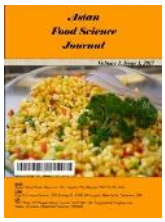

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Production of Volatile Compounds by *Lactococcus lactis* sp. Strain at Different Growth Phases

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Authors' contributions

This work was carried out in collaboration between all authors. Authors DBDS, ZNF, AR, and FAF designed the study, performed the statistical analysis, wrote the protocol and first draft of the manuscript. Authors ZNF and AR managed the analyses of the study. Author FAF managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The present study aimed at identifying secondary metabolites produced during the growth of *Lactococcus lactis* sp. strain
Study Design: The bacteria strain was grown at different time intervals.
Place and Duration of Study: The work was done at the Microbiology and Fermentation Technology Department, Central Food Technological Research Institute (CFTRI), India, between May 2014 and May 2015.
Methodology: The strain was grown in M17 broth for 56 h to determine the growth curve. Produced volatile compounds were studied by Gas Chromatography and Mass Spectrophotometer (GC-MS) at different phases of culture growth.
Results: Results showed that lag phase was extended for about six h. Exponential phase (log phase) lasted for the next 6 to 18 h, stationary phase 18 to 40 h, and decline phase started after 40 to 48 h. GC-MS analysis of the volatile compounds resulted in the identification of 18, 20, 18, and

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17 compounds respectively during the lag phase, the logarithmic phase, the stationary phase and the decline phase. Out of the total of 50 volatiles compounds identified, 4 compounds *viz*., Benzyl alcohol; Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methyl propyl)-; 5-Isopropylidene-3,3-dimethyl-dihydrofuran-2-one, and Cyclohexane, 1,3,5-trimethyl-2-octadecyl- were present at different peaks area in all growth phases of the cultured strain. Numerous of these volatile compounds produced revealed to possess biological activities.

Conclusion: The incubation period affected the production of volatile compounds. *L. lactis* was observed to produce wide-ranging bioactive compounds for human therapeutic applications as well as various industrial applications. Also, many secondary metabolites were produced by the strain at the logarithmic phase.

Keywords: Lactococcus lactis sp.; growth phases; gas chromatography and mass spectrophotometer (GC-MS); volatile compounds; biological activities.

1. INTRODUCTION

Several small molecules available worldwide on drug market can be traced back to or were inspired by natural products [1]. Microorganisms from environments have gained considerable attention during recent years. Most of the natural products currently used in the market as therapeutic agents or as health supplements are derived from terrestrial organisms such as plants, animals and microorganisms. Microbial secondary metabolites are the most important sources of natural compounds with bioactivity potentials [2]. Working on probiotic bacteria may lead to the discovery of bioactive compounds with a very large spectrum of action. Probiotic microorganisms produce a great variety of secondary metabolites as a result of metabolic activities. They are used throughout the world in foods manufacturing for their technological properties and for the beneficial effects they provide to consumers. Some probiotics used have been shown to confer many health benefits to humans. These beneficial effects could be due to the secondary metabolites produced by the probiotic bacteria. Few studies have been carried out in this domain on some microorganisms. Vanaja et al. [3], Sohini and Agrawal [4] investigated in the production of volatile compounds with an adapted culture of *Lactobacillus plantarum* sp., Narasaiah [2] on *Streptomyces albus* CN-4, Priya and Anandaraj [5] on *Streptomyces tuirus.*

Generally, the strains from the *L. lactis* species have many interesting technological properties such as lactic acid, exopolysaccharide and flavor production [6,7]. Many strains from this specie have been used for centuries in cheesemaking, as they acidify the milk and significantly contribute to the flavor of dairy products [8]. In the quest for new therapeutic compounds, produced by lactic acid bacteria, *L. lactis* sp. was chosen to identify the volatile compounds produced at different growth phases. This work aimed at identifying secondary metabolites produced by *L. lactis* sp. strain isolated from a fermented maize beverage "Sha'a" of Cameroon.

2. MATERIALS AND METHODS

2.1 Starter

The starter used was the stock culture of *L. lactis* obtained from the Laboratory of Biochemistry, Medicinal Plant, Nutrition and Food science (LABPMAN) of the University of Dschang-Cameroon, isolated from a fermented maize beverage "Sha'a" of Cameroon. The culture mediums used for bacteria growth and cells count were M17 broth and agar respectively.

2.2 Growth Curve of *Lactococcus lactis* sp.

L. lactis culture (2.5 ml) was inoculated into a conical flask (500 ml) containing 250 ml sterilized M17 broth. Inoculated media (1%) containing 1×10^6 CFU/ml was incubated in an INCUCELL V incubator at optimum temperature (37°C), for growth and samples were removed and shacked with New Brunswick™ Innova® 2000 (150 tr/min) at different time intervals (0^{th}, 2th, 4^{th}, 6^{th}, 8^{th},10^{th}, 12^{th}, 14^{th}, 16^{th}, 18^{th}, 20^{th}, 24^{th}, 28^{th}, 32th, 36^{th}, 40^{th}, 44^{th}, 48^{th}, 56^{th} h). The number of viable bacteria was counted on an M17 agar plate.

2.3 Volatile Compound Analysis

2.3.1 Extraction of volatile compounds

5 ml of M17 broth culture were taken at different growth phase, mixed with 5 ml dichloromethane

(DCM), kept in a freezer for 24 h, and then macerated in a mortar. The homogenized culture in DCM solvent was transferred to a separating funnel. The bottom dichloromethane layer was taken in the dry tubes. The solution was dried by adding a pinch of anhydrous sodium sulphate ($Na_2SO_4.10H_2O$). The dried solution was transferred in a graduated test tube. It was sealed with cellophane tape. The cells were stored in a freezer (-20°C). The sample was concentrated to 1 ml using Nitrogen concentrator, and 1 µl was injected into a Gas Chromatograph (GC 8,000 Series, Fisons Instruments).

2.3.2 Analysis of volatile compounds

The concentrated extract was analyzed for volatile compounds using a gas chromatography-mass spectrophotometer (GC-MS). The gas chromatograph (Perkin Elmer Auto system XL) was interfaced to a mass spectrometer (Perkin Elmer Turbo Mass Gold). The separation of volatiles was carried out in ELITE 1 non-polar capillary column (30 m X 0.25 mm (ID); 0.25 µm film thickness). One microliter of the extract was injected (split ratio 1:10) and the carrier gas was helium at a flow rate of 1 ml min^{-1}. The oven temperature was held at 100°C for 6 min, heated at 4°C min^{-1} to 150°C, then at 8°C min^{-1} to 220°C and held at 220°C until an approximate run time of 40 min. The mass spectrophotometer was operated in the electron impact mode, and mass spectra were taken using an ionization voltage of 70 eV. The mass scan range was 40-400 AMU, with a scanning speed of 2.0 sec. Data acquisition and generation of chromatograms and spectra were done with Turbo Mass software [3].

2.3.3 Identification and quantification of volatile compounds

The identification of volatile compounds was performed by comparing the mass spectra with standard mass spectra database from the NIST Ver. 2.1 2009 Mass Spectra Library, Gaithersburg, Maryland, USA. The relative percentage of each volatile compound was calculated by comparing the peak area with the total area as follows:

Compound peak area= (Relative percentage of each volatile compound/ Total compounds peak area) × 100

3. RESULTS AND DISCUSSION

3.1 Growth Curve of *Lactococcus lactis* sp.

Growth curve of *L. lactis* is established in Fig. 1. It was observed that from 0 to 6 h the variation of the bacteria concentration (log 10 CFU ml^{-1}) varied a bit. From 6 to 18 h, there was an increase in bacteria concentration in the medium, and from 18 to 40 h during this extended period, the concentration did not change significantly, the curve remained constant, and from 40, and above, the bacteria concentration drops considerably. According to the growth curve, lag phase extended about six h. At this time, the bacteria had to adapt to the medium. The exponential phase (log phase) lasted for the next 6 to 18 h. After that, the growth of bacteria turned to stationary phase (18-40 h). Decline phase started after 40 to 48 h.

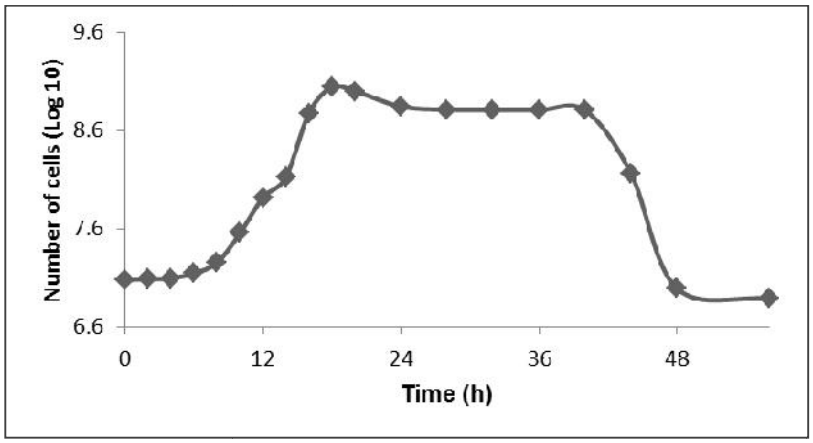

Fig. 1. Growth curve of *L. lactis* sp.

3.2 Volatile Compounds Production at Different Growth Phases of *Lactococcus lactis* sp.

GC-MS chromatograms of the volatile compounds produced at different growth phases of the bacteria are shown in Fig. 2, 3, 4, and 5. Compounds identified with their retention time, molecular formula, molecular weight and peak area are presented in Table 1, 2, 3, and 4.

The GC-MS chromatogram of the peaks of the compounds detected with varied retention times (5.61 - 38.96) and varied peak area (0.30 - 19.29) at the lag phase is shown in Fig. 2. Chromatogram GC-MS analysis showed the presence of eighteen compounds (Table 1) with four predominant peaks. Benzyl alcohol (RT: 5.86) was the first major peak that was detected, the second 1,2-Cyclopentanedione, 3,3,5,5-tetramethyl- (RT: 31.64), followed by l-Proline, N-alloxycarbonyl-, tridecyl ester (RT: 33.10), Pyrrolo[1,2-a]pyrazine-1,4-dione, and hexahydro-3-(2-methylpropyl)- (RT: 33.35)

Twenty different compounds with varied retention times (5.09 - 38.32) and varied peak area (0.29 - 17.53) at the logarithmic phase were identified (Fig. 3 and Table 2). Seven predominant peaks corresponding to Benzyl alcohol (RT: 5.84), 1,2-Cyclopentanedione, 3,3,5,5-tetramethyl- (RT: 31.66), Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methyl propyl)- (RT: 33.37), Nonanoic acid (RT: 33.60), 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester (37.20), Butanoic acid, 2-propenyl ester (37.53), and 2-Propenamide (37.69) were determined.

Eighteen volatile compounds with varied retention times (5.08 - 38.25), and varied peak area (0.28 - 21.27) at the stationary phase were detected (Fig. 4 and Table 3). Four predominant peaks corresponding to Benzyl alcohol (RT: 5.96), 1,2-Cyclopentanedione, 3,3,5,5-tetramethyl- (RT: 31.63), Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methyl propyl)- (RT: 33.34), Nonanoic acid (RT: 33.60), and n-Hexadecanoic acid were identified.

Seventeen compounds with varied retention times (5.05 - 39.93), and varied peak are (0.19 - 23.44) at decline phase were determined (Fig. 5 and Table 4). Four predominant peaks corresponding to Benzyl alcohol (RT: 5.86), Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)- (RT: 31.63), Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- (RT: 33.34), and 5-Isopropylidene-3,3-dimethyl-dihydrofuran-2-one (RT: 33.48) were identified.

These tables revealed that a total of 50 different volatile compounds were detected namely 1-Hexanol, 2-ethyl-; Benzyl alcohol; Benzoic acid, cyclohexyl ester; 1,2-Cyclopentanedione, 3,3,5,5-tetramethyl-; Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-; l-Proline, N-alloxycarbonyl-, tridecyl ester; Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-; 5-Isopropylidene-3,3-dimethyl-dihydrofuran-2-one, and Cyclohexane, 1,3,5-trimethyl-2-octadecyl- were present at different peak area in all growth phases of the cultured strain.

The major volatile compound detected at the lag phase was Benzyl alcohol (19.29%) known to possess antioxidant activity; Butanoic acid, 2-propenyl ester used as a flavoring agent at the logarithmic phase and Benzyl alcohol at the stationary and decline phases (21.27 and 23.43% respectively). The next compound was Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methyl propyl)- with peak area (8.93; 7.71; 9.33, and 9.64% respectively for lag, log, stationary and decline phases) known to possess antioxidant activity and antimicrobial activity. These three main compounds could be used in the food industry for food preservation or can be beneficial for human health.

Nonetheless other authors have found some of the volatile compounds identified in this study with other strains. Pyrrolo[1,2-a]pyrazine-1, 4-dione, hexahydro -3-(2-methylpropyl), and Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl) have been identified as second metabolite compounds produced by the strain *Streptomyces albus* CN-4 by Narasaiah. [2]. Sanjenbam and Kannabiran [9] identified the pyrrolo[1,2-a] pyrazine-1,4- dione,hexahydro-3-(phenylmethyl)- (PPDHP) extracted from *Streptomyces* sp. VITPK9; Krishnan et al. [10] isolated the compound, 2- benzene dicarboxylic acid, mono 2- ethylhexyl ester (DMEHE) from marine-derived actinomycete *Streptomyces* sp.

Based on Table 1, 2, 3, and 4, it can be concluded that *L. lactis* produced more volatile compounds with biological activity at the logarithmic phase.

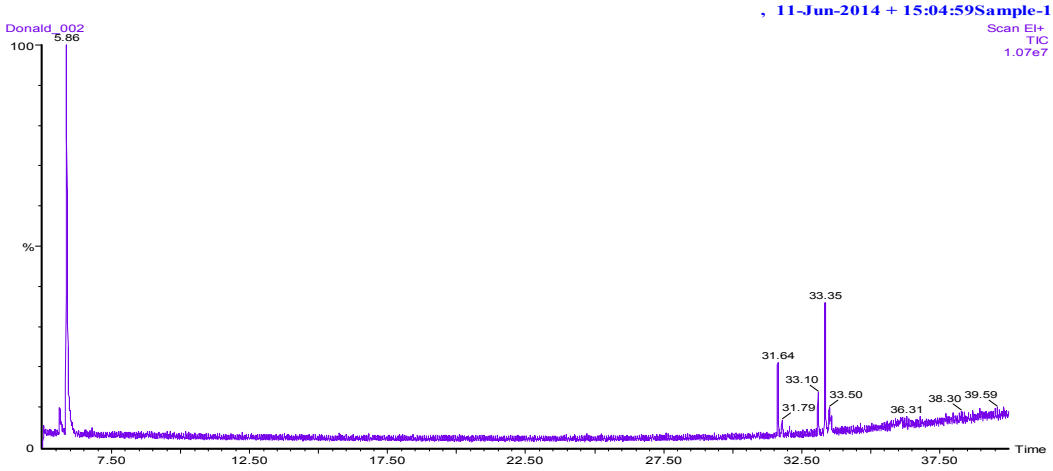

Fig. 2. Gas chromatogram of volatile compounds production at the lag phase

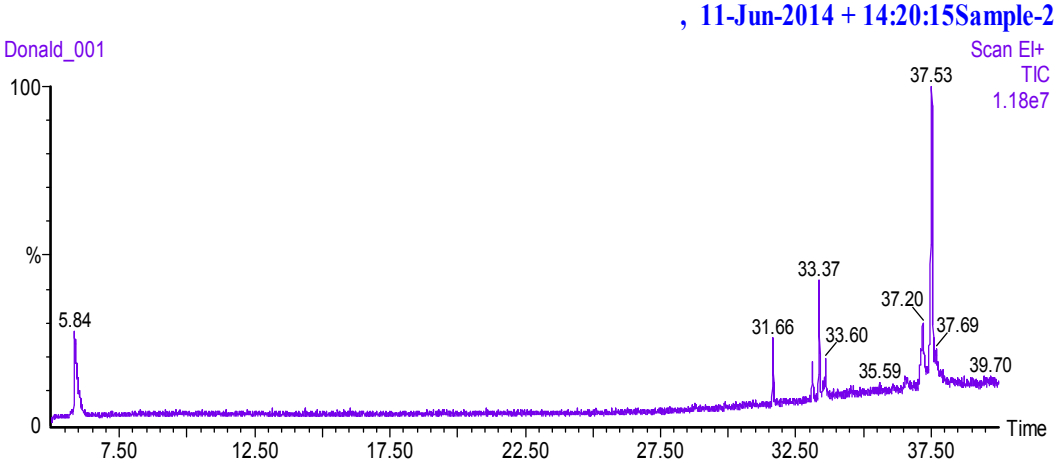

Fig. 3. Gas chromatogram of volatile compounds production at the logarithmic phase

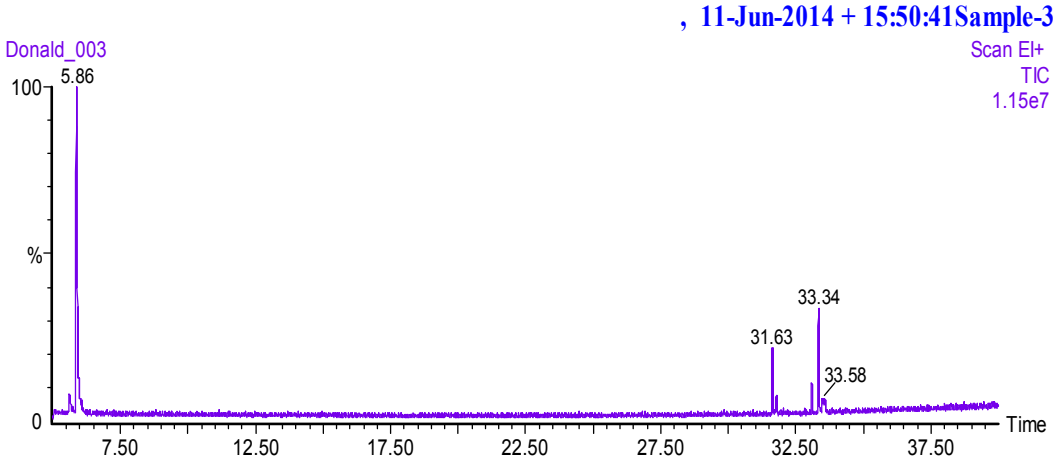

Fig. 4. Gas chromatogram of volatile compounds production at the stationary phase

Table 1. Volatile compounds production at the lag phase

N°	RT (min)	Name of the compounds	Molecular formula	MW (g/mol)	Yield (%)	Activity
1	5.61	1-Hexanol, 2-ethyl-	$C_8H_{18}O$	130,23	1.57	Flavouring agent [11]
2	5.86	Benzyl alcohol	C_7H_8O	108.14	19.29	Antioxidant [12]
3	6.06	Benzoic acid, cyclohexyl ester	$C_{13}H_{16}O_2$	204.27	0.46	Antioxidant [13]
4	31.64	1,2-Cyclopentanedione, 3,3,5,5-tetramethyl-	$C_9H_{14}O_2$	154.21	2.65	Antimicrobial [14]
5	31.79	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	$C_{14}H_{16}N_2O_2$	244.29	0.68	Antioxidant and antifungal [9]
6	33.10	l-Proline, N-alloxycarbonyl-, tridecyl ester	$C_{22}H_{39}NO_4$	381.56	1.28	Antitumor [15]
7	33.35	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	$C_{11}H_{18}N_2O_2$	210.27	4.26	Antioxidant [16], antimicrobial [17]
8	33.50	5-Isopropylidene-3,3-dimethyl-dihydrofuran-2-one	$C_9H_{14}O_2$	154.21	0.73	Unknown
9	35.74	S-Methyl 2-methylpropanethioate	$C_5H_{10}OS$	118.19	0.31	Flavor and fragrance agent [15]
10	35.82	Cyclohexane, 1,3,5-trimethyl-2-octadecyl-	$C_{27}H_{54}$	378.72	1.27	Anticancer [18]
11	35.99	2-Thiophenecarboxylic acid, 5-(-1,1-dimethylethoxy)-	$C_9H_{12}O_3S$	200.25	0.36	Antimicrobial [15]
12	36.07	Pentanoic acid, 2-hydroxy-, methyl ester	$C_6H_{12}O_3$	132.16	0.43	Antioxidant [15]
13	36.10	(2S,3S)-(-)-3-Propyloxiranemethanol	$C_6H_{12}O_2$	116.16	0.67	Antimicrobial [15]
14	36.22	1,3,5-Triazine, 2-[(1-methylethyl)thio]-4,6-bis(trichloromethyl)-	$C_8H_7Cl_6N_3S$	389.94	0.46	unknown
15	37.74	Dodecane, 5,8-diethyl-	$C_{16}H_{34}$	226.45	0.37	Used for Tetany, Pulmonary edema, Muscle weakness [19]
16	37.99	3-Piperidinol	$C_5H_{11}NO$	101.15	0.30	Antimicrobial and antitubercular [20]
17	38.40	Cyclobutanol	C_4H_8O	72.11	0.34	Unknown
18	38.96	Heptadecane, 9-hexyl-	$C_{23}H_{48}$	324.64	0.79	antibacterial [21]

Data are means of triplicate values; RT: Retention time; MW: Molecular weight

Table 2. Volatile compounds production at the logarithmic phase

N°	RT (min)	Name of the compounds	Molecular formula	MW (g/mol)	Yield (%)	Activity
1	5.09	Ethylene oxide	C_2H_4O	44.05	0.41	Antioxidant [22]
2	5.17	Pyridine, 2-tridecyl	$C_{18}H_{31}N$	261.45	0.32	Unknown
3	5.24	2-Thiophenecarboxylic acid, 5-(1,1-dimethylethoxy)	$C_9H_{12}O_3S$	200.25	0.36	Flavouring agent [15]
4	5.72	2-butanone, 1-(acetyloxy)-	$C_6H_{10}O_3$	130.14	0.46	Flavouring agent [15]
5	5.84	Benzyl alcohol	C_7H_8O	108.14	9.43	Antioxidant [12]
6	31.66	1,2-Cyclopentadione, 3,3,5,5-tetramethyl-	$C_9H_{14}O_2$	154.21	2.20	Antioxidant [14]
7	33.11	L-leucine, N-cyclopropylcarbonyl-, methyl ester	$C_{11}H_{19}NO_3$	213.27	1.22	Antitumor [15]
8	33.37	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	$C_{11}H_{18}N_2O_2$	210.27	3.78	Antioxidant [16], antimicrobial [17]
9	33.52	5-Isopropylidene-3,3-dimethyl-dihudrofuran-2-one	$C_9H_{14}O_2$	154.21	0.51	unknown
10	33.60	Nonanoic acid	$C_9H_{18}O_2$	158.24	1.53	Treatment of epilepsy [23]
11	36.55	Butyric acid, 3-tridecyl ester	$C_{17}H_{34}O_2$	270.46	0.54	Flavouring agent [15]
12	36.64	Cyclohexane, 1,3,5-trimethyl-2-octadecyl-	$C_{27}H_{54}$	378.72	0.47	Anticancer [18]
13	36.72	Dimethylamine	C_2H_7N	45,08	0.29	Antimicrobial [24]
14	36.89	3-Dodecanol, 3,7,11-trimethyl-	$C_{15}H_{32}O$	228.41	0.29	Unknown
15	37.20	1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	$C_{16}H_{22}O_4$	278.34	4.91	Anticancer activity [9]
16	37.53	Butanoic acid, 2-propenyl ester	$C_7H_{12}O_2$	128.17	17.53	Flavouring agent [15]
17	37.69	2-Propenamide	$C_2{}^{13}CH_5NO$	72.07	1.38	Antiproliferative [25]
18	37.76	Cyclopropane, 1-ethyl-2-methyl-, cis-	C_6H_{12}	84.16	0.57	Unknown
19	38.24	Decanoic acid, 3-methyl-	$C_{11}H_{22}O_2$	186.29	0.32	Antimicrobial and antifungal [26]
20	38.32	Heptadecane, 9-hexyl-	$C_{23}H_{48}$	324.64	0.50	Antibacterial [21]

Data are means of triplicate values.
RT: Retention time; MW: Molecular weight

Table 3. Volatile compounds production at the stationary phase

N°	RT (min)	Name of the compounds	Molecular formula	MW (g/mol)	Yield (%)	Activity
1	5.08	1-Hexanol, 6-amino-	$C_6H_{15}NO$	117.19	0.42	antimicrobial and antifungal [27]
2	5.25	Benzoic acid 3-methyl-4-(1,3,3,3-tetrafluoro-2-methoxycarbonyl-propenylsulfanyl)-phenyl ester	$C_{19}H_{14}F_4O_4S$	414.37	0.44	Antimicrobial [15]
3	5.34	Allantoic acid	$C_4H_8N_4O_4$	176.13	0.42	Antimicrobial [15]
4	5.42	Glutaraldehyde	$C_5H_8O_2$	100,11	0.40	Antimicrobial activity [28]
5	5.50	Oxalic acid, allyl butyl ester	$C_9H_{14}O_4$	186.21	0.31	Antimicrobial [15]
6	5.61	Oxalic acid, allyl heptyl ester	$C_{12}H_{20}O_4$	228.29	2.09	Acidifier [15]
7	5.72	Ethylene oxide	C_2H_4O	44.05	0.62	Antioxidant activity [22]
8	5.96	Benzyl alcohol	C_7H_8O	108.14	21.27	antioxidant activity [12]
9	6.06	1,2-Ethanediol, diformate	$C_4H_6O_4$	118.09	0.65	Antimicrobial activity, sedatives, and anesthetics [15]
10	6.13	1,3-Cyclopentanedione, 4,4-dimethyl-	$C_7H_{10}O_2$	126.15	0.42	Unknown
11	29.85	3-Pyrrolidin-2-yl-propionic acid	$C_7H_{13}NO_2$	143.18	0.28	Antimicrobial [29] Antioxidant [30]
12	31.63	1,2-Cyclopentadione, 3,3,5,5-tetramethyl-	$C_9H_{14}O_2$	154.21	2.84	Antimicrobial [14]
13	31.78	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	$C_{14}H_{16}N_2O_2$	244.29	1.02	Antioxidant and antifungal [9]
14	33.10	l-Proline, N-alloxycarbonyl-, tridecyl ester	$C_{22}H_{39}NO_4$	381.56	1.35	Antitumor [15]
15	33.34	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	$C_{11}H_{18}N_2O_2$	210.27	5.14	Antioxidant [16], antimicrobial [17]
16	33.54	5-Isopropylidene-3,3-dimethyl-dihydrofuran-2-one	$C_9H_{14}O_2$	154.21	0.42	Unknown
17	33.58	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.43	0.50	Antioxidant, hypocholesterolemic activity [31]
18	38.25	Cyclohexane, 1,3,5-trimethyl-2-octadecyl-	$C_{27}H_{54}$	378.72	0.34	Anticancer [18]

Data are means of triplicate values.
RT: Retention time; MW: Molecular weight

Table 4. Volatile compounds production at the decline phase

N°	RT (min)	Name of the compounds	Molecular formula	MW (g/mol)	Yield (%)	Activity
1	5.05	Acetaldehyde	C_2H_4O	44.05	0.45	Antimicrobial activity [32]
2	5.21	Urea,N-ethyl-N',N'-dimethyl-N-nitroso-	$C_5H_{11}N_3O_2$	145.16	0.36	Antitumor [15]
3	5.30	2-n-propoxyamphetamine	$C_{12}H_{19}NO$	193.28	0.27	Anti-diabetic activity [33]
4	5.36	Docosanoic acid, 8,9-dihydroxy-, methyl ester	$C_{23}H_{46}O_4$	386.61	0.22	Antimicrobial [15]
5	5.61	1-Hexanol, 2-ethyl	$C_8H_{18}O$	130.23	1.64	Flavouring agent [11]
6	5.86	Benzyl alcohol	C_7H_8O	108.14	23.44	Antioxidant [12]
7	6.10	Pentacosane, 13-phenyl-	$C_{31}H_{56}$	428.78	0.37	Anticancer Drug [34]
8	8.77	Dodecane, 5,8-diethyl	$C_{16}H_{34}$	226.44	0.20	Used for Tetany, Pulmonary edema, Muscle weakness [19]
9	29.87	3-Amino-s-triazole	$C_2H_4N_4$	84.08	0.21	Used as an opposite sex attractant compound [35]
10	31.63	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	$C_{14}H_{16}N_2O_2$	244.29	2.90	Antioxidant and antifungal [9]
11	32.05	Cyclohexane, 1,3,5-trimethyl-2-octadecyl-	$C_{27}H_{54}$	378.72	0.27	Anticancer [18]
12	33.10	l-Proline, N-alloxycarbonyl-, tridecyl ester	$C_{22}H_{39}NO_4$	381.56	1.36	Antitumor [15]
13	33.34	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	$C_{11}H_{18}N_2O_2$	210.27	4.78	Antioxidant [16], antimicrobial [17]
14	33.48	5-Isopropylidene-3,3-dimethyl-dihydrofuran-2-one	$C_9H_{14}O$	154.21	11.06	Unknown
15	33.58	Oxalic acid, allyl isobutyl ester	$C_9H_{14}O$	186.21	0.26	Unknown
16	39.85	Cholest-4-ene-3,6-dione	$C_{27}H_{42}O_2$	398.63	0.19	anti-obesity, hypocholesterolemia activity [36]
17	39.93	Ethyne, fluoro-	C_2HF	44.03	0.22	Unknown

Data are means of triplicate values.
RT: Retention time; MW: Molecular weight

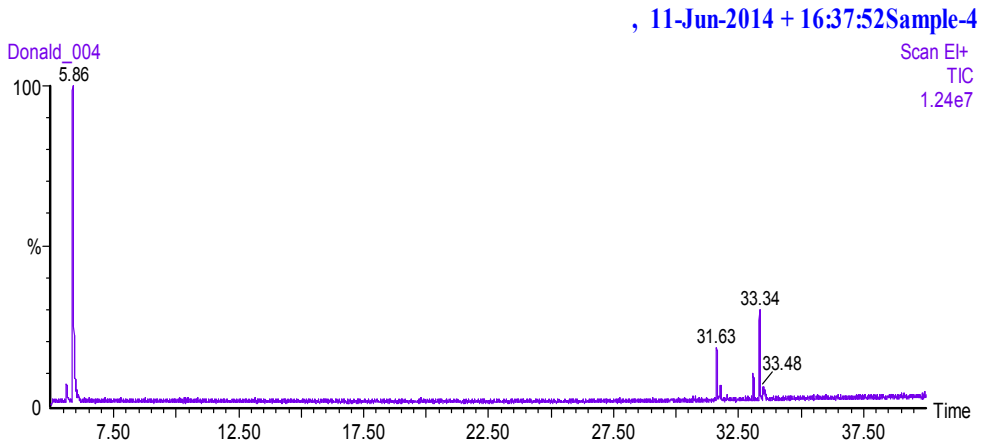

Fig. 5. Gas chromatogram of volatile compounds production at the decline phase

4. CONCLUSION

At the end of the present study, many secondary metabolites with biological activities were produced at different growth phases of *L. lactis*. The presence of these secondary metabolites and their biological activities in *L. lactis* make it a potentially valuable source of novel bioactive compounds. For some of them, their biological activities were unknown. However, the isolation of individual constituents and subjections to different biological test will give us exact information about their biological activity. More volatile compounds were found during the logarithmic phase. This strain could be collected at this phase and used as a probiotic to formulate functional food.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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