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Identification of bacteria using volatile organic compounds

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Abstract: The rapid diagnosis of respiratory infections has always been an important goal for medical professionals, because rapid and accurate diagnosis leads to proper and timely treatment, and consequently, reduces the costs of incorrect and long-term treatments, and antibiotic resistance. The present study was conducted with the aim of detecting volatile organic compounds (VOCs) in three bacteria: *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*. Headspace of the studied bacteria, after separately culturing in two types of liquid medium in three different time-periods, was extracted by solid phase microextraction and analysed by gas chromatography mass spectrometry The analysis results of the VOCs produced by the studied bacteria indicate that some VOCs are common and some are unique in each bacterium. *1-penten-3-ol*, *levomenthol*, and *2-octyl-1-ol* for *P. aeruginosa*, *cyclohexene, 4-ethenyl*, and *cis-Dihydro-α-terpinyl acetate* for *A. baumannii* and *1,3-butadiene*, *butyraldehyde*, *longifolene*, *octyl acetate*, *tridecanol*, *dodecenal*, *(E)-2-hexyl ester*, *butanoic acid*, and *5,5-dodecadinyl-1 12-diol* for *K. pneumoniae* were identified as unique VOCs for each bacterium. Finally, it can be said that an accurate and rapid bacterial detection method can be achieved by using a tool that can detect bacterial VOCs. However, more studies are needed to design a tool for which all aspects have been assessed, so that it can give us a more complete pattern for the use of these compounds as biomarkers.

Key words: Bacteria; VOC; SPME; GC-MS; Biomarker.

Introduction

Pneumonia is one of the most deadly nosocomial infections, which imposes a high economic burden on both the patient and the health system (1). Considering the reports on respiratory infections after hospitalization, various a range of prevalence (9% to 46%) has been reported, according to the type of communities (2). The incidence of pneumonia is higher in cold seasons (3). Lower respiratory infections, especially pneumonia, are a significant cause of children mortality (4), such that out of every 1,000 live births in developing countries, 12-20 children die of pneumonia under the age of five years (5). In recent years, pneumonia has been considered as one of the most important infections causing mortality (6). The first step to proper treatment of children is the accurate and timely identification of causative bacteria. Ideally, a bacterial infection should be diagnosed as soon as possible. Bacterial detection methods can be divided into three major groups: 1) Traditional methods based on bacteriological tests, 2) Molecular methods, 3) Detection of specific volatile compounds, produced by bacterial metabolism (7).

Traditional methods are still used, among other methods, due to their availability. But they should be followed step by step until the final diagnosis, and typically include Gram staining, culturing in the general media, and then specific media, single-colony culture, antibiogram, and biochemical methods, respectively. As is evident, this process is time-consuming and has some limitations; for example, bacterial culture usually takes several days to become positive, especially in patients who have recently received antibiotics for infections, it also lacks sufficient specificity and sensitivity (8). Bacteria have certain metabolism, some of which leads to the production of VOCs (9-11). The identification and detection of VOCs require advanced technology, which has medical diagnostic value (10, 12-13). These techniques include gas chromatography-mass spectrometry (GC-MS), selected flow tube ion mass spectrometry (SIFT-MS), and ion-molecule reaction mass spectrometry (IMR-MS) (14-15). GC-MS is considered as the reference method for isolation, identification, and detection of VOCs. These compounds can be detected and tested *in vitro* (in culture medium, or directly on the patient's sample) or in the patient's exhaled breath, and can be used as a rapid, non-invasive diagnostic method (12).

The following goals can be met through the detection of bacteria-specific VOCs and their use for diagnosis: 1) Proving the absence of pathogens [with high sensitivity and negative predictive value (NPV)] and thus not starting antibiotic treatment; 2) specific identification of a strain [with high specificity and positive predictive value (PPV)], and thus starting an appropriate antibiotic treatment; and 3) differentiation of pathogenic strains of bacteria and therefore, proper treatment based on the type of contaminant strains (16).

In several previous studies, VOCs of *P. aeruginosa* have been used as a diagnostic method in patients with cystic fibrosis, which was useful, but different biomarkers have been considered (17-20). This issue indicates that the same biomarkers have not yet been used for diagnosis, even for this bacterium, on which more studies have been carried out.

This study was performed with the purpose of *in vitro* detection of specific VOCs of a number of pathogenic organisms of the respiratory tract, including *P. aeruginosa*, *A. baumannii*, and *K. pneumoniae*, using GC-MS techniques in order to obtain molecular markers appropriate for early detection of the bacterial agents causing respiratory tract infections.

Materials and Methods

Cultivation of bacteria and conditions before GC-MS injection

In this study, the standard strains of *P. aeruginosa* (ATCC 27853) *A. baumannii* (ATCC 19606) and *K. pneumoniae* (ATCC 700683) were used. To prepare before the injection of bacterial headspace into GC-MS, first, a 24 hour incubation was performed in nutrient agar medium at 37°C, and all three bacteria were cultured in two types of liquid medium, including Mueller Hinton broth (MHB) and a richer medium, tryptic soy broth (TSB). And then, bacterial headspace was injected into GC-MS device after two, four, and 24 hours. The bacteria were shaken in 150rpm at 37°C during the culture time. [Final optical density at 600nm (OD600) > 0.5 for all samples]. Also, to provide conditions that increase the absorption of the compounds, after the time-periods and before the injection of headspace, 2ml of NaCl 36% was added. Then each bottle was placed on a stirring heater at 70°C (21), and a solid phase microextraction (SPME) syringe containing a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) coated fibre that absorbs non-polar compounds, was suspended at the top of the bottle for 30 minutes. After 30 minutes, the bacterial headspace was injected into the GC-MS. Moreover, before the expose of the SPME syringe to the headspace of bacterial samples, it was placed in the injector of the device for two minutes, for resorption of possible VOCs that had been absorbed into the fibre (22).

For both types of the media and in all three periods of time, a sterile liquid medium was used as the negative control (this study using a total of six negative control samples), and their headspace was injected into the GC-MS device under the same conditions considered for bacterial samples.

GC-MS

To study the bacterial volatile compounds, a Thermo-Finnigan Trace GC-MS system (Thermo Quest-Finnigan Co.) equipped with a DB-5 column (60m length,; 0.25mm inner diameter, and 0.25µm film thickness,) with helium carrier gas at a flow rate of 1.1ml/min, was used under the temperature programme consisting of the following steps: start at 50°C, increase at a rate of 10°C/minute up to 250°C. The GC-MS was set in splitless mode, and quadrupole ion trap, with ionization energy of 70eV used in the filament.

To analyze the GC-MS data, quartz index (RI) was calculated for each chromatographic peak, and then NIST 14 Mass Spectral Library (NIST14/2014/EPA/NIH) was used to identify each compound according to its RI. Also, extensive studies were performed by a phytochemist, to determine if the compound is organic. In this way, some information was obtained on the type of VOCs produced by the three mentioned bacteria.

Results

The analysis of the VOCs of the studied bacteria is shown in the following tables. Table 1: VOCs identified in *P. aeruginosa* are separately shown in the TSB and MHB, as well as the time intervals of two, four, and 24 hours after the culture.

Twenty VOCs of *P. aeruginosa* were identified after two hours' culture in TSB medium, and 25 compounds were identified after two hours' culture in MHB culture. The compounds that were produced by this bacterium in both media and this period of time include *benzaldehyde, decene, 2-Heptanone, 2-ethenyl-6-methyl-Pyrazine, 2,6-dibutyl-2,5-cyclohexadiene-1,4-dione,* 1- (1, 5-dimethyl-4-hexenyl) -4-methyl- benzene*, zingiberene, 2,5-bis(1,1-dimethylethyl)-Phenol, heptadecane, Cedran-1,8-diol, 3-Propionyloxypentadecane, 2-methyl-2-Undecanethiol, 4-t-butyl-2-(1-methyl-2-nitroethyl) cyclohexane, octacosane, dibutyl phthalate, phthalic acid,* and *butyl ester.* Certain specific VOCs, including *2,3-Pentanedione, naphthalenol, 1,3-heptadiene-3-yne* and *α-acetoxydihydrocoumarin,* were produced by *P. aeruginosa* only in TSB medium and in this time-period. Also, *carbamic acid, β-terpinyl acetate, 1-penten-3-ol, 4 (1,1) dimethylcyclohexane, neryl acetate, τ-cadinene, bisabolene* and *β-sesquiphellandrene* were produced by this bacterium after growth in MHB medium.

A total of 14 VOCs of *P. aeruginosa* were identified after four hours of culture in TSB medium, and 11 compounds after four hours' culture in MHB medium. The compounds produced by this bacterium in both media during this time-period include *thiophene, 1,2-butadiene, ethyl butanoate, 2,3-pentandione, 2-methyl-1-propanol, 1-Decyne, cis-dihydro-α-terpinyl acetate, cis-dihydro-α-terpinyl acetate, caryophyllene* and *β-santalol*. Certain VOCs, such as *2-acetyl-1-pyrroline, dimethyl sulfone, 1-methoxy-2-propanol, butyraldehyde,* and *2-Methyl tetradecanet*, were produced by P. aeruginosa only in TSB medium during this time-period. Also, *1-penten-3-ol* and *2-heptanone* were produced by this bacterium under the same conditions, after growth in MHB medium.

Fifteen VOCs of *P. aeruginosa* were identified after 24 hours of culture in TSB medium, and 11 compounds after 24 hours of culture in MHB medium. The compounds produced by this bacterium in both media in this time-period include *thiophene, 1, 2-butadiene, ethyl butanoate, 2,3-pentandione, 2-methyl-1-propanol, isopentyl acetate, levomenthol, cis-dihydro-α-terpinyl acetate, β-santalol.* The VOCs *dimethyl sulfone, 2-phenyl, 2H-tetrazole-5-carboxylic acid, 1-decyne, indole, caryophyllene* and *2-methyl tetradecane* were produced by

Table 1. Volataile compound produced by P.aeruginosa at 2, 4 and 24 hourse after culture in TSB and MB medium.

P. aeruginosa in MB				*P. aeruginosa* in TSB			
2h after culture							
Area%	**RI**	**Tn**	**Compound**	**Area%**	**RI[2]**	**Tn[1]**	**Compound**
0.31	973.26	5.53	Benzaldehyde	1.96	964.17	5.36	Benzaldehyde
15.11	996.79	5.97	Carbamic acid	18.68	1069.3	7.54	2,3-Pentanedione
14.7	1029.3	6.67	β-Terpinyl acetate	3.03	1089	7.97	Decene
1.21	1058.72	7.31	Ocimene	1.2	1199.5	10.42	2-Heptanone
1.24	1088.99	7.97	Decene	6.98	1226.4	11.04	Naphthalenol
0.51	1177.48	9.93	1-Penten-3-ol	8.07	1305.1	12.82	1,3-Heptadiene-3-yne
0.07	1199.55	10.42	2-Heptanone	13.91	1419.1	14.82	2-ethenyl-6-methyl-Pyrazine
0.26	1394.9	14.23	4(1,1)dimethyl-Cyclohexane	2.39	1474.2	16.29	2,6-dibutyl-2,5-cyclohexadiene-1,4-dione
5.97	1419.1	14.82	2-ethenyl-6-methyl-Pyrazine	4.32	1486.9	16.63	1-(1,5-dimethyl-4-hexyl-4-methyl-Benzene
0.22	1462.55	15.98	Neryl acetate	1.79	1493.3	16.8	α-Acetoxydihydrocoumarin
1.11	1474.53	16.3	2,6-dibutyl-2,5-cyclohexadiene-1,4-dione	1.63	1496.3	16.88	Zingiberene
18.83	1486.89	16.63	1-(1,5-dimethyl-4-hexyl-4-methyl-Benzene	2.18	1515.2	17.28	2,5-(1,1-dimethylethyl)-Phenol
8.33	1496.25	16.88	Zingiberene	11.25	1594.9	18.85	Heptadecane
2.92	1500.51	16.99	τ-Cadinene	1.41	1666.3	20.19	Cedran-1,8-diol
7.07	1508.12	17.14	Bisabolene	2.17	1687.7	20.59	3-Propionyloxypentadecane
0.52	1515.23	17.28	2,5-(1,1-dimethylethyl)-Phenol	1.18	1694.1	20.71	2-methyl-2-Undecanethiol
6.87	1524.37	17.46	β-Sesquiphellandrene	8.02	1700.6	20.83	4-t-butyl-2-(1-methyl-2-nitroethyl)cyclohexane
5.09	1594.92	18.85	Heptadecane	1.77	1797.7	22.49	Octacosane
0.71	1666.31	20.19	Cedran-1,8-diol	3.43	1872.4	23.76	Dibutyl phatalate
1.43	1687.7	20.59	3-Propionyloxypentadecane	1.63	1967.3	25.32	Phtalic acid, butyl ester
0.48	1694.12	20.71	2-methyl-2-Undecanethiol				
4.06	1700.58	20.83	4-t-butyl-2-(1-methyl-2-nitroethyl)cyclohexane				
0.65	1797.66	22.49	Octacosane				
1.53	1871.76	23.75	Dibutyl phatalate				
0.81	1966.67	25.31	Phtalic acid, butyl ester				
P. aeruginosa in MB				*P. aeruginosa* in TSB			
4h after culture							
Area%	**RI**	**Tn**	**Compound**	**Area%**	**RI**	**Tn**	**Compound**
0.11	1012.14	4.89	Thiophene	22.94	918.81	1.49	2-Acetyl-1-pyrroline
0.07	1036.1	5.64	1,2-Butadiyene	7.51	923.65	1.67	Dimethyl sulfone
1.04	1046.65	5.97	Ethyl butanoate	6.01	1012.14	4.89	Thiophene
0.88	1072.84	6.79	2,3-Pentandione	3.96	1035.78	5.63	1,2-Butadiyene
0.20	1118.08	8.11	2-methyl-1-propanol	5.29	1046.01	5.95	Ethyl butanoate
17.74	1176.54	9.63	1-Penten-3-ol	8.48	1071.88	6.76	2,3-Pentandione
0.44	1185.38	9.86	2-Heptanone	2.82	1116.54	8.07	2-methyl-1-propanol
75.35	1195.38	10.12	1-Decyne	0.65	1136.54	9.54	1-Methoxy-2-propanol
3.62	1303.52	12.91	cis-Dihydro-α-terpinyl acetate	1.36	1189.23	9.96	1-Decyne
0.06	1419.81	15.52	Caryophyllene	0.40	1288.8	12.54	Butyraldehyde
0.07	1695.68	20.99	β-Santalol	2.62	1300.44	12.84	cis-Dihydro-α-terpinyl acetate
				0.29	1404.72	15.2	2-Methyl tetradecane
				1.72	1417.92	15.48	Caryophyllene
				1.91	1693.51	20.95	β-Santalol
P. aeruginosa in MB				*P. aeruginosa* in TSB			
24 after culture							
Area%	**RI**	**Tn**	**Compound**	**Area%**	**RI**	**Tn**	**Compound**
0.12	1012.78	4.91	Thiophene	4.81	921.50	1.59	Dimethyl sulfone
0.12	1036.42	5.65	1,2-Butadiyene	3.55	990.86	4.17	2H-Tetrazole-5-carboxylicacid, 2-phenyl
1.20	1046.33	5.96	Ethyl butanoate	8.67	1012.46	4.9	Thiophene
0.62	1072.52	6.78	2,3-Pentandione	5.20	1036.1	5.64	1,2-Butadiyene
3.25	1118.08	8.11	2-methyl-1-propanol	17.46	1046.01	5.95	Ethyl butanoate
0.15	1127.31	8.35	Isopentyl acetate	28.69	1072.2	6.77	2,3-Pentandione
21.45	1175	9.59	Levomenthol	2.32	1117.31	8.09	2-methyl-1-propanol
0.42	1184.23	9.83	2-Heptanone	1.47	1127.69	8.36	Isopentyl acetate
54.83	1193.46	10.07	2-octyl-1-ol	1.86	1173.85	9.56	Levomenthol
17.47	1303.08	12.9	cis-Dihydro-α-terpinyl acetate	5.71	1189.62	9.97	1-Decyne
0.37	1694.59	20.97	β-Santalol	10.66	1300.44	12.84	cis-Dihydro-α-terpinyl acetate
				0.48	1325.99	13.42	Indole
				3.39	1418.87	15.5	Caryophyllene
				0.63	1491.04	17.03	2-Methyl tetradecane
				4.27	1694.05	20.96	β-Santalol

P. aeruginosa only in TSB medium during this period of time. Also, *2-heptanone* and *2-octyl-1-ol* were produced by the bacterium under the same conditions, after growth in only MHB medium.

Table 2: The VOCs detected in *A. baumannii* are separately shown in the TSB and MHB media as well as in the time periods of two, four, and 24 hours after culture

Thirty-one VOCs of *A. baumannii* were identified after two hours of culture in TSB medium, and 28 VOCs after two hours of culture in MHB medium. The compounds produced by this bacterium in both media during this time-period include *benzaldehyde, 1,2-butadiene, 3-methyl-1,5-heptadiene, 1,3-heptadiene-3-yne, 2-ethenyl -6-methyl pyrazine, neryl acetate, 2,6-dibutyl-2,5-cyclohexadiene-1,4-dione, 1-(1,5-dimethyl-4-*

Table 2. Volataile compound produced by A.baumannii at 2, 4 and 24 hourse after culture in TSB and MB medium.

A. baumannii in MB				*A. baumannii* in TSB			
2h after culture							
Area%	**RI**	**Tn**	**Compound**	**Area%**	**RI**	**Tn**	**Compound**
3.9	973.26	5.53	Benzaldehyde	5.79	917.11	4.48	2,5-dimethyl Pyrazine
44.77	994.65	5.93	Phenol	10.61	964.17	5.36	Benzaldehyde
0.18	1032.1	6.73	1,2-Butadiyene	0.13	1032.11	6.73	1,2-Butadiyene
0.35	1173.9	9.85	3-Methyl-1,5-heptadiene	23.76	1069.27	7.54	2,3-Pentandione
0.82	1177.9	9.94	1-Penten-3-ol	3.65	1090.83	8.01	1-Decyne
0.83	1199.5	10.42	2-Heptanone	0.11	1107.21	8.37	Methyl isopropyl Hexenal
9.9	1305.1	12.82	1,3-Heptadiene-3-yne	2.64	1174.32	9.86	3-Methyl-1,5-heptadiene
4.53	1419.1	14.82	2-ethenyl-6-methyl-Pyrazine	5.75	1226.84	11.05	Naphthalenol
0.29	1462.5	15.98	Neryl acetate	21.97	1304.46	12.81	1,3-Heptadiene-3-yne
0.42	1474.5	16.3	2,6-dibutyl-2,5-cyclohexadiene-1,4-dione	1.33	1331.85	13.24	Indole
3.63	1486.9	16.63	1-(1,5-dimethyl-4-hexyl-4-methyl-Benzene	3.41	1419.1	14.82	2-ethenyl-6-methyl-Pyrazine
0.65	1492.9	16.79	α-Acetoxydihydrocoumarin	0.41	1448.31	15.6	pentylhexyl Benzene
1.42	1496.3	16.88	Zingiberene	0.29	1457.3	15.84	Pentylethyl Pyrol
0.57	1508.1	17.14	Farnezol	0.26	1462.55	15.98	Neryl acetate
0.74	1515.2	17.28	2,5-(1,1-dimethylethyl)-Phenol	0.4	1474.53	16.3	2,6-dibutyl-2,5-cyclohexadiene-1,4-dione
0.17	1524.4	17.46	Cedrene	0.28	1479.78	16.44	2,6-bis(1,1-dimethylethyl)-4-methyl-phenol
0.25	1547.7	17.92	Tetradecanol	0.7	1486.89	16.63	1-(1,5-dimethyl-4-hexyl-4-methyl-Benzene
7.86	1594.9	18.85	Heptadecane	0.82	1492.88	16.79	α-Acetoxydihydrocoumarin
0.17	1624.1	19.4	Cedrol	0.5	1496.25	16.88	Zingiberene
0.12	1643.3	19.76	2,6,10-trimethyl-Pentadecane	1.22	1515.23	17.28	2,5-(1,1-dimethylethyl)-Phenol
2.53	1666.8	20.2	Cedran-1,8-diol	0.06	1547.72	17.92	Tetradecanol
3.14	1687.7	20.59	3-Propionyloxypentadecane	4.82	1594.92	18.85	Heptadecane
0.77	1694.1	20.71	2-methyl-2-Undecanethiol	0.06	1624.6	19.41	Cedrol
7.57	1700.6	20.83	4-t-butyl-2-(1-methyl-2-nitroethyl)cyclohexane	1.08	1666.84	20.2	Cedran-1,8-diol
0.52	1752	21.71	2-(phenylmethylene)-Octanal	0.62	1688.24	20.6	3-Propionyloxypentadecane
0.84	1797.7	22.49	Octacosane	0.52	1694.65	20.72	2-methyl-2-Undecanethiol
1.06	1872.4	23.76	Dibutyl phatalate	6.23	1700.58	20.83	4-t-butyl-2-(1-methyl-2-nitroethyl)cyclohexane
0.44	1966.7	25.31	Phatalic acid, butyl ester	0.15	1752.05	21.71	2-(phenylmethylene)-Octanal
				0.29	1798.25	22.5	Octacosane
				1.09	1872.94	23.77	Dibutyl phatalate
				0.11	1967.9	25.33	Phatalic acid, butyl ester
A. baumannii in MB				*A. baumannii* in TSB			
4h after culture							
Area%	**RI**	**Tn**	**Compound**	**Area%**	**RI**	**Tn**	**Compound**
1.55	1013.09	4.92	Cyclohexene 4-ethenyl	15.67	920.43	1.55	2-Acetyl-1-pyrroline
1.44	1036.74	5.66	1,2-Butadiyene	6.84	924.46	1.7	Dimethyl sulfone
4.88	1046.64	5.97	Ethyl butanoate	5.72	1012.77	4.91	Cyclohexene 4-ethenyl-
4.63	1073.16	6.8	2,3-Pentandione	2.33	1036.42	5.65	1,2-Butadiyene
0.39	1192.30	10.04	1-Decyne	8.73	1046.64	5.97	Ethyl butanoate
86.86	1306.60	12.98	cis-Dihydro-α-terpinyl acetate	16.78	1072.20	6.77	2,3-Pentandione
0.26	1419.81	15.52	Caryophyllene	0.91	1172.69	9.53	Levomenthol
				1.30	1189.23	9.96	1-Decyne
				7.11	1300.44	12.84	cis-Dihydro-α-terpinyl acetate
				3.74	1417.92	15.48	Caryophyllene
				0.72	1490.56	17.02	2-Methyl tetradecane
				5.13	1693.51	20.95	β-Santalol
A. baumannii in MB				*A. baumannii* in TSB			
24h after culture							
Area%	**RI**	**Tn**	**Compound**	**Area%**	**RI**	**Tn**	**Compound**
18.65	1013.41	4.93	Cyclohexene 4-ethenyl-	10.87	918.81	1.49	2-Acetyl-1-pyrroline
0.69	1022.04	5.2	Thiophene	2.11	991.66	4.2	2H-Tetrazole-5-carboxylicacid, 2-phenyl
8.79	1036.74	5.66	1,2-Butadiyene	5.91	1012.14	4.89	Cyclohexene, 4-ethenyl-
12.32	1046.64	5.97	Ethyl butanoate	4.05	1035.78	5.63	1,2-Butadiyene
7.97	1072.84	6.79	2,3-Pentandione	7.34	1046	5.95	Ethyl butanoate
0.75	1117.69	8.1	2-methyl-1-propanol	13.69	1072.20	6.77	2,3-Pentandione
0.77	1173.84	9.56	Levomenthol	0.83	1116.53	8.07	2-methyl-1-propanol
0.68	1191.53	10.02	1-Decyne	1.33	1173.84	9.56	Levomenthol
47.25	1301.76	12.87	cis-Dihydro-α-terpinyl acetate	0.16	1183.84	9.82	1-Decyne
0.15	1325.99	13.42	Indole	42.19	1301.15	12.86	cis-Dihydro-α-terpinyl acetate
0.23	1405.66	15.22	1,9-Decadiene	0.35	1325.55	13.41	Indole
0.47	1418.86	15.5	Caryophyllene	1.76	1418.39	15.49	Caryophyllene
0.23	1587.5	18.97	2-Methyl tetradecane	0.19	1485.37	16.91	5.5-Dodecadinyl-1, 12-diol
1.04	1694.59	20.97	β-Santalol	0.31	1491.03	17.03	2-Methyl tetradecane
				0.33	1587.5	18.97	(E)-2-hexyl ester- Butanoic acid
				0.26	1658.91	20.31	2-Decenal
				1.71	1693.51	20.95	β-Santalol

hexenyl)-4-methyl-Benzene,α acetoxydihydrocoumarin, zingiberene, 2,5-bis-(1,1-dimethylethyl) phenol, tetradecanol, heptadecane, cedrol, cedran-1,8-diol, 3-propionyl oxypentadecane, 2-methyl-2-Undecanethiol, 4-t-Butyl-2-(1-methyl-2-nitroethyl) cyclohexanone, 2-(phenylmethylene)-octanal, Octacosane, dibutyl phthalate, phthalic acid, and butyl ester. The VOCs *2,5-dimethyl pyrazine, 2,3-pentandione, 1-decyne, isopropyl methyl hexenal, naphthalenol, indole, pentylhexyl benzene,* and *2,6-dibutyl-2,5-cyclohexadiene-1,4-dione* were produced by *A. baumannii* only in TSB medium in this period of time. Also, the VOCs phenol, 1-penten-3-ol, 2-heptanone, and *cedrene*, were produced in the same conditions, only after growth in MHB medium.

Twelve VOCs of *A. baumannii* were identified after four hours of culture in TSB medium, and seven VOCs after four hours of culture in MHB medium. The compounds produced by this bacterium in both media during this time-period include *4-ethenyl cyclohexene, ethyl butanoate, 1,2-butadiene, 2,3-pentandione, 1-decyne, cis-dihydro-α-terpinyl acetate, caryophyllene. The VOCs 2-acetyl-1-pyrroline, dimethyl sulfone, levomenthol, 2-methyl tetradecane,* and *β-santalol,* were produced by *A. baumannii in* TSB medium during this time-period.

Seventeen VOCs of *A. baumannii* were identified after 24 hours of culture in TSB medium, and 14 VOCs after 24 hours' culture in MHB medium. The compounds produced by this bacterium in both media during this time-period include *4-ethenyl cyclohexene, 1,2-butadiene, ethyl butanoate, 2,3-pentandione, 2-methyl-1-propanol, levomenthol, 1-decyne, cis-dihydro-α-terpinyl acetate, indole, aryophyllene, 2-methyl tetradecane, 2-decenal* and *β-santalol.* The VOCs *2-acetyl-1-pyrroline, 2-phenyl, 2H-tetrazole-5-carboxylicacid, 12-diol 5,5-dodecadinyl-1,* and *(E)-2-hexyl ester-butanoic acid* were produced only in TSB medium during this time-period. Also, the VOCs *thiophene* and *1,9-decadiene* were produced by this bacterium in the same conditions in MHB medium.

Table 3: VOCs detected in *K. pneumoniae* are separately shown in the TSB and MHB media as well as in time-periods of two, four, and 24 hours after culture.

Thirty-three VOCs of *K. pneumoniae* were identified after two hours of culture in TSB medium, and 28 compounds after two hours' culture in MHB medium. The compounds produced by this bacterium in both media during this time-period included *1,3-butadiene, ethyl butanoate, 3-methyl-1,5-heptadiene, butyraldehyde, dimethylethyl cyclohexanol, 1,9-decadiene, 2-ethenyl-6-methyl-pyrazine, longifolene, octyl acetate, 2,6-bis-(1,1-dimethylethyl)-4-methyl-phenol, zingiberene, 2,5-(1,1-dimethylethyl)-phenol, (E)-2-hexyl ester-butanoic acid, cedran-1,8-diol, 3-propionylo eicosane xypentadecane, β-santalol, 4-t-butyl-2-(1-methyl-2-nitroethyl) cyclohexane,* and *dibutyl phatalate.* The VOCs *2,5-dimethyl pyrazine, cyclohexene 4-ethenyl, ocimene, methone, dimethyl octenal, naphthalenol, dodecenal, 1,3-heptadiene-3-yne, (z)-2-octene-1-ol, 6-methyl-5-hepten-2-one, β-sesquiphellandrene,* and *eicosane,* were produced by *K. pneumoniae* in TSB medium during this period of time. Also, *carbamic acid, 12-diol, 5,5-dodecadinyl-1, cadinene, β-sesquiphellandrene, cedrol, butyl ester,* and *phthalic acid,* were produced by this bacterium under the same conditions in MHB medium.

Forty-eight VOCs of *K. pneumoniae* were identified after four hours' culture in TSB, and 46 compounds after four hours' culture in MHB medium. The compounds produced by this bacterium in both media during this time-period include *2-phenyl, 2H-tetrazole-5-carboxylicacid, carbamic acid, 1,3-butadiene, ethyl butanoate, 2,3-pentandione, 1-decyne, methone, dimethyl octenal, 3-methyl-1,5-heptadiene, 2-heptanone, 2-octyl-1-ol, dodecenal, butyraldehyde, (z)-4-decan-1-ol, 1,3-heptadiene-3-yne, indole, (z)-2-octene-1-ol, 6-methyl-5-hepten-2-one, 2-hexan-1-ol, dimethylethyl cyclohexanol, 1,9-decadiene, 2-ethenyl-6-methyl-pyrazine, longifolene, 2,3-hexandione,1,5-decadiene, octyl acetate, 5,5-dodecadinyl-1, 12-diol, β-santalol, 2,5-(1,1-dimethylethyl)-phenol, tetradecanol, (E)-2-hexyl ester-butanoic acid, dodecenol, cedrol, cedran-1,8-diol, tridecanol, 4-t-butyl-2-(1-methyl-2-nitroethyl)cyclohexane, octacosane, 1,2-benzenedicarboxulic acid, dibutyl phthalate, phthalic acid,* and *butyl ester* .The VOCs *2,5-dimethyl pyrazine, benzaldehyde, 2-methyl-1-propanol, 3-propionyloxypentadecane, 2-(phenylmethylene)-octanal,* and *eicosane,* were produced by *K. pneumonia* only in TSB medium. The VOCs *naphthalenol, cadinene,* and *benzophenone* were produced by this bacterium in the same conditions in MHB medium.

Twenty-five VOCs of *K. pneumoniae* were identified after 24 hours' culture in TSB medium, and 30 VOCs after 24 hours' culture in MHB medium. The compounds produced by this bacterium in both media during this time-period include *2,3-pentandione, 3-methyl-1,5-heptadiene, dodecenal, butyraldehyde, (z)-4-decan-1-ol, 1,3-heptadiene-3-yne, indole 2-ethenyl-6-methyl-pyrazine, longifolene, octyl acetate, 2,6-bis(1,1-dimethylethyl)-4-methyl-phenol, zingiberene, cadinene, 2,5-(1,1-dimethylethyl)-phenol, (E)-2-hexyl ester-butanoic acid, 2,6,10-trimethyl-pentadecane, tridecanol, 3-propionyloxypentadecane, 2-methyl-2-undecanethiol, 4-t-butyl-2-(1-methyl-2-nitroethyl) cyclohexane, octacosane,* and *dibutyl phatalate.* The VOCs *methyl isopropyl hexenal, 2-octyl-1-ol,* and *1,5-decadiene* were produced by *K. pneumoniae* only in TSB medium. Also, *carbamic acid, 1,3-butadiene, 1-decyne, dimethylethyl cyclohexanol, caryophyllene, β-sesquiphellandrene,* and *cedran-1,8-diol,* were produced by this bacterium under the same conditions in MHB medium.

A total of 45 types of VOCs were identified for *P. aeruginosa* (Table 1) in three different time periods of two, four, and 24 hours, in which bacterial headspace was injected into the GC-MS device. Among them, 19 compounds (42%) were produced in the headspace after growth in one of the two media. In the case of *A. baumannii*, a total of 52 types of compound were identified (Table 2), 19 (36%) of which were produced in the headspace after growth in one of the two media. Also, in *K. pneumonia*, 57 compounds were identified (Table 3), of which 11 (19%) compounds were produced in the headspace after growth in one of the two media.

Discussion

All organisms naturally produce general and specific

Table 3. Volataile compound produced by K.penumonia at 2, 4 and 24 hourse after culture in TSB and MB medium.

K. penumonia in MB				*K. penumonia* in TSB			
2h after culture							
Area%	**RI**	**Tn**	**Compound**	**Area%**	**RI**	**Tn**	**Compound**
17.89	997.861	5.99	Carbamic acid	5.03	917.11	4.48	2,5-dimethyl Pyrazine
4.71	1029.4	6.67	1,3-Butadiyene	0.69	1011.5	6.28	Cyclohexene, 4-ethenyl-
3.06	1045.9	7.03	Ethyl butanoate	0.81	1033	6.75	1,3-Butadiyene
2.95	1090.8	8.01	1-Decyne	0.97	1047.7	7.07	Ethyl butanoate
1.41	1177.5	9.93	3-Methyl-1,5-heptadiene	1.29	1075.2	7.67	Ocimene
3.3	1291.8	12.55	Butyraldehyde	10.19	1091.7	8.03	1-Decyne
2.11	1394.9	14.23	Dimethylethyl Cyclohexanol	1.97	1156.8	9.47	Methone
0.57	1416.5	14.75	1,9-Decadiene	0.39	1168.9	9.74	Dimethyl Octenal
3.87	1418.7	14.81	2-ethenyl-6-methyl-Pyrazine	2.33	1177.9	9.94	3-Methyl-1,5-heptadiene
1.36	1428.8	15.08	Longifolene	0.29	1227.7	11.07	Naphthalenol
0.61	1474.2	16.29	Octyl acetate	1.24	1268.8	12.02	Dodecenal
0.66	1476	16.34	2,6-bis(1,1-dimethylethyl)-4-methyl-phenol	4.05	1293.1	12.58	Butyraldehyde
8.03	1486.5	16.62	5.5-Dodecadinyl-1, 12-diol	3.1	1305.1	12.82	1,3-Heptadiene-3-yne
7.38	1495.9	16.87	Zingiberene	0.73	1331.8	13.24	(z)-2-Octene-1-ol
1.61	1500	16.98	Cadinene	0.43	1347.1	13.48	6-Methyl-5-hepten-2-one
1.06	1514.7	17.27	2,5-(1,1-dimethylethyl)-Phenol	0.54	1396.8	14.26	Dimethylethyl Cyclohexanol
3.34	1523.9	17.45	β-Sesquiphellandrene	0.29	1413.5	14.67	1,9-Decadiene
7.13	1594.4	18.84	(E)-2-hexyl ester- Butanoic acid	2.25	1419.9	14.84	2-ethenyl-6-methyl-Pyrazine
0.85	1624.1	19.4	Cedrol	0.3	1429.6	15.1	Longifolene
3.39	1666.3	20.19	Cedran-1,8-diol	3.23	1476.8	16.36	Octyl acetate
0.8	1672.2	20.3	Tridecanol	9.81	1480.5	16.46	2,6-bis(1,1-dimethylethyl)-4-methyl-phenol
1.59	1687.7	20.59	3-Propionyloxypentadecane	1.71	1495.1	16.85	Zingiberene
1.98	1694.1	20.71	β-Santalol	0.46	1514.2	17.26	2,5-(1,1-dimethylethyl)-Phenol
6.06	1700	20.82	4-t-butyl-2-(1-methyl-2-nitroethyl)cyclohexane	0.18	1525.4	17.48	β-Sesquiphellandrene
3.84	1751.5	21.7	2-(phenylmethylene)-Octanal	1.99	1595.9	18.87	(E)-2-hexyl ester- Butanoic acid
1.86	1797.7	22.49	Octacosane	0.43	1667.4	20.21	Cedran-1,8-diol
2.33	1871.3	23.75	Dibutyl phatalate	0.29	1675.9	20.37	Tridecanol
1.01	1962.6	25.31	Phatalic acid, butyl ester	1.2	1688.8	20.61	3-Propionyloxypentadecane
				0.69	1695.2	20.73	β-Santalol
				2.48	1701.8	20.85	4-t-butyl-2-(1-methyl-2-nitroethyl)cyclohexane
				0.62	1798.8	22.51	Octacosane
				0.23	1872.9	23.77	Dibutyl phatalate
				0.16	1967.9	25.33	Eicosane
K. penumonia in MB				*K. penumonia* in TSB			
4h after culture							
Area%	**RI**	**Tn**	**Compound**	**Area%**	**RI**	**Tn**	**Compound**
2.55	975.401	5.57	2H-Tetrazole-5-carboxylicacid, 2-phenyl	0.47	925.66	4.64	2,5-dimethyl Pyrazine
9.64	991.979	5.88	Carbamic acid	0.27	950.80	5.11	Benzaldehyde
4.43	1030.28	6.69	1,3-Butadiyene	0.86	975.40	5.57	2H-Tetrazole-5-carboxylicacid, 2-phenyl
0.4	1059.63	7.33	Ethyl butanoate	0.96	992.51	5.89	Carbamic acid
0.71	1073.39	7.63	2,3-Pentandione	1.75	1030.28	6.69	1,3-Butadiyene
6.26	1092.2	8.04	1-Decyne	0.46	1047.7	7.07	Ethyl butanoate
1.82	1157.21	9.48	Methone	1.12	1075.23	7.67	2,3-Pentandione
0.65	1168.92	9.74	Dimethyl Octenal	9.46	1092.66	8.05	1-Decyne
8.2	1177.93	9.94	3-Methyl-1,5-heptadiene	0.24	1125.69	8.77	2-methyl-1-propanol
0.35	1192.79	10.27	2-Heptanone	0.87	1157.21	9.48	Methone
0.37	1223.38	10.97	Naphthalenol	0.2	1170.72	9.78	Dimethyl Octenal
0.62	1246.32	11.5	2-octyl-1-ol	1.78	1178.83	9.96	3-Methyl-1,5-heptadiene
2	1276.62	12.2	Dodecenal	0.2	1193.24	10.28	2-Heptanone
1.68	1287.88	12.46	Butyraldehyde	0.22	1246.75	11.51	2-octyl-1-ol
1.99	1293.07	12.58	(z)-4-Decan-1-ol	20.6	1276.62	12.2	Dodecenal
0.36	1297.84	12.69	1,3-Heptadiene-3-yne	1.46	1286.15	12.42	Butyraldehyde
0.63	1308.92	12.88	Indole	3.15	1293.94	12.6	(z)-4-Decan-1-ol
0.48	1337.58	13.33	(z)-2-Octene-1-ol	0.2	1298.27	12.7	1,3-Heptadiene-3-yne
0.35	1347.77	13.49	6-Methyl-5-hepten-2-one	0.6	1309.55	12.89	Indole
0.38	1370.7	13.85	2-Hexan-1-ol	0.55	1333.12	13.26	(z)-2-Octene-1-ol
1.39	1396.82	14.26	Dimethylethyl Cyclohexanol	0.13	1348.41	13.5	6-Methyl-5-hepten-2-one
0.46	1413.86	14.68	1,9-Decadiene	0.29	1371.34	13.86	2-Hexan-1-ol
6.74	1420.6	14.86	2-ethenyl-6-methyl-Pyrazine	0.68	1394.27	14.22	Dimethylethyl Cyclohexanol
0.57	1429.96	15.11	Longifolene	0.77	1413.86	14.68	1,9-Decadiene
1.4	1440.82	15.4	2,3-Hexandione	5.3	1420.6	14.86	2-ethenyl-6-methyl-Pyrazine
0.77	1463.3	16	1,5-Decadiene	0.54	1428.84	15.08	Longifolene
1.81	1476.78	16.36	Octyl acetate	0.46	1440.82	15.4	2,3-Hexandione
3.51	1482.77	16.52	5.5-Dodecadinyl-1, 12-diol	0.32	1464.04	16.02	1,5-Decadiene
0.58	1490.64	16.73	Zingiberene	3.47	1477.15	16.37	Octyl acetate
3.28	1494.01	16.82	β-Santalol	12.72	1481.27	16.48	5.5-Dodecadinyl-1, 12-diol
0.21	1509.14	17.16	Cadinene	2.47	1495.88	16.87	β-Santalol

1.6	1514.72	17.27	2,5-(1,1-dimethylethyl)-Phenol	0.82	1515.23	17.28	2,5-(1,1-dimethylethyl)-Phenol
0.65	1567.01	18.3	Tetradecanol	0.45	1567.51	18.31	Tetradecanol
8.52	1596.45	18.88	(E)-2-hexyl ester- Butanoic acid	5.76	1596.95	18.89	(E)-2-hexyl ester- Butanoic acid
0.37	1609.63	19.13	Dodecenol	0.18	1610.7	19.15	Dodecenol
0.58	1625.13	19.42	Cedrol	0.14	1625.67	19.43	Cedrol
0.71	1634.76	19.6	Benzophenone	0.79	1663.1	20.13	Cedran-1,8-diol
1.13	1667.91	20.22	Tridecanol	1.07	1667.91	20.22	Tridecanol
0.95	1687.7	20.59	Cedran-1,8-diol	0.43	1676.47	20.38	3-Propionyloxypentadecane
5.63	1702.34	20.86	4-t-butyl-2-(1-methyl-2-nitroethyl)cyclohexane	3.2	1702.92	20.87	4-t-butyl-2-(1-methyl-2-nitroethyl)cyclohexane
0.73	1799.42	22.52	Octacosane	0.36	1749.71	21.67	2-(phenylmethylene)-Octanal
0.36	1808.77	22.68	1,2-Benzenedicarboxulic acid	0.83	1799.42	22.52	Octacosane
1.39	1873.53	23.78	Dibutyl phatalate	0.29	1808.82	22.68	1,2-Benzenedicarboxulic acid
1.45	1968.52	25.34	Phatalic acid, butyl ester	0.91	1874.12	23.79	Dibutyl phatalate
				0.71	1968.52	25.34	Phatalic acid, butyl ester
				0.16	1999.38	25.84	Eicosane
***K. penumonia* in MB**				***K. penumonia* in TSB**			
24h after culture							
Area%	**RI**	**Tn**	**RI**	**Area%**	**RI**	**Tn**	**RI**
12.76	996.7914	5.97	Carbamic acid	1.95	1069.7	7.55	2,3-Pentandione
7.02	1029.358	6.67	1,3-Butadiyene	0.71	1101.8	8.25	Methyl isopropyl Hexenal
1.2	1046.789	7.05	Ethyl butanoate	1.09	1177	9.92	3-Methyl-1,5-heptadiene
11.89	1091.284	8.02	2,3-Pentandione	0.69	1200	10.43	2-octyl-1-ol
1.75	1173.874	9.85	3-Methyl-1,5-heptadiene	2.69	1274	12.14	Dodecenal
0.44	1199.55	10.42	1-Decyne	1.78	1281	12.3	Butyraldehyde
1.47	1275.758	12.18	Dodecenal	2.11	1292.2	12.56	(z)-4-Decan-1-ol
2.97	1281.818	12.32	Butyraldehyde	0.44	1297.8	12.69	1,3-Heptadiene-3-yne
5.95	1292.208	12.56	(z)-4-Decan-1-ol	1.5	1301.9	12.77	Indole
3.21	1302.548	12.78	Indole	0.57	1412.7	14.65	2-ethenyl-6-methyl-Pyrazine
0.57	1394.904	14.23	Dimethylethyl Cyclohexanol	3.68	1419.1	14.82	Longifolene
1.91	1416.105	14.74	Caryophyllene	0.39	1427.3	15.04	1,5-Decadiene
1.51	1419.101	14.82	2-ethenyl-6-methyl-Pyrazine	2.58	1476	16.34	Octyl acetate
1.16	1426.966	15.03	Longifolene	22.92	1480.1	16.45	2,6-bis(1,1-dimethylethyl)-4-methyl-phenol
0.31	1439.326	15.36	2,3-Hexandione	5.22	1494.8	16.84	Zingiberene
4.16	1476.03	16.34	Octyl acetate	0.63	1502.5	17.03	Cadinene
4.36	1480.899	16.47	2,6-bis(1,1-dimethylethyl)-4-methyl-phenol	0.42	1515.2	17.28	2,5-(1,1-dimethylethyl)-Phenol
4.08	1486.517	16.62	5.5-Dodecadinyl-1, 12-diol	5.44	1594.9	18.85	(E)-2-hexyl ester- Butanoic acid
7.95	1494.757	16.84	Zingiberene	5.48	1658.8	20.05	2,6,10-trimethyl-Pentadecane
1.73	1502.03	17.02	Cadinene	9.45	1677	20.39	Tridecanol
1.01	1515.228	17.28	2,5-(1,1-dimethylethyl)-Phenol	0.8	1688.2	20.6	3-Propionyloxypentadecane
1.2	1524.365	17.46	β-Sesquiphellandrene	5.06	1695.2	20.73	2-methyl-2-Undecanethiol
4.38	1594.416	18.84	(E)-2-hexyl ester- Butanoic acid	3.24	1700.6	20.83	4-t-butyl-2-(1-methyl-2-nitroethyl)cyclohexane
0.47	1666.31	20.19	Cedran-1,8-diol	1.36	1798.2	22.5	Octacosane
0.65	1674.866	20.35	Tridecanol	1.13	1872.4	23.76	Dibutyl phatalate
0.88	1687.701	20.59	3-Propionyloxypentadecane				
1.49	1694.652	20.72	2-methyl-2-Undecanethiol				
2.82	1700.585	20.83	4-t-butyl-2-(1-methyl-2-nitroethyl)cyclohexane				
0.83	1797.661	22.49	Octacosane				
1.46	1872.353	23.76	Dibutyl phatalate				

volatile compounds, which result from their metabolism (18). In this study, VOCs of three standard strains of gram-negative bacteria, including, *P. aeruginosa, A. baumannii* and *K. pneumonia*, were investigated in MHB and TSB media in three time periods of two, four, and 24 hours. In most previous studies, only one type of culture medium (often TSB) had been used (23-27). But in this study, VOCs produced by bacteria under the same conditions were studied both in a simple medium (MHB) and an enriched medium (TSB), to reveal possible similarity or difference between the culture in two types of medium. As shown in the present study, more than 50% of the detected compounds were produced by growth in both culture media. Some of the difference between the two compounds produced in both of the media can be a result of their different chemical structures.

The investigation and comparison of the VOCs detected in these three bacteria showed that a number of compounds are common among bacteria (e.g., *2,3-pentanedione, ocimene, β-santalol, zingiberene, octacosane*, etc.), which can result from the release from the medium, as well as production of common general compounds.

Although some VOCs identified in this study are seen among the compounds produced by other bacteria, they include a higher percentage in one of them, which can be the result of metabolism in one of them and the result of medium in another one. For example, *benzaldehyde* is produced by all three studied bacteria, but with different amounts; in *P. aeruginosa,* it comprises 1.96% of the compounds after two hours' culture in TSB medium; in *K. pneumoniae*, 0.27% of the compounds after four hours' culture in TSB medium; but in *A. baumannii* 10.61% after two hours' culture in TSB medium.

In this study, a number of VOCs were also identified, which were exclusively produced by only one of the bacterium. The VOCs only produced by *P. aeruginosa* (Table 1) include *decene, β-terpinyl acetate, 4 (1,1) dimethyl-cyclohexane, τ-cadinene,* and *bisabolene,* which were identified in the headspace after two hours of culture; *thiophene, 1-methoxy-2-propanol, 1-penten-3-ol,* which were identified in the headspace after four hours of culture; and *thiophene, levomenthol,* and *2-octyl-1-ol,* which were identified in headspace after 24 hours of culture. Among them, *4(1,1) dimethyl-cyclohexane* and *1-methoxy-2-propanol* compounds comprise less than 1% of the compounds. *Thiophene* was produced in both four hours and 24 hours of culture, and can be considered an important biomarker. Although, *1-penten-3-ol* is among the compounds identified in a two-hour period of time in both *A. baumannii* in addition to and *P. aeruginosa,* but in the four-hour period of time, it comprises 17.74% of the compounds exclusively in *P. aeruginosa,* which indicates that this increase is the result of metabolism. Although, *levomenthol* and *2-octyl-1-ol* have been respectively produced by *A. baumannii* (< 2%) and *K. pneumoniae* (< 1%), their production in *P. aeruginosa* is much more than in other bacteria (21.54% and 54.83%, respectively).

The VOCs produced by *A. baumannii (Table 2)* include *phenol, cedrene, pentylethyl pyrol, farnezol, pentylhexyl benzene* and *benzaldehyde,* which were detected in the headspace two hours after the culture; *cyclohexene 4-ethenyl, cis-dihydro-α-terpinyl acetate* four hours *after the culture*; and *cyclohexene 4-ethenyl-, cis-dihydro-α-terpinyl acetate* 24 hours after the culture. Among these, the VOCs *cedrene, pentylethyl pyrol, farnezol,* and *pentylhexyl* benzene comprise less than 1% of the compounds. *cyclohexene 4-ethenyl* and *cis-dihydro-α-terpinyl acetate,* were also respectively produced by *K. pneumoniae* (< 1%) and *P. aeruginosa* (< 18%), but both of these compounds were produced by *A. baumannii* during four hours in a 24-hour period of time, and their production rate remained stable or even increased.

Cyclohexene 4-ethenyl was produced in the four-hour period of time by culturing in the TSB and MHB media (5.72% and 1.55%, respectively), but its production respectively increased to 5.91% and 18.65% in a 24-hour period of time. *Cis-Dihydro-α-terpinyl acetate* was produced in the four-hour period of time by culturing in the TSB and MHB media (7.11% and 86.86%, respectively), but its production respectively increased to 42.19% and 47.25. The production of these two compounds was higher in *A. baumannii*, as compared to *P. aeruginosa* and *K. pneumoniae,* from which it can be deduced that it is the result of the metabolism of *A. baumannii.*

The VOCs only produced by *K. pneumoniae* (Table 3) include *1,3-butadiene, methone, dimethyl octenal, butyraldehyde, dimethylethyl cyclohexanol, (z)-2-octene-1-ol, 6-methyl- 5-hepten-2-one, longifolene, octyl acetate, (E)-2-hexyl ester-butanoic acid, 12-diol, 5,5-dodecadinyl-1, cadinene, dodecenal, eicosane, 2,6-bis-(1,1-dimethylethyl)-4-methyl-phenol,* and *tridecanol,* which were detected in the headspace two hours after the culture; and *1,3-butadiene, methone, dimethyl octenal, dodecenal, butyraldehyde, (z)-4-decan-1-ol, (z)-2-octene-1-ol, 6-methyl-5-hepten-2-one, 2-hexan-1-ol, dimethylethyl cyclohexanol, longifolene, 2,3-hexandione, 1,5-decadiene, octyl acetate, 5,5-dodecadinyl-1, 12-diol, (E)-2-hexyl ester-butanoic acid, dodecenol, 1,2-benzenedicarboxulic acid, benzophenone, eicosane,* and *tridecanol,* which were detected in the headspace after four hours of culture; and *butyraldehyde, (z)-4-decan-1-ol, longifolene, 1,5-decadiene, octyl acetate, 2,6-bis-(1,1-dimethylethyl)-4-methyl-phenol, cadinene, 2,6, 10-trimethyl-pentadecane, (E)-2-hexyl ester-butanoic acid, tridecanol, dodecenal, 1,3-butadiene, 5.5-dodecadinyl-1 12-diol,* and *dimethylethyl cyclohexanol,* which were detected in the headspace after 24 hours of culture. The VOCs *dimethyl octenal, 1,5-decadiene, eicosane, benzophenone, (z)-2-Octene-1-ol, 6-methyl-5-hepten-2-one, 2-hexan-1-oll,* and *1,2-benzenedicarboxulic acid* included less than 1% of VOCs. The VOCs *2,6-bis-(1,1-dimethylethyl)-4-methyl-phenol, 5,5-dodecadinyl-1 12-*diol and *(E)-2-hexyl ester-butanoic acid* were also detected in *A. baumannii.* But their production level was less than 1%. The level of *butyraldehyde* production in *P. aeruginosa* was less than 1%. Some of the detected VOCs released from this bacterium, including *methone* and *dimethylethyl cyclohexanol* were produced two and four hours after culture also *(z)-4-decan-1-ol* was produced four and 24 hours after culture , and *2,6-bis-(1,1-dimethylethyl)-4-methyl-phenol* was produced two and 24 hours after culture periods of time. Also, a number of compounds produced in all three periods of time, including *1,3-butadiene, butyraldehyde, longifolene, octyl acetate, tridecanol, dodecenal, (E)-2-hexyl ester-butanoic acid,* and *5,5-dodecadinyl-1 12-diol,* can be more important as biomarkers for *K. pneumoniae.*

The previous studies on bacterial VOCs had different designs; for example Filipiak et al. (2012) used multibed sorption tubes to collect headspace and compared VOCs of *P. aeruginosa* and *Staphylococcus aureus* bacteria (26).

In another study by Zhu et al. (2013), secondary electrospray ionization-mass spectrometry (SESI-MS) breath analysis was used to study mouse models infected with different respiratory bacteria, including *P. aeruginosa* and *K. pneumonia,* and finally declared that this method could detect lung infection caused by different bacteria and that different bacteria have various VOCs patterns (28).

In a study by Kunze et al. (2013), the effect of changes caused by *P. aeruginosa* and *E. coli,* was investigated on VOCs produced by multi-capillary columnion mobility spectrometry (MCC-IMS) method, and it was concluded that even adjacent culture of bacteria could lead to production of unique compounds (29).

In a study by Gao et al. (2016) the VOCs of *A. baumannii* in infection status, colonization in the lung, and in-vitro were assessed, it was concluded that the compounds profile of this bacterium differed across the three states (30).

According to the findings of the present study, unique VOCs can be introduced as biomarkers for *P. aeruginosa*, *A. baumannii*, and *K. pneumoniae*. This study suggests that thiophene, 1-penten-3-ol, levomenthol and 2-octyl-1-ol, can be considered more as biomarkers for *P. Aeruginosa*; *cyclohexene 4-ethenyl* and *cis-dihydro-α-terpinyl acetate* for *A. Baumannii*; and 1,3-butadiene, *butyraldehyde, longifolene, octyl acetate, tridecanol, dodecenal, (E)-2-hexyl ester-butanoic acid,* and *5,5-dodecadinyl-1 12-diol* for *K. pneumonia.*

We should speak more cautiously about the type of compounds proposed as biomarkers. *In-vivo*, *in-vitro*, infection, or colonization in the respiratory tract without causing infection, can all affect the production of VOCs. In this study and various other studies, it was determined that this method can be used as an accurate and non-invasive method to identify bacteria. However, more studies are needed to design a tool for which all aspects have been assessed (for example assessment and detection of VOCs of more types of bacteria in different states), so that it can give us a more complete pattern for the use of these compounds as biomarkers.

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