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Mutual correlation between different microorganisms and bacterial indicators and their effect on bottled water quality in Egyptian market

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Abstract : The main objective of this study is to detect the correlation between presence of microorganisms and bacterial indicators and some physicochemical properties of bottled water samples. During the period from January to June, 2015, monthly samples (n= 54) were collected from markets in Cairo, Egypt which produced by 6 commercial brands (A, B, C, D, E and F).Isolation and identification of classical bacterial indicators, total molds and coliphages as well as sensitivity test for 12 types of antibiotics for *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Bacillus subtillus* were carried out. The physico-chemical characteristics namelypH, total dissolved salts (TDS), electrical conductivity (Ec), nitrate and ammonia concentration, were determined in bottled water samples. In addition, all microorganisms (except coliphage and fungi) were examined for survival in bottled water in the presence of both inoculation (10 – 100 cfu / 100ml) and without inoculation for 6 months of storing at room temperature. Result showed that, all samples were free from bacterial indicators. Also, result revealed that heterotrophic plate counts (HPCat 37ºC and 22ºC) and some physicochemical characteristics were found to be complying with the Egyptian Standard and within International Standard for drinking water. Moreover, results of microbial and physicochemical analysis were safe according to Egyptian Standards for drinking water, after 6 months of storingfor some bottled water samples at room temperature. Also, results showed that positive correlation between HPC and physicochemical characteristics with absence of bacterial indicators. Although bacterial indicators were absent, some bacteria have been isolated and identified including *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Bacillus subtillus.* These bacteria were multi-antibiotic resistant (MAR) when studied against to antibiotic (12 kinds) sensitivity test. *Pseudomonas aeruginosa,* and *Bacillus subtillus* were more survive than other microbes examined including bacterial indicators, yeast and *Staphylococcus aureus* when inoculating with $\log_{10}$about 1 to 2 cfu/100ml for 6 monthsat room temperature in some samples. Positive correlation (*r= 0.856*) were observed in this studybetween time (storing period) and counts of microbes (before and after storing) where recorded. Statistical analysis of results showed no significant correlation between bacterial isolates and absence of bacterial indicators. Moreover, in this investigation, observed correlation of both filamentous fungi and yeasts with free bacterial indicators as well as presence of HPC bacteria were found to be significant (*r= 0.856*). The results showed that the presence of HPC bacteria, negatively correlated with nitrate (r=-0.06), while positively correlated with other physicochemical parameters.
This work concluded that, it is necessary to regularly monitor bottled water to protect public health of consumers.
Key words : Classical bacterial indicators, Bacteria, Total molds, Coliphages, Antibiotic Resistant bacteria, Bottled water, Physico-chemical characteristics, Storing time.

Introduction

Using bottled water in the world has been steadily growing annually by 12%, although tap water has low price compared with bottled water **(Rosemann,[1]).** In Egypt, bottled water is considerable sources soft drink for consumers. Generally, bottled water is not sterile and should be free from pathogenic microorganisms and chemical pollution **(Warbuton, etal[2] and Saleh, etal[3]).** However, bottled water taste does not indicate safeness. Also, **Warburton,[4;5]** reported that bottled water may contain microorganisms like in surface water and therefore some microbial can able to grow may harm the consumer health.

Sources of bottled water may be wells, spring or other sources that it must be safe with or without treatment **(Ramalho, etal[6]).** Moreover, the treatment used in bottled water depends on the initial quality of water **(Warbuton and Austin,[7]).** In addition, bottled water may be faced some condition leading to re-contamination e.g. steps of manufacture, during the storage and selling periods **(Legnani, etal[8] and WHO,[9;10]).**

Some studies **(Hunter,[11]; Schindler, etal[12] and Legnani, etal[8])** reported that, the presence of *Pseudomonas aeruginosa*, staphylococci, total yeast and coliphage indicate the pollution with organic material or domestic wastewater and may be due to the contamination during the bottling process. In addition, **Schindler, etal[12],** used *Pseudomonas aeruginosa* in bottled water as an indicator of suitability for drinking water and good manufacturing processes in some countries (Canada, France, Germany, United States and others). Moreover, **Ali, etal[13]** concluded that, staphylococci group can be considered as bacterial indicators showing a significant relation with physicochemical characteristic and phytoplankton biomass. Moreover, in absent bacterial indicators, the presence of coliphage in water used as a more specific index of fecal pollution **(EL-Abagy, etal[14]).**

Yamaguchi, etal[15]), found that, the presence of yeasts was not correlated, with bacterial indicators and filamentous fungi in bottled mineral water samples. **FSAI,[16]** reported that there is no direct correlation in bottled water between bacterial indicator counts and number of pathogenic bacteria. Moreover they concluded that in absence of bacterial indicators mean that the water free from pathogenic bacteria but it possible present other pathogenic microorganisms too **(Ashbolt, etal[17]).** Also, in Bangladesh, **Ahmed, etal[18]** noted that, there were no correlations recorded between presence or absence *Pseudomonas aeruginosa* and *Salmonella* spp., and numbers of bacterial indicators in bottled water. In addition, in India **Venkatesan, etal[19]** found that 33.3% of bottled water samples, examined bacteriologically, samples were failed according to the **WHO[20]**for drinking water standard although these samples were free from bacterial indicators. Moreover, there were some pathogenic bacteria detected in absence of bacterial indicators like *Pseudomonas* spp., *Staphylococcus aureus* and *Bacillus* spp. then, they reported that no significantly correlations between presence or absence of pathogenic bacteria and bacterial indicators (**Venkatesan, etal[19]**).

Several studies **(Rivera, etal[21]; Warburton,[4]; Warburton, etal[22]; Kerr, etal[23] and Leclerc & Moreau,[24])** suggested that there were strong correlation between several human diseases and the presence of enteric bacteria, protozoa or viruses in absence of bacterial indicators. These microorganisms could be multiply during storage causing infective diseases for consumers. **Jeena, etal[25]**studied the correlation between total heterotrophic bacterial load (THB) and coliform bacteria in bottled drinking water. It was noted that, there are linear relationship between THB and coliform bacteria. Moreover, they concluded that, the presence of any loads of bacteria especially resistant to some antibiotics (malty-antibiotic resistant), may be out break diseases for consumers. While in Iran **Farhadkhani, etal[26]**studied correlation analyses between THB population, temperature, total organic carbon and electric conductivity in bottled and tap water, they found a significant effect.

Also, the presence of nitrate, nitrite or ammonia with high concentration (more level than **WHO[20]**may harm the consumers as well as produce a bad taste of water. This may be lead to methemoglobinemia and death for infants **(Abouleish,[27]).** In addition, some studies **(Hirondel and Hirondel,[28]; Mesa, etal[29] and WHO,[10])** reported that, by increasing the concentration of nitrate, nitrite or ammonia than permissible standard for drinking water due to water contamination with microorganisms especially bacteria. **Yasin, etal[30]** noticed that, the presence of aerobic microorganisms were positively correlated with total suspended solids (TSS), turbidity and dissolved oxygen (DO) while, negatively correlated with electric conductivity (Ec), total dissolved solid (TDS) and pH. Also, dissolved oxygen is one of the important of water quality where it is the correlation with

water body gives direct and indirect information e.g. bacterial activity or photosynthesis and other of stratification, etc **(Premlata,[31]).**

On the contrary, **Rabee, etal[32],** noticed that there were no correlation between physicochemical parameters and microbial loads in some bottled water. Where authors observed that, according to international standard specifications for bottled and drinking water Iraqi, physicochemical properties were accepted but microbial loads were not. Also, data from groundwater analysis in South Africa by **Palamuleni and Akoth[33],** concluded that, physicochemical parameters were within acceptable permissible levels while, microbial loads (spring season) were higher according to World Health Organization (WHO) and Department of Water Affairs (DWAF) as well as this type of water may pose severe health risk causing diseases to consumers.

Thus, the aim of the present work is to correlate the presence of some microbes and bacterial indicators to assess the quality of bottled water to gauge the safety by examining biological and physicochemical quality as well as comparing with the Egyptian Standards in order to protect public health consumers.

Materials and Methods

Water samples

The present study was extended for 12 months period (from January to December, 2015) on a total of 324 bottled water samples of 6 commercial water producing brands in Egypt. The bottled water samples were obtained from local markets in Cairo, Egypt. The samples were opened in the laboratory and analyzed within 2 to 3 hours according to **APHA,[34].** The production date of all samples was the same for the samples of each brand. These bottles have a validity date of one year and volume of was 1.5 L each. The bottled water samples were designated from A to F (6 specimens each). Bottled water samples (54 bottles) were collected, divided into 4 groups, first 12 bottles (2 bottles from each brand) for microbial examinations. The second group 6 bottles (one bottles from specimens each) were examined for physico-chemical analysis. The third group (control) 18 bottles (3 bottles from each brand) were stored at room temperatures to detect the effect of storage on their quality for 6 months, for microbial (12 bottles) and physico-chemical (6 bottles) characteristics. The 4th group, was the same conditions of group 3th but inoculating every bottles with some microbes with count about 10 to 50 cfu/100ml, to follow up their survival in bottled water at room temperatures for 6 months. After storing, (groups 3rd and 4th) water samples were examined for bacterial and physico-chemical parameters characteristics.

Microbial examination:

In this investigation, water samples were examined for total bacterial counts at 22ºC and 37ºC, using poured plate. While, total coliform, fecal coliform and fecal streptococci as well as *Pseudomonas aeruginosa* (confirmed by used PA agar) were detected by using MPN (Most Probable Number) methods according to **APHA[34].**

On other hand, 100ml were filtrated from each bottled water sample. Samples were analyzed for detection and enumeration of total staphylococci group, total yeast and fungi, by using membrane filter technique (0.45µm pore size and 47mm diameter) and the filtrate were transferred onto the surface plates of mannitol salt agar, Yeast Malt Agar and Sabouraud Dextrose Agar, respectively **(APHA[34]).**

Moreover, detection and enumerated of coliphage (pfu) (plaques forming unit/100 ml) test was carried out according **APHA,[34].** In addition, bacterial isolates were identified according to **APHA,[34].**

Survival bacterial experiment:

The source of microbial isolates (which used in the entire work) was from groundwater samples. Isolates (*E. coli, Streptococcus faecalis, Staphylococcus aureus, Pseudomonas aeruginosa* and *Bacillus subtillus* as well as yeast) were detected and identified (except yeast) according to **APHA,[34].**

A loop-full from one specific colony for each microbial isolate was transferred to 5 ml Tripticase Soya broth tube and incubated at 37°C for 24 hours (yeast for 2 days). After incubation, the tubes were centrifuged at 5000 rpm for 15 minutes. The obtained pellets were separately transferred to 5 ml sterile saline water and then

vortexed. The washing, centrifugation and vortexing steps were repeated three times then the microbial solution was ready for use.

By using dilution method, number of colony forming units in each of the obtained microbial suspension was determined using plate count agar for bacteria and yeast malt agar for yeast, according to **APHA,[34].** Finally, the inoculums count was about 10 to 50 cfu/100 ml for each bottled water tested.

Physicochemical Analysis:

In this study, the samples were analyzed for water quality physicochemical parameters such as, pH, total dissolved salts (TDS), electrical conductivity (Ec), nitrate and ammonia, were determined according to standards procedures described in APHA, 2015.

Antibiotic bioassay:

The sensitivity of the strains against various antibiotics were determined by using antibiotic discs; Coloxicillin (30 µg), Naldixic Acid (30 µg), Gentamycin (10 µg), kanamycin (30 µg), Ampicillin (30 µg), Amoxicillin (30 µg), Erythromycin (15 µg), Tetracycline (30 µg), Nitrofurantion (200µg), Lincomycine (15 µg), Rifamipicin (50 µg), and Chloramphinicol (30 µg), Mueller Hinton Agar (MHA) by Himedia (M173) was used to evaluate the bacteria tested for antibiotic resistance. One colony from pure culture (24 hours age grown on triptcase soy agar) put onto 2 ml sterile distilled water tubes and vortex for 5 minutes. By sterile pipette 0.2 ml from these tubes put onto Mueller Hinton Agar plates. Streaking it by rod glass, then draying plates and by forceps antibiotic discs were putted and plates were incubated for 24 hours at 37^{o}C. After incubation, the present growth resistanc made zone but the absent was sensitive as well as the plates control which contain Mueller Hinton Agar without antibiotic was tested. Antibiotics which used in the present investigation were obtained from Alkan Medical Company, Egypt..

Statistical Analysis:

Statistical analysis was performed as for two factorial randomized complete block design according to **Gomez and Gomez[35].**

Results and Dissections

I- Examination of Heterotrophic Plate Counts (HPC) in bottled water

In many parts of world, the quality of drinking bottled water varies from place to another and from company to other in the same country. The present investigation of bacterial and chemical characteristics of bottled water identifies the safety of water quality which may cause direct diseases for consumers. Generally, the quantity of microbes in bottled water depended on the disinfection process of natural spring which used in the produced bottled water **(Nsanze, etal[36]).**

Table (1): Statistical analysis of total viable bacterial count (cfu / 100ml) at 37ºC and 22ºC from six types of bottled water companies (A, B, C, D, E and F) collected from Egyptian market at different conditions (I, II, III and IV) during 2015.

Para-meter	Total bacteria viable count (cfu / 100ml) at:							
	37ºC				22ºC			
	1	Ii	III	IV	1	Ii	III	IV
A								
MIN	24	18	107	65	18	13	89	52
MD	37.5	37.5	143.5	79	29.5	25.5	122.5	65
MAX	44	47	213	89	36	41	199	79
AV	36	36.33	149.33	78.67	28	26.17	130.83	65.17
SD	7.72	10.76	41.02	9.75	7.13	11.89	40.24	8.57
B								
MIN	66	52	119	98	55	43	107	92
MD	76	67	149	114	64	55	122	97
MAX	84	111	177	127	81	98	153	113
AV	75.17	72.33	149.67	114.17	66.17	62.67	125.33	99.67
SD	8.08	21.95	25.26	10.98	9.75	21.29	16.49	8.62
C								
MIN	33	52	116	122	25	44	105	107
MD	69	74.5	167	149.5	60	68.5	154	121.5
MAX	93	117	214	188	82	105	186	139
AV	66.33	79.83	165.67	150.33	57.83	71.16667	149	121.67
SD	20.86	25.10	46.89	22.51	19.65	23.46	36.62	10.98
D								
MIN	28	17	126	38	18	11	109	32
MD	34.5	30	168	55.5	26.5	25.5	120.5	44.5
MAX	44	59	211	88	35	49	175	64
AV	34.67	33.83	171	58.17	27.17	27.33	135.33	45.83
SD	6.31	15.61	31.79	17.70	6.18	14.24	29.52	11.94
E								
MIN	38	33	117	55	29	28	108	46
MD	48.5	51.5	161.5	68.5	39.5	42	142.5	56
MAX	67	66	216	94	54	51	189	85
AV	50.5	49.33	168	72.5	40.33	40.17	147	62.5
SD	10.48	13.25	36.33	17.31	8.50	9.37	28.02	17.68
F								
MIN	21	14	139	49	14	9	118	38
MD	29.5	36	183.5	68.5	20.5	26.5	157	54
MAX	46	59	197	81	34	41	167	62
AV	31.17	35.17	178.17	65.17	22.33	24.67	151.83	51.33
SD	8.94	16.81	21.31	11.91	7.35	11.91	18.24	9.26

I = examined during 2-3 hours, II = examined after storing for 6 months at room temperature, III = examined after inoculum with bacteria, IV = examined after storing with inoculum by bacteria, A, B, C, D, E and F=Bottled water companies, SD = Standard Deviation, AV = average, MD = Median, MAX = maximum, MIN = minimum

Heterotrophic Plate Counts (HPC) examined in water could be considered as potential indicators for sanitation and safety as well as in some cases may indicate presence of pathogenic bacteria. In this study, statistical results analysis of total viable bacterial count at 37ºC and 22ºC were recorded in **Table 1**. Results showed that, total viable bacterial averages count at 37ºC and 22ºC were accepted according to the Egyptian Standard (2007) for drinking water (less than 50 cfu /ml). The higher average count (cfu / 100ml) at 37ºC and 22ºC were 75.17 and 66.17, respectively in samples of group B. But the lower average count (cfu / 100ml) at 37ºC and 22ºC were observed in samples of group F with counts of 31.17 and 22.33, respectively. The results were line with **Abd El-Salam, etal**[37] in Egypt, who examined 84 samples of bottled water (from 14 brands)

using standard methods of HPC comparing them with the Egyptian Standards. Authors found that 8 brands (57.14 %) were accepted but the remaining were not, where 7.14 % from all samples were exceeded 10^3 and others exceeded 10^2 according to Egyptian Regulation. HPC in this study were lower than that recorded by **Osman, etal[38]**in Egypt who counted (cfu / 100ml) HPC at 37ºC and 22ºC from 4 brands of bottled water collected from Egyptian markets. The average range of their results was from varied from 258 to 118 and from 105 to 216 at 37ºC and 22ºC, respectively.

Also, in the present study, the result of HPC were not in agreement with **Khaniki, etal[39]2010** in Iran, who observed that, HPC in 35 bottled water samples collected from markets. Their results showed that, the standard deviation and mean values of their results were 2.07and 3.14 cfu /100ml, respectively, as well as this data was acceptable according to Iranian Regulation. This data was lower than our study **(Table 1).**

Also, data of our investigation were lower than that obtained by **Venkatesan, etal[19]**in India, who detected HPC at 35ºC in 5 brands of bottled water collected from Indian market. The mean count of HPC varied from 230 to 3400 cfu/100ml. In addition, they reported that although coliform bacteria were not detected in all sample of bottled water, but this type of water unfit for human consumption.

In the other side, in this study, after 6 months of storing at room temperature, the higher average count (cfu / 100ml) at 37ºC and 22ºC were 79.83 and 71.17, respectively in samples of group C. In the contrary, the lower average count (cfu / 100ml) at 37ºC and 22ºC were in group D (33.83) and group F (24.67) samples, respectively.

After inoculation with some microbes in some bottled water samples (before storing), the maximum value of total viable bacterial count (cfu / 100ml) at 37ºC was 214 in samples of group C while, at 22ºC was 189 in samples of group E. On the other hand, the minimum was observed in samples of group A which was 107 and 89 cfu / 100ml at 37ºC and 22ºC, respectively.

After 6 months of storing at room temperature, these count (maximum) at 37ºC and 22ºC were decreased reaching 188 cfu / 100ml (with count reduction by 12.96 %) and 85 cfu / 100ml (with count reduction by 55.03 %), respectively. while, the minimum counts were decreased until reaching 65 cfu /100ml (39.25% reduction) and 52 cfu / 100ml (41.57% reduction) at 37ºC and 22ºC, respectively. This means that during storage, bacteria can be able to survive in bottled water depending on suitable temperature, pH and enough nutrients (organic matter) as well as type of microorganism for regrowth **(Stickler[40]; Guerzoni, etal[41]; Warburton[5] and John and Rose,[42])**. Moreover, the data showed the correlation between time (storing period) and counts (before and after storing) were recorded positive correlation (r= 0.856).

The data were targeted the same goal with that obtained by **Akinde, etal[43]**, in Nigeria who noticed that HPC (initial count was 102 cfu / 100 ml) was increased gradually within four weeks but reached zero level after 4 months from 10 brands of bottled water.

Also, data in this study were in a good agreement with **Mardani, etal[44]**in Iran who concluded that, by increasing the storage period of bottled water at room temperature leading to the increase in the count of microbial loads depending on some condition (organic matters, temperatures and pH).

2- Examination of bacterial indicators in bottled water

The absence of bacterial indicators in water indicating the safety of water but present these bacteria indicate contaminated by fecal matter **(WHO[10]).** Data given in **Table (2)** indicated that, all samples were free from bacterial indicators. This means that, this water were safe biologically according to **Egyptian Standard[45]**for drinking water. But in this study was detected HPC for bacteria in absent bacterial indicators. So, bottled water is not necessarily safe in absent bacterial indicators because may be exposed to another source leading to re-contaminated **(Warburton[5]).** Also, data showed that no correlation between absence of bacterial indicators and safety for human consumers.

This data was in same target with **Obiri-Danso, etal[46]**in Ghana who observed bacterial indicators were absent in bottled water (3 brands) samples (n=8) but counted HPC from 1 to 460 cfu / ml. Authors concluded that, this type of water may be contained pathogenic bacteria. Also, data were in a line with **Jeena, etal[25]** in India who noticed in 105 samples of bottled water samples (35 brands) that increased HPC due to appeared (indicating a linear relationship) coliform bacteria. Authors observed that, counts of HPC less than 100 cfu / ml while total fecal was zero tolerance for coliforms. But more than this count (HPC), tested positive for coliforms. Also, they isolated some pathogenic bacteria (*Staphylococcus* spp. and *Aeromonas* spp.) in free bacterial

indicators. Moreover, this investigation was in line with **Al-Zahrani and El-Hamshary**[47] in Saudi Arabia, who examined (17 brands) of 51 bottled water samples for bacterial analysis. Authors found that, although all samples were free from bacterial indicators but detected some pathogenic bacteria.

On other hand, this study examined the bacterial indicators for 6 months storing at room temperature for all bottled water samples. Results reveled that, from maximum counts (23 MPN-index / 100ml) were reached zero count. This means that, bacterial indicators were not able to survive in these conditions. This may be due to the lack in organic matters, pH and incubation temperatures **(John and Rose,**[42]**).** Moreover, statistical analysis indicated that negative significant correlation between counts and time for bacterial indicators.

Table 2: Statistical analysis of total coliform and fecal streptococci count (MPN-index/100ml) from six types of bottled water brands (A, B, C, D, E and F) collected from Egyptian market at different conditions (I, II, III and IV) during 2015.

Para-meter	MPN-index / 100ml) at:							
	Total coliform				Fecal streptococci			
	1	Ii	III	IV	1	Ii	III	IV
A								
MIN	0	0	11	0	0	0	11	0
MD	0	0	16.5	0	0	0	16	0
MAX	0	0	23	0	0	0	23	3
AV	0	0	16.17	0	0	0	16	0.67
SD	0	0	4.36	0	0	0	4.34	1.21
B								
MIN	0	0	15	0	0	0	11	0
MD	0	0	19.5	1	0	0	14	0.5
MAX	0	0	23	5	0	0	23	4
AV	0	0	19.5	1.5	0	0	15.17	1
SD	0	0	3.33	1.87	0	0	4.54	1.55
C								
MIN	0	0	12	0	0	0	13	0
MD	0	0	16.5	1	0	0	16	1
MAX	0	0	18	2	0	0	18	1
AV	0	0	16	1.17	0	0	16	0.83
SD	0	0	2.28	0.75	0	0	1.90	0.41
D								
MIN	0	0	12	0	0	0	11	0
MD	0	0	16	0	0	0	16	1
MAX	0	0	23	0	0	0	23	1
AV	0	0	17.17	0	0	0	17.33	0.67
SD	0	0	4.07	0	0	0	4.76	0.52
E								
MIN	0	0	12	0	0	0	11	0
MD	0	0	17	0	0	0	16	0
MAX	0	0	23	0	0	0	23	0
AV	0	0	17.5	0	0	0	15.83	0
SD	0	0	4.03	0	0	0	4.27	0
F								
MIN	0	0	12	0	0	0	11	0
MD	0	0	17	0	0	0	12	0
MAX	0	0	23	0	0	0	23	0
AV	0	0	18	0	0	0	14.33	0
SD	0	0	4.16	0	0	0	4.79	0

I = examined during 2-3 hours, II = examined after storing for 6 months at room temperature, III = examined after inoculum with bacteria, IV = examined after storing with inoculum by bacteria, A, B, C, D, E and F=Bottled water companies, SD = Standard Deviation, AV = average, MD = Median, MAX = maximum, MIN = minimum

Results of **Sakyi and Asare,**[48]in Ghana studied viability of total coliform and *E. coli* in sterilized tap water. They found that, complete log_{10} count reduction (initial count log_{10} 4.5 cfu / 100ml) observed at the 7th day for both bacterial strains. Also, data were in agreement with **El-Leithy etal**[49] in Egypt who followed the survival *E. coli* (strain O157:H7 ; ATCC 35150) at room temperature in sterilized ground water with initial log_{10} count 6. Authors found that complete log_{10} reduction occurred at the 84th day.

Moreover, **Tandon, etal**[50]noticed that, fecal streptococci in groundwater was reduced from 10 to 120-fold through 12 to 48 hours, they concluded the decreased count due to unsuitable conditions for growth. In addition, authors reported that, the rate of reduction depend upon the enumeration conditions.

Data in this study showed that, it is not necessary absent bacterial indicators in bottled water indicated on fit water for drinking.

3- Examination of some new indicators in bottled water

Although all samples were free from bacterial indicators but some pathogenic bacteria were detected **(Tables 3 and 4). Table (3)** shows samples of groups E and F brands were free from *Pseudomonas aeruginosa* and *Bacillus subtillus* (after 6 months of storing at room temperature). On the other hand, in samples of group B, 7 and 15 cfu/100 ml for *Pseudomonas aeruginosa* and *Bacillus subtillus* were recorded as higher values, respectively. After 6 months of storing at room temperature from samples of group B, the count were 12 and 13 cfu/100 ml for *Pseudomonas aeruginosa* and *Bacillus subtillus*, respectively. Also, in this study, after inoculation with different types of bacteria, the count of *Pseudomonas aeruginosa* was 23 while, *Bacillus subtillus* was 45 cfu/100 ml in samples of group B. After 6 months at of storing at room temperature, the count of *Pseudomonas aeruginosa* was fixed but *Bacillus subtillus* was reached 39 cfu / 100 ml. Data indicated that, these types of bacteria were able to survive in bottled water for several months. In addition, no significant correlation between presence of bacterial indicators and the previously mentioned bacteria.

These results were confirmed by that obtained by **Abou-Ali**[51] in Egypt who found that, bacterial indicators were not detected in tested bottled water while the most prevalent bacteria were *Bacillus* spp. and *Pseudomonas aeruginosa*. Moreover, **Tamagnini and Gonzalez,**[52]also observed that *Pseudomonas aeruginosa* can multiply and reach to very high count after water bottling.

Table 3 Statistical analysis of *Pseudomonas aeruginosa* and *Bacillus subtillus* count (cfu/100ml) from six types of bottled water brands (A, B, C, D, E and F) collected from Egyptian market at different conditions (I, II, III and IV) during 2015.

Para-meter	Total bacterial viable count (cfu / 100ml)							
	Pseudomonas aeruginosa				*Bacillus subtillus*			
	1	**Ii**	**III**	**IV**	**1**	**Ii**	**III**	**IV**
A								
MIN	0	0	12	15	0	0	16	15
MD	0	0.5	17	16	0	0	24	21.5
MAX	1	3	23	18	2	2	31	29
AV	0.17	0.83	17.5	16.33	0.5	0.5	23.5	22
SD	0.41	1.17	4.04	1.37	0.84	0.84	5.86	5.87
B								
MIN	0	0	11	11	0	0	27	23
MD	3	4.5	16	15.5	4.5	4	37	33.5
MAX	7	12	23	23	15	13	45	39
AV	3	4.33	17.33	16.17	5.83	5	37	32
SD	2.83	4.41	4.77	4.99	6.68	5.73	7.35	7.21
C								
MIN	0	0	16	12	0	0	33	31
MD	1	1	21	18	5.5	5	45	40.5
MAX	2	3	23	18	13	11	49	42
AV	0.83	1	20	16.67	5.83	5	42.33	37.67
SD	0.75	1.10	3.22	2.42	4.53	3.90	6.68	5.20
D								
MIN	0	0	15	11	0	0	34	19
MD	0	0	17	14	0	0	40.5	37
MAX	1	1	23	23	1	1	48	47
AV	0.17	0.17	18.17	15	0.17	0.17	40.33	35.5
SD	0.41	0.41	3.19	4.47	0.41	0.41	5.39	9.59
E								
MIN	0	0	18	11	0	0	32	18
MD	0	0	22	16	0	0	39	26.5
MAX	0	0	25	18	0	0	52	38
AV	0	0	21.33	14.83	0	0	39.83	27
SD	0	0	2.88	2.71	0	0	7.52	7.43
F								
MIN	0	0	18	12	0	0	32	25
MD	0	0	22	16	0	0	38	33.5
MAX	0	0	23	18	0	0	44	41
AV	0	0	21	16	0	0	38	32.67
SD	0	0	2.21	2.20	0	0	4.45	5.52

I = examined during 2-3 hours, II = examined after storing for 6 months at room temperature, III = examined after inoculum with bacteria, IV = examined after storing with inoculum by bacteria, A, B, C, D, E and F=Bottled water companies, SD = Standard Deviation, AV = average, MD = Median, MAX = maximum, MIN = minimum

Table 4: Statistical analysis of *Staphylococcus aureus* (cfu / 100ml) and coliphage (PFU/100ml) count (cfu / 100ml) from six types of bottled water brands (A, B, C, D, E and F) collected from Egyptian market at different conditions (I, II, III and IV) during 2015.

Para meter	viable count / 100ml							
	Staphylococcus aureus (cfu)				Coliphage (PFU)			
	1	Ii	III	IV	1	Ii	III	IV
A								
MIN	0	0	12	0	0	0	ND	ND
MD	0	0	17	0	0	0	ND	ND
MAX	0	0	23	0	0	0	ND	ND
AV	0	0	17.17	0	0	0	ND	ND
SD	0	0	3.60	0	0	0	ND	ND
B								
MIN	0	0	16	0	0	0	ND	ND
MD	1.5	0	18	0.5	1.5	0	ND	ND
MAX	2	0	23	1	2	0	ND	ND
AV	1.33	0	18.67	0.5	1.33	0	ND	ND
SD	0.82	0	2.80	0.55	0.82	0	ND	ND
C								
MIN	0	0	16	0	0	0	ND	ND
MD	0	0	21	0.5	0	0	ND	ND
MAX	0	0	23	1	0	0	ND	ND
AV	0	0	20.33	0.5	0	0	ND	ND
SD	0	0	2.81	0.55	0	0	ND	ND
D								
MIN	0	0	16	0	0	0	ND	ND
MD	0	0	19.5	0	0	0	ND	ND
MAX	1	0	23	1	1	0	ND	ND
AV	0.17	0	19.67	0.33	0.17	0	ND	ND
SD	0.41	0	3.08	0.52	0.41	0	ND	ND
E								
MIN	0	0	16	0	0	0	ND	ND
MD	0	0	17.5	0	0	0	ND	ND
MAX	0	0	23	0	0	0	ND	ND
AV	0	0	18.17	0	0	0	ND	ND
SD	0	0	2.48	0	0	0	ND	ND
F								
MIN	0	0	12	0	0	0	ND	ND
MD	0	0	17.5	0	0	0	ND	ND
MAX	0	0	23	0	0	0	ND	ND
AV	0	0	17.33	0	0	0	ND	ND
SD	0	0	3.71	0	0	0	ND	ND

ND = not detected PFU = plaques forming unit I = examined during 2-3 hours, II = examined after storing for 6 months at room temperature, III = examined after inoculum with bacteria, IV = examined after storing with inoculum by bacteria, A, B, C, D, E and F=Bottled water companies, SD = Standard Deviation, AV = average, MD = Median, MAX = maximum, MIN = minimum

Brillard, etal[53] in France, concluded that *Bacillus* spp. can survive in sterilized tap water for several months because it able to transform from vegetative cell to spore form. In addition, **Anthony, etal**[54] reported that spores of *Bacillus* spp. contained protective compounds (Dipicolinic acid). These compounds protect it from surrounding unsuitable condition for regrowth (organic matters, pH and temperature) for several years.

Regarding to **Table (4),** the samples of groups B and D of bottled water contains staphylococci, with average of 1.33 and 0.17 cfu/100 ml, while others samples tested were free. After 6 months of storing at room temperature, staphylococci were not detected in all samples. While, the stored samples containing 23 cfu

/100ml of staphylococci bacteria was decreased until reaching 1 cfu/100 ml in samples of groups B, C and D. This data might be to the quality of bottled water for each brand **(John and Rose[42]).**

Moreover, some reports **(Evans[55] and WHO[56])** concluded that the presence of *Staphylococcus* spp. in water can be considered as complementary tests for the evaluation of water pollution.

Result in this study, were in agreement with **Serre, etal[57]**, in France who studied survival of *Staphylococcus aureus* in sterile tap water stored at room temperature (20 to 25°C) for 6 months with $\log_{10}$ 7, 5 and 3 cfu/ml. Authors found that, after 9 days all $\log_{10}$ count reached zero. Also, **Venkatesan, etal[19]** in India, isolates *Staphylococcus aureus* from 15 samples of bottled water in absence of bacterial indicators.

In this study,the variation of survival of staphylococci bacteria between 6 brands may be too due to the quality of water **(John and Rose[42]).**

Data given in **Table (4)** revealed that, coliphage was not detected in all types of bottled water except some samples of group B. In this study, the maximum value of coliphage was being obtained in type B (2 pfu/100 ml). This data was in line with **Ehlers, etal[58]** in South Africa, who examined 10 brands after product (8 local and 2 imported) for 3 month (at 1, 30 and 90 days) for somatic and F-RNA coliphages. Authors found that all samples (n = 30) were free from somatic and F-RNA coliphages. Also, the results in this study was in agreement with **Osman, etal[38]** who collected bottled water from Egyptian markets (groups A, B, C and D brands) 4 times during 2009. Authors found that all samples (n = 144) were free from coliphage except A sample (average was 6 pfu/100 ml).

The presence of coliphage indicates the pollution with organic material or domestic wastewater and may be due to the contamination during the bottling process **(El-Abagy, etal[14]; Hunter[11], and Legnani, etal[8]).**

Moreover, coliphage was used as a more specific index of fecal pollution **(El-Abagy, etal[14] and Grabow[59]**). Also all types of bottled water brands were compiled (except B brands) with the bacteriological standards presented by **WHO[9]**and **Egyptian Standard[45]**for drinking water.

Finally, data showed that, there is no correlation between bacterial indicators and presence or absent of *Staphylococcus aureus* as well as coliphage.

4- Examination of total molds in bottled water

Regarding with to the additional indicators in bottled water, data given in **Table (5)** reveals that, some types of bottled water containing yeast and fungi. Higher count of yeast was observed in samples of C (15 cfu / 100 ml). But maximum count of fungi was detected in samples of groups C, D and F (2 cfu / 100 ml). From the aforementioned of the total averages molds results, it could be concluded that, some bottled water samples were not complied with the microbiological standards presented in the **Egyptian Standard[45].** But after storing, all fungi were disappeared at room temperature for 6 months.

This data presented in this study were in agreement with **Gonçalves, etal[60]** who reported that, the role bacteria and yeast could have inhibition effect for the filamentous fungi by competing for the nutrient as well as toxin production in drinking water samples. Also, data was supported by the results of Yamaguchi, et al[15] in Brazil who counted yeast and fungi in 22 from 60 bottled water samples. Their counts were 100 and 50 cfu / 100 ml, respectively, in absence of fecal coliform.

Statistical analysis of correlation between yeast and HPC (22^{o}C), in group C samples, showed that significant coefficient (r = 0.8917) but correlation weakerbetween fungi and HPC (22^{o}C), (r=0.1159, r=0.1808 and r= 0.2389) for groups C, D and F samples, respectively. On the other side, correlation between storage (6 months) and yeast was significant coefficient (r =0.9819) but negative significant coefficient with fungi. Also, data of yeast, after inoculation (10 - 50 cfu/100ml) and storing at room temperature for 6 months was significant coefficient (r= 0.95) with HPC (22^{o}C), but not correlation between the counts of initial and at the end of the experiment. Moreover, in this investigation, correlation of filamentous fungi and yeasts was significant (r= 0.9) high **(Yamaguchi, etal[15]).**

Table 5 Statistical analysis of total molds (yeast and fungi) viable count (cfu / 100ml) from six types of bottled water brands (A, B, C, D, E and F) collected from Egyptian market at different conditions (I, II, III and IV) during 2015.

Para-meter	Total molds viable count (cfu / 100ml)							
	Yeast				Fungi			
	1	Ii	III	IV	1	Ii	III	IV
A								
MIN	0	0	39	0	0	0	ND	ND
MD	0	0	44	3	0	0	ND	ND
MAX	3	0	49	6	2	0	ND	ND
AV	0.83	0	43.83	3	0.5	0	ND	ND
SD	1.33	0	3.55	2.76	0.84	0	ND	ND
B								
MIN	0	0	36	1	0	0	ND	ND
MD	0	0	42.5	5	0	0	ND	ND
MAX	12	2	48	11	2	0	ND	ND
AV	3.33	0.5	42.33	5.17	0.5	0	ND	ND
SD	5.32	0.84	4.13	3.66	0.84	0	ND	ND
C								
MIN	0	0	37	2	0	0	ND	ND
MD	1	0	40	11.5	0.5	0	ND	ND
MAX	15	1	45	24	1	0	ND	ND
AV	3.5	0.33	40.67	12.83	0.5	0	ND	ND
SD	5.86	0.52	3.27	9.13	0.55	0	ND	ND
D								
MIN	0	0	39	4	0	0	ND	ND
MD	2	0	43.5	11	1	0	ND	ND
MAX	5	0	48	16	2	0	ND	ND
AV	2.33	0	43.67	10.17	0.83	0	ND	ND
SD	1.86	0	3.56	4.17	0.75	0	ND	ND
E								
MIN	0	0	41	4	0	0	ND	ND
MD	0.5	0	46	8.5	0	0	ND	ND
MAX	2	0	49	17	1	0	ND	ND
AV	0.8333	0	45.67	10.33	0.33	0	ND	ND
SD	0.9831	0	2.94	5.09	0.52	0	ND	ND
F								
MIN	0	0	33	6	0	0	ND	ND
MD	0.5	0	38.5	15.5	1	0	ND	ND
MAX	4	0	41	28	2	0	ND	ND
AV	1	0	37.5	15.33	0.83	0	ND	ND
SD	1.56	0	2.93	7.9	0.74	0	ND	ND

ND = not detected I = examined during 2-3 hours, II = examined after storing for 6 months at room temperature, III = examined after inoculum with bacteria, IV = examined after storing with inoculum by bacteria, A, B, C, D, E and F=Bottled water companies, SD = Standard Deviation, AV = average, MD = Median, MAX = maximum, MIN = minimum

Also data of correlation between yeast and HPC (22^{o}C) is in agreement with **Yamaguchi, et al**[15]who reported that, a significant positive correlation between yeasts and HPC. In addition, some studies **(Evans**[55]**and WHO,**[56]**)** reported that, the occurrence of yeast or fungi in bottled water is considered unsuitable for consumers and these microorganisms are new indicators for contamination of water.

5- Examination of bacteria isolated for some antibiotics

In this study, about 70 isolates were [*Pseudomonas aeruginosa* (24 isolates) *Bacillus subtilus* (36 isolates) and *Staphylococcus aureus* (20 isolates)] examined against various antibiotics. The result showed that,

these bacteria were multi-antibiotic resistant (MAR) with ratio of 70.83, 66.67 and 80 % for *Pseudomonas aeruginosa, Bacillus subtilus* and *Staphylococcus aureus*, respectively**(Table 6).** Where, *Pseudomonas aeruginosa* was sensitive to Ampicillin, Chloramphinicol, Coloxicillin and Erythromycin but resistance to remaining (8 types) tested antibiotic. In addition, *Bacillus subtilus* was sensitive to Amoxicillin, Tetracyclin and Oxytetracyclin while, resistant to other (9 types) tested antibiotic. Moreover, *Staphylococcus aureus* was sensitive to Kanamycin, Tetracyclin, Oxytetracyclin and Amoxicillinwhile while it was resistant to other (8 types) tested antibiotic **(Table 7).** This indicates that, these bacteria may be pathogenic and caused diseases for human consumers.

In addition, MAR bacteria from bottled water are considerable as source of antibiotic resistant bacteria which has negative effect on public health **(Falcone-Dias, etal[61]).**

Similar study conducted by **Poonia, etal[62]** in India who isolated some bacteria (19 bacterial species from 225 isolates) from springs and streams water samples. Isolates were Including *Pseudomonas* spp. *(n=41)* which examined for different antibiotics discs (12 antibiotics {ampicillin (10 µg), amoxicillin/clavulanic acid (20/10 µg), cefixime (5 µg), tetracycline (30 µg), ceftazidime (30 µg), ofloxacin (5 µg), amikacin (30 µg), gentamicin (10 µg), piperacillin/tazobactam (100/10 µg), imipenem (10 µg), chloramphenicol (30 µg), and trimethoprim/sulfamethoxaole (1.25/23.75 µg)}. They found *Pseudomonas* spp. (6 strains) multi-antibiotic resistance (MAR) to 3 or more (ratio, 14.6 %) of antibiotics using standard disc diffusion method.

Also, **Oluyege, etal[63]**isolated 36 strains including, *Staphylococcus aureus* (n=8), *Pseudomonas* spp. (n=4) and *Bacillus* spp. (n=6) from 6 samples of bottled water in Nigeria. Authors conducted bioassay antibiotics (8 types of antibiotics {Augmentin

Table 6: Percentage of MAR by individual bacterial species

	Number of antibiotics resistance bacteria (%)			
Bacterial isolates	**0 (%)**	**1 (%)**	**2 (%)**	**3 or more (%)**
Psuedomonas aeruginosa	1 (14.17)	3 (12.5)	3 (12.5)	17 (70.83)
Bacillus subtilus	3 (8.33)	2 (5.56)	7 (19.44)	24 (66.67)
Staphylococcus aureus	0 (0)	1 (5)	3 (15)	16 (80)

Table 7: Antibiotic resistance patterns in bacteria isolated from bottled water samples

	Antibiotic resistance patterns of the bacterial isolates (%)		
Bacteria Antibiotics	***Psuedomonas aeruginosa (NS=24)***	***Bacillus subtilus (NS=36)***	***Staphylococcus aureus (NS=20)***
Ampicillin	0 (0)	20 (55.56)	12 (60)
Chloramphinicol	0 (0)	14 (38.89)	9 (45)
kanamycin	4 (16.67)	4 (11.11)	0 (0)
Naldixic acid	21 (87.5)	29 (80.56)	14 (70)
Tetracycline	13 (17)	0 (0)	0 (0)
Lincomycin	14 (58.33)	11 (30.56)	15 (75)
Gentamycin	19 (79.17)	13 (36.11)	2 (10)
Coloxicillin	0 (0)	34 (94.44)	20 (100)
Oxytetracyclin	5 (20.83)	0 (0)	0 (0)
Nitrofurantion	22 (91.67)	19 (52.78)	17 (85)
Amoxicillin	23 (95.83)	0 (0)	0 (0)
Erythromycin	0 (0)	21 (58.33)	14 (70)

Notes: (NS = Number of isolates

(30µg), Chloramphenicol (30µg), Ofloxacin (5µg), Gentamycin (10µg), Cotrimoxazole (25µg), Nitrofurantoin (300µg), Tetracycline (30µg), and Nalcillin (30µg)} all isolates were resistant for different of antibiotics. Their results of previously mentioned isolates were multiple antibiotic resistance (MAR) to the 8 types of antibiotics. Moreover, authors concluded that, these isolate may have serious public health

risk.Generally, this study indicates that most of bacterial isolates from bottled water which produced under some brands in Egypt were containing pathogens and may be due to affect the public health of the consumers without the presence of bacterial indicators.

6- Physico-chemical analysis

Table (8) shows some physicochemical characteristics of bottled water samples. Electrical conductivity (Ec), pH, total dissolved substances (TDS), nitrate and ammonia concentration were measured in our laboratory according to **APHA[34].**Results showed that, physicochemical characteristics of all bottled water samples were complying with the **Egyptian Standards[45]** for drinking water as well as **WHO[9].** The averages of Ec, TDS, nitrate and ammonia from all samples were 0.538 (dS/cm), 129.5 (mg/l), 11 (mg/l) and 0.138 (mg/l), respectively. After 6 months of storing at room temperature, some values were increased to be 0.717 dS/cm, 229.67mg/l and 0.208 mg/l for Ec, TDS, and ammonia, respectively. While, the pH value still in the range of 7.31-7.35 and the average nitrate was decreased from 11 to 7.23 mg/l **(Table 8).** On the other hand, physicochemical parameters were studied after inoculation with 10-100 cfu/ml for each microbe in some bottled water samples (of 6 brands) for 6 months storing at room temperature. After storing, the level of pH, Ec, TDS, nitrate and ammonia were 7.68, 1.77 (dS/cm), 344.33 (mg/l), 35.33 (mg/l) and 0.58 (mg/l), respectively. Although, this type of water were contaminated by this study but physicochemical properties were limit with national and international standard set for drinking water. In this investigation, statistical analysis of correlation between HPC (37^{o}C) and physicochemical characteristics indicated that positive correlation with Ec (r= 0.174) TDS (r= 0.053) and ammonia (r=0.140). On other side, in the presence of bacteria (HPC), negative correlation with nitrate *(r=-0.06*). Moreover, data observed that, no significant coefficient between presence of bacteria (HPC) and pH value.

In another study by **Aydin[64]**, in Turkey, who recorded conductivities lower and higher values of groundwater samples were 463 and 1460 µS/cm, respectively compared with international standard for drinking water. Also nitrate was ranged from 1.1 to 15 mg/l. These values are within maximum permissible limit. In addition, authors concluded that, the highest values due to re-contamination with organic matters.

Similar results were obtained in another study by **Abd El-Salam, etal[37]**in Egypt, who recorded the physicochemical characteristics in 84 samples of bottled water (from 14 brands). Authors found that, TDS was ranged from 198 to 438 mg/l as well as pH in compliance with the Egyptian standard (6.5 – 8.5).

On the other hand, these data were in agreement with **Rabee, et al[32]**in Iraq, who detected Ec (59-597 µS/cm) and TDS (ranged from 18 to216 mg/l) within international standard for drinking water from 42 samples (14 brands).

Also, these data were in line with **Farhadkhani, et al[26]**in Turkey, who observed from statistical analysis a significant effect between Ec and total bacterial count in bottled water samples. Moreover, authors found that, there was not any significant effect of pH on the microbial quality from those samples.

Results in this investigation in a good agreement with **Duwiejuah, et al[65]**in Ghana, who examined the effect of storage for three months at room temperature (27^{o}C) on the

Table 8: Statistical analysis of some physicochemical parameters examined in six types of bottled water brands (A, B, C, D, E and F) collected from Egyptian market at different conditions (I, II, III and IV) during 2015.

	physicochemical parameters				
Parameters	**pH (6.5 - 8.5)**	**Ec dS/cm (1.4)**	**TDS (1000 ppm)**	**Nitrate (45 ppm)**	**Ammonia (0.5 ppm)**
I					
MIN	6.9	0.3	92	3.1	0.03
MD	7.35	0.55	117	9.32	0.145
MAX	7.8	0.9	181	22.4	0.24
AV	7.35	0.58	129.5	11	0.14
SD	0.32	0.25	37.87	7.76	0.1
II					
MIN	6.8	0.4	134	2.9	0.05
MD	7.4	0.7	243.5	7.6	0.2
MAX	7.7	1.1	277	12.4	0.38
AV	7.32	0.72	229.67	7.23	0.21
SD	0.32	0.26	54.26	3.66	0.15
III					
MIN	7	0.3	94	3.2	0.03
MD	7.3	0.65	155.5	10.5	0.205
MAX	7.4	1.1	175	18.2	0.6
AV	7.25	0.67	142.17	10.18	0.23
SD	0.16	0.30	34.34773	6.534345	0.20
IV					
MIN	7.5	1.2	235	24.7	0.39
MD	7.65	1.5	347.5	34.8	0.505
MAX	7.9	1.7	417	44.5	0.9
AV	7.68	1.47	344.33	35.33	0.58
SD	0.15	0.20	66.45	8.40	0.22

Note: Ec = electric conductivity TDS = Total dissolve salts I = examined during 2-3 hours, II = examined after storing for 6 months at room temperature, III = examined after inoculum with bacteria, IV = examined after storing with inoculum by bacteria, A, B, C, D, E and F=Bottled water companies, SD = Standard Deviation, AV = average, MD = Median, MAX = maximum, MIN = minimum

Quality of sachet-vended water (six packs or bags) for 2 brands. Authors concluded that, according to World Health Organization results of physicochemical characteristics within limits for drinking water. Thus, there was no significant correlation between time (storage) and physicochemical characteristics.

Conclusion and Recommendation

This study concluded that:

- According to recommended limits of Egyptian Standard and International Standard for drinking water, biological and physico-chemical characteristics of all bottled water were accepted.
- According to microbial and physicochemical analyses, samples of groups E and F were found to be more quality compare with other samples.
- Negative coefficient was observed between HPC with nitrate while, positive with other physico-chemical.
- There were no correlation between the absence of bacterial indicators and water quality.
- Bacterial isolates (*Pseudomonas aeruginosa, Staphylococcus aureus, and Bacillus subtillus*) from water were multi-antibiotic resistant for some antibiotic.
- *Pseudomonas aeruginosa* and *Bacillus subtillus* were more survive than other microbial tested for further 6 months at room temperature in bottled water.
- In spite of bacterial indicators were absent but *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Bacillus subtillus* were isolated from some bottled water.

- There were no correlation between the absence of bacterial indicators and bacterial isolates.
- In addition, there should be further studies and periodic monitoring of the quality for stored bottled water.

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