

Isolation, Identification and Selection of Lactic Acid Bacteria Cultures for Production of Food Aroma & Flavour Compounds

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Abstract: Over the past few years, the increasing demand for natural products in the food industry has encouraged remarkable efforts towards the development of biotechnological processes for the production of flavour compounds. A total of 30 cultures were isolated from laban rayeb (natural sour milk), raw milk and soft & Ras cheeses obtained from different regions in Egypt. Lactic acid bacteria strains (LAB) were identified as *Lactobacillus casei* , *Lactobacillus plantarum*; *Lecuoconostoc mesentroides*; *Enterococcus faecium* ; *Lactobacillus bulgaricus*; *Lactococcus lactis subsp lactis* and *Lactococcus lactis subsp diacetylactis*. For flavour production , MRS and M17 media, sterilized milk with and without amino acid (methionine , leucine , phenylalanine) were used .The headspace volatiles of lactic acid bacteria (LAB) with media MRS or M17 and with sterilized milk with or without amino acids were collected and subjected to GC and GC-MS analysis. The major component was ethylacetate which comprises (83.791). in *Lactobacillus casei*; (81.99%) in *Lactobacillus plantarum*; (78.99%) in *Lecuoconostoc mesentroides*; (74.30%) in *Enterococcus faecium* and (74.04%) in *Lactobacillus bulgaricus*; (59.50%) in *Lactococcus lactis subsp lactis*, while acetadehyde was the most abundant constituent (38.26%) in *Lactococcus lactis subsp diacetylactis*, also it comprised the highest concentration in 2,3-butanedione (18.15%). In case of fermented milk, the major component was ethyl butanoate. Which comprised 67.56%, 67.29% in *Leucoconostoc mesentroides* and *Lactobacillus plantarum* and comprised 57.33%, 54.30% in *Enterococcus faecium* and *Lactobacillus casei*, also comprised 49.74%, 48.32% in *Lactobacillus bulgaricus* and *Lactococcus diacetylactis* compared to 24.35% in control milk sample. Also LAB cause a remarkable increase in ethylhexanoate. Addition of 0.25% phenylalanine causes increase in 2,3-butanedione percentage in most sample since comprised 33.89% in *Lactobacillus plantarum*, 25, 64% in *Lactobacillus casei* and 18.03% in *Lactococcus diacetylactis* compared to 15.08% in control sample. Strecker aldehydes 2/3-methylbutanal;phenylacetaldehyde and the two dicarbonyls 2,3-butanedione and 2,3-pentanedione were represented with remarkable concentration as the effect of leucine, phenylalanine and methionine addition which considered the main precursors of key aroma compounds.

Key words: Lactic acid bacteria , aroma & flavour , Amino acids,GC-MS volatile components.

INTRODUCTION

Flavour development in dairy fermentations, most notably cheeses, results from a series of biochemical processes in which the starter cultures provide the enzymes. Particularly the enzymatic degradation of proteins (caseins) leads to the formation of keyflavour components, which contribute to the sensory perception of dairy products. More specifically, caseins are degraded into peptides and amino acids and the latter are major precursors for volatile aroma compounds. The formation of aroma in cheese is a complex process carried out mainly by cheese microflora. Biochemical reactions lead to the release of aroma compounds from lactose, milk fat and proteins. The topic has been extensively reviewed elsewhere (Dumont & Adda, 1979 ; Fox, *et al*, 1995 ; Christensen, *et al*, 1999; Cristiani & Monnet, 2001; Yvon & Rijnen, 2001; Spinnler, *et al.*, 2002; Marilley & Casey, 2004) The enzymatically formed aroma compounds found in cheese are very diverse and include alcohols, acids, esters, ketones, pyrazines. furans, phenolic compounds and free fatty acids. Currently, consumer demand for a diversification of cheese products is increasing and requires the development of innovative products. New starter and adjunct cultures 'are seen as a promising means to improve the attractiveness of cheese products. Higher scores for flavour quality were attributed to Manchego (Poveda, *et al.*, 2003), washed-curd (Hynes *et al.*, 2003), Feta (Katsiari, *et al.*, 2002) and Cheddar (Fenelon, *et al.*, 2002 and Lynch, *et al.*, 1996) cheese manufactured with the addition of adjunct cultures to cheesemilk. Although Centeno, *et al.*, (2002) found that the flavour quality of ewes' raw milk cheese was impaired by the addition of wild strains of *Lactococcus lactis*, the abundance of volatile compounds was increased in the presence of these wild strains.

Recently, the use of amino acids by 71 strains of the genus *Lactococcus* and *Lactobacillus* were measured (Liu, *et al.*, 2003) revealing differences in their aroma-forming ability.

Williams, *et al.*, (2001) tested the capacity of 152 lactic acid bacteria retrieved from Cheddar cheese to catabolise protein hydrolysates and individual amino acids in the presence of α -ketoglutarate.

Several studies have shown that the production of volatile compounds varies considerably between bacterial isolates and is probably strain dependant (Ayad, *et al.*, 1999; Drake *et al.*, 1999; Mauriello *et al.*, 2001; Tavaría *et al.*, 2002 and Williams *et al.*, 2001).

Fatty acids, esters, aldehydes, alcohols, ketones, and sulphur compounds have been reported to be important contributors to the final taste and aroma of cheese (Bosset & Gauch, 1993 and Curioni & Bosset, 2002). Most of the volatile flavour components detected are formed during ripening, some being derived from catabolism of the FAAs released during proteolysis. For instance, reduction of the corresponding aldehydes formed by Strecker degradation of Val and Leu can give rise to 2-methylpropan-1-ol and 3-methylbutan-1-ol, respectively (Smit *et al.*, 2000).

In another paper, Fox & Wallace (1997) observed that the products of amino acid catabolism make a greater contribution to flavour than do the amino acids themselves. Much is now known of the enzyme systems involved in the conversion of caseins to FAAs, but only recently has attention been turned to the enzymes of starter bacteria involved in amino acid catabolism (Engels *et al.*, 1996 and Smit *et al.*, 2000). Dias & Weiner (1998) reported the conversion of Met to thiols by lactococcal enzymes, catabolism of Met and Cys to sulphur compounds being essential to the formation of Cheddar cheese flavour (Urbach, 1993).

The major flavour compounds that are released during lipolysis are free fatty acids (FFA), which directly affect cheese flavour. FFA can also be transformed by microorganisms to other and often more potent flavour compounds, including methyl ketones, lactones, esters, secondary alcohols and aldehydes, which also directly affect flavour in different cheeses (Collins, *et al.*, 2003; Abd El-Mageed 1997).

Indigenous LAB isolated from ewe's and goat's milk and artisanal cheeses manufactured in the provinces of northwest Argentina were identified as enterococci, lactococci, leuconostoc and lactobacilli. *Enterococcus faecium* and *Lactobacillus plantarum* were the most frequently isolated species from ewe's and goat's milk and cheeses (Medina *et al.*, 2001; Oliszewski, 2006). The contribution of cheese microflora to the formation of flavour compounds occurs via the esterase/lipase systems of lactic and propionic acid bacteria, non-starter LAB (NSLAB), surface microorganisms, yeasts and moulds (McSweeney & Soursa, 2000).

Moreover, some volatile compounds arise from microbial amino acid catabolism (Tavaria & Malcata, 2003). We have previously demonstrated that LAB isolated from goat's and ewe's dairy products present very complex intracellular esterolytic systems able to release C2-C6 fatty acids and thus they actively contribute to flavour development in these products. (Abeijon Mukdsi *et al.*, 2006; Abeijon Mukdsi *et al.*, 2009a and Oliszewski *et al.*, 2007)

Amino acid catabolism by the microflora is a major process for the formation of a large number of key aroma compounds in Swiss-type cheeses such as gruyere and emmental (Friedrich & Acree 1998; Preininger & Grosch 1994 and Rychlik & Bosset 2001). However, the amino acid catabolic pathways in the different bacteria present in these cheeses are not well known. This knowledge could lead to the development of cultures with optimized aromatic properties. Amino acid catabolism by *Lactococcus lactis* and mesophilic lactobacilli has been extensively studied recently (Amarita, *et al.*, 2001; Gummalla & Broadbent 1999; Tammam, *et al.*, 2000 and Yvon & Rijnen, 2001).

The microflora of Swiss-type cheese consists mainly of propionibacteria and thermophilic LAB, especially *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *lactis*, and *Streptococcus thermophilus*. Recently, Thierry *et al.*, 2002 have shown that propionibacteria are capable of producing isovaleric acid from leucine in vitro and are mainly responsible for its production in Swiss cheese. However, propionibacteria do not seem to be capable of producing aldehydes from amino acids, while these compounds are very important for Swiss cheese aroma. Among the thermophilic LAB, only amino acid conversion by *L. helveticus* was partially studied. In vitro studies have shown that *L. helveticus* can produce acetaldehyde from threonine (Hickey, *et al.*, 1983) volatile sulfur compounds from methionine, and, in the presence of ex-KG, benzaldehyde from phenylalanine (Klein, *et al.*, 2001). Under simulated cheese-ripening conditions, *L. helveticus* cells mainly produces carboxylic acids and hydroxyacids from phenylalanine and tyrosine (Gummalla & Broadbent 2001).

The aim of this study is to use lactic acid bacteria to produce different dairy aroma notes by fermentation on media or on raw milk besides addition of some amino acids to study their effects on the volatile components produced and on their yield.

MATERIALS And METHODS

Isolation of LAB Strains:

Thirty samples of Laban Rayeb (Natural sour milk), raw milk and soft & Ras cheeses were collected from different locations in Egypt. MRS and M17 agar media (Oxoid) were used to isolate *Lactobacilli* and *Lactococci* respectively. Kanamycin Aesculin Azide Agar Base (Oxoid) was used for *Enterococci*. Typical colonies were

picked and further purified in three successive passage on MRS agar and M17 agar .Cultures were routinely maintained on appropriate solid media at 4⁰ C.

Identification of LAB Strains:

Lactic acid bacteria organisms were identified according to the criteria described by Hardi(1986), Kandler & Weiss (1986) and Mundt (1986).

Screen for Flavour Production:

Medium and Growth Conditions:

For flavour production, the following media were tested for seven LAB isolates *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactococcus lactis subsp lactis*, *Lactococcus lactis subsp diacetylactis*, *Enterococcus faecium*, *Leuconostoc mesenteroides*:

- 1- MRS broth or M17 media were prepared and 10 % of the inoculum of LAB strains was inoculated separately and incubated at 35°C or 30°C for 48 hours according to LAB type.
- 2- Sterilized milk was inoculated with LAB strains separately and incubated at 35°C or 30°C for 48 hours according to LAB type.
- 3- Sterilized milk was supplemented with amino acids (leucine, phenylalanine and methionine) and inoculated with LAB strains separately and incubated at 35°C or 30°C for 48 hours according to LAB type.

Sensory Evaluation:

The organoleptic properties of the fermented milk samples with LAB and different amino acids were evaluated by 10 experienced assessors. The panelists were asked to evaluate the sample according to their quality characteristics (colour, flavour, taste, consistency and overall acceptability) on 1-10 hedonic scale, where 1 was dislike extremely and 10 was like extremely Abd-El-Mageed & Raghieb (2006) in comparison with the control milk sample which was given the highest scores (10) for all sensory attributes. The means scores were compared for statistical differences using Tukey's test (Steel & Torrie, 1980).

Isolation of Headspace Volatiles:

The volatiles in the headspace of each sample under investigation were isolated by using a dynamic headspace system. The samples were purged for 1 h with nitrogen gas (grade of N2 < 99.99) at a flow rate 100 ml/min. the headspace volatiles were swept into cold traps containing diethyl ether and pentane (1:1, v/v) and held at -10°C. The solvents containing the volatiles were dried over anhydrous sodium sulfate for 1 h. the volatiles were obtained by evaporation of the solvents under reduced pressure Abd El-Mageed (2007).

Gas Chromatographic (GC) Analysis:

GC analysis was performed by using Hewlett-Packard model 5890 equipped with a flame ionization detector (FID). A fused silica capillary column DB-5 (60mx0.32 mm id,) was used. The oven temperature was maintained initially at 50°C for 5 min, then programmed from 50 to 250°C at a rate of 4°C/min. Helium was used as the carrier gas, at flow rate of 1.1 ml/min. The injector and detector temperatures were 220 and 250°C, respectively. The retention indices (Kovats index) of the separated volatile components were calculated using hydrocarbons (C₈-C₂₂, Aldrich Co.) as references.

Gas Chromatographic - Mass Spectrometric (GC-MS) Analysis:

The analysis was carried out by using a coupled gas chromatography Hewlett-Packard model (5890)/ mass spectrometry Hewlett-Packard MS (5970). The ionization voltage was 70 eV, mass range m/z 39-400 a.m.u. The GC condition was carried out as mentioned above. The isolated peaks were identified by matching with data from the library of mass spectra (National Institute of Standard and Technology) and compared with those of authentic compounds and published data (Adams ,2001). The quantitative determination was carried out based on peak area integration.

RESULTS AND DISCUSSION

Identification of Various Lactic Cultures:

Thirty Cultures were isolated from various sources. Isolates were identified to the following genera (Table 1).

Table 1: The percentage distribution of different genus of LAB.

Genus	Number	%
<i>Lactococcus</i>	9	30 %
<i>Enterococcus</i>	4	13 %
<i>Lactobacillus</i>	15	50 %
<i>Leuconostoc</i>	2	7 %

The LAB isolates were classified into the genera *Enterococcus*, *Leuconostoc*, *Lactococcus* and *Lactobacillus* based on their morphology and biochemical characters Table (1) shows the percentage distribution of different genus of LAB . Of the cultures, 50 % belonged to the genus *Lactobacillus*.

Table 2: identification using morphological, phenotypic and biochemical characteristics.

Species	Number
<i>Lactobacillus plantarum</i>	8 (27 %)
<i>Lactobacillus bulgaricus</i>	6 (20 %)
<i>Lactobacillus casei</i>	1 (3 %)
<i>Lactococcus lactis subsp lactis</i>	7 (23 %)
<i>Lactococcus lactis subsp diacetylactis</i>	2 (7 %)
<i>Enterococcus faecium</i>	4 (13 %)
<i>Leuconostoc mesentroides</i>	2 (7 %)

Table (2) reveals the presence of relatively high number of lactobacilli in the Egyptian milk and dairy products, 27% of the identified cultures were *Lactobacillus plantarum*. Twenty three percent of identified strains were *L. lactis* subsp. *lactis*.

Volatile Components in Headspace of Media MRS or M17 with Lactic Acid Bacteria (LAB) Incubated at 37°C for 48 hr:

The separated volatile compounds as identified by comparison of their mass spectra with library (National Institute of standard and Technology) and compared with Kovats indexs (KI) of standard compounds run under similar conditions (Adams 2001), are cited together with their area percentages in Table (3). Fifteen compounds were identified including 5 aldehydes, 4 alcohols, 2 ketones and 3 esters.

Table 3: Volatile Components Identified in Headspace of Media (MRS, M17) with lactic Acid Bacteria (LAB) incubated at 37°C for 48 hrs*.

Peak No.	KI ^a	Components	Control MRS	Sample (1)	Sample (2)	Sample (3)	Sample (4)	Sample (5)	Sample (6)	Methods of Identification ^b
1	581	Acetaldehyde	6.00	2.18	5.40	4.80	5.10	7.87	38.26	MS, KI
2	600	1-Propanol	-	1.49	0.37	-	-	0.26	9.35	MS, KI
3	616	2,3-butanedione	-	3.60	2.06	1.89	1.87	2.79	18.15	MS, KI
4	630	2-Methyl butanal	10.67	0.59	0.73	-	0.89	0.83	1.57	MS, KI
5	644	Ethyl acetate	-	74.04	81.99	83.97	78.99	74.30	8.65	MS, KI
6	729	2,3-pentanedione	-	1.30	1.28	1.44	1.94	1.53	1.45	MS, KI
7	774	3-Methyl butanol	-	1.19	2.00	1.78	0.89	0.91	1.12	MS, KI
8	802	Hexanal	1.90	0.86	0.97	1.09	0.89	1.35	1.57	MS, KI
9	821	Unknown	79.98	-	-	-	-	-	-	MS, KI
10	844	Ethyl butanoate	-	2.76	2.41	2.20	2.47	1.24	1.38	MS, KI
11	884	Hexanol	-	0.99	0.74	0.80	0.61	-	4.46	MS, KI
12	1016	Ethyl hexanoate	-	8.65	0.76	0.96	2.80	1.74	3.43	MS, KI
13	1053	(Z)3-octen-1-ol	-	1.42	1.24	0.35	1.22	0.89	2.63	MS, KI
14	1163	Nonanal	1.44	0.34	0.54	0.25	0.67	1.44	2.07	MS, KI
15	1215	Decanal	-	0.58	0.24	0.46	0.65	4.84	2.88	MS, KI

*Values expressed as relative area percentages to total identified components.

-: Not detected

Compounds listed according to their elution on DB5 column

a: Kovats index

b: Compounds identified by GC-MS(MS) and/or by comparison of MS and KI of standard compound run under similar conditions

Key of samples

1. Media + *Lactobacillus bulgaricus*

3. Media + *Lactobacillus casei*

5. Media + *Enterococcus faecium*

2. Media + *Lactobacillus plantarum*

4. Media + *Leuconostoc mesentroides*

6. Media + *Lactococcus diacetylactis*

The major component in media lactic acid broth was ethylacetate which comprise (83.97%) in *Lactobacillus casei*; (81.99%) in *Lactobacillus plantarum*. (78.99%) in *Leuconostoc mesentroides*; (74.30%) in *Enterococcus faecium*; (74.04%) in *Lactobacillus bulgaricus*. These results are in accordance with Chamba & Irlinger, (2004) ; Cabral,(2005) and Abeijon Mukdsi, *et al.*, (2009). Although sample lactococcus diacetylactis showed drastic decrease in concentration of ethylacetate (8.65%), it comprised the highest percentage of acetaldehyde (38.26%) the second major component by comparing with other samples Table (3). Also the same sample comprised the highest value of 2,3-butanedione (18.15%), this compound is known as sugar degradation

products (Ledle & Schleicher, 1990) and can undergo condensation reaction via *Millard* reaction. These results are in accordance with Chammas *et al.*, (2006). Ethyl butanoate comprised approximately remarkable concentration in most sample while ethyl hexanoate showed remarkable increase in *Lactobacillus bulgaricus* sample (8.65%) and in *Lactococcus diacetylactis* (3.43%) Table (3). These results are in accordance with Abeijon Mukdsi *et al.*, (2009 b).

Volatile Components in Headspace of Fermented Milk with Lactic Acid Bacteria (LAB) and Phenylalanine:

Fourteen compounds were identified in fermented milk samples with LAB and in the same samples + 0.25% phenylalanine compared with control milk sample are cited together with their percentages in Table (4).

The typical gas chromatograms of the volatiles of control milk and fermented milk with LAB and the same samples + 0.25% phenylalanine are shown in Figures (1 - 7).

The identified compounds are recorded (Table 4) including 3 aldehydes, 3 ketones, 1 alcohols, and 7 esters. From Table (4) we can show the addition of lactic acid bacteria cause a sharp decrease in most identified compounds in most samples while cause a great increase in esters compounds especially the major one ethyl butanoate which comprised 67.56%, 67.29% in *Leuconostoc mesentroides* and *Lactobacillus plantarum* and comprised 57.33%, 54.30% in *Enterococcus faecium* and *Lactobacillus casei*, also comprised 49.74%, 48.32% in *Lactobacillus bulgaricus* and *Lactococcus diacetylactis* compared to 24.35% in control milk sample i.e addition of LAB cause double and triple folded increase in major ester compared to control one Table (4). Also LAB cause a remarkable increase in ethylhexanoate with the increase ranged from three to seventh folded than its percentage in control milk sample Table (4). These results are in accordance with Abeijon Mukdsi *et al.*, (2009b) who reported that ethylester of the straight-chain acids C₂-C₁₀ are most frequently found (Liu, *et al.* ,2004). These esters which are potent flavour compounds at less than 5 ppm. are important for development of the characteristic "fruity" type flavours such as ethyl butanoate and ethylhexanoate (Moio &Addeo 1998; Abd El Mageed & Ragheb 2005, 2006). Esters may also mask the impact of off-flavours (e.g. pungent, sharp) imparted by high levels of short-chain FFA. Excessive levels of ethyl esters of short-chain FFA (typically ethylbutanoate and ethylhexanoate) cause a fruity flavour defect in some raw and pasteurized milks, and Cheddar cheese (Horwood *et al.*, 1987 and Whitfield *et al.*, 2000).

The same trend are shown in fermented milk samples with LAB + 0.25% phenylalanine as mentioned before except for the compounds 2,3-butanedione, addition of phenylalanine causes increase in its percentage in most samples since comprised 33.89% in *Lactobacillus plantarum*, 25.64% in *Lactobacillus casei* and 18.03% in *Lactococcus diacetylactis* compared to 15.08% in control sample Table (4).

Table 4: Volatile Components Identified in headspace volatiles of Fermented milk with lactic acid bacteria (LAB) and phenylalanine.

Peak No.	K _I ^a	Components	Contro 1	volatiles. Of fermented milk samples with LAB						Volatiles of the same fermented milk samples with LAB + 0.25% phenylalanine						Methods of identification ^b
				1	2	3	4	5	6	7	8	9	10	11	12	
1	581	Acetaldehyde	*25.79	-	4.72	6.88	3.70	-	4.13	0.23	27.73	-	0.87	1.27	1.51	MS, KI
2	616	2,3-butanedione	15.08	8.20	15.17	18.47	12.05	10.39	7.38	9.79	33.89	25.64	11.69	12.70	18.03	MS, KI
3	644	Ethylacetate	6.49	0.18	1.88	2.67	2.40	1.99	1.45	-	7.30	0.58	0.21	0.71	0.86	MS, KI
4	711	2,3-pentanedione	12.71	3.74	1.51	1.28	2.66	5.33	3.93	5.86	0.99	2.85	6.20	7.25	1.36	MS, KI
5	754	3-Methyl butanol	2.27	0.93	0.42	1.27	0.50	3.27	1.09	1.84	2.05	3.29	1.90	1.36	0.09	MS, KI
6	777	Ethyl-2-methyl propanoate	1.38	0.06	-	8.39	0.05	0.05	0.06	0.03	10.47	2.17	0.41	0.62	-	MS, KI
7	832	Methyl valerate	-	0.64	0.19	-	0.04	0.14	0.75	0.19	-	0.64	0.30	0.50	0.09	MS, KI
8	851	Ethyl butanoate	24.35	49.74	67.29	54.30	67.56	57.33	48.32	59.53	11.66	54.12	64.09	65.84	70.96	MS, KI
9	891	Ethyl-2-methyl butanoate	-	4.88	-	-	-	-	-	-	-	-	-	-	-	MS, KI
10	902	Ethyl pentanoate	-	14.06	-	-	-	-	-	-	-	-	-	-	-	MS, KI
11	1003	Octanal	0.15	8.93	3.21	1.38	5.84	7.20	4.94	10.42	-	3.21	6.79	3.49	1.37	MS, KI
12	1016	Ethyl hexanoate	1.09	7.24	3.07	1.11	4.25	4.39	3.37	6.06	2.16	0.77	6.15	2.83	1.24	MS, KI
13	1100	Nonanone	-	0.37	0.16	1.44	0.02	1.27	0.45	0.97	1.30	0.30	0.06	2.16	1.13	MS, KI
14	1215	Decanal	-	0.17	1.35	1.18	0.76	3.60	12.71	3.05	1.20	3.83	1.14	-	1.99	MS, KI

Values expressed as relative area percentages to total identified components

-: not detected

Compounds listed according to their elution on DB5 column

a: Kovats index

b: Compounds identified by GC-MS(MS) and/or by comparison of MS and KI of standard compound run under similar conditions

Key of samples

1. Milk + *Lactobacillus bulgaricus*

2. Milk + *Lactobacillus plantarum*

3. Milk + *Lactobacillus casei*

4. Milk + *Leuconostoc mesentroides*

5. Milk + *Enterococcus faecium*

6. Milk + *Lactococcus diacetylactis*

2,3-Butanedione and 2,3-pentanedione have a buttery aroma (Wu & Cadwallader 2002).

On contrary as mentioned before addition of 0.25% phenylalanine causes increase in percentage of acetaldehyde, ethylacetate, ethyl-2-methylpropanoate in *Lactobacillus plantarum* only since comprised (27.73%; 7.3% and 10.47%) compared to 25.79%; 6.49% and 1.38% in control milk sample Table (4) this increase is in this only in sample accompanied by drastic decrease in the major compound ethylbutanoate, (11.66%) compared to 24.35% in control milk sample. These results are in accordance with (Rotsachakal *et al.*, 2009) who found that fermented samples with LAB incubated at 30°C for 3 days, resulted in a decrease in aldehydes and sharp increase in esters and alcohols on the 1st day. The most prominent aroma compounds identified by their odour-active values were ethyl butanoate (buttery, ripe fruit note), ethyl-2-methyl propanoate (fruity), 2,3-butanedione (yoghurt-like), octanal (fatty-fruity), ethyl butanoate, ethyl-2-methyl propanoate and octanal derived from microbial activity in fermented samples.

Volatile Components and Sensory Evaluation Identified in Headspace of Fermented Milk with LAB and Different Amino Acids (Phenylalanine, Methionine and Leucine):

Fifteen volatile compounds were isolated in headspace of fermented milk with (*Lactococcus diacetylactis*; *leuconostoc mesentroides* and *lactobacillus plantarum*) with 0.5% (phenylalanine, methionine and leucine) for each are cited with their area percentages in Table (5).

The typical gas chromatograms of the above mentioned volatile samples are shown in Figures (8- 12). the identified compounds different not only qualitatively but also quantitatively due to the addition of these amino acids. The compounds identified including 6 aldehydes, 3 alcohols, 2 ketones and, 2 esters and 2 phenyl compounds. As shown from Table (5) the strecker aldehydes 2-methyl butanal; 3-methyl butanal; phenylacetaldehyde and the two dicarbonyls 2,3-butanedione and 2,3-pentanedione were represented with remarkable concentration in Table (5). The formation of these compounds is mainly depending on the precursor amino acids (Yvon & Rijnen 2001) who reported that branched-chain amino acids (Leu) aromatic amino-acid Phe. and Meth. being the main precursors of key aroma compound.

Also from Table (5) 1-propanol represent with high concentration in all samples and considered the second major compound. This alcohol derived from Met. metabolism (Collin, *et al.*, 2003). Acetaldehyde also represented in Table (5) with remarkable concentration in all samples. Acetaldehyde is one of the most common aldehydes found in dairy products. Branched aldehydes as 3-methyl butanal which derived from leu quickly converted to corresponding alcohol by lactobacilli present in the adjunct culture two esters were identified in Table (5) ethyl butanoate and ethylhexanoate, the major one was ethylbutanoate which comprised very high concentration in all sample ranged between 35.26% - 86.71% (Table 5).

The importance contribution of esters to cheese aroma is not in doubt, since short-chain esters have a perception threshold to times lower than the alcohols from which they are derived. (Martinez-castro, *et al.*, 1991).

Table 5: Volatile Components Identified in headspace volatiles of fermented milk with lactic acid bacteria (LAB) and different Amino Acids.

Peak No	KI ^a	Components	Fermented milk+ <i>Lactococcus diacetylactis</i> +0.5% A.A.			Fermented milk+ <i>Leuconostoc mesentroides</i> +0.5% A.A.			Fermented milk+ <i>Lactobacillus plantarum</i> +0.5% A.A.			Methods of Identification
			0.5% Ph.Al.	0.5% Meth.	0.5% Leu.	0.5% Ph.Al.	0.5% Meth.	0.5% Leu.	0.5/ Ph.Al.	0.5% Meth.	0.5% Leu.	
1	581	Acetaldehyde	5.41	4.82	8.09	15.70	13.16	3.70	8.26	4.96	10.35	KI, MS
2	600	1-Propanol	9.53	15.49	14.96	21.79	31.93	12.16	10.91	8.97	13.89	KI, MS
3	616	2,3-butanedione	3.17	1.92	3.26	6.56	4.74	1.57	5.46	3.10	5.74	KI, MS
4	630	2-Methyl buanal	2.22	1.54	0.30	6.36	-	6.73	4.15	5.35	1.94	KI, MS
5	638	3-Methyl buanal	5.48	-	1.31	-	-	0.60	-	-	-	KI, MS
6	697	1-Butanal	0.88	-	-	0.31	-	1.10	0.45	0.33	0.22	KI, MS
7	729	2,3-pentanedione	0.79	0.44	0.09	0.78	-	3.21	3.14	4.68	5.30	KI, MS
8	802	Hexanal	0.16	0.20	0.17	0.46	-	1.12	0.31	0.24	0.26	KI, MS
9	851	Ethyl butanoate	65.28	68.71	67.72	46.71	49.93	35.26	60.60	58.02	56.77	KI, MS
10	866	2-hexenal	-	-	0.89	-	-	2.96	-	-	0.55	KI, MS
11	875	1-Hexanal	-	-	0.88	-	-	0.63	-	-	-	KI, MS
12	1003	Octanal	4.14	3.27	1.27	0.52	-	11.14	3.29	7.56	2.72	KI, MS
13	1016	Ethyl hexanoate	2.50	3.13	0.92	0.41	-	10.59	2.43	5.29	2.09	KI, MS
14	1039	phenylaceticacid	-	-	-	-	-	4.65	0.10	0.28	-	KI, MS
15	1046	phenylacetaldehyde	0.05	0.13	0.06	-	-	1.99	0.16	0.09	0.16	KI, MS

values expressed as relative area percentages to total Identified comparts

Phenylalanine "Ph.Al", Methionine "Meth" and Leucine "Leu"

-: not detected

Compounds listed according to their elution on DB5 column

a: Kovats index

b: Compounds identified by GC-MS(MS) and/or by comparison of MS and KI of standard compound run under similar conditions

Sensory Evaluation:

The organoleptic properties of the fermented milk samples with LAB and different amino acids were evaluated by 10 experienced assessors (Table 6).

Table 6: Sensory evaluation of fermented milk with phenyl alanine(ph.Al), Methionene(Meth.) and Leucine(Leu).

samples	buttery	ripe cheese	cheesy	yogurt	milk
Fermented milk+ <i>Lactococcus diacetylactis</i> +0.5% Ph.Al.	9±0.2	3±1.02	3±0.78	4±0.23	3±0.14
Fermented milk+ <i>Lactococcus diacetylactis</i> +0.5% Meth.	8±1.07	2±0.24	3±0.12	3±0.42	3±0.35
Fermented milk+ <i>Lactococcus diacetylactis</i> +0.5% Leu.	2±0.7	1±0.23	3±0.12	5±0.7	9±1.02
Fermented milk+ <i>Leuconostoc mesentroides</i> +0.5% Ph.Al.	2±0.2	2±0.3	3±0.98	9±1.03	4±1.04
Fermented milk+ <i>Leuconostoc mesentroides</i> +0.5% Meth.	2±0.07	1±0.04	2±0.14	8±0.7	2±0.23
Fermented milk+ <i>Leuconostoc mesentroides</i> +0.5% Leu.	3±0.87	4±0.25	3±0.62	8±1.03	4±0.35
Fermented milk+ <i>Lactobacillus plantarum</i> +0.5% Ph.Al.	3±0.03	8±1.02	9±1.45	4±0.45	4±0.35
Fermented milk+ <i>Lactobacillus plantarum</i> +0.5% Meth.	9±1.32	2±0.2	3±0.01	1±0.01	2±0.12
Fermented milk+ <i>Lactobacillus plantarum</i> +0.5% Leu.	2±0.03	2±0.04	2±0.04	2±0.07	8±1.02

In conclusion, from the aforementioned results, *Leuconostoc mesenteroids*, *Lactobacillus plantarum* and *Lactococcus diacetylactis* were chosen for further studies to produce flavour on pilot plant scale.

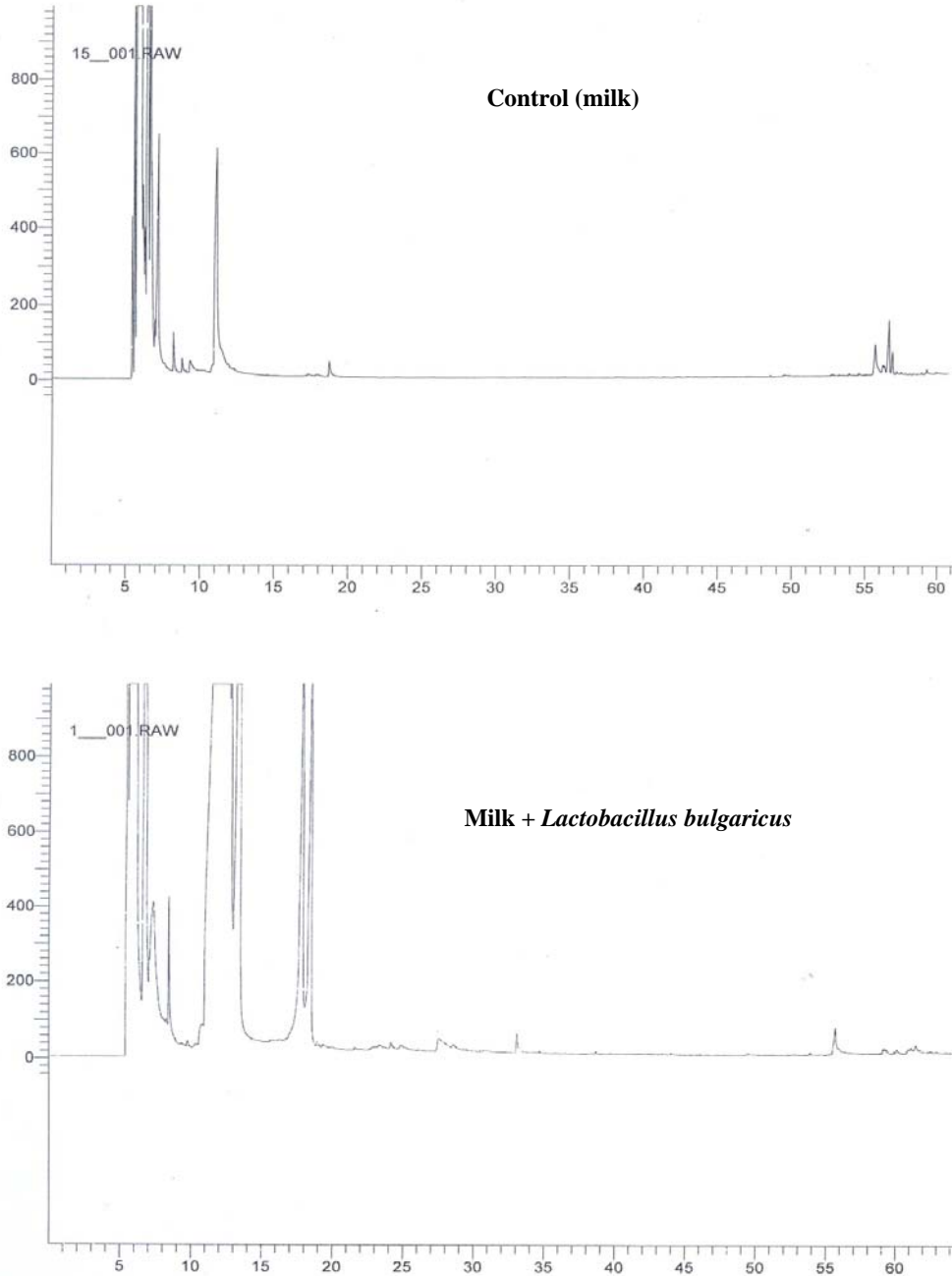


Fig. 1: Gas chromatograms of the volatile components isolated in headspace of control milk and fermented milk + *Lactobacillus bulgaricus*.

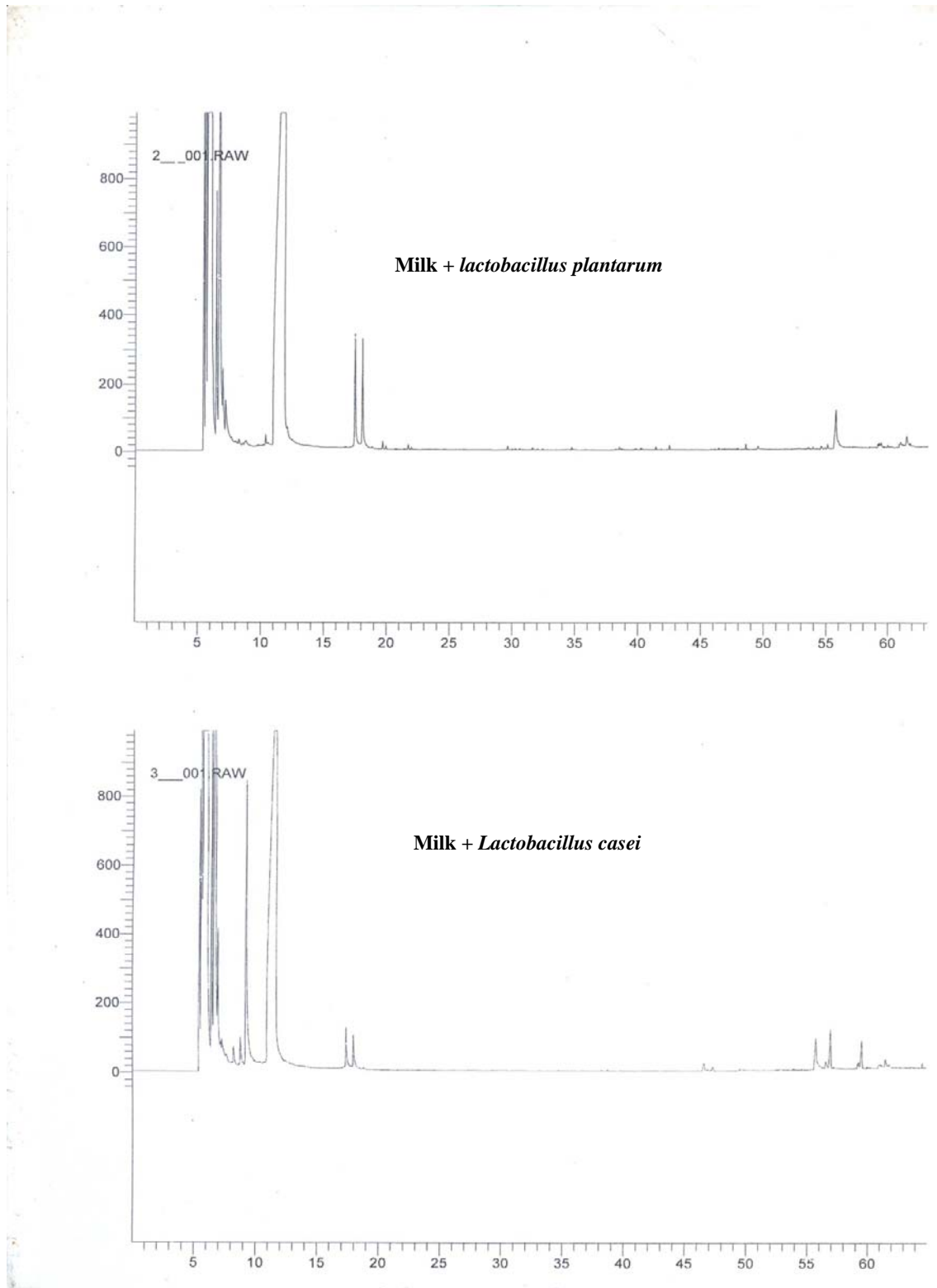


Fig. 2: Gas chromatograms of the volatile components isolated in headspace of fermented milk + *Lactobacillus plantarum*, milk + *Lactobacillus casei*

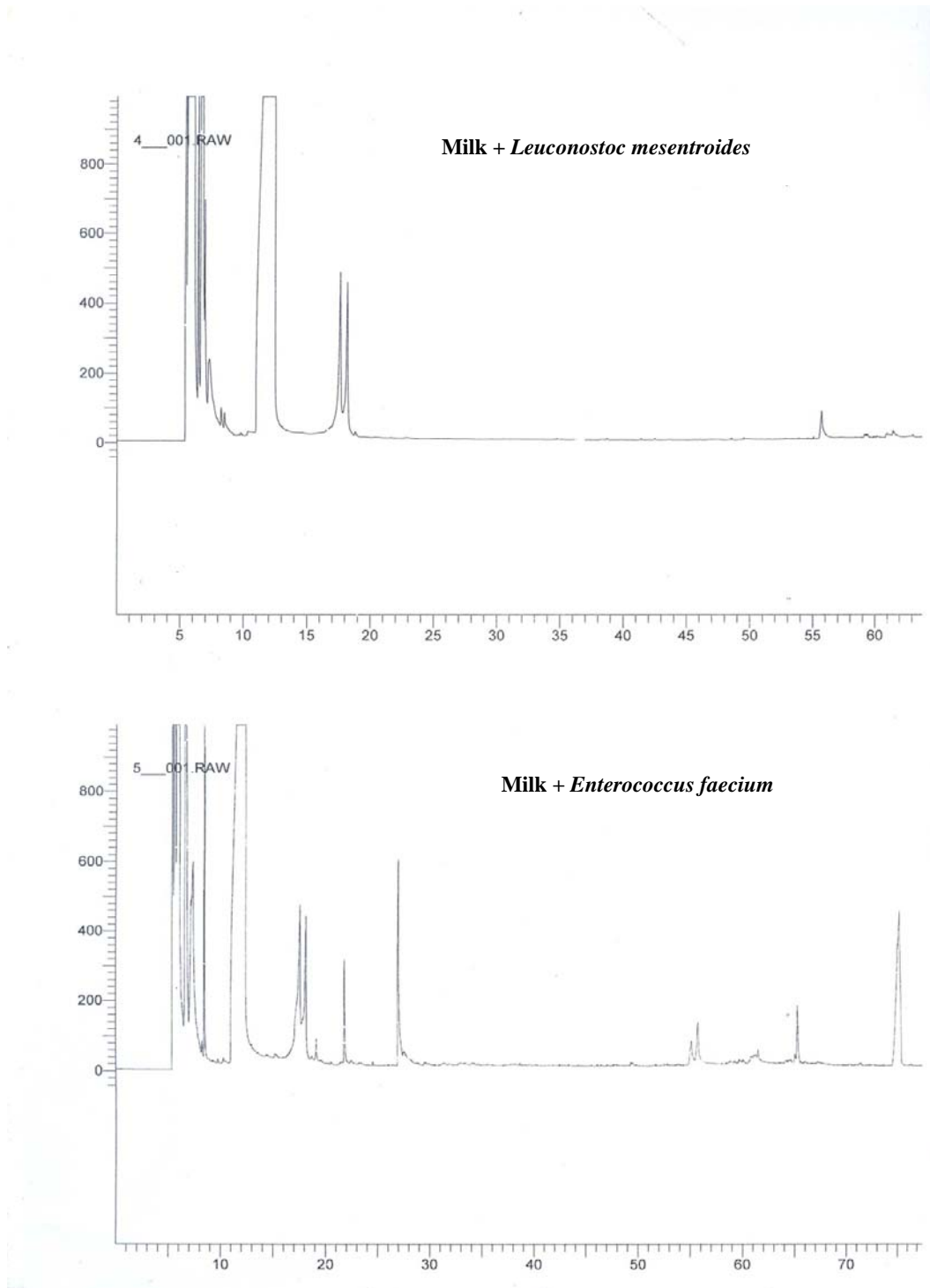


Fig. 3: Gas chromatograms of the volatile components isolated in the fermented milk + *Lactobacillus plantarum* and milk + *Lactobacillus casei*.

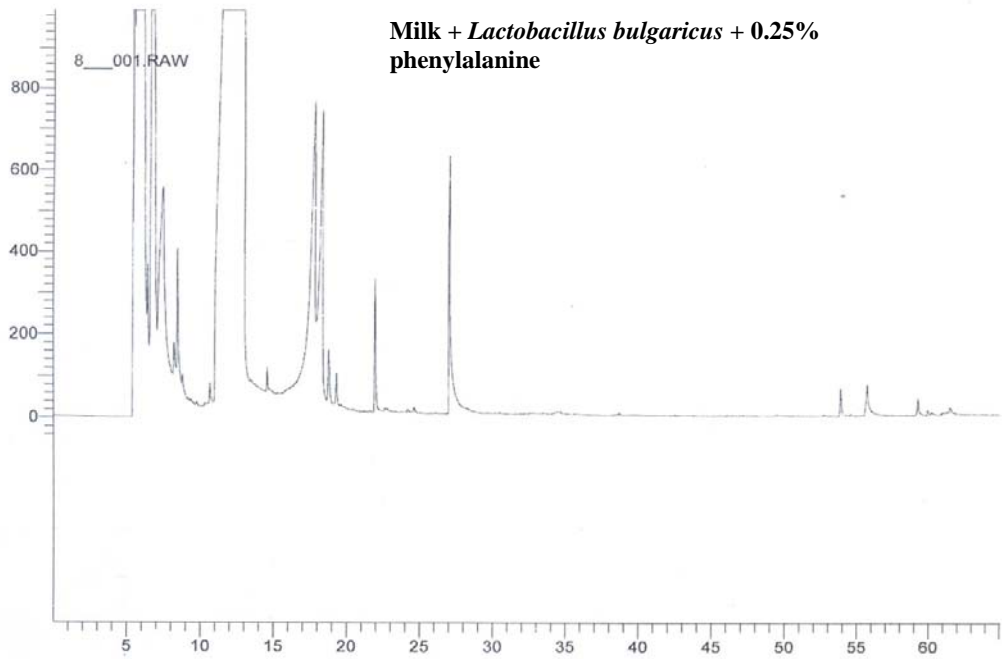
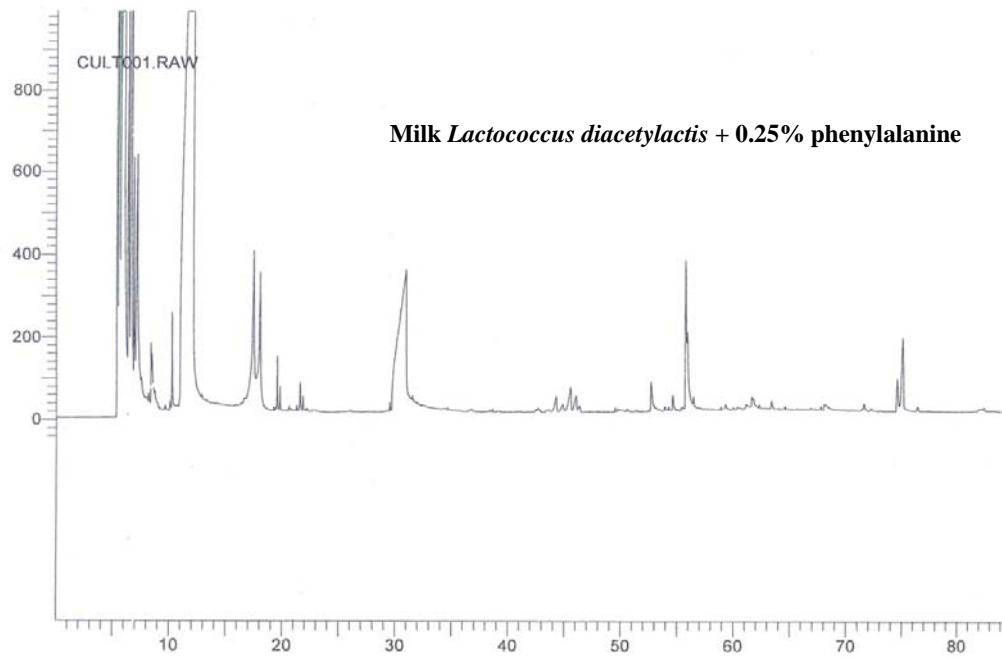


Fig. 4: Gas chromatograms of the volatile components isolated in headspace of fermented milk with *Lactococcus diacetylactis* and fermented milk with *Lactobacillus bulgaricus* + 0.25% phenylalanine.

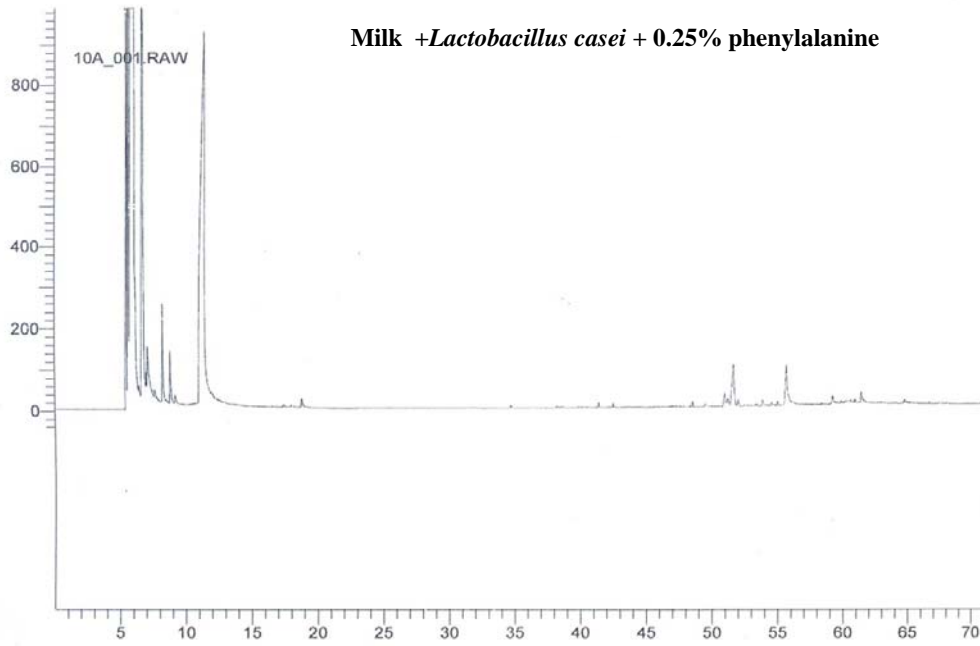
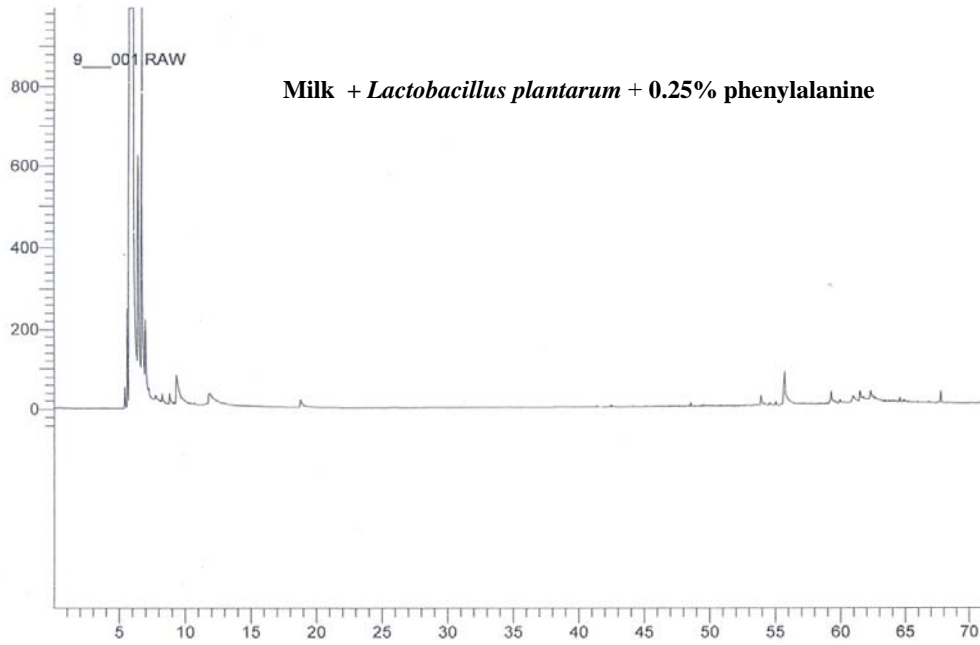


Fig. 5: Gas chromatograms of the volatile components isolated in headspace of fermented milk with *Lactobacillus plantarum* and *Lactobacillus casei* + 0.25% phenylalanine for each.

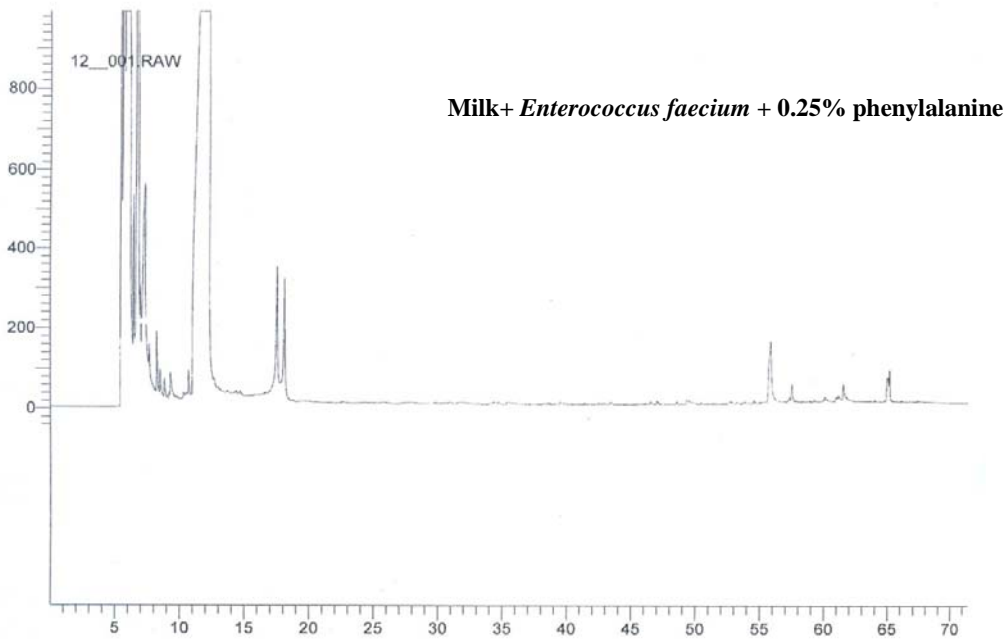
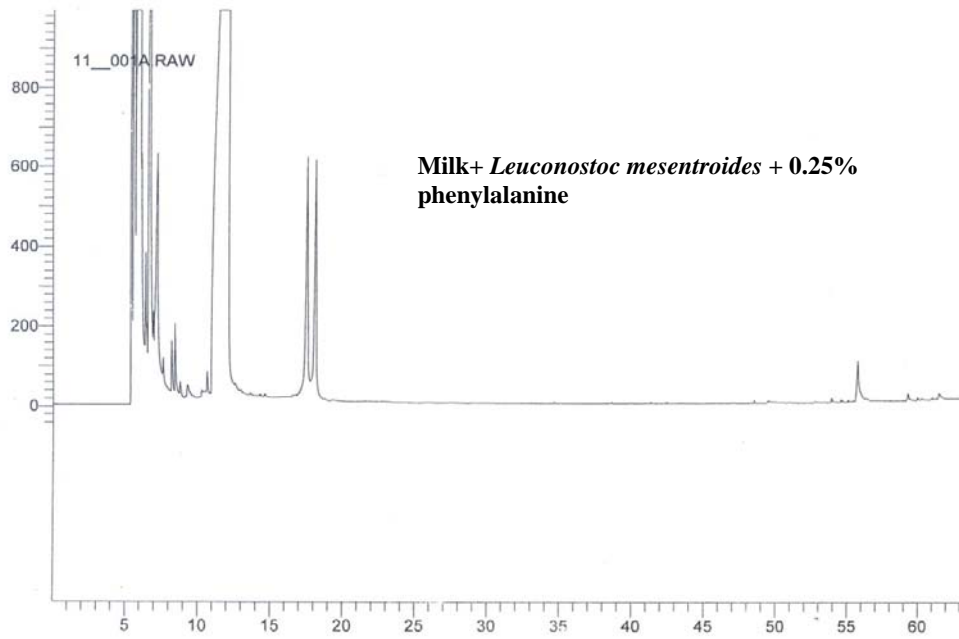


Fig. 6: Gas chromatograms of the volatile components isolated in headspace of fermented milk with *Leuconostoc mesentroides* and *Enterococcus faecium* +0.25% phenylalanine fro each.

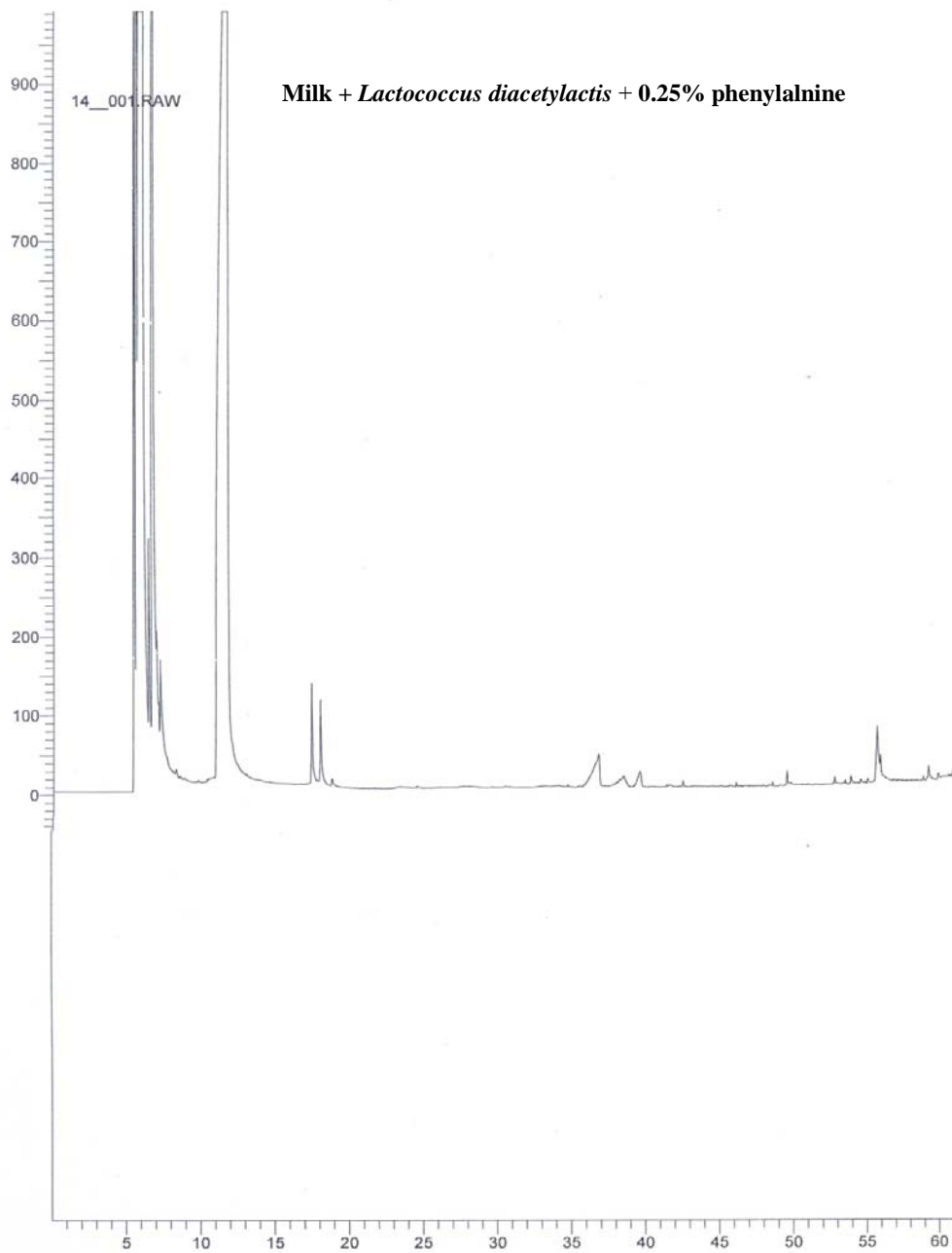


Fig. 7: Gas chromatograms of the volatile components isolated in headspace of fermented milk with *Lactococcus diacetylactis* and 0.25% phenylalanine.

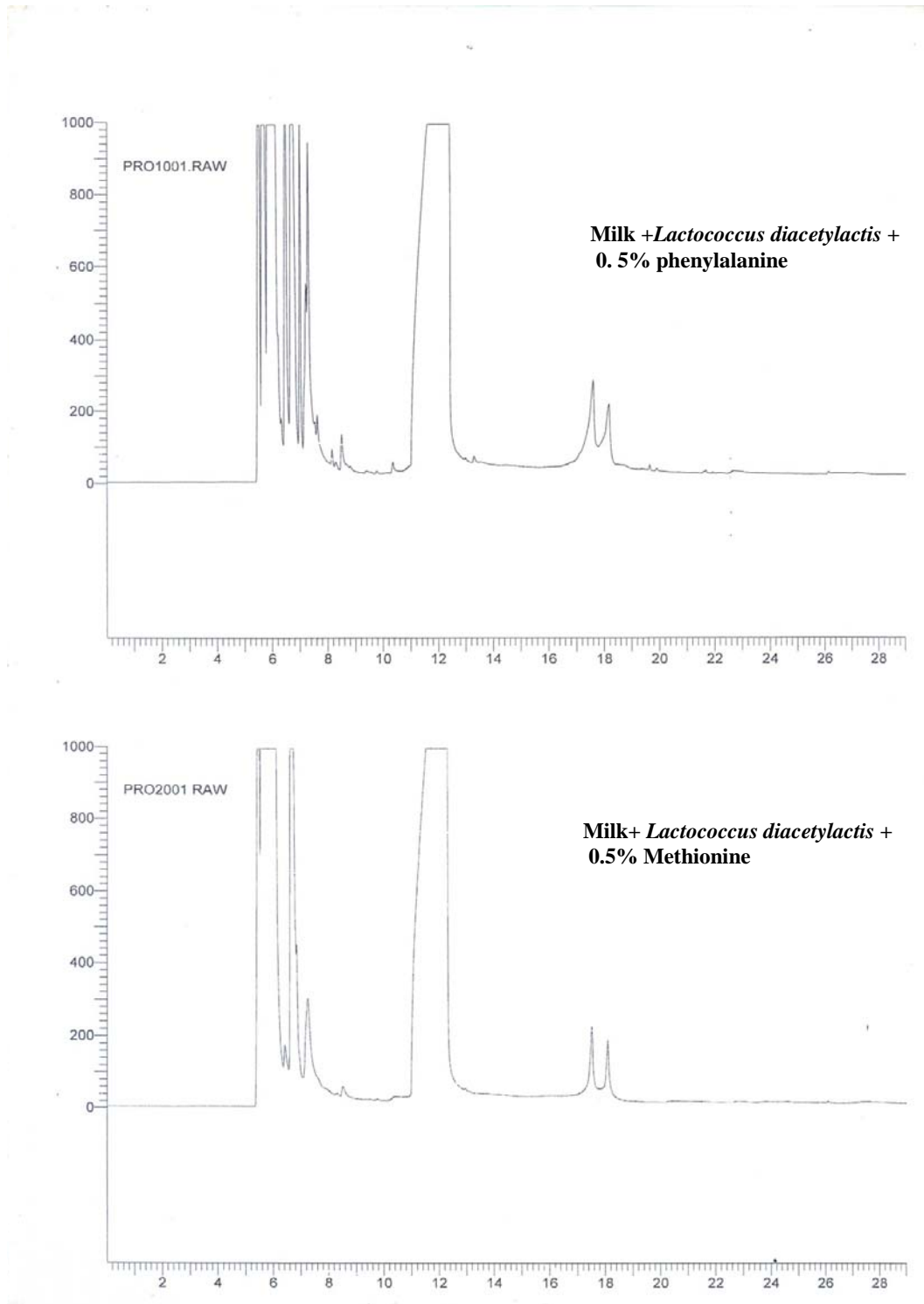


Fig. 8: Gas chromatograms of the volatiles isolated in headspace of fermented milk with *Lactococcus diacetylactis* and 0.5% phenylalanine in sample (1) and 0.5% Methionine in sample (2).

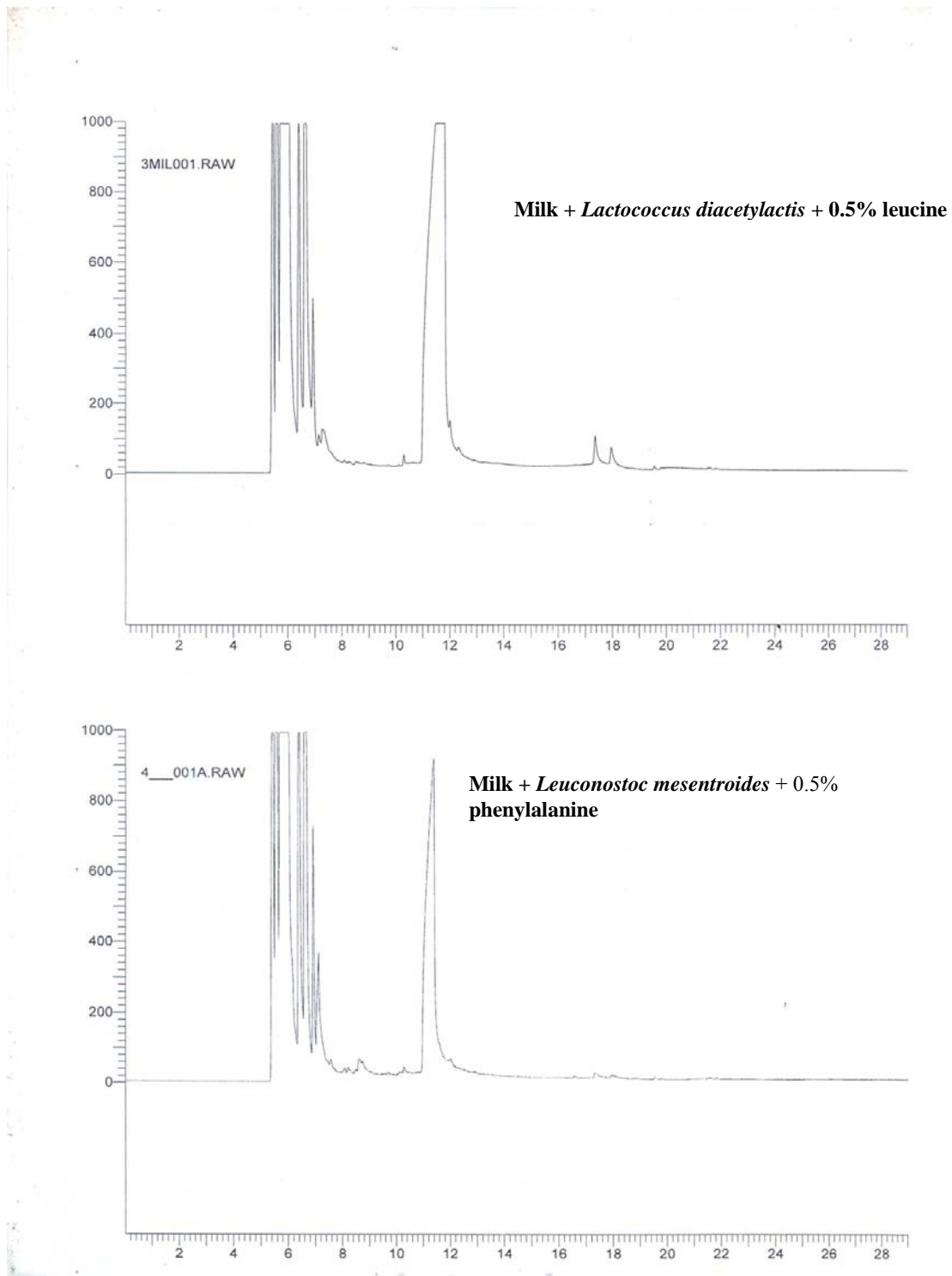


Fig. 9: Gas chromatograms of the volatiles isolated in headspace of fermented milk with *Lactococcus diacetylactis* and 0.5% leucine in sample (3) While fermented milk with *Leuconostoc mesenteroides*+0.5% phenylalanine in sample (4).

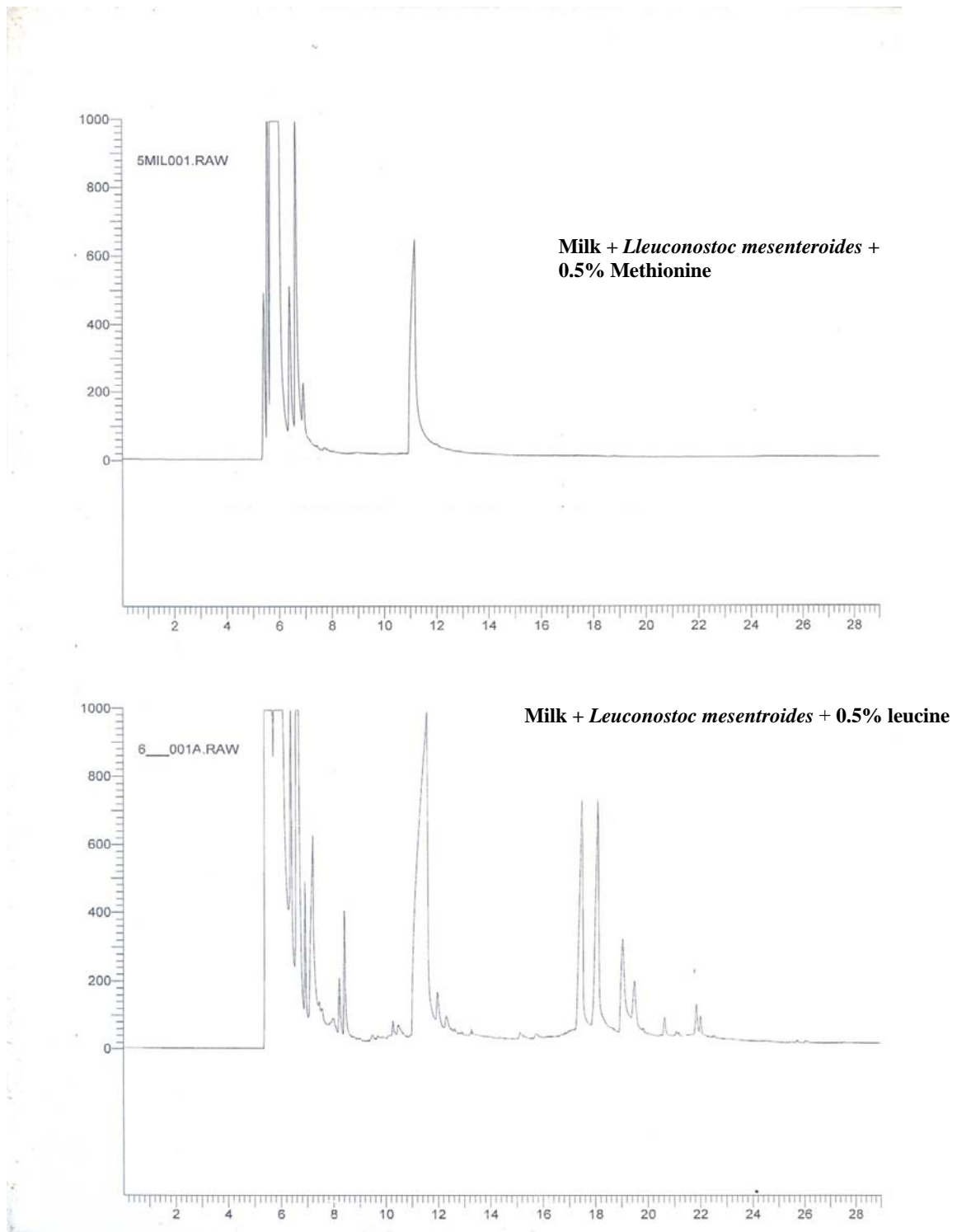


Fig. 10: Gas chromatograms of the volatiles isolated in headspace of fermented milk with *Leuconostoc mesenteroides* and 0.5% Methionine in sample (5) while 0.5% leucine in sample (6).

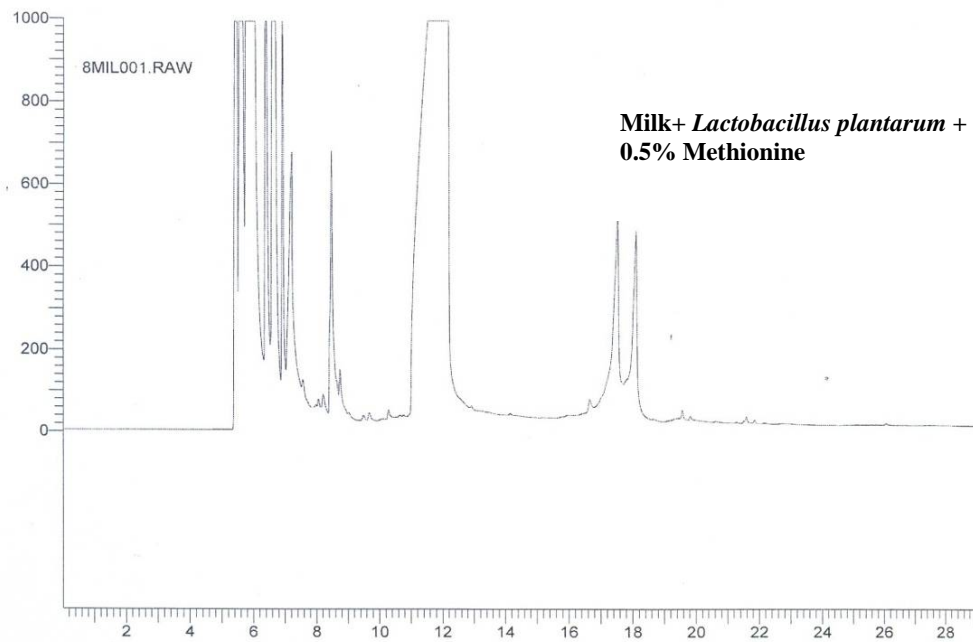
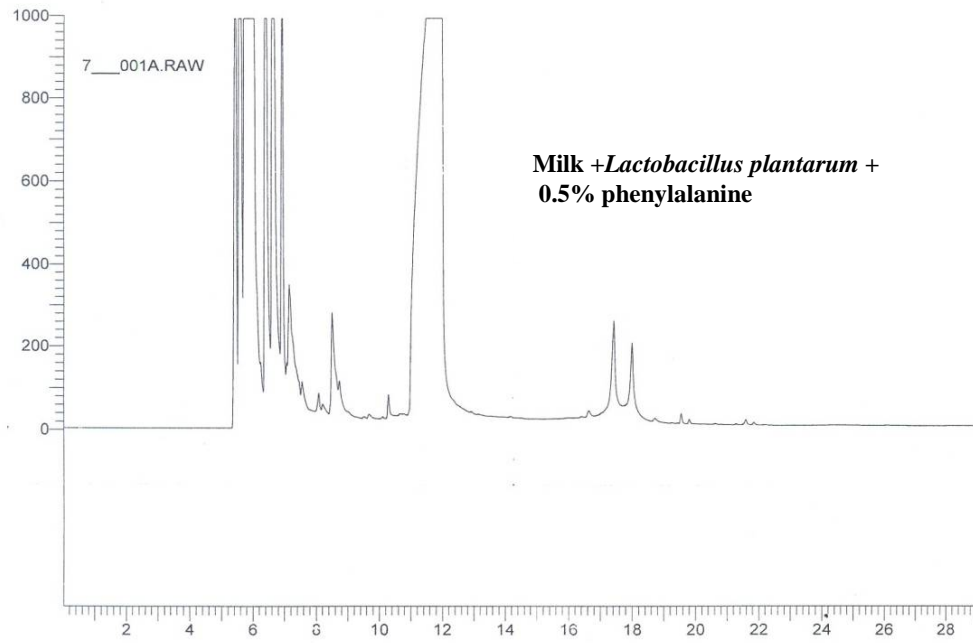


Fig. 11: Gas chromatograms of the volatiles isolated in headspace of fermented milk with *Lactobacillus plantarum* and 0.5% phenylalanine in sample (7) while 0.5% Methionine in sample (8).

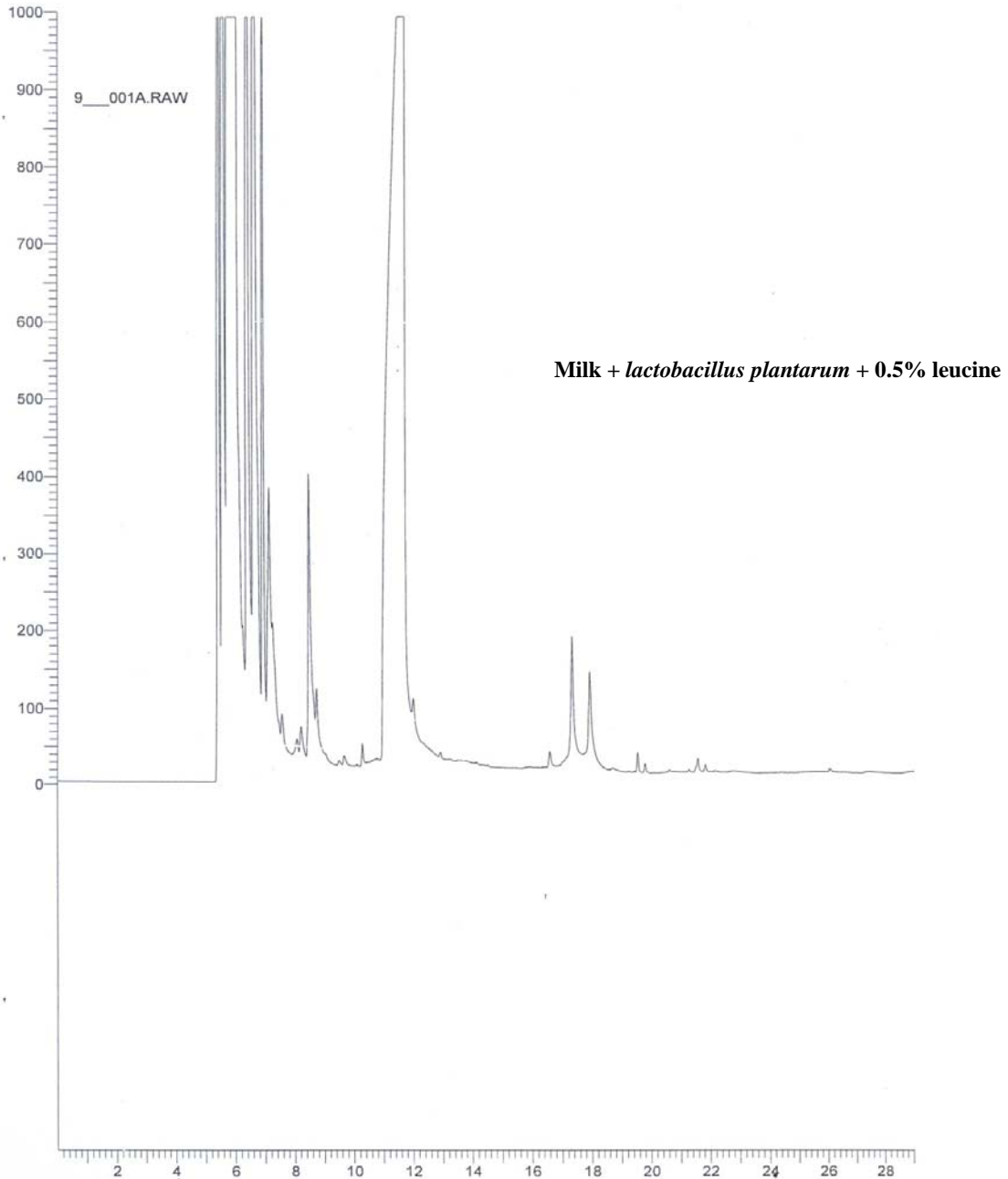


Fig. 12: Gas chromatogram of the volatiles isolated in headspace of fermented milk with *lactobacillus plantarum* and 0.5% leucine.

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