MICROBIOLOGICAL QUALITY OF BOTTLED WATER IN FREESTANDING DISPENSERS

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT:

Nowadays, bottled water has become part of the lifestyle, replacing tap water, and water from freestanding dispensers is increasingly being used. The main goal of this research was to determine the health suitability of bottled water in freestanding dispensers. The research included the microbiological analysis of a total of 100 samples of bottled water from freestanding dispensers. The samples were mostly taken in the wider area of the city of Doboj (doctor's offices, shops, public buildings), and one dispenser in the city of Tuzla. Water samples were taken twice, in the period from April to June 2022. Water samples from 6 different manufacturers (Vivia, Kristal, Nevra, Gora, Aqua doria, Aqua team) were analyzed. 9% of water samples (9/100) were microbiologically correct. Microbiologically defective samples contained a higher total number of bacteria at 22°C and 37°C, as well as a higher number of coliform bacteria than the maximum allowed values. No significant differences in microbiological quality were found between older and/or recently installed water dispensers, as well as in terms of environmental conditions, while visible differences were observed between dispensers that were regularly hygienically maintained.

KEYWORDS: dispensers; bottled water; coliform bacteria; biofilms; total bacteria count

INTRODUCTION

During the past decade, in many regions of the world there has been a significant increase in the consumption of drinking water obtained from various sources, instead of tap water. One of these alternative sources is water from dispensers, which are popular in office buildings, medical offices and commercial shops. Dispensers are often presented as systems that are able to improve some water characteristics and are easy to use and maintain [1]. The quality of such a water source is questioned and has the potential to cause waterborne epidemics, especially in sensitive and immunocompromised individuals [2]. Bacteria present in bottled water can multiply due to high ambient temperatures. It is also possible for pathogens to enter bottled water through contaminated dispensing systems. Freestanding bottled water dispensers are tanks where water is stored in a bottle. As the water from the dispenser is discharged, the bottle is filled with room air that has its own transient microbiota. The volume of potentially contaminated air increases over time. Until the moment of replacement and installation of a new one, the bottle could have up to 19 liters of air [2]. Water contains different nutrients: organic substances such as carbohydrates, fats and proteins, and inorganic substances such as calcium, potassium, magnesium, iron, manganese [3]. Nutrient concentration affects the concentration of microorganisms in water, and the higher their concentration, the higher the concentration of microorganisms [4]. Pathogens can get into bottled water if faucets are not properly cleaned or maintained, which is a major drawback of freestanding dispensers. Various inorganic and organic substances present in bottled water provide favorable conditions for the development of microorganisms. Although water itself is not a rich source of nutrients, bacteria are incredibly resourceful in their ability to use them. Certain types of bacteria, such as *Pseudomonas aeruginosa*, can feed on components found in plastic packaging, as well as on rubber caps present in the dispenser [5], bacteria can attach to the inside of the bottle and multiply. After the contact of bacterial cells with the substrate, changes in gene expression occur, and the extracellular matrix begins to form and biofilms are formed [6]. Biofilms can be defined as highly structured and complex communities of bacteria connected by an extracellular matrix that they secrete themselves [7]. Given that the use of bottled water dispensers is very common, their

improper maintenance can pose a health risk, especially for people with a weaker immune system. It is important to have insight into the suitability of drinking water from freestanding dispensers and thus point out the importance of regular sanitization in order to achieve an optimal state of chemical and microbiological quality of drinking water consumed through dispensers.

MATERIALS AND METHODS

A total of 100 samples of bottled water from 6 different producers Vivia, Kristal, Nevra, Gora, Aqua doria, Aqua tim were microbiologically analyzed, which in the paper are marked with alphabetical letters (A, B, C, D, E, F), from freestanding dispensers in the area of the city of Doboj and its surroundings (doctor's offices, shops, public buildings), as well as a water dispenser installed at the Faculty of Science and Mathematics of the University of Tuzla. Samples for determining microbiological parameters were taken in sterile glass bottles, volume 1L with a metal cap. A cold chain was provided for all collected samples during transport and they were stored in refrigerators at a temperature of ± 4°C. The samples were analyzed within 24 hours. Sampling was carried out twice, in the period from April to June 2022. The first (I) sampling was the day after the installation of the balloon, and the second (II) sampling of the bottled water was performed at the end of the consumption of the water from the balloon. The time period from I and II measurements was 6-12 days. The volume of balloons on freestanding dispensers was 18.0L in all dispensers, except for one, whose balloon volume was 15L. The analysis was carried out in the Laboratory for Genetics and Microbiology of the Faculty of Science and Mathematics, University of Tuzla.

MEMBRANE FILTRATION METHOD

A standard membrane filtration technique was used to estimate the abundance of the bacterial population in water with a low content of suspended particles. Before microbiological testing, the work surfaces were disinfected with 70% ethanol. After each new sample, the funnel of the membrane filtration device was sterilized by immersing it in a beaker with 70% ethanol, after which the inner sides and edges of the funnel and the support for the filter paper were burned with a gas burner. A sample volume of 100 mL was filtered through a sterile membrane filter with a pore diameter of 0.45 μm, and placed on a nutrient medium and incubated at the appropriate temperature. After incubation, the grown colonies were counted and the number of colonies per 1 mL of water sample was calculated.

METHOD FOR DETERMINING THE TOTAL BACTERIA COUNT IN 1ML AT 22°C AND 37°C

Determination of the total bacteria count (TBC) was performed according to the BAS EN ISO 6222:2010 standard [8]. The method is suitable for microbiological examination of all waters, and refers to the determination of living microorganisms in water by counting colonies formed inside or on the surface of the nutrient medium after aerobic incubation at temperatures of 22°C and 37°C. The number of microorganisms is determined using the standard pouring technique (pour plate) with the use of samples from decimal dilutions. The number of microorganisms was determined by incubating Petri plates at two different temperatures, 22°C and 37°C, for 24 hours. The colonies present on each plate were counted, and the CFU per mL of sample were calculated for each incubation temperature. The number of aerobic heterotrophs, as CFU/mL, is calculated according to the formula:

$$C_s = Z/V_{tot} \; x \; V_s$$

where Cs is the number of formed colonies in the tested volume, Z is the sum of the counted colonies on solid substrates with or without membrane filters resulting from dilution d1, d2, or resulting from special volumes of the test sample (initial sample or dilution), Vs - the volume that is chosen to express the concentration of microorganisms in the sample, Vtot - calculated total volume of the initial, original sample that was seeded on solid substrates and whose colonies were counted.

DETECTION AND DETERMINATION OF THE NUMBER OF COLIFORM BACTERIA AND *E.COLI*

Determination of the presence and abundance of coliform bacteria and *E. coli* using the membrane filtration technique was carried out according to the BAS EN ISO 9308-1:2015 standard [9]. The method is based on membrane filtration of a 100 mL water sample, incubation of the concentrate after membrane filtration, on Coliform Chromogenic Agar (CCA), at a temperature of 37°C, and colony assessment after a confirmatory test. After incubation, all colonies that give a positive β-D-galactosidase and β-D-glucuronidase reaction are counted. The appearance of a dark blue to purple color is understood as the presence of *E. coli*. All colonies that give a positive β-

D-galactosidase reaction (pink to red color) are then counted as likely non-*E.coli* coliforms. To confirm probable coliform bacteria other than *E. coli*, an oxidase test is performed. The number of *E.coli* and coliform bacteria present in 100 mL of the sample is calculated from the total number of confirmed colonies from the membrane filter. The coliform count is the sum of all oxidase negative pink to red colonies plus all dark blue to purple *E.coli* colonies.

DETECTION AND DETERMINATION OF THE NUMBER OF INTESTINAL ENTEROCOCCI

Detection and determination of the number of intestinal enterococci using the membrane filtration technique was carried out according to the standard BAS EN ISO 7899-2:2003 [10]. The detection and determination of the total number of intestinal enterococci is based on the filtration of a 100 mL water sample through a sterile membrane filter with pores of 0.45 μm. The filter is then placed on the surface of SBA (Slanetz Bartley Medium) and incubated (24h at 37°C), after which chestnut, red or pink colonies are counted, as typical colonies. The detection of enterococci is carried out by transferring the filter with colonies to a plate with Bile Aesculin Azide Agar, which is heated to 44°C, and incubating for 24 hours at 44±0.5°C. After incubation, all dark brown or black colonies are counted as enterococci colonies.

DETECTION AND QUANTIFICATION OF *PSEUDOMONAS AERUGINOSA*

Detection and determination of the number of *P. aeruginosa* using the membrane filtration technique was carried out according to the standard BAS EN ISO 16266:2009 [11]. The method is based on membrane filtration of a certain volume of water sample, incubation of the concentrate after membrane filtration on a certain selective medium (*Pseudomonas agar* base/CN - agar), at a temperature of 37°C, and colony assessment after a confirmatory test. After the required incubation (24h), all blue-green colonies are counted as confirmed *P. aeruginosa* colonies. After that, the filter is viewed under a UV lamp and all colonies that fluoresce are counted, as probable *P. aeruginosa* colonies, which are then proven with Acetamide broth. All red-brown pigmented colonies are also counted, as probable *P. aeruginosa* colonies, which are confirmed by Oxidase test, Acetamide broth and King's B medium. The growth is read after 22±2 hours due to the possibility of overgrowth and merging of colonies, which can happen with a reading of 44±4 hours.

RESULTS

During this research, 50 bottles of bottled water from freestanding dispensers, from six different manufacturers, were analyzed through two measurements (100 samples in total). Bacteria from the genus *Enterococcus sp., E. coli and P. aeruginosa* were not isolated in any sample. The presence of aerobic mesophilic and psychrophilic bacteria (total number of bacteria at 22°C and 37°C) and coliform bacteria was recorded in the water samples.

TOTAL NUMBER OF BACTERIA AND COLIFORM BACTERIA IN SAMPLES OF BOTTLED WATER IN FREESTANDING DISPENSERS OF MANUFACTURER A

The measured values of the total bacteria count (TBC) in a milliliter of the sample (CFU/mL) of manufacturer A was from 3.78×10^2 to 5.05×10^2 at a temperature of 22°C, while at a temperature of 37°C these values ranged between 2.25×10^2 and 1.83×10^3. Coliform bacteria were not isolated. The sample analysis indicates that the TBC exceeds the Maximum Allowed Concentration (MAC) specified in the Official Gazette of Bosnia and Herzegovina 30/12. The MAC for TBC is set to 100 colonies at 22°C and 20 colonies at 37°C, with no presence of other coliform bacteria [12]. One sample of manufacturer A was analyzed.

TOTAL NUMBER OF BACTERIA AND COLIFORM BACTERIA IN SAMPLES OF BOTTLED WATER IN FREESTANDING DISPENSERS OF MANUFACTURER B

Determination of microbiological parameters of bottled water in freestanding dispensers of manufacturer B included 7 samples. The dispenser of the mentioned manufacturer was not regularly hygienically maintained. The measured values of the total bacteria count in a milliliter of sample (CFU/mL) of producer B were from 3.1×10^1 to 1.40×10^4 at a temperature of 22°C, while at a temperature of 37°C these values ranged between 1.9×10^1 and 1.95×10^4. Coliform bacteria were not isolated. All analyzed samples show that the TBC is significantly higher compared to the maximum allowed concentrations.

TOTAL NUMBER OF BACTERIA AND COLIFORM BACTERIA IN SAMPLES OF BOTTLED WATER IN FREESTANDING DISPENSERS OF MANUFACTURER C

Determination of microbiological parameters of bottled water in freestanding dispensers of producer C included 11 samples. The dispensers of the mentioned

manufacturer were not regularly hygienically maintained, with the exception of two (samples 14 and 17). The measured values of the total bacteria count in a milliliter of the sample of producer C were from 2.2 x 10^1 to 1.27 x 10^4 at a temperature of 22°C, while at a temperature of 37°C these values ranged between 1.2 x 10^1 and 2.21 x 10^1. Coliform bacteria were detected in two samples and their values ranged from 1.34 x 10^2 to 5.45 x 10^2. Analysis of the samples shows that the TBC and coliform bacteria are significantly higher than MAC except for samples 14 and 17, which are microbiologically correct.

TOTAL NUMBER OF BACTERIA AND COLIFORM BACTERIA IN SAMPLES OF BOTTLED WATER IN FREESTANDING DISPENSERS OF MANUFACTURER D

Determination of microbiological parameters of bottled water in freestanding dispensers of manufacturer D included 10 samples. The dispensers of the mentioned manufacturer were not regularly hygienically maintained, with the exception of four (samples 22, 23, 26 and 29). The measured CFU/mL values of manufacturer D ranged from 6 to 3.80 x 10^4 at a temperature of 22°C, while at a temperature of 37°C these values ranged between 3 and 3.06 x 10^4. Coliform bacteria were detected in two samples and their values ranged from 4 to 4.40 x 10^3. The analysis of the samples shows that the TBC and coliform bacteria are significantly higher compared to the MAC, with the exception of samples 22, 23, 26 and 29, which are microbiologically correct.

TOTAL NUMBER OF BACTERIA AND COLIFORM BACTERIA IN SAMPLES OF BOTTLED WATER IN FREESTANDING DISPENSERS OF MANUFACTURER E

Determination of microbiological parameters of bottled water in freestanding dispensers of producer E included 11 samples. The dispensers of the mentioned manufacturer were not regularly hygienically maintained, with the exception of one (sample 35). The measured values of the total bacteria count in a milliliter of the sample of manufacturer E were from 3.4 x 10^1 to 6.30 x 10^4 at a temperature of 22°C, while at a temperature of 37°C these values ranged between 2 and 4.58 x 10^4. Coliform bacteria were detected in six samples and their values ranged from 1 to 1.08 x 10^4. The analysis of the samples shows that the TBC and coliform bacteria are significantly higher than the MAC, except for sample 35, which is microbiologically correct.

TOTAL NUMBER OF BACTERIA AND COLIFORM BACTERIA IN SAMPLES OF BOTTLED WATER IN FREESTANDING DISPENSERS OF MANUFACTURER F

Determination of microbiological parameters of bottled water in freestanding dispensers of manufacturer F included 10 samples. The dispensers of the mentioned manufacturer were not regularly hygienically maintained, with the exception of two (samples 43 and 45). The measured values of the total bacteria count in a milliliter of the sample from manufacturer F were from 6 to 2.19 x 10^4 at a temperature of 22°C, while at a temperature of 37°C these values ranged between 4 and 5.88 x 10^4. Coliform bacteria were detected in five samples and their values ranged from 1 to 7.10 x 10^3. The analysis of the samples shows that the TBC and coliform bacteria are significantly higher than the MAC, except for samples 43 and 45, which are microbiologically correct.

A SUMMARY OF THE RESULTS OF THE MICROBIOLOGICAL ANALYSIS OF BOTTLED WATER

Table 1. Summary of the number of defective samples of bottled water from freestanding dispensers of different manufacturers.

	First sampling (50 samples)				Second sampling (50 samples)		
Total number of analyzed samples		TBC at 22 °C	TBC at 37 °C	Coliform bacteria	TBC at 22 °C	TBC at 37 °C	Coliform bacteria
Manufacturer A	1	1	1	0	1	1	0
Manufacturer B	7	5	6	0	6	7	0
Manufacturer C	11	7	9	2	9	9	2
Manufacturer D	10	6	6	2	6	6	2
Manufacturer E	11	8	9	5	9	10	6
Manufacturer F	10	7	7	6	8	8	6
Total number of defective water samples	41/50	34	38	12	39	41	16

Out of a total of 100 analyzed samples of bottled water from freestanding dispensers (I and II sampling), 9% of the samples were bacteriologically correct.

Table 1 shows the number of defective samples of bottled water from freestanding dispensers from different manufacturers. The samples showed an increased total bacteria count and coliform bacteria (over MAC).

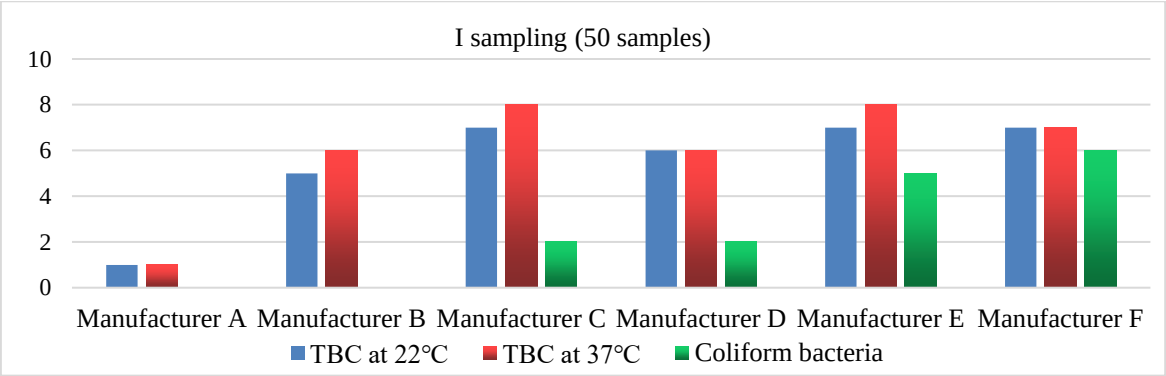

Figure 1. Summary of the number of defective samples of bottled water from freestanding dispensers during the first sampling.

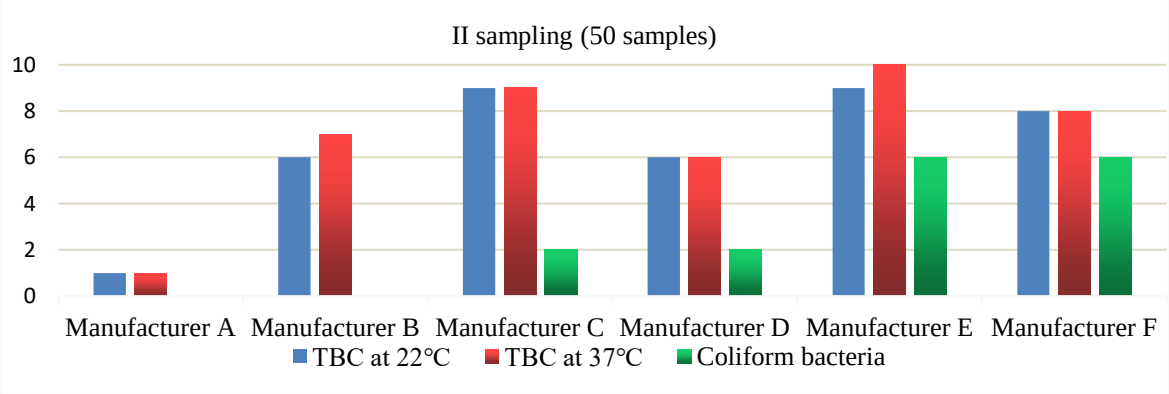

Figure 2.. Summary of the number of defective samples of bottled water from freestanding dispensers during the second sampling.

The first sampling (Figure 1) included 50 samples during which TBC was detected at 22°C significantly higher than MAC from 34 samples, and significantly higher TBC at 37°C from 38 samples, while coliform bacteria were detected in 15 samples. All the mentioned samples were microbiologically defective, i.e. 76% (38/50). The second sampling (Figure 2) included 50 samples during which TBC was detected at 22°C significantly higher than MAC from 39 samples, and significantly higher TBC at 37°C from 41 samples, while coliform bacteria were detected in 16 samples. All the mentioned samples were microbiologically defective, i.e. 82% (41/50)

DISCUSSION

To our knowledge, very few studies have been conducted on the microbiological quality of bottled water from dispensers. Testing of the quality of bottled water in freestanding dispensers began in 2005 by the German Federal Institute for Risk Assessment. After a microbiological analysis of all publicly available dispensers (799), the results indicated that a third of the water samples were contaminated with bacteria [13]. The results in our research show a high percentage of defective samples already after the first sampling (the day after the balloon placement). The bacteriological quality of the water at the end of the balloon was worse compared to the water at the very installation of the freestanding dispenser balloon. Such results were expected considering the initial number of bacteria, as well as the fact that during the discharge of water from dispenser, the bottle is filled with air from the room, which can also be contaminated with various microorganisms. Over time, the volume of potentially contaminated air in the bottle increases. Even running water does not wash away microorganisms, because bacteria in the form of biofilm adhere to the exit openings and can spread even against the direction of flow and continue to

multiply in the water. The results in this research show that the surfaces of the bottle and the dispenser favored the excessive growth of bacteria and the formation of biofilms. In microbiologically defective samples, an increased total bacteria count and coliform bacteria (via MAC) was found. Coliform bacteria are the most suitable group of indicator bacteria for evaluating the hygienic quality of water [3]. *Enterococcus spp., P. aeruginosa* and *E. coli* were not detected in any of the 100 samples of bottled water from freestanding dispensers. Absence of *Enterococcus spp*. and *E. coli*, which are considered to be indicators of fecal contamination, makes the water satisfactory and safe without health implications [13]. Observing the results for the mentioned parameters, regardless of the institution where the dispenser is installed and which manufacturer is represented, it is clearly observed that the concentration of bacterial cells increases with the reduced volume of water in the balloon. Also, when water is used less often from the balloon, there is an increased concentration of bacteria. As an example, we can take sample number 4 of manufacturer C, where a period of 7 days passed from the first to second sampling, and sample number 6 of manufacturer D, where this period was 12 days. No significant differences were found in the number of tested bacteria between older and/or more recently installed water dispensers, as well as in terms of environmental conditions. The environmental conditions of the rooms where the freestanding dispensers were installed (temperature, light) did not affect the number of bacteria. The devices were not placed in illuminated places, and the temperature in all locations was approximately uniform. Visible differences were observed between dispensers that were regularly and those that were occasionally hygienically maintained. Samples that were microbiologically correct were taken from dispensers that are disinfected every 3 to 6 months. Water coolers must be thoroughly cleaned and disinfected to prevent biofilm formation. It has also been proven that disinfection of microfiltered water dispensers with hydrogen peroxide allows obtaining water with TBC levels in accordance with drinking water regulations (≤100 CFU/ml) [14].

CONCLUSION

Out of a total of 100 bottled water samples analyzed, 9 samples were microbiologically correct (9%). In the bottled water samples of all tested manufacturers, the presence of the total bacteria count above the MAC was recorded in 82% and coliform bacteria in 32% of the samples.

Enterococcus sp., Escherichia coli and *Pseudomonas aeruginosa* were not isolated in any water sample. No significant differences were found in the number of tested bacteria between older and/or recently installed water dispensers. Water samples taken from dispensers that are regularly hygienically maintained are microbiologically correct. The results highlight the importance of a periodic disinfection procedure (every 3 to 6 months) of the water cooler monitoring system, in order to keep the level of microbial contamination under control.

REFERENCES

[1] German Bundesinstitut für Risikobewertung. Hygienemängel bei Wasserspendern. Deutschland, 2005.

[2] Stafford, D., A microbiologist's view on bottle-fed water cooler safety. Department Microbiology & Biotechnology, Canada, 2021.

[3] World Health Organization. Nutrients in drinking water. World Health Organization. 210, 2005.

[4] Duraković S. Primjenjena mikrobiologija. Prehrambeno tehnološki inžinjering, Zagreb, 1996.

[5] World Health Organization. Guidelines for drinking water quality: fourthedition. World Health Organization. 564, 2011.

[6] Kostakioti, M., Hadjifrangiskou, M., Hultgren, S. J. Bacterial Biofilms: Development, Dispersal and Therapeutic Strategies in the Dawn of the Postantibiotic Era. Cold Spring Harbor Perspectives in Medicine, 3, a010306, 2013.

[7] Aguilar, C., Eichwald, C., Eberl, L. Multicellularity in Bacteria: From Division of Labor to Biofilm Formation. In: Ruiz-Trillo, I., Nedelcu, A. (eds). Evolutionary Tranistions to Multicellular Life. Advances in Marine Genomics, 2, 79-95, 2015.

[8] Bosanskohercegovački standard - BAS EN ISO 6222:2010. Water quality-Enumeration of culturable micro-organisms - Colony count by inoculation in a nutrient agar culture medium, 2010.

[9] Bosanskohercegovački standard - BAS EN ISO 9308-1:2015. Water quality -Enumeration of Escherichia coli and coliform bacteria - Part 2, 2015.

[10] Bosanskohercegovački standard - BAS EN ISO 7899-2:2000. Waterquality – Detection and enumeration of intestinal enterococci – Part 2: Membrane filtrationmethod, 2000.

[11] Bosanskohercegovački standard - BAS EN ISO 16266:2009. Water quality - Detection and enumeration of Pseudomonas aeruginosa - Method by membrane filtration, 2009.

[12] Pravilnik o zdravstvenoj ispravnosti vode za piće (Offical Gazette of Bosnia and Herzegovina 30/12).

[13] Buffet-Bataillon, S., Bonnaure-Mallet, M., De La Pintière, A., Defawe, G., Gautier- Lerestif, A.L., Fauveau, S., Minet, J. Heterotrophic bacterial growth on hoses in a neonatal water distribution system. J Microbiol Biotech, 20(4): 779, 2010.

[14] Zanetti F, De Luca G, Sacchetti R. Control of bacterial contamination in microfiltered water dispensers by disinfection. Int J Food Microbiol, 128 (3), 446–452, 2009.