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Comparative study of the cellular fatty acids of different *Staphylococcus* species isolated from human blood in Iraq

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Abstract

The cellular fatty acid composition of ten different clinical *Staphylococcus* species namely*: S. aureus, S. haemolyticus, S. cohnii, S.lentus, S. auricularis, S. hominis, S. lugdunensis, S. epidermidis, S. equorum, S. warneri* were analyzed by high Performance liquid chromatography (HPLC). Results indicated that the fatty acid compositions of the staphylococci were qualitatively similar but differ quantitatively with respect of relative percentages. All the *Staphylococcus* species understand contained $C_{14:0}$, $C_{15:Br}$, C_{16}, $C_{17:Br}$, C_{18}, C_{19} and C_{20} .The quantitative data obtained were examined by cluster analysis , a dendrogram obtained from the analysis showed two major clusters and one member cluster. The higher similarities between the tenth isolates were 53.5. The successful use of the computerized HPLC analysis in this study demonstrated its appropriate application for identification of *Staphylococcus* species.

Keywords: *Staphylococcus* species, Fatty acids profile, HPLC, Euclidean distance.

Introduction

Both of coagulase–positive and coagulase–negative *Staphylococcus* sp . considered among the most important agents of nosocomial infections (Ragbetli *et al*., 2016 ; Karmakar *et al*., 2016). The genus *Staphylococcus* comprises 47 different species (Prax *et al*., 2013). Therefore, identification of this pathogen is needed to provide useful information for the diagnosis of infections caused by this bacterial pathogen. Identification in clinical laboratories based usually on using different biochemical tests. (UK standards, 2014).Several automated systems including MicroScan, Vitek2 and Crystal GP are available today achieve the identification of this pathogen (Kim *et al*., 2008).

For epidemiological typing of *Staphylococcus* spp. several methods have been employed including (biochemical characterization) that could be achieved by using conventional method or by using automated systems (Bobenchik *et al*., 2014). Antibiogram or antimicrobial susceptibility pattern (Hemamalini *et al*., 2015) serotyping or serological typing (Sutter *et al*., 2011). Molecular techniques including DNA-DNA hypridization and plasmid profile have been used for strain characterization. (Tong *et al*., 2015; Costa *et al.,* 2016). The fatty acid profiles of *Staphylococcus* spp. had been used by many authors for identification of bacteria (Li *et al*., 2010; Morey, 2013). In the present study the fatty acid composition of ten various *Staphylococcus* spp. clinical isolates were determined using HPLC and the quantitative data obtained were examined by cluster analysis.

Materials and Methods

Bacterial cultures: The tenth *Staphylococcus* species namely: *S.aureus, S. haemolyticus, S. cohnii, S. lentus, S. auricularis, S. hominis, S. lugdunensis, S. epidermidis, S. equorum, S. warneri* were isolated from human blood and were diagnosed by using ID – GPC card and $Vitek_2$ system by the present authors (F. Ahmed and L. Said).

Media and growth conditions: Each of the tenth *Staphylococcus* spp. isolates was grown in 25 ml trypticase soy broth with dextrose. Preparation of cultures were done by incubation of the previously mentioned medium with 10^8 colony - forming unit from an 18-hrs old culture, rotary shaker (20) rpm at $34C^{o}$ for 24 hrs. was used for incubation according to the method of (Durham & Kloos , 1978) with modification .

Fatty acid analysis: The fatty acids were analyzed by the method described by (Sehat *et al*., 1998) with modifications. *Staphylococcus* sp. cells were collected and 50 mg of potassium oxalate, 10ml ethyl alcohol were placed in blender jar for homogenized for 3min , the jar content were poured in 250ml centrifuge tube, 10ml at diethyl ether and 10ml petroleum ether were added , the content and were mixed for 1min, after each addition of solvent . The mixture was centrifuged at 1700 rpm for 7min at room temperature. The lower phase was re extracted two more times with 10 ml petroleum ether and diethyl ether (1: 1 vol : vol). The combined organic phases were transferred to 500 ml separating funnel containing 25ml distilled water, and 20ml saturated NaCl, the organic layers were washed with 50ml distilled water, the emulsion were removed by adding 2-5ml NaCl, the organic allowed to stand for 5min to settle down, the liquid extracted were evaporated to 10ml by rotary evaporator at 37^{0}C.

Detection of fatty acid by HPLC: The high performance liquid chromatography (HPLC), model Shimadzu 10 AV- LC equipped with binary delivery pump model LC- 10 A Shimadzu, the eluted peaks were monitored by Shimadzu SPD 10 A VP detector were used to detect the fatty acid and there concentration according to below conditions:

The mixture of fatty acid were separated on FLC (Fast Liquid Chromatographic) column, 3µm particle size, (50 × 2.0mm LD) C– 8DB column. Mobile phase were: acetonitrile: tetrahydrofuran (THF): 0.1% phosphoric acid in THF (50.4: 21.6: 28, V/V). Detection: UV set at 215 nm, Flow rate 1.5 ml / min., temp: 40^{0}C. The data were recorded on Shim-pack C- R8A (Shimadzu, Kyoto, Japan)

Statistical analysis: The similarity index were calculated between individual fatty acid profile by using the complete linkage method which is one of the hierarchical Agglomerative the algorithm (Sneath and Sakai, 1973) the relatedness among *Staphylococcus* sp. isolates based on their fatty acid composition was assessed with cluster analysis (Euclidean distance) by using the computer and according to statistical program Statistical Package for Social Sciences (SPSS) version 10 and according to the equation :

$$\text{Distance (dij)} = \sqrt{\sum(\text{xi} - \text{xj})2}$$

Distance (dij) = the distance between two strains (Euclidean distance).

i = Epithet strain rate i

j = Epithet strain rate j

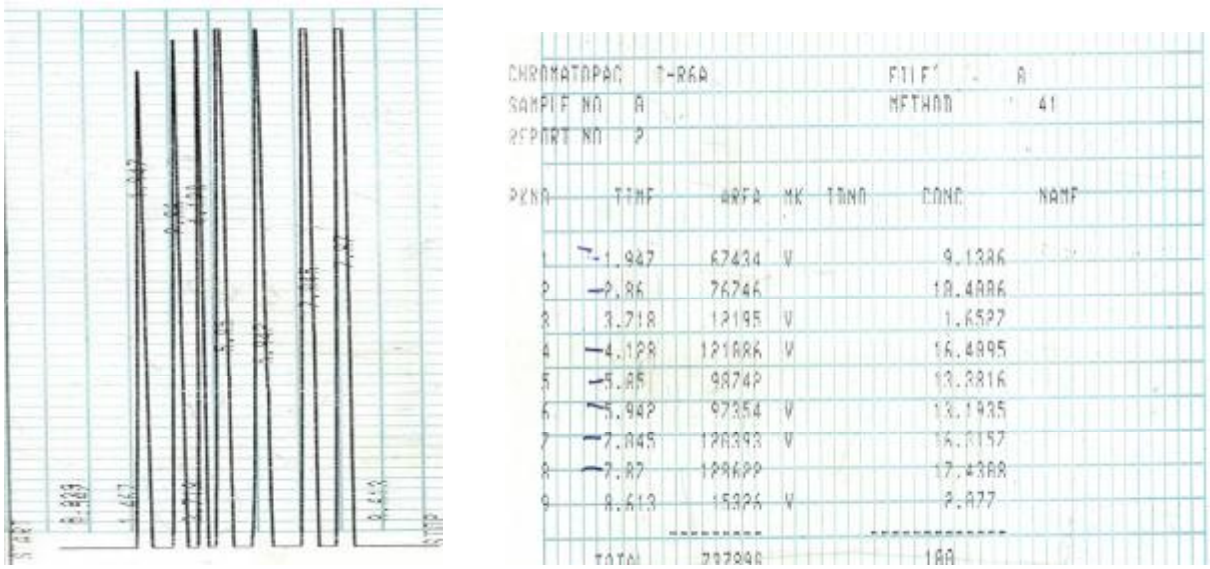
Results and Discussion

The standard fatty acids and their concentration shown in (Table-1) were used for the detection of fatty acids type in *Staphylococcus* sp.

Table (1): The standard fatty acids and their concentration

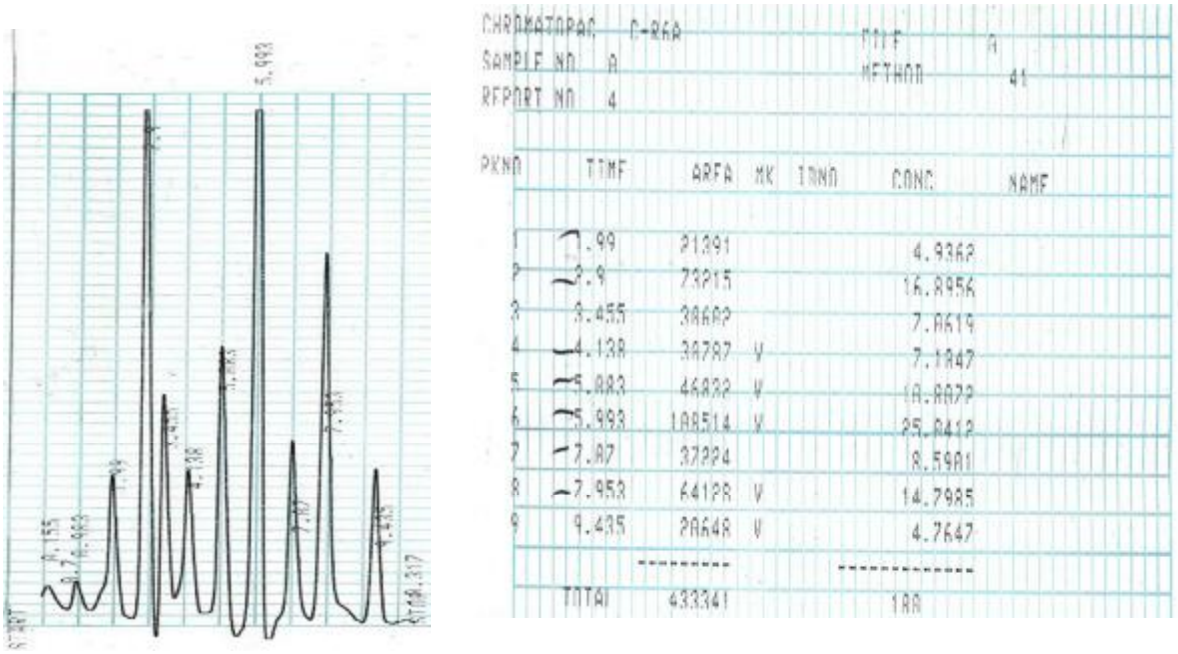
No.	standard fatty acids	Retention time minute	Area	Concentration 25 µg / ml
1	Tetradecanoate C14:0	1.94	67434	25 µg / ml
2	Pentadecanoate: C15 : Br	2.86	76746	25 µg / ml
3	Hexadecanoate C: 16	4.12	121086	25 µg / ml
4	Heptadecanoate C 17 : Br	5.05	98742	25 µg / ml
5	Octodecanoate C 18	5.94	97354	25 µg / ml
6	Nanodecanoate C19	7.04	120393	25 µg / ml
7	Eicosanoate C 20	7.87	128622	25 µg / ml

The cells of isolates were collected and the fatty acids were extracted according to the method mentioned before .The analysis of the fatty acids were done by using HPLC method to detect fatty acids according to the retention time for each . Different chromatograms were obtained as shown in (Figures 2-11).



PEAK	TIME	AREA	MK	TANG	CONC	NAME
1	1.987	67434	V		9.1336	
2	2.86	76746			10.4006	
3	3.718	12195	V		1.6527	
4	4.128	121686	V		16.4895	
5	5.85	98749			13.3816	
6	5.992	97354	V		13.1935	
7	7.045	120393	V		16.3152	
8	7.87	129629			17.4388	
9	8.613	15926	V		2.077	
	TOTAL	737898			100	

(**Figure 1) shows the HPLC chromatogram of the standard fatty acids used.**



PEAK	TIME	AREA	MK	TANG	CONC	NAME
1	1.99	21391			4.9362	
2	2.9	73915			16.8956	
3	3.455	38682			7.8619	
4	4.138	38787	V		7.1847	
5	5.883	46832	V		10.8079	
6	5.993	108514	V		25.0412	
7	7.87	37994			8.5981	
8	7.953	64198	V		14.7985	
9	9.435	20648	V		4.7647	
	TOTAL	433341			100	

Figure (2) HPLC chromatogram of the fatty acids of *S. aureus* .

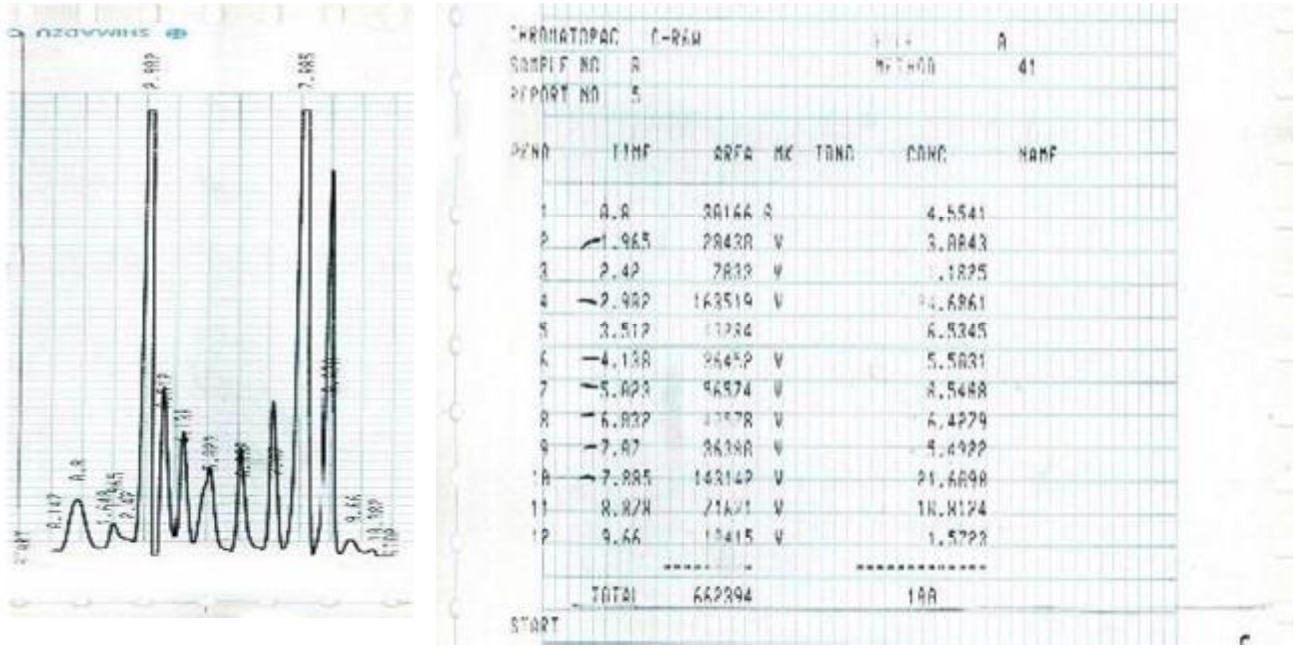


Figure (4): HPLC chromatogram of the fatty acids *S.cohnii*.

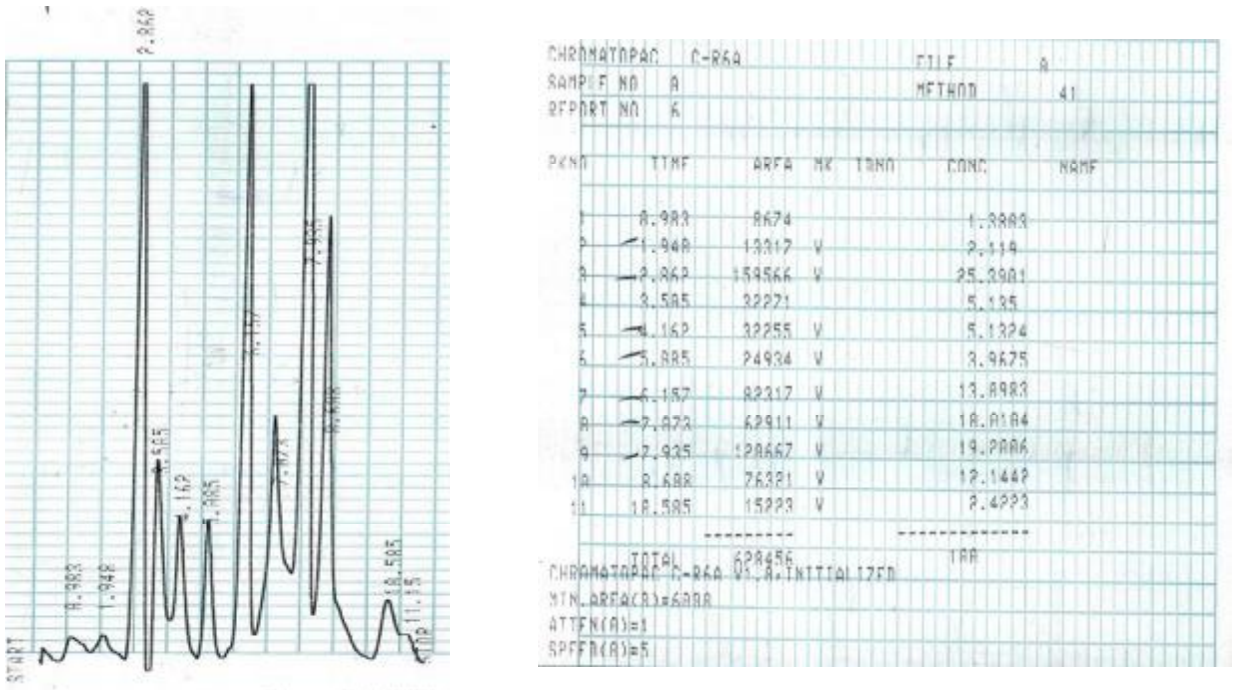
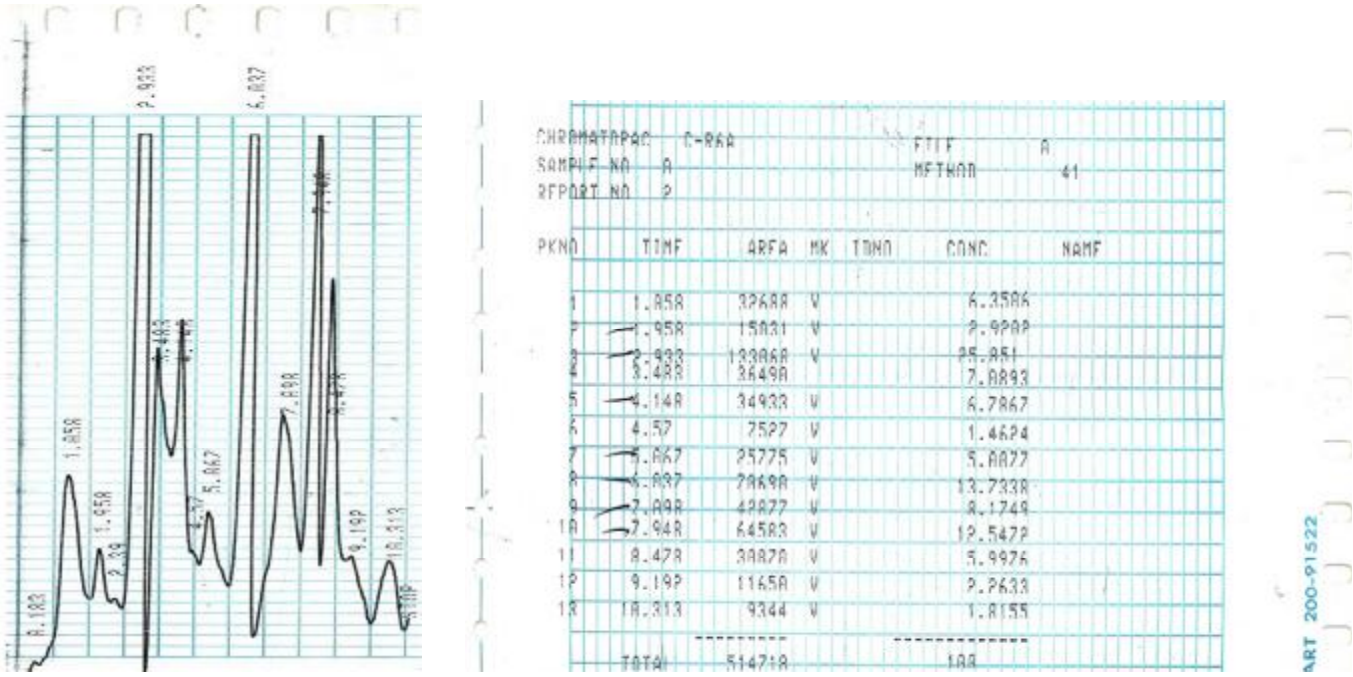
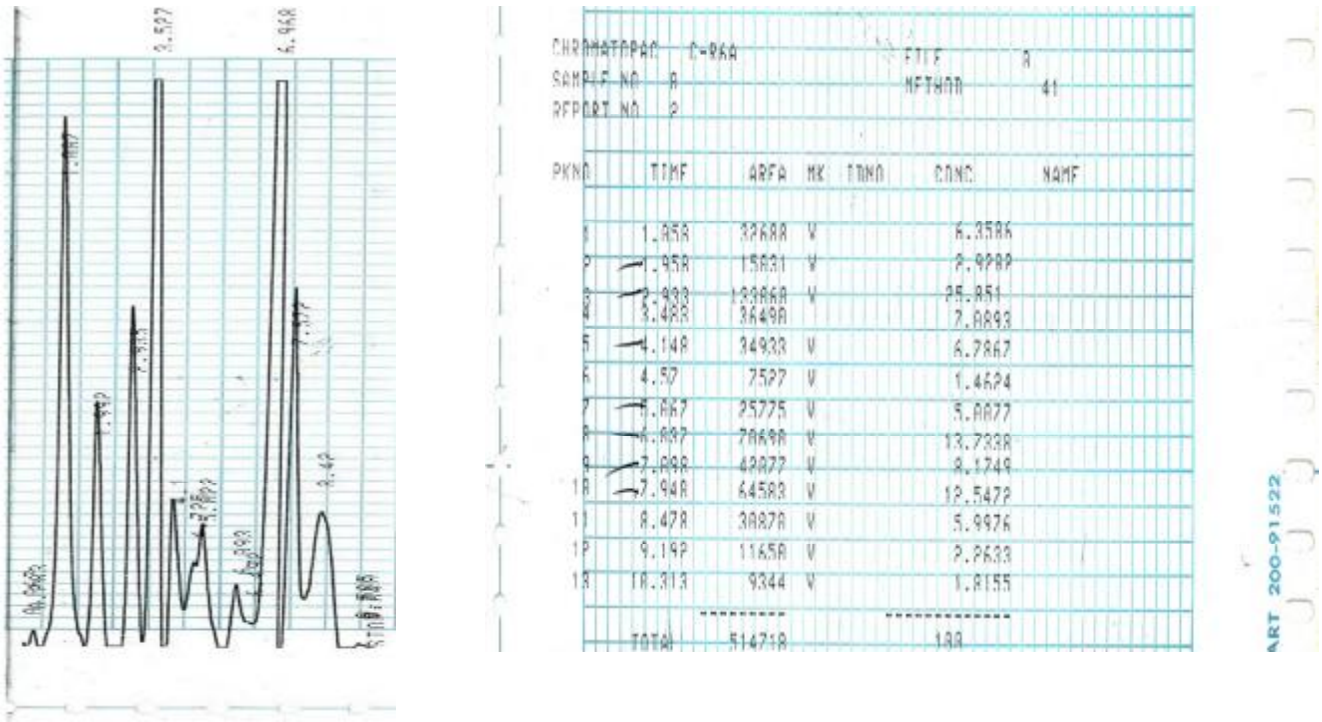


Figure (5): HPLC chromatogram of the fatty acids of *S. lentus.*



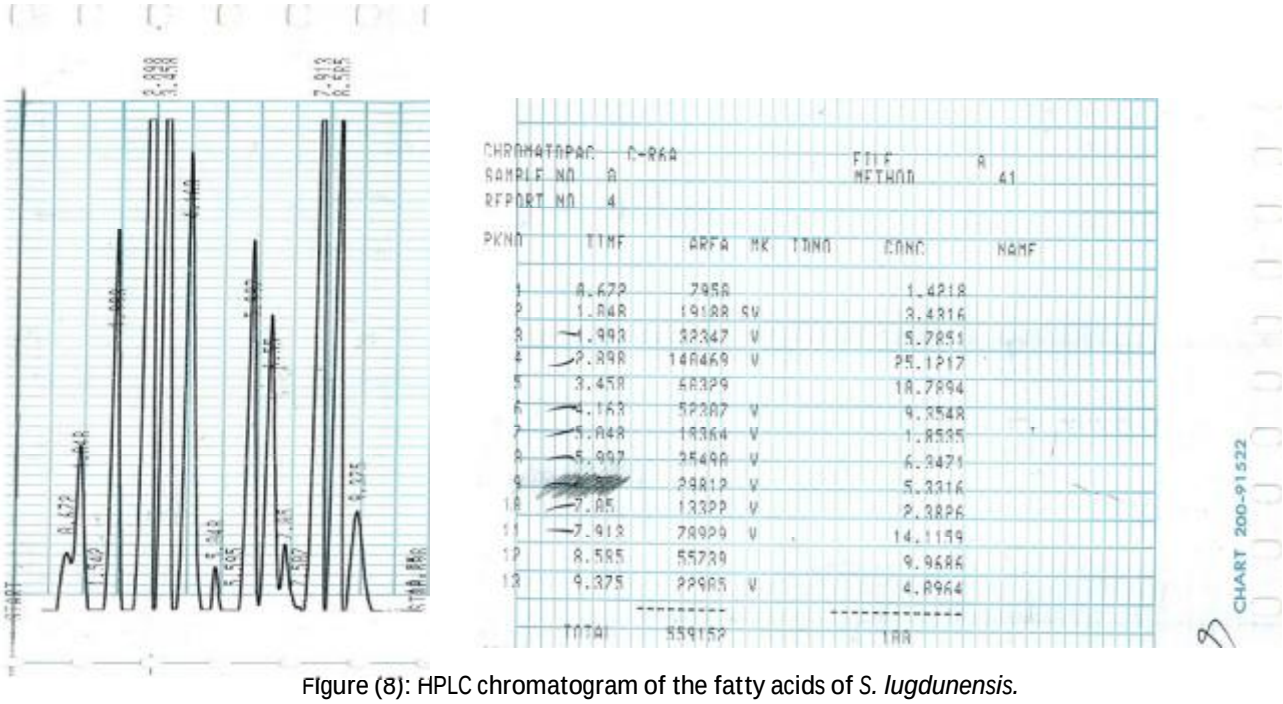
PKNO	TIME	AREA	MK	TUNO	CONC	NAME
1	1.858	32688	V		6.3586	
2	1.958	15831	V		2.9989	
3	2.993	139868	V		25.851	
4	3.488	36498			7.0893	
5	4.148	34933	V		6.7867	
6	4.57	7527	V		1.4624	
7	5.867	25775	V		5.0077	
8	6.897	70698	V		13.7338	
9	7.898	42877	V		8.1749	
10	7.948	64583	V		12.5472	
11	8.478	30876	V		5.9976	
12	9.198	11658	V		2.2633	
13	10.313	9344	V		1.8155	
	TOTAL	514718			100	

Figure (6): HPLC chromatogram of the fatty acids of *S. auricularis.*



PKNO	TIME	AREA	MK	TUNO	CONC	NAME
1	1.858	32688	V		6.3586	
2	1.958	15831	V		2.9989	
3	2.993	139868	V		25.851	
4	3.488	36498			7.0893	
5	4.148	34933	V		6.7867	
6	4.57	7527	V		1.4624	
7	5.867	25775	V		5.0077	
8	6.897	70698	V		13.7338	
9	7.898	42877	V		8.1749	
10	7.948	64583	V		12.5472	
11	8.478	30876	V		5.9976	
12	9.198	11658	V		2.2633	
13	10.313	9344	V		1.8155	
	TOTAL	514718			100	

Figure (7): HPLC chromatogram of the fatty acids of *S. hominis.*



CHROMATOPAC C-R6A FILE A
SAMPLE NO 0 METHOD 41
REPORT NO 4

PKNO	TIME	AREA	MK	TANO	CONC	NAME
1	0.672	7958			1.4218	
P	1.048	19188	SV		3.4316	
3	1.993	32347	V		5.7851	
4	2.998	140469	V		25.1217	
5	3.458	58329			10.7894	
6	4.163	52387	V		9.3548	
7	5.048	10364	V		1.8535	
8	5.997	35498	V		6.3471	
9	[illegible]	29812	V		5.3316	
10	7.05	13322	V		2.3826	
11	7.913	78929	V		14.1159	
12	8.585	55739			9.9686	
13	9.375	22985	V		4.1964	
	TOTAL	559152			100	

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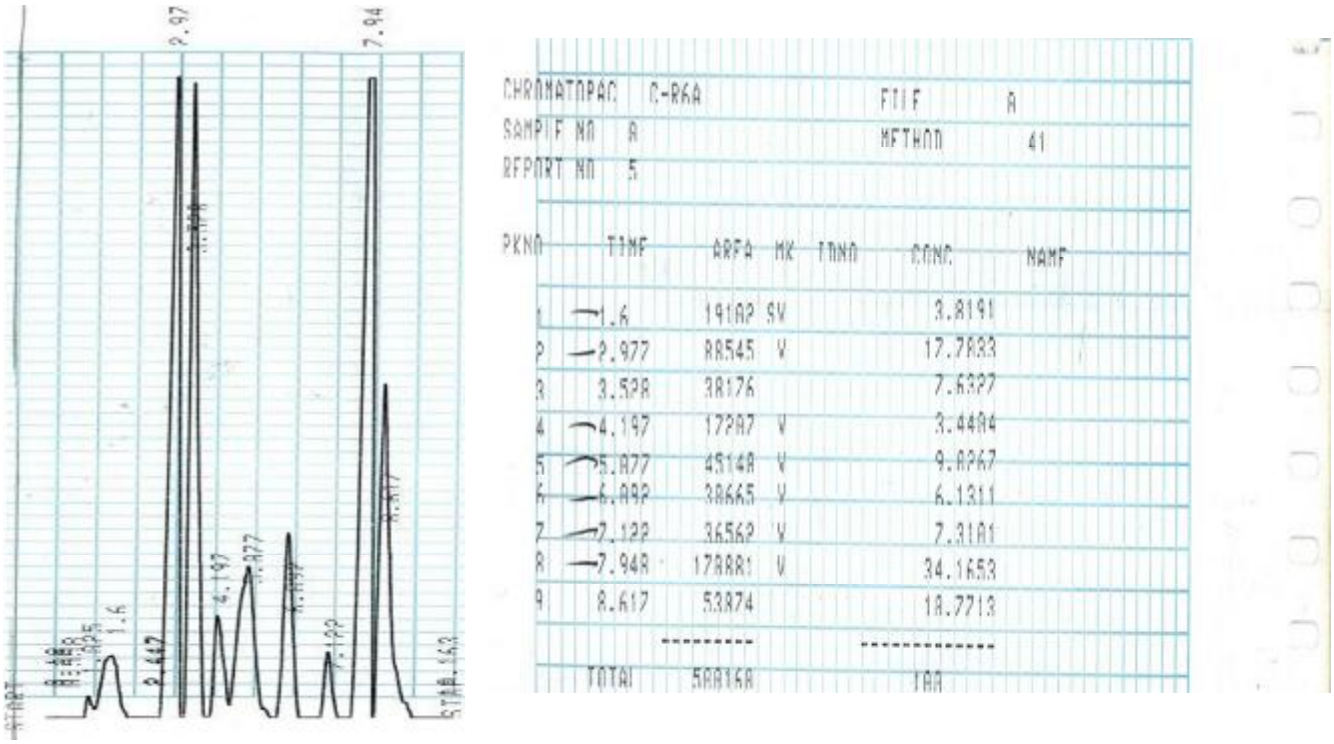
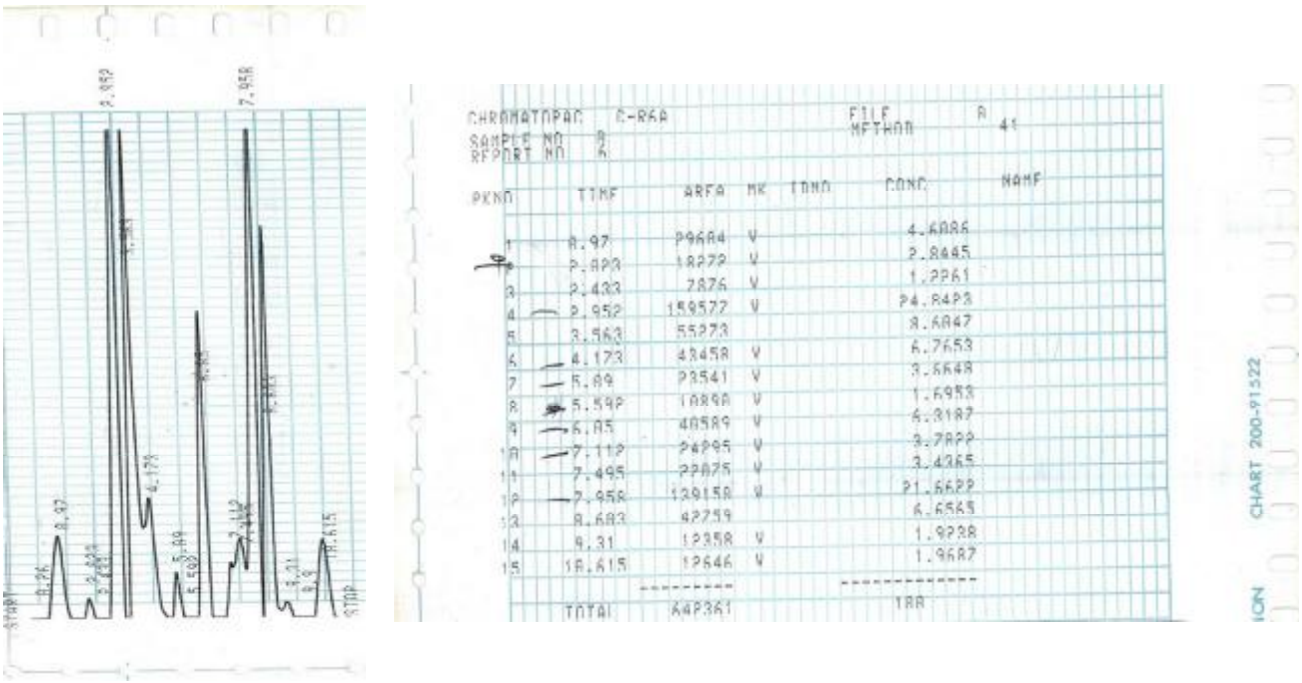
Figure (8): HPLC chromatogram of the fatty acids of *S. lugdunensis.*



CHROMATOPAC C-R6A FILE A
SAMPLE NO A METHOD 41
REPORT NO 5

PKNO	TIME	AREA	MK	TANO	CONC	NAME
1	1.6	19182	SV		3.8191	
P	2.977	88545	V		17.7833	
3	3.528	38176			7.6327	
4	4.197	17287	V		3.4484	
5	5.877	45148	V		9.0967	
6	6.892	30665	V		6.1311	
7	7.122	36562	V		7.3101	
8	7.948	170881	V		34.1653	
9	8.617	53874			10.7713	
	TOTAL	500126			100	

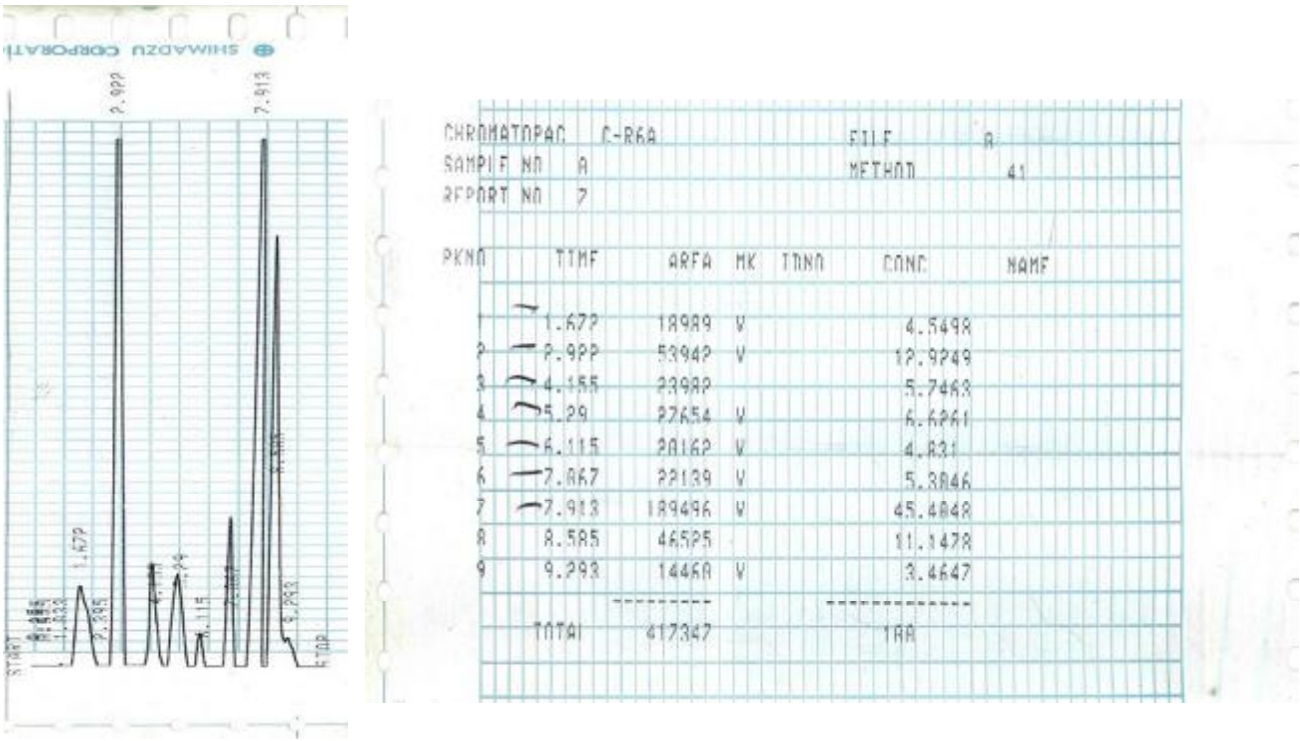
Figure (9): HPLC chromatogram of the fatty acids of *S.epidermidis.*



CHROMATOPAC C-R6A FILE A
SAMPLE NO A METHOD 41
REPORT NO 6

PKNO	TIME	AREA	MK	TUNO	CONC	NAME
1	8.97	P9684	V		4.6086	
2	P.899	18272	V		P.8645	
3	P.433	7876	V		1.2961	
4	P.952	159572	V		P4.8493	
5	3.563	55273			8.6847	
6	4.173	43458	V		6.7653	
7	5.89	P3541	V		3.5648	
8	5.599	10899	V		1.6953	
9	6.05	40589	V		6.3187	
10	7.112	24295	V		3.7899	
11	7.495	22875	V		3.4365	
12	7.958	139158	V		P1.6629	
13	8.683	42759			6.6565	
14	9.31	12358	V		1.9238	
15	10.615	12646	V		1.9687	
	TOTAL	649361			100	

Figure (10): HPLC chromatogram of the fatty acids of *S. equorum.*



CHROMATOPAC C-R6A FILE A
SAMPLE NO A METHOD 41
REPORT NO 7

PKNO	TIME	AREA	MK	TUNO	CONC	NAME
1	1.672	18989	V		4.5498	
2	P.999	53949	V		12.9249	
3	4.155	23989			5.7463	
4	5.29	27654	V		6.6261	
5	6.115	20162	V		4.831	
6	7.867	22139	V		5.3046	
7	7.913	189496	V		45.4848	
8	8.585	46525			11.1478	
9	9.293	14468	V		3.4647	
	TOTAL	417347			100	

Figure (11): HPLC chromatogram of the fatty acids of *S. warneri*.

HPLC had been used by many researchers to determine the fatty acids composition of many bacteria, a study by (Yamamoto *et al*., 1998) used HPLC to detect the fatty acids composition of 11 species of thermophilic clostridia. Another research conducted by (De Baere *et al*., 2013) had used a HPLC-UV method for the quantitative determination of some short-chain fatty acids and lactic acid produced by some intestinal bacteria. The RT for the fatty acids $C_{\cdot 14:0}$, $C_{15:Br}$, C_{16} , $C_{17:Br}$, $C_{18,}C_{19}$ and C_{20} were 1.94 , 2.86 , 4.12 , 5.05 , 5.94 , 7.04 and 7.87 respectively. Result in Table (2) shows different concentrations of fatty acids types that were extracted and detected in each isolate.

Table (2): Fatty acids standard and fatty acids concentration in different *Staphylococcus* sp.

N	Sample	C14:0 1.94		C15:Br 2.86		C16 4.12		C17:Br 5.05		C18 5.94		C19 7.04		C20 7.87	
		A	Conc. (mg/mL) %	A	Conc. (mg/mL) %	A	Conc. (mg/mL) %	A	Conc. (mg/mL) %	A	Conc. (mg/mL) %	A	Conc. (mg/mL) %	A	Conc. (mg/mL) %
	Standard	67434	25	76746	25	121086	25	98742	25	97354	25	120393	25	128622	25
1	***S.aureus***	30502	11.30 (6.37)%	163681	53.31 (31.34) %	50647	10.45 (5.89)%	86629	21.93 (12.37)%	137222	35.23 (19.88)%	48902	10.15 (5.72)%	142637	27.72 (15.64) %
2	***S.haemolyticus***	21391	7.93 (8.09)%	73215	23.84 (24.32) %	30787	6.35 (6.47)%	46832	11.85 (12.09)%	108514	27.86 (28.42)%	37224	7.72 (7.87)%	64128	12.46 (12.71) %
3	***S.cohnii***	20430	7.57 (5.86)%	163519	53.26 (41.29) %	36452	7.52 (5.83)%	56574	14.32 (11.10)%	42578	10.93 (8.47)%	36380	7.55 (5.85)%	143142	27.82 (21.57) %
4	***S.lentus***	13317	4.93 (3.8)%	159566	51.97 (40.76) %	32255	6.65 (5.21)%	24934	6.31 (4.94)%	82317	21.13 (16.57)%	62911	13.06 (10.24) %	120667	23.45 (18.39) %
5	***S.auricularis***	15031	5.57 (5.45)%	133060	43.34 (42.46) %	34933	7.21 (7.06)%	25775	6.52 (6.38)%	70690	18.15 (17.78)%	42077	8.73 (8.55)%	64583	12.55 (12.29) %
6	***S.hominis***	17560	6.51 (12.44) %	23995	7.81 (14.93) %	15288	3.15 (6.02)%	20701	5.24 (10.01)%	12902	3.31 (6.32)%	98211	20.39 (38.97) %	30374	5.90 (11.27) %
7	***S.lugdunensis***	32347	11.99 (12.18) %	140469	45.75 (46.51) %	52307	10.79 (10.96) %	10364	2.62 (2.66)%	35490	9.11 (9.26)%	13322	2.76 (2.80)%	78929	15.34 (15.59) %
8	***S.epidermidis***	19102	7.08 (7.11)%	88545	28.84 (28.96) %	17207	3.55 (3.56)%	45148	11.43 (11.47)%	30665	7.87 (7.90)%	36562	7.59 (7.62)%	170881	33.21 (33.35) %
9	***S.equorum***	18272	6.77 (5.82)%	159577	51.98 (44.74) %	43458	8.97 (7.72)%	23541	5.96 (5.12)%	40589	10.42 (8.96)%	24295	5.04 (4.33)%	139150	27.04 (23.27) %
10	***S.warneri***	18989	7.03 (8.45)%	53942	17.57 (21.13) %	23982	4.95 (5.95)%	27654	7 (8.41)%	20162	5.17 (6.21)%	22139	4.59 (5.52)%	189496	36.83 (44.29) %

The fatty acids composition of the *Staphylococcus* spp. isolates were qualitatively similar , all species had the fatty acids C_{14} , $C_{15:Br}$, C_{16} , $C_{17:Br}$, C_{18} , C_{19} , C_{20}. Results obtained in this study is in a good agreement with the results reported by several authors based on various growth media and conditions (Durham *et al*., 1978; Donnell *et al*., 1985; Kotilainen *et al*, 1991). (Durham *et al*., 1978) had found out that different *Staphylococcus* sp. had fatty acids which were in the range of C_{14} to C_{20}. And mentioned the presence of fatty acids which were in the range of C_{14} to C_{20} in 100 strains representing different species of *Staphylococcus* analyzed by gas-liquid chromatography. The study conducted by (O' Donnell *et al*., 1985) revealed the detection of straight chain and methyl branched iso and anteiso fatty acids of between 12 and 22 carbons in all the strains of coagulase–positive staphylococci and coagulase–negative staphylococci by using both of gas chromatography and analytical and preparative thin – layer chromatography in their study. Another study performed by (Kotilainen *et al.,* 1991) conducted that by using gas liquid chromatography a total of 21 different fatty acid were detected in their chromatograms among them the fatty acids in range C_{12} to C_{20}. A recent study by (Saichek *et al*. ,2016) revealed that various *Staphylococcus* spp. contained fatty acids which were the range C_3 to C_{26} their study had mentioned that even – numbered fatty acids C_{14} , C_{16} , C_{18} were more prevalent in methicillin resistant *S. aureus* isolates while odd – numbered fatty acids C_{13} , C_{17} , C_{19} and C_{21} were more prevalent in MSSA isolates. Results appeared in (Table-2) shows that the same fatty acids were present in all the analyzed *Staphylococcus* spp. isolates understudy , while different concentrations of each fatty acid found in different isolates this result is in agreement with the result obtained by (Saichek *et al*. ,2016).As shown InTable (2) among the tenth *Staphylococcus* spp. the isolate *S.lugdunensis* was characterized by high concentrations of the fatty acids ($C_{14:0}$) and ($C_{16:0}$), *S.aureus* was characterized by high concentrations of fatty acids $C_{15:0}$, $C_{17:0}$, $C_{18:0}$, *S.hominis* was characterized by high concentrations of the fatty acids $C_{19:0}$ and *S.warneri* was characterized by the high concentrations of the fatty acids $C_{20:0}$, while isolate *S.lentus* was characterized by low concentrations of fatty acids $C_{14:0}$, *S.hominis* was characterized by low concentrations of $C_{15:0}$, $C_{16:0}$, $C_{18:0}$, $C_{20:0}$ and *S.lugdunensis* was characterized by low concentrations of $C_{17:0}$, $C_{19:0}$. *S.aureus* isolate contained a relatively large percentage of $C_{15:\ Br}$ fraction, this result is in agreement with the result obtained by (Parsons *et al*., 2011) as shown in (Figure 12).

S.warneri isolate contained a relatively large percentage of C_{20} , this result is in agreement with the result obtained by (Durham and Kloos., 1978) as shown in (Figure 13).

S. epidermidis isolate contained a relatively large percentage of C_{20}, this result is in agreement with the result obtained by (Wieser and Busse., 2000) as shown in (Figure 14).

S.hominis isolate contained large amount of fatty acid C_{19} ; this result disagree with the result obtained by (Kotilainen *et al*., 1991) when they had found out that the largest amount of fatty acids in their *S.hominis* isolate was $C_{:20}$. as shown in (Figure 15). *S.haemolyticus* isolate contained relatively large amount of $C_{:18}$, this result disagree with the result obtained by (Kotilanen *et al*,1991) when they had found out that the largest amount of fatty acids in their *S.haemolyticus* isolate was $C_{:17}$ as shown in (Figure 16).

S.cohnii, S.lentus, S.auricularis, S.lugdunensis, S.equorum contained relatively large amount of $C_{:15}$, this result agree with the results obtained by (Tornabena *et al*., 1970; Crompton *et al*., 2014) as shown in Figures (17-21).

Numerical analysis of fatty acid profile: A similarity matrix (Table 3) and a dendrogram (Figure 22) were obtained from the numerical analysis based on (Euclidean distance) clustering.

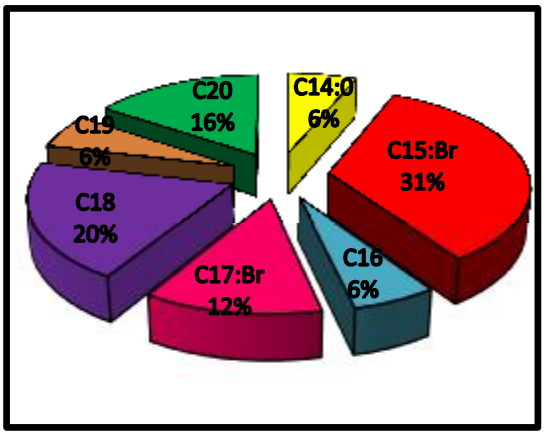


Figure (12): The percentages of different fatty acids in *S.aureus* isolate

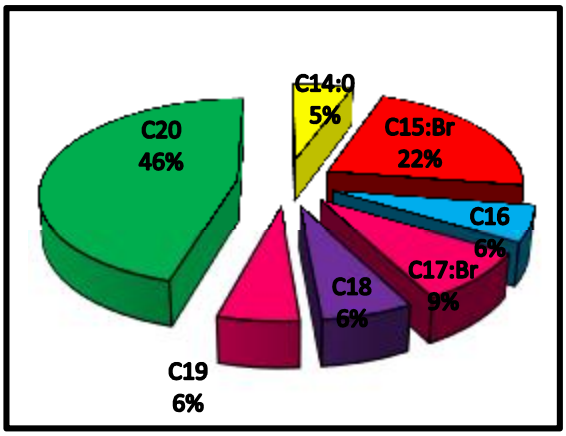


Figure (13): The percentages of different fatty acids in *S.warneri* isolate

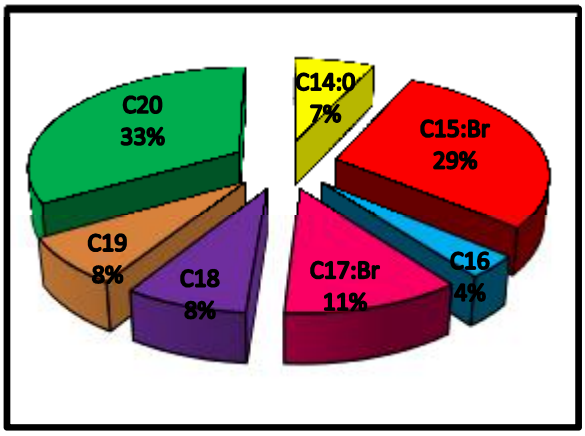


Figure (14): The percentages of different fatty acids in *S.epidermidis* isolate

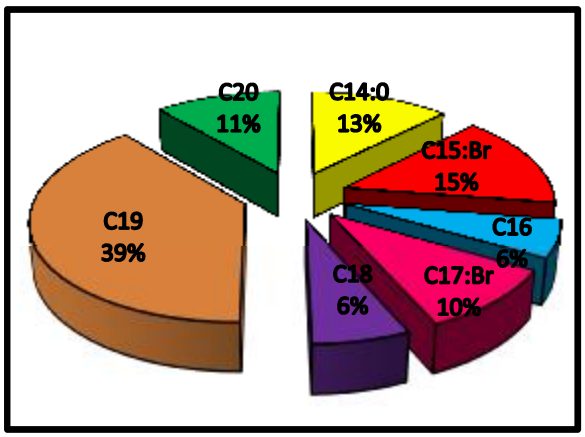


Figure (15): The percentages of different fatty acids in *S.hominis* isolate

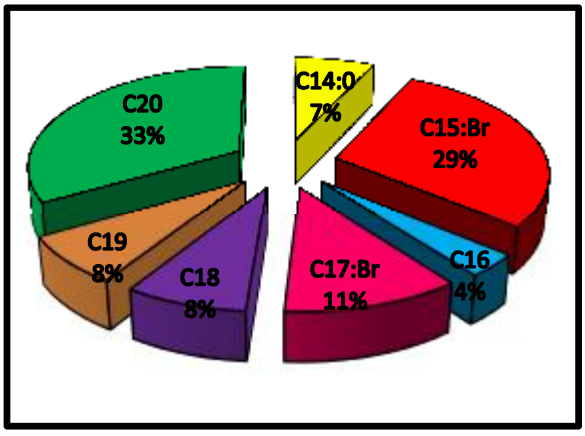


Figure (14): The percentages of different fatty acids in *S.epidermidis* isolate

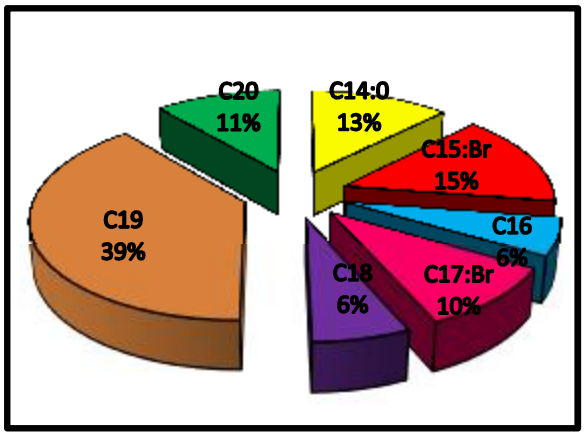


Figure (15): The percentages of different fatty acids in *S.hominis* isolate

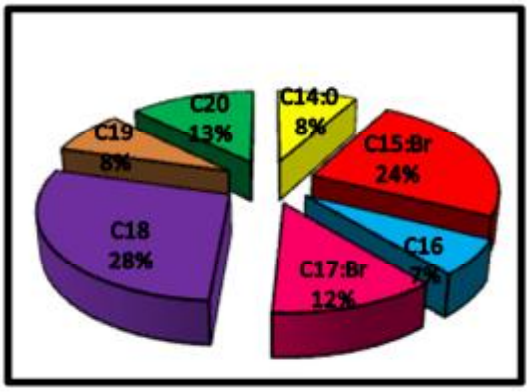


Figure (16): The percentages of different fatty acids in *S.haemolyticus* isolate

Table (3): Similarity matrix based on numerical analysis of fatty acids composition of *Staphylococcus* sp. isolates

	VAR00001	VAR00002	VAR00003	VAR00004	VAR00005	VAR00006	VAR00007	VAR00008	VAR00009	VAR00010
VAR00001	.000	41.946	26.994	24.404	33.102	68.705	38.419	44.238	31.476	55.887
VAR00002	41.946	.000	37.359	32.026	22.589	33.454	31.407	29.392	36.860	34.411
VAR00003	26.994	37.359	.000	15.068	21.214	53.589	20.082	25.673	9.024	38.176
VAR00004	24.404	32.026	15.068	.000	14.889	51.432	20.837	29.605	14.171	41.190
VAR00005	33.102	22.589	21.214	14.889	.000	41.010	14.167	27.997	19.048	38.036
VAR00006	68.705	33.454	53.589	51.432	41.010	.000	44.385	37.570	52.139	36.216
VAR00007	38.419	31.407	20.082	20.837	14.167	44.385	.000	27.948	14.908	36.727
VAR00008	44.238	29.392	25.673	29.605	27.997	37.570	27.948	.000	25.415	13.341
VAR00009	31.476	36.860	9.024	14.171	19.048	52.139	14.908	25.415	.000	36.400
VAR00010	55.887	34.411	38.176	41.190	38.036	36.216	36.727	13.341	36.400	.000

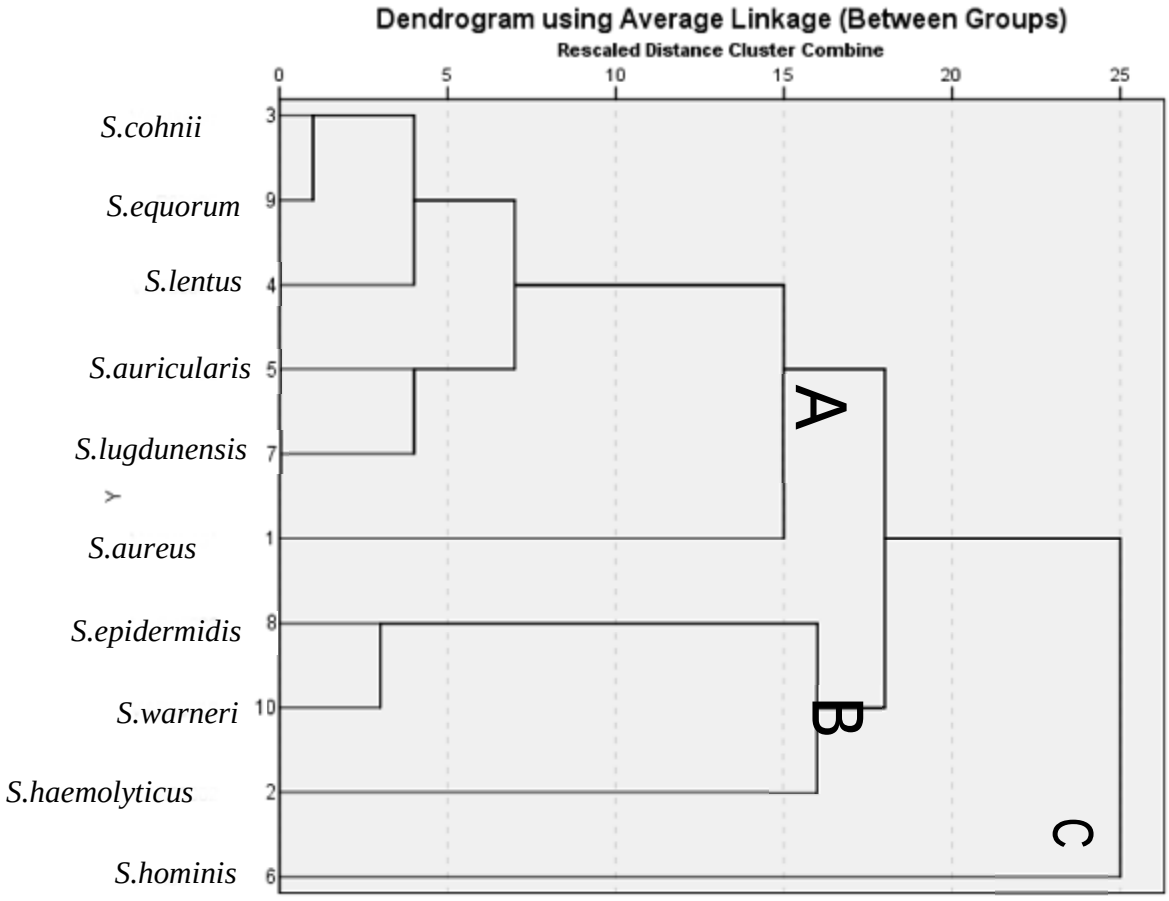


Figure (22): A dendrogram showing the relationships between *Staphylococcus* spp. isolates based on the analysis of fatty acids compositions using Euclidean distance and complete linkage method

As shown in Figure (22) the tenth different *Staphylococcus* sp. were separated into three major clusters, two clusters (A and B) and one single member cluster (C) .Cluster (A) *contained S. cohnii, S. lentus, S. auricularis, S. lugdunensis, S. equorum* and *S. aureus*, the inter group average similarity was 26.9%. Cluster (B) *contained S. warneri, S. haemolyticus, S. epidermidis,* the inter group average similarity was 29.39% . Cluster (C) consist of *S.hominis* linked to the other isolates in a significantly at 53.5% level. The lower linkage between the tenth isolates were 9.02%. As shown in Table (4) the similarities values between *Staphylococcus* species were 9.02, 13.34, 14.16, 14.61, 18.49, 18.49, 30.87, 31.90, 34.97, and 46.50% were between isolates *S. cohnii* and *S. equorum*, *S. epidermidis* and *S. warneri, S. auricularis and S. lugdunensis, S. cohnii* and *S. lentus*, *S. cohnii and S. auricularis, S. aureus* and *S. cohnii; S. haemolyticus and S. epidermidis; S. aureus and S. haemolyticus, S. aureus* and *S. hominis*, respectively. Many authors used Euclidean distances to differentiate between isolates depending on their fatty acid profile (Diogo *et al*., 1999; Lanoiselet *et al*.,2005). Euclidean distance had been used by (Saichek *et al*., 2016) to draw a dendrogram showing the differences between *Staphylococcus* spp.isolates depending on their fatty acid profile.

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