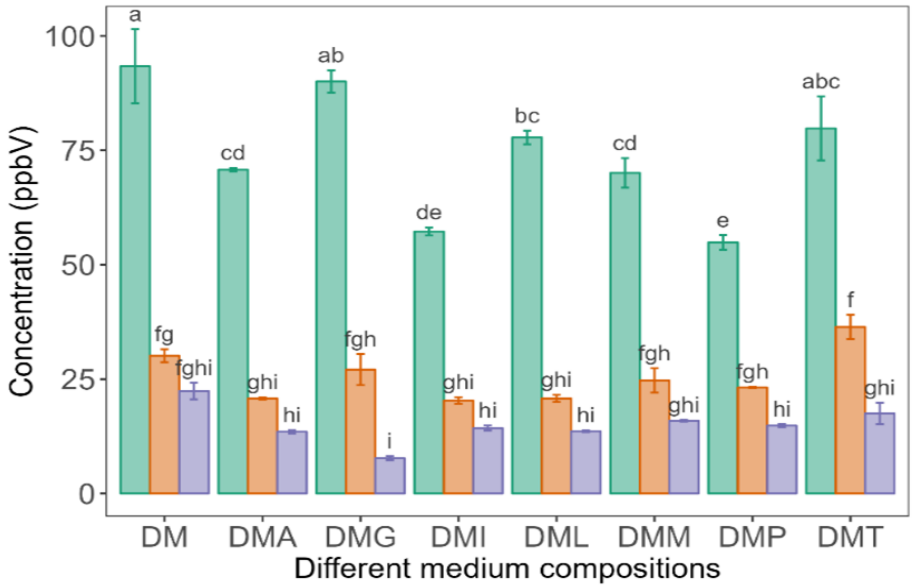

Figure 6. Mean concentration (ppbV) of m/z 89.058 (t.i. acetoin, ethyl acetate, and butyric acid) across different medium compositions after 7 days of fermentation by LB672 (■), LP100 (■), and PP100 (■) at 25 °C. Values are presented as mean ± standard error (n = 3). Different superscript lowercase letters represent significant differences between different medium compositions fermented by different LAB strains according to Tukey's test at $p < 0.05$.

4. Conclusions

The present study demonstrated that VOCs produced during fermentation analysed by PTR-ToF-MS, HS-SPME-GC-MS, and fastGC-ToF-MS were influenced by DM compositions (AA addition), and LAB strains. The addition of Thr, Met, and Ile AAs to the DM, noticeably impacted the relative concentrations of VOCs after LB672, LP100, and PP100 fermentation, suggesting the presence of specific AA catabolic enzymes in these strains. Understanding how specific VOC generation responds to varying medium compositions and LAB strains will facilitate the production of the VOC required to improve the flavour profiles of plant-based fermentation products or analogue products. Therefore, further investigations utilising different commercial LAB strains are needed to more broadly understand how AAs or medium compositions impact the generation of fermentation-related VOCs. This would provide more information about which LAB strain and which AA combination, or medium compositions work best together to produce target VOCs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10060317/s1, Table S1: title; The VOCS (m/z) detected by PTR-ToF-MS after fermentation by different LAB strains across different medium compositions at 25 °C that significantly ($p < 0.05$) distinguished between different medium compositions (M), LAB strains (S), and fermentation time (0 and 7 days) (T) and their interaction effects.

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