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Bacteriological quality of bottled natural mineral waters commercialized in Hungary

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ABSTRACT

The purpose of the present study was to examine the bacteriological quality of bottled natural mineral waters sold in Hungary because, in recent years, there has been a tremendous increase in consumer demand for these products in the country. In total, 492 samples of domestic and imported brands of carbonated and non-carbonated mineral waters (246 samples each) were purchased from retail outlets, and were then screened for the presence of the following indicator and potentially pathogenic bacteria: spore-forming sulfite-reducing anaerobes (clostridia), total coliforms, *Escherichia coli*, *Enterococcus* spp., and *Pseudomonas aeruginosa*. Heterotrophic plate counts (HPC) were also determined by incubation at 37 °C for 24 h and at 22 °C for 72 h. The data generated were compared to the reference criteria set by Directive 2009/54/EC of the European Parliament and the Council of the European Union on the exploitation and marketing of natural mineral waters. The results showed that 5.3% and 10.2% of the carbonated and non-carbonated mineral water samples tested, respectively, were positive for at least one of the specific indicator or potentially pathogenic bacteria. Overall, a total of 38 sample units (7.7%) failed to comply with the standards set by Directive 2009/54/EC. None of the samples were judged non-compliant with current regulations on the basis of the presence of HPC bacteria at levels reaching or exceeding the acceptability limit (i.e., 100 CFU/ml at 22 °C or 20 CFU/ml at 37 °C) because the analyses were not carried out within 12 h after bottling, as required by Directive 2009/54/EC. The findings of this study highlight the need for a more stringent self-control by some producers of mineral water. In addition, a more systematic surveillance by the official authorities of food control is also necessary.

1. Introduction

Although natural mineral waters have been consumed since Roman times, only the 20th century has seen the emergence of the natural mineral water industry and the drinking of these products on a large scale as an alternative to tap water and non-alcoholic beverages (Stickler, 1989). In recent years, there has been a tremendous increase in consumer demand for bottled mineral waters worldwide, including in Hungary. Hungarians are increasingly coming to realize the importance of a healthy lifestyle and, for this reason, appreciate the beneficial nutritional properties of mineral waters. According to official statistics, the amount of bottled mineral water consumed in the Republic of Hungary has quadrupled over the past 10 years. Specifically, between 1999 and 2009, the average per capita consumption of bottled mineral water has increased from 28 L to 110 L (Hungarian Mineral Water Association and Product Board, 2010). A well-defined group of the population fulfills its need for drinking water almost exclusively with bottled mineral water (Somlai et al., 2002). Making up approximately two-thirds of the total mineral water consumption, carbonated mineral water is considerably more popular than non-carbonated in Hungary. Dozens of both domestic and imported brands are marketed nationally (Hungarian Mineral Water Association and Product Board, 2010).

With the significant increase in bottled mineral water consumption over the last decade, there has been a growing concern about the microbiological quality of such products. Two groups of bacteria are found in natural mineral water. Autochthonous bacteria are the natural flora of water. They are generally psychrotrophic and oligocarbotrophic, and they multiply rapidly in the bottled water. Allochthonous bacteria, which are contaminants, mostly enter the water during bottling, although they can also be present in the raw water. Their viability in the bottled water is generally poor because of the low nutrient level (Mavridou, 1992). Typically, the autochthonous flora is of no concern to a healthy population (Rosenberg, 2003). Overall experimental and

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epidemiological data show that autochthonous bacteria in natural mineral waters have never brought about detectable pathological disorders in humans. Since the existence of European regulations dating from 1980 (Council of the European Communities, 1980), no outbreak or single case of disease due to the consumption of natural mineral water has been recorded in the European Union (Leclerc & Moreau, 2002).

Natural mineral water is defined by Directive 2009/54/EC (European Parliament & Council of the European Union, 2009) as microbiologically wholesome water originating in an underground water table or deposit and emerging from a spring tapped at one or more natural or bore exits. It may not be subjected to any treatment aside from the separation of unstable constituents and the elimination, introduction or reintroduction of carbon dioxide. Any treatment likely to change the viable colony count of the natural mineral water is strongly prohibited. The total colony count, often referred to as heterotrophic plate count (HPC), measured within 12 h following bottling should not exceed 100 CFU/ml after incubation at 20–22 °C for 72 h and 20 CFU/ml after incubation at 37 °C for 24 h. Directive 2009/54/EC (European Parliament & Council of the European Union, 2009) also makes it a condition that, at source and during marketing, a natural mineral water shall be free from: (a) parasites and pathogenic microorganisms, (b) *Pseudomonas aeruginosa*, enterococci, *Escherichia coli* and other coliforms in any 250 ml sample examined, and (c) sporulated sulfite-reducing anaerobes in any 50 ml sample examined. For this reason, in addition to the implementation of various hygiene measures during the capture and packaging of mineral water, special care should also be taken during transport and storage of the filled bottles in order to ensure conditions that exclude the possibility of microbial contamination and proliferation and protect the product and its package from deterioration and damage (Filho, Sant'ana, & Cruz, 2008).

The objective of the present study was to examine the bacteriological quality and safety of bottled natural mineral waters sold in Hungary. Although several qualitative studies of the microbiology of bottled mineral waters have been carried out in various countries (Da Silva et al., 2008; Tsai & Yu, 1997; Venieri, Vantarakis, Komninou, & Papapetropoulou, 2006; Warburton et al., 1998; Zeenat, Hatha, Viola, & Vipra, 2009), this is the first representative report on this issue in Hungary insofar as the samples tested covered all of the main brands available in retail stores in the country.

2. Materials and methods

2.1. Sampling

From September 2009 through March 2010, a total of 492 samples of bottled natural mineral waters comprising 452 samples of 27 domestic brands and 40 samples of 8 brands imported from Austria, Croatia, France, Germany, and Italy were purchased from retail outlets located all over Hungary. Upon collection, carbonated and non-carbonated mineral waters (246 samples each), sealed in 1.5 L to 2.0 L polyethylene terephthalate (PET) bottles, were taken to the microbiological laboratory and stored at refrigeration temperature in the original bottle as purchased from food stores until tested. All samples were processed within 24 h after collection.

2.2. Bacteriological analysis

To detect the presence of spore-forming sulfite-reducing anaerobes (clostridia), total coliforms, *E. coli*, *Enterococcus* spp., and *P. aeruginosa*, bottled mineral waters were examined by filtration of 50 ml (clostridia) or 250 ml sample volumes through mixed cellulose esters membranes of 0.22 μm pore size and 47 mm diameter (Millipore, Bedford, MA, USA) followed by plating on selective media. In bottled water, bacteria are exposed to various stress factors which reduce their cell diameter. Although various standard methods generally 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 (Leclerc & Moreau, 2002; Tsai & Yu, 1997).

For total coliforms, membranes were placed on m-Endo Agar LES (Merck, Darmstadt, Germany) followed by aerobic incubation at 35 °C for 24 h. For *E. coli*, membranes were transferred on Chromocult® TBX (Tryptone Bile X-Glucuronide) Agar (Merck) and plates were incubated aerobically at 44 °C for 24 h. *Enterococcus* spp. were isolated by plating the membranes on Membrane-filter *Enterococcus* Selective Agar according to Slanetz and Bartley (Merck) and by incubating aerobically at 35 °C for 48 h. *Enterococcus* spp. isolates were confirmed by transferring the membrane filters with sterile forceps onto Bile Aesculin Azide Agar (Merck) plates preheated at 44 °C. Enterococci hydrolyzed aesculin on this medium within 2 h. To detect *P. aeruginosa*, membranes were plated onto *Pseudomonas* CN Selective Agar supplemented with 200 mg/l cetrimide and 15 mg/l nalidixic acid (Merck), and were incubated aerobically at 36 °C for 48 h. Suspect colonies of *P. aeruginosa* were confirmed by the oxidase test, acetamide solution and King's B Medium (Merck) according to Hungarian Standard MSZ EN 12780:2003 (Hungarian Standards Institution, 2003). For enumeration of sulfite-reducing clostridia, membrane filters were placed on Tryptose Sulfite Cycloserine Agar (Merck) followed by anaerobic incubation at 37 °C for 24 h. Anaerobic conditions were generated using anaerobic culture jars (2.5 L) and AnaeroGen AN 25 sachets (Oxoid, Basingstoke, UK).

For enumeration of heterotrophic bacteria, the pour plate method was chosen, using 1 ml of mineral water sample and mixing with melted Plate Count Agar (Casein–peptone Dextrose Yeast Agar; Merck) tempered at 44 °C. Two sets of plates were prepared for all samples. One set was incubated aerobically at 37 °C for 24 h and the other set at 22 °C for 72 h. All colonies were counted and the results were expressed as colony-forming units (CFU) per milliliter of the water sample.

The results were compared to the reference criteria contained in Directive 2009/54/EC of the European Parliament & the Council of the European Union (2009) on the exploitation and marketing of natural mineral waters (Table 1).

3. Results and discussion

Table 2 shows that out of the 246 non-carbonated mineral water samples examined, 187 (76.0%) had a 22 °C-HPC below 100 CFU/ml, whereas at 37 °C as many as 193 (78.4%) samples contained heterotrophic microorganisms at less than 20 CFU/ml. In other words, 59 (24.0%) and 53 (21.6%) of the non-carbonated sample units incubated at 22 °C and 37 °C, respectively, failed to comply

Table 1
Current microbiological limits for bottled natural mineral waters as established in Directive 2009/54/EC of the European Parliament & the Council of the European Union (2009).

Parameter	Parametric value
Total coliforms	Non-detectable (ND)/250 ml
Escherichia coli	ND/250 ml
Enterococcus spp.	ND/250 ml
Pseudomonas aeruginosa	ND/250 ml
Spore-forming sulfite-reducing anaerobes (clostridia)	ND/50 ml
Heterotrophic plate count at 20–22 °C[a]	$\leq 1.0 \times 10^2$ CFU/ml
Heterotrophic plate count at 37 °C[a]	$\leq 2.0 \times 10^1$ CFU/ml
Parasites and pathogenic microorganisms	Absent (in full product volume)

[a] To be measured within 12 h after bottling.

Table 2
Heterotrophic plate counts (HPC) of non-carbonated and carbonated mineral water samples tested at 22 °C or 37 °C.

HPC (CFU/ml)	No. (%) of non-carbonated samples at		No. (%) of carbonated samples at	
	22 °C	37 °C	22 °C	37 °C
HPC < 20	178 (72.3)	193 (78.4)	238 (96.8)	238 (96.8)
20 ≤ HPC < 100	9 (3.7)	4 (1.6)	2 (0.8)	3 (1.2)
HPC ≥ 100	59 (24.0)	49 (20.0)	6 (2.4)	5 (2.0)
Total	246 (100)	246 (100)	246 (100)	246 (100)

with the standards set by the EU legislation for the HPC levels of bottled mineral waters.

Carbonated mineral waters proved to be superior in terms of HPC to the non-carbonated brands tested, in that only 6 (2.4%) samples reached or exceeded the legal limit of 100 CFU/ml measured at 22 °C, and 8 (3.2%) samples had a 37 °C-HPC of ≥20 CFU/ml (Table 2).

Of all the 492 mineral water samples examined, 13.2% had HPC ≥ 100 CFU/ml at 22 °C and 12.4% had HPC ≥ 20 CFU/ml at 37 °C (Table 2). It should be noted, however, that the microbiological analyses did not take place within 12 h after bottling, as is required by EU Directive 2009/54/EC (European Parliament & Council of the European Union, 2009). Furthermore, the samples in this study were purchased from retail outlets where they were stored at approximately 20 °C for up to several months after bottling, conditions that may have allowed proliferation of their microbiota (Leclerc & Moreau, 2002; Venieri et al., 2006).

The counts for heterotrophic bacteria obtained in the present research were below those reported by various authors. Tsai & Yu (1997) evaluated 88 domestic and 44 imported samples of non-carbonated mineral water sold in Taiwan, and found that 45 domestic samples (51.1%) and 29 imported samples (60.4%) were above the HPC limit of 200 CFU/ml established for the product in Taiwan. Zeenat et al. (2009) examined the microbial quality of 75 samples of bottled mineral water belonging to three domestic brands in Fiji and, according to their findings, between 28% and 68% of the samples were out of compliance with HPC standards.

Heterotrophs are microorganisms that use organic compounds to cover most or all of their carbon requirements, and include species in the genera *Pseudomonas*, *Aeromonas*, *Alcaligenes*, *Acinetobacter*, *Klebsiella*, *Flavobacterium*, *Chromobacterium*, and many others (Filho et al., 2008). High levels of HPC in non-carbonated mineral waters are usually indicative of the natural proliferation of autochthonous bacteria present in low numbers in the source water, although sometimes contamination within the bottling plant is considered to be responsible (Kassenga, 2007; Manaia, Nunes, Morais, & Da Costa, 1990; Stickler, 1989). The rapid growth of bacteria after the mineral water is bottled may be due to oxygenation of the water during processing, the increase in surface area provided by the bottle, the increase in temperature, and the amount of nutrients arising from the bottle (Warburton, 2000; Warburton et al., 1992). It is also worth mentioning that the autochthonous bacteria in mineral water are capable of multiplying well even with a very limited supply of nutrients (Tsai & Yu, 1997).

There is no evidence either from epidemiological studies or from correlation with occurrence of waterborne pathogens that HPC values alone directly relate to any health risk. However, specific strains of microbial species that may be a part of HPC microbiota can cause infection in certain vulnerable individuals, i.e., the very young, the elderly, the immune suppressed population, and pregnant women (Nsanze, Babarinde, & Al Kohaly, 1999; Warburton, 2000).

The fact that the carbonated mineral water samples tested in this study were generally found to have low bacterial numbers is not unexpected because carbonation is known to decrease the pH of water and, in turn, has an antibacterial effect (Insalata, 1952; King & Nagel, 1967; Venieri et al., 2006; Warburton, 2000; Warburton et al., 1998).

As shown in Table 3, 6.3% of the 492 samples tested were non-compliant for total coliforms, 1.4% contained *E. coli*, and 1.2% were contaminated with *Enterococcus* spp. Interestingly, the percentage of sample units positive for enterococci was higher in carbonated waters than in non-carbonated ones, which is just the opposite of what was found for total coliforms and *E. coli*. In the present study, the occurrence of coliform bacteria was considerably higher than that of *E. coli*, but the isolation of *E. coli* from seven brands, with all the positive samples being non-carbonated mineral water, also has health implications because this species is considered to be an indicator of recent fecal contamination (Da Silva et al., 2008; Edberg, Rice, Karlin, & Allen, 2000; Leclerc & Moreau, 2002).

These results parallel the non-compliance found in a Greek study, where the prevalence of total coliforms, *E. coli*, and *Enterococcus* spp. were 11%, 1%, and 1.2%, respectively (Venieri et al., 2006), and also correspond to reports by Bharath et al. (2003) that 5.2% of 344 samples of bottled water marketed in Trinidad and Tobago contained coliforms and 1.5% were positive for *E. coli*. As opposed to these findings, Tsai & Yu (1997) detected no total coliforms or enterococci in 136 samples of bottled non-carbonated mineral water in Taiwan and, similarly, Kokkinakis, Fragkiadakis, and Kokkinaki (2008) found no coliforms, *E. coli* or enterococci in 240 samples of Greek bottled water. By contrast, according to the results of Da Silva et al. (2008), as much as 40% of 77 samples of bottled mineral water from water dispensers in Brazil contained coliform organisms.

Table 3 also indicates that seven (1.4%) out of the 492 sample units examined were found to contain *P. aeruginosa*, and six of the samples contaminated with *P. aeruginosa* were non-carbonated mineral water. There are several studies in the literature on the occurrence of *P. aeruginosa* in bottled mineral waters, but the data differ widely among various investigators. Values ranging from 0% to 11% are generally reported (Jayasekara, Heard, Cox, & Fleet, 1998; Manaia et al., 1990; Massa, Fanelli, Brienza, & Sinigaglia, 1998; Tsai & Yu, 1997; Venieri et al., 2006); however, Da Silva et al. (2008) and Rivilla & González (1988) isolated *P. aeruginosa* from 58.4% and 37.5% of Brazilian and Spanish bottled mineral water samples, respectively.

Pseudomonas aeruginosa is usually an indicator of contamination during the bottling process (Rosenberg, 2003). There is minimal risk of *P. aeruginosa* infection for the general population if the pathogen is present in bottled water. It needs to be ingested at

Table 3
Occurrence of indicator and potentially pathogenic bacteria in the bottled mineral waters tested in this study.

Type of mineral water	No. of samples tested	No. (%) of samples positive for				
		Total coliforms	*Escherichia coli*	*Enterococcus* spp.	*Pseudomonas aeruginosa*	Spore-forming sulfite-reducing anaerobes
Non-carbonated	246	23 (9.3)	7 (2.8)	2 (0.8)	6 (2.4)	0 (0.0)
Carbonated	246	8 (3.2)	0 (0.0)	4 (1.6)	1 (0.4)	1 (0.4)
Total	492	31 (6.3)	7 (1.4)	6 (1.2)	7 (1.4)	1 (0.2)

a rate of 5.0×10^3 to 1.0×10^4 CFU/ml in 2 L of daily intake to have even a 1:10,000 chance of colonization of the gut (Rusin, Rose, Haas, & Gerba, 1997). Despite this fact, the presence of *P. aeruginosa* in mineral water is considered unacceptable because it is an opportunistic pathogen capable of causing infections in immunocompromised consumers (Hunter, 1993). So far, there have been no recorded outbreaks or infection cases traced to the presence of *P. aeruginosa* in bottled mineral water (Fok, 2005).

As for the occurrence of spore-forming sulfite-reducing anaerobes, only one brand (0.2%) of carbonated mineral water tested positive for this group of bacteria (Table 3). Relatively few authors have examined bottled mineral waters for clostridia. Fewtrell, Kay, Wyer, Godfree, and O'Neill (1997) analyzed 1082 samples of bottled water and found that only one sample (0.1%) contained sulfite-reducing anaerobes. Similar observations were reported by Da Silva et al. (2008), who detected no *Clostridium* spp. in any of 99 samples of bottled mineral water in Brazil. Sulfite-reducing clostridia are common components of the intestinal microbiota of humans and other mammals and, therefore, their presence or absence is widely used to evaluate the sanitary quality of water. Because spore-forming sulfite-reducing anaerobes are generally seldomly detected in natural mineral waters, it is arguable whether such an indicator really makes any sense in a regulation.

All things considered, 5.3% and 10.2% of the carbonated and non-carbonated mineral water samples tested, respectively, were found to contain detectable levels of at least one of the following indicator or potentially pathogenic bacteria: total coliforms, *E. coli*, *Enterococcus* spp., *P. aeruginosa*, or spore-forming sulfite-reducing anaerobes. This means that a total of 38 sample units (7.7%) failed to comply with the standards set by Directive 2009/54/EC (European Parliament & Council of the European Union, 2009). None of the samples were judged non-compliant with current regulations on the basis of the presence of HPC bacteria at levels reaching or exceeding the acceptability limit of 100 CFU/ml at 22 °C or 20 CFU/ml at 37 °C, because all the analyses were performed well after 12 h following bottling.

4. Conclusions

Contrary to what many people believe, bottled mineral water is not free from microorganisms. Most of these microbes, measured by the heterotrophic plate count, come from the source water itself. They are not problematic for healthy consumers and do not result in microbial spoilage or flavor impairment of the products. In contrast, potential pathogens typically indicate contamination at the source or during the bottling process. The presence of opportunistic pathogens such as *P. aeruginosa* in mineral waters underscores the importance of caution regarding the safety of these products, especially for health compromised individuals; however, the risks should by no means be exaggerated. The major factors and practices that influence the changes in microbial populations and contamination of bottled mineral waters are well researched and understood. In addition, current legislation in Hungary and in most European countries requires food business operators to put in place, implement and maintain a permanent procedure or procedures based on HACCP principles. Yet, the results of this study highlight the need for a more stringent self-control by some producers of mineral water, and a more systematic surveillance by the official authorities of food control is also necessary.

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