Isolation and 16s rRNA Identification of Micro Organisms from Drinking Water

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ABSTRACT

Water is one of the most important elements for all forms of life and is indispensable in the maintenance of life on Earth and essential for the composition and renewal of cells. Water represents 70% of our body, participates in the composition of our tissues, and transports the most diverse substances throughout our organism. Notwithstanding, human beings increasingly continue to pollute the reserves which remain, provoking illnesses that can jeopardize the population [http://www.sabesp.com.br] web site Bioaccumulation, sewerage, agricultural, industrial, radioactive, and thermal residues are the principal polluters of water on our planet.

INTRODUCTION

Earth consists of approximately 70% surface area covered with water and the remaining is a land that has only 2% water which is drinkable (Anthony *et al.,* 1980). Water is an important chemical molecule containing feature of life it can be dissolved into organic compounds, salts, inorganic compounds and gases that are involved in metabolic processes because it is a universal solvent and due to that it provides stability to membrane system, macromolecules, hemostasis, transportation and thermal regulation of body (Armstrong *et al.,* 2007; Yehiaa *et al*., 2020).

All cells of the body contain water as an important component. The water content of a single cell is 45% to 95% and microorganism contains 80% of body weight as water and human contains water i-e 70% of their body weight.

It is a thermal regulator of the human body and the normal human body contains 42 liters of water in them (Botkin& Keller, 2005; El-Gaied *et al.,* 2019). Whenever 2.7 liters of water loss from the body it can lead to headache, dehydration, and weakness. Water is equally important and critical for both humans and the environment and it is a key issue in the form of drinking water (Bourne & Seager 2001; Ahmed *et al.,* 2019). Dams, canals, and wells show the importance of water and the impact of human beings on the water cycle. Environmental effects like the migration of peoples and animals, land losses, change of environmental factors, depletion of biological resources show that these activities are noticeable (Buyckx, 2007; Bahareth et al., 2019). Pathogenic contamination of water is also an important threat to living organisms. In Asian regions peoples, those are living near to rivers are at high risk of their lives because of sewage pollution which is directly disposed of from chemical factories and septic tanks that are the main reservoir of pathogens involves in water-borne diseases (Ehsan, *e t a l*, 2015; Mohamed *et al*., 2018). Developing regions lack in the provision of safe drinking water to their peoples and in Africa and Asia almost 800 million individuals using unsafe drinking water which results in the suffering of individuals from water-borne diseases Farooq, 2006; Salheldeen *et al.,* 2017). The aim of this study was to isolate and identify environmental bacteria from various raw water sources as well as the drinking water distribution system in Makkah.

Water samples from different sites were analyzed for the presence of bacteria as well as other microorganisms.

MATERIALS AND METHODS
Sources of Water Samples:

Twenty-seven water samples were collected from different water sources within Makkah city and analyzed for bacterial contamination. There were four sources of water included in this study: governmental sea desalinated water, drinkable wells water, non-drinkable wells water, and small commercial desalination water factories.

Isolation of Bacterial Species from Water Samples:

By using the inoculation needle, the water samples were streaked for the growth of isolated colonies on nutrient agar. Then the plates were incubated at 37 °C for 24 hrs for bacteria. After 24 hrs the colonies grown on the plates were examined for their morphology and the same type of colonies was used for grams staining. Characterization of Bacterial species the isolated bacterial culture further identified and then characterized by using standard Microbiology methods.

Purification of Colonies:

Colonies were purified by twice subculturing using the streaking plate method. Young cultures were used for Gram staining. The bacteria were picked and streaked onto nutrient agar slants to make sample cultures and for PCR purposes.

Gram Stain:

One drop of saline was mixed with a single colony on a slide and fixed with gentle heat. Crystal Violet Oxalate (Atlas, UK) was poured on the slide for 2-3 minutes. Gram's Iodine (mordant) was poured on the slide for 2-3 minutes. Alcohol decolorized was poured on the slide for 1 minute. Safranin counterstain was poured on the slide for 2-3 minutes. In each step, the slide was washed with distilled water. The slide was examined microscopically according to (Mihdir *et al.,* 2016). Gram-positive bacteria were blue or violet while gram-negative bacteria were pink or (Abulreesh *et al.,* 2016) red

Extraction of Genomic DNA by the Boiling Method:

1ml from each isolate was put in 1.5 Eppendorf and centrifuge for 5 min the pellet was suspended in 100 µl and put the Eppendorf for 5 min in boiling water and put it immediately after boiling in ice after 5 min centrifuge for 60 sec and take 2 µl for PCR.

Isolation of DNA:

One bacterial colony was suspended in 1 ml of lysis buffer and 15 µl of

proteinase K (200 µg/ml), then the mixture was vortexed. The mixture was incubated at 56 °C for 30 min, followed by 95°C for 10 min. An equal volume of ice-cold isopropanol was used to precipitate the DNA. Then, the DNA pellet was washed twice with 70% ethanol, dried, and resuspended in 50 µl of TE buffer (Atia et al., 20016).

16S rRNA Identification of Bacterial Isolates:

The 16S rRNA universal primers named 27F 5$^/$(AGAGTTTGATCCTGGCTCAG) 3$^/$ and 1492R5/ (TACGGTTACCTTGTTACGACTT) 3/ were used to amplify the genomic DNA of the bacterial isolates. The PCR was performed according to the instruction manual of Qiagen (PCR Kit). The PCR programme was 1 min of denaturation at 94°C, followed by 25 cycles of 96 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, with a final expansion under 72 °C for 10 min. The PCR product was analyzed on 1% (w/v) agarose gels. A 100 kb marker was used as a DNA marker using TBE as a buffer. Finally, the PCR product was purified using a QIAquick PCR purification kit (Qiagen) and eluted in 50 µl Tris–HCl before the sequence (Osman *et al., 2015).*

Electrophoresis of PCR Products:

PCR products were separated by electrophoresis on 1% (w/v) agarose gel. Electrophoresis was conducted in a horizontal Pharmacia Biotech equipment system (model Hoefer HE 99X; Amersham Pharmacia Biotech, Sweden) for 2 h at 60V using 1x TAE buffer (40mM Tris, 1mM EDTA, and 20mM glacial acetic acid, pH 8.0). The Gel contained a 100 bp DNA molecular weight marker (Fermentas, USA). A Gene Genius Bioimaging system (Syngene, Synoptics, UK) was used to capture the images.

Sequencing of the PCR Product and Gene Homology:

The purified PCR products were sequenced using a PRISM BigDye Terminator v3.1 Cycle Sequencing Kit. The sequencing primers were 785F 5$^/$(GGATTAGATACCCTGGTA)3$^/$ and907R 5$^/$(CCGTCAATTCCTTTAAGTTT) 3$^/$.

DNA samples containing the extension products were added to Hi-Di formamide. The mixture was incubated at 95 °C for 5 min, followed by 5 min on ice and then analyzed by the ABI Prism 3730XL DNA Analyzer (Applied Biosystems). The gene homology and related sequences were carried out by public databases BLAST at the NCBI server (El-Menofy *et al., 2014).*

Sequence Similarity and Phylogenetic Analysis:

Twenty-four partial 16S rRNA gene sequences have been scanned for similarity using BLAST in the National Central Bank Database (Mohamed *et al.,* 2015). The taxonomic hierarchies of the test sequences were obtained by defining the nearest neighbors on the basis of common words (percent) between test sequences and query sequences. The 24 rRNA sequences, as well as their closely related hits in the GenBank database, were aligned using the "Clustal W" algorithm (Salaheldin *et al.,* 2016a) using the default parameters. Phylogenetic analyses of partial gene sequences were executed by MEGA X (Salaheldin *et al.,* 2016b). The evolutionary background was derived from the use of the Maximum Likelihood test and the Tamura-Nei model. (EL-Ghareeb *et al.,* 2012). The tree with the highest log likelihood (-21143.14) is shown. To calculate the evolutionary distances through 1000 bootstraps, initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms (Osman, 2012) to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then by selecting a topology with a higher log probability value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of trees in which the associated taxa clustered together is shown next to the branches with a cutoff value of 70%. This analysis involved 47 nucleotide sequences. In total, there were 1664 positions in the final dataset.

RESULTS AND DISCUSSION
Water Samples Culturing:

Table (1) showed the number of plates which have grown. Fourteen water samples out of 27 water samples were clean (have no growth on the 3 plates), 6 of them have a growth on all 3 plates, 1 has on 2 plates, and 6 have a growth on one plate Figure (1).

Table 1: show the number of plates which have growth for each water samples

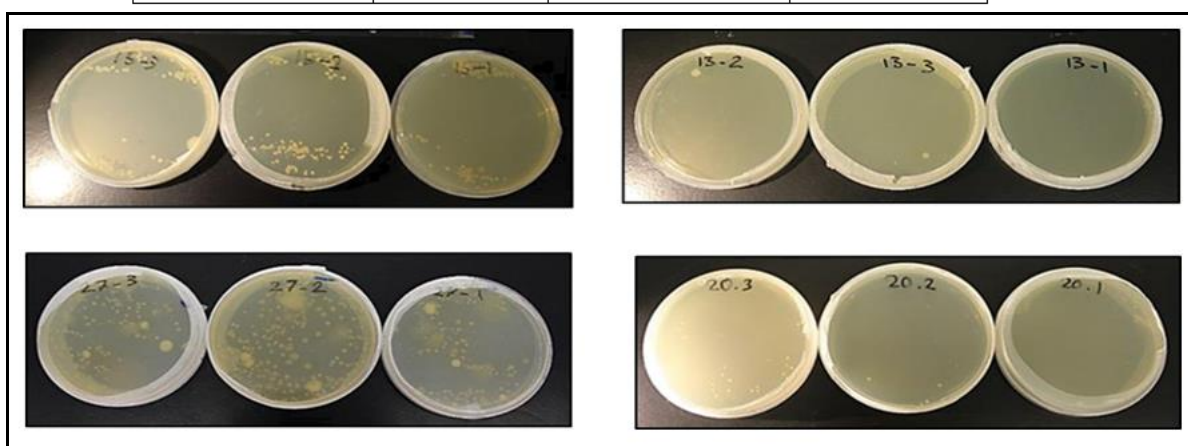| Sample number	Growth	Sample number	Growth
1	-	15	3 plates
2	-	16	1 plates
3	1 plate	17	1 plate
4	1 plate	18	-
5	-	19	-
6	1 plate	20	3 plates
7	-	21	-
8	-	22	3 plates
9	-	23	-
10	-	24	-
11	3 plates	25	1 plate
12	3 plates	26	-
13	2 plates	27	3 plates
14	-		

Fig.1: showing the bacterial growth on plates.

Molecular Characterization of Bacterial Isolates:

The 16S rRNA universal primers were used to amplify the genomic DNA of the bacterial isolates. The PCR products were electrophorized by 1% agarose gel, stained by ethidium bromide, and visualized by a UV transilluminator. According to the results, 35 PCR products of approximately 1,500 bp were visualized (Fig. 2).
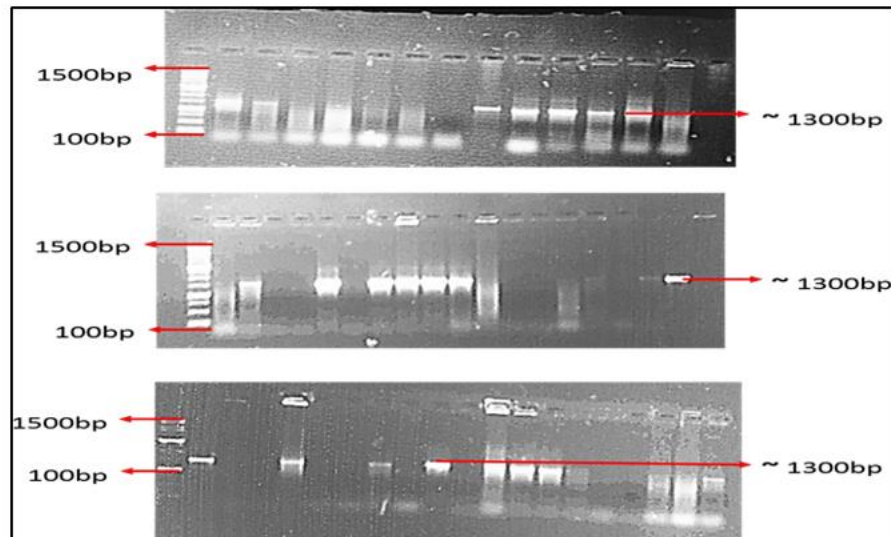
Fig.2: Ethidium bromide stained agarose gel resolving the PCR amplification fragment of 16S rRNA gene 1.3Kb from all isolates. (~1300 kbp)16S rRNA gene as PCR product. Lane M: DNA marker (100bp).

Phylogenetic Tree:

The phylogenetic dendrogram was constructed to know the genetic relationship between the bacterial isolates. The identification of the isolates was represented in Table 2 and their phylogenetic dendrogram as shown in Figure (3).

Table 2: 16S rRNA sequences analysis of the isolates.

| No	Isolate number	Best match description	Identity %
1	2	Pseudoxanthomonas_mexicana. Arabic, Gram-negative, non-spore-forming, rod-shaped bacteria with one polar flagellum. Isolated from human urine and urban riverside soil	97
2	5	Microbacterium sp. Gram-positive, nonspore-forming, rod-shaped bacteria. Marine habitat	96
3	7	Tsukamurella_pulmonis. Gram-positive Bacilli and aerobic bacterium. Cause pneumonia	97
4	8	Acidovorax sp., aerobic mesophilic Gram-negative bacterium OR beta proteobacterium Plant pathogen	97
5	9	Microbacterium trichothecenolyticum. Marine habitat, gram-positive, rod-shaped, commonly found in dairy products and are characterized by marked resistance to heat. Not normally pathogenic	96
6	10	Limnobacter thioxidans. Thiosulfate-oxidizing strains, Gram-negative bacterium with a single polar flagellum	96
7	14	Limnobacter thiooxidans. Thiosulfate-oxidizing strains, Gram-negative bacterium with a single polar flagellum	96
8	16	Sphingopyxis ummariensis Gram-negative, motile, rod-shaped and yellow-pigmented capable of growing on polyethylene glycol Isolated from hexachlorocyclohexane (HCH) dump site OR Estrogen-degrading bacterium.	97 or 98
9	17	Cupriavidus taiwanensis. Gram-negative, nitrogen-fixing bacterium	97
10	20	Acidovorax sp., aerobic mesophilic Gram-negative bacterium	97
11	23	Acidovorax sp., aerobic mesophilic Gram-negative bacterium	97
12	26	Sphingopyxis macrogoltabida Gram-negative, motile, rod-shaped and yellow-pigmented capable of growing on polyethylene glycol Isolated from hexachlorocyclohexane (HCH) dump site OR Kartchner_Caverns	96 or 96
13	28	Staphylococcus hominis. Gram-positive coagulase-negative, producing thioalcohol compounds that contribute to body odour	96
14	30	No significant similarity	
15	32	Lysinibacillus sp., Gram-positive, mesophilic, rod-shaped bacterium commonly found on soil.	95
16	33	Terribacillus halophilus OR Terribacillus aidingensis., Gram-positive aerobic and non-motile bacterium	89 or 88
17	36	Pseudomonas alcaligenes, Gram-negative, motile with polar monotrichous flagellation	96
18	38	Limnobacter thioxidans,, Thiosulfate-oxidizing strains, Gram-negative bacterium with a single polar flagellum	96
19	39	Acinetobacter junii, gram-negative	97
20	40	Pseudomonas alcaligenes, Gram-negative	96
21	46	Ideonella sp. Gram-negative OR Aquincola_sp., Gram-negative	95 or 95
22	48	Sphingomonas leidyi, Gram-negative, rod-shaped	95
23	49	Sphingopyxis_sp,Gram-negative, motile, rod-shaped OR Sphingomonas_sp. Gram-negative, rod-shaped	95
24	50	Sphingopyxis_sp.,Gram-negative, motile, rod-shaped OR Sphingomonas_sp. Gram-negative, rod-shaped	89
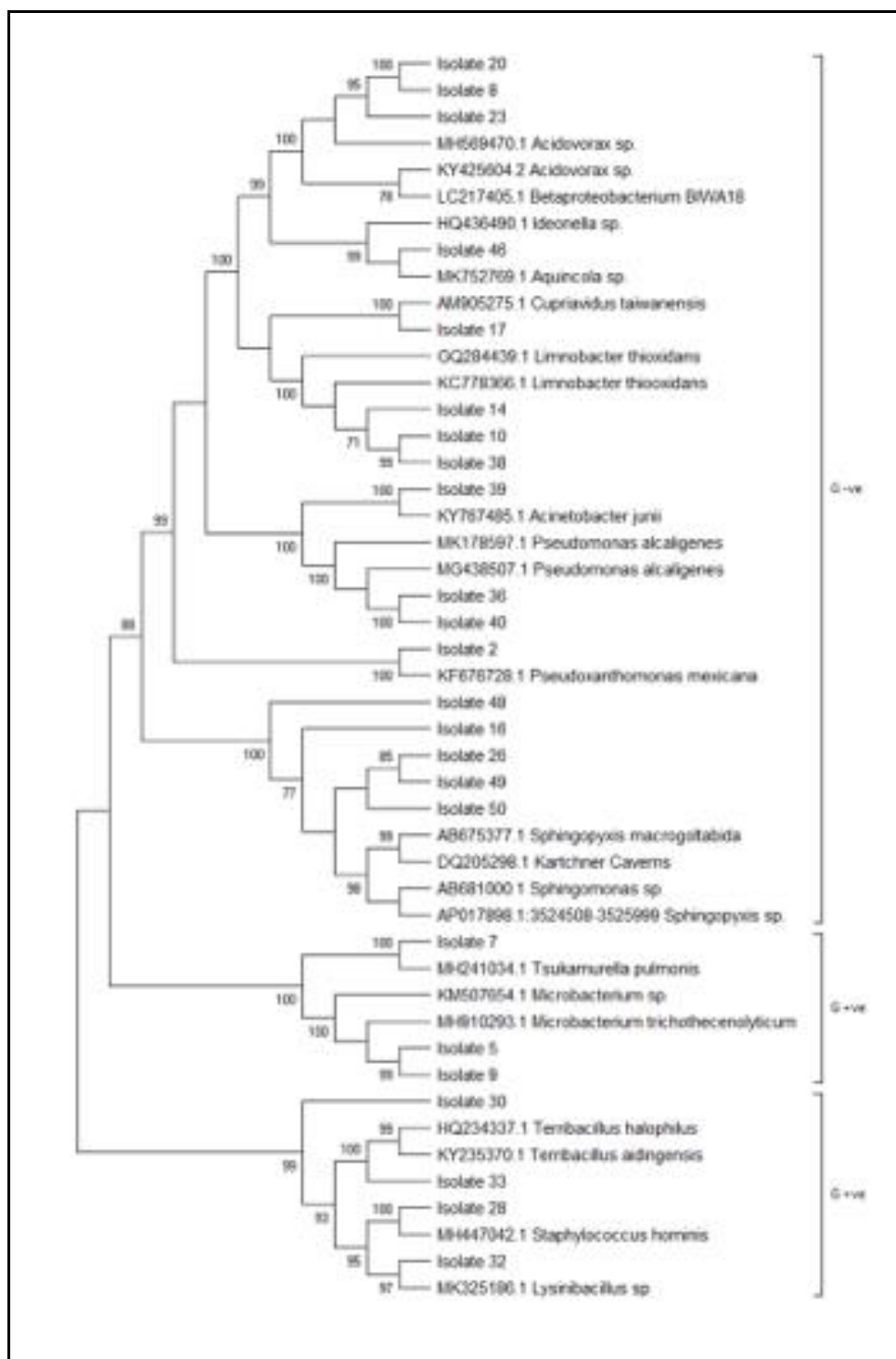
Fig.3: Phylogenetic analysis of the 16S rRNA gene sequences. Maximum Likelihood tree of twenty-four partial 16S rRNA gene sequences along with their closest relatives in the GenBank database (each sequence is shown as the accession number and scientific name) was constructed using MEGAX. Bootstrap values shown next to the nods are calculated based on 1000 resampled datasets.

Molecular Characterization and Genetic Identification of Isolates:

Sequencing of 16S- rRNA gene as a PCR based technique was used to identify the selected bacterial isolates. According to the alignment at the National Center for Biotechnology Information (NCBI), the 24 sequences of isolates in (Table 2) were

identified as Pseudoxanthomonas-mexicana, Microbacterium sp, Tsukamurella-pulmonis, Acidovorax sp., Microbacterium trichothecenolyticum, Limnobacter thioxidans, Sphingopyxis ummariensis, Cupriavidus taiwanensis. Acidovorax sp, Sphingopyxis macrogoltabida, Staphylococcus hominis, Lysinibacillus sp.,.Terribacillus halophilus OR Terribacillus aidingensis., Pseudomonas alcaligenes, Limnobacter thioxidans, Acinetobacter junii, Pseudomonas alcaligenes, Ideonella sp. Isolate number 30 has no similarity with any bacterial isolates on NCBI which indicates that it is anew bacterial isolate. Many researchers employed 16S-rRNA biotechnology to identify isolated bacterial species from water and other environments (Osman *et al.,* 2016).

Sequence Analysis and Phylogeny:

Isolates 8, 20 and 23 are closely related to each other, their closest relative in the database is the genus *Acidovorax*. Nether the similarity search nor the phylogenetic analysis were able to predict a specific species for any of the three isolates. However, the phylogenetic analysis pointed out that isolate 23 is distanced from isolates 8 and 20 as indicated by its long branch length.

1. The similarity search result showed that isolate 48 belong to genus *Sphingomonas* with identity score 97%. However, the phylogenetic analysis grouped it with isolates 16, 26, 49, and 50 where they are branched from the same node with the genera *Sphingopyxis,* and *Kartchner*, indicating that the new isolates are related to but distinct from their closest genera.

2. Isolate 2 16S rRNA gene sequence was found to be highly similar to that of *Psedoxanthomonas mexicana* with a similarity score 97% which was also supported by the phylogenetic analysis. Likewise, isolate 17 is anticipated to be a member of *Cupriavidus taiwanensis* with strong bootstrap support. Isolate 39 was found to be a member of *Acinetobacter junii*. While isolate 36 and 40 were proposed to be *Pseudomonas alcaligenes*.

Also, isolate 7 was identified as *Tsukamurella pulmonis*. Isolate 5 and 9 were recognized as members of genus *Micobacterium*, however, the similarity score as well as the phylogenetic analysis were unable to determine the specific species. Isolate 33 was identified as a member of genus *Terribacillus* but no conclusive result about the species, isolate 28 was found to be *Staphylococcus hominis* while isolate 32 was found to be a member of genus *Lysinibacillus* but the species could not be predicted.

3. The phylogenetic analysis was able to correlate isolate 46 to genus *Aquincola* with strong bootstrap value (99%) separating it from genus *Ideonella* that was suggested by the similarity search by the same identity score (95%) as genus *Aquincola*.

4. Interestingly, isolate 30 has no significant similarity with any of the bacterial isolates recorded in the database, and its branch length, in the phylogeny, is significantly longer than its closest relative. Further analysis is required before proposing a new genus.

Conclusion

bottled water is an important source of drinking water in the KSA, which has limited resources of freshwater. Continuous monitoring of water quality and effectiveness of the treatment processes, and obeying regulations, are required to ensure that the water quality meets the set standards and to meet the increasing demand for good quality tap or bottled water. The study revealed that most of the chemical parameters were within the World Health Organization limits (WHO) regardless of the brand name. No *E. coli* was detected throughout the study period. There is a need for a rigorous inspection and follow-up of water bottling facilities and municipal water so that only those companies which consistently produce water of acceptable bacteriological quality are allowed to produce water for public consumption. It is recommended that Saudi Food and Drugs Authority (SFDA) should promulgate the standardized method of the

bottled water industry in order to increase its characters and shelf life. Also, the results of these analyses indicated the need to improve Hazard Analysis Critical Control Points (HACCP) systems, in order to continuously monitor the water supply source in bottling plants and at supermarkets.

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