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Original Article

Impact of Tween 80 on Fatty Acid Composition in Two Bacterial Species

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Abstract

Tween 80 is a well-known non-ionic emulsifier which is used in pharmaceutical and food industries. Due to its widespread applications it is needed to understand how it affects bacteria. The current study aimed to investigate the effect of Tween 80 on the fatty acid content of bacteria. Three bacterial isolates were used in this study: two isolates of *Serratia marcescens* and one isolate of *Acinetobacter baumannii*. The analysis of fatty acid components of bacterial lipids was performed, followed by the assessment of the effect of Tween 80 on fatty acid content by adding it to the culture medium in three concentrations: 0.1%, 0.5% , and 1.0 % .The results indicated that the Tween 80 has the ability to change the fatty acid content present in these bacteria by the appearance and disappearance of three fatty acids, including Elaidic acid 18.1 trance 9, Oleic acid 18.1 cis 9, and erucic acid 22.1, which belong to Mono-Unsaturated Fatty Acids (MUFA), in the presence of different concentrations of Tween 80. The results also demonstrated that the p-value was significant in two situations, the first belonged to *S. marcescens* at 0.5 % concentration for all groups of FAs (Saturated fatty acids, Monounsaturated fatty acids, and polyunsaturated fatty acids), and the second was for *S. marcescens* just in MUFA in all concentrations of Tween 80.

Keywords: Fatty acids, *Serratia marcescens*, *Acinetobacter baumanniim*, Tween 80

1. Introduction

Serratia marcescens (S. marcescens) is a waterborne bacterium which is responsible for urinary tract infections and nosocomial pneumonia. The contamination of medical devices that have been exposed to contaminated water is the main spreading pathway of these bacteria. *Serratia* is used in industries for the production of 2,3-butanediol which is a biomass alcohol as a replacement for petroleum products (1).

Acinetobacter are Gram-negative, oxidase- negative, non-fermentative bacteria. The genus *Acinetobacter* consists of 51 named species and 11 unnamed species, 26 cases of which are pathogenic to human. They are the second- most- common nonfermenters isolated from clinical specimens after *Pseudomonas aeruginosa*. Multidrug-resistant *Acinetobacter baumannii (A. baumannii)* produces epidemics in hospitals and increases mortality among patients with systemic infections. The identification of *acinetobacter* spp. can be performed accurately by molecular methods which may be irrelevant in diagnostic laboratories (2).

Numerous time-consuming techniques are used for bacterial identification; therefore, alternative methods should be developed for professionals. The determination of Cellular Fatty Acid (CFA) composition by Gas Chromatography (GC) may provide an alternative method to differentiate between the bacterial species. As early as 1963, Abel, Deschmertzing (3) demonstrated that the normalization of the bacterial fatty acid analysis method with GC is

required for parallel comparison of CFA results obtained in different laboratories (4).

Fatty acids (FAs) which are important components in bacterial cell membranes determine the different structures of lipids and lipid A (5). Tween 80 is commonly added to microbiological growth media to provide an exogenous oleic acid which constitutes the lipophilic group of Tween 80. Moreover, oleic acid is one of the most prevalent FAs in many organisms and is present in a variety of food products (6). Tween 80 contains a polyethoxylated sorbitan esterified to the naturally occurring fatty acid, oleic acid (Figure 1). The addition of tween 80 to the medium provides a controlled method of delivering a physiologically relevant amount of a specific FA to the bacteria.

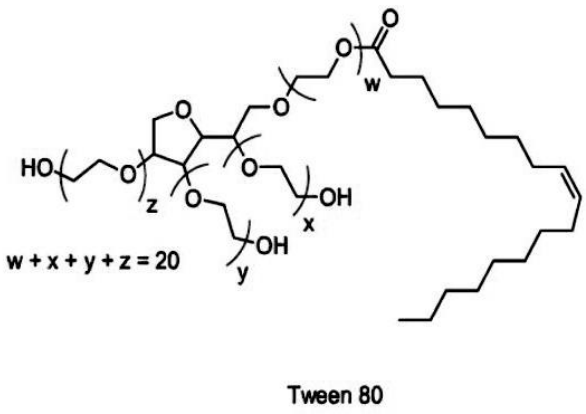

Figure 1. Chemical composition of Tween 80 (7)

Furthermore, growth in the presence of Tween 80 can modify membrane composition (6). In light of the aforementioned issues, the present study aimed to assess the relationship between the supplementation of the growth medium with Tween 80 and the membrane FA composition in two different bacterial species: *S. marcescence* and *A. baumannii*.

2. Materials and Methods

2.1. Bacterial Isolates

Two isolates of *Serratia marcescens* named *S. marcescens* (1) and *S. marcescens* (2) were obtained from the Department of biology/ college of sciences/ university of Mosul/ Iraq, and one isolate of *A. baumannii* was obtained from a previous study (8). The source of all the referred bacteria was from clinical samples.

2.2. Culture

After the incubation of overnight fresh bacterial cells in Brain-Heart Infusion Broth, a loop-full of cells was cultured by streaking method on:

a- Nutrient agar (without any addition, for control)

b- Nutrient agar (With 0.1 % of Tween 80)

c- Nutrient agar (With 0.5 % of Tween 80)

d- Nutrient agar (With 1.0 % of Tween 80). All these cultures were made in triplicate.

2.3. Extraction and Esterification of Fatty Acids

The extraction process was dependent on dos Santos, Moreira (9), and the esterification was achieved by Boron trifluoride-Methanol depending on Hodge, Simpson (10).

2.4. Analysis of Fatty Acids

The analysis was performed in the Ministry of Sciences and Technology/ Baghdad/ Iraq. A number of 15 standard FAs as demonstrated in table 1 were used to measure their retention times and then used to calculate the concentrations of FAs presented in tested bacteria. Capillary Gas Chromatography (CGC) apparatus (SHIMADZU CORPORATion-2010-JAPAN) was used by injecting 1 µl of standerd and extracted bacterial FAs in the capillary column (SP 2480, long: 30 m; 0.25 mm diameter) at 70ºC-250ºC, N_2 was the used gas, and the measurement time was 40 min.

Table 1. Standard fatty acids used in the study

No.	Standard Fatty acids	
1-	Butyric acid	4:0
2-	undecanoic acid	11:0
3-	Myristic	14:0
4-	Palmatic	16:0
5-	Hepadecanoic	17:0
6-	Stearic	18:0
7-	Elaidic	18:1 trance 9
8-	oleic acid	18.1 cis 9
9-	Linoleic acid	18:2
10-	Arachidic acid	20:0
11-	Eicosenoic acid	20:1
12-	Linolenic acid	18:3
13-	Erucic acid	22:1
14-	Arachidonic	20:4
15-	Tricosanoic acid	23:0

2.5. Statistical Analysis

The data were analyzed in SPSS software (version 18) using T-test. A p-value less than 0.05 was considered statistically significant.

3. Results

3.1. Composition of Fatty Acids

Figure 2 displays the results of standard FAs separated by CGC, and the retention time for each standard FA was recorded from the attached report. All FAs were diagnosed in each bacterial sample through the comparison of retention times with those found in bacterial samples. Figure 3 demonstrates FAs analysis of *S. marcescens* (1), *S. marcescens* (2), and *A. baumannii* by CGC.

The FAs in each bacterium *S. marcescens* (1), *S. marcescens* (2), and *A. baumannii* were estimated by the injection of 1 µl from the sample (prepared for measurement after its esterification) in the CGC apparatus, and performing the comparison of retention time.

Figure 4 presents the FAs percentages of three bacterial isolates.

As depicted in figure 4, the fatty acid profiles of three isolates were almost similar to each other with some differences. Nonetheless, the results pointed out that the FAs presented in *S. marcescens* were revealed in different and varying percentages. The highest value was for: 1) saturated FAs arachidic acid 20:0 in both *S. marcescens* 1 and *A. baumannii* with a percentage of (1.3966) and (2.237), respectively, 2) unsaturated FA : linolenic 18:3 had come in the second rank 1.378 , 1.284 and 1.2534 for *S. marcescens* 1 , *S. marcescence* 2, and *A. baumannii*, respectively. On the other hand, the lowest value was for oleic acid 18:1 in a percentage equal to 0.0032 for *S. marcescence* (1), while *S. marcescens* 2 and *A. baumannii*, as well as *S. marcescens* 1, had very low values for myristic acid 14:0 with a percentage equal to 0.0096, 0.012 , and 0.0122, respectively.

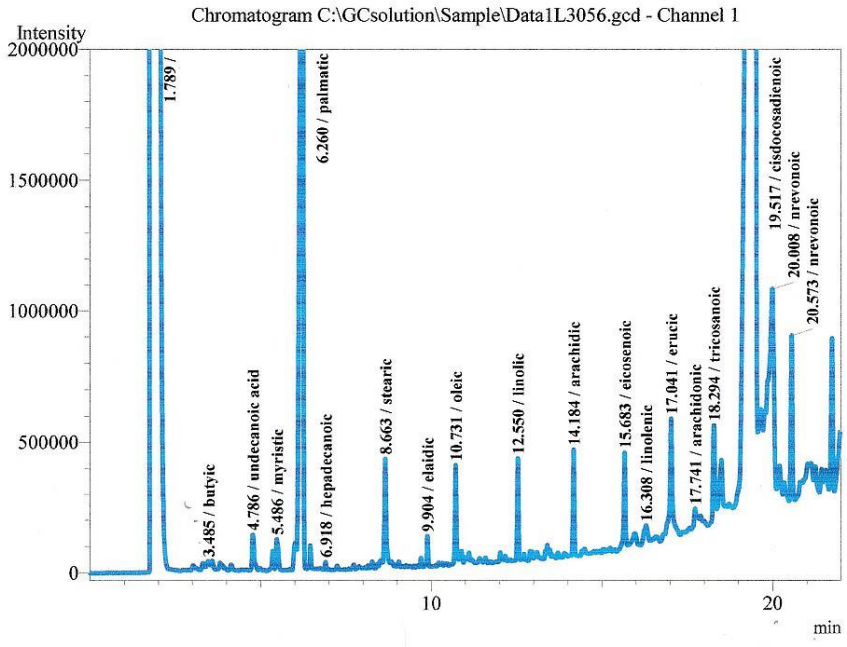

Figure 2. Standard fatty acid analysis by Capillary Gas Chromatography

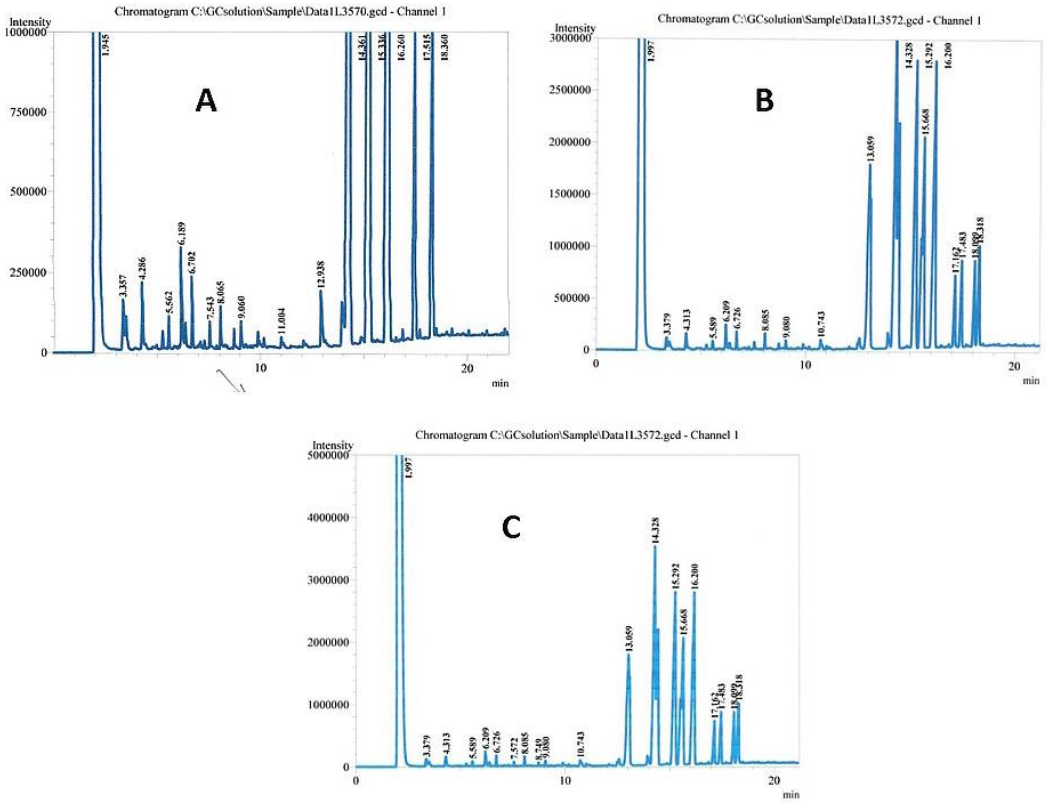

Figure 3. Fatty acid analysis of:A=*S.marcescens* (1), B=*S.marcescens* (2), and C=*A.baumannii* without the addition of Tween 80 (control)

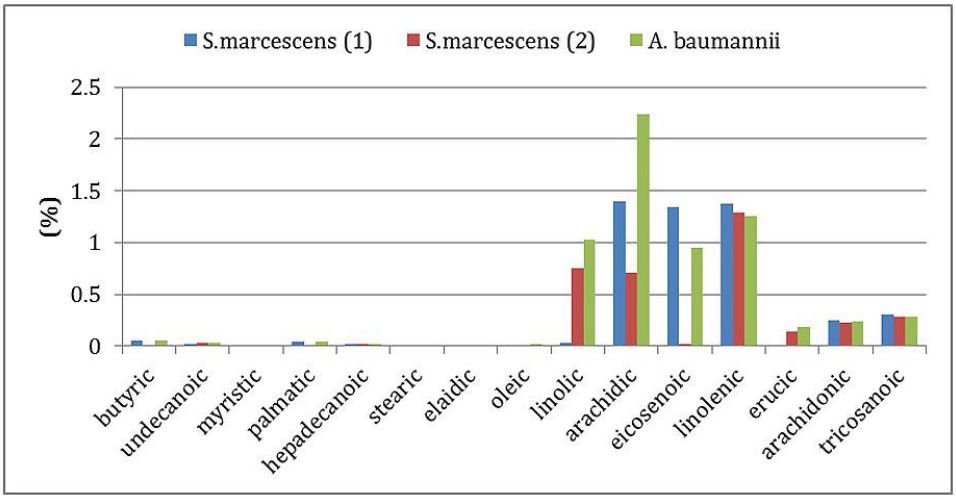

Figure 4. Percentages of fatty acids of three bacterial isolates

3.2. Impact of Tween 80 on Fatty Acid Content

The results indicated that changes have occurred in the appearance and disappearance of three FAs (Elaidic acid 18.1 trance 9, Oleic acid 18.1 cis 9, and erucic acid 22.1) in the presence of different concentrations of Tween 80. As illustrated in table 2: in *S. marcescence* (1) and S. *marcescence* (2), elaidic acid appeared in two concentrations of Tween 80 after it was completely absent in the original content (control); nonetheless, there was no effect at all in *A. baumannii*.

For oleic acid, it disappeared in *S. marcescence* (2) and *A. baumannii* in two concentrations (0.1 and 1.0 % concentration of Tween 80, respectively, after it was present in control in detectable value). For erucic acid, there is a difference between the two species of *Serratia*, this FA appeared in 0.5 % concentration. After its absence in the control in *S. marcescens* (1), it disappeared in all concentrations of Tween 80 after its presence in control in *S. marcescens* (2). However, *A. baumannii* did not demonstrate any changes for both elaidic and erucic acid.

The impact of different concentrations of Tween 80 on all other FAs used in this study have been observed generally as groups to provide the best insight into the effect in general. These groups are 1) Total Saturated FAs (TSF), 2) total Mono-Unsaturated FAs (MUFA), and total Poly-Unsaturated FAs (PUFA). Figures 5 and 6 demonstrate total percentages for total (SFA), total (MUFA), and total (PUFA) for *S. marcescens* (1) and *S. marcescens* (2), respectively.

Table 2. Impact of different concentrations of tween 80 on fatty acid contents in three bacteria

	***S. marcescens* 1**				***S. marcescens* 2**				***A. baumannii***			
Tween 80% / Fatty acids	**C**	**0.1**	**0.5**	**1.0**	**C**	**0.1**	**0.5**	**1.0**	**C**	**0.1**	**0.5**	**1.0**
Elaidic acid	X	0.0046	X	0.0038	X	0.0049	0.0079	X	X	X	X	X
Oleic acid	0.0032	0.003	0.0324	0.0038	0.0146	X	0.0058	0.0042	0.0177	0.0032	0.005	X
Erucic acid	X	X	0.0939	X	0.1384	X	X	X	X	X	X	X

C= control; X= abscent

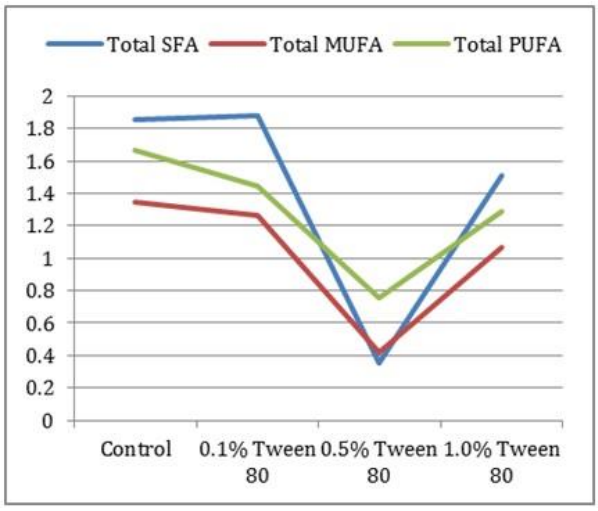

Figure 5. Percentages of total (SFA), total (MUFA), and total (PUFA) for *S. marcescens* (1)

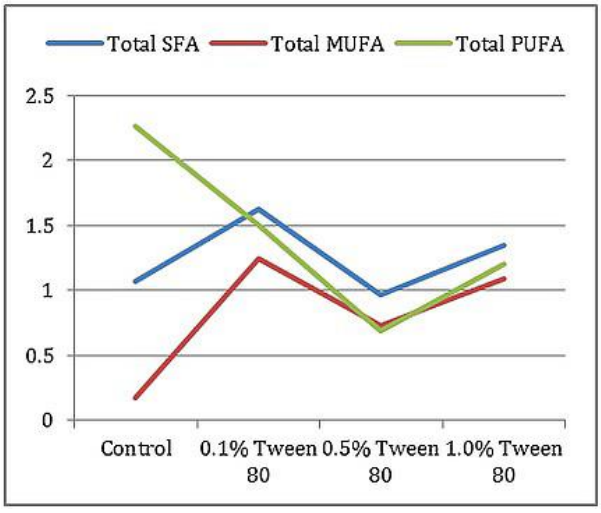

Figure 6. Percentages of total (SFA), total (MUFA), and total (PUFA) for *S. marcescens* (2)

The results were similar between *S. marcescence* (1) and (2) in which all types of FAs (SFAs, MUFA, and PUFA) decreased only in concentration 0.5 % of Tween 80. Figure 7 demonstrates the percentages for: total (SFA), total (MUFA), and total (PUFA) for *A. baumannii*.

In *A. baumannii*, the results were different from *Serratia* spp., and all types of FAs decreased in the concentration 1 % of Tween 80. Moreover, following statistical analysis, the significant value was calculated at $P \leq 0.05$ % for the three concentrations of Tween 80 for SFA, MUFA, and PUFA as demonstrated in table 3.

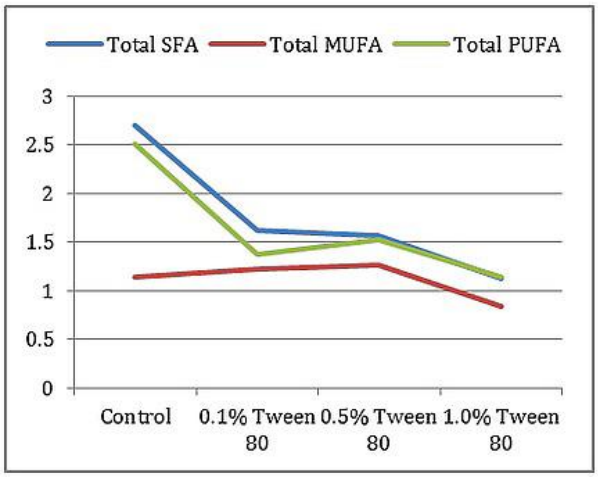

Figure 7. Percentages of total (SFA), total (MUFA), and total (PUFA) for *A. baumannii*

Table 3. Significance of Saturated Fatty acids, Mono-Unsaturated Fatty acids, and Poly-Unsaturated fatty acids, in three concentrations of Tween 80, as compared to control at $P \leq 0.05$

Concentration of Tween 80 / Type of Fatty acids	0.1%	P-value	0.5%	P-value	1%	P-value
***S. marcescens* (1)**	**mean±st. error**		**mean±st. error**		**mean±st. error**	
Saturated Fatty acids	0.2346±0.1595	0.844	0.0440±0.0144	0.041	0.1890±0.1401	0.755
Mono-Unsaturated Fatty acids	0.3163±0.3137	0.888	0.1053±0.0662	0.055	0.2676±0.2650	0.636
Poly-Unsaturated fatty acids	0.4819±0.3790	0.822	0.2517±0.1493	0.094	0.4288±0.3233	0.557
***S. marcescens* (2)**	**mean±st. error**		**mean±st. error**		**mean±st. error**	
Saturated Fatty acids	0.2032±0.1428	0.510	0.1211±0.0836	0.772	0.1680±0.1145	0.767
Mono-Unsaturated Fatty acids	0.3107±0.3091	0.037	0.1816±0.1770	0.052	0.2719±0.2705	0.039
Poly-Unsaturated fatty acids	0.5025±0.3740	0.564	0.2307±0.1434	0.419	0.4006±0.3160	0.767
A. baumannii	**mean±st. error**		**mean±st. error**		**mean±st. error**	
Saturated Fatty acids	0.2025±0.1448	0.377	0.1951±0.1498	0.396	0.1406±0.0964	0.191
Mono-Unsaturated Fatty acids	0.3071±0.3061	0.526	0.3168±0.3151	0.493	0.2106±0.2106	0.935
Poly-Unsaturated fatty acids	0.4602±0.3460	0.778	0.5065±0.3840	0.594	0.3802±0.2896	0.926

$P=\leq 0.05$

The *P*-value was significant in two positions in Table 3: the first one belonged to *S. marcescens* (1) at 0.5 % concentration for both SFA and MUFA; the second one was for *S. marcescens* (2) only in MUFA in all concentrations of Tween 80.

4. Discussion

Each bacterial strain possesses a unique fatty acid profile when grown on a given culture medium at a certain temperature (11). It has been noted that the identification obtained by FA profile sometimes is not in agreement with biochemical criteria in strains of some bacterial genera (12). The FAs profile for the three bacterial isolates under study was relatively similar in different concentrations (Figure 4), and it may be attributed to the isolates which are clinical in sources. Studies on fatty acid composition of prokaryotic cell membrane demonstrate the variance of responses that the cell may use to adjust the fluidity of the cellular membrane when placed under certain conditions (11).

The results of the present study disagree with those obtained by Chao, Guo (4) who reported that the most significant cellular FA in *A. baumannii* is 18:1 ω9c. The other major CFAs in their studies are 16:0. This disprepncy can be ascribed to difference in the sources and locations of isolate, variying depending on its FAs requirement and adaptation. The proportions of 12:0, 17:1ω 8c, 3- OH- 12:0, 17:0, and 18:0 are relatively lower. In their study, the minor fatty acids detected in the different strains of *A.baumannii* were 10:0, unknown 12.484, 13:0, and since all these FAs were not used in our standard FAs, we can not compare them.

One of the long-chain types of FAs obtained in this study was eicosenic acid (20:1). Halliwell and Gutteridge (13) had previously claimed that the long chain Fas, such as eicosapentaenoic acid (EPA, C20:5), controled membrane fluidity. Moreover, in addition to an unexpected new function of FAs, they are involved in resistance against extracellular oxidants, such as hydrogen peroxide.

In the same context, Hassan, Uddin (14) used the same FAs and the results showed different percentages which can be attributed to the used strain of *S. marsescence* (glacial ice), and the fatty acid that appeared was indicative of its adaptation to this cold environment; nonetheless, the strains used in the current study were mesophiles. Moreover, they did not use both 18:3 and 20:0 that demonstrated higher values in the results of the current study (14). The appearance and disappearance of three FAs (Elaidic, Oleic and erucic) could be as a response to environmental changes represented by the addition of Tween 80 to the medium; therefore, it can be concluded that Tween 80 had a significant impact on the content of FAs.

Elaidic acid is a white crystalline unsaturated acid with chemical composition: $C_{17}H_{33}COOH$ with the double bond (between carbon atoms 9 and 10) in *trans* configuration obtained from oleic acid by isomerization. The salts and esters of this colorless oily solid are called elaidates. This compound has attracted assiduous attention since it is a major trans fat found in hydrogenated vegetable oils, and trans fats have been implicated in heart disease. The name of the elaidinization reaction comes from elaidic acid, and its name comes from the ancient Greek word ἔλαιον (*elaion*) which means oil (15).

Wu and Shah (16) showed no correlations between cell surface hydrophobicity and the metabolism of elaidic acid by lactobacilli. Moreover, elaidic acid was able to influence cell surface hydrophobicity, and the decrease in hydrophobicity was more obvious in *Lactobacillus paracasei* and *Lactobacillus casei*, as compared to that in other tested lactobacilli species. Their study suggested that elaidic acid could change physiochemical surface properties of lactobacilli (16).

The presence of Oleic acid in three bacterial species and in almost all concentrations of Tween 80 (although it was in barely measurable) might be due to the fact

that tween 80 is considered the source of oleic acid (17). In addition, oleic acid moiety of Tween 80 can be incorporated into the cell membrane, affecting cell membrane properties (18). Erucic acid was another monounsaturated omega-9 fatty acid obtained in the present study. It is produced by the elongation of oleic acid via oleoyl-coenzyme A and malonyl-CoA. The presence of this FA in bacteria was rarely mentioned in literature David, Both (19).

The FAs in bacteria are synthesized in the type II fatty acid biosynthesis pathway by a set of enzymes. Additional enzymes could further modify the structure of FAs in response to environmental changes. The fatty acid composition of the bacterial membrane depends on a number of factors, including growth temperature, pH, growth phase, cultivation medium composition, and NaCl concentration (20). Accordingly, another factor may be added and alter FAs, and it is Tween 80 compound based on the results of the present study.

When the growth conditions change, prokaryotic cells can alter their FA composition to adjust the fluidity of the membrane by a series of strategies. For instance, the effect of unsaturation is growth at different temperatures, pH, pressure, salinity, in the presence of organic solvents, and the effect of polyunsaturation is growth in deep-sea rapid adaptation to increased salinity and extreme conditions (11).

Furthermore, regarding Oleic acid, the results of the present study disagreed with those obtained by Reitermayer, Kafka (6) who reported that S. *marcescens* (1) was slightly affected in the presence of Tween 80. As for *S. marcescens* (2), the concentration of 0.5% of Tween 80 gave less oleic acid concentration than the control. This may be explained by the transcriptomic response of these bacteria to the supplementation of the culture medium with Tween 80, and a significant down regulation of genes was found to be involved in the fatty acid biosynthesis pathway. This can be ascribed to the fact that Tween 80 leads to the downregulation of FAs biosynthesis at increasing concentration. The mechanism by which Tween 80 affects a specific microorganism is probably complex and could include many elements.

From enzymatic changes of FA to de novo synthesis, cells attempt to make quick changes with the least energy possible. Nevertheless, FAs in the environment can be a threat to the stability and function of bacterial cell membranes (11). The permeabilizing ability of Tween 80 depends on the specific lipid composition of the membrane (both inner and outer for Gram-negative bacteria), membrane proteins, as well as the properties of peptidoglycan layer and surrounding extracellular substrates. Therefore, this effect will be strain-dependent and may explain the different effects detected for varying strains (21).

Along the same lines, Marsh (22) indicated that high concentrations of PUFA allow the adaptation of barophilic bacteria to the low temperature and high hydrostatic pressure of the deep-sea environment (22). Based on the results of the current study, the total PUFA is estimated in low concentrations since these bacteria were grown in normal temperature and pressure conditions. More data is needed to obtain a basic scientific understanding of lipid regulation/metabolism by the addition of other molecules to change the FAs composition in bacterial cell. Moreover, further studies are suggested to use more bacteria to identify modified FA and phospholipid composition, depending on environmental niche (23).

Several *A. baumannii* genomes encode multiple genes predicted to have FA desaturase activity, homologues to enzymes that could be responsible for altering stereochemical conformation of the FAs post-incorporation, thereby alleviating membrane FA disorder and lowering permeability (24, 25). New antibiotics that target bacterial FA biosynthesis have been developed since 1980s (26). Free FA acting upon the cellular membrane hold up the electron transport chain and oxidative phosphorylation, although the mode of action is not completely understood. The FAs as free or in monoacylglycerides are encouraging

antibacterial agents with therapeutic applications for medicine and human health (11, 27).

In bacterial cell membranes, unsaturated fatty acids, usually containing one or two double bonds, occur as frequently as saturated FAs. However, some limited groups of bacteria have been demonstrated to produce distinct unsaturated fatty acids that have chain lengths longer than 20 carbons and contain at least four double bonds. Among them, we can refer to arachidonic acid (20:4), eicosapentaenoic acid (20:5), and docosahexaenoic acid (22:6), which are collectively termed long-chain polyunsaturated fatty acids (28).

The two major modes of long-chain PUFA synthesis in nature are 1) aerobic desaturation and elongation of SFAs, 2) anaerobic pathway for de novo synthesis of long-chain PUFA. The *pfa* genes that are responsible for the production of long chain PUFA demonstrate a broad variety of gene structure (29, 30). Tween 80 is no longer a mere source of carbon, rather it is commonly used as an emulsifier both in research and industrial microbiology. Fatty acids are involved in affecting cellular growth, motility, biofilm formation and virulence (31, 32). The effect of Tween 80 on vegetative cells includes the enhancement of bacterial growth and protection of cells against harsh environmental conditions, including acidity, bile salts, freeze-drying, and nutrient depletion (4).

The main role of the cellular envelope in prokaryotes is the protection of these organisms from the surrounding environment. Fatty acids are vital components in cell membranes of bacteria, determining the different structures of lipids and lipid A. (5). The present study aimed to analyze the fatty acid composition of different strains of *S. marcescens* (1), *S. marcescens* (2), and *A. baumannii* to determine the differentiation capability of FA patterns, then observing the effects of Tween 80 molecules on this pattern. It has been elucidated that Tween 80 exerts an effect on FAs content in bacterial cells, especially in MUFA type, for both *S. marcescens* and *A. baumannii*. Furthermore, the concentration of this substance plays a major role in appearance and disappearance of these FAs. The obtained results also demonstrated that the p-value was significant in two situations, the first one belonged to *S. marcescens* (1) at 0.5 % concentration for all groups of Fas, and the second one was for *S. marcescens* (2) just in MUFA in all concentrations of Tween 80.

Authors' Contribution

Study concept and design: G. A. M. and S. T. D.
Acquisition of data: G. A. M. and S. T. D.
Analysis and interpretation of data: G. A. M. and S. T. D.
Drafting of the manuscript: G. A. M. and S. T. D.
Critical revision of the manuscript for important intellectual content: G. A. M. and S. T. D.
Statistical analysis: G. A. M. and S. T. D.
Administrative, technical, and material support: G. A. M. and S. T. D.

Conflict of Interest

The authors declare that they have no conflict of interest.

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