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Microbiological Quality of Carbonated and Non-Carbonated Mineral Water Stored at Different Temperatures

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A b s t r a c t

The microbiological quality of five brands of carbonated and non-carbonated mineral water sold in Poland was studied. The study was carried out on the survival of heterotrophic bacteria at 22 and 37°C (pour plate technique) in the samples of mineral waters stored at 4 and 22°C. The one hundred ten bottles (twenty two bottles of each of the five brands) of carbonated and uncarbonated mineral waters with different levels of dissolved solids and organic content were chosen to microorganisms study. Ten bottles of mineral water were studied initially. Fifty bottles were stored at 4°C, the other fifty were kept at 22°C. The haemolysing bacteria in 1 cm^3; *E. coli*, *P. aeruginosa* and *A. hydrophila* in 250 cm^3 of mineral water were unidentifiable. Total viable count of heterotrophic bacteria at 22 and 37°C in 1 cm^3 of mineral water was the highest respectively for brand T and for brands T and M; the lowest for brand Ż. Initially, approximately 29% of 110 water samples (respectively 4% of carbonated and 55% of uncarbonated) had bacterial counts greater than Ministry of Health's standards, notwithstanding the number of water samples which doesn't perform requirements grew up to 47% (respectively 36% of carbonated and 58% of uncarbonated) when the time of TVC 37 and 22°C incubation was elongated from 1 and 3 days to 3 and 14 days respectively. The temperature of storage was inessential for the numbers of studied microorganisms. The most important factors were the brand, time of storage and the carbonating or non-carbonating of water. The highest numbers of the bacteria analysed were detected in non-carbonating water, irrespective of the water brand and temperature of storage.

K e y w o r d s: mineral water, heterotrophic bacteria, survival

Introduction

The bottled water industry in Poland is currently experiencing an annual growth rate 10% (83% during last 8 years) (Erazmus-Rogóż, 2005). The dramatic increase in the consumption of bottled water has been prompted by consumers' concern over increasing water pollution, and by their objection to offensive tastes or odours from municipal water supplies, and to fluoride, chlorine, and other additives. In Poland, like in certain parts of the world, disinfection and sterilisation (or otherwise treatment to remove or destroy microorganisms) of commercially available mineral waters is not permitted (Regulation of the Ministry of Health 2004; Warburton *et al.*, 1992). Therefore, they generally have high heterotrophic bacteria counts a few days after bottling that should result only from an increase of present in the source water. The microbiological quality of bottled, especially noncarbonated mineral waters has been of considerable interest in recent years due to increasing consumer preference for these products over potable tap water. Mineral water sampled directly from aquifers has a natural microbial population of about 20 cfu cm^{-3}. This population increases to approximately 10^2–10^5 cfu cm^{-3} after bottling due to the change in ecological condition (Hunter, 1993). The microflora of the water consists of indigenous species originating from the aquifers and species that enter as contaminants during processing and bottling. The indigenous bacteria can survive in bottled waters for many years (Biziagos *et al*., 1988; Szewzyk *et al*., 2000; Kirow, 1997), and there may be secondary growth, as some species die and provide nutrients for the growth of the others. Thus it is very important to investigate the microbiological quality of bottled mineral water even after storage for long time from bottling.

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Materials and Methods

Bottled water. The one hundred ten bottles (1.5 dm^3) (twenty two bottles of each of the five brands), all with the same expiry date, of carbonated and uncarbonated mineral waters in polyethyleneterephthalate bottles (PET) with different levels of dissolved solids and organic content (Table I) were chosen to study every group of microorganisms and purchased directly from manufacturers. Ten bottles were studied initially. The next ten bottles of mineral water (five bottles of carbonated water and five bottles of non-carbonated water) were stored for eight months (266 days) at 4°C, the other samples were stored at 22°C. Viable counting of studied bacteria was repeated after 0, 1, 3, 6, 10 and 38 weeks of the storage.

Microbiological analyses. Microbiological analyses involved determination of:

1. total viable count of heterotrophic bacteria in 1 cm^3 of water onto the agar-bullion medium after 3, 7 and 14 days of incubation at 22°C;
2. total viable count of heterotrophic bacteria in 1 cm^3 of water onto the agar-bullion medium after 24, 48 and 72 hours of incubation at 37°C;
3. total viable count of haemolysing bacteria in 1 cm^3 of water onto the agar-bullion medium with 5% sheep blood added, after 24 and 48 hours of incubation at 37°C;
4. number of *Escherichia coli* bacteria in 250 cm^3 of water on m-FC media (Merck) after 24 hours of incubation at 44.5°C;
5. number of *Pseudomonas aeruginosa* bacteria in 250 cm^3 of water on an agar medium with cetrimide (Merck) and on King A medium (Burbianka and Pliszka, 1983) after 24, 48 and 72 hour incubation at 42°C and 37°C respectively;
6. number of *Aeromonas hydrophila* bacteria in 250 cm^3 of water on mA agar (Rippey and Cabelli, 1979) after 24 and 48 hours of incubation at 37°C.

Table I
Analytical characteristics of five brands of bottled water (information on label of bottle – mg dm^{-3})

Brand		Saturation CO_2	Total mineral components	HCO_3^-	SO_4^{2-}	Cl^-	F^-	SiO_2	Ca^{2+}	Mg^{2+}	Na^+	K^+
N	C[1)]	6000	700	483.6	–	32	7.1	32	111.9	23.3	12.5	5.1
	N[2)]	–	700	483.6	–	7.1	0.24	32	111.9	23.3	12.5	5.1
Ż	C	6000	309.98	200.4	–	–	–	–	61.24	6.9	7.23	–
	N	–	185.84	109	–	4.6	0.07	–	27.73	8.18	8	–
B	C	6000	615	–	80	300	–	–	120	85	30	–
	N	–	–	–	<180	<180	–	–	75–95	35–45	<20	–
M	C	6000	2070.6	1525.5	21.2	14.2	–	–	212.8	85.1	171	13.3
	N	–	644.5	439.3	31.6	5.3	–	–	121.4	21.4	3.3	1.3
T	C	4000	263	159.87	–	7.09	0.19	–	43.61	5.83	8	1.6
	N	–	263	159.87	10	7.09	0.19	–	43.61	5.83	8	1.6

[1)] – carbonated water; [2)] – non-carbonated water

Each bottle was aseptically opened. Microbial numbers were estimated by decimal dilution in ¼ strength Ringer's solution. For total viable count of heterotrophic and haemolysing bacteria pour plates with 1cm^3 of water sample (or diluted water sample) were prepared with the chosen media. For other groups of bacteria sterile Millipore membrane filters with pores of 0.45 µm in diameter were used to condense the bacteria from water samples; next filters were placed on the surface of the selective media in Petri plates. The water samples were incubated at a specified temperature for the desired period of time. After the incubation typical colonies were counted. To determine the number of *E. coli* bacteria, blue coloured colonies were counted. The occurrence of *P. aeruginosa* was verified under the light of a Wood UV lamp; colonies which produced pyocyanin were counted. For the determination of the number of *A. hydrophila* bacteria, yellow coloured colonies were counted.

Statistical evaluation. In order to obtain information concerning potential differences between bacteria numbers for various brands of water, for carbonated/uncarbonated water, for water stored at different time and temperature, a single factor analysis of variance (ANOVA) was conducted, verifying the hypothesis of the equality of means ($H_0: x_1 = x_2 = ... = x_5$) at the level of significance $\alpha = 0.05$, assuming that the variance for the numerousness of the bacteria groups under study are uniform. The uniformity of variance was tested with Levene's test. If the test proved significant, the hypothesis was rejected. Next, the Kruskal-Wallis' test was applied, which is a non-parametric equivalent of the analysis of variance (Stanisz, 1998). Estimation by Spearman' correlation between numbers of studied groups of microorganisms received during whole time of water storage and some chemical compounds in mineral water were used in this study too.

Results and Discussion

Bacterial numbers. The haemolysing bacteria in 1 cm^3, *E. coli*, *P. aeruginosa* and *A. hydrophila* in 250 cm^3 of mineral water were undetected.

Total viable count of heterotrophic bacteria at 22 and 37°C in 1 cm^3 of mineral water was the highest for noncarbonated water stored at 22°C and noncarbonated water stored at 4°C respectively (Fig. 1). The numbers of studied microorganisms were the lowest for carbonated water stored at 22°C (detailed results can be obtained from the authors). In our study the number of heterotrophic bacteria at 22°C in noncarbonated water increased during the first three weeks, decreased during the next seven weeks and increased during the next days (Figure 1). Number of these microorganisms decreased (except the tenth week) in carbonated water during the whole time of storage. The number of heterotrophic bacteria at 37°C decreased during the first week, increased during the next five weeks, decreased during the next four weeks and increased during the next days in uncarbonated water. It was unidentifiable in carbonated water stored at both temperatures after this time.

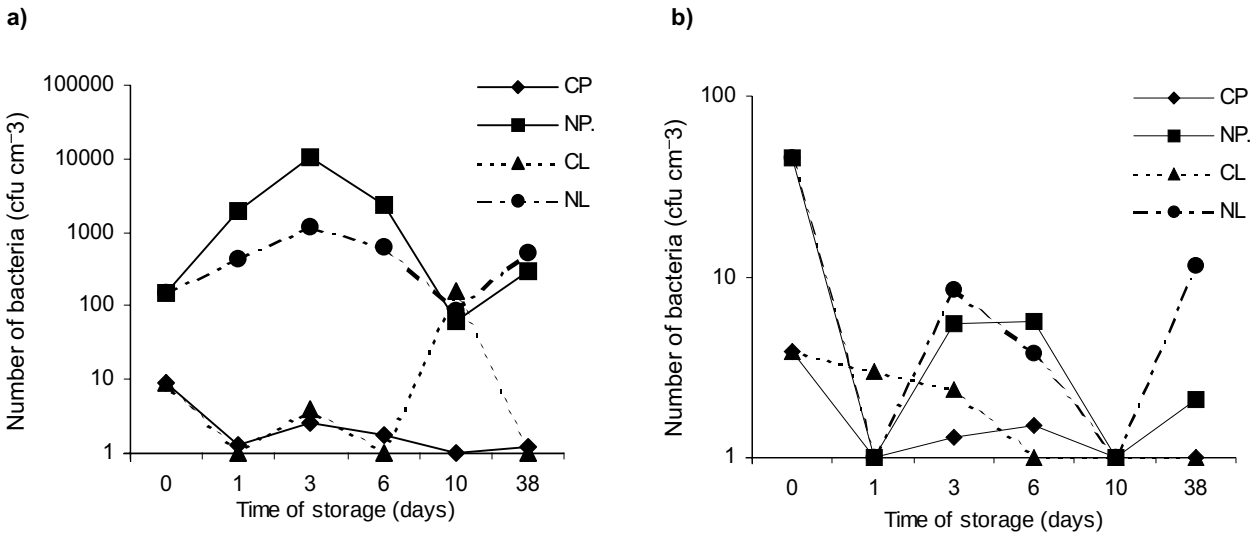

Fig. 1. Number of heterotrophic bacteria a) at 22°C and b) at 37°C in carbonated and non-carbonated mineral water during eight-month storage in 4 and 22°C. CP – carbonated water stored at 22°C; NP – non-carbonated water stored at 22°C; CL – carbonated water stored at 4°C; NL – non-carbonated water stored at 4°C

Microbes have evolved longer than any other living organisms, so in all probability, the non-spore-forming heterotrophic bacteria must have developed mechanisms to survive long periods when no energy or nutrients were available (Leclerc and Moreau, 2002). Morita (1997) described four pattern of starvation-survival. The most frequently pattern noted which might be representative for most environmental bacteria shows an initial increase in cell number due to fragmentation followed by a decline. The starvation pattern with time occurs in three stages. During the first stage lasting 14 days, large fluctuations in plate counts were noted. In the second stage (14–70 days) the colony count decreased by 99.7%. The third stage was marked by a stabilization of viable cells. Microorganisms in bottled water may multiply and exceed 10^5 cfu cm^{-3} after storage (Hunter *et. al.*, 1990).

Usually the maximum bacterial density was observed in room temperature stored samples. Nevertheless, storage at low temperatures, such as that of refrigeration, does not stop bacterial multiplication. The same results received Leclerc and da Costa (1998) in their studies of natural mineral waters.

According Polish and European regulations, the heterotrophic plate counts of mineral waters were assessed (immediately after bottling and during the next 12 hours) at two recovery temperatures: 22°C for 72 h and 37°C for 24 h. The 37°C plate count was believed to give an incubation of fast-growing bacteria more likely to be related to pathogenic types and the 22°C plate count was used for enumeration of characteristic water bacteria that tend to develop slowly. However a lot of microorganisms from these both groups need longer time of incubation to their growing. In our study microorganisms growing at 22 and 37°C were the most numerous after 14 and 3 days' incubation respectively. Initially, approximately 29% of water samples (respectively 4% of carbonated and 55% of uncarbonated) had bacterial counts greater than Ministry of Health's standards (2004), notwithstanding the number of water samples which doesn't exceed requirements grew up to 47% (respectively 36% of carbonated and 58% of uncarbonated) when the time of TVC 37 and 22°C incubation was elongated from 1 and 3 days to 3 and 14 days respectively. The one-day' incubation of TVC 37°C was appeared to short to isolation that group of microorganisms. Unfortunately longer-time' procedure is not only time consuming but also expensive.

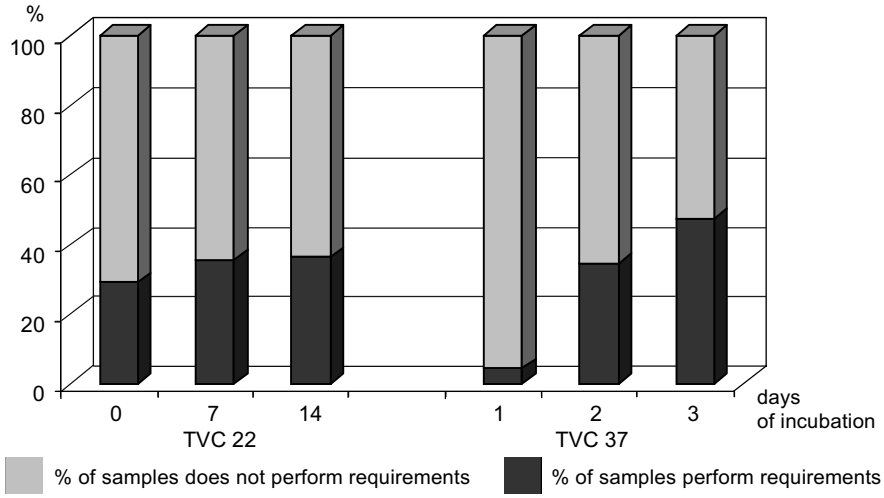

Fig. 2. Percent of mineral water samples performed requirements of Regulation of the Ministry of Health (2004). TVC 22 and TVC 37 – heterotrophic bacteria recovered from carbonated and non-carbonated mineral water respectively at 22 and 37°C for different incubation periods.

Statistical evaluation. This paper presents only the general statistical relationships; the detailed results can be obtained from the authors.

There were significant differences between the numbers of heterotrophic bacteria growing at 22 and 37°C (Figure 3) recovered from the different brands of bottled mineral water (respectively $p = 0.0000$ and $p = 0.0002$). The mean numerousness of the tested bacteria was the highest respectively in T and T and M brands, the lowest in B and Ż brands. Armas and Sutherland (1999) obtained significant differences in microbial numbers between the brands of water too.

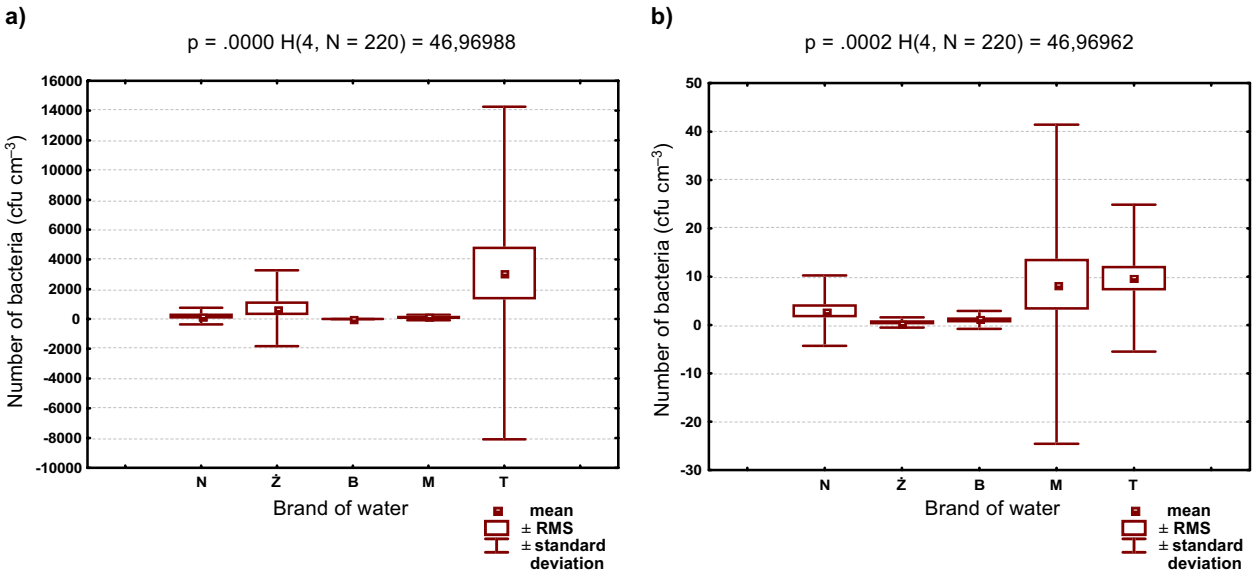

Fig. 3. Averages numbers (± standard deviation and ± random mean square-RMS) of heterotrophic bacteria a) at 22°C and b) at 37°C in different brands of carbonated and non-carbonated mineral water (as in Table I) during half-year storage. Independent variable (assembling): brand of water. ANOVA test of Kruskal-Wallis ranges.

There were significant differences between the numbers of tested microorganisms initially presented in the water and those after storage for 8 months (for respectively $p = 0.0209$ and $p = 0.0000$) (Figure 4). The mean numbers of the tested bacteria were the highest on third week for bacteria growing at 22°C and initially presented for bacteria growing at 37°C. Enumeration of TVC 22 and 37°C heterotrophic bacteria recovered from mineral water balanced from 0 to 1.5×10^4 and 70 cfu in 1 cm^3 respectively.

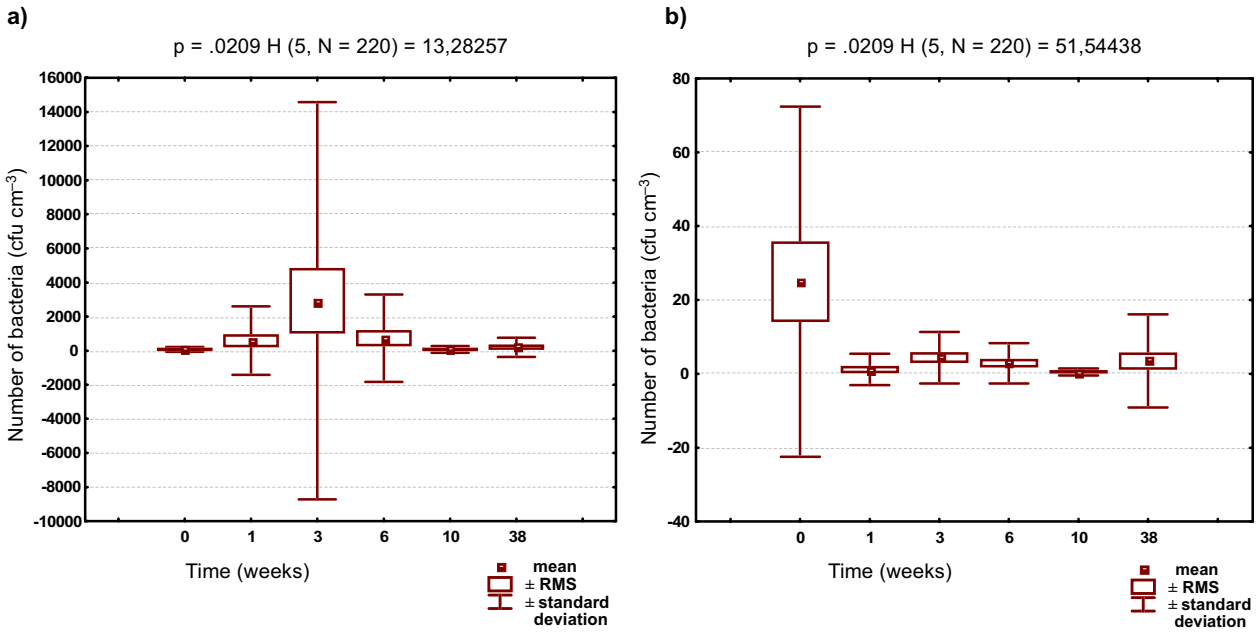

Fig. 4. Averages numbers (± standard deviation and ± random mean square-RMS) of heterotrophic bacteria a) at 22°C and b) at 37°C inoculated into mineral water during storage – total viable counts (cfu cm^{-3}). Independent variable (assembling): time. ANOVA test of Kruskal-Wallis ranges.

There were significant differences between the numbers of studied microorganisms recovered from the carbonated and uncarbonated water (respectively $p = 0.000$ and $p = 0.0081$). The mean numbers of the tested bacteria were the highest in uncarbonated water stored at temperatures 22°C. There were significant differences between the numbers of studied microorganisms isolated from different brand of water stored in different temperatures (respectively $p = 0.000$ and $p = 0.0017$). The mean numerousness of the tested bacteria was the highest in uncarbonated mineral water stored at room temperature, respectively T and M brands (Figure 5).

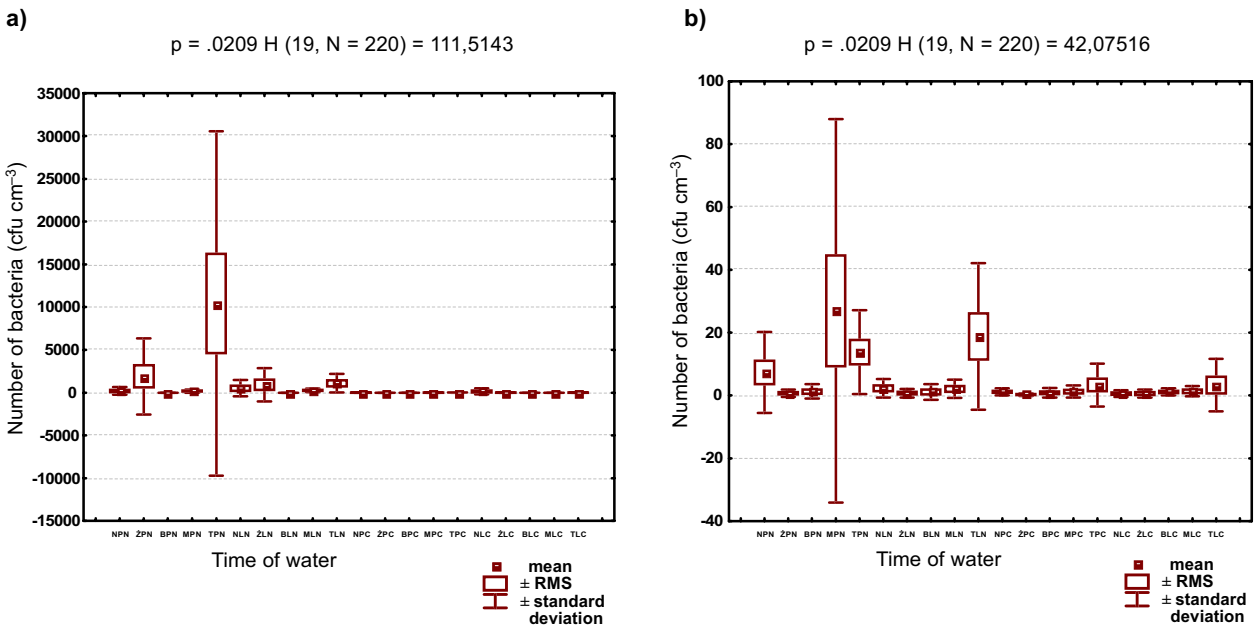

Fig. 5. Averages numbers (± standard deviation and ± random mean square-RMS) of heterotrophic bacteria a) at 22°C and b) at 37°C inoculated into mineral water during eight-month storage at 4 and 22°C. Independent variable (assembling): type of water (brand + temperature of storage + carbonated/uncarbonated). ANOVA test of Kruskal-Wallis ranges. First letters N, Ż, M, B and T – mean brand of water; second letter – C, N – mean carbonated/uncarbonated water; third letters – L, P – mean temperatures of storage – 4/22°C.

Table II
Statistic estimation by Spearman' correlation between numbers (cfu cm^{-3}) of studied groups of microorganisms received during whole time of water storage and some chemical compounds (mg dm^{-3}) in mineral water (TVC 22°C – total viable counts at 22°C, TVC 37°C – total viable counts at 37°C). BD eliminated in couple. Important correlations ($p < 0.05000$) marked

Variable	A22	A37
A22	1.000000	*0.369601*
A37	*0.369601*	1.000000
CO_2	*–0.440194*	*–0.181508*
Total mineral components	–0.004003	0.009774
HCO_3^-	–0.050388	0.002941
SO_4^{2-}	*–0.524169*	*–0.393409*
Cl^-	*–0.377332*	–0.090177
F^-	0.047757	0.026940
Ca^{2+}	0.005785	0.008276
Mg^{2+}	*–0.250448*	–0.101267
Na^+	*–0.281518*	–0.021108
K^+	*–0.443067*	–0.131429

There were no significant differences between the numbers of tested microorganisms recovered from the water stored at temperatures 4 and 22°C. The same results obtained Bharath *et al.* (2003) who did not find statistically significant differences between prevalence of aerobic bacteria in bottled water stored at room and refrigeration temperature (respectively 25 and 4°C) and air-conditioned environments (18°C).

According statistic estimation by Spearman' correlation the sulphate ion and carbon dioxide content was correlated negative with TVC 22°C and TVC 37°C (Table II). Carbonation is known to decrease pH of the water and in turn have a bactericidal effect on the bacteria (Caroli 1985). The chlorine, magnesium, sodium and potassium content were correlated negative with TVC 22°C only.

Conclusions

The number of heterotrophic bacteria at 22 and 37°C into bottled mineral carbonated/uncarbonated water depended on the time of storage and decreased in carbonated water during storage or was at the same level as initial presented in uncarbonated water. Percent of mineral water samples which doesn't perform requirements of Regulation of the Ministry of Health Initially approximately 29% of water samples had bacterial counts greater than Ministry of Health's standards, notwithstanding the number of water samples perform requirements grew up to 47% when the time of incubation increased. The kind of brand and the carbonating or uncarbonating of water was the most important factors for the survival/growth of microorganisms. The most numerous bacteria were detected in uncarbonated water, irrespective of the water brand or temperature of storage. Higher counts of the bacteria were typically observed in uncarbonated water samples stored at room temperature (about 22°C). Because mineral uncarbonated water can be identified as a significant risk factor for bacteria-associated diseases, survival of heterotrophic bacteria (especially growing at 37°C) in water during storage at different time and temperature is a very important research question.

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