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MICROBIOLOGICAL INVESTIGATION OF BOTTLED MINERAL AND DRINKING WATERS SOLD IN ESKİŞEHİR (TURKEY) MARKETS

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ABSTRACT

The microbiological quality of bottled natural spring and mineral water, purchased at different markets in Eskişehir (Turkey) markets, was investigated. Applying the membrane filtration method, the aliquots of water samples were analyzed for the presence and enumeration of total coliforms, *Escherichia coli*, *Enterococcus* spp., *Aeromonas hydrophila*. Aerobic bacteria were counted as Heterotrophic Bacteria Count (HPC) ml^{-1} by incubation at 22 and 37 ^{o}C. While *Bacillus* species in bottled mineral water samples have also been determined, any bacteria or contamination in bottled drinking water samples have not found.

Keywords: VITEK, Eskişehir, *Bacillus*, Mineral and drinking water.

ESKİŞEHİR (TÜRKİYE) MARKETLERİNDE SATILAN ŞİŞELENMİŞ MİNERAL VE İÇME SULARININ MİKROBİYOLOJİK İNCELENMESİ

ÖZ

Eskişehir (Türkiye)'deki farklı marketlerden toplanmış olan şişelenmiş doğal ve mineral suların mikrobiyal kalitesi incelenmiştir. Membran filtrasyon tekniği uygulanarak su örneklerinin kalitesi; toplam koliform, *Escherichia coli*, *Enterococcus* spp., *Aeromonas hydrophila*'nın varlığı ve miktarı yönünden analiz edilmiştir. Aerobik bakteriler 22 ve 37 ºC'de inkübasyonu sonucunda ml'deki heterotrofik bakteri sayısı (HPC) olarak belirlenmiştir. Şişelenmiş mineral su örneklerinde *Bacillus* türleri belirlenirken, şişelenmiş içme suyu örneklerinde herhangi bir bakteri ya da kontaminasyon bulunmamıştır.

Anahtar Kelimeler: VITEK, Eskişehir, *Bacillus*, Mineral ve içme suyu.

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1. INTRODUCTION

The consumption of bottled water in Turkey has gained in popularity and the sales volume rises rapidly. Turkish people consumed about 78 L bottled water per capita in 2002 (Çelik, 2003). There was 229 domestic brands of bottled water (excluding fruit flavored bottled waters) recognized by the Turkish Ministry of Health (Güler, 2007). Several types of bottled water are produced in Turkey: natural mineral water, natural spring water, drinking water and processed drinking water (Güler, 2007). Consumers may have various reasons for purchasing bottled drinking water, such as offensive taste and odor (Venieri et al., 2006), convenience or fashion, but for many consumers, safety and potential health benefits are important considerations (Armas and Sutherland, 1999).

There have been a number of reports describing the bacterial contamination of bottled water (Bischofberger et al., 1990; Manaia et al., 1990; Guyard et al., 1999; Leclerc and Moreau, 2002; Bharath et al., 2003; Venieri et al., 2006; Zamberlan da Silva et al., 2008).

In the European Community (Directives 80/777/EEC and 98/83/EC of the European Parliament and of the Council), natural mineral water is microbiologically unaltered water and thus clearly distinguishable from ordinary drinking water. The microbiological quality of bottled waters is defined by the Turkish legislation in parallel with the European Community directives, according to which total coliforms, fecal coliforms, *Escherichia coli*, *Enterecococcus* spp., *Pseudomonas aeruginosa*, fecal *Streptococcus* should not be detectable in any 250 ml bottled water sample analyzed, while Heterotrophic Plate Count, at 22 ^{o}C, and 37 ^{o}C, should not exceed 100/ml, and 20/ml cfu, respectively (Resmi Gazete, 2004; 2005). 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 (Loy et al., 2005).

The water quality is often related to the degree of bacterial contamination. Mineral drinking water is characterized by its bacterial microflora, chemical and physical composition. The quantity of bacteria in commercial mineral water is generally dependent on the disinfecting process of natural spring water used at the factory (Nsanze et al., 1999; Zamberlan da Silva et al., 2008). Spring water contains a natural microbiota composed mainly of species of the genera *Achromobacter*, *Flavobacterium*, *Alcaligenes*, *Acinetobacter*, *Cytophaga*, *Moraxella* and *Pseudomonas* (Nsanze et al., 1999; Zamberlan da Silva et al., 2008). The number of bacteria recovered at the source is generally very low, about 10 cfu ml^{-1}, but it can evolve rapidly to high numbers during storage (Armas and Sutherland, 1999). In the absence of treatment with chlorination or ozonation, bacterial multiplication may occur for 1 to 3 weeks after bottling, and the bacterial count can reach 10^3-10^5 cfu ml^{-1}at 37 ^{o}C (Tsai and Yu, 1997; Armas and Sutherland, 1999; Guyard et al., 1999). The rapid growth of bacteria after the water is bottled may be due to oxygenation of the water during the process, the increased surface area from the bottle, the increase in temperature during storage and the trace amounts of nutrients arising from the bottle (Leclerc and Moreau, 2002; Venieri et al., 2006).

The purpose of present study was to determine the microbial quality of commonly available brands of bottled natural spring and mineral water in Turkey.

2. MATERIALS AND METHODS

2.1 Water Samples

A total of 15 different brands of both natural mineral and natural spring water in a variety of different containers (plastic and glass, clear and colored) were purchased from a variety of retailers around the Eskişehir, Turkey markets. Table 1 lists mark of water and types of containers. Samples were transported in cool (2-8 ºC) and dark conditions for analysis within 6h of purchase.

2.2 Isolation and Susceptibility to Antibiotics

Samples of water were also filtered through Millipore membrane filter (0.45 µm pore size, 47 mm diameter). The membrane was placed on the following selective media: Violet Red Bile Agar with 4-methylumbelliferyl-β-D-glucuronide (MUG) (VRBA with MUG, Oxoid), m-Enterococcus Agar (Difco), Aeromonas Isolation Agar Base (Fluka) for enumeration of *E. coli* and total coliforms, fecal coliforms, *Aeromonas hydrophila*, respectively. The plates were incubated at 37 ºC for 24-48 h. For the enumeration of heterotrophic bacteria pour plate count method was chosen, using 1 ml of water sample and mixing wilt melted Plate Count Agar (Fluka) tempered at 45 ^{o}C.

Table 1. Water types, brands, containers and pH.

Water type	**Brand**	**Container**	**Material**	**pH**
Natural Spring Water	A	Clear	Plastic	7.6
	B	Clear	Plastic	7.6
	C	Clear	Plastic	7.6
	D	Clear	Plastic	7.6
	E	Clear	Plastic	7.6
	F	Clear	Plastic	7.6
Natural Mineral Water	G (Aroma with morello)	Colored	Glass	2.9
	H (Aroma with apple)	Colored	Glass	4.2
	I	Colored	Glass	8.4
	J (Aroma with lemon)	Colored	Glass	3.3
	K	Colored	Glass	9.2
	L	Colored	Glass	6.9
	M (Aroma with lemon)	Clear	Plastic	4.0
	N	Colored	Glass	5.9
	O	Colored	Glass	5.7

Two sets of plates were prepared for all samples. One set was incubated at 22 ºC for 72 h and the other set at 37 ºC for 24 h.

For determination of the resistance of obtained bacteria from samples, 11 isolates were grown overnight in Nutrient agar (NA) plate, and plates including to NA were inoculated by 100 µl of the culture adjusted to 0.5 McFarland and sprayed to all sample on the plate surface. After inoculating, antibiotic discs were placed to these plates and incubated at 37 ºC for 48 hours. After incubation, susceptibility to antibiotics was determinate with measuring to inhibition zones and considered according to HiMedia and Oxoid instruction sheet (Table 2).

2.3 Identification

Cell morphology, endospore properties, gram staining reaction, oxidase and catalase tests of these isolates was determined with basic microbiological methods (Table 3). After the determination to cellular properties, the isolated microorganisms were identified, according to laboratory manager Vitek manual (2004), depending on their biochemical characteristics using standardized identification system VITEK (BioMérieux, Basingstoke, UK) (Table 4). Results of VITEK system were exported for analysis in .NTS files and imported into NTSYSpc 2.1. Clustering analysis was performed by the unweighted pair group method with arithmetic averages (UPGMA) method based on the SM coefficient (Figure 1).

3. RESULTS AND DISCUSSION

A total of 15 bottles were purchased, of which 6 were drinking water and 9 were natural mineral water. It was detected no bacteria in the bottled drinking water but obtained 11 bacterial strains from bottled mineral waters. Table 3 lists properties of total colony counts, gram staining reaction, endospore, oxidase and catalase of natural mineral water samples. All of the obtained isolates were determined as Gram positive and with endospore. By means of these properties, these bacteria have resistance against to environmental conditions such as low storage temperature, different pH values i.e. (Leclerc and Moreau, 2002).

According to Leclerc and Moreau (2002); after bottling, the number of viable counts increases rapidly, attaining 10^4-10^5 cfu ml^{-1} within 3-7 days. During the following weeks, the bacterial counts decrease slowly or remain fairly constant. At the end of 2 years storage, colony counts are still about 10^3 cfu ml^{-1}. In addition, Loy et al. (2005).

Table 2. Results of the susceptibility to antibiotics.

Antibiotic	Symbol	Isolates										
µg disc $^{-1}$		M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11
Ampicilin (10)	A	R	R	R	R	R	I	R	R	R	R	R
Ciprofloxacin (5)	Cf	S	S	S	S	S	S	S	S	S	S	S
Erytromycin (15)	E	S	I	S	I	S	S	S	S	I	I	I
Gentamicin (10)	G	S	S	S	S	S	S	S	S	S	S	S
Penicilin G (10)	P	R	R	R	I	I	S	S	S	R	R	R
Rifampicin (5)	R	I	I	S	R	S	S	S	S	I	R	R
Vancomycin (30)	Va	S	S	S	S	S	S	S	S	S	S	S

S: Sensitive; I: Intermediate; R: Resistant.

The inhibition zone size (diam in mm) interpretation was based on HiMedia and Oxoid instruction sheet (the following values are upper and lower cut-off lines for R and S, respectively): 28 and 29 for A; 15 and 21 for Cf; 13 and 23 for E; 12 and 15 for G; 19 and 28 for P; 16 and 20 for R; 14 and 17 for Va.

Table 3. Cellular properties of isolates.

Mark	**Total Colony Counts (cfu/ml)**	**Isolate No**	**Morphology**	**Endospore**	**Gram Staining Reaction**	**Catalase**	**Oxidase**
G	**3**	**M1**	Bacil	+	Gr(+)	-	+
		M2	Bacil	+	Gr(+)	+	+
		M3	Bacil	+	Gr(+)	+	+
H	**1**	**M4**	Bacil	+	Gr(+)	+	+
I	**1**	**M5**	Bacil	+	Gr(+)	+	+
J	**1**	**M6**	Bacil	+	Gr(+)	+	+
K	**2**	**M7**	Bacil	+	Gr(+)	+	+
		M8	Bacil	+	Gr(+)	+	+
L	**3**	**M9**	Bacil	+	Gr(+)	+	+
		M10	Bacil	+	Gr(+)	+	+
		M11	Bacil	+	Gr(+)	-	+
M	No growing						
N	No growing						
O	No growing						

Table 4. Results of VITEK system.

Isolate No	Isolate Name	Similarity (%)	Used Carbon Sources
M1	*Bacillus subtilis*	99	SUC, GLU, INO, ARA, XYL, MAN, SAL, INU, RIB, MLT, TRE, PLA, KCN, NCL, MEN, NAA, PAS, ESC
M2	*Bacillus subtilis*	99	SUC, GLU, INO, ARA, XYL, MAN, SAL, INU, MLT, TRE, PLA, AMY, KCN, NCL, MEN, NAA, PAS, ESC
M3	*Bacillus subtilis*	99	SUC, TZR, GLU, ARA, XYL, MAN, SAL, INU, MLT, TRE, PLA, KCN, NCL, MEN, NAA, PAS, ESC
M4	*Bacillus pumilus*	99	SUC, TAG, GLU, MAN, SAL, TRE, KCN, MEN, ESC
M5	*Bacillus amyloliquefaciens*	96	SUC, GLU, MAN, SAL, INU, MLT, TRE, KCN, NCL, MEN, NAA, PAS, ESC
M6	*Bacillus subtilis*	88	SUC, GLU, ARA, MAN, SAL, INU, MLT, TRE, PLA, KCN, NCL, MEN, NAA, PAS, ESC
M7	*Bacillus subtilis*	81	SUC, TZR, GLU, ARA, XYL, MAN, SAL, INU, MLT, TRE, PLA, KCN, NCL, MEN, NAA, PAS, ESC
M8	*Bacillus subtilis*	98	SUC, GLU, INO, ARA, MAN, SAL, INU, MLT, TRE, PLA, KCN, NCL, MEN, NAA, PAS, ESC
M9	*Bacillus alvei*	95	GLU, NAG, MEN, PAS, ESC
M10	*Bacillus licheniformis*	92	SUC, TAG, GLU, ARA, MAN, SAL, AGA, MLT, TRE, PLA, SOR, NAG, KCN, NCL, MEN, NAA, PAS, ESC
M11	*Bacillus alvei*	94	GLU, NAG, MEN, PAS, ESC

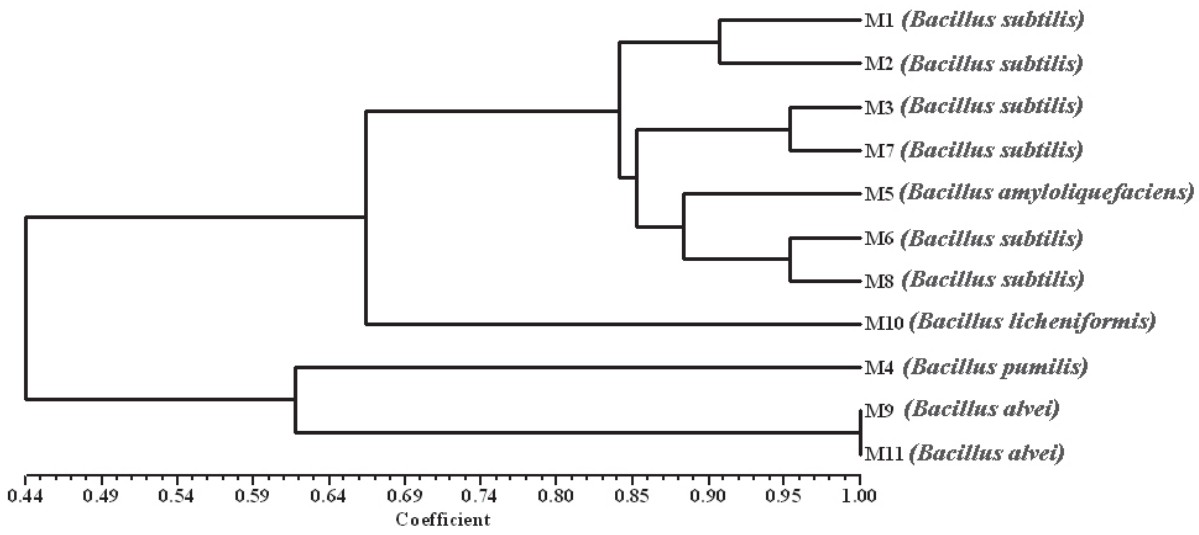

Figure 1. Dendogram based on UPGMA cluster analysis of VITEK results. All isolates were compared according to their capabilities of using to 31 different carbon sources.

Stated that natural mineral water is microbiologically unaltered water and thus clearly distinguishable from ordinary drinking water. As parallel to these papers, in our study we isolated 11 bacteria from total 9 bottled natural mineral water. We obtained 3 isolates from sample G and L, 2 isolates from sample K and 1 isolate from sample H, J and I. But we couldn't detect bacterial isolate from sample M, N and O. Our natural mineral water samples have different pH properties. While the natural mineral water samples with aroma have low pH between 2 and 4, the other natural mineral water samples have pH values changing between 5 and 9. Although there are wide pH ranges, we couldn't find correlation between pH values and isolate amount or species.

The results for susceptibility of the isolates to 7 different antibiotics are shown in Table 2. While all tested isolates were generaly showned sensitivity (S) to tested antibiotics, ten isolates were resistant to Ampicilin, six isolates were resistant to Penicilin G, and three isolates were resistant to Rifampicin.

These 11 bacterial isolates were identified by VITEK system and all of the isolated were determined as five different *Bacillus* species. In addition, capabilities of using to different carbon sources of these species were determined by this VITEK system (Table 4). While 6 isolates were identified as *Bacillus subtilis* with percentage identification between 81-99%, 2 isolates were identified as *Bacillus alvei* with percentage identification between 94-95% and other isolates were identified as *Bacillus pumilus* (id. 99%), *Bacillus amyloliquefaciens* (id. 96%), and *Bacillus licheniformis* (id. 92%).

The isolates that were identified as different *Bacillus* species were shown very high coefficient. Six isolates (M1, M2, M3, M7, M6, and M8) were identified as *B. subtilis*. From these isolates, while M1 and M2 isolates have 90% coefficient value, M3 and M7 isolates have 96% coefficient value, M6 and M8 isolates have 96% coefficient value. Isolate M5 was identified as *B. amyloliquefaciens* that have vegetative rods, gram positive cells. This isolate was shown 88% coefficient value to M6 and M8 isolates that were identified as *B. subtilis* (Figure 1). These two isolates are closely related and *B. amyloliquefaciens* were reported as *B. subtilis* at the first time by Fukumoto (1943a, b). But *B. amyloliquefaciens* was found as a different species from *B. subtilis* by Welker and Campbell (1967). According to this comparison, *B. amyloliquefaciens* differentiate from *B. subtilis* with good growth in 10% NaCl broth, positive hydrolysis of starch, good growth at 30 to 40 ºC, no good at 50 ºC and very high α-amylase production. The other species that is closely related with *B. subtilis* is *B. licheniformis*. *B. licheniformis* is a member of *B. subtilis* group and distinguishes from *B. subtilis* with propionate positive, grows in temperatures up to 55 ºC and is facultatively anaerobic (Fritze, 2004). Isolate M10 were identified as *B. licheniformis* with 66% coefficient to our other *B. subtilis* isolates (Figure 1). M9 and M11 isolates were identified as *B. alvei* with 100% coefficient. M4 isolate that was identified as *B. pumilus* with %62 coefficient value to these isolates (Figure 1). The *B. pumilus* distinguishes from *B. alvei* with no growth in anaerobic agar, growth at 50 ºC and in 7% NaCl and no starch hydrolyzed. In addition, this species distinguishes from *B. subtilis* with NO_3 reduced to NO_2 and no starch hydrolyzed (Slepecky and Hemphill, 2006).

Although the origins of these species are commonly soil habitats, *B. subtilis* is find soil and water habitats. *B. subtilis*, *B. licheniformis*, *B. alvei* and *B. pumilus* are causing some human disease. *B. subtilis*, *B. alvei* and *B. pumilus* are causative organisms of some secondary infections such as otitis, mastoiditis, infected subdural hematoma and hematogenous dissemination from a urinary tract infection. Only one case of postoperative ventriculitis due to *B. licheniformis* has been reported (Farrar and Reboli, 2006).

The present study describes the bacterial composition of bottled drinking and natural mineral water from Eskişehir, Turkey markets. In the investigated total 6 samples for bottled drinking water, we found no bacterial isolates. This type waters generally prove from groundwater and expose a lot of purification steps such as physical, chemical and microbiological. In addition, the selling of these waters is controlled by government systems. Because of this reason, bottled drinking waters have a high quality. For the public health, this result of bottled drinking water is very well and unsurprising.

In this stage of our study, we have reached to our aim of determining to cultivable bacterial community of bottled drinking and natural mineral water of Eskişehir, Turkey markets. Further studies should include applying molecular identification techniques and investigation of non-cultivable bacterial population.

4. CONCLUSION

Natural mineral water can be clearly distinguished from drinking water in distribution networks by physical, chemical and microbiological characteristics. From its origin, natural mineral water naturally contains bacterial populations in starvation/survival state with only few parts being cultivable. After bottling, in the absence of any disinfection treatment, the revivable total colony count may only be that resulting from the normal increase in the bacteria content which it had at source. With natural mineral waters that are untreated, the problem of risk management must be discussed specifically.

REFERENCES

Armas, A.B. and Sutherland, J.P. (1999). A survey of the microbiological quality of bottled water sold in the UK and changes occurring during storage. *Int. J. Food Microbiol*. 48, 59-65.

Bharath, J., Mosodeen, M., Motilal, S., Sandy, S., Sharma, S., Tessaro, T., Thomas, K., Umamaheswaram, M., Simeon, D. and Adesiyan, A.A. (2003). Microbiological quality of domestic and imported brands of bottled water in Trinidad. *Int. J. Food Microbiol*. 81, 53-62.

Bischofberger, T., Cha, S.K., Schmitt, R., Konig, B. and Schmit- Lorenz,W. (1990). The bacterial flora of non-carbonated natural mineral water from the springs to reservoir and glass and plastic bottles. *Int. J. Food Microbiol*. 11, 51-72.

Çelik, P. (2003). Milliyet Business: İlk sucumuz Hamidiye'ydi (in Turkish). Link accessed October 2009 (http://www.milliyet.com.tr/2003/09/01/business/bus06.html).

Farrar, W.E. and Reboli, A.C. (2006). The genus *Bacillus*– medical. The Prokaryotes: A Handbook on the Biology of Bacteria. Vol. 4 (Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.H. and Stackebrandt, E. eds), Third Edition, 609-630. Springer, New York.

Fritze, D. (2004). Taxonomy of the genus *Bacillus* and related genera: the aerobic endosporee-forming bacteria. *Phytopathology* 94(11), 1245-1248.

Fukumoto, J. (1943a). Studies on the production of bacterial amylase. I. Isolation of bacteria secreting potent amylases and their distribution (in Japanese). *J. Agr. Chem. Soc. Japan* 19, 487-503.

Fukumoto, J. (1943b). Studies on the production of bacterial amylase. II. Bacterial and physiological nature (in Japanese). *J. Agr. Chem. Soc. Japan* 19, 634-640.

Guyard, S., Mary, P., Defives, C. and Hornez, J.P. (1999). Enumeration and characterization of bacterial in mineral water by improved direct count method. *J. Appl. Microbiol*. 86, 841-850.

Güler, C. (2007). Characterization of Turkish bottled waters using pattern recognition method. *Chemometr. Intell. Lab*. 86, 86-94.

Laboratory Manager Vitek Manual (2004). TML/MSH Microbiology Department Policy & Procedure Manual. (http://microbiology.mstinai.on.ca/manual/vitek/mi_vitek.pdf).

Lau, O. and Luk, S. (2002). A survey on the composition of mineral water and identification of natural mineral water. *Int. J. Food Sci. Tech*. 37, 309-317.

Leclerc, H. and Moreau, A. (2002). Microbiological safety of natural mineral water. *FEMS Microbiol*. Rev. 26, 207-222.

Loy, A., Beisker, W. and Meier, H. (2005). Diversity of bacteria growing in natural mineral water after bottling. *Appl. Environ. Microbiol*. 71(7), 3624-3632.

Manaia, C.M., Nunes, O.C., Morais, P. and Da Costa, M.S. (1990). Heterotrophic plate cohunts and the isolation of bacteria from mineral waters on selective and enrichment media. *J. Appl. Bacteriol*. 69, 871-876.

Nsanze, H., Babarinde, Z. and Al Kohaly, H. (1999). Microbiological quality of bottled drinking water in the UAE and the effect of storage at different temperatures. *Environ. Int.* 25(1), 53-57.

Resmi Gazete. (2004). Doğal mineralli sular hakkında yönetmelik. Resmi Gazete no. 25657, 1.12.2004.

Resmi Gazete. (2005). İnsani tüketim amaçlı sular hakkında yönetmelik. Resmi Gazete no. 25730, 17.2.2005.

Slepecky, R.A. and Hemphill, H.E. (2006). The Genus *Bacillus*-Nonmedical. The Prokaryotes: A Handbook on the Biology of Bacteria. Vol. 4 (Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.H. and Stackebrandt, E. eds), Third Edition, 530-562. Springer, New York.

Tsai, G.J. and Yu, S.C. (1997). Microbiological evaluation of bottled uncarbonated mineral water in Taiwan. *Int. J. Food Microbiol*. 37, 137-143.

Venieri, D., Vantarakis, A., Komninou, G. and Papapetropoulou, M. (2006). Microbiological evaluation of bottled non-carbonated (''still'') water from domestic brands in Greece. *Int. J. Food Microbiol*. 107, 68-72.

Welker, N.E. and Campbell, L.L. (1967). Unrelatedness of *Bacillus amyloliquefaciens* and *Bacillus subtilis*. *J. Bacteriol*. 94(4), 1124-1130.

Zamberlan da Silva, M.E., Getirana Santana, R., Guilhermetti, M., Camargo Filho, I., Harue Endo, E., Ueda-Nakamura, T., Vataru Nakamura, C. and Dias Filho, B.P. (2008). Comparison of the bacteriological quality of tap water and bottled mineral water. *Int. J. Hyg. Environ. Health* 211, 504-509.