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A GC-MS Based Metabolic Profiling of Probiotic Lactic Acid Bacteria Isolated from Traditional Food Products

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Abstract

A GC-MS based metabolic profiling was carried out to study metabolic differences of lactic acid bacteria isolated from different food sources. Metabolic fingerprinting is a non-targeted procedure where all detectable peaks are considered to establish sample classification. A total of 40 compounds were identified as major metabolites contributing to the difference among five different probiotic lactic acid bacteria. Some of the metabolites identified in this study have been reported as a defrosting agent, antioxidant, flavour agent, antimicrobial, natural food additive, anti-inflammatory, anti-sleep disorder agent and anti-cancer agents. These results suggest that GC-MS based metabolomic analysis is a useful tool to facilitate future investigations into the characterization of probiotic lactic acid bacteria.

Keywords: Lactic acid bacteria, metabolic profiling, GC-MS, traditional food products, probiotics

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INTRODUCTION

Lactobacillus and *Pediococcus* is a gram-positive facultative anaerobic bacterium found widely in fermented food products and can be investigated for the metabolites present in them by GC-MS based metabolic profiling.

Metabolic fingerprinting is explained as the semi-quantitative investigation of extracellular (exo-metabolome) and intracellular (endo-metabolome) metabolites, respectively (Villas-Boas *et al*., 2005). These metabolic profiles GC-MS has consistently been the most favoured analytical technique for the analysis of metabolites present in distinct biological samples. Gas chromatography and mass spectrophotometry (GC-MS) present the high chromatographic resolution ascribed to the high sensitivity and specificity of mass spectrophotometry (Villas-Boas *et al*., 2005). In comparison with other techniques, GC-MS can produce a comparably high reproducibility, high resolution, high-quality sensitivity, and good- throughput analysis, which can be used for analyzing the metabolic products, inclusive of carbohydrates, fatty acids, organic acids, and amino acids (Park *et al*., 2016). Derivatization is necessary before the investigation by GC-MS as most of the metabolites are non-volatile (Schummer *et al*., 2009). Nowadays, most adaptable derivatization technique is silylation, which has the ability to derivatize compounds having polar functional groups by mixing TMS (Trimethylsilyl) reagent to develop TMS Compounds (Nordström A, 2004). However, for the study of different biological metabolic fingerprinting, N, O-bis (trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane is generally used as silylation reagent and derivatization method with GC-MS (Alreshidi *et al*., 2015; Li *et al*., 2013). For the study of metabolites in the microbial sample, derivatization based GC-MS technique can be applied to identify the intracellular as well as extracellular metabolites of *Lactobacillus* and *Pediococcus* species.

Liquid injection GC-MS provides an inexpensive and simple option for the analysis of metabolites produced by probiotic lactic acid bacteria. As the concentration of metabolites varies widely in different probiotic lactic acid bacteria, it is required to develop the analytical plan that would permit concurrent quantification of them in a single run utilizing comparably a small amount of sample along with minimum sample preparation. GC-MS has high sensitivity and hence can be used for the investigation of less common samples that might only be available in minute quantity. In this study, direct-injection GC-MS methodology was used for the profiling of different metabolites, which has the high specificity of mass spectrometry along with the high reproducibility and high resolution of gas chromatography.

The results of this study provide reference data for interpreting the differences in the metabolite profiles of different probiotic lactic acid bacteria isolated from different food sources.

MATERIALS AND METHODS

Collection of food samples

Food items viz. Dosa batter, jalebi batter, maida dough, sauerkraut and soymilk were used in this experiment. Dosa batter, Jalebi batter, Soymilk were collected from a local market in New Delhi, India. Maida dough and Sauerkraut were prepared at home. The food samples were taken in a sterilized bag and stored at -4° until use.

Strain isolation

1 g or 1 ml of food sample were added into 9 ml of 0.85% (w/v) normal saline. After homogenization, serial dilutions were prepared upto 10^{-9} with 0.85% (w/v) normal saline and 0.1 ml decimal of appropriate dilutions were plated onto de Man, Rogosa, Sharpe (MRS) agar medium (Himedia, India) (de Man *et al.*, 1960). The agar plates were incubated at 35° for 24 h under anaerobiosis. Morphologically different colonies were picked and re-streaked onto MRS agar plates up to purity. Glycerol stocks of strains were preserved at -20°.

Probiotic Lactic Acid Bacteria

L. plantarum DB-2, *L. fermentum* J-1, *P. acidilactici* M-3, *L. plantarum* SK-3 and *P. pentosaceus* SM-2 were isolated from Dosa batter, Jalebi batter, Maida dough, Sauerkraut, and Soymilk and were identified in own previous studies with NCBI accession no. MK246169, MK353735, MK461878, MK246167 and MK461882 respectively (Chaudhary and Saharan, 2019) (Table 1). Probiotic attributes such as acid tolerance (Liong and Shah, 2005), bile tolerance (Walker and Gilliland, 1993), antibiotic susceptibility (Thirabunyanon *et al*., 2009), hemolytic activity (Harrigan, 1998), gelatinase activity (Harrigan,

1990), autoaggregation (Del Re *et al*., 2000), co-aggregation studies (Del Re *et al*., 2000), hydrophobicity (Rosenberg *et al*., 1980), bacteriocin production (Papagianni and Anastasiadou, 2009), lactic acid and hydrogen peroxide production (AOAC, 1995), exopolysaccharide production (Mora *et al*., 2002) were studied on all the five isolates. Bacterial isolates were identified at the genomic level by using 16 S rRNA gene technique (Hashem *et al*., 2010; Bollag and Edelstein, 1991).

Metabolic fingerprinting

Metabolites were extracted by following the method given by Coucheney *et al*. (2008) with minor modifications.

Sampling for metabolic fingerprinting

Microbial suspensions (20 ml) of isolate (grown in MRS broth for 24 h at 35°C) were disrupted using pulsed, high-frequency sound waves (>20kHz) for five cycles (30 sec. run; one min break) to extract intracellular metabolites. Suspensions procured after sonication were centrifuged for 10 min at 10,000 rpm in order to segregate the extra as well as intracellular metabolites from the cells.

Extraction for metabolic fingerprinting

The supernatant was removed and the metabolites were extracted with a Methanol: Water: Chloroform mixture (2: 0.8: 1): 2.5 ml of cold chloroform and 5 ml of cold methanol (-20°C) and the phases were allowed to separate. Metabolic fingerprints were assessed in the aqueous phase after freeze-drying while in the organic phase, the dried chemical extracts obtained after complete evaporation of the solvent was used.

Generation of metabolic fingerprints and data processing

Derivatization of dried samples

The derivatization method given by Mastrangelo *et al.* (2015) and Park *et al*. (2019) was used in this study for the evaluation of metabolic fingerprinting. The freeze-dried samples and dried chemical extracts were methoxymated using methoxyamine in pyridine solution and trimethylsilylated by BSTFA (N,O-Bis(trimethylsilyl) trifluoroacetamide) with 1 % chlorotrimethylsilane (TMCS).

GC-MS analysis

Derivatives from *Lactobacillus* and *Pediococcus* strains were analysed using Thermo Trace 1300GC coupled with Thermo TSQ 800 Triple Quadrupole MS (Pogacic *et al*., 2015). Six-microlitre aliquots were injected in splitless mode. The samples were warmed to 65°C for 15 min and the metabolites were extracted before being adsorbed on the trap at 35°C. The trap was heated at 250°C for 0.1 min, leading to desorption of the metabolites. Metabolites were then separated on TG 5MS column (30m X 0.25mm, 0.25µm thickness) with column makeup of 5% diphenyl; 95% dimethyl polysiloxane. The temperature of the oven was initially 50°C, maintained for 3 min. The temperature was increased at 15°C/min to 220°C. The mass spectrometer was operated in the scan mode within a mass range of *m/z* 30 to 700. The quadruple mass spectrophotometry parameters were set to the conditions: ion source temperature of 230°C, split flow of 40 ml/min, carrier flow of 1.5 ml/ min, injector temperature and MS transfer line temperature of 250°C. Ionization was done by electronic impact at 70 eV. All samples were analysed in the same GC-MS and injected in a randomized order over the GC-MS run. Blank samples (boiled deionized water) were injected after every sample to verify the instrumental carryover.

Data pre-processing and data analysis

The GC-MS raw data files were converted to netCDF format. Further, the raw

Table 1. Genotyping of screened lactic acid bacterial strains

Name of isolate	Source	Identity (%)	16S rRNA identification	Accession number
DB-2	Dosa batter	99	*Lactobacillus plantarum*	MK246169
J-1	Jalebi batter	99	*Lactobacillus fermentum*	MK353735
M-3	Maida dough	97	*Pediococcus acidilactici*	MK461878
SK-3	Sauerkraut	100	*Lactobacillus plantarum*	MK246167
SM-2	Soymilk	99	*Pediococcus pentosaceus*	MK461882

Table 2. Metabolic profile of biocomponents identified in solvent extract of probiotic lactic acid bacteria isolated by GC-MS

S.No	Compound	Five isolates	Four isolates	Three isolates	Two isolates	One isolate
1	Acetic acid, anhydride with formic acid					■
2	4-amino-1-butanol					■
3	Benzaldehyde,2,4,dimethyl	■■■■■				
4	Benzoic acid	■■■■■				
5	Decane	■■■■■				
6	DL-2,3-Butanediol				■■	
7	Dodecane	■■■■■				
8	Dodecanoic acid	■■■■■				
9	Eicosanoic acid			■■■		
10	Ethanamine, 2-propoxy-				■■	
11	Ethanol, 2-(2-propenyloxy)				■■	
12	2-Ethoxyethylamine					■
13	Ethylamine					■
14	Formamide					■
15	Geranyl isovalerate	■■■■■				
16	Hexadecane	■■■■■				
17	Hexadecane,2,6,11,15 tetramethyl	■■■■■				
18	n-hexadecanoic acid	■■■■■				
19	2-Hydrazino ethanol			■■■		
20	Hydroperoxide, heptyl					■
21	Hydroperoxide, 1-methylhexyl					■
22	Hydroperoxide, pentyl				■■	
23	Isopropyl Alcohol	■■■■■				
24	Isopropyl myristate		■■■■			
25	L-Lactic acid	■■■■■				
26	Methane, nitroso-		■■■■			
27	Methyltartronic acid				■■	
28	9 Octadecenamide, (Z)	■■■■■				
29	4-Penten-2-ol					■
30	Phenol,2,4,bis (1,1 dimethylethyl)	■■■■■				
31	Propanoic acid, 2-hydroxy-, methylester					■
32	2-Propanol,1-Hydrazino	■■■■■				
33	Propylene glycol		■■■■			
34	Pyrrolo [1,2 a] pyrazine 1,4 dione, hexahydro 3 (2 methylpropyl)	■■■■■				
35	R-(-)-1,2 propanediol		■■■■			
36	Squalene				■■	
37	Tetracosane		■■■■			
38	Tetradecane	■■■■■				
39	Tetradecanoic acid		■■■			
40	Undecane	■■■■■				

■ represents *Lactobacillus plantarum* DB-2
■ represents *Lactobacillus fermentum* J-1
■ represents *Pediococcus acidilactici* M-3
■ represents *Lactobacillus plantarum* SK-3
■ represents *Pediococcus pentosaceus* SM-2

data were processed to time- and mass- aligned chromatographic peak areas with the XCalibur 2.2SP1 with Foundation 2.0SP1. Metabolites were identified by comparison of mass spectra and retention times with those of authentic standards from NIST 2.0 Mass Spectral Library (Mastrangelo *et al.*, 2015).

RESULTS AND DISCUSSION

Metabolic fingerprinting

The exo- and endo-metabolites of probiotic isolates in culture supernatant provides a window for explaining the comprehensive nature of metabolites. The metabolite profiling provides information about the nutritional as well as of toxic effects, if any of metabolites on the interactive effects of the dietary components on the health of the gut of the host.

In total, 40 metabolites were detected from the culture supernatants of these five isolates. Out of 40 compounds obtained in the solvent phase, only 17 compounds viz. Isopropyl alcohol; L-lactic acid; 2-Propanol, 1-hydrazino; Decane; Benzaldehyde, 2,4, dimethyl; Dodecane; Tetradecane; Phenol, 2,4 bis (1,1 dimethylethyl); Hexadecane, 2,6,11,15 tetramethyl; Hexadecane; Geranyl isovalerate; Pyrrolo [1,2a] pyrazine 1,4 dione, hexahydro 3 (2 methylpropyl); n-Hexadeconic acid; 9 Octadecenamide, (Z); Benzoic acid; Undecane; Dodecanoic acid were common to all the tested isolates as described in Table 2. Out of 17 compounds, Isopropyl alcohol; Dodecane, Hexadecane, Tetradecane and Pyrrolo [1,2a] pyrazine 1,4 dione, hexahydro 3 (2 methylpropyl); Benzaldehyde, 2,4, dimethyl and Geranyl isovalerate; Phenol, 2,4 bis (1,1 dimethylethyl); Hexadecane, 2,6,11,15 tetramethyl; n-Hexadeconic acid; 9 Octadecenamide, (Z), have been reported as defrosting agent, antioxidant, flavour agent, antimicrobial, natural food additive, anti-inflammatory, anti-sleep disorder agent, respectively (Bharat *et al*., 2013).

All five bacterial strains secreted different metabolites. The metabolites, their retention time, peak area, area % and peak height are represented in Table 3 and the GC-MS chromatograms are shown in Figure 1.

Total scans for *L. plantarum* DB-2 was 5709. Total run time was 19.42 min (5.00 – 24.42 min) (Figure 1a). The maximum peak area of 19659374.61 at the peak height of 7235909.56 was observed with 85.29% area covered at retention time 10.53 min (Table 3a, Figure 1b).

L. fermentum J-1 has total of 5713 scans. Total run time was 19.43 min (5.00 – 24.43 min) (Figure 1c). The maximum peak area of 863376582.00 at the peak height of 157335492.57 was observed with 54.03% area covered at retention time 11.37 min (Table 3b, Figure 1d).

P. acidilactici M-3 has total of 5715 scans. Total run time was 19.44 min (5.00 – 24.44 min) (Figure 1e). The maximum peak area of 31245106.39 at the peak height of 9971964.56 was observed with 58.41% area covered at retention time 10.70 min (Table 3c, Figure 1f).

L. plantarum SK-3 has total of 5719 scans. Total run time was 19.45 min (5.00 – 24.45 min) (Figure 1g). The maximum peak area of 55699133.56 at the peak height of 13468063.78 was observed with 92.21% area covered at retention time 10.49 min (Table 3d, Figure 1h).

P. pentosaceus SM-2 has total of 5860 scans. Total run time was 19.93 min (4.50 – 24.43 min) (Figure 1i). The maximum peak area of 1586768.57 at the peak height of 739977.40 was observed with 68.57% area covered at retention time 10.13 min (Table 3e, Figure 1j).

Five metabolic compounds were distributed in four of the selected isolates viz. Isopropyl myristate; Methane, nitroso-; Propylene glycol; R-(-)-1,2 propanediol and Tetracosane. Compounds- Methane, nitroso-; Propylene glycol; R-(-)-1, 2 propanediol were present in *L. plantarum* DB-2, *P. acidilactici* M-3, *L. plantarum* SK-3 and *P. pentosaceus* SM-2. A metabolite - Isopropyl myristate was secreted by *L. fermentum* J-1, *P. acidilactici* M-3, *L. plantarum* SK-3 and *P. pentosaceus* SM-2. Compound - Tetracosane was reported in *L. plantarum* DB-2, *L. fermentum* J-1, *P. acidilactici* M-3 and *P. pentosaceus* SM-2. Out of five metabolic compounds, Isopropyl myristate has been reported with medicinal activity against skin disorders. Out of 40, 3 metabolic compounds i.e. Eicosanoic acid, 2-Hydrazino ethanol and Tetradecanoic acid have been reported in three of the selected isolates. A metabolite - Eicosanoic acid was secreted by *L. fermentum* J-1, *P. acidilactici* M-3, and *P. pentosaceus* SM-2. Compound - 2-Hydrazino ethanol was found in supernatant of

Table 3. Metabolic profile of biocomponents identified in solvent extract of probiotic Lactic acid bacteria isolates by GC-MS

a) *Lactobacillus plantarum* DB-2

Retention Time	Peak Area	Area %	Peak Height
9.63	13668.39	0.06	23789.56
9.65	5965.64	0.03	13938.29
9.71	16405.98	0.07	28881.92
10.09	13022.90	0.06	27200.06
10.12	10917.74	0.05	18554.92
10.36	532337.99	2.31	147518.20
10.43	89582.31	0.39	148261.31
10.53	19659374.61	85.29	7235909.56
10.63	1922104.73	8.34	757552.20
10.77	785784.33	3.41	319137.44

L. plantarum DB-2, *P. acidilactici* M-3 and *P. pentosaceus* SM-2. A metabolic compound - Tetradecanoic acid was present in *L. fermentum* J-1, *P. acidilactici* M-3 and *P. pentosaceus* SM-2 (Table 2).

Six out of 40 metabolic compounds viz. DL-2, 3-Butanediol; Ethanamine, 2-propoxy-; Ethanol, 2-(2-propenyloxy); Hydroperoxide, pentyl; Methyltartronic acid and Squalene were present in two of the selected isolates. Metabolites - DL-2, 3-Butanediol and Methyltartronic acid were secreted by *L. plantarum* DB-2 and *L. plantarum* SK-3. Compounds - Ethanamine, 2-propoxy-; Ethanol, 2-(2-propenyloxy) and Hydroperoxide, pentyl were found in the supernatant of *P. acidilactici* M-3 and *L. plantarum* SK-3.

Retention Time	Metabolites	Molecular formula	Cas No.
9.63	Methane, nitroso-	CH_3NO	18501-20-7
	Formamide	CH_3NO	75-12-7
	Dodecane	$CH_3(CH_2)_{10}CH_3$	112-40-3
	DL-2,3-Butanediol	$C_4H_{10}O_2$	6982-25-8
9.65	2-Propanol, 1-hydrazino-	$C_3H_{10}N_2O$	18501-20-7
	Isopropyl alcohol	C_3H_8O	67-63-0
	Propylene glycol	$C_3H_8O_2$	57-55-6
9.71	L-Lactic acid	$C_3H_6O_3$	79-33-4
	Acetic acid, anhydride with formic acid	$C_3H_4O_3$	2258-42-6
	R-(-)-1,2-propanediol	$C_3H_8O_2$	4254-14-2
10.09	Methyltartronic acid	$C_4H_6O_5$	595-98-2
	Dodecanoic acid	$C_3H_{10}N_20$	18501-20-7
	2-Hydrazinoethanol	$C_2H_8N_{20}$	109-84-2
10.12	Tetradecane	$CH_2(CH_3)_{10}CH_2$	629-59-4
	Benzoic acid	C_6H_5COOH	65-85-0
	Undecane	$C_{11}H_{24}$	1120-21-4
10.36	Hexadecane, 2,6,11,15, tetrame-thyl	$C_{20}H_{42}$	504-44-9
	Benzaldehyde, 2,4, dimethyl	$C_9H_{10}O$	15764-16-6
	Phenol, 2,4, bis (1,1 di-methylethyl)	$C_{14}H_{22}O$	96-76-4
10.43	Decane	$C^{10}H_{22}$	124-18-5
	Formamide	CH_3N0	75-12-7
	Tetracosane	$C_{24}H_50$	646-31-1
10.53	Hexadecane	$C_{16}H_{34}$	544-76-3
	Geranyl isovalerate	$C_{15}H_{26}O_2$	109-20-6
	Pyrrolo [1,2a] pyrazine 1,4 dio-ne, hexahydro 3 (2 methylpro-pyl)	$C_{11}H_{18}N_2O_2$	5654-86-4
10.63	n-Hexadeconic acid	$C_{16}H_{32}O_2$	57-10-3
	9 Octadecenamide, (Z)	$C_{18}H_{35}NO$	301-02-0
	L-Lactic acid	$C_3H_6O_3$	79-33-4
10.77	Methane, nitroso-	CH_3NO	865-40-7
	Ethylamine	C_2H_7N	75-04-7
	2-Ethoxyethylamine	$C_4H_{11}NO$	110-76-9

Metabolite – Squalene was synthesized in the culture supernatant of *P. acidilactici* M-3 and *P. pentosaceus* SM-2 (Table 2). Squalene is a triterpine compound, originally found in shark liver oil and has a high therapeutic potential. Squalene is a therapeutic agent having anti-cancerous, anti-tumour, chemo-preventive, antioxidant, and sunscreen properties along with anti-microbial activity (Ezhilan and Neelamegam, 2012). In industry, it is scarcely produced by plant sources along with the shark liver oil. Microbial production of Squalene is an attractive alternative to label the issue with an objective to increase its productivity and purity by using biotechnological interventions. Although, a detailed study is required for the commercialization of the production of Squalene from native probiotic bacteria.

9 metabolites out of 40 were produced by only one selected isolate viz. Acetic acid, anhydride with formic acid; 4-amino-1-butanol; 2-Ethoxyethylamine; Ethylamine; Formamide; Hydroperoxide, heptyl; Hydroperoxide, 1-methylhexyl; 4-Penten-2-ol and Propanoic acid, 2-hydroxy-, methylester. Compounds - Acetic acid, anhydride with formic acid; 2-Ethoxyethylamine; Ethylamine; Formamide were produced by *L. plantarum* DB-2. Metabolite - 4-amino-1-butanol was found to be synthesized by *L. plantarum* SK-3. Metabolite - Hydroperoxide, heptyl was present in *P. acidilactici* M-3. Compounds - Hydroperoxide, 1-methylhexyl and Propanoic acid, 2-hydroxy-, methylester were secreted

b) *Lactobacillus fermentum* J-1

Retention Time	Peak Area	Area %	Peak Height
5.04	2763275.83	0.17	4173857.72
11.30	152563796.70	9.55	141697072
11.37	863376582.00	54.03	157335492.57
11.53	497177369.61	31.12	129599071.95
11.61	81982240.17	5.13	26851053.15

Retention Time	Metabolites	Molecular Formula	Cas No.
5.04	Hydroperoxide, pentyl	$C_5H_{12}O_2$	74-80-6
	Ethanol, 2-(2-propenyloxy)-	$C_5H_{10}O_2$	111-45-5
	Hydroperoxide, 1-methylhexyl	$C_7H_{16}O_2$	762-46-9
	Decane	$C_{10}H_{22}$	124-18-5
	Undecane	$C_{11}H_{24}$	1120-21-4
11.30	Benzaldehyde, 2,4, dimethyl	$C_9H_{10}O$	15764-16-6
	Benzoic acid	C_6H_5COOH	65-85-0
	2-Propanol, 1-hydrazino-	$C_3H_{10}N_2O$	18501-20-7
	Isopropyl alcohol	C_3H_8O	67-63-0
	L-Lactic acid	$C_3H_6O_3$	79-33-4
11.37	Dodecane	$C_{12}H_{26}$	112-40-3
	Phenol, 2,4 bis (1,1dimethylethyl)	$C_{14}H_{22}O$	96-76-4
	Propanoic acid, 2-hydroxy-,methyl ester, (ñ)-	$C_4H_8O_3$	2155-30-8
	Dodecanoic acid	$C_{12}H_{24}O_2$	143-07-7
	Tetradecane	$CH_2(CH_3)_{10}CH_2$	629-59-4
11.53	Hexadecane	$C_{16}H_{34}$	544-76-3
	Geranyl Isovalerate	$C_{15}H_{26}O_2$	109-20-6
	Pyrrolo [1,2 a] pyrazine 1,4 dione, hexahydro 3 (2 methylpropyl)	$C_{11}H_{18}N_2O_2$	5654-86-4
	n-Hexadeconic acid	$C_{16}H_{32}O_2$	57-10-3
	2-Hydrazinoethanol	$C_2H_8N_2O$	109-84-2
11.61	Eicosanoic acid	$C_{20}H_{40}O_2$	506-30-9
	Tetracosane	$C_{24}H_{50}$	646-31-1
	Isopropyl myristate	$C_{17}H_{34}O_2$	110-27-0
	9 Octadecenamide, (Z)	$C_{18}H_{35}NO$	301-02-0
	Tetradecanoic acid	$C_{14}H_{28}O_2$	544-63-8
	Hexadecane, 2,6,11,15 tetrame-thyl	$C_{20}H_{42}$	504-44-9

by *L. fermentum* J1. Metabolite - 4-Penten-2-ol was produced by *P. pentosaceus* SM-2 (Table 2).

Probiotics has a vital role in well-being and health beyond basic nutrition. Probiotics have potential health benefits such as antimicrobial activity, antimutagenic, anticarcinogenic activities, constipation, regulation of immune function, improving gastrointestinal health, reducing lactose intolerance, and allergenic diseases such as food allergy, etc. Due to these health benefits, probiotic bacteria and their metabolites are gaining importance in pharmaceutical preparation, food products, medicines and dietary supplements (Da Cruz *et al*., 2009; Settanni and Moschetti, 2010; Fernandez *et al*., 2013). Production of different primary and secondary metabolites by the probiotic bacteria, employs their biological effects directly or by modifying the immune system. The metabolites of lactic acid bacteria provide therapeutic benefits in preventing and curing various diseases and also responsible for the development of flavour, aroma and texture in food products (Chen *et al*., 2014). The metabolites produced by lactic acid bacteria in this study

c) *Pediococcus acidilactici* M-3

Retention Time	Peak Area	Area %	Peak Height
5.02	3774941.17	7.06	3718077.48
10.58	2981495.15	5.57	1994601.20
10.63	1773985.26	3.32	1572423.15
10.70	31245106.39	58.41	9971964.56
10.80	9650577.59	18.04	3715383.92
11.00	4064008.32	7.60	1071788.04

Retention Time	Metabolites	Molecular Formula	Cas No.
5.02	Hydroperoxide, pentyl	$C_5H_{12}O_2$	74-80-6
	Hydroperoxide, heptyl	$C_7H_{16}O_2$	764-81-8
	Ethanol, 2-(2-propenyloxy)-	$C_5H_{10}O_2$	111-45-5
	Benzoic acid	C_6H_5COOH	65-85-0
10.58	Dodecane	$C_{12}H_{26}$	112-40-3
	Undecane	$C_{11}H_{24}$	1120-21-4
	Isopropyl alcohol	C_3H_8O	67-63-0
	Propylene Glycol	$C_3H_8O_2$	57-55-6
	L-Lactic acid	$C_3H_6O_3$	79-33-4
10.63	R-(-)-1,2-propanediol	$C_3H_8O_2$	4254-14-2
	Tetradecane	$CH_2(CH_3)_{10}CH_2$	629-59-4
	Phenol, 2,4 bis (1,1dimethylethyl)	$C_{14}H_{22}O$	96-76-4
	Hexadecane, 2,6,11,15 tetra-methyl	$C_{20}H_{42}$	504-44-9
	Benzaldehyde, 2,4, dimethyl	$C_9H_{10}O$	15764-16-6
10.70	2-Propanol, 1-hydrazino-	$C_3H_{10}N_2O$	18501-20-7
	Dodecanoic acid	$C_{12}H_{24}O_2$	143-07-7
	Methane, nitroso-	CH_3NO	865-40-7
	Tetracosane	$C_{24}H_50$	646-31-1
	Decane	$C_{10}H_{22}$	124-18-5
10.80	Eicosanoic acid	$C_{20}H_{40}O_2$	506-30-9
	Hexadecane	$C_{16}H_{34}$	544-76-3
	Isopropyl myristate	$C_{17}H_{34}O_2$	110-27-0
	Geranyl isovalerate	$C_{15}H_{26}O_2$	109-20-6
	n-Hexadeconic acid	$C_{16}H_{32}O_2$	57-10-3
11.00	Ethanamine, 2-propoxy-		
	Pyrrolo [1,2 a] pyrazine 1,4 dione, hexahydro 3 (2 methylpropyl)	$C_{11}H_{18}N_2O_2$	5654-86-4
	Tetradecanoic acid	$C_{14}H_{28}O_2$	544-63-8
	9 Octadecenamide, (Z)	$C_{18}H_{35}NO$	301-02-0
	Squalene	$C_{30}H_50$	111-02-4

have been remarked with various functional as well as therapeutic properties viz. food additives, flavouring agents, anti-inflammatory, anti-cancerous, potential against skin disorders, cholesterol-lowering and anti-diarrheal properties and endorse their huge potential in food and pharmaceutical industries.

Many researchers have observed similar metabolic products in many lactic acid bacteria influencing human metabolism when incorporated into the system as probiotics or the food fermented by the respective bacteria. Cagno *et al*. (2009) observed tomato juice fermented with *L. plantarum* POM1 and POM35 for the effect of metabolites produced by the bacterial starters (by GC-MS technique) on the sensory and health-promoting properties of tomato juice and found a large number of volatile compounds in fermented tomato juice as compared to the control. Lee *et al*. (2009) isolated the metabolites from lactic acid bacteria in grape wines through GC-based metabolic profiling and ^{1}H-NMR and identified various organic acids such as lactic acid, tartaric acid and volatile compounds such as benzoic acid, dodecanoic acid, dodecamethyl cyclohexasiloxane, isopropyl myristate, tetradecanoic acid, 1-hexadecanol, hexadeconoic acid and octadecanoic acid responsible for wine odour and flavour. Vanaja *et al*. (2011) observed the metabolites secreted by *L. plantarum* LPcfr and found compounds viz. hexadecane, hexadeconic acid, dodecanal having antifungal and antioxidant properties and fatty acids such as stearic and palmitic acids having antifungal properties. Sheela *et al*. (2012) found antimicrobial compounds (fatty acids such as palmitic acid and stearic acid and

d) *Lactobacillus plantarum* SK-3

Retention Time	Peak Area	Area %	Peak Height
9.75	625128.64	1.03	279968.97
10.33	87526.28	0.14	137242.54
10.42	1067849.36	1.77	1455637.02
10.45	898971.70	1.49	1508377.52
10.49	55699133.56	92.21	13468063.78
10.69	2027689.17	3.36	774610.72

Retention Time	Metabolites	Molecular Formula	Cas No.
9.75	Isopropyl alcohol	C_3H_8O	67-63-0
	L-Lactic acid	$C_3H_6O_3$	79-33-4
	2-propanol, 1-hydrazino	$C_3H_{10}N_2O$	18501-20-7
	Benzoic acid	C_6H_5COOH	65-85-0
10.33	2-Hydrazinoethanol	$C_2H_8N_20$	109-84-2
	Dodecane	$C_{12}H_{26}$	112-40-3
	Benzaldehyde, 2,4, dimethyl	$C_9H_{10}O$	15764-16-6
	Undecane	$C_{11}H_{24}$	1120-21-4
10.42	Propylene glycol	$C_3H_8O_2$	57-55-6
	Tetradecane	$CH_2(CH_3)_{10}CH_2$	629-59-4
	Dodecanoic acid	$C_{12}H_{24}O_2$	143-07-7
	Hexadecane, 2,6,11,15 tetra-methyl	$C_{20}H_{42}$	504-44-9
10.45	R-(-)-1,2-propanediol	$C_3H_8O_2$	4254-14-2
	Hexadecane	$C_{16}H_{34}$	544-76-3
	Isopropyl myristate	$C_{17}H_{34}O_2$	110-27-0
	Decane	$C_{10}H_{22}$	124-18-5
10.49	Methane, nitroso-	CH_3NO	865-40-7
	Phenol, 2,4 bis (1,1dimethylethyl)	$C_{14}H_{22}O$	96-76-4
	Geranyl isovalerate	$C_{15}H_{26}O_2$	109-20-6
	n-Hexadeconic acid	$C_{16}H_{32}O_2$	57-10-3
10.69	Ethanamine, 2-propoxy-	$C_5H_{13}NO$	42185-03-5
	4-Amino-1-butanol	$C_4H_{11}NO$	13325-10-5
	Pyrrolo [1,2 a] pyrazine 1,4 dione, hexahydro 3 (2 methylpropyl)	$C_{11}H_{18}N_2O_2$	5654-86-4
	9 Octadecenamide, (Z)	$C_{18}H_{35}NO$	301-02-0

Phenol, 2, 4-bis (1, dimethylethyl)) after GC-MS in symbiotic cow milk beverage fermented with *L. kefiranofaciens*, *Candida kefir* and *Saccharomyces boulardii*. The fermented cow milk symbiotic beverage had the greater anti-diarrhoeal effect as compared to other milk beverages, thereby indicating the role of antibacterial metabolites produced by the probiotic bacteria. Padmavathi *et al*. (2014) studied the anti-biofilm efficacy and anti-quorum sensing of the metabolites produced by marine *Vibrio alginolyticus* G16 against *Serratia marcescens* and found the active compound as phenol, 2, 4-bis (1,1-dimethylethyl) after purification and mass spectrometric analysis.

Sheela *et al*. (2015) studied the biocontrol efficacy of symbiotic strawberry juice and found most of the metabolites were of antibacterial, anti-diarrhoeal (17-pentatriacontene) and of antioxidative nature. Effect of probiotics and prebiotics was evaluated on the metabolites isolated from human microbiota and found these metabolites helping in the regulation of colonic cell proliferation, inactivation of toxic compounds and enhancement of health-promoting properties (Vitali *et al.,* 2012). Hong-Xin *et al*. (2015) used GC-MS analysis to identify aroma compounds viz. decane, dodecanoic acid, hexadecanoic acid, hexanoic acid 4-hexadecyl ester and phenol 2,4

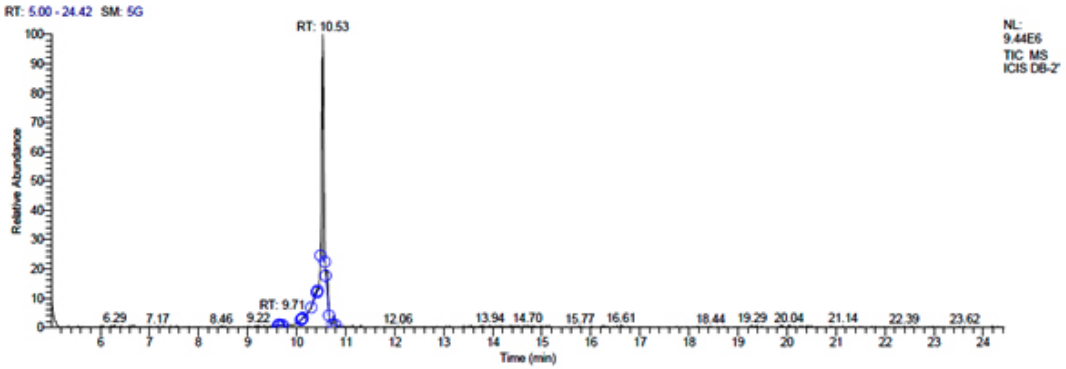
e) *Pediococcus pentosaceus* SM-2

Retention Time	Peak Area	Area %	Peak Height
9.72	216757.74	9.37	98425.02
9.79	210048.41	9.08	89220.41
9.82	72569.37	3.14	142459.44
9.86	36228.30	1.57	51748.50
10.13	1586768.57	68.57	739977.40
10.20	191626.29	8.28	115859.62

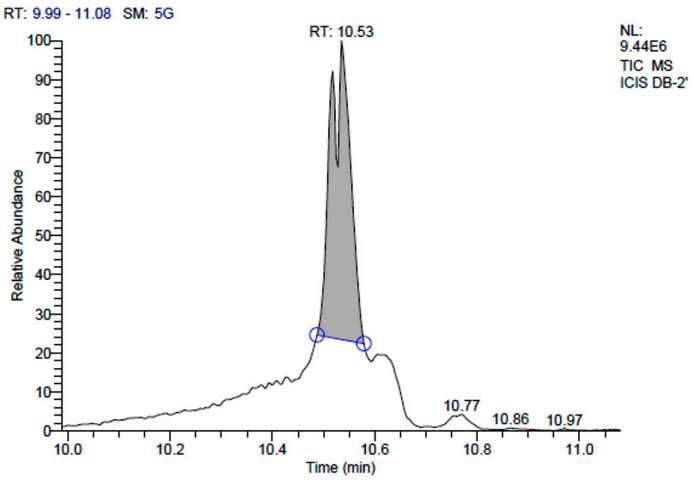
Retention Time	Metabolites	Molecular Formula	Cas No.
9.72	Methyltartronic acid	$C_4H_6O_5$	595-98-2
	L-Lactic acid	$C_3H_6O_3$	79-33-4
	2-propanol, 1-hydrazino	$C_3H_{10}N_2O$	18501-20-7
	Benzoic acid	C_6H_5COOH	65-85-0
9.79	R-(-)-1,2-propanediol	$C_3H_8O_2$	4254-14-2
	DL- 2,3 –Butanediol	$C_4H_{10}O_2$	6982-25-8
	Dodecane	$C_{12}H_{26}$	112-40-3
	Decane	$C_{10}H_{22}$	124-18-5
	Benzaldehyde, 2,4, dimethyl	$C_9H_{10}O$	15764-16-6
9.82	Propylene glycol	$C_3H_8O_2$	57-55-6
	Tetradecane	$CH_2(CH_3)_{10}CH_2$	629-59-4
	Dodecanoic acid	$C_{12}H_{24}O_2$	143-07-7
	Phenol, 2,4 bis (1,1dimethylethyl)	$C_{14}H_{22}O$	96-76-4
	Hexadecane, 2,6,11,15 tetra-methyl	$C_{20}H_{42}$	504-44-9
9.86	Isopropyl Alcohol	C_3H_8O	67-63-0
	Hexadecane	$C_{16}H_{34}$	544-76-3
	Tetracosane	$C_{24}H_{50}$	646-31-1
	Tetradecanoic acid	$C_{14}H_{28}O_2$	544-63-8
	Undecane	$C_{11}H_{24}$	1120-21-4
10.13	Methane, nitroso-	CH_3NO	865-40-7
	Isopropyl myristate	$C_{17}H_{34}O_2$	110-27-0
	Geranyl isovalerate	$C_{15}H_{26}O_2$	109-20-6
	n-Hexadeconic acid	$C_{16}H_{32}O_2$	57-10-3
	Eicosanoic acid	$C_{20}H_40O_2$	506-30-9
10.20	4-Penten-2-ol	C5H10O	625-31-0
	Pyrrolo [1,2 a] pyrazine 1,4 dione, hexahydro 3 (2 methylpropyl)	$C_{11}H_{18}N_2O_2$	5654-86-4
	9 Octadecenamide, (Z)	$C_{18}H_{35}NO$	301-02-0
	Squalene	$C_{30}H_50$	111-02-4

bis (1,1-dimethylethyl) during ripening period (180 days) of cheese fermented with *L. casei* LC2W and found these metabolites help in accelerating the ripening process.

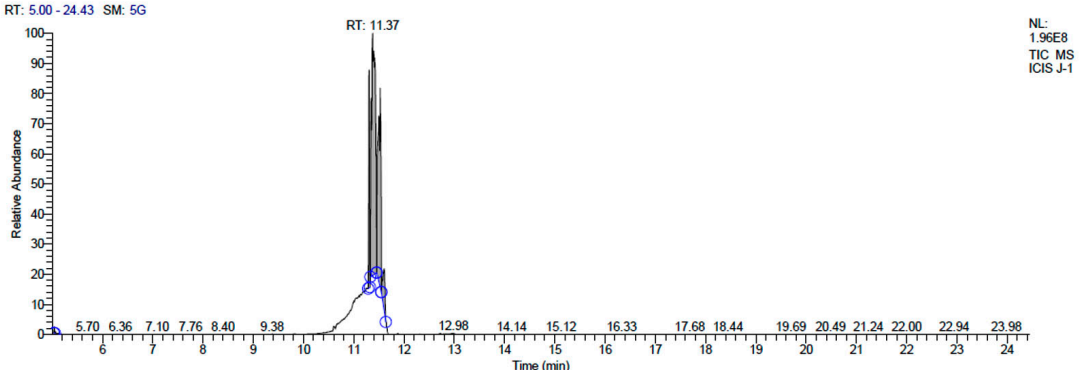
The reliability of the technique in investigating the effect of metabolic compounds on colonic metabolic signature has been recently



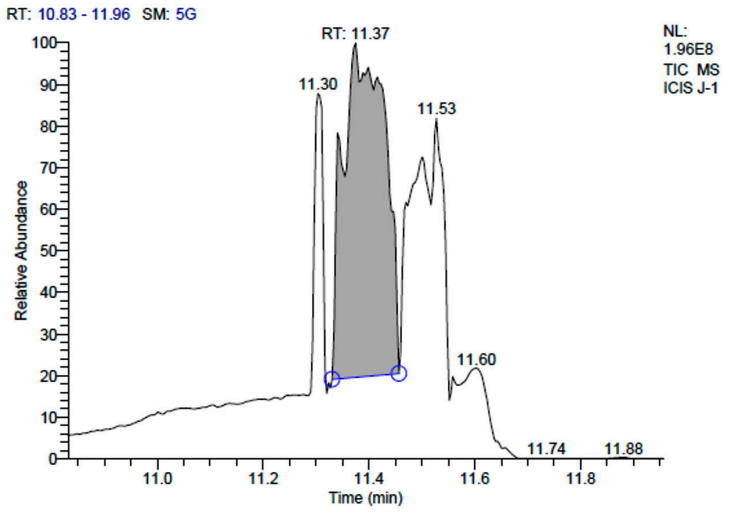
(a) GC-MS chromatogram of metabolites secreted by *L. plantarum* DB-2



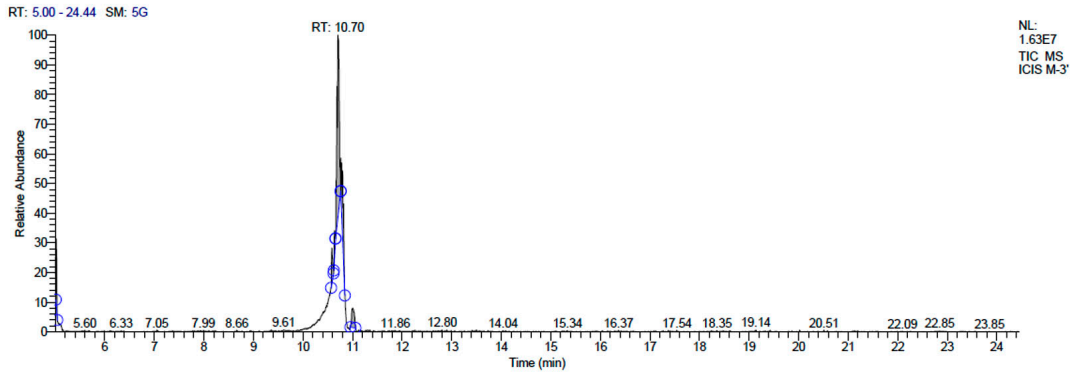
(b) GC-MS chromatogram of *L. plantarum* DB-2 at retention time 10.53 min



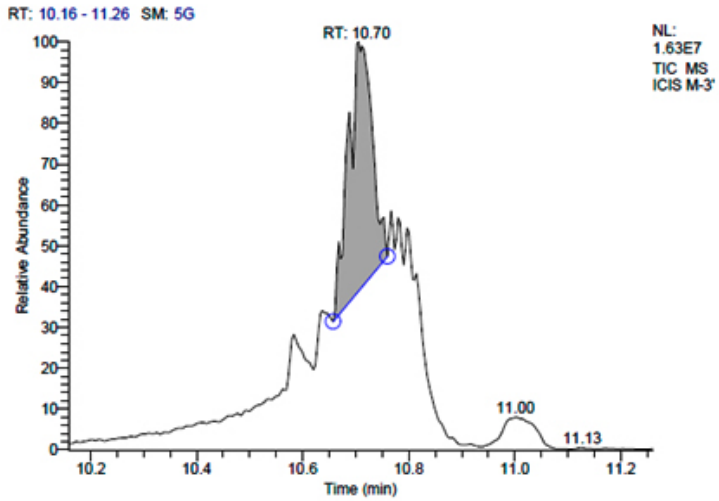
(c) GC-MS chromatogram of metabolites secreted by *L. fermentum* J-1



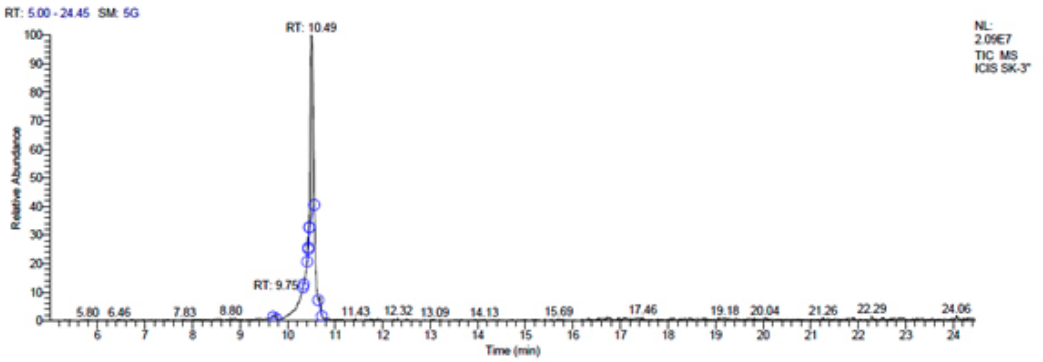
(d) GC-MS chromatogram of *L. fermentum* J-1 at retention time 11.37 min



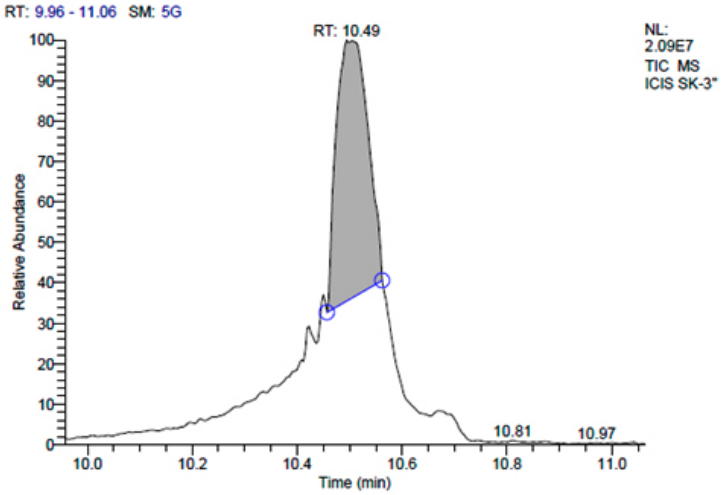
(e) GC-MS chromatogram of metabolites secreted by *P. acidilactici* M-3



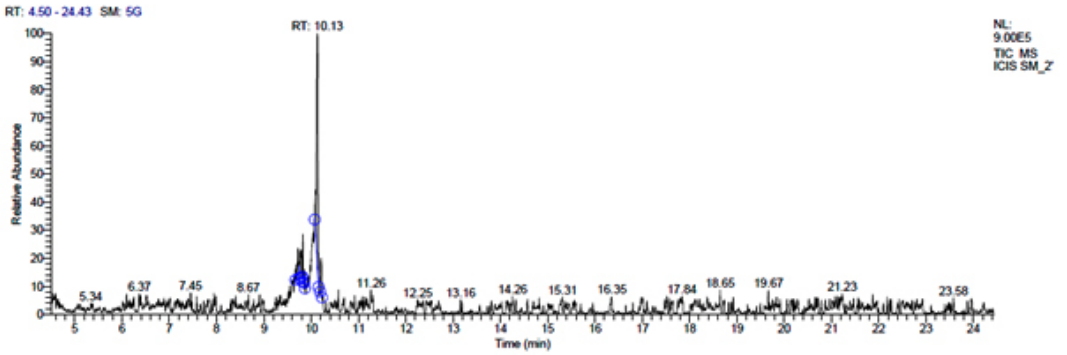
(f) GC-MS chromatogram of *P. acidilactici* M-3 at retention time 10.70 min



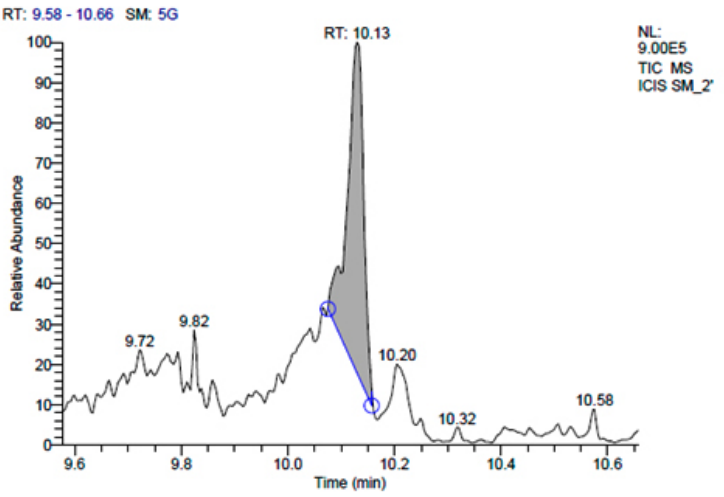
(g) GC-MS chromatogram of metabolites secreted by *L. plantarum* SK-3



(h) GC-MS chromatogram of *L. plantarum* SK-3 at retention time 10.49 min



(i) GC-MS chromatogram of metabolites secreted by *P. pentosaceus* SM-2



(j) GC-MS chromatogram of *P. pentosaceus* SM-2 at retention time 10.13 min

Fig. 1. GC-MS chromatograms of metabolites secreted by Lactic acid bacteria

studied (Maccaferri *et al*., 2010, Vitali *et al*., 2010, Garner *et al*., 2007).

CONCLUSION

Utilization of probiotic bacteria and their metabolites in medicines, dietary supplements, pharmaceutical preparation and food products are enhancing due to their potential health benefits such as anticarcinogenic activities, constipation, reducing lactose intolerance, regulation of immune function, allergenic diseases such as food allergy, etc. These attributes of probiotic lactic acid bacteria have been described by the production of different primary and secondary metabolites that exert their biological effects either directly or by modifying the immune system.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

None.

AUTHORS' CONTRIBUTION

AC conceived and planned the experiments, performed the analysis and wrote the manuscript. KV and BSS supervised the research project.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This research does not contain any studies with human participants or animals performed by any of the authors.

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