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Microbiological evaluation of bottled non-carbonated ("still") water from domestic brands in Greece

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Abstract

The microbiological quality of 1527 samples of bottled noncarbonated ('still') mineral water, purchased from retail outlets and derived from 10 manufacturing companies in Greece, was investigated during the period 1995–2003. Applying the membrane filter technique, the aliquots of water samples (250 ml) were analyzed for the presence and enumeration of total coliforms, *Escherichia coli*, *Enterococcus* spp. and *Pseudomonas aeruginosa*. Also, aerobic bacteria were counted as Heterotrophic Plate Count (HPC) ml^{-1} at 22 and 37 °C. Positive samples for the parameters tested varied significantly among brands with an overall percentage of 13.95% bottled water samples noncompliant with the Greek water regulation. Microorganisms isolated from the samples tested were identified as species of *Pseudomonas*, *Aeromonas*, *Pasteurella*, *Citrobacter*, *Flavobacterium*, *Providencia* and *Enterococcus.* The most frequent isolated microorganism during the period of the study was *P. aeruginosa*. Generally, bacterial load of the samples tested ranged in low levels. The purpose of the current study was to evaluate the microbiological quality of the bottled water provided by domestic brands in the Greek market during the period 1995–2003.

Keywords: Bottled water; Microbiological quality; Faecal; *P. aeruginosa*

1. Introduction

Over the past decade the consumption of bottled water in Greece has increased tremendously, demanding the continuous surveillance of public health services (Fewtrell et al., 1997; Papapetropoulou, 1998). The manufacturers' successful promotion has led to the general perception that bottled water is pure and impeccably clean water, safe for human consumption (Bharath et al., 2003). In part, this increasing market probably reflects the public's scepticism about the quality of tap water (Hunter, 1993; Papapetropoulou et al., 1997; Tamagnini and González, 1997), considering the frequent pollution of municipal water supplies, offensive taste and odor as well as fluoride and chlorine (Bharath et al., 2003). In addition, bottled water has been marketed as ideal for infants and immuno-compromised individuals in order to avoid potential pathogens which must be absent in 250 ml of the bottled water (Papapetropoulou, 1998).

Nevertheless, bottled water is rarely completely free of microorganisms (Rosenberg, 2003). Natural mineral water is not sterilized, pasteurized or otherwise treated to remove or destroy microorganisms (Armas and Sutherland, 1999; Nsanze et al., 1999). The number of bacteria recovered at the source is generally very low but there are many reports that viable counts increase, notably in still water after 1–3 weeks of storage (Moreira et al., 1994; Armas and Sutherland, 1999; Leclerc and Moreau, 2002; Bharath et al., 2003). Relating the water quality to the degree of bacterial contamination, concerns have been raised about its microbial quality (Warburton et al., 1998a,b) and standards have been established to protect the public from waterborne disease outbreaks.

The microbiological quality of bottled waters is defined by the Greek legislation in harmonization with the European Community (EC) Directive of 1980, according to which total coliforms, *Escherichia coli*, *Enterococcus* spp. and *Pseudomonas aeruginosa* should not be detectable in 250 ml of any bottled water. As it has already been reported, predominant bacteria in bottled waters include species of *Pseudomonas* (Jayasekara et al., 1998; Legnani et al., 1999; Obiri-Danso et

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al., 2003), some of which are known opportunists (Warburton et al., 1998a,b). The heterotrophic plate count (HPC) standard of bottled mineral water within the EC sets an upper acceptable limit of 100 colony forming units (cfu) ml^{-1} (European Community Directive, 1980). The HPC of bottled mineral water has been evaluated by several studies (Tsai and Yu, 1997; Parrington and Sharpe, 1998; Bartram et al., 2004; Reasoner, 2004) which have shown that still mineral water has much higher counts in general compared to carbonated mineral water (Hunter, 1993; Rosenberg, 2003). The rapid growth of bacteria after the water is bottled may be due to oxygenation of the water during the process, the increased surface area from the bottle, the increase in temperature during storage and the trace amounts of nutrients arising from the bottle (Leclerc and Moreau, 2002).

The purpose of the present study was to evaluate the microbiological quality of 1527 bottled water samples, provided in the Greek market by 10 domestic brands from 1995 to 2003 and to survey the current status of the industry as well as their compliance with Greek water regulation. Furthermore, we overviewed the potential enhancement of bottled water manufactured in Greece during the period 1995–2003.

2. Materials and methods

For this study, bottled water was defined as any potable water that is manufactured, distributed or offered for sale, which is sealed in food grade bottles or other containers and is intended for human consumption (Warburton et al., 1992; Warburton et al., 1998a,b).

2.1. Water samples

In total, 1527 samples of still bottled water sealed in polyvinyl chloride (PVC) bottles derived from 10 different domestic brands, sold all over Greece, were analyzed. All samples were purchased from retail outlets in Patras (western Greece) during the period 1995–2003.

2.2. Microbiological analysis

All water samples were tested for the presence and enumeration of total coliforms, *E. coli*, *Enterococcus* spp., *P. aeruginosa* and heterotrophic plate count according to International Standards Organization (ISO) techniques. Though the designation "faecal streptococci" appear both in the E.U directives and in the Greek legislation, the generic name *Enterococcus*, established more than two decades ago, has been used throughout this article.

To detect the presence of total coliforms, *E. coli*, *Enterococcus* spp. and *P. aeruginosa* bottled waters were examined by filtration of samples through nitrocellulose membranes (0.45 ìm pore size, 47 mm diameter, Pall–Gelman Laboratory) followed by plating on selective media. The membrane filtration method is considered the most flexible method for qualitative and quantitative studies of bottled water (Reasoner, 2004).

A 250 ml of each sample was filtered through the membrane filter for the determination and enumeration of total coliforms, *E. coli*, *Enterococcus* spp. and *P. aeruginosa*. For total coliforms, membranes were plated on m-Endo Agar Les (Difco, product code: 273620, MI, USA) followed by incubation at 37 °C for 48 h. For *E. coli*, membranes were transferred on Tryptone Bile X-Glucuronide medium — T.B.X. (OXOID LTD., product code: CM945, Basingstoke, Hampshire, England) and plates were incubated at 44 °C for 24 h. *Enterococcus* spp. were isolated by plating the membranes on Slanetz and Bartley medium (OXOID, product code: CM377) and by incubating at 37 °C for 48 h. *Enterococcus* spp. isolates were confirmed by transferring the membranes onto Bile-Aesculin-Azide agar (OXOID, product code: CM888), preheated at 44 °C. Enterococci hydrolyse aesculin on this medium in 2 h. To detect *P. aeruginosa*, membranes were plated onto Pseudomonas Agar Base (OXOID, product code: CM559) with cetrimide and were incubated at 37 °C for 48 h. Typical colonies of *P. aeruginosa* were confirmed with King's medium and acetamide broth, according to European Standard prEN 12780:2001.

For the enumeration of heterotrophic bacteria pour plate count method was chosen, using 1 ml of water sample and mixing with melted Water Plate Count Agar-ISO (OXOID, product code: CM1012) tempered at 44 °C. Two sets of plates were prepared for all samples. One set was incubated aerobically at 37 °C for 48 h and the other set at 22 °C for 72 h. All colonies were counted as colony forming units (cfu) per milliliter of the water sample.

The isolated microorganisms were identified depending on their biochemical characteristics and using standardized identification systems API 20E and API 20NE (BioMérieux, 69280 Marcy-l'Étoile, France). Furthermore, *E. coli* isolates were tested whether they belonged to the O157 serogroup. The diagnostic reagent used was *E. coli* O157 Latex agglutination test (OXOID, product code: DR 620M).

2.3. Statistical analysis

The chi-square (χ^2) test was used to determine whether statistically significant differences existed in the prevalence of total coliforms, *E. coli*, enterococci, *P. aeruginosa* and aerobic bacteria in bottled water. Finally, the following hypothesis was examined: there was no significant difference between the numbers of microorganisms recovered from the different brands of bottled water. The program used for the statistical evaluation was SPSS, version 12.0.

3. Results

In total, 1527 samples of still mineral water in polyvinyl chloride bottles were analyzed for the presence and enumeration of total coliforms, *E. coli*, *P. aeruginosa*, *Enterococcus* spp. and heterotrophic plate count at 22 and 37 °C. All water samples studied, derived from the 10 domestic brands were not expired. The number of samples from each brand analyzed during the period 1995–2003 is shown in Table 1.

Table 1
Number of bottled water samples from the 10 domestic brands studied during the period 1995–2003

Brand code	Number of samples analyzed from 1995 to 2003									Total
	1995	1996	1997	1998	1999	2000	2001	2002	2003	
A	6	24	1	6	10	18	5	5	17	92
B	39	51	25	20	13	49	44	17	17	275
C	28	32	41	45	64	49	28	19	10	316
D	3	0	0	0	1	33	20	0	10	67
E	0	2	3	2	4	0	0	0	9	20
F	0	0	0	0	0	4	13	22	0	39
G	16	26	34	2	0	0	0	0	0	78
I	30	45	52	87	31	6	0	0	0	251
J	35	23	7	7	10	0	0	0	0	82
K	60	71	37	41	27	48	8	4	11	307
Total	217	274	200	210	160	207	118	67	74	1527

The prevalence of total coliforms, *E. coli* and enterococci is shown in Table 2. Overall, there was a variety of the microbiological profile among the samples tested from the 10 brands. The differences were statistically significant for total coliforms ($\chi^2=189.8$; $p<0.001$) and *Enterococcus* spp. ($\chi^2=19.8$; $p<0.05$) while for *E. coli* they were not ($\chi^2=7.11$; $p>0.05$). *E. coli* isolates were detected in low numbers and none of them was typed as O157 strains, which may survive and multiply in bottled water (Warburton et al., 1998a,b).

The prevalence of *Pseudomonas* spp. as well as *P. aeruginosa* in water samples from the various brands is shown in Table 3. Some brands had relatively high percentages of positive samples for *Pseudomonas* spp., but still they complied with the standing Greek regulation as only *P. aeruginosa* must not be detectable in 250 ml of each sample of bottled water. Positive samples for *P. aeruginosa* varied significantly among brands ($\chi^2=46.5$; $p=0.001$).

Table 4 shows the prevalence of aerobic bacteria in the samples tested, counted as cfu ml^{-1} at 37 and 22 °C. According to Greek legislation the limits stated for heterotrophic plate count in bottled water are 20 and 100 cfu ml^{-1} after incubation at 37 and 22 °C, respectively. From the 1527 water samples tested, 87.9% had HPC $\leq$ 100 cfu/ml at 22 °C and 85.6% had HPC $\leq$ 20 cfu/ml at 37 °C. To these results, it should be taken into account that the microbiological analysis did not take place within 12 h after bottling, as it is stated by Greek water regulation.

Table 2
Prevalence of total coliforms, *E.coli* and *Enterococcus* spp. in sampled bottled water

Brand code	No. of samples tested	No. (%) of samples positive for		
		Total coliforms	*E.coli*	*Enterococcus* spp.
A	92	5 (5.4)	0 (0)	0 (0)
B	275	10 (3.6)	3 (1.1)	0 (0)
C	316	19 (6)	3 (0.9)	7 (2.2)
D	67	2 (3)	0 (0)	0 (0)
E	20	0 (0)	0 (0)	0 (0)
F	39	0 (0)	0 (0)	0 (0)
G	78	6 (7.7)	2 (2.6)	1 (1.3)
I	251	87 (34.7)	5 (2)	8 (3.2)
J	82	5 (6)	0 (0)	0 (0)
K	307	35 (11)	3 (1)	2 (0.7)
Total	1527	167 (11)	16 (1)	18 (1.2)

Table 3
Prevalence of *Pseudomonas* spp. and *Pseudomonas aeruginosa* in sampled bottled water

Brand code	No. of samples tested	No. (%) of samples positive for	
		Pseudomonas spp.[a]	*Pseudomonas aeruginosa*
A	92	3 (3.3)	2 (2.2)
B	275	24 (8.7)	22 (8)
C	316	14 (4.4)	9 (2.8)
D	67	10 (14.9)	6 (8.9)
E	20	0 (0)	0 (0)
F	39	12 (30.7)	0 (0)
G	78	3 (3.8)	1 (1.3)
I	251	47 (18.7)	25 (9.9)
J	82	15 (18.3)	14 (17)
K	307	20 (6.5)	11 (3.6)
Total	1527	151 (9.9)	90 (5.9)

[a] *Pseudomonas* species isolated from positive samples were: *P. diminuto*, *P. fluorescens*, *P. maltophilia*, *P. putida*, *P. stutzeri*, *P. testo alcaligenes*, and *P. vesicularis*.

Other bacteria isolated from water samples included species of *Pseudomonas* like *P. stutzeri* recovered from 14 bottles (0.9%), *P. testoalcaligenes* from 12 bottles (0.8%), *P. putida*, *P. maltophilia* and *P. diminuto* from 2 bottles (0.1%), *P. fluorescens* and *P. vesicularis* recovered from 1 bottle (0.07%). *Aeromonas hydrophila* was isolated from 4 samples of the bottled water tested (0.3%). Other microorganisms recovered were *Citrobacter freundii* from 4 bottles (0.3%), *Flavobacterium breve* and *Pasteurella* spp. from 2 bottles (0.1%), *Providencia alcalifaciens* from 1 bottle (0.07%) and *Enterococcus cloacae* from 9 bottles (0.6%).

Many isolates could not be identified with the API system due to the limitations of the database (Armas and Sutherland, 1999) which are often mentioned, especially when we are referring to environmental isolates. Although the API system has already been used for the identification of bacteria from bottled water (Mannaia et al., 1990; Mavridou, 1992) additional tests are usually necessary as this system is designed for the discrimination of species of clinical origin.

Screening the microbiological quality of the water samples tested from 1995 to 2003, the parameters analyzed show a variance depicted in Fig. 1. Overall, the highest percentages of positive samples for total coliforms were recorded in 1997 and 1998 when the majority of them were derived from brand I (64.4% in 1997 and 72.5% in 1998). *E. coli* was almost absent

Table 4
Heterotrophic plate count (cfu ml^{-1}) of the samples tested at 22 and 37 °C

HPC (cfu ml^{-1})	No. (%) of samples at	
	22 °C	37 °C
HPC $\leq$ 20	1248 (81.7)	1307 (85.6)
20 < HPC $\leq$ 100	94 (6.2)	93 (6.1)
100 < HPC $\leq$ 1000	156 (10.2)	109 (7.1)
HPC > 1000	29 (1.9)	18 (1.2)

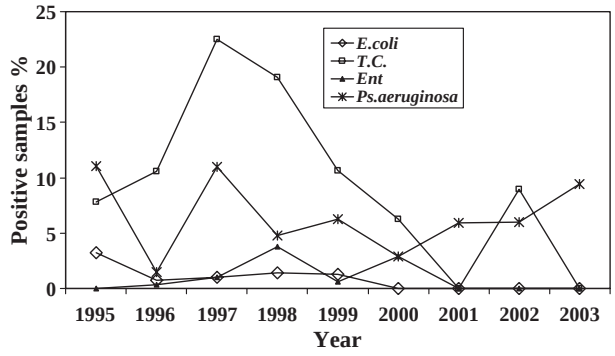

Fig. 1. Distribution of positive samples of bottled water for total coliforms (T.C.), *E. coli*, *Enterococcus* spp. (Ent) and *Pseudomonas aeruginosa* from 1995 to 2003.

in the bottled water ranging in low percentages with its peak recorded in 1995, in samples from various brands. In 1998, 75% of the positive samples derived from brand I and from 2000 to 2003 *E. coli* were not detectable in any sample of bottled water. *Enterococcus* spp. was also detected in very few bottles. Positive samples were recorded from 1996 to 2000. In 1998, brand I represented 75% of the contaminated samples and in 2000 enterococci were detectable in bottled water from brand C. For *P. aeruginosa*, most positive samples were recorded in 1995, 1997 and 2003. In 1995, 45.8% of the contaminated samples belonged to brand J and 25% to brand I, in 1997, 63.6% of the positive samples derived from brand I and finally all contaminated samples in 2003 were derived from brand B.

4. Discussion

Worldwide, sales of bottled water increase every year (Penland and Wilhelmus, 1999) because of the general belief that it is safe and free of all impurities (Bharath et al., 2003). Natural mineral water cannot be subjected to any type of disinfection that modifies or eliminates its biological components (Leclerc and Moreau, 2002); therefore it must come from an unadulterated, protected source and adhere to stringent quantitative and qualitative regulations (Rosenberg, 2003). Nevertheless, several studies have shown that bottled water is not sterile as it may contain various pathogens like coliforms, *Pseudomonas* spp., *E. coli*, *Campylobacter* or even mycobacteria (Mavridou et al., 1994; Papapetropoulou et al., 1997; Tsai and Yu, 1997; Warburton et al., 1998a,b; Armas and Sutherland, 1999; Bharath et al., 2003; Obiri-Danso et al., 2003, Evans et al., 2003).

In our study, a very low proportion of the samples tested were unsuitable for drinking according to the standing Greek regulation, as they were contaminated with one or more of the microbial parameters analyzed. Nevertheless, this finding should be gauged taking into account the fact that the technique was applied with nitrocellulose membranes of 0.45 μm pore size. In bottled water, bacterial cells are exposed to stresses reducing thus their cell diameter. Though ISO techniques indicate the use of 0.45 μm filters many authors recommend membranes of 0.22 μm pore size in order to isolate larger proportion of bacterial cells, including the stressed ones (Tamagnini and González, 1997; Tsai and Yu, 1997; Leclerc and Moreau, 2002).

The presence of total coliforms, *E. coli* and *Enterococcus* spp. indicates incidence of contamination and potential presence of pathogenic enteric microorganisms (Bharath et al., 2003). Among the species of *Pseudomonas* isolated, the most important is *P. aeruginosa*, considering that to date in Greece it is the only one unacceptable criterion in bottled water and is used as a process management indicator in the production. Its presence means contamination during the bottling process or that the source has become polluted by organic material (Legnani et al., 1999; Rosenberg, 2003; Bartram et al., 2004).

The variance of positive samples for the parameters tested including *P. aeruginosa* among the tested brands implies malfunction of certain bottling plants or temporary contamination from an indigenous or extraneous factor, especially considering the fact that in Greece all plants follow the same procedure of preparation of bottling water (Mossel and Struijk, 2004).

Confirming the documented heterogeneity of the microbial flora of bottled water (Hunter, 1993), apart from *Pseudomonas* species, other microorganisms were isolated from the samples tested, including *A. hydrophila*, which is prohibited by regulations of many countries. Many isolates remained unidentified with API galleries due to their limitations (Armas and Sutherland, 1999). Mineral water ecosystems exhibit a high degree of phenotypic and genetic microbial diversity that cannot always be supported by species identification (Leclerc and Moreau, 2002).

As far as heterotrophic plate counts (HPC) at 22 and 37 °C are concerned, the majority of the samples had the limits of 100 and 20 cfu ml^{-1}, respectively, as Greek water legislation specifies these as the recommended limits for aerobic colony count but within 12 h after bottling. It should also be taken into account that the samples of the study were purchased from retail outlets, where they were stored at approximately 20 °C for one to two months after bottling, conditions that allow proliferation of their autotrophic microbial flora (Leclerc et al., 1985; Parrington and Sharpe, 1998; Bartram et al., 2004). Nevertheless, HPC were kept in low numbers and although there has been considerable discussion as to the health importance of these organisms their measurements are always recommended (Rosenberg, 2003).

In the current study, for HPC measurements, the pour plate (PP) method was applied as it is the official method of the European Community Directive (1980). The drawbacks of this method include the stress and the injury of bacteria when mixed with the melted agar medium, the limited volume of the sample that can be analyzed and the fact that some very fastidious microbes do not grow at all or only very poorly in the agar medium (Parrington and Sharpe, 1998; Ramalho et al., 2001; Reasoner, 2004). Yet, the pour plate technique is preferable as it tends to favour the recovery of fast growing mesophilic and psychotrophic bacteria (Armas and Sutherland, 1999).

Screening the microbiological results of the samples tested over the period 1995–2003, the parameters varied significantly among brands. Generally, what the present study demonstrates is an overall reduction of total coliforms, *E. coli* and enterococci from 1995 to 2003. As far as *P. aeruginosa* is concerned it had a prevalence lower than that of other studies (Mavridou et al., 1994; Bharath et al., 2003).

The findings of this investigation should be evaluated considering that the water tested was still and sealed in plastic containers, contributing to increased bacterial growth in the water. Carbonation of bottled water can lower the pH of the product and thus microorganisms are less likely to grow (Warburton, 2000). Furthermore, bacteria usually occur in greater numbers in plastic containers than in glass bottles as plastic tends to be more permeable to external oxygen and extraneous vapours (Moreira et al., 1994; Rosenberg, 2003).

Concluding, the findings of the current study demonstrate that the status in the Greek bottled water industry has become better over the years, considering the overall reduction of contaminated samples from 1995 to 2003. At present, manufacturing companies have applied Hazard Analysis and Critical Control Points (H.A.C.C.P.) as well as ISO (International Organization for Standardization) guidelines in order to stringent manufacturing standards, to control the production process and to establish the quality of the final product. Thus, apart from sporadic cases with increased colony counts and broad variety of bacteria recovered from some brands, bottled water provided in the Greek market meets high quality standards.

Nevertheless, there is a need for the inclusion of H.A.C.C.P in the process of bottling by the companies for the improvement of the microbiological quality of bottled water, the development of media supporting fastidious types of microbes indigenous to bottled water and to develop new indicator principles.

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