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RESEARCH ARTICLE

Degradation of Bacterial Water Quality in Drinking Water after Bottling

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Abstract:

Background:

The consumption of bottled water globally, including Iran, has increased tremendously in recent years. This study was designed to assess the bacteriological quality of bottled water and its compliance with the drinking water regulations. In addition, we evaluated bottled waters for the presence of a variety of genera of bacteria and the effect of storage duration on the extent of bacterial contamination.

Methods:

Four hundred samples of bottled water belonging to ten different Iranian brands with various production dates were purchased from supermarkets in Gorgan, Iran, from 2017 to 2018. Bacterial quality of bottled water was assessed using heterotrophic plate count (HPC) followed by usual biochemical tests for identification of bacterial genera, and by the API system.

Results:

The average HPC of bottled water was 9974 colony-forming units per milliliter (CFU/ml). Twelve genera were isolated, among which *Bacillus* spp. and *Escherichia coli* were the most and least abundant, respectively. Statistical analysis showed that there was a positive association between water quality and storage duration so that the highest microbial load occurred within the first to third months after bottling. Furthermore, the highest rate of contamination was observed in May when ambient air temperatures commonly reached 40 °C.

Conclusion:

The bacterial quality of bottled water was not according to the standard of drinking water quality. This study demonstrated the variation in bacterial levels after bottling, which indicates the presence of waterborne heterotrophic bacteria, some of which can pose severe health risks to consumers.

Keywords: Bacteria, Drinking water, Heterotrophic plate count, Water Quality, Bottled Water, *Escherichia coli*.

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1. INTRODUCTION

The production and consumption of bottled drinking water are increasing all around the world with public concern rising regarding the safety and quality of the water, particularly with respect to the possibility of water-borne diseases [1]. Similar to other countries, there is an increasing demand for consumption of bottled water among Iranian people [2]. The most important factors affecting the consumption of bottled water are organoleptic chemicals such as nitrate and nitrite, hardness, taste, odor and risk of water-borne diseases [3, 4]. Bottled water is drinking water packaged in glass or plastic bottles, most often after advanced treatment processes including microfiltration, reverse osmosis, ultraviolet radiation and/or ozonation [5 - 7]. For this reason, many individuals, especially elderly persons, pregnant women, children and immune-compromised individuals, prefer to drink bottled water for assured microbiological quality and safety of the water [8, 9]. Contrary to expectations, some studies have detected waterborne bacteria such as *Escherichia coli (E. coli)*, *Pseudomonas aeruginosa*, *Staphylococcus* spp., *Micrococcus* spp. and *Corynebacterium* spp. in bottled water [1, 10].

The fecal indicator bacterium *E. coli* has been adopted by the World Health Organization (WHO) and many countries as the standard for assessing the microbial safety of drinking water [11, 12]. The number of heterotrophic bacteria as

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measured by the Heterotrophic Plate Count (HPC) is also used as a criterion of microbiological water quality assessment [6]. For example, in the United States of America, when a distribution system sample does not have detectable chlorine residual, the HPC concentration less than 500 CFU/mL (using plate count agar) can be a surrogate for a detectable disinfectant residual [13]. Heterotrophic Plate Count is also used as a process management indicator in municipal systems in particular, when chloramine is used as the distribution system disinfectant [14], and for bottled water production [15]. Heterotrophic bacteria are a group of non-pathogenic and pathogenic microorganisms including, *Escherichia coli*, *Bacillus* spp, *Corynebacterium* spp, *Streptococcus* spp, *Enterobacter* spp, *Streptomyces* spp, and opportunistic bacterial pathogens (*i.e*., *Klebsiella* spp, *Legionella* spp, *Moraxella* spp, *Pseudomonas* spp.) [6]. Previous clinical and epidemiological studies have not documented that the consumption of water with high HPC poses a significant health risk to consumers [6, 16, 17]. Nevertheless, in a survey conducted by Pavlov *et al* ., a possible link was observed between HPC bacteria and the incidence of gastroenteritis due to opportunistic bacterial pathogens [18].

Contamination of drinking water is one of the most important public health concerns and it is necessary to know the microbial quality of bottled water. The HPC method is a reliable and cost-effective procedure for assessing the bacterial quality of drinking water, particularly for water with high quality such as bottled water [16, 17, 19]. This study was design to assess the bacteriological quality of bottled water and its compliance with the drinking water regulations. In addition, we evaluated bottled waters for the presence of a variety of genera of bacteria and the effect of storage duration on the extent of bacterial contamination.

2. MATERIALS AND METHODS

2.1. Bottled Water Samples

Four hundred samples of bottled water (500ml and 1.5Lit) produced by ten different Iranian brands were collected from numerous supermarkets in Gorgan, Iran, during the period May 2017 to April 2018. The number of samples was determined based on the sample size formula for descriptive studies (n=(z_(1-□/2)^2)/d^2), and a comparative study in Isfahan [3]. In our study, samples were randomly taken from the refrigerator from which they were ordinarily purchased by consumers. All the samples were transferred to the microbiology laboratory in the school of public health of Golestan university of medical sciences in an insulated cooler (T≤ 5 °C) within 2 hours after collection and were analyzed upon arrival at the laboratory. This laboratory was highly efficient due to a number of factors, including modern equipment and suitable testing facilities. The HPC values were categorized into seven subgroups depending on the time difference between bottling at the factory and the testing date (≤30, 31-60, 61-90, 91-120, 121-150, 151-180, and >180 days).

2.2. HPC Bacteria Analysis

For heterotrophic bacteria, the samples were vortexed for 15 seconds and ten-fold serial dilutions were prepared for each sample, using sterile 0.85% saline. The spread plate method using R2A agar (Merck, Germany) was used for enumeration of HPC bacteria as described in Standard Methods, except that the plates were incubated at 28 °C for at least 72 h and up to 7 days [20]. All the experiments were carried out in duplicate and the mean values were recorded as total heterotrophic bacteria (CFU/ml) for each sample.

2.3. Identification of Heterotrophic Bacteria

The isolated bacteria from R2A plates were identified using several biochemical tests, including gram-staining, oxidase production and catalase tests, glucose and lactose fermentation, hydrogen sulfide production, indole production, methyl red, Voges-Proskauer, citrate utilization, motility, urea hydrolysis, ONPG hydrolysis, ornithine and lysine decarboxylase, arginine dihydrolase, gelatin hydrolysis, Schaeffer-Fulton staining, all according to standard methods [20]. In addition, to confirm the bacteria, the API 20E/NE, Staph, Coryne and CHB Medium tests were performed in a manner conforming to the manufacturer's protocol (BioMérieux, 69280, Marcy I'Etoile, France).

2.4. Statistical Analysis

The data was analyzed using IBM SPSS Statistics v16. Descriptive statistical parameters (mean and standard deviation) were used to describe the heterotrophic bacteria population in different bottled water brands. The Chi-square test was used to determine the variation in the bacterial population after bottling. A p-value of <0.05 was considered significant.

3. RESULTS

The mean HPC level of bottled water was 9974 CFU/ml and ranged from less than one up to 2×10^5 CFU/ml. The highest and lowest amount of bacterial contamination was observed in brands 8 and 7, respectively (Table **1**). The statistical analysis revealed a significant difference between some brands and the amount of bacterial contamination ($p<0.001$).

In Table **2**, the results of HPC are presented based on the storage duration. Our study revealed that the highest percentage of samples with HPC higher than 500 CFU/ml was found within the first to ninety days after bottling. The statistical analysis also showed a difference between the HPC value and the duration of storage ($p<0.001$).

Ten genera of HPC bacteria were identified from the HPC plates. *Bacillus* spp. were the predominant genera followed by *Acinetobacter* spp., *Pseudomonas* spp*., Micrococcus* spp., *Enterobacter* spp*., Flavobacterium* spp., *Corynebacterium* spp., *Stenotrophomonas* spp., *Staphylococcus* spp., and *Escherichia coli*. A statistically significant difference was not observed between the presence of the bacterial genera and bottle brands ($p=0.428$) (Table **3**); whereas, a positive association was found between the HPC level of >1000 CFU/ml and the presence of bacteria listed above ($p<0.001$) (Table **4**). We observed a positive association between storage duration and HPC ($p<0.001$).

4. DISCUSSION

This study assessed the quality of different brands of Iranian bottled water with respect to microbiological quality. We found that more than 84% and 98% of the samples had a HPC of over 500 and 100 CFU/ml, respectively. These values were much higher than the recommended microbiological value for bottled water (HPC<500 CFU/ml) in the USA [9] and some European countries (HPC<100 CFU/ml) [21]. However, it should be noted that higher values for HPC will occur on minimal nutrient media such as R2A as opposed to standard plate count agar [22]. Nevertheless, the American Public Health Association (APHA) recommended that R2A agar in combination with a longer incubation period and lower incubation temperature can improve the recovery of stressed and chlorine-tolerant bacteria. This medium may yield higher counts than high-nutrient formulations. However, it is preferable to other media such as m-HPC agar and Plate count agar [20]. Attempted cultivation on plate count agar can result in nutrient shock resulting in lower concentrations. Regulatory norms are based on plate count agar, primarily as a matter of tradition.

Other studies have also reported the high HPC in bottled water. Moazeni *et al*., and Kouchesfahani *et al*., reported high numbers of HPC bacteria in bottled water sold in Isfahan and Tehran [2, 3]. The quantity of heterotrophic bacteria in bottled water is generally dependent on the type of source water, the availability of organic carbon, the autochthonic microorganisms and methods employed in the disinfection process [6].

Variations in heterotrophic bacterial populations revealed that storage duration affected the rate of bacterial contamination. In the current survey, the highest rate of samples with HPC higher than 1000 CFU/ml and the presence of heterotrophic bacteria were found within the first to ninety days following bottling (Tables **2** and **4**). Our finding is consistent with the findings of other studies, which reported that the number of HPC bacteria can increase logarithmically during storage time to 10^5 CFU/ml [10, 23]. Zeenat *et al*., revealed that death and autolysis of microflora provide nutrients and further support to the growth of heterotrophic bacteria to high levels after prolonged storage [24].

All bottled water had a variety of genera of heterotrophic bacteria. The results of the biochemical tests for speciation showed that *Bacillus* spp. and *Pseudomonas* spp. were detected in 61.8 and 34.5 percent of all samples. The presence of these bacteria in bottled water is due to failure to de-contaminate (properly disinfect) the source water, or the introduction of contaminants during the bottling process [25]. The WHO reported that *Bacillus* spp., *Pseudomonas aeruginosa*, *Acinetobacter* spp., *Staphylococcus* spp. and other pathogenic bacteria may be found in drinking waters [6]. Both norms and customer expectations require that drinking water that leaves a bottling plant must be free from opportunistic pathogens [26].

The presence of *Pseudomonas* spp. is countrary the standards established by the European Union water regulations, citing that this bacterium should not be detected in 250 ml bottled water samples [2]. The level of *Pseudomonas* spp. detected in the present study was in line with what detected by Kouchesfahani *et al*., who described samples purchased from retailers in Tehran, Iran [2]. In that study, contamination with *E. coli* and *Enterobacter* spp. was also observed in 4.5% and 27.5% of the samples, respectively. Although, these bacteria are considered nonpathogenic, their presence in bottled water indicates a failure in disinfecting the source water or contamination within the bottling plant [27]. Farhadkhani *et al*., and Otterholt *et al*., showed a significant association between the heterotrophic bacteria level and the presence of opportunistic bacteria [28, 29]. Fortunately, *Vibrio chlorae*, *Salmonella* spp., *Shigella* spp. and *Aeromonas* spp. were not found in any of the bottled water samples in our study.

Table 1. Heterotrophic bacterial count (CFU/ml) of bottled water of different brands.

Brand Code	No. of Samples	Mean±SD	Minimum	Maximum
1	40	14483.0±36237.3	62.0	1.70×10^5
2	40	9489.4±12667.2	130.0	5.80×10^4
3	40	4730±8911.0	134.0	5.60×10^4
4	40	5233.8±13372.0	100.0	8.20×10^4
5	40	10752.7±29486.2	337.0	1.80×10^5
6	40	10443.2±17442.0	< 1.0	9.50×10^4
7	40	2568.2±3414.6	<1.0	1.70×10^4
8	40	18860.8±42351.9	153.0	2.00×10^5
9	40	11397.9±24324.7	95.0	1.50×10^5
10	40	11825.5±19704.6	424.0	1.00×10^5
Total	400	9974.43±23855.24	<1.0	2.00×10^5

Table 2. Heterotrophic bacterial count (CFU/ml) result for bottled water stored at different duration (days).

During Storage	Number of Samples with HPC, HPC/ml (%)				
	<100	**100-500**	**500-1000**	**>1000**	**Total**
≤30	1 (0.2)	14 (3.5)	4 (1.0)	48 (12.0)	67 (16.8)
31-60	5 (1.2)	25 (6.3)	10 (2.5)	60 (15.0)	100 (25)
61-90	1 (0.2)	8 (2.0)	7 (1.8)	75 (18.8)	91 (22.8)
91-120	1 (0.2)	1 (0.2)	5 (1.2)	55 (13.9)	62 (15.5)
121-150	0 (0.0)	1 (0.2)	2 (0.5)	31 (7.8)	34 (8.5)
151-180	0 (0.0)	0 (0.0)	3 (0.8)	8 (2.0)	11 (2.8)
>180	0 (0.0)	3 (0.8)	4 (1.0)	28 (7.0)	35 (8.8)
Total	8 (2.0)	52 (13.0)	35 (8.8)	305 (76.2)	400 (100.0)

Table 3. Bacteria isolated from bottled water at different heterotrophic bacterial count (CFU/ml).

Bacteria Genera	Number of Samples with HPC, HPC/ml (%)				
	<100	**100-500**	**500-1000**	**>1000**	**Total**
Escherichia coli	0 (0.0)	3 (0.8)	2 (0.5)	13 (3.2)	18 (4.5)
Staphylococcus spp.	3 (0.8)	6 (1.5)	3 (0.8)	26 (6.5)	38 (9.5)
Stenotrophomonas spp.	0 (0.0)	2 (0.5)	2 (0.5)	54 (13.5)	58 (14.5)
Corynebacterium spp.	1 (0.2)	6 (1.5)	7 (1.8)	60 (15.0)	74 (18.5)
Flavobacterium spp.	1 (0.2)	9 (2.3)	5 (1.3)	72 (18.0)	87 (21.8)
Enterobacter spp.	4 (1.0)	10 (2.5)	7 (1.8)	89 (22.2)	110 (27.5)
Micrococcus spp.	2 (0.5)	18 (4.5)	11 (2.8)	92 (23.0)	123 (30.8)
Pseudomonas spp.	5 (1.2)	25 (6.2)	13 (3.2)	95 (23.9)	138 (34.5)
Acinetobacter spp.	3 (0.8)	23 (5.8)	12 (3.0)	115 (28.8)	153 (38.2)
Bacillus spp.	4 (1.0)	34 (8.5)	21 (5.2)	188 (47.1)	247 (61.8)

Table 4. Bacteria isolated from bottled water at different storage duration (days).

Bacteria Genera	Number of Samples with Storage Duration, HPC/ml (%)							
	≤30	**31-60**	**61-90**	**91-120**	**121-150**	**151-180**	**>180**	**Total**
Escherichia coli	2 (0.5)	4 (1.0)	3 (0.8)	4 (0.8)	2 (0.5)	1 (0.2)	2 (0.5)	18 (4.5)
Staphylococcus spp.	4 (1.0)	9 (2.2)	10 (2.5)	7 (1.8)	4 (1.0)	1 (0.2)	3 (0.8)	38 (9.5)
Stenotrophomonas spp.	8 (2.0)	15 (3.8)	9 (2.2)	8 (2.0)	5 (1.2)	1 (0.2)	12 (3.0)	58 (14.5)
Corynebacterium spp.	15 (3.8)	14 (3.5)	18 (4.5)	10 (2.5)	7 (1.8)	3 (0.8)	7 (1.8)	74 (18.5)
Flavobacterium spp.	18 (4.5)	21 (5.2)	17 (4.2)	9 (2.2)	9 (2.2)	4 (1.0)	9 (2.2)	87 (21.8)
Enterobacter spp.	21 (5.2)	31 (7.8)	24 (6.0)	15 (3.8)	8 (2.0)	3 (0.8)	8 (2.0)	110 (27.5)
Micrococcus spp.	18 (4.5)	39 (9.8)	26 (6.5)	21 (5.2)	11 (2.8)	2 (0.5)	6 (1.5)	123 (30.8)
Pseudomonas spp.	27 (6.8)	35 (8.8)	32 (8.0)	16 (4.0)	15 (3.8)	4 (1.0)	9 (2.2)	138 (34.5)
Acinetobacter spp.	32 (8.0)	36 (9.0)	36 (9.0)	27 (6.8)	11 (2.8)	2 (0.5)	9 (2.2)	153 (38.2)
Bacillus spp.	4 (10.5)	57 (14.2)	61 (15.2)	42 (10.5)	21 (5.2)	6 (1.5)	18 (4.5)	247 (61.8)

Our findings also indicated that storage influenced the bacterial quality of bottled water. The highest and lowest rate of contamination was observed in months of May and October when ambient air temperatures are relatively high and low compared to other months. The environmental temperature could be one important reason for the growth of heterotrophic bacteria in bottled water. Falcone-Dias *et al*., reported that the regrowth of bacteria increases at temperatures higher than 37 °C [30]. In our study area, ambient environmental air temperatures and air temperatures inside stores commonly reached to 40 °C and occasionally 50 °C (in summer). This finding is similar to the result obtained by Nsanze *et al*., who reported a significant association between high numbers of HPC and bottled water stored at 25 °C and 37 °C [23].

CONCLUSION

The quality of bottled water reported in this study is of concern for the health of consumers. In our study, all the samples were collected only from supermarkets and not from the manufacturers. Therefore, it is difficult to determine whether the contamination was a result of improper disinfection of the source water, or contamination in the bottling plants; both could result in increased bacterial concentrations over time. The findings point to the need for

monitoring of bottled water as well as the adoption of Hazard Assessment Critical Control Points (HACCP) programs, which focus on the most vulnerable aspects of food and water processing to ensure consumer safety.

LIST OF ABBREVIATIONS

E. coli	=	*Escherichia coli*
WHO	=	World Health Organization
HPC	=	Heterotrophic Plate Count
CFU	=	Colony-Forming Unit
ONPG	=	Ortho-Nitrophenyl-β-Galactoside
API	=	Analytical Profile Index
HACCP	=	Hazard Assessment Critical Control Points

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used in the study that is the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data supporting the findings of the article is available in the manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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