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Validation of the Identification of Some Foodborne Enteric Bacteria by Fatty Acid Profiles and Real-Time PCR

Rudwan BADR AL–DEEN[1*], Bassam ALOKLAH[2]

[1,2]National Commission for Biotechnology (NCBT), Damascus, SYRIA

[1]https://orcid.org/0000-0002-5921-0809, [2]https://orcid.org/0000-0001-6533-7374

[*]Corresponding Author Rudwanbadr@gmail.com

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ABSTRACT

Foodborne pathogenic bacteria are considered as a main cause of infection and death. Real time PCR (RT-PCR) assay can be used to identify pathogens using species-specific primers. The composition of fatty acids can be used to identify foodborne pathogenic bacteria, as a rapid method, by means of gas chromatography connected with mass spectrometry (GC-MS). We compared between RT-PCR and the composition of cellular fatty acids (CFAs), to identify four foodborne pathogenic bacteria (*Escherichia coli*, *Enterobacter cloacae*, *Proteus mirabilis* and *Salmonella enterica*). Unique primers designed by us were used for identifying the four pathogenic bacterial species using RT-PCR. Moreover, the traditional method for analysis (CFA) of bacteria was modified by addition a concentration step at the end of the process. Results revealed that RT-PCR was superior to CFA profiles, and the accuracy of RT-PCR method was 100%; while it was between 81% for *E. cloacae* and *P. mirabilis*, and 100% for *S. enterica* in CFA profiles.

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INTRODUCTION

Some foods such as meat, poultry and dairy are considered the main source of pathogenic bacteria. These types of foods are suitable for growing a lot of bacterial species, for example, the species pertaining to the following genera, *Salmonella*, *Campylobacter* and *Listeria*, (Swaminathan and Feng, 1994). According to World Health Organization, more than 93 million individual suffer from foodborne illnesses, and 155 thousand people die annually because of foodborne pathogens (Salam and Tothill, 2009; Freitas et al., 2014; Oh et al., 2017).

Some foodborne bacteria may cause severe illnesses, such as the infection of intestinal tract, arthritis, renal infections and central nervous problems (Qiao et al., 2020). Hence, several conventional methods, such as, ELISA, serological tests and PCR are used for identifying pathogenic bacteria (Kumar et al., 2016).

The technics based on nucleic acid are methods used to detect a particular part of the DNA or RNA of the bacteria. Aiming to investigate the targeted DNA or RNA chain,

the nucleic acid chain of the bacteria is hybridized with the complementary sequence (Zhao et al., 2014). Toxins can be produced by a lot of bacteria, such as *Clostridium botulinum*, *Staphylococcus aureus*, *Vibrio cholerae* and *Escherichia coli* O157, therefor they considered pathogenic bacteria (Akbulut et al., 2004; Fusco et al., 2011; Son et al., 2014). The last pathogens can be detected by different types of PCR, just as RT-PCR or quantitive PCR (qPCR), multiplexed PCR (mPCR), and other PCR methods (Yadav et al., 2020).

Real-Time PCR or quantitative PCR is a kind of PCR, in which the amplification process may be shown directly throughout PCR run. In this technic, the number of the copy of amplified sequence is determined by fluorescence amount, which is proportional to the number of amplified copies (Omiccioli et al., 2009). The quantitative PCR technic relays on measuring the fluorescence. The principal fluorescent method used in quantitative PCR is SYBR green, which is the simplest fluorescent method among the others. Low fluorescence signal is emitted as SYBR green insert into the furrows of DNA (Hein et al., 2001), so the fluorescence signal may be improved by inserting a fluorescent dye in DNA's furrows (Fukushima et al., 2003).

The composition of Bacterial fatty acid is relatively a new method for the identification of bacteria. One of the methods used currently based on preparation of the methyl esters of bacterial fat, followed by gas chromatographic analysis (Cody et al., 2015). The first apparatus commercially available in 1990s, namely "MIDI Sherlock" analyzes fatty acids, and uses the obtained data for identification of the bacteria. The MIDI method uses a procedure including simultaneously saponification and esterification process, followed by gas chromatographic analysis, applied to bacterial colonies that had been grown under special adjusted conditions (Cody et al., 2015).

The current research aimed to investigate two rapid methods used to identifying foodborne bacteria. The first method is real time polymerase chain reaction (RT-PCR) using a novel primer designed by us and used for the first time by real time PCR technic. The other method is cellular fatty acid profiles, which contain some important modification of Sasser (1990) method, including a concentration step of methylated fatty acids to make them detectable by GC-MS.

MATERIALS AND METHODS

Identification of Enteric Foodborne Bacteria

Salmonella strains were isolated according to Hariggan (1998). Briefly, 25 g of food product was added into 225 mL of Buffered Peptone Water (BPW) (SRL, Sisco Research Laboratories, Mumbai, India), mixed well, and incubated at 37°C. Ten mL of primary enrichment medium (BPW) was added into 100 mL of Rappaport-

Vassiliadis medium (RV) (Merck, Germany). The last medium was incubated at 42°C for 24 h, then one loopful of RV medium was streaked onto each of two plates, namely: xylose lysine desoxycholate (XLD) and Hektoen Enteric agar (HEA) (HiMedia, India). Isolation of other Enteric bacteria (except for *Salmonella*) was performed according to Kilonzo-Nthenge (2008) method, as following: 25 g of the food products was weighted into sterile bag, and 225 mL of BPW was added. The bag with its contents was then incubated at 37°C for 20 h. After incubation period passed, 200 μL of BPW culture was streaked onto MacConkey agar plate (Criterion, Hardy Diagnostics, Santa Maria, CA, USA) and the plates were then incubated at 37°C for 24 h.

Extraction and Purification of DNA

DNA of the bacteria was extracted from cultures, which were grown on Luria Bertani Broth (LBB) (Sigma-aldrich, Chemie, Gmbh, Germany) at 37°C for 24 h. by means of the kit namely "Wizard® Genomic DNA Purification" (Promega, Madison, USA) by following the instructions of manufacturer. The DNA purity was determined by calculating the values of optic densities OD_{260} to OD_{280} nm, as measured spectrophotometrically (Optizen 3000 Plus, Mecasys Inc., Korea). The ratio between 1.8 and 2.1 was considered optimal to RT-PCR reaction (Findlay, 2011). The concentration of DNA was measured according to Jain (2004), using following equation:

DNA concentration=optical density at 260 nm × dilution factor × 50

Primers Design and Sequences

Primers were designed according to data obtained from the Gene Bank (website: http://www.ncbi.nlm.nih.gov/tools/primer-blast/). These primers are used for the first time to identify bacterial species (*Escherichia coli*, *Enterobacter cloacae*, *Proteus mirabilis* and *Salmonella enterica*) by RT-PCR technic. However, these primers are used in former research (Badr Al-Deen et al., 2014) by traditional PCR-acrylamide gel electrophoresis.

Identification of Isolated Bacteria by RT-PCR

DNA Preparation for RT-PCR Analysis

Extracted and purified DNA of each isolates were analyzed by means of RT-PCR (Spartan DX-12TM, Spartan Bioscience Inc., Ottawa, Canada), using quantitive PCR Master Mix kit (Maxima SYBR Green, Thermo Scientific) according to manufacturer's instructions. One μL of forward primer and one μL of reverse primer (at concentration of 25 $pmol.\mu L^{-1}$) was mixed with 12.5 μL of SYBR Green Master Mix, 1 μL of extracted DNA (at concentration of 25 $pmol.\mu L^{-1}$) and 9.5 μL of free DNase deionized water.

Thermal Program for Amplification Target Genes

The amplification of target gene of *E. coli* (*uid*A) was performed as following: One cycle initial denaturation at 92°C for 10 minutes; then 35 cycles: denaturation at 94°C for 1 minute, then annealing at 58.8°C for 1 minute, and finally extension at 72°C for 45 seconds. The same thermal cycle was used to amplify the target genes of *E. cloacae* (rpoB), *P. mirabilis* (*aad*) and *S. enterica* (*inv*A) but annealing temperatures were 60.8, 50.3 and 50.9°C, respectively. The melting temperatures (T_m) of amplicons were determined using increased temperatures ranged from 55 to 95°C at 0.1°C per step.

Fatty Acid Profiles of The Enteric Bacteria

Bacteria Growth and Biomass Harvesting

All strains of studied bacteria were grown on Trypticase Soy Agar (TSA) (Merck, Germany) at 37°C for 24 hours. The cells of the bacteria were harvested by means of 4mm loop to achieve a biomass of about 40 mg, which is used to prepare fatty acid methyl ester.

Preparation of Cellular Fatty Acids Methyl Ester (Fames)

The cells of the bacteria were harvested by means of a wire loop (4 mm diameter) to obtain about 40 mg of bacterial cells. The biomass was placed in 13×100 mm screw cover glass test tube. One mL of Reagent A (45 g sodium hydroxide, 150 mL methanol, 150 mL distilled water) was added to the glass test tube, which containing the cells of the bacteria. The tubes were securely closed with the screw caps lined with Teflon and then vortexed for some seconds and immersed in a boiling water bath (Memmert, Germany) for 5 minutes with vortexing occasionally for 5-10 seconds and but pack to the water bath to complete boiling for 30 minutes.

The tubes were cooled, and then 2 mL of Reagent B (325 mL of HCl 6 N, 275 mL methanol) were added. The tubes were recapped and vortexed for few seconds. The tubes were but in water bath adjusted at 80°±1°C for 10±1 minutes. (This step is sensitive for each of the time and the temperature). Then 1.25 mL of Reagent C (200 mL hexane, 200 mL of methyl tert-butyl ether) was added to the tubes after cooling, and finally the tubs were tumbled gently by means of a clinical rotator (Stewart, UK) for approximately 10 minutes.

The lower aqueous phase was withdrawn using Pasteur pipet and discarded. Three mL of Reagent D (10.8 g sodium hydroxide, 900 mL distilled water) were added to the solution remaining in the tube, which was tumbled for about 5 minutes. Two third the organic solution was transferred using Pasteur pipet into a GC vial to be injected in the GC-MS (Sasser, 1990). However, the concentration of the methylated fatty acids in the organic phase was very low and they can not be detected by GC-MS technic, so we modified the method of Sasser (1990), by evaporate the organic phase-using a slow current of pure Nitrogen-into about 40 µL to concentrate the methylated fatty acids to reach the detection limits of GC-MS, and transfer the concentrated

organic phase into GC vial equipped with central cone (100 µL capacity), so we could obtain the ideal chromatograms shown in Figure 2.

FAMEs Analysis Using GC-MS

FAMEs were analyzed by means of gas chromatograph coupled with mass spectrometer (Agilent, USA) and capillary column (DB-1, 30 m length, 0.25 mm inner diameter, 0.20 µm film thickness; Agilent, USA). Oven temperature was programmed as shown in table 1. The carrier gas was Helium, which was used at 1.0 $mL.min^{-1}$ rate flow. Autosampler temperature was adjusted at 250°C, and spilt ratio was adjusted at 1:50, and injection volume was 1.0 µL. Fatty acids methyl esters were identified according their retention times compared with those of the standard (methyl ester mix of bacteria's fatty acids, 47080-U, Supelco, Germany), see Figure. 1.

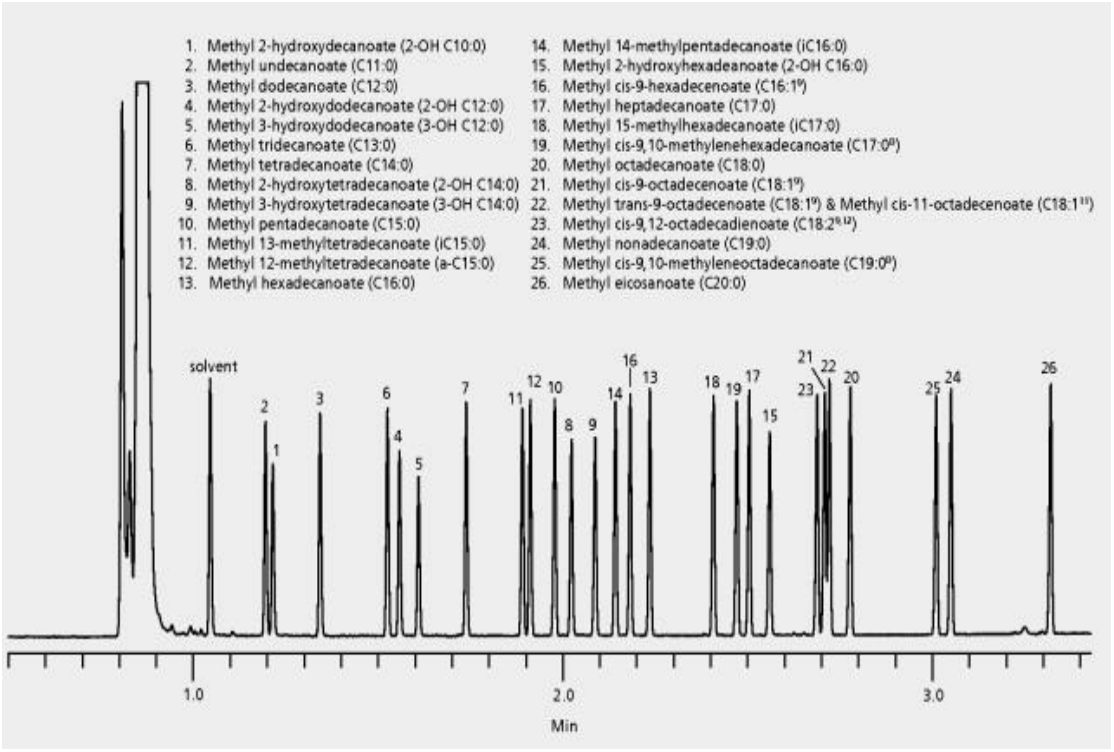

Figure 1. Fatty acid methyl esters of bacteria (47080-U, Supelco, Germany)

Table 1. Oven temperature program

Rate (°C.min^{-1})	Set point (°C)	Hold time (min)	Run time (min)
-	125	2	2
5	150	4	11
4	250	5	41
13	290	0	44

RESULTS and DISCUSSION

Foodborne Species, Which Are Isolated From Different Foodstuffs

Samples were collected from local market, Damascus-Syria, include poultry, red meats, milk, dairy products, fruits, vegetables, grains, legumes and nuts. Table 2 illustrates the bacterial species obtained in current research, there numbers and food sources of them.

Table 2. Bacterial species, there numbers and food sources

Isolates	Isolates No.	Food sources
Escherichia coli	69	Poultry meat (wing, legs, gizzard), parsley
Enterobacter cloacae	53	Cucumber, celery, peach, persimmon, apricot, cheese.
Proteus mirabilis	26	Poultry meat, berry, carrot, milk.
Salmonella enterica	8	Poultry meat (liver, gizzard, wing)

Table 2 showed that, *E. coli* represented the most frequent isolate (69 isolates) among the 4 species; while *S. enterica* was the least frequent isolate (8 isolates only). The poultry products were the main reservoirs of *S. enterica*, while other food products were free of it. *Enterobacter cloacae* was isolated from plants (Cucumber, celery, peach, persimmon and apricot) in addition to cheese, and was absent in poultry products and milk. Poultry products and milk, in addition to some plants (berry and carrot) were the main reservoirs of *P. mirabilis*.

Identification of Isolated Bacteria Using RT-PCR

Foodborne bacteria were identified using novel primers designed by us and used by RT-PCR in the current research, while sterile, DNase free, deionized water was used as a control instead of forward and reverse primers (refer to material and methods section). Figure 2 show fluorescence curve, with threshold cycle value for each of the four species studied. The melting temperatures of the amplicons to ensure the annealing of the primers in the correct sequence of the target gene.

Figure 2 revealed that the threshold cycle (C_t) was 11.6 for *E. coli*; 24.7, 10.9 and 16.3 for *E. cloacae*, *P. mirabilis* and *S. enterica*, respectively. While the melting temperatures of the amplicon (T_m) were 82.3, 83.3, 83.7 and 81.9 for *E. coli*, *E. cloacae*, *P. mirabilis* and *S. enterica*, respectively.

A few molecular methods had been used to identify *E. coli* (Godambe et al., 2017). For example, Chen and Griffiths (1998) used *usp*A gene to detect *E. coli*; while *uidA* gene revealed to be a unique gene to detect *E. coli* and some researchers used it for detection of *E. coli* and identifying it (Bej et al., 1991; Maheux et al., 2009). Liang et al. (2022) reported that *Salmonella* spp. can be detected using *inv*A gene by means of multiplex qRCR in the presence of *E. coli* and *S. aureus* in bird's nest, donkey gelatin and wolfberry. Mollet et al. (1997) found that phylogenetic trees resulted from the

sequencing of *rpoB* gene came in accordance with the classification currently used for *Enterobacteriaceae*, and it was more suitable than 16S rDNA.

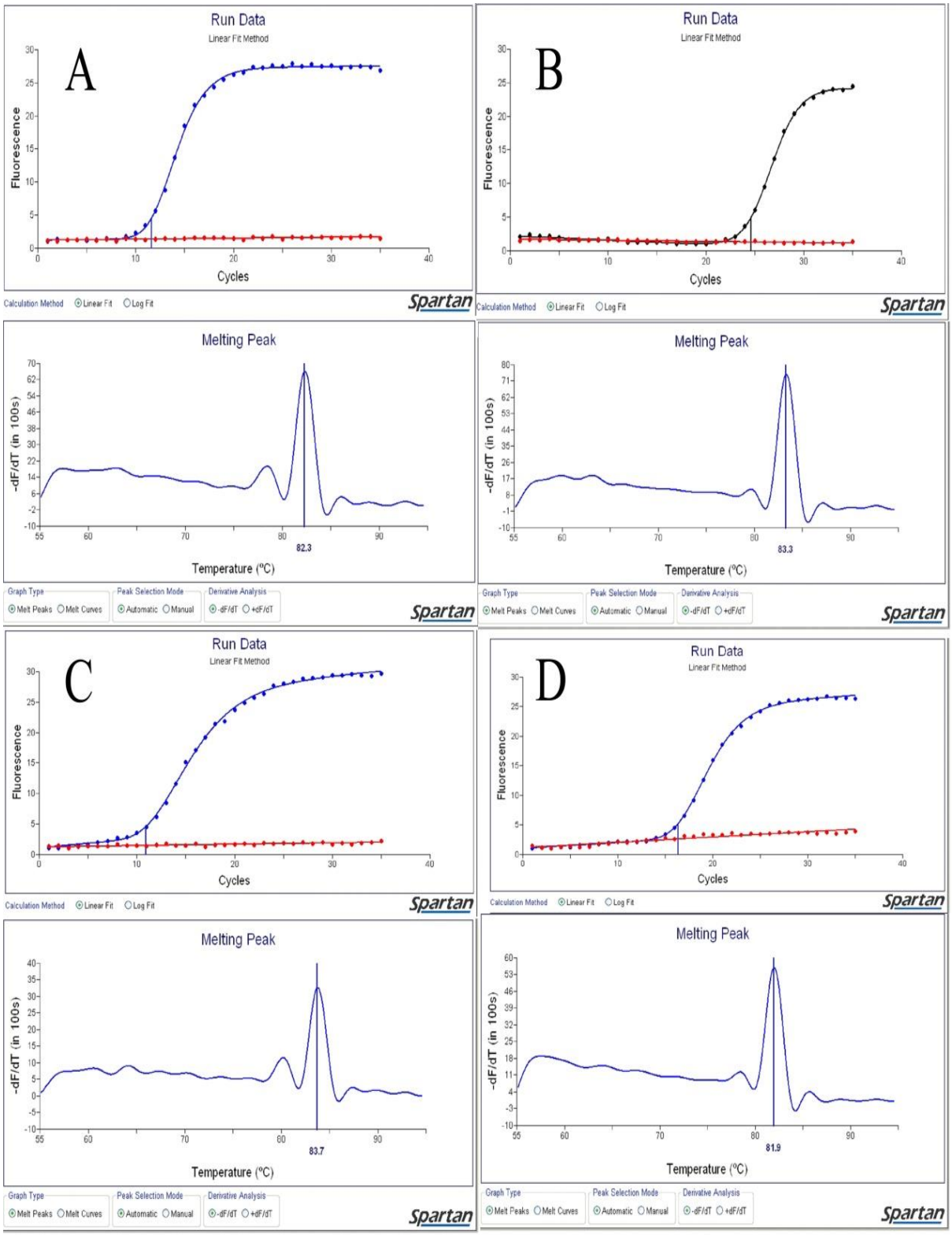

Figure 2. Results of target gene amplification, the upper figure of each pair shows the fluorescence curve, and the lower one shows the melting curve: A. *E. coli*; B. *E. cloacae*; C. *P. mirabilis* and D. *S. enterica*

Hoffmann et al. (2003) reproduced this fact in their study. However, the last researchers used another sequences of target *uidA*, *inv*A, and rpoB genes, which are different from that used in the current research.

Identification of Isolated Bacteria by Fatty Acid Profiles

The analysis of fatty acid composition of bacteria's cells using GC-MS revealed the existence of 23 different fatty acids containing 10 to 20 carbon atoms. Table 3 shows distribution of fatty acids in *Enterobacteriaceae* members, where the presence of saturated, straight chain fatty acids, namely C10:0, C12:0-aldehyde, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0; unsaturated fatty acids, namely C14:1$^{(9)}$, C14:1$^{(11)}$, C16:1$^{(9)}$, C16:1$^{(11)}$, C18:1$^{(8)}$, C18:1$^{(9)}$, C18:1$^{(11)}$, C19:1, C20:1; hydroxyl fatty acids, namely C14:0-2OH, C14:0-3OH; and finally cyclic fatty acids, namely C17:0 $^{\Delta}$ and C19:0 $^{\Delta}$.

Table 3. Fatty acid profiles of the studied species, belong to *Enterobacteriaceae* family

NO.	Fatty acid	*E. coli*	*E. cloacae*	*P. mirabilis*	*S. enterica*
1	C10:0	0.02	-	0.12	0.18
2	C12:0-aldehyde	-	-	3.14	-
3	C12:0	3.18	4.01	1.54	8.62
4	C13:0	0.06	0.22	0.07	0.17
5	C14:1$^{(9)}$	0.19	0.1	0.36	0.32
6	C14:1$^{(11)}$	0.15	0.08	0.21	0.26
7	C14:0	12.88	15.31	17.58	18.21
8	C15:0	0.32	0.63	0.76	0.44
9	C14:0-2OH	-	-	-	1.12
10	C14:0-3OH	6.37	13.14	12.56	8.55
11	C16:1$^{(9)}$	1.22	7.71	1.66	3.52
12	C16:1$^{(11)}$	0.08	-	-	0.4
13	C16:0	34.36	28.84	27.73	30.19
14	C17:0$^{\Delta}$	23.65	15.82	17.76	20.11
15	C17:0	0.21	0.77	0.16	0.52
16	C18:2	0.05	0.62	-	0.13
17	C18:1$^{(8)}$	0.21	-	0.07	-
18	C18:1$^{(9)}$	4.12	9.21	6.61	1.18
19	C18:1$^{(11)}$	0.16	0.1	0.38	1.2
20	C18:0	0.87	0.27	0.41	1.26
21	C19:1	0.03	-	0.65	-
22	C19:0$^{\Delta}$	10.16	1.98	8.22	2.91
23	C20:1	0.01	-	0.03	-

Escherichia coli strains characterized by the high percentage of C16:0, C17:0 $^{\Delta}$ and C19:0 $^{\Delta}$, compared with other species, and the recorded percentage of these fatty

acids were 34.36, 23.65 and 10.16%, respectively (see Figure 3. A). This result was compatible with that recorded by Richards et al. (1997), who found that the fatty acids C16:0 (33.4%), C17:0 Δ (22.2%) and C19:0 Δ (11.9%) represent the main fatty acids in *E. coli*. The percentage of C16:0 was close to that recorded by Li et al. (2010), Cahoon et al. (1996) and Korachi et al. (2010) (34.88, 34.7 and 35.3%, respectively). However, the fatty acid profiles were disagreed with that recorded in other research (Whittaker et al., 2005), where the percentage of fatty acids were: 37.8, 15.2 and 13.9% for C16:0, C17:0Δ and C19:0Δ, respectively. The difference between the last study and current study is due to the different culture medium, which is used to grow the biomass, TSA in our study vs Brain Hart Infusion (BHI) in the study of Whittaker et al. (2005). This result indicates to the great importance of standardizing the growth conditions, including the growth medium, which is used in preparation of biomass.

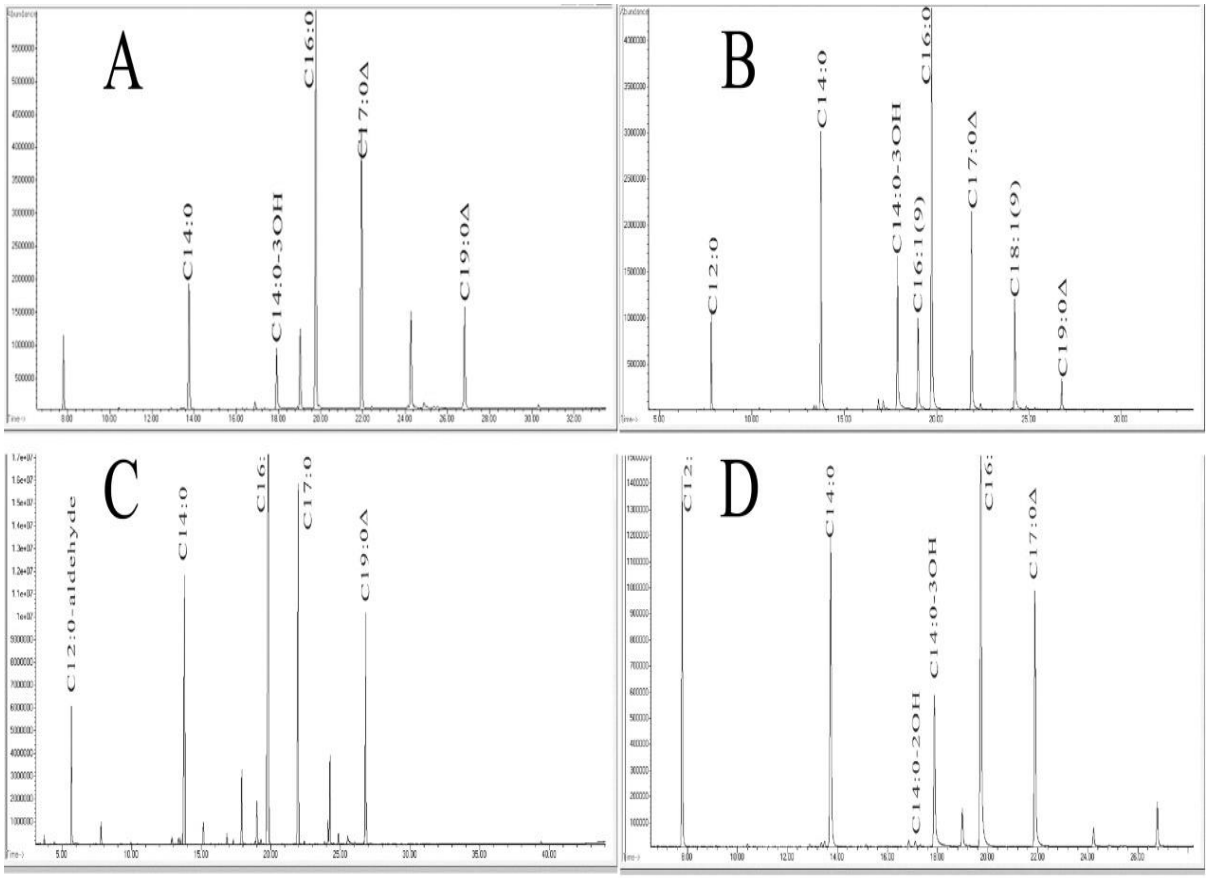

Figure 3. Fatty acid profiles in: A. *E. coli*; B. *E. cloacae*; C. *P. mirabilis* and D. *S. enterica*

The main fatty acids in *E. cloacae* isolates were C16:0, C17:0 Δ, C14:0 and C14:0-3OH, where their percentages were: 28.8, 15.82, 15.31 and 13.14, respectively, (see Fig 3-B). Table 3 shows the high percentage of C18:1(9) and C16:1(9) in *E. cloacae* Strains (9.21 and 7.71%, respectively), compared with other *Enterobacteriaceae* members. Whittaker et al. (2007) and Bøe and Gjerde (1980) proved the high percentage of C16:0, C14:0, C18:1 and C14:0-3OH in *E. cloacae*, while Madhaiyan et al. (2010) recorded the major

fatty acid in *Enterobacter* sp. included C16:0, C18:1 and C17:0$^{\Delta}$. The main fatty acids in *P. mirabilis* were C16:0, C17:0$^{\Delta}$, C14:0 and C14:0-3OH (27.73, 17.76, 17.58 and 12.56, respectively). The current study proved the presence of C12:0-aldehyde in a percent of 3.14% (see Fig 3-C), while it did not recorded by Cherniavskaia and Vasiurenko (1984), because they did not use MS in their study.

Results revealed the unique composition of fatty acid in *S. enterica*, where this species characterized in the presence of C14:0-2OH (1.12%), while this fatty acid was absent in other *Enterobacteriaceae* members. This result was compatible with that recorded in Whittaker et al. (2005) research, as they prove the presence of this fatty acid only in *S. typhimurium* among other *Enterobacteriaceae* members; and the study by Wollenweber et al. (1983), in the presence of C14:0-2OH in *Salmonella* sp. The other main fatty acids were C16:0, C17:0$^{\Delta}$ and C14:0 (30.19, 20.11 and 18.21%, respectively.

The Accuracy of The Two Methods and the Overall Evaluation of Them

The accuracy of the two methods (number of correctly identified isolates compared with the total number of the studied isolates), as well as the lengthy, technical difficulties in sample preparation and total costs. Table 4 illustrates the accuracy of the fatty acid profiles and RT-PCR, which is calculated from the following equation:

$$\text{Accuracy \%} = \frac{\text{Number of correctly identified isolates}}{\text{Total number of the isolates}} \times 100$$

Table 4. Accuracy% of identification methods

Isolates	RT-PCR			GC-MS Fatty Acid profiles		
	No. CII*	No. TI**	Accuracy%	No. CII	No. TI	Accuracy%
E. coli	69	69	100	69	57	83
E. cloacae	53	53	100	53	43	81
P. mirabilis	26	26	100	26	21	81
S. enterica	8	8	100	8	8	100

*Number of correctly identified isolates. **Number of total isolates.

Table 4 proved the absolute accuracy of RT-PCR method compared with fatty acid profiles methods, which its accuracy ranged from 81% for *E. cloacae* and *P. mirabilis* to 100% for *S. enterica*. However, RT-PCR method were more expensive than GC fatty acid profiles, while the two methods were the same labor intensive and lengthy, although the two methods are considered as rapid methods for identifying pathogens including Enteric bacteria.

CONCLUSION

Enteric bacteria can be identified using traditional methods (include morphological, physiological and biochemical methods), which are considered as the reference methods. However, the traditional methods are lengthy, effortful, and expensive, and can give misleading results, because they depend on a lot of tests, and any false negative (or positive) test can affect the identification completely. RT-PCR method can be a good alternative of traditional methods, which is more accurate and less labor intensive and lengthy, and need less technical experience. However, primers design is critical criteria, which can affects the results seriously. Primers used in current study might be considered as trustful primers for identifying the four enteric bacteria separately using RT-PCR method. However, they can not be used as multiplex RT-PCR method because melting temperatures of amplicons are very close to each others. Fatty acid profiles of bacterial cells using GC-MS technic may be considered as a rapid low cost method for identification pathogens including enteric bacteria. However, this method are less accurate compared with RT-PCR technic, but more accurate than traditional methods. *Salmonella enterica* was unique with the presence of C14:0-2OH and can be distinguished easily form other enteric bacteria by GC-MS fatty acid profiles using this unique character.

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Conflict of Interest

The authors declare that there are no competing interests.

Authors Contribution

The authors contributed equally to the manuscript

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