Bacterial diversity of domestic and imported mineral bottled water in Syria

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ABSTRACT

A total of 370 bottles of noncarbonated natural mineral water (domestic and imported) with different bottling dates, were collected directly from the markets in Syria one word one year 2006. All natural waters collected were cultured on R2A medium and incubated at 22 C° for 5–7 days. Viable cultivable bacteria were counted. Bacterial diversity was approached with tentative identification of the strains isolated using biochemical and enzymatic criteria (with the aid of API 20 NE identification system for nonfermenters).

The highest bacterial densities were observed among imported waters and oldest bottles of both sources (i.e. domestic and imported waters). Obviously, increases in bacterial numbers showed to be positively related to age of bottle since 53.33% of the samples of >180 d of bottling showed as high as 10^4 cfu ml^{-1}. Of the193 strains isolated, 154 (79.79%) were classified to the genus/species level. These were predominated by gram negative bacilli represented by *Pseudomonas* and pseudomonas-like bacteria with an overall percentage of 57.82 % of Gram-negatives and 44.04% of all isolates. Differences were observed in the pattern of species distribution between the two sources of the studied water.

The high number reported of heterotrophic bacteria raises the question as to whether these waters are hygienically produced and conditions where they stored and consequently the real or valid expiry date. The predominance of *Pseudomonas* and pseudomonas-like bacteria may has potential health risks and should be considered properly.

Keywords: Bottled mineral water, Domestic, Imported, HPC, Bacteria, Diversity.

التنوع البكتيري في المياه المعدنية المعبأة المحلية والمستوردة في سورية

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الملخص

جمعت 370 عبوة مياه معدنية طبيعية غير مكربنة (محلية ومستوردة) ذات تواريخ تعبئة مختلفة من الأسواق السورية مباشرةً خلال العام 2006.

زرعت جميع عينات المياه المعدنية الطبيعية على وسط R2A وحضنت عند الدرجـة $22\ C^{\circ}$ مـدة 5-7 أيام. تم تعداد البكتيريا الحية في المزارع وتحديد التنوع الجرثومي من خلال المطابقة البيوكيميائية والإنزيمية للسلالات المعزولة (بمـساعدة نظـام المطابقـة 20 NE identification system لغيـر المخمرات).

لوحظت الكثافات البكتيرية الأعلى في المياه المستوردة وفي العبوات الأقـدم مـن كـلا المـصدرين (المحلية والمستوردة).

أظهرت الزيادات في الأعداد البكتيرية علاقة إيجابية مع عمر العبوة, إذ إن 53.33% مـن العينـات التي مضى على تعبئتها أكثر من 180 يوم أعطت أعداداً بحدود 10^4 وحدة مـستعمراتية فـي المليليتـر الواحد.

من أصل 193 سلالة تم عزلها, صنفت 154 سلالة (أي 79.79%) إلـى مـستوى جـنس/نـوع , سيطرت عليها العصيات سالبات غرام ممثلةً بالزوائف والبكتيريا الشبيهة بـالزوائف مـع نـسبة عامـة مقدارها 57.82% من سالبات غرام ونسبة 44.04% من مجموع العزلات. لوحظت اختلافات في تـوزع الأنواع في كلا المصدرين للمياه المدروسة. يطرح العدد المرتفع من غيريات التغذية استفهاماً هـل هـذه المياه تُنتج في ظروف صحية؟ وما الظروف التي تخزن بها؟ ومن ثم يجب تحديد الزمن الحقيقي لانتهـاء صلاحيتها. إن سيطرة الزوائف والبكتيريا الشبيهة بالزوائف يمكن أن يشكل خطراً صحياً كامناً يجب أخذه بالحسبان.

الكلمات المفتاحية: مياه معدنية معبأة, محلية, مستوردة, تعداد غيريات التغذية HPC, بكتريا, تنوع.

Introduction

Bottled water is considered drinking-water under some regulatory schemes. It may derive from "pristine" sources (natural mineral water) or from processed waters. They may contain or have added carbon dioxide that will restrict growth potential, but typically no long-lasting disinfectant residual is present. Bottled waters could represent a specific growth situation for microbial flora. (WHO 2003).

Microbiologically, natural mineral water is distinguishable from ordinary drinking water by its native microflora, i.e. contains the indigenous microbial flora present at the source (Leclerc and Costa 1998) and constancy of composition concerning certain mineral salts and trace elements. With some exceptions, it is prohibited to subject natural mineral water to any treatment. For this reason, natural or drilled underground sources of natural mineral water must be protected from pollution to guarantee the original microbiological purity and the chemical composition of essential components of the mineral water. Because natural mineral water is an oligotrophic environment (0.1 µg ml^{-1} carbon), its count of viable bacteria is very low, i.e. as low as 10 colony forming unit (cfu) ml^{-1} (Ferreira *et al.*, 1994). However, after bottling, this population reaches 10^3-10^5 bacteria $ml^{-}1$ in few days (2-7d) with occasionally, a maximum of 10^7 bacteria $ml^{-}1$ (Warburton *et al*., 1992).

This natural bacterial community appears to be highly preserved throughout the bottling process (Vachée *et al*., 1997a). Buttiaux and Boudier 1960 were the first to show that within one week after bottling and storage at ambient temperatures the natural microbial flora of the water starts to multiply and gives rise to an increase in cfu up to 10^4 to 10^5 ml^{-1}. Various research groups confirmed this bacterial growth phenomenon by quantifying the bacteria present in natural mineral waters at the source and at several points in time after bottling and storage at different temperatures (Warburton *et al*., 986, Gonzalez *et al*., 1987, Bischofberger *et al*., 1990, Morais and da Costa 1990, Ferreira *et al*., 1994).

Regarding the bacterial community composition of bottled natural mineral water, and having that this water is characterized by an autochthonous microbial flora (Hunter1993), most available information resulted mainly from cultivation-based methods (Gonzalez *et al*., 1987, Guillot and Leclerc 1993, Vachée *et al*., 1997a) although deficiency of such methods in reflecting the true and whole bacterial community and diversity (Alexander *et al*., 2005). Collectively, cultivation-based studies indicate that this community is dominated by aerobic heterotrophs of Gamma class of *Proteobacteria* followed by *Alpha* and *Beta* classes.

The aim of the present study is to determine the composition of the bacterial community in bottled mineral waters that has being consumed in Syria as well as to recover the potential effect of time of bottling on the bacterial content of these bottled waters.

Materials and Methods

Sample collection: A total of 370 bottles of noncarbonated natural mineral water samples (9 brands, labeled A to I) with different production (bottling) dates, were collected from the local retail markets in Syria throughought the year 2006 (from 20 January to 10 December); 125 bottles of them are produced by local companies and labeled A,B and C. The rest of the bottles, (i.e.245 bottles) were imported to the Syrian market and labeled D, E, F, G, H and I. All Natural mineral water packaged in 0.5- and 1.5-liter of bottles that made from polyethylene terephthalate (PET) were purchased directly from the retail Syrian markets. Mineral water bottles were stored at room temperature (20 to 22°C) prior to investigation. Bottles were vigorously agitated before analysis.

Bacteriological media and culture conditions:

Heterotrophic (viable) plate counts (HPC): Using R2A medium (Reasoner and Geldreich 1985), portions of samples (usually 100µl) or their 10-fold dilutions (using the same autoclaved mineral water as a dilute solution) were plated and incubated at 22° C for 5–7 days. Colonies were counted and identified.

Membrane filtration. As well as plating method, volumes up to 1L of sample were filtrated using cellulose membrane filter of 0.45 µm pores (Millipore *Ltd*) and pads saturated with R2A medium, incubation periods and temperatures were as in HPC.

For quantitative detection of *Aeromonas* spp and *Pseudomonas* spp (and by necessity *P. aeruginosa*), water samples were filtered then, filters were placed directly on culture plates of ampicillin dextrin agar (Havelaar and Vonk, 1988) and mPA agar (Hi Media *Ltd*), respectively. All plates were incubated for 48 h at 30°C.

Identification and classification. For identification purposes from R2A medium, 24 h-sub-cultures were prepared on Trypticase Soy Agar (oxoide *Ltd*). Isolates of each type of bacterial colony were tested by Gram stain, for oxidase production and glucose assimilation; further identification was out with the API 20 NE identification system for nonfermenters (Bio Mereux). All tests were carried out at 22°C.

In addition to the API 20E identification system and above tests, further tests were carried out aerobically at 30°C for identification of the typical colonies for *Aeromonas* spp and *Pseudomonas* spp.

For *Aeromonas* spp isolates, these tests were: the oxidation/fermentation (O/F), sensitivity to vibriostatic agent O/129, gas production from glucose, H_2S production from cysteine, and

esculin hydrolysis (Popoff 1984, Namdari and Bottone 1989, Carnahan *et al*., 1991) ; and the tests : oxidation/fermentation (O/F), motility, growth at 41.50° C , characteristic growth on King's A' agar, denitrification, arginine dihydrolase, and gelatin liquefaction for identification of the *Pseudomonas* spp isolates (Murray *et al*., 1999).

Results

In total, 370 samples of domestic (three brands A, B and C) and imported (6 brands D, E, F, G, H and I) noncarbonated natural mineral water in PET bottles were collected directly from the local retail markets in Syria throughought the year 2006. All samples were analyzed bacteriologically to determine their bacterial diversity and viable colony forming unit (cfu) through HPC test at 22°C using R2A culture medium. The number and ages of samples from each brand analyzed are shown in table 1.

Table 1. Number and age of samples from each brand analyzed.

Age of bottle (days)	No. of samples analyzed of each brand									Total
	A	B	C	D	E	F	G	H	I	
10-30	8	8	7	7	6	8	6	7	6	59
30-60	7	8	6	6	7	7	6	6	6	70
60-90	7	7	7	6	6	7	8	7	7	75
90-120	8	8	7	7	7	7	6	7	7	48
120-180	6	6	6	6	7	8	6	7	7	73
> 180	4	8	7	8	7	8	8	6	7	45
Total	40	45	40	40	40	45	40	40	40	370

The cell concentrations of the samples studied are shown in table 2. Overall, there was clear variety of the bacterial counts among the samples tested. Furthermore, and as shown from table 2 below, there was clear difference between domestic and imported water bottles.

Table 2. Bacterial concentrations in bottled mineral waters studied

Brand	No. of samples	Cell concentration as cfu* ml^{-1} (%)					
		0	0-10	$10-10^2$	10^2-10^3	10^3-10^4	$>10^4$
A	40	10 (25)	17 (42.5)	12 (30)	1 (2.5)	0 (0)	0 (0)
B	45	11 (24.44)	15 (33.33)	15 (33.33)	4 (8.89)	0 (0)	0 (0)
C	40	17 (42.5)	18 (45)	5 (12.5)	0 (0)	0 (0)	0 (0)
D	40	1 (2.5)	3 (7.5)	6 (15)	13 (32.5)	17 (42.5)	0 (0)
E	40	0 (0)	0 (0)	4 (10)	14 (35)	2 (5)	20 (50)
F	40	2 (5)	4 (10)	8 (20)	12 (30)	14 (35)	0 (0)
G	45	3 (6.67)	6 (13.33)	7 (15.55)	17 (37.78)	4 (8.89)	8 (17.78)
H	40	4 (10)	5 (12.5)	9 (22.5)	9 (22.5)	3 (7.5)	10 (25)
I	40	0 (0)	2 (5)	4 (10)	8 (20)	10 (25)	16 (40)
Total	**370**	**48 (12.97)**	**70 (18.92)**	**70 (18.92)**	**78 (21.08)**	**50 (13.51)**	**54 (14.59)**

* cfu: colony forming unit.

Higher bacterial counts were reported for samples from some imported brands. The cell concentrations of these samples are shown in table 3. Obviously, the samples from the brands E, G, H and I have showed increased bacterial counts. The highest counts were found in water from brands E and I; so, 6.34% of their samples contained as high as 10^8 ml^{-1} (See table 3).

Table 3. High bacterial concentrations recorded in samples from some imported brands.

Brand	No. of samples	Cell concentration as cfu* ml^{-1} (%)				
		10^4-10^5	10^5-10^6	10^6-10^7	10^7-10^8	>10^8
E	40	3 (7.5)	2 (5)	4 (10)	4 (10)	7 (17.5)
G	45	5 (11.11)	3 (6.67)	0 (0)	0 (0)	0 (0)
H	40	3 (7.5)	4 (10)	3 (7.5)	0 (0)	0 (0)
I	40	1 (2.5)	3 (7.5)	2 (5)	4 (10)	6 (15)
Total	**205**	**12 (5.85)**	**12 (5.85)**	**10 (4.88)**	**8 (3.90)**	**13 (6.34)**

* cfu: colony forming unit

Table 4 and figure 1 show the differences observed in the bacterial content between samples of domestic and imported mineral waters. As shown in table 4, about 44.45% of samples of imported brands showed bacterial cells more than 10^3 ml^{-1} compared with only 4% of samples of domestic brands having counts ranging between 10^2-10^3 ml^{-1}.

Table 4. Bacterial counts in domestic mineral waters compared with imported ones.

Mineral water	No. of samples	Cell concentration as cfu* ml^{-1} (%)					
		0	**0-10**	**10-10^2**	**10^2-10^3**	**10^3-10^4**	**> 10^4**
Domestic	125	38 (30.4)	50 (40)	32 (25.6)	5 (4)	0 (0)	0 (0)
Imported	245	10 (4.08)	20 (8.16)	38 (15.51)	73 (29.79)	50 (20.41)	54 (22.04)

* cfu: colony forming unit

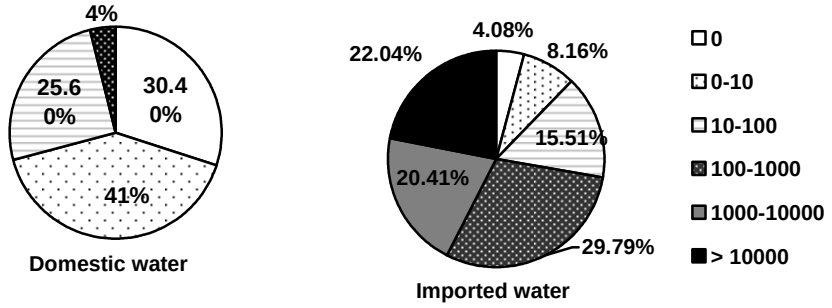

Fig 1. Percent distribution of samples of domestic and imported water according to their contents of heterotrophic bacteria (as cfu ml^{-1}).

Table 5 shows the composition of bacterial community found in the samples tested from domestic and imported mineral water. These genera and species have been resulted from tentative identification process of colonies obtained mainly by HPC test and using the API 20 NE identification system for nonfermenters.

Table 5. Groups of bacteria isolated and species identified from bottled mineral water studied.

	Genus /Species	Mineral water		
		Domestic	**Imported**	**Total**
Gram negatives	*Pseudomonas* spp	10 (9.61)	13 (14.61)	23 (11.92)
	Psd.aerogenosa	4	8	12
	Psd.fluorescence	2	2	4
	Psd.putida	1	1	2
	Burkholderia spp	9 (8.65)	12 (13.48)	21 (10.88)
	Burkh.cepacia	3	8	11
	Aeromonas spp	8 (7.69)	10 (11.23)	18 (9.33)
	Aer.hydrophila	2	4	6
	Aer.caviae	2	2	4
	Alcaligenes spp	6 (5.77)	9 (10.11)	15 (7.77)
	Alc.faecalis	3	5	8
	Acinetobacter spp	5 (4.81)	5 (5.62)	10 (5.18)
	Comamonas spp	2 (1.92)	6 (6.74)	8 (4.14)
	Com.acidovorans	2	4	6
	Acidovorax spp	7 (6.73)	0 (0)	7 (3.63)
	Sphingomonas spp	6 (5.77)	0 (0)	6 (3.11)
	Sph.paucimobilis	2	0	2
	Serratia spp	0 (0)	5 (5.62)	5 (2.59)
	Ser.fonticola	0	4	4
	Ralstonia spp	4 (3.84)	0 (0)	4 (2.07)
	Cytophaga spp	4 (3.84)	0 (0)	4 (2.07)
	Moraxella spp	3 (2.88)	0 (0)	3 (1.55)
	Flavobacterium spp	1 (0.96)	0 (0)	1 (0.52)
	Flav.aquatile	1	0	1
	Brevundimonas spp	1 (0.96)	0 (0)	1 (0.52)
	Brev.vesicularis	1	0	1
	Non-identified	**12 (11.54)**	**9 (10.11)**	**21 (10.88)**
Gram positives	*Corynebacterium* spp	8 (7.69)	4 (4.49)	12 (6.22)
	Micrococcus spp	6 (5.77)	4 (4.49)	10 (5.18)
	Micr.luteus	6	4	10
	Arthrobacter spp	7 (6.73)	2 (2.25)	9 (4.66)
	Staphylococcus spp	0 (0)	5 (5.62)	5 (2.59)
	Staph.epidermidis		5	5
	Bacillus spp	0 (0)	1 (1.12)	1 (0.52)
	Non-identified	**5 (4.81)**	**4 (4.49)**	**9 (4.66)**
Total		**104 (53.88)**	**89 (46.11)**	**193**

Figure 2 and figure 3 show bacterial composition in samples from domestic and imported bottled mineral waters, respectively. Obviously, the domestic water has more bacterial diversity compared with imported one.

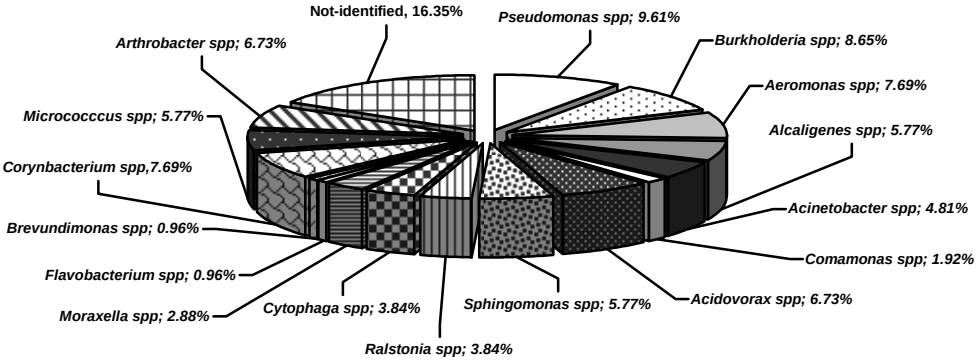

fig 2 . Bacterial composition of domestic bottled mineral water.

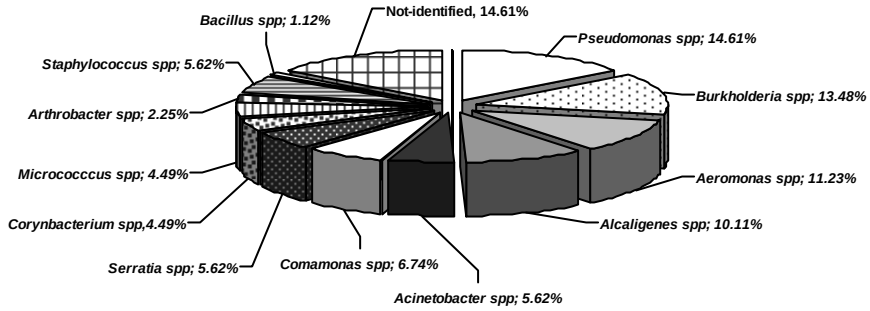

fig 3 . Bacterial composition of imported bottled mineral water.

In order to investigate the relationships expected between age of the tested bottles and their contents of bacteria, the resulted bacterial cell concentrations of tested samples were categorized according to

age of bottle measured by days (See table 6 and figure 4). Until 90-120 days of bottling the increase of bacterial content of samples was obvious. So, water samples of 10-120 days of bottling have showed bacterial concentrations ranged between 0-10^3 cfu ml^{-1}. However, with some exceptions, all samples with more than 120 days of bottling have showed bacterial numbers more than 10^2 cfu ml^{-1}. Furthermore, and as shown from table 6, 42.37% of these samples have had as high as 10^4 cfu ml^{-1}.

able 6. Concentrations of bacteria in mineral waters according to bottle ages.

Age of bottle (days)	No. of samples	Cell concentration (cfu* ml^{-1})					
		0	**0-10**	**10-10^2**	**10^2-10^3**	**10^3-10^4**	**> 10^4**
10-30	59	15 (25.42)	28 (47.46)	11 (18.64)	5 (8.47)	0 (0)	0 (0)
30-60	70	18 (25.71)	20 (28.57)	23 (32.86)	9 (12.86)	0 (0)	0 (0)
60-90	75	11 (14.67)	17 (22.67)	22 (29.33)	22 (29.33)	3 (4)	0 (0)
90-120	48	4 (8.33)	5 (10.42)	9 (18.75)	17 (35.42)	9 (18.75)	4 (8.33)
120-180	73	0 (0)	0 (0)	5 (6.85)	20 (27.40)	22 (30.14)	26 (35.62)
> 180	45	0 (0)	0 (0)	0 (0)	5 (11.11)	16 (35.56)	24 (53.33)
Total	370	**48 (12.97)**	**70 (18.92)**	**70 (18.92)**	**78 (21.08)**	**50 (13.51)**	**54 (14.59)**

* cfu: colony forming unite

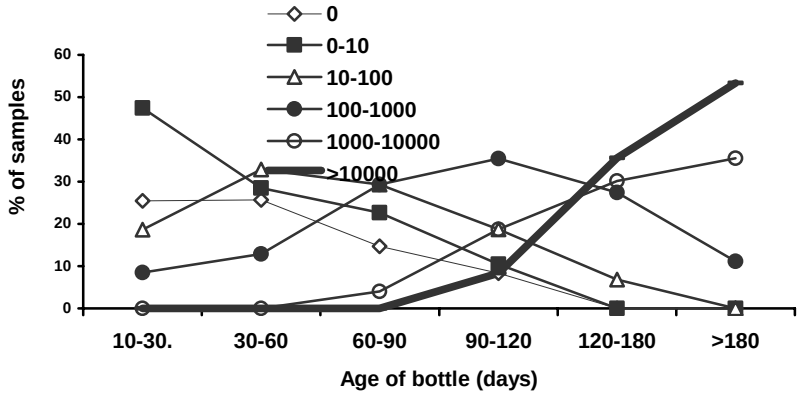

Fig 4 . Percent distribution of bottled minearal water samples (by their HPC as cfu/ml) according to bottle age (by days).

Discussion

Although disadvantages that limit cultivation methods in drinking water analysis like limited count obtained (approximately 1% of the viable bacteria are not cultivable) and the long time of incubation required (5–7 days at 22°C), the use of adequate media particularly those with low nutrient levels (e.g., R2A), which are better suited to the needs of water microflora, increase the recoverable populations of waterborne microorganisms that can be determined by the cultivation method and consequently enhance the productivity of such methods (Reasoner and Geldreich 1985). In the same context, and although controversial differences in definition of medium used and conditions of incubation, as well as results interpretation from one country to another (WHO 2003), cultivation methods are still required either because the sanitary significance of viable counts (or colony count) of waterborne bacteria (Nichols et al., 1995) or because these colony counts are still encountered as mandatory values for the colony counts of water by several countries guidelines (WHO 2003).

From a quantities viewpoint, it is important to emphasize that bacterial counts yielded by membrane filtration technique were higher as compared with that of spread or plating method using R2A medium. However, plating method had showed remarkable efficiency to support the development the pigmented colonies of heterotrophic bacteria. Hence, the spreading plates had mostly been selected to isolate our strains for study and identification.

Actually, the majority of our isolated strains was pigmented bacteria and predominated by yellow or carotene- like pigmented colonies followed by orange and red pigmented ones respectively. The pigmented colonies were more common in domestic water.

There appeared to be an inverse relationship between the production of pigments by bacteria originated from mineral water of bottle and bacterial density or contamination of this water, i.e., when the diversity of pigmented forms was high, the density of bacteria or plate count was low, and vice versa. So, water samples with counts of 102 ml-1 or higher appeared to be free of pigmented colonies.

Having that the legislations of mineral water may differ from one country to another, the European regulations represented by European Community (EC) Directive of 1980 for natural mineral water allow with 100 cfu ml-1 after incubating at 20-22°C for 72h as upper acceptable limit of HPC at spring or 12h of bottling (European Community 1980).

By taking this limit into account, and although our study is not dedicated to assess the quality of the studied mineral water upon microbiological criteria and related standards, we can verify that the majority of samples from imported mineral water, (i.e.,72.24 % of samples) can be classified as rejected or unacceptable waters for drinking purpose. By contrast, samples from domestic mineral water showed to fulfill the requirements of that directive, since 96% of the samples contained less or equal to100 cfu ml-1. (See table 4).

Worldwide, high viable counts of planktonic bacteria in bottled mineral waters have been reported. For instance, in one study of three leading commercial brands of European mineral water, the counts ranged from 1.8×10^4 cfu ml^{-1} to 1.2×10^5 cfu ml^{-1}. (Jones *et al.,* 1999); similar results had been found by other previous separated studies (Warburton et al., 1986, Hunter et al., 1990, Ferreira et al., 1994). This is in agreement with the results of our finding although the highest bacterial densities were observed among imported waters. So, the overall mean HPC count in imported bottled water was $8.3 \times 10^3 \pm 2.1 \times 10^4$ cfu ml^{-1}, as compared with $9.2 \times 10^1 \pm 1.1 \times 10^2$ cfu ml^{-1} for domestic bottles. Furthermore, some samples showed as high as 10^9 cfu ml^{-1} (See table 3).

It's clear that there was dramatic increase of bacterial density as age of bottle increased. So the significant increases in the number of bacterial population appeared with more aged bottles of water since 53.33% of the samples of >180 d of bottling showed as high as 10^4 cfu ml^{-1} (See table 6 and figure 4).

However, the initial elevation in bacterial numbers had occurred with more fresh bottles (i.e. 10-30 d of bottling).

Partially, this result is in agreement with published observations about elevating in the bacterial number after short periods of bottling (Leclerc and Moreau 2002). In quantitative studies of bacterial populations in mineral water after bottling, the bacterial number has reached up10^4–10^5 cfu ml^{-1} after one week of bottling (Buttiaux and Boudier 1960), to 10^5–10^6 cfu ml^{-1} after three days of storage (Gonzalez *et al.,* 1987) and to 10^4–10^5 within 3-7 days of bottling (Leclerc and Costa 1998).

Several attempts were made to explain this phenomenon, many studies have suggested that the autochthonous bacteria compared with allochthonous ones are well adapted to the oligotrophic conditions of bottled water; so their numbers rapidly increase after bottling (Schmidt-Lorenz 1976, Leclerc *et al.,* 1985). Other suggested reasons include oxygenation of the water during the process, the increased

surface area from the bottle and the increase in temperature during storage (Leclerc and Moreau 2002).

The low amount of dissolved organic carbon in mineral water after bottling has been suggested to be the factor that might prompt the growth of bacteria in the bottled mineral water (Leclerc and Moreau 2002).

Regarding the composition of bacterial community in water studied and identification of grown isolates and strains up to species level, difficulties have been raised because little information available about taxonomy of such species although the majority of them are oxidative gram negative bacilli. Hence, the first step in the tentative identification process was Gram stain. The API 20 NE identification system was mainly used for the identification of oxidative non-fermenter isolates. With some exceptions, this system hasn't given satisfactory results and there always have been some failure or non-interpretable results. As a result, out of 147 gram-negative isolates, the system gave only 61 (i.e.41.50%) well identified strains to species level. However, this rate decreased for Gram-positives since of 46 gram-positive isolates, 15 (i.e.32.61%) were identified as species.

As shown in table 5, out of the 193 strains isolated, 163 (84.45%) were classified to the genus/species level as well as 30 (i.e.15.54%) non-identified isolates. The identified isolates can be divided into two categories, gram-negatives represented by 126 strains distributed on members of 14 genera and gram-positives consisted of 37 species and 5 genera. As shown from that table, there is clear predominance of gram-negatives. This is in full agreement with data reported by many authors (Morais and da Costa 1990, Mavridou 1992, Ferreira et al., 1994).

Overall, the fourteenth well identified gram-negative genera are ordered according to their decreased frequency of isolation as follows: Pseudomonas, 23 strains (15.65%); Burkholderia, 21 strains (14.28%); Aeromonas, 18 strains (12.24%); Alcaligenes, 15 strains (10.20%); Acinetobacter, 10 strains (6.80%); Comamonas, 8 strains (5.44%); Acidovorax, 7 strains (4.76%); Sphingomonas, 6 strains (4.08%); Serratia, 5 strains (3.40%); Ralstonia, 4 strains (2.72%); Cytophaga, 4 strains (2.72%); Moraxella, 3 strains (2.04%); Flavobacterium, 1 strain (0.68%); and Brevundimonas, 1 strain (0.68%); as well as 21 (14.28%) non-identified isolates.

The gram-positive strains were included in the genera: Corynebacterium, 12 strains (26.09%); Micrococcus, 10 strains (21.74%); Arthrobacter, 9 strains (19.56%); Staphylococcus, 5 strains

(10.87%); and Bacillus, 1 strain (2.17.3%); as well as 9 (19.56%) non-identified isolates.

Differences in the pattern of species distribution and diversity were observed between the two sources of the studied water. As shown in table 5, figure 2 and figure 3, the domestic water has revealed more bacterial diversity among Gram-negatives. Of the fourteen genera, thirteen were recovered from domestic against only seven genera from imported mineral water. However, as indicated above, the imported water has mostly given the highest HPC compared to domestic water. It is important to denote that these elevated HPC numbers have mainly arisen from pseudomonas like bacteria.

Available data of bacterial structure and diversity for natural mineral waters obtained by cultivation- based studies suggest that the most frequently isolated microorganisms from bottled mineral water were aerobic heterotrophs belonging mainly to the Gamma but also to the Alpha and Beta subclasses of Proteobacteria as well as members of the genera Cytophaga–Flavobacterium– Bacteroides (Leclerc and Moreau 2002, WHO 2003).

According to Leclerc and Costa (Leclerc and Costa 1998), the Gram-negative genera most isolated from natural mineral waters belong to Pseudomonas, Acinetobacter and Alcaligenes; in addition to les common genera such as Caulobacter, Spherotilus, Leptothrix, Xanthomonas, Vibrio and Aeromonas. However, other studies have revealed the isolation of other less important species belonging to genera: Comamonas, Burkholderia, Ralstonia, Stenotrophomonas, Sphingomonas, Acidovorax, Brevundimonas and Paucimonas. (Schwaller and Schmidt-Lorenz 1980, Bischofberger et al., 1990, Manaia et al., 1990, Vachée et al., 1997a).

Our results revealed the predominance of Pseudomonas and pseudomonas like bacteria in both sources of mineral water studied. Therefore, strains of Pseudomonas spp, Acidovorax spp, Alcaligenes spp, Brevundimonas spp, Burkholderia spp, Comamonas spp, Sphingomonas spp, and Ralstonia spp have been frequently isolated with overall percentage of 57.82 % of Gram-negatives and 44.04% of all isolates. It is noteworthy that in 1993, Guillot and Leclerc have reported in one study that 40% of isolates originated from mineral water were Pseudomonas species (Guillot and Leclerc 1993).

Having the predominance of Psd.aerogenosa in our study, two other species of pseudomonas have been mainly identified Psd.fluorescence and Psd.putida.

According to EC Directive, mineral bottled waters must be free of Pseudomonas aeruginosa in any 250-ml sample (European Community 1980). The presence of Psd. aeruginosa is unacceptable because this species is an opportunistic pathogen, has been implicated in foodborne and waterborne diseases, and is now considered a primary pathogenic (Warburton 1993).

Some pathogenic like allochthonous bacterium Psd.aeruginosa can be adapted metabolically and survive for long time in the mineral water although their poor status of nutrients (Legnani *et al.,* 1999). Furthermore, Psd.aeruginosa is also capable of multiplying abundantly in low-nutrient water (Gonza´lez *et al.,* 1987, Moreira *et al.,* 1994) and can, therefore, colonize bottled waters.

The species Psd.fluorescens together with Psd.putida have been frequently reported in other studies of mineral water (Morais and da Costa 1990, Morais *et al.* 1997, Vachée *et al.* 1997b, Defives *et al.* 1999).

In our study, and although the isolation of Alcaligenes spp from mineral water has been reported (Gonzalez et al., 1987, Manaia et al., 1990, Guillot and Leclerc 1993), the worthwhile related point was the high frequency of occurrence of the species Alc.faecalis. So, of fifteen strains of Alcaligenes spp isolated from domestic and imported water, eight were identified as Alc.faecalis.

Two strains of Sphingomonas spp that only isolated from domestic mineral water were identified as Sph.paucimobilis. Environmentally, Sphingomonas spp have been found in sea water (Schut *et al.,* 1997) and as ultramicrocells in other aquatic environments (Eguchi *et al.,* 1996) and as autochthonous flora of natural mineral water (Bischofberger *et al.,* 1990); the species Sph.paucimobilis has been isolated from mineral water (Guyard *et al.,* 1999).

Most strains of Comamonas spp isolated from two sources of mineral water were identified as Com.acidovorans. The species Com.acidovorans has been frequently isolated from mineral water (Schwaller and Schmidt-Lorenz 1980, Gonzalez *et al.,* 1987, Manaia *et al.,* 1990, Guillot and Leclerc 1993, Vachée *et al.,* 1997a).

The seven strains of Acidovorax spp isolated from domestic mineral water were identified to genus level only. However, Acidovorax spp have been isolated from mineral water (Schwaller and Schmidt-Lorenz 1981, Zheng and Kellog 1994).

The only one strain of Brevundimonas isolated from domestic mineral water was identified as Brev. vesicularis. This specie has been

frequently isolated from mineral water (Guillot and Leclerc 1993, Vachée *et al.,* 1997a, Defives *et al.,* 1999); as well as, but and to less extent, the species Brev.diminuta (Guyard *et al.,* 1999) and as Psd. diminuta (Edberg *et al.,* 1997).

Similarly, strains of Acinetobacter spp seemed to be distributed in both two sources of mineral water with overall percentage of 6.80%.

The Acinetobacter spp are opportunistic pathogens that may cause urinary tract infections, pneumonia, bacteraemia, secondary meningitis and wound infections. However, Acinetobacter spp. are ubiquitous inhabitants of soil, water and sewage environments (WHO 2004) and have been isolated from mineral water (Leclerc 1994).

Relatively, there was considerable occurrence of Aeromonas spp since eighteen strains recovered from the two sources of water were identified as Aeromonas. Of these, six strains were as Aer.hydrophila and four as Aer.caviae.

While Aeromonas spp are suspected causative agents of wound and gastrointestinal infections, they are widespread in surface waters and may contaminate bagged and mineral waters due to poor hygienic practices of producers. Therefore, as with Psd.aeruginosa, their presence in these situations is indicative of induced faecal pollution and hence, its significance as quality indicators (Coroler *et al.,* 1996, Leclerc and Moreau 2002). However, Aeromonas spp have been isolated frequently from mineral water (Schwaller and Schmidt-Lorenz 1981, Zheng and Kellog 1994).

As attempts to understand the frequent occurrence of certain species of bacteria such as Pseudomonas spp and Aeromonas spp in mineral water, studies showed that the levels of organic carbon needed by many of them are as little as mineral water contains. For examples, it has been found that level needed of organic carbon for growth of Aeromonas hydrophila is10μgl^{-1}, and 25μgl^{-1} for Psd.aeruginosa (Van der Kooij *et al.,* 1980, 1982).

Although Flavobacterium spp have been reported as frequent isolates in mineral water (Schwaller and Schmidt-Lorenz 1981, Zheng and Kellog 1994), Flavobacterium spp occurred for one time in our domestic mineral water and was identified as Flav.aquatile.

Although Moraxella spp can cause infections of the eye and upper respiratory tract, it is considered as constituent of the autochthonous flora of bottled water (Schmidt-Lorenz 1976) and has been isolated from mineral water (Defives *et al.,* 1999). This is in agreement with

the our results, where isolation of three strains of Moraxella spp were made from domestic mineral water.

Of the five strains of Serratia spp isolated from imported mineral water, four were identified as Serratia fonticola. Serratia fonticola is psychrotrophic, purely environmental coliform that widely distributed in water and mostly originate from vegetable or small animal sources (Leclerc *et al.*, 2001).

Collectively, the above prevalence results indicate that seven genera were isolated only from domestic mineral water that are Acidovorax, Sphingomonas spp, Ralstonia spp, Cytophaga spp, Moraxella spp, Flavobacterium, and Brevundimonas spp. These against only one genus Serratia spp isolated from imported water.

Although the Gram-positive bacteria have been reported in natural mineral waters, they are not considered as constituents of autochthonous flora of mineral water in origin. Therefore, they are thought to be derived from external sources like bottling plant or directly from humans because they are partially considered as normal inhabitants of human beings. So, the species belonging to genera Bacillus, Staphylococcus and Micrococcus are rarely isolated from mineral water (Leclerc *et al.*, 1985). However, representatives of other Gram-positive bacteria like Coryebacterium, Arthrobacter, and Calvibacter have been reported with significant numbers (Schwaller and Schmidt-Lorenz 1980, 1981, Gonzalez *et al.*, 1987, Bischofberger *et al.*, 1990, Guillot and Leclerc 1993, Zheng and Kellog 1994, Vachée *et al.*, 1997a).

In the current study, in addition to Corynebacterium spp, Arthrobacter spp, Micrococcus spp that were isolated only from domestic water, two other genera Bacillus, and Staphylococcus were isolated from imported water.

All strains of Staphylococcus were identified as Staph.epidermidis and all strains of Micrococcus as Micr.luteus while no species were identified within the other three genera Corynebacterium, Arthrobacter and Bacillus.

While (Leclerc and Costa 1998) have reported the isolation of the genera Bacillus spp, Staphylococcus spp and Micrococcus spp from mineral water, others have reported members of Arthrobacter and Corynebacterium (Schwaller and Schmidt-Lorenz 1980, Gonzalez *et al.*, 1987, Bischofberger *et al.*, 1990, Guillot and Leclerc 1993).

Finally, and from health point of view, it is important to indicate that WHO has considered the following bacterial species as causatives

of waterborne nosocomial infections: Pseudomonas aeruginosa, Aeromonas hydrophilia, Burkholderia cepacia, Stenotrophomonas maltophilia, Serratia marcescens, Flavobacterium meningosepticum, Acinetobacter calcoaceticus (WHO 2002). However, there do not appear to have been any outbreaks of infectious illness associated with high concentrations of HPC bacteria in bottled waters. (Gonzalez *et al.,* 1987).

Conclusion

Bottled mineral water, is an important additional source of drinking water with increased consumption rate, but in many instances, is subject to microbiological contamination. Contamination may be induced by unprotected source, during bottling operations and unsuitable storage conditions. Therefore, there is need to set strategies for long term microbiological monitoring of this kind of drinking water sources.

In this study, the high number reported of heterotrophic bacteria (especially in imported water) raises the question as to whether these water are hygienically produced and conditions where they stored and consequently the real or valid expiry date. The predominance of Pseudomonas and pseudomonas like bacteria may has potential health risks and should be considered properly.

Our result revealed that there might be somewhat relationship between rate of pigmented colonies and level of the contamination of bottled mineral water. Therefore, our initial observations suggest that the presence of more pigmented colonies may be considered as a primary indicator of purity of analyzed mineral water.

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