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FRONT MATTER

Title: Identification of microbial indicators and free living protozoa in natural mineral water using cultural and molecular methods.

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Keywords: natural mineral water; free living protozoa; Nontuberculous mycobacteria; Legionella; qPCR

Abstract

Italian Directives recommends the good quality of natural mineral waters but literature data assert a potential risk from several microorganisms colonizing wellsprings and mineral water bottling plants. Aim of study is the evaluation of the risk related to the presence of microorganisms from spring waters (SW) and bottled mineral waters (BMW) samples. Routine microbiological indicators, further microorganism as *Legionella* spp., Nontuberculous mycobacteria (NTM), protozoa (FLA) and physical-chemical parameters were assessed in 24 SW and 10 BMW samples performing culture methods and molecular tests as PCR and qPCR. In 33 out of 34 samples no cultivable bacteria were counted with the exception of 83 CFU/L of *Mycobacterium gilvum*, detected in one warm rich-mineralized SW. qPCR showed the presence of Legionella qPCR units in 24% of samples (mean $2.9x10^2 \pm 1.7x10^2$ qPCR units/L) and NTM qPCR units in 18% of samples (mean $5.7x10^3 \pm 4.1x10^3$ qPCR units/L). *Vermamoeba vermiformis* and *Acanthamoeba polyphaga* were recovered respectively in 70% of BMW samples (counts from $1.3x10^3$ to $1.2x10^5$ qPCR units/L) and 42% of SW samples (counts from $1.1x10^3$ to $1.3x10^4$ qPCR units/L). *Vahlkampfia* spp. was detected in 42% of SW and 70% of BMW samples (mean $1.3x10^4$ $\pm 2.9x10^3$ qPCR units/L). Considering the presence FLA as possible reservoir for protection

bacteria we suggest the importance of microbiological risk assessment in natural mineral waters despite the absence of cultivable bacteria.

Introduction

European Regulation (EC) No 54/2009 [1] concerns the general principles and requirements of natural mineral water safety. Natural mineral water in its state at source may not be the subject of any addition of chemical products, as disinfectants, and may not be the subject of any treatment such as the separation of unstable elements, as iron and sulphur compounds, in order to not alter the original water composition and property in ions constituents.

During the last decades an increase of bottled mineral water (BMW) consumption was observed in Italy and over 200 liters are consumed yearly by every person [2]. In this country there are about 140 companies bottling over 260 mineral water brands and the large production and consumption of natural mineral water is due to the high number of Italian springs and the good organoleptic quality of bottled water. Therefore, more than 30% of Italian people preferred to drink mineral water, despite being more expensive in comparison to domestic tap water [3]. In Italy, collection and distribution of natural mineral water is regulated by Legislative Decree 176/2001 and Ministerial Decree of 10 February 2015 [4-5], which concerns the evaluation criteria for geological, chemical, microbiological and pharmacological characteristics of natural mineral waters. Regarding the microbiological parameters spring water (SW) and BMW must be bacteriologically pure with a *microbial facies* (environmental microorganisms) free from changes during the production cycle phases, from production to distribution and sale. Routine microbiological analysis performed in SW and BMW are aimed at ensuring the absence of *E.coli* and further coliform bacteria, faecal streptococci, *Pseudomonas aeruginosa, Staphylococcus aeureus,* sulfate-reducing bacteria, and further parasites or pathogenic microorganisms in cases of suspected contaminations [4-5].

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Literature data [6-7] suggest widespread presence of other microbial hazards in SW and BMW such as non-tuberculous mycobacteria (NTM), *Legionella* spp., fungi, free living amoebae (FLA), ect. NTM and *Legionella* spp. are widely present in soil, freshwater, hot springs and replicates in a large range of temperature (20-45°C). These bacteria can adapt and resist to stressful environmental conditions because of their ability to enter in a viable but nonculturable state showing a low metabolic activity despite their cell integrity [8]. Under favourable conditions within FLA, which are considered as natural primary hosts, waterborne bacteria can recover their vegetative state and regain pathogenic potential [9-10]. The World Health Organization has been classified several NTM species and *Legionella pneumophila* as emerging pathogens transmitted by water that may cause severe pneumonia and health-care associated pathologies, including skin and soft tissues infections, mostly in immunocompromised people [11-13]. Gastrointestinal effect are associated with water exposure which may be contaminated by FLA and in less common cases by *Legionella pneumophila* sg 1 (Linee guida legionellosi 2015) and some NTM species as *Mycobacterium avium* [11], which are capable of survival and growth in phagocytic FLA as *Acanthamoeba* spp. *Vermamoeba* spp. with consequent increasing of virulence and resistance to environmental stress [14-15]. Therefore, pathogens like *Giardia* spp., *Cryptosporidium* spp., *Entaomoeba* spp., are recognized as important waterborne disease pathogens and are frequently associated with severe gastrointestinal illness, while amoebiasis outbreaks have been reported especially in South-American countries [16-17]. Moreover, skin and respiratory tract infections are linked to waterborne bacteria, fungi and virus present in baths waters. In particular, old and immuno-suppressed people receiving bath thermal treatments with contaminated natural mineral water may be subjected to infections occurrences. *Pseudomonas aeruginosa* has proved responsible for cases of skin infections related to the contact with spa water while *Legionella pneumophila* has been the cause of outbreaks of pneumonia when the water is nebulized in the form of aerosol for respiratory hydrotherapy [18].

The aim of this research is to assess the microbiological quality of SW and BMW samples of different Italian brands, searching for microbial indicators and microorganisms not routinely searched according to current regulations.

Materials and Methods

Setting: The study was performed during the period from April 2016 to November 2016 on 34 natural mineral water samples belonging to 11 different brands. Samples were subdivided for their productive state (SW and BMW). In details, the research was performed on 24 SW and 10 BMW samples, which were classified according to their fixed residue at 180°C (FR).

Therefore, 12 minimally-mineralized (FR≤50 mg/L), 10 oligo-mineralized (FR=51-1499 mg/L) and 12 rich-mineralized (FR≥1500 mg/L) water samples were collected and analyzed for the routine microbiological test recommended by Legislative Decree 176/2001 and Ministerial Decree of 10 February 2015. Further microbiological tests as *Legionella* spp., NTM, and FLA were performed in the same SW and BMW samples.

SW samples were collected directly from the environmental springs, localized in the Tuscany, Italy (Tuscan-Emilian Appennines, Apuan Alps, Val d'Orcia and Tuscan archipelago). BMW samples were collected at the storage zone, after the bottling procedure and before being transported to the points of sale. Temperatures of the storage zones were 10-15°C. BMW samples were collected and analyzed ten days later the day of the production.

Table I show details about the 11 water brands collected and analyzed at different steps of the production chain.

Table I: Information about the correspondence between the number and type of spring water (SW) and bottled mineral water (BMW) samples and their geographical position for each brand.

BRANDS	NUMBER OF SW AND BMW SAMPLES COLLECTED	TYPE OF WATER	GEOGRAPHICAL POSITION
BRAND 1	2 SW	Minimally-mineralized	Tuscan-Emilian Appennines
BRAND 2	2 SW	Minimally-mineralized	Tuscan-Emilian Appennines
BRAND 3	2 SW; 2 BMW	Minimally-mineralized	Tuscan-Emilian Appennines
BRAND 4	1 SW	Minimally-mineralized	Apuan Alps
BRAND 5	1 SW	Minimally-mineralized	Apuan Alps
BRAND 6	2 SW	Minimally-mineralized	Tuscan-Emilian Appennines
BRAND 7	4 SW; 4 BMW	Oligo-mineralized	Tuscan-Emilian Appennines
BRAND 8	2 SW	Oligo-mineralized	Tuscan-Emilian Appennines
BRAND 9	2 SW	Rich-mineralized	Tuscan archipelago
BRAND 10	2 SW	Rich-mineralized	Tuscan-Emilian Appennines
BRAND 11	4 SW; 4 BMW	Rich-mineralized	Val d'Orcia

Routine microbiological and physical-chemical analysis: As suggested by Italian Regulations three liters of each SW and BMW samples were collected and mixed in a sterile container. The mixtures were analyzed for the detection of the total viable counts at 22 and 37°C [19] in two 1 ml aliquots using Plate Count Agar (Oxoid Ltd, Basingstoke, Hampshire, UK) as medium. Coliform bacteria, *E.coli* [20] and faecal streptococci [21] were enumerated filtering two 250 ml aliquots using Colilert 250 Test (Idexx, US) and Slanetz Bartley Agar (Biolife, Italy), respectively. *Pseudomomas aeruginosa* [22] and *Staphylococcus aeures* [23] were searched by one 250 ml aliquot using CN Pseudomoas Agar (Biolife, Italy) and Mannitol Salt Agar (Biolife, Italy) Sulfate-reducing bacteria [23] were enumerated filtering one 50 ml aliquot using Sulphite Polymyxin Sulfadiazine (SPS) Agar (Biolife, Italy). Filtrations were performed through a 0.45 µm membrane (Nalgene, USA), which were layered on the respective culture media before being incubated at the proper temperature and for the requested period. At the same time some physical-chemical water parameters as temperature, pH, and conductivity were measured. For BMW samples temperature values reported in bottle labels were considered.

***Legionella* spp. search:** *Legionella* spp. isolation in SW and BMW samples was performed in accordance with a standard procedure [24]. One liter of water was filtrated through a 0.2 µm membrane (Millipore, Billerica, MA), which was subsequently immersed in 10 ml of the same water and sonicated for 5 minutes, allowing the detachment of cells from the membrane and their suspension in water. Suspension was subjected to a thermal inactivation treatment at 50°C for 30 minutes with the aim to select *Legionella* spp., inactivating all microbial species not resistant to high temperature. Afterwards 0.1 ml of the suspension was seeded in triplicate on Legionella BMPA selective medium (Oxoid Ltd, Basingstoke, Hampshire, UK) and the plates were incubated at 37°C for 7-10 days within jars in which a modified atmosphere (2.5% CO_2). Suspected Legionella colonies grown on the medium were subjected to specie and serogroup identification analysis using a multi-purpose latex agglutination test (Legionella Latex Test, Oxoid Ltd, Basingstoke, Hampshire, UK).

NTM search: SW and BMW samples of one liter were centrifuged at 5.000 x g for 20 min. Pellets were suspended in 1 ml of sterile distilled water, and 0.1 ml samples were spread on the surface of Middlebrook 7H10 agar medium (BBL Microbiology Systems, Cockeysville, Md.) containing 0.5% (vol/vol) glycerol and 10% oleic acid–albumin enrichment. Plates were incubated at 37°C and examined after 21 days of incubation. Following the incubation period the total number of colonies was treated with an acid-fast staining [25].

From one to five acid-fast colonies per plate were identified by sequencing of the *hsp65* gene (439 bp). DNA was extracted using QIAamp DNA Mini Kit (Qiagen) and for each Polymerase Chain Reaction (PCR) 50 µL of mix were prepared with 31.25 µL of water; 5 µL of 10X PCR Buffer (15 mM MgCl2), 1 µL of dNTPs mix (10 µM); 1.25 µL of Tb11 (5'-ACCAACGATGGTGTGTCCAT) (20mM); 1.25 µL of Tb12 (5'-CTTGTCGAACCGCATACCCT) (20mM); 0.25 µL of HotStarTaq DNA Polymerase (5U/µL); and 10 µL of extracted DNA (HotStarTaq DNA Polymerase, Qiagen, United States). The reaction was subjected to 45 cycles of amplification (1 min at 94°C, 1 min at 60°C, 1 min at 72°C); followed by 10 min of extension at 72°C [26]. 10 µL of the amplified PCR mixture was loaded to a 1% agarose gel with ethidium bromide. A 1.5 Kb ladder was used to compare amplified PCR product.

After electrophoretic run, applied at 110 V for 30 minutes, *hsp65* gene amplification results were visualized in UV transilluminator. Amplified *hsp65* gene was sequenced in outsourcing (GATC, Biotech, Germany) and sequence alignment was performed by BioEdit Version 7.0.0. Sequences identification was obtained by Basic Local Alignment Search Tool (BLAST) Database.

FLA search: To detect FLA cells one liter of SW and BMW samples was filtered through a 0.2 µm membrane (Millipore, Billerica, MA), which was suspended in 10 mL of Page's modified Neff's Ameoba Saline (PAS) [27]. 3 mL of suspension were centrifugated at 750xg for 20 minutes and then 1 ml of sample was seeded on non-nutrient agar-*E. coli* ATCC 11229. After two hours, any excess liquid was gently pipetted off and the plates were closed in a polythene bag and incubated at 37°C for 7 days. The cells were observed daily by inverted microscope with a 20X objective. Trophozoite plaques or out-growths from deposit inocula were gently scraped from the plate and added to 1 mL of PAS [28]. Differentiation of FLA strains was performed by Giemsa-Romanowsky staining [29] applied on 100 µl of suspension while the other 900 µl were analysed by multiplex PCR assay to identified FLA strains. Briefly, DNA was extracted using QIAamp DNA Mini Kit (Qiagen) and multiplex PCR on the *18S rRNA* gene was performed using two pairs of primers (Amo_1400_F5'ATGCCGACCARSGATYMGGAG3'/Amo_1540_R5'CAAGSTGCYMG GGGAGTCAT3' and Vahl_560_F5'AGGTAGTGACAAGMYRTAGYGACT3'/Vahl_730_R5'GGGCGTTTTA ACTACARCAGTATTA3'), which allowed the amplification of 130 bp fragment for Acanthamoeba DNA, a 50 bp fragment for Echinamoeba DNA and Hartmannella DNA, and a 150 bp fragment for Vahlkampfiidae DNA. The run was performed using the following protocol: initial denaturation step at 94°C for 15 minutes, and then 35 cycles at 94°C for 30 seconds, 62°C for 1 minute, and 72°C for 1 minute, followed by a final elongation step at 72°C for 10 minutes [30]. Electrophoretic run and sequencing methods are described above.

qPCR tests: To perform the quantification of Legionella, NTM and FLA qPCR units, all SW and BMW samples were subjected to a qPCR, in accordance with the protocol of the SsoAdvanced SYBR Green Supermix (Bio-rad) using the CFX96 qPCR detection system (Bio-rad). DNA extraction (QIAamp DNA Mini Kit, Qiagen) was performed on 500 µL of concentrated water, which was prepared through filtration of one liter of water as described for *Legionella* spp. search. To detect Legionella qPCR units the *mip* gene (558 bp) was amplified with one pair of primer (mip595R5'-CATATGCAAGACCTGAGGGAAC/mip58F5'-GCTGCAACCGATGCCAC). Briefly, 12.5 µl of Supermix were added to 5 µL DNA template in a 25 µL volume, with 0.3 µM of each primer. Reaction conditions were 98°C for 2 minutes, followed by 40 cycles of 98°C for 2 seconds, 55°C for 20 seconds, and 72°C for 20 seconds [31]. NTM and FLA qPCR units were detected with the same protocols applied for qualitative PCR on *hsp65* and *18S rRNA* gene, respectively.

CFX96 qPCR detection system (Bio-rad) can detect and quantify the copy number of target genes present in 5 µL of DNA sample and each samples and standard control were amplified in triplicate.

Results are expressed as qPCR units per litre (qPCR units/L). The limit of detection (LOD) and quantification (LOQ) of the qPCR assay were 5 qPCR units/well and 25 qPCR units/well, respectively. The presence of PCR inhibitors in extracted DNA was considered if there was no amplification of the internal control (standard gene). In case of inhibition, extracted DNA was diluted in sterile water (1:5 and 1:10) and then amplified again.

Standard curves were obtained by decimal dilutions of the plasmid, which was obtained by cloning procedure of Legionella, NTM and FLA genes. Genes were amplified and cloned in a plasmid vector (pGEM-T Easy Vector System, Promega). Vector-insert ratio 1:3 was calculated by Biomath calculator program (Promega, Italy) and the insert was quantified by spectrophotometric reading at 260/280 nm and 260/230 nm. Bacterial transformations were obtained in *E.coli* JM HighEfficienty Competent Cells (Promega, Italy).

Statistical analysis: Correlation tests were performed and Pearson's coefficients were calculated with the aim of analyzing the correlations between physical-chemical parameters (temperature, pH, conductivity) and qPCR units belonging to *Legionella* spp., NTM, Amoebozoa and Vahlkampfiidae. These tests were independently applied for SW and BMW samples. Therefore, we considered the following ranges of values: 0-0.3 (weak correlation); 0.3-0.7 (moderate correlation); 0.7-1 (strong correlation). The statistical analysis was carried out using the SPSS software package, version 17.0.1.

Results

Routine microbiological and physical-chemical results: In all 34 natural mineral water samples, all microbiological parameters respected the limits suggested by Italian Regulations. Therefore, in SW samples we detected total viable counts at 22°C lower than 20 CFU/ml (mean value 4.8±1.3 CFU/ml) and total viable counts at 22°C lower than 5 CFU/ml (mean value 1.2±0.1 CFU/ml), respectively. Furthermore, in BMW samples total viable counts at 22°C lower than 10^2 (mean value 14.9±3.1 CFU/ml) and total viable counts at 37°C lower than 20 CFU/ml (mean value 3.2±0.7 CFU/ml) were always obtained. In accordance to the same Regulations, Coliform bacteria, *E.coli, Pseudomonas aeruginosa, Staphylococcus aureus* and sulfate-reducing bacteria were not isolated in natural mineral water samples. Physical-chemical results showed temperature values ranging from 10.7 to 42°C. A mean pH value of 6±0.24 was observed in water samples, while conductively values ranged from 43 to 3765 µS. No statistically significant difference of physical-chemical results was observed between SW and BMW samples (Table 2).

Table II: Mean Temperature, pH and conductivity values obtained in minimally-mineralized, oligo-mineralized and rich-mineralized spring water (SW) and bottled mineral water (BMW) samples.

	Mean Temperature (°C)	*Mean pH*	*Mean Conductivity (µS)*
Minimally-mineralized SW	**11.30±0.70**	**6.29±0.10**	**76.70±3.20**
Oligo-mineralized SW	**12.90±0.13**	**5.80±0.01**	**1104.00±0.84**
Rich-mineralized SW	**35.20±10.10**	**5.90±0.05**	**3316.00±476.00**
Minimally-mineralized BMW	**12.00±0.70**	**6.00±1.00**	**46.50±3.00**

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Oligo-mineralized BMW	**12.60±0.14**	**5.80±0.09**	**1110.00±1.37**
Rich-mineralized BMW	**28.30±11.70**	**6.10±0.06**	**3195.00±551.00**

***Legionella* spp. results:** No viable and cultivable Legionella cells were isolated in SW and BMW samples. Legionella qPCR units were detected in 2 out of 24 (8%) and in a 7 out of 10 (70%) SW and BMW samples, respectively. Overall, qPCR showed the presence of *mip* gene in 9 out of 34 (24%) samples, with a mean count of $2.9x10^2 \pm 1.7x10^2$ qPCR units/L. The highest percentage of positive *mip* gene amplification and the hightest Legionella qPCR units/L counts were recovered in rich-oligo-mineralized bottled water samples. The amplification efficiency was mean SLOPE= -3.155 (±0.17); mean E=1.49 (±0.12). Figure 1 and Figure 2 show Legionella qPCR units, conductivity and temperature values detected in 34 water samples. In facts, the presence of minerals in waters represent an important growing factor for the microorganisms, which may use that elements as nutrition and survival factor. Therefore bacteria needs specific physical-chemical parameters as pH and temperature to perform their metabolic activities in the environment.

NTM results: Cultivable NTM cells were not detected in natural mineral water, with the exception of 83 CFU/L of *Mycobacterium gilvum* isolated in one rich-mineralized SW sample (similarity value of 97,9%) having a temperature of 42°C, pH 5.8 and conductivity of 3740 µS. These physical-chemical values represent the optimal environmental conditions for the NTM growth and colonization in wellsprings. Moreover, qPCR results showed the presence of NTM qPCR units in 6 out of 34 (18%) samples, with a mean count of $5.7x10^3 \pm 4.1x10^3$ qPCR units/L. In details, the *hsp65* gene was amplified in 3 out of 24 (13%) SW samples and in 3 out of 10 (30%) BMW samples.

The highest percentage of positive *hsp65* gene amplification was detected in rich-oligo-mineralized bottled water samples while the hightest NTM qPCR units/L counts were recovered in the rich-mineralized spring positive to cultivable *Mycobacterium gilvum*. The amplification efficiency was mean SLOPE= -3.123 (±0,14); mean E=1.09 (±0.15). Figure 1 and Figure 2 show NTM qPCR units, conductivity and temperature values detected in 34 water samples.

Figure 1: Legionella, NTM, FLA qPCR units (qPCR/L) and Conductivity values detected in 34 water samples.

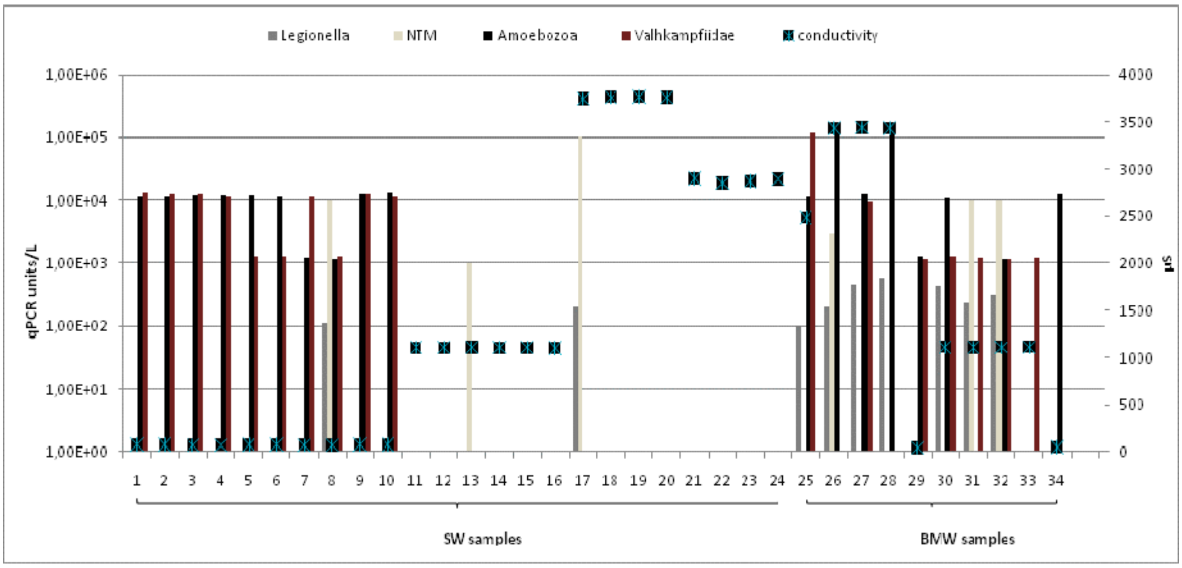
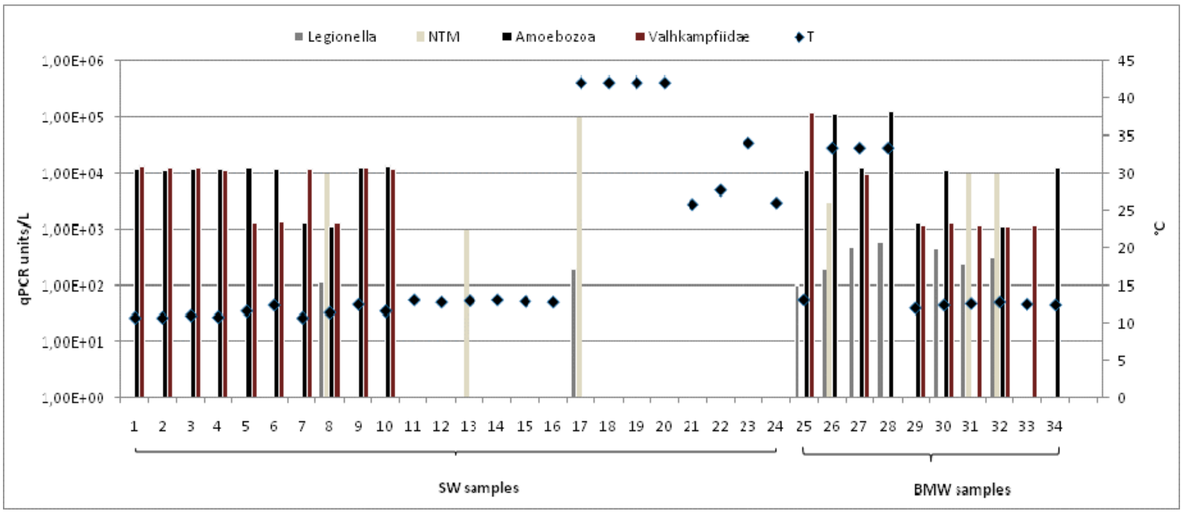


Figure 2: Legionella, NTM, FLA qPCR units (qPCR units/L) and Temperature values (T) detected in 34 water samples.



FLA results: Cultivable method allowed the detection of viable FLA in 17 out of 34 (50%) of water samples analyzed, showed shapes resembling trophozoites and cyst cells by inverted microscope with a 20X objective. Shapes were confirmed by Giemsa-Romanovsky staining. Identification of FLA was completed detecting the presence of Amoebozoa DNA *(Acanthamoeba polyphaga* and *Vermamoeba vermiformis)* and Vahlkampfiidae DNA *(Vahlkampfia inornata)* (similarity values from 97,2 to 98,1%).

qPCR results showed the amplification and sequencing of *18S rRNA* gene (50 bp) related to *Vermamoeba vermiformis* in 7 out of 10 (70%) BMW samples with counts ranging from $1.3x10^3$ to $1.2x10^5$ (mean count of $3.9x10^4$) qPCR units/L. Among these BMWs 2 out of 7 (29%) were minimally-mineralized water, further 2 out of 7 (29%) were oligo-mineralized water and 3 out of 7 (42%) were rich-mineralized waters. An amplification and sequencing of *18S rRNA* gene (130 bp) belonging to *Acanthamoeba polyphaga* was recovered in 10 out of 24 (42%) SW samples with counts ranging from $1.1x10^3$ to $1.3x10^4$ (mean count of $9.9x10^3$) qPCR units/L. All these springs were minimally-mineralized waters.

Furthermore, an amplification of *18S rRNA* gene (150 bp) belonging to the genus *Vahlkampfia inornata* was showed in 10 out of 24 (42%) SW and in 7 out of 10 (70%) BMW samples, with a mean count of $1.3x10^4 \pm 2.9x10^3$ qPCR units/L.

The amplification efficiency was mean SLOPE= -3.261 (±0.21); mean E=1.02 (±0.09). Figure 1 and Figure 2 show Amoebozoa and Vahlkampfiidae qPCR units, conductivity and temperature values detected in 34 water samples.

Statistical results: In SW samples statistical results showed moderate correlations between the low physical-chemical parameters (conductivity; temperature) and the high FLA qPCR units (mean r=0.582±0.09; p=0.007±0.005). In SW samples a strong correlation was detected between the increase of Amoeboza and Vahlkampfiidae qPCR units (r=0.772; p=0.001) and between the increase of NTM and Legionella qPCR units (r=0.916; p=0.001). In BMW samples we detected strong correlations between the high physical-chemical parameters (conductivity; temperature) and the high Legionella qPCR units (mean r=0.74±0.07; p=0.017±0.01). At last, a further strong correlation was detected for

Legionella and Amoebozoa qPCR units (r=1; p=0.001), proving the strong relationship between these microorganism in the environment.

Discussion

The natural mineral water quality is a requirement aimed at ensuring the water safety for exposed people and the cited Directives represent the only legislative tool which requires the assessment and the management of water risk [1, 4-5]. The same documents mention the possible microbiological hazards present in environmental habitats such as springs, soils, ect, ensuring a tight control of bottled waters for consumers. Despite microbiological parameters, such as the total viable counts at 22 and 37°C, *E.coli*, coliform bacteria, fecal streptococci, *Pseudomonas aeruginosa, Staphylococcus aureus* and sulfate-reducing bacteria, are considered for routine tests, no detailed mention is reported for further microbiological hazards as Legionella, NTM, and other environmental opportunistic human and animal pathogens, which may colonize springs and bottling plants [32-33]. The growth of these microorganisms could be due to the presence of biological reservoirs and free living protozoa, which are considered to be a natural hosts of waterborne bacteria. These pathogens can benefit from symbiosis for replication, spread (amoebae are vectors of *Legionella* spp. and NTM), protection, virulence and resuscitation of viable non-culturable cells [8-10].

In this study we evaluated the possible presence of viable cells of and qPCR units belonging to *Legionella* spp., NTM, and FLA in SW and BMW samples. The lack of cultivable bacteria isolation in almost all samples may be due to the environmental physical-chemical conditions, which limit the widespread colonization of bacteria in springs and bottling plants and the absence of cultivable Legionella cells could be due to the nutritional requirements of the bacteria, which needs specific growing conditions despite its widespread in environment [23]. As an exception, 83 CFU/L of *Mycobacterium gilvum* were detected in only one warm rich-mineralized SW sample. *Mycobacterium* *gilvum* does not represent an hazard because it is a saprophyte detected with a low concentration [34]. Cultivable FLA (*Acanthamoeba polyphaga, Vermamoeba vermiformis* and *Vahlkampfia inornata),* were detected in half of the water samples. Molecular tests, performed with PCR and qPCR, suggest the presence of high qPCR units belonging to *Legionella* spp., *Mycobacterium* spp., Amoebozoa and Vahlkampfidiiae genus. In this study we recognize the absence of specific tests as qPCR with the nucleic acid-binding dye ethidium monoazide bromide (EMA-qPCR), aimed to show that qPCR units belongs to viable and not damaged cells. This test proves that bacterial copy number of target genes belongs to viable but non cultivable cells but some authors demonstrated that EMA in high quantities (10-20 µg/ml) was identified as bactericidal and affected the quantification of viable cells [35-36].

Bacteria qPCR units, showed in Figure 1 and Figure 2, are often associated with the presence of protozoan *18S rRNA* gene, mostly in BMW samples. Moreover, the percentage of samples positive to FLA qPCR units is higher compared to the percentage of samples positive to Legionella and NTM genes. This data assert the possible role of FLA as reservoirs of waterborne bacteria, which may increase their resistance to environmental conditions, as described in further studies performed on drinking water and other environments [10, 13]. This statement may be demonstrated in future studies aimed to evaluate the endosymbiosis between intracellular bacteria and FLA using molecular tests as immunofluorescence techniques and electron microscopy. Another issue confirmed by this study concerns the importance of physical-chemical parameters measured in water samples. In accordance with further studies [37-38], the temperature, pH and conductivity parameters are some of important environmental factor influencing the bacterial community in wellsprings, mostly for *Mycobacterium* spp. and *Legionella* spp., which appears to be cultivability in natural mineral waters [39-40]. Despite our data suggest a low infectious risk, the lack of chemical disinfection treatment may support a microbial colonization in any points of the natural mineral water distribution plants.

Conclusions

In conclusion, the absence of natural mineral water disinfections highlights the importance of microbiological control plans for SWs and BMWs in accordance to the international food hygiene Regulations. Our study suggests the lack of viable and cultivable waterborne bacteria despite the presence of cultivable FLA, which may be a bacteria reservoir for protection and resuscitation activity. For this reason we highlight the need of routine microbiological tests aimed to ensure the water safety, mostly for high risk people such as immuno-suppressed people. Therefore, to avoid the occurrence of biological risk factors a wider assessment of microbial indicators presence is recommended.

Supplementary materials

Table I: Information about the correspondence between the number and type of spring water (SW) and bottled mineral water (BMW) samples and their geographical position for each brand.

Table II: Mean Temperature, pH and conductivity values obtained in minimally-mineralized, oligo-mineralized and rich-mineralized spring water (SW) and bottled mineral water (BMW) samples.

Figure 1: Legionella, NTM, FLA qPCR units (qPCR units/L) and Conductivity values detected in 34 water samples.

Figure 2: Legionella, NTM, FLA qPCR units (qPCR units /L) and Temperature values (T) detected in 34 water samples.

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Authors contributions

Angelo Baggiani conceived and designed the experiments. Michele Totaro, Beatrice Casini and Paola Valentini performed the experiments and wrote the paper. Pier Luigi Lopalco and Mario Miccoli analyzed the data.

Conflicts of interest

The authors declare that they have no competing interests.

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