Bacteriological quality and risk assessment of the imported and domestic bottled mineral water sold in Fiji

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ABSTRACT

Considering the popularity of bottled mineral water among indigenous Fijians and tourists alike, a study was carried out to determine the bacteriological quality of different bottled waters. A risk assessment was also carried out. Seventy-five samples of bottled mineral water belonging to three domestic brands and 25 samples of one imported brand were analysed for heterotrophic plate count (HPC) bacteria and faecal coliforms. HPC counts were determined at 22°C and 37°C using R2A medium and a membrane filtration technique was used to determine the faecal coliform (FC) load in 100 ml of water on mFC agar. Between 28 and 68% of the samples of the various domestic brands failed to meet the WHO standard of 100 colony forming units (cfu) per 100 ml at 22°C and 7% of these also tested positive for faecal coliforms. All imported bottled mineral water samples were within WHO standards. A risk assessment of the HPC bacteria was carried out in terms of beta haemolytic activity and antibiotic resistance. More than 50% of the isolates showed beta haemolytic activity and were multi-drug resistant. While the overall quality of the product was generally good, there is a need to enforce stringent quality standards for the domestic bottlers to ensure the safety of consumers.

Key words | bacteriological quality, beta haemolytic activity, drug resistance, mineral water, risk assessment

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INTRODUCTION

Increased public awareness about waterborne disease outbreaks and lack of safe drinking water supply during travel has resulted in an increased demand for bottled drinking water. The market demand and the profits from this business are so overwhelming that the number of new bottling companies starting up increases on a regular basis. The proliferation of new brands calls for safety controls as:

> …the natural waters, suitable for direct bottling must be clear, colourless, and free from objectionable taste and odour. There should be no detectable organic matter present and the water must be of the highest bacteriological quality–virtually sterile–and should remain in this condition during the collection and bottling processes (Windle-Taylor 1976).

In countries with pristine natural waters preserved deep in aquifers, companies have also started marketing natural mineral water as a ready-to-consume commodity. In Fiji there is very high demand for this product and bottled mineral waters are also being imported from various countries. Although it is considered safe to consume without any heat processing, bottled mineral water is not a sterile product and can contain naturally occurring bacteria as well as those introduced during manufacturing or consumer handling. Also it is understood that risk of contamination by certain materials in contact with the

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water encourages the growth of saprophytic bacteria. Heterotrophic plate count (HPC) bacteria are commonly used to assess the bacteriological quality, the limit for which ranges from 100 to 500 cfu ml^{-1} depending on legislation in different countries (WHO 2001).

Since the bottled water is a ready-to-consume commodity and is consumed in large volumes, the risk of ingesting large numbers of bacteria is rather high. It has been reported that the HPC bacteria may include potential pathogens and opportunistic bacteria that can cause infections, especially in persons with a compromised immune system (Pavlov *et al.* 2004). Epidemiological studies conducted to examine the potential risk associated with HPC bacteria in bottled drinking water have yielded both negative and positive correlation (Payment *et al.* 1994). It is recognised that the component of the general population which is at risk of being infected by HPC bacteria is increasing worldwide.

Increasing drug resistance in HPC bacteria from non-carbonated mineral waters is another concern (Massa *et al.* 1995). As the bottled drinking water is consumed without any heat processing, the high total heterotrophic bacteria (THB) load with possible opportunistic pathogens, especially multiple antibiotic resistant forms, can pose a definite health risk to immunologically compromised individuals. These drug-resistant bacteria can also act as reservoirs of resistance plasmids that can freely exchange with pathogenic bacteria in the gastrointestinal tract. In the present investigation an attempt has been made to evaluate the bacteriological quality in terms of HPC bacteria and faecal coliforms (FC) of the three leading domestic brands and one imported brand of bottled mineral water currently sold in various outlets in Fiji. HPC bacteria were characterised to genera level and the risk associated with the HPC bacteria was assessed in terms of drug resistance and beta haemolytic activity.

METHODS

Collection of samples

Seventy-five samples of domestic and 25 samples of imported bottled mineral water were collected from various retail outlets and supermarkets in Suva, Fiji. While 80% of the samples from outlets were stored at 5°C, the remaining samples from the other outlets were stored at room temperature. The samples were analysed for heterotrophic plate count and coliforms.

Analysis of heterotrophic bacteria

Using a sterile pipette, 1 and 0.1 ml of water samples were aseptically dispensed into sterile Petri dishes. Approximately 20 ml of sterile R2A medium (TSA, Merck, Germany) was poured into each plate and rotated clockwise and anticlockwise in order to uniformly mix the water samples with the plating media. The plates were left to solidify. Each sample was plated in duplicate. One set of plates was incubated at 37°C while the other set was incubated at 22°C. The plate counts were determined after 48 hours and the results were expressed as number of cfu per 100 ml of water.

Analysis of faecal coliforms (FC)

FC were analysed by the membrane filtration method: 100 ml of water sample was filtered through sterile 0.45μm bacteriological filter (Sartorius, Germany) using a sterile membrane filter assembly. After filtration, the filter was aseptically removed using sterile forceps, placed on sterile mFC agar (Merck, Germany) plates and incubated at 37°C for 24 hours. After incubation, the plates were observed for typical FC-like colonies and the results expressed as number of FC per 100 ml.

Characterisation of heterotrophic plate count bacteria

After HPC determination, morphologically different colonies were picked up at random, restreaked to ensure purity and maintained on TSA slants at room temperature. Forty-two isolates were chosen for further characterisation. The selected HPC isolates were characterised according to Gram staining, spore staining, motility, Kovac's oxidase, catalase and oxidation/fermentation (O/F) tests.

Young cultures of less than 20 hours old were taken for Gram staining. After staining, the slides were observed under an oil immersion objective of a light microscope (Olympus, CH-20i). Gram-positive, rod-shaped bacteria were subjected

to endospore staining by the Schaeffer-Fulton method and examined for the presence of endospores using an oil immersion objective of a light microscope.

Gram-negative bacterial isolates and Gram-positive, non-spore forming rods were subjected to motility testing using semisolid agar (nutrient agar with 0.3% agar). Kovac's oxidase test was performed on sterile filter paper strips impregnated with 1% solution of tetramethyl paraphenylene diamine dihydrochloride. The mode of utilisation of carbohydrate glucose was determined using Hugh and Leifson's (O/F) medium (Merck, Germany). The catalase test was determined by using 3% hydrogen peroxide (H_2O_2). The specific reactions of the isolates were recorded and the isolates were grouped into different genera (Buchanan & Gibbons 1974; Prescott *et al.* 2002).

Antibiotic sensitivity testing

Thirty-three HPC isolates were subjected to antibiotic sensitivity testing against ten antibiotics using standard methodology (Bauer *et al.* 1966). The antibiotics and the concentrations used were amikacin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), gentamycin (10 μg), nalidixic acid (30 μg), novobiocin (30 μg), oxytetracycline (30 μg), penicillin-G (10 units), streptomycin (10 μg) and vancomycin (30 μg). All the antibiotic discs used were Oxoid (England) brand. The cultures were inoculated into sterile brain heart infusion broth (BHIB, Oxoid, England) and enriched by overnight incubation at 37°C. Enriched cultures were then streaked over sterile Mueller Hinton agar (MHA, Oxoid, England) plates. After 15 minutes of pre-diffusion time, the antibiotic discs were dispensed over the seeded agar plates (five antibiotic discs per plate) which were then incubated at 37°C for 16–18 hours. After incubation, the diameter of inhibition zones was measured, and following comparison with the zone interpretive chart of Kirby-Bauer, the isolates were grouped as resistant or sensitive.

Multiple antibiotic resistance (MAR) indexing of the isolates

The MAR index when applied to a single isolate is defined as a/b, where a represents the number of antibiotics to which the isolate was resistant and b represents the number of antibiotics to which the isolate was exposed. A MAR index value higher than 0.2 is considered as multiple antibiotic resistant.

Statistical testing

The results were subjected to a χ^2 test to determine the significance of the variations in HPC bacteria of imported and domestic bottled waters and to Pearson correlation to test the correlation between HPC bacteria and presence of coliforms.

RESULTS AND DISCUSSION

The bottled mineral water samples analysed in the present investigations were classified according to the HPC load (Table 1). All the samples belonging to the imported brand and 72% of the leading domestic brand had an HPC count lower than the WHO standard of 100 cfu ml^{-1} when incubated at 22°C, while only 32–36% of the samples from the other two domestic brands were within this limit. The HPC count at 37°C was slightly higher when compared with that at 22°C, probably indicating the mesophilic nature of the contaminant or the natural flora of the bottled mineral waters. The mean HPC counts of imported bottled mineral water were significantly lower ($p = 0.015$) when compared with those of the domestic brands. However, the variation in the HPC of domestic brands of bottled mineral water was not significant ($p = 0.174$).

The HPC load of the samples analysed in the present study was much lower than those reported in the bottled, uncarbonated mineral waters sold in retail outlets in Nigeria (Ogan 1992), Taiwan (Tsai & Yu 1997) and India (Radhakrishna *et al.* 2003). Our results were comparable to the bacteriological quality of imported and domestic brands of bottled water sold in Trinidad, West Indies (Bharath *et al.* 2003). However, the prevalence of faecal coliform bacteria in our samples was slightly higher, although it was restricted to one domestic brand. We observed significantly higher levels of HPC in domestic brands when compared with imported brands of mineral water, which was similar to previous observations (Bharath *et al.* 2003). The significant difference in the HPC load of imported and domestic

Table 1 | Classification of bottled mineral water samples according to the heterotrophic plate count (HPC) bacteria

Brand	No. of samples analysed	% of samples with HPC < 100 cfu ml^{-1}		% of samples with HPC 100–1,000 cfu ml^{-1}		% of samples with HPC > 1,000 cfu ml^{-1}		Mean HPC (cfu ml^{-1})	
		22°C	37°C	22°C	37°C	22°C	37°C	22°C	37°C
Domestic 1	25	72	80	6	20	1	0	109.64	137.95
Domestic 2	25	36	22.73	11	63.64	5	13.64	512.28	526.00
Domestic 3	25	32	32	9	36	8	32	704.84	826.04
Imported	25	100	86.36	0	13.64	0	0	23.8	49.77

bottled mineral water could be a result of the stringent quality standards set for bottled water destined for export. Such dual standards are seen mostly in developing countries where manufacturers exploit the lack of awareness of consumers. Consistently low HPC levels (< 100 cfu ml^{-1}) observed in the case of a leading domestic brand of bottled mineral water, marketed internationally, further strengthen the observation that dual standards exist in same country. Hence it is recommended that equally stringent standards must be applied to commodities that are meant for domestic consumption, especially when they are ready to eat/consumed without any further processing. The HPC bacterial loads we encountered in our samples were also comparable to those in bottled drinking water sold on the streets of Kumasi, Ghana (Obiri-Danso *et al.* 2003). However, the same researchers have observed a high level of HPC in factory-bagged sachet water and in hand-filled, hand-tied, bagged water from the same location.

Table 2 represents the HPC count of those bottled mineral water samples that tested positive for faecal coliforms. There was a strong correlation between high heterotrophic plate counts and positive faecal coliform detection. All those samples that had tested positive for faecal coliforms had an HPC count higher than the prescribed limit and ranged between 5.9×10^2 and 4.35×10^3 cfu ml^{-1}. Although there is dispute (Hatha *et al.* 1998) about the correlation between the high total aerobic plate count and faecal coliforms in food samples, there was found to be strong correlation in the case of bottled mineral waters (Jeena *et al.* 2006). FC were detected in only one of the domestic brands. Four out of the six production batches tested of this particular brand tested positive for FC, indicating poor process control during bottling. Although there was considerable variation in the HPC of the other two domestic brands of bottled mineral water, neither tested positive for FC. We analysed samples originating from five out of eight production batches to assess the variation between the batches as an indicator of process integrity. However, the variation in the HPC between different production batches was not found to be significant, indicating good process control by all three domestic bottlers of mineral water.

The bacteriological quality of mineral water is of great importance because such water is used by many consumers and is considered to be safer than tap water. However, bottled mineral water is not sterile and may contain the indigenous microflora, since such waters are not subjected to disinfection or treatments to remove them. There can be changes in the bottled water microflora arising from contamination or growth of indigenous microorganisms previously stressed, dormant or starved as a result of an altered environment. However, researchers vary in their opinion about the risk posed by the HPC bacteria in bottled mineral water/drinking waters. While the potentially pathogenic features of HPC bacteria isolated from treated and untreated drinking water are highlighted by some of researchers (Rusin *et al.* 1997; Pavlov *et al.* 2004), others (Allen *et al.* 2004; Edberg & Allen 2004) are of the opinion that such a risk is not supported by clinical evidence. Although naturally occurring HPC bacteria are reported to have low invasiveness and cytotoxicity, the opportunistic pathogens among them pose a threat to immunologically compromised individuals.

In the present study, 20 samples of the leading domestic brand were stored for one year and analysed to compare the HPC with that of fresh samples. Mean HPC of stored samples (364 cfu ml^{-1}) was slightly higher than that of fresh samples (138 cfu ml^{-1}). The findings suggest that, although

Table 2 | Heterotrophic plate count of the bottled mineral water samples that were tested positive for faecal coliforms and the percentage incidence of various genera

Sample No.	No. of FC per 100 ml	HPC at 37°C (cfu ml^{-1})	Genera of HPC bacteria	Percentage of occurrence
1	1	840	*Bacillus*	26.7
2	134	1,412	*Corynebacterium*	26.7
3	66	939	*Micrococcus*	17.8
4	33	4,356	*Kurthia*	17.8
5	73	590	*Klebsiella*	6.6
6	67	946	*Pseudomonas*	2.2
7	40	750	*Staphylococcus*	2.2

the shelf life of bottled mineral waters is 2 years, it is better to choose the fresh product. The findings were comparable to that of Sefcova (1997) who observed an elevated count of mesophilic and psychrophilic bacteria in uncarbonated bottled water stored for a period of one year. Increased oxygenation during bottling and the increased surface area provided by the bottle could be the reasons for the altered growth. Death and autolysis of some members of the original microflora during storage could also provide nutrients and support growth of other species: for example, heterotrophic enteric pathogens. This can promote the growth of some health significant organisms that may be present in very low concentrations initially but rise to harmful levels after prolonged storage. Chemical components could also leach or migrate from the container walls into the water, and may then support growth of bacteria. Biofilm formation in bottles of water after prolonged storage also plays an important role in the survival and growth of bacterial flora. The viability of many heterotrophs is enhanced when they become incorporated in biofilms (Banning *et al.* 2003).

Characterisation of HPC bacteria revealed that *Bacillus* and *Corynebacterium* were the predominant genera followed by *Micrococcus* and *Kurthia* (Table 2). High prevalence of Gram-positive bacteria in non-carbonated natural mineral waters has been reported (Armstrong *et al.* 1981; Massa *et al.* 1995) although a number of researchers reported Gram-negative bacteria as the predominant genera. *Pseudomonas* and *Staphylococcus* were also detected in the mineral water samples analysed during this study. Many researchers have reported the prevalence of *Pseudomonas*, more specifically *Pseudomonas aeruginosa*, in bottled mineral waters. The presence of *Pseudomonas* in bottled mineral water is considered important as this genus is oligocarbotolerant and can therefore multiply in mineral water of extremely low nutrient level after adaptation. *P. aeruginosa* is used as an indicator of potentially pathogenic bacteria which are also able to grow in low nutrient conditions normally prevalent in drinking water (Hambsch *et al.* 2004). However, the oral infectious dose for *P. aeruginosa* is of the order of 10^8 to 10^9 cells, which is very unlikely to be reached in mineral water samples. *Staphylococcus* and *Pseudomonas* are considered as important causative agents of nosocomial infections and the ability of these opportunistic pathogens to cause infections in immunocompromised individuals cannot be ruled out. Another important genus encountered was *Klebsiella*, which is considered a secondary pathogen. *Klebsiella* was isolated from the samples that were positive for faecal coliforms. Contamination in the water samples could have originated from the source, from the equipment that is used to pump water from the source or during bottling. Exposure of water to air and contact with workers during bottling are also potential sources of contamination.

Thirty-three isolates of HPC bacteria selected randomly from the bottled mineral water samples were subjected to drug sensitivity testing. Nearly 50% of the isolates were resistant to gentamycin and streptomycin (Figure 1). Overall drug resistance levels were low when compared with our previous observations in pathogenic bacteria such as *Salmonella*, *Aeromonas* and *Escherichia coli* isolates from various environmental samples (Hatha *et al.* 1999, 2005). The lowest resistance was to chloramphenicol and ciprofloxacin. Resistance to nalidixic acid was relatively high. In our previous studies (Ruiz *et al.* 1999) on

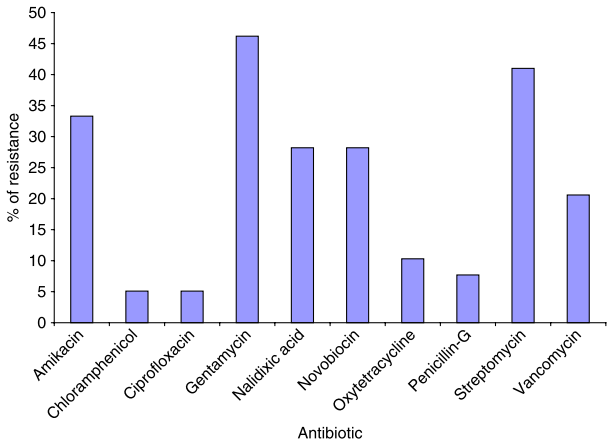

Figure 1 | Percentage of drug resistance among the heterotrophic plate count (HPC) bacteria from bottled mineral water.

Salmonella typhimurium resistance to nalidixic acid and chloramphenicol, we found several resistance mechanisms including a single mutation in the gyrA gene (conferring nalidixic acid resistance), and production of chloramphenicol acetyl transferase. Resistance to nalidixic acid and chloramphenicol was lower than that encountered among the HPC bacteria from non-carbonated mineral waters sold in Italy (Massa *et al.* 1995). However, resistance to gentamycin and streptomycin was higher when compared with the findings of the above researchers. The differences in resistance level to antibiotics are expected and may be attributed to variations in the selection pressures at different geographical locations. In general, antibiotic usage in Fiji is restricted mostly to humans as animal husbandry practices are relatively low except for a major poultry farm operation. Pressure on natural resources and resultant contamination is also less of a problem because of the low population density in Fiji.

Multiple antibiotic resistance (MAR) index, resistance patterns and beta haemolytic activity of the HPC isolates are presented in Table 3. MAR index varied between 0 and 0.5 and 22 different drug resistance patterns were observed. The isolates with a MAR index greater than 0.2 were considered as multi drug resistant. The prevalence of MAR bacteria (39%) was lower than that encountered among the HPC bacteria from non-carbonated mineral waters (Massa *et al.* 1995). Our findings were comparable to the observations of Armstrong *et al.* (1981) among HPC bacteria from treated drinking water, although not from mineral water which was not subjected to any kind of treatment. It is also argued that the water treatment procedures such as disinfection and purification and the distribution of water result in an increased selection of MAR bacteria in drinking water. From this perspective, bottling of mineral water at

Table 3 | MAR index, resistance pattern and beta haemolytic activity of the heterotrophic plate count (HPC) bacteria from bottled mineral water

Culture no.	MAR index	Resistance pattern*	Beta haemolytic activity
1	0.0	–	−
2	0.1	S	+
3	0.1	S	+
4	0.1	S	+
5	0.4	AkPSVa	−
6	0.2	AkVa	−
7	0.3	NvPVa	−
8	0.3	CnPVa	+
9	0.2	NaNv	+
10	0.3	NaSVa	+
11	0.0	–	−
12	0.3	CNaNv	+
13	0.1	Na	−
14	0.5	CCnNaNvS	+
15	0.1	Na	−
16	0.2	NvS	+
17	0.2	CnS	+
18	0.1	Cn	+
19	0.5	AkCnNvSVa	−
20	0.2	CnS	+
21	0.5	AkCnNaNvS	+
22	0.3	CnSVa	+
23	0.3	CnNvS	+
24	0.3	AkCipS	−
25	0.1	Cn	−
26	0.2	AkCn	+
27	0.2	AkCn	+
28	0.1	Ak	+
29	0.1	Ak	+
30	0.1	Cip	−
31	0.2	AkCn	+
32	0.0	–	−
33	0.2	CnOt	+

*Ak, amikacin; C, chloramphenicol; Cip, ciprofloxacin; Cn, gentamycin; Na, nalidixic acid; Nv, novobiocin; P, penicillin G; Ot, oxytetracycline; S, streptomycin; Va, vancomycin.

source is an advantage, provided it is carried out without any secondary contamination. The rise of bacterial resistance to drugs is a superb example of biological evolution since prokaryotes exhibit a diverse and multi-dimensional nutritional and physiological range, helping them to occupy almost all the possible niches on Earth.

Beta haemolytic activity (Table 3) among the HPC bacteria from bottled mineral water was found to be high (63%). The high percentage of positive beta-haemolytic activity detected among the HPC bacteria in the different bottled water sources indicate that the strains of bacteria pose a significant threat to individuals in terms of cellular aerobic processes since haemolysis bursts the red blood cells and releases haemoglobin. Once this happens, the blood oxygen concentration decreases, and the individual's overall cellular activity decreases because of the unavailability of oxygen within cells.

Excessive HPC bacteria can be an indicator of the level of general bacterial contamination in bottled water. However, high numbers of HPC bacteria exceeding specified limits tends not to be perceived as a serious violation of water quality specifications and is widely accepted. There is growing concern because of the accumulation of evidence suggesting that HPCs may indicate the presence of organisms of serious health significance. The results of the present study show that, despite some variations in quality among the domestic producers, overall bacteriological quality of imported and domestic bottled mineral water marketed in Fiji is relatively good when compared with reports from other parts of the world. However, enforcement of stringent quality standards by government authorities is necessary to ensure that consumers receive a continuing safe supply of bottled water.

CONCLUSIONS

The study revealed a relatively good quality of bottled mineral water available in Fiji. While all the imported brands had superior bacteriological quality, there was significant variation in the quality of the product, especially in terms of presence of FC, produced by various domestic bottlers. It is highly recommended that the Fiji government introduces a system of random audits of bottling processes and final products of the various producers of bottled waters in Fiji. Labelling of the bottling source should also be made mandatory for all the bottlers. Risk assessment studies revealed that the HPC bacteria from this product could pose a health hazard, especially to immunocompromised individuals. Since bottled waters with a high organic load could facilitate the development of biofilms on the walls of the containers during extended storage, survival kinetics of indicator and pathogenic bacteria in these biofilms may be studied in detail to fully assess the risk associated with this product.

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