Mineral water: a microbiological approach

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ABSTRACT

The microbiological quality of bottled mineral water of various domestic brands sold in Brazil was investigated, with particular focus on the heterotrophic plate count (HPC). Neither total coliforms nor *Escherichia coli* were found in any 1.5 L bottle samples. Total coliforms were found in 2.9% of the small bottles, while in 20 L bottles the presence of total coliforms and *E. coli* was demonstrated in 15.5 and 2.4% of samples, respectively. *Pseudomonas aeruginosa* was detected in 4.3, 4.5 and 9.5% of small, 1.5 and 20 L bottles, respectively. In 36.4% of the samples of 1.5 L bottles, the HPC was above 500 cfu/mL. This percentage of samples with an HPC above 500 cfu/mL increased to 52.0 and 61.9% in small and 20 L bottles, respectively. Higher contamination by total coliforms, *E. coli*, *P. aeruginosa* and HPCs occurred in 20 L bottles. In conclusion, several samples in this study were outside the international quality standard for mineral water and the large number of samples with high HPCs shows that more work must be done on the use of HPC in mineral water and the damaging effects that these microorganisms may cause to humans. The bottled mineral water was confirmed as a particularly important public health problem, due to the poor microbiological quality of the products that are marketed.

Key words | *Escherichia coli*, heterotrophic plate count, mineral water, *Pseudomonas aeruginosa*, total coliforms

INTRODUCTION

Sales of bottled mineral water have been increasing all over the world. Various factors may have contributed to this increase: dissatisfaction with the odor and taste of tap water due to chlorine, greater consumer concern about the safety of tap water, or the use of bottled mineral water as a substitute for other beverages (Warburton 2000). In addition, mineral water has been marketed as ideal for immunosuppressed people, infant formula preparation and nursery drinking water (Warburton 1993).

However, it may not be true to say that the microbiological quality of mineral water is superior to that of the public water supply, despite its origin in underground springs (Hunter 1993). Bottled mineral water has been implicated in outbreaks of cholera (Blake et al. 1977), reported incidences of typhoid fever and traveler’s diarrhea (Rosenberg 2003). There have also been reports of potential pathogens, such as enteric bacteria (Schindler 1994), protozoa (Salazar et al. 1982), acid-fast bacteria (Papapetropoulou et al. 1997) and amoeba (Rosenberg 2003), being detected in bottled mineral water. Fungi (Cabral & Fernández Pinto 2002) and antibiotic resistant bacteria (Massa et al. 1995; Mary et al. 2000; Messi et al. 2005) have also been detected in bottled mineral water. In Brazil some studies have detected the presence of *Cryptosporidium* oocysts (Franco & Cantusio Neto 2002), *Pseudomonas aeruginosa* (P. aeruginosa) and high counts of heterotrophic bacteria (Franco & Cantusio Neto 2002; Da Silva et al. 2008) in bottled mineral water. All these reports demonstrate the need to ensure good microbiological quality for these products.

Mineral waters have autochthonous microorganisms that begin to multiply rapidly soon after bottling. Since natural mineral water is not sterilized or pasteurized to remove
or destroy microorganisms before bottling, the bottled mineral water is not completely free of microorganisms (Rosenberg 2003). The bacterial population is approximately 10–100 colony-forming units per mL of sample (cfu/mL) in the underground source and, after filling, this population increases to approximately 10^5–10^6 cfu/mL in about 1–3 weeks (Legnani et al. 1999).

Several international organizations such as the Codex Alimentarius Commission, in their specific regulations for bottled mineral water, advocate the absence of pathogenic microorganisms and establish limits for certain indicator microorganisms such as coliforms and P. aeruginosa, but there are no limits for the heterotrophic plate count (HPC) (Codex Alimentarius Commission 1985).

Studies have led to the detection of HPCs in bottled mineral water at levels far exceeding international standards established for potable water in public water supply (Reasoner 2004). All pathogenic and opportunistic bacteria are heterotrophic, and most can grow on media designed to determine HPC in drinking water (Allen et al. 2004). In this light it is suggested that HPC should be treated as a parameter of quality for mineral water, as is the case for public water supply. On the other hand, there is insufficient clinical and epidemiological evidence to conclude that a high HPC in drinking water poses a risk to consumer health (Edberg & Allen 2004; Stelma Jr et al. 2004). The fact is that high levels of microbial growth can affect the taste and odor of drinking water and may well indicate the presence of nutrients and biofilms (Sartory 2004). It may also be a sign that some event has interfered with the normal production of the drinking water. There is some evidence that the presence of autochthonous non-pathogenic bacteria can cause changes that encourage an increase in the number of pathogens that can be present in biofilms (Bartram et al. 2004). It is also known that an HPC greater than 500–1,000 cfu/mL in drinking water can interfere with coliform analysis, resulting in false negatives when lactose-based media are used. Therefore it is believed that the HPC should be used as a process management indicator in bottled water production (Allen et al. 2004).

The consumption of bottled mineral water has increased around the world and some studies conducted in Brazil and other countries have shown that mineral water can contain microorganisms harmful to human health. In addition, there is still much debate about the use of HPC as a parameter of quality for bottled mineral water; as such, it still requires attention. Based on these studies it is possible to hypothesize that bottled mineral water could represent a serious public health problem. The current study was designed to test such a hypothesis, whether bottled mineral waters may represent a particularly important public health problem, due to the poor microbiological quality of the products that are marketed. Based on this hypothesis, the microbiological quality of various brands of domestic bottled mineral waters sold in Brazil, with particular emphasis on the HPC, was assessed and compared against the different volumes sold in the country.

**MATERIALS AND METHODS**

**Samples**

In all, 263 samples of bottled mineral water from 37 Brazilian brands, sealed in plastic bottles, were analyzed. All the bottled mineral water originated from natural springs and cannot be sterilized, pasteurized or otherwise treated to remove or destroy microorganisms. All samples of bottled mineral water were purchased from retail outlets in Araraquara city (State of São Paulo, Brazil), as follows: 84 samples in 20 litre (L) bottles belonging to 21 brands, 110 samples in 1.5 L bottles belonging to 22 brands and 69 samples in small bottles (0.33–0.60 L) belonging to 17 brands. Each of the samples were from different batches. All bottles when sampled were in perfect state, without the formation of biofilms. The analyses were carried out on the day the samples arrived. The bottles were cleaned externally with detergent and ethanol (70% in water) and homogenized manually before analysis of the samples. All the samples were analyzed in triplicate.

**Microbiological analysis**

**Determination of total coliforms and *Escherichia coli***

To determine the presence of total coliforms and *E. coli* the Enzyme Substrate Test was used according to the standard methods for the examination of water and wastewater.
(APHA et al. 1998). Dehydrated Colilert® medium was added to 100 mL of a sample in a disposable sterile bottle and incubated at 35°C for 24 h. Following incubation, the samples were read for yellow color indicating coliform β-galactosidase activity and fluorescence as a result of the action of β-glucuronidase from E. coli. An ultraviolet (UV) lamp (at 365 nm) was used to identify the fluorescence.

**Determination of P. aeruginosa**

The presence of P. aeruginosa was determined by the multiple tube method according to standard methods (APHA et al. 1998). For the presumptive test, 10 mL aliquots of each sample were poured into each of 10 tubes with 10 mL double asparagine broth and incubated at 35°C for 48 h. The production of pyoverdin in the asparagine broth detected by fluorescence under a UV lamp (at 365 nm), meant that the presumptive test was positive. A confirmatory test was carried out by transferring 0.1 mL inocula from the positive asparagine broth tubes into acetamide broth and incubating the tubes at 35°C for 24–36 h. Tubes that developed an alkaline reaction (purple coloration) were considered positive for the confirmatory test.

**Determination of HPC**

The pour-plate technique was used to determine the HPC as described in the standard methods (APHA et al. 1998). Petri dishes were inoculated with 1 mL of sample and then PCA, previously melted and brought to 44°C, was added. All plates were incubated at 35°C for 48 h. All colonies formed were enumerated using a colony counter (CP600 Plus, Phoenix, São Paulo, Brazil) and the result expressed in cfu/mL.

**RESULTS AND DISCUSSION**

The current study was planned with the aim of assessing the microbiological quality of Brazilian bottled mineral water. To fulfil this objective, standardized tests were used in order to verify the presence of indicator microorganisms of quality, such as total coliforms, E. coli, P. aeruginosa and HPC.

Figure 1 shows that neither total coliforms nor E. coli were found in any 1.5 L sample. However, in small bottles the presence of total coliforms was noted in 2.9% of the samples. On the other hand, for the 20 L bottles the presence of both total coliforms and E. coli was confirmed in 15.5 and 2.4% of samples, respectively. The results were also divided into brands of tested products (Table 1), and the 20 L bottle brands showed a large number contaminated by total coliforms (38.1%). The presence of total coliforms and E. coli in samples of bottled mineral water indicates that there was some contamination in the product. The specific presence of E. coli indicates fecal contamination and possible occurrence of enteric pathogens (Bharath et al. 2005). Positive samples were also found for total coliforms and E. coli in mineral water in other studies, such as in those of Bharath et al. (2003) and Venieri et al. (2006).

Figure 2 shows that in 4.3 and 4.5% of samples of small and 1.5 L bottles, respectively, the presence of P. aeruginosa was demonstrated. In the 20 L bottles P. aeruginosa was found in 9.5% of the samples. The results for each product brand was also analyzed (Table 1) and in all volumes of bottles analyzed a considerable number of brands were found to be contaminated by P. aeruginosa. In the studies of Venieri et al. (2006), Marzano et al. (2011) and Varga (2011) samples positive for P. aeruginosa were found as in this study. The presence of P. aeruginosa in bottled mineral water indicates that the contamination occurred during bottling or at source (Legnani et al. 1999; Rosenberg 2003; Bartram et al. 2004) and the presence of this bacteria can result in a general deterioration of the water quality affecting

![Figure 1](https://iwaponline.com/ws/article-pdf/12/5/556/416849/556.pdf)
According to Rosenberg (2003), mineral water is recommended to individuals with heart and kidney diseases, so that the presence of *P. aeruginosa* (which is an opportunistic pathogen) in such water products is of concern. The high prevalence of these bacteria in bottled water can be explained by their ability to adhere to the bottling system surfaces (forming sources of contamination) and to the bottle surface (Hunter 1993). Another factor favoring the presence of this bacterium is its great capacity to multiply in water with low nutrient content (Legnani et al. 1999). Therefore, additional control measures are needed to protect the source and the cleaning of the bottling system, to ensure the absence of *P. aeruginosa* in the final product.

The code of hygienic practice for collecting, processing and marketing of natural mineral waters (Codex Alimentarius Commission 1985) sets no limits for HPCs in bottled mineral water. However, HPC monitoring of these products varies with the country and the specific source of the mineral water, and most countries including the United States, Australia and Brazil have not established limits for bottled mineral water (WHO 2003). Thus, we rely on the limit of 500 cfu/mL, established for drinking water delivered by public water supply in the United States (USEPA 2002); this limit is also followed in Brazil for public supply water, and is also used in other studies about bottled mineral water (Da Silva et al. 2008).

Figure 3 shows that 36.4% of the samples from 1.5 L bottles exhibited counts above 500 cfu/mL. In small and 20 L bottles, respectively, this percentage rises to 52.0 and 61.9% of samples with HPCs above 500 cfu/mL. Dividing the results for the product brands (Table 1), it was observed that in the 1.5 L bottles, 40.9% of the brands showed HPC above 500 cfu/mL. Furthermore, in small and 20 L bottles HPCs above 500 cfu/mL were observed in 70.6 and 100% of the brands analyzed, respectively. Table 2 shows the

<table>
<thead>
<tr>
<th>Microorganism evaluated</th>
<th>0.33–0.60</th>
<th>1.5</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>Absence</td>
<td>Presence</td>
<td>Absence</td>
</tr>
<tr>
<td>Total coliforms</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>94.1</td>
<td>5.9</td>
<td>100</td>
</tr>
<tr>
<td>Heterotrophic plate count</td>
<td>88.2</td>
<td>11.8</td>
<td>81.8</td>
</tr>
</tbody>
</table>

Figure 2 | Percentage of samples that meet the standard for *Pseudomonas aeruginosa*.

Figure 3 | Percentage of samples that meet the standard for heterotrophic plate count.
distribution of HPCs in the samples. Most of the samples had counts equal to or less than 500 cfu/mL (48.7%) or above 2,000 cfu/mL (37.4%) in all volumes analyzed. This shows that when the HPC exceeded 500 cfu/mL, a great multiplication of bacteria probably must have occurred, raising the HPC to a concerning level. Most of the bacteria recovered in the HPC are not pathogenic. However, some such microorganisms may be opportunistic pathogens, capable of causing damage to the health of immunosuppressed individuals (Stelma Jr et al. 2004). High HPC may also cause water quality deterioration, with the formation of biofilms and the development of unpleasant odors and tastes (Sartory 2004). Therefore, HPC measurement is always recommended, although there is still much discussion about its importance to health (Rosenberg 2003). It is believed that HPC should be used as an indicator in bottled water production (Allen et al. 2004). In this study, as in that of Massa et al. (2001) bottled mineral water samples were found with counts above 500 cfu/mL.

In this study, higher contamination by total coliforms, E. coli, P. aeruginosa and HPC occurred in 20 L bottles (Table 1). This was probably due to the reuse of bottles without appropriate cleaning and disinfection (Kokkinakis et al. 2008; WHO 2008; Marzano et al. 2011) whereas the 20 L bottles are returnable. Some Pseudomonas strains were isolated from water used to rinse the returnable bottles of one mineral water company, representing a risk of contamination to that water (Legnani et al. 1999). The small bottles also had a high HPC in this study. According to Bischofberger et al. (1990), the multiplication of bacteria after bottling is possible because they adhere to the inner surface of the bottles where nutrients are adsorbed and concentrated. Zobell & Anderson (1936) believe that small bottles facilitate such propagation more, owing to the larger bottle surface area in contact with a given volume of water. If we analyze the brands separately, these numbers are worse. In the case of HPC, all brands of 20 L bottles had at least one sample with counts above 500 cfu/mL. In addition, approximately 70% of brands of small bottles tested were found to have counts above 500 cfu/mL.

Given the high HPC found in bottled mineral waters, the salient point for the consumer is the health significance of the bacteria present. As it is known that high numbers of opportunistic pathogens among the bacteria of HPC could be present (Stelma Jr et al. 2004), this shows the need to perform more detailed studies about the potential health risk of heterotrophic bacteria. Moreover, little is known about the composition of the HPCs of mineral water and about their multiplication after bottling. Therefore further studies about the microbiological characteristics of bottled mineral waters may bring additional clues, in order not to underestimate the microbiological risks associated with their consumption and thereby improve their quality standards.

### Table 2 | Heterotrophic plate count in different bottles of mineral water

<table>
<thead>
<tr>
<th>Mineral water bottle (L)ą</th>
<th>0.33–0.60</th>
<th>1.5</th>
<th>20</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean of HPC (cfu/mL)ą</td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>≤500</td>
<td>29</td>
<td>42</td>
<td>72</td>
<td>60</td>
</tr>
<tr>
<td>500 &lt; HPC ≤ 1,000</td>
<td>2</td>
<td>2.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1,000 &lt; HPC ≤ 1,500</td>
<td>5</td>
<td>7.3</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>1,500 &lt; HPC ≤ 2,000</td>
<td>2</td>
<td>2.9</td>
<td>4</td>
<td>3.3</td>
</tr>
<tr>
<td>&gt;2,000</td>
<td>31</td>
<td>44.9</td>
<td>38</td>
<td>31.7</td>
</tr>
<tr>
<td>Total</td>
<td>69</td>
<td>100</td>
<td>120</td>
<td>100</td>
</tr>
</tbody>
</table>

### CONCLUSION

Several samples in this study were outside the international quality standard for mineral water, as they were contaminated by total coliforms (15 samples; 5.7%), E. coli (two samples; 0.76%) and P. aeruginosa (16 samples; 6.08%). For HPC, 132 samples (50.19%) had counts above...
500 cfu/mL, making them of questionable quality for human consumption. This large number of samples with high HPCs shows that more studies should be carried out on the use of HPCs in mineral water testing and the damaging effects that these microorganisms may cause to humans. Additional measures would be welcome and could be taken, in order to ensure mineral water of good quality, among which we mention: protection of sources, stringent hygienic-sanitary care for the personnel and equipment involved in the production and cleaning of containers.

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