

Article

Evaluation of Microbial Communities of Bottled Mineral Waters and Preliminary Traceability Analysis Using NGS Microbial Fingerprints

Federica Carraturo ^{1,*}, Carmela Del Giudice ², Mariacristina Compagnone ³, Giovanni Libralato ¹,
Maria Toscanesi ⁴, Marco Trifuoggi ⁴, Emilia Galdiero ¹ and Marco Guida ¹

¹ Department of Biology, University of Naples Federico II, Via Cinthia 21, I-80126 Naples, Italy; giovanni.libralato@unina.it (G.L.); emilia.galdiero@unina.it (E.G.); marco.guida@unina.it (M.G.)

² Department of Neuroscience, Reproductive and Odontostomatological Sciences, University of Naples Federico II, Via Pansini 5, I-80138 Naples, Italy; delgiudicecarmela93@gmail.com

³ Department of Chemical Engineering and Food Technologies, Faculty of Sciences, University of Cádiz, 11510 Cádiz, Spain; mariacristina.compagnone@uca.es

⁴ Department of Chemical Sciences, University of Naples Federico II, Via Cinthia 21, I-80126 Naples, Italy; maria.toscanesi@unina.it (M.T.); marco.trifuoggi@unina.it (M.T.)

* Correspondence: federica.carraturo@unina.it; Tel.: +39-081-2534625

Abstract: The microbiological monitoring of mineral bottled waters results is crucial for the prevention of outbreaks in consumers. European and International regulations establish the quality of water intended for human consumption in order to preserve human health from the negative effects deriving from water contamination. Advanced methods targeting the faster detection of potential pathogens in drinking water may consent to the creation of an early warning system, enhancing water quality management. This study aimed to suggest the implementation of standard water quality evaluations, based on the characterization of the microbial composition of mineral bottled water brands, contributing to the periodic control of the water's microbiological stability along with the shelf life, and, consequently, the stability of the supplying sources. Bottled water microbiota analysis was combined with the qualitative and quantitative evaluation of microbial loads in time, and the monitoring was performed in two seasons and two different storage conditions for a total of sixty days. The employment of molecular microbiology techniques (NGS and Sanger sequencing), compared to standardized cultural methods and integrated with metagenomic analysis, combining chemical and physical indicators for each sample, allowing for the generation of specific fingerprints for mineral bottled waters, pointing at simplifying and improving the foreseen risk assessment strategies to ensure the adequate traceability, quality and safety management of drinking water.

Keywords: mineral water; shelf life; NGS; microbiota; fingerprints; traceability



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1. Introduction

Mineral bottled water cannot be considered sterile due to naturally occurring microorganisms [1], which as a fingerprint, can sometimes identify the specific water source [2]. In such a scenario, bacteria may be slightly present and harmless, as suggested by the fact that disease outbreaks associated with bottled water consumption are infrequent [3]. Currently, the microbiological controls of mineral waters are oriented towards the determination of the saprophytic microbial load, the identification of pathogenic flora and the search for specific indicators of environmental or human contamination.

In order to avoid water sources contamination due to pathogens and consequent risks for human health, water bottling plants and natural mineral water are frequently monitored [3,4]. Hygienic monitoring of potential pathogens in bottled water results articulated because harmful bacteria tend to be present intermittently and in very low amounts. Therefore, it is considered more effective to monitor the water for indicator

microorganisms, which may be present at higher concentrations. Directive 2009/54/EC in EU [4], which forbids disinfection treatments by defining terms of use and marketing of mineral bottled water, establishes that allochthonous germs, such as total Coliforms, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, must be absent in 250 mL and spore-forming sulfite reducers anaerobes must be absent in 50 mL. In the United States of America, Title 21 of Code of Federal Regulation §165.110 [5] only establishes limits for Coliforms (1 CFU/100 mL) and *Escherichia coli* (absence in 100 mL). Moreover, after bottling, the values of heterotrophic colony count (HPC), with a set limit of 100 CFU/mL at 20–22 °C for 72 h and 20 CFU/mL at 37 °C for 24 h, are determined [5]. The indicated values are measured within 12 h after the bottling process. Despite the fact that European and International regulations establish the exclusive research of the culturable bacteria in mineral bottled waters [4,5], these matrices contain further microorganisms, which are not easily detectable employing the standardized cultural methodologies [6–8], thus underestimating the real quantity and quality of bacteria contained in the drinking bottled water, additionally representing a risk for consumers. Recent studies suggest the employment of culture-independent methods to characterize the dynamics of the microbial community over time inside the bottle [7,9]: the studies report bacterial reactivation along with bottled water storage, together with the succession of microbial communities along with shelf life, probably because of the exploitation of dead microorganisms' organic matter [9,10]. Furthermore, it was demonstrated that bottle material might play a major role in the variations in water microbiota, showing that PET and PVC plastic mainly support the increased replication of microorganisms [11], highly influencing the shelf life determined by the producers [12]. Routine evaluations of mineral water (organoleptic, chemical and microbiological parameters) suggested focusing on the microbial facies representing the fingerprint of natural water [13]. Advanced microbiological characterizations with molecular fingerprinting in mineral bottled waters may indeed contribute to identifying potential not regulated pathogens (e.g., *Acinetobacter* spp., *Campylobacter jejuni*, etc.) and their variations in water sources. Studies focused on the microbial composition of bottled mineral drinking waters are extremely scarce, including research on shelf-life evaluation, as well as sources and water fingerprints characterization.

The present study, based on an innovative methodological approach, is directed at exploiting fingerprinting data and characterized viable microorganisms in bottled water samples to suggest the integration of standardized cultural protocols foreseen by international laws. The study includes molecular analysis, useful in detecting potential pathogens, which are not currently monitored. Based on the purpose, the research conducted had two aims: the first, analyzing the variations in the microbial communities of bottled mineral waters using Sanger sequencing; the second proposes the use of NGS methodology to support a traceability analysis, disposing of microbial fingerprints. The first approach aimed to evaluate the qualitative and quantitative variations in microbial communities during part of the storage time (60 days) of six brands of Italian mineral bottled water (three natural and three naturally sparkling water, stored in PET bottles). The microbial composition of the mineral bottled water under analysis was characterized in two sampling seasons (winter and spring). One specific batch for each of the six mineral bottled water brands, purchased in one store after 3 months since production, was analyzed. PET bottles were exposed to different temperatures, and analyses were conducted using culture-dependent (heterotrophic total bacterial count) and culture-independent (16S rRNA Next Generation Sequencing) methods, at multiple time points, covering two seasons. The study aimed to assess the dynamics of microbial concentrations while storing bottled waters, testing changes in protocols (compared to UNI EN ISO 6222:2001) [14] and improving the ability for microbial recovery. The second phase of the study consisted of characterizing and using molecular microbiology fingerprints, from which it may be possible to compare microbiota of newly produced mineral bottled water batches: the suggested methodology may be considered as an upgraded traceability approach, useful for water brand production and distribution processes.

2. Materials and Methods

2.1. Sample Collection and Experimental Design

Three batches of six Italian commercial brands of bottled mineral water were purchased in February 2019 from one store (Naples, Italy), and the analysis started within 24 h at the Hygiene Laboratories of the Department of Biology, University of Naples Federico II. One batch for each brand was selected, ensuring that the production date was comprised within 3 months prior to purchasing. The selection included three naturally carbonated natural mineral water (indicated in the text as “sparkling”), packaged in 1.5 L polyethylene terephthalate (PET) bottles (named A, D and E) and three natural mineral water (indicated in the text as “flat”), packaged in 2 L PET bottles (named B, C and F). The bottled water brands are sourced from geographically independent Italian water sources. The waters classification was made accordingly with [4,11]. Production and expiration dates were reported on the bottles by the producing company. The expiration dates for flat waters were indicated between June and July 2020 (maintaining an 18-month shelf life); for naturally sparkling water, the shelf life was due between November and December 2019 (12-month shelf life). The analyses were conducted from February to May 2019: the storage analysis was conducted from mid-February to mid-April 2019 (60 days, covering two seasons), and the NGS analysis was conducted on the purchasing day, corresponding to day 1 of the shelf-life analysis.

2.2. Storage Evaluation

The microbial dynamics of mineral bottled waters under evaluation were evaluated based on the reported shelf life, in two seasons (winter and spring) and two storage conditions for a total of 60 days, including the temperature simulating the shopkeepers' conservation of environmental light conditions (variable temperature (VT); i.e., bottled water can be exposed to extreme temperatures in shops or storage rooms) and a controlled constant temperature (constant temperature (CT); 22 ± 2 °C, in the dark).

For the shelf-life analysis, performed in two seasons (winter and spring), in two storage conditions (variable and controlled temperature), after 1, 5, 10, 15 and 30 days each season following samples purchase, 60 bottles of each brand were analyzed in triplicate (for a total of 360 bottles).

The change in total bacterial count in time was evaluated, analyzing samples at five intervals for 30 days in winter (from 19 February to 20 March 2019) and spring (from 21 March to 19 April 2019) seasons. Microbiological analysis and Sanger sequencing analysis were performed on samples under evaluation.

2.2.1. Method Selection for the Enumeration of Culturable Microorganisms

For microbiological analysis and Sanger sequencing, one bottle of each brand was analyzed in triplicate (i.e., three bottles from the same batch; six brands in triplicate and two methods for a total of 36 bottles). Total bacterial count (TBC) was performed employing three different culture methods. Samples were first processed using the standard pour plating protocol (pouring 1 mL sample in enrichment solid medium, UNI EN ISO 6222:2001) [14]; the second step consisted of modifying the UNI EN ISO 6222:2001 standardized method: in aseptic conditions, samples were analyzed using the membrane filtration technique, instead of using pour plating method, employing sterile nitrocellulose membranes with 0.22 µm porosity (instead of 0.45 µm). Filtration was carried out aseptically, filtering 1 L of the sample [15]. For each sample, one nitrocellulose membrane was transferred on Plate Count Agar (PCA), then incubated at 30 ± 2.5 °C for 72 h; another nitrocellulose membrane was transferred in a sterile plastic tube containing 10 mL sterile deionized water. The tube was mixed for 20 s using a vortex, and a 100 µL aliquot was spread plated on PCA solid medium (UNI EN ISO 7218:2013). Plates were incubated at 30 ± 2.5 °C for 72 h; colonies were counted, and the results were expressed in CFU/L. The temperature selected is a mean value of the temperatures required by the ISO method described in UNI EN ISO 6222:2001 (22 °C for environmental microorganisms, 37 °C for

potential pathogens) for evaluating the total heterotrophic microorganisms count in mineral waters [14]. Morphologically different colonies were isolated on the PCA medium and sub-cultured for molecular identification.

2.2.2. DNA Extraction, Amplification and Sanger Sequencing

Isolated morphologically different colonies underwent DNA extraction: in particular, at least three colonies, each with different morphology, were selected for the molecular characterization. Each colony was picked up and reconstituted with 70 µL Milli-Q Type 1 Ultrapure Water. DNA was extracted through denaturation at 98 °C for 10 min, and supernatant recovered after centrifuged samples at 8000 rpm for 5 min at 4 °C [16]. PCR reactions were carried out in a TECHNE Prime Thermal Cycler using universal primers complementary to the V3 and V6 conserved regions of 16 S rRNA gene (700 bp amplicon size) [17]. The selected oligos were V3_F (5'-CCAGACTCCTACGGGAGGCAG-3') and V6_R (5'-TCGATGCAACGCGAAGAA-3'). Each PCR reaction was carried out in 200 µL sterile vials using VWR Chemicals reagents for a final volume of 55 µL of which 5.5 µL PCR Key Buffer Tripton Free (Tris-HCl pH 8.5, KCl, 15 mM $MgCl_2$), 1.0 µL dNTP 12 µM, 0.22 µL forward primer and 0.22 µL reverse primer both 50 µM, 0.5 µL Taq polymerase, 1.3 µL DNA and 47 µL sterile deionized water. The incubation conditions consisted of an initial denaturation at 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 62 °C for 30 s and 72 °C for 30 s. Afterward, a final extension of 72 °C for 5 min was performed. The amplified DNA samples were analyzed on 1.5% agarose gel, stained with GelRed (Nucleic acid gel stain—BIOTIUM), employing a 100 bp DNA ladder as a reference. PCR products were shipped to an external service (Biofab Research srl, Rome, Italy) for purification and sequencing. The sequence data were compared to NCBI Sequence Database sequences [18].

2.2.3. Next-Generation Sequencing Using Illumina MiSeq and Data Analysis

Next-generation sequencing evaluations were performed on the purchasing day, corresponding to shelf-life analysis' day 1, about 3 months after production. As stated before, NGS analysis was employed to suggest a traceability method for the water sources. The resulting microbiota was compared to the analysis conducted using Sanger sequencing. The extraction of the total DNA from water samples was carried out to sequence the metagenome of the sample. The extraction was carried out using CTAB protocol [19] with some variations: as described below, an initial step was added, allowing the concentration of bacteria on a nitrocellulose membrane and the detachment of the concentrated microorganisms, using glass beads and CTAB buffer. For NGS evaluations, 6 bottles, each commercial brand, were analyzed in triplicate (for a total of 108 bottles (i.e., 6 brands in triplicate (6 bottles per replicate)).

For each bottled water commercial brand, 10 L volumes were filtered, and microorganisms were concentrated on nitrocellulose membranes (0.22 µm) under aseptic conditions. The membranes were transferred into sterile 2 mL vials containing 500 mg of glass beads (1–2 mm diameter); the membrane was cut into four parts, and CTAB extraction protocol was carried out. Each sample was reconstituted with 700 µL of CTAB extraction buffer (200-mM Tris-HCl (pH 8.0), 25-mM EDTA (pH 8.0), 250-mM NaCl, 10% CTAB) and 1.4 µL β-mercaptoethanol. Each vial was mixed for two minutes; samples were incubated in a water bath for 30 min at 65 °C and then placed on ice for 15 min at −20 °C. One volume of chloroform: isoamyl alcohol (24:1) was added; the solution was mixed and later centrifuged at 10,000 rpm for 10 min. The aqueous phase was recovered in new tubes and chloroform: isoamyl alcohol (24:1) step was repeated, tubes were centrifuged at 10,000 rpm for 10 min and the aqueous phase was recovered. A 70% volume of ice-cold isopropanol was added, and samples were stored for 45 min at −20 °C, allowing DNA precipitation. Samples were centrifuged at 13,300 rpm, at 4 °C for 15 min, the supernatant was discarded, and the pellet was washed twice with 500 µL of 80% ethanol. The supernatant was discarded, and the DNA was dried and then resuspended in 30–40 µL Milli-Q Type 1 Ultrapure Water. The extracted DNA samples were amplified with PCR using the V3 and V4 primers,

complementary to the V3–V4 variable region of the bacterial 16S rRNA gene (500 bp) [20], and gel electrophoresis was performed to verify templates concentration. Sequencing was performed by an external service employing the MiSeq Illumina platform, using 2 × 300 bp paired-end, for 600 cycles, following the manufacturer’s instructions (Illumina Inc., San Diego, CA, USA).

2.2.4. Physical and Chemical Analysis

On the purchasing day, aliquots from the samples were submitted to physical and chemical evaluations required by regulations and guidelines: bicarbonate (APAT CNR IRSA 2010 Man 20 2003), calcium, magnesium, sodium, potassium (APAT CNR IRSA 3030 Man 20 2003), chlorides, fluorides, nitrates, sulfates (APAT CNR IRSA 4020 Man 20 2003), pH (APAT CNR IRSA 2060 Man 20 2003), conductivity (APAT CNR IRSA 2030 Man 20 2003), total dissolved solids (APHA Methods for water Ed 23rd 2017 2540 C) and free carbon dioxide (APAT CNR IRSA 4010 Man 20 2003) were quantified [21]. The collected data were employed for the metagenomic evaluations and association analysis. Physical and chemical analyses were not performed during storage but only on d₀, testing one bottle for each of the six mineral bottled water types.

2.3. Data Analysis

Shannon diversity indices were calculated, normalizing the reads, calculating the relative abundance of OTU sequences in a sample and normalizing the minimum number of sequences in a sample (18,000). FastQC v0.11.9 was used to perform some quality control checks on raw sequence data. Pre-analysis trimming, adapter removal and filtering steps were performed by Cutadapt 2.8 version, with trimming thresholds value set to 25 (quality cut-off values encoded as ASCII, Phred quality + 33). A 97% threshold was chosen with the intended purpose that the resulting OTUs could be interpreted as a proxy for bacterial species; the database employed is SILVA Release 132. UniFrac distance metrics were calculated using the Phyloseq R package [22]. The Kruskal–Wallis test was used to assess differences between OTUs and taxonomic classifications of the different bottled water groups. Discriminant analysis was performed using linear discriminant analysis in order to discriminate between mineral water groups: an analysis of differential proportions was performed to detect the most and least differentially expressed OTUs. Differences in the group’s communities retrieved from the Illumina experiment were assessed by anosim using weighted UniFrac distance and ANOVA using the Bray Curtis distance (Mothur) [9]. An analysis of β -diversity using unweighted UniFrac, a phylogenetic-based multidimensional scaling analysis, clustered with the statistical significance of the samples according to each of the six commercial brands, was conducted [9]. Principal component analysis (PCA) of the NGS data was performed to analyze differences in microbial compositions between the 6 bottled water brands and the available physical–chemical data and depicted via biplot diagrams. Statistical analysis was carried out using XLStat Version 2019.

3. Results and Discussion

3.1. Physical and Chemical Characterization

According to the hydrochemical facies, based on the analysis performed on the purchasing day (d₀), samples present a dominant cation type corresponding to the magnesium bicarbonate (A, B, C, E and F), demonstrating that weak acids exceed strong acids, and alkaline earth exceed alkalis. These assumptions are not valid for sample D, which belongs to a mixed type where there is not any clear dominant type of cations or anions (Figure 1).

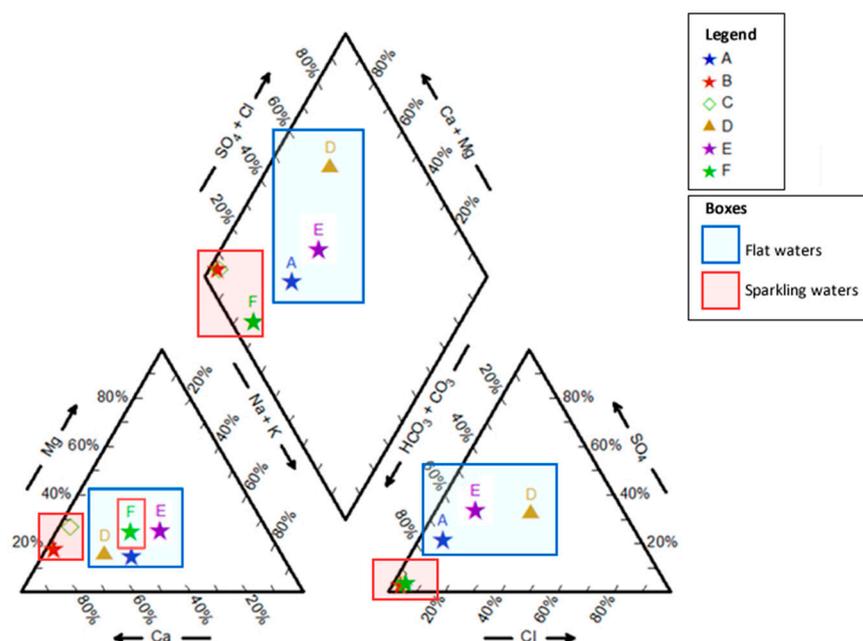


Figure 1. Classification results of the six mineral bottled water brands examined using the Piper diagram. The data elaboration consented to the association between the majority of chemical and physical indicators (calcium, magnesium, sodium, potassium, bicarbonates, sulfates, chlorides). Results of parameters not shown in the Piper are available in Table S1 in Supplementary Materials.

As for the physico-chemical indicators not shown in the Piper diagram in Figure 1 (nitrates, fluorides, total dissolved solids, conductivity, pH, free CO_2), the results are available in Table S1 in Supplementary Materials. The six mineral bottled water brands sensibly differ in conductivity and free CO_2 values, whereas naturally sparkling and flat water sub-groups results are similar, and in the nitrates outcomes, reporting values over 7 mg/L for samples D (sparkling) and F (flat), and below 3.4 mg/L for the others.

3.2. Selection of Microbiological Method

In order to obtain the best microorganisms' recovery in the shelf-life study of mineral bottled waters, the standard cultural protocol, which is pour plating method (1 mL sample), UNI EN ISO 6222:2001 [14], was compared to two additional approaches: the first consisted on filtering 1 L water and plating the membrane on agarised media; the second foresaw 1 L water filtration on the membrane for microorganisms concentration, followed by spread plating of 100 μL aliquots. Hence, 1 L replicates each bottled water sample (instead of 1 mL for pour plating and 100 mL for membrane filtration) for each analysis method (pour plating, membrane filtration, filtration and spread plating) that underwent cultural analysis. The results of the comparison between the three cultural methods employed in the present research are available in Table 1: the standardized method and the membrane filtration method, evidence a lower recovery number, with respect to the proposed method, consisting of the addition of spread plating, suggesting that the modified method holds higher recovery rate compared to standardized cultural methods in use.

The outcomes showed that the suggested modified protocol, consisting of the addition of the spread plating method step after the membrane filtration, holds higher detection limits than the standard protocol (pour plating): for this reason, it was employed for shelf-life analyses. It must be specified that the batches employed for the experiments summarized in Table 1 are different from the batches evaluated in storage analysis: for this reason, the d_0 value results are different.

Table 1. Results expressed in CFU/L were obtained by quantitative analysis using the three methods. Limit of detection: 1 CFU/L (whereas a plate was free of microorganisms, the value < 1 was indicated).

Sample Code	Type of Water	Pour Plating (Standard) [CFU/L]	Membrane Filtration [CFU/L]	Filtration + Spread [CFU/L]
A	Sparkling	<1	8 ± 1.15	67 ± 2.96
B	Flat	<1	333 ± 6.24	19,300 ± 28.14
C	Flat	<1	<1	<1
D	Sparkling	<1	302 ± 4.58	13,200 ± 28.87
E	Sparkling	<1	<1	<1
F	Flat	<1	14 ± 1.76	9260 ± 61.10

3.3. Bottled Water Storage Evaluation

Microbial communities change significantly after bottling and during storage [23]. The variations in the microbial communities along the storage may represent a health risk for consumers, whereas inadequate storage of bottled mineral waters may lead to the proliferation of pathogenic microorganisms, whose monitoring is not foreseen by current laws. In order to verify the potential microbial changes, in the present study, a 2-month shelf-life assessment of the bottled waters under analysis (over the 12–18 months of storage) was performed during storage. The analysis of qualitative and quantitative microbial communities' variations in branded bottled water was assessed considering two storage methods: constant temperature (coded as CT) and variable environmental temperature (coded as VT), in winter and spring seasons, using membrane filtration technique followed by spread plating, and the modified proposed cultural method, which resulted in the highest microorganisms recovery rate.

3.3.1. Microbiological Analysis

The changes in microbial load in time, comparing the two described storage methods, during the two seasons considered are shown in Table 2.

During the shelf-life evaluation, samples A, D, E (i.e., naturally sparkling bottled waters) were free of culturable microorganisms in both seasons and storage methods. Sample B, stored at different conditions in the two considered seasons, registered similar trends in the microbial load. The only exception in B was about storage data from variable winter temperatures: a marked decline in the number of microorganisms between day 5 and day 10 was described. The decrease may depend on the low temperatures recorded between day 5 and day 10, which is 5 °C lower than the mean of the 30 days of the analysis. The same batches, stored in the spring season, registered, in general, higher microbial loads, together with a 2-log increase until day 15 at a constant temperature, probably due to higher temperatures, especially considering the batch stored at variable (environmental) temperatures. The microbial loads of sample C registered a decrease on day 10 at variable winter temperature, compared to storage at constant temperatures, thus reporting the same changes in microbial load for both the storage methods along the 30 days. Moreover, in this case, the reason may be linked to the lower temperatures. In spring, the trend of the microbial load was similar in the two storage methods. Sample F, in winter, showed a similar trend both at constant and variable temperatures; in samples exposed at variable temperature, microbial load remained mainly stable. Sample F storage at a constant temperature, in winter, registered a microbial load decrease to 0 CFU/L until day 10, followed by an increase at day 15 and again a 0 CFU/L concentration at day 30; at variable temperatures, microbial loads decreased to 0 CFU/L after 5 days storage. Such microorganism intermittent growth may be justified by the fact that constant temperature was 7 °C higher than the mean of variable temperatures. The shelf-life evaluation allowed establishing that constant temperature storage is preferred to variable temperature storage, considered the lower microbial loads variability registered at constant temperature conditions (Table 2). In most cases, excluding sparkling water, where no viable cells were isolated, the microbial count reaches values of about 10⁴ CFU/L, in agreement with previous studies [9,23], remaining constant after 30 days, compared to initial analysis results.

3.3.2. Characterization of Isolated Microorganisms

The evaluation of the microbial facies isolated from the shelf-life investigation was characterized using Sanger sequencing: the molecular identification resulted essential to detecting especially pathogenic bacteria, providing the opportunity to dispose of both genus and species information for each isolated bacterium, thus producing additional information to microbiological evaluations. The microorganisms identified are described in Table 3. Additional information on identified microorganisms in water samples B, C and F, comparing the obtained results with available bibliography, are described in Tables S2 and S3 in Supplementary Materials.

Table 3. Results of Sanger sequencing on colonies isolated from the bottled waters during the shelf-life analysis conducted in winter and spring seasons: codes B, C and F indicate the 3 brands of flat waters, codes A, D and E indicate the 3 brands of sparkling waters. No colonies were isolated from sparkling waters. Microorganisms in bold evidence the germs isolated in both winter and spring evaluations.

Type of Water	Sample	Microorganisms in Winter	Microorganisms in Spring
Flat	B	<i>Acidovorax delafieldii</i>	<i>Acidovorax delafieldii</i>
		<i>Acidovorax facilis</i>	<i>Aquabacterium commune</i>
		<i>Aquabacterium parvum</i>	<i>Blastomonas natatoria</i>
		<i>Brevundimonas vesicularis</i>	<i>Delftia acidovorans</i>
		<i>Caulobacter henricii</i>	<i>Ensifer adhaerens</i>
		<i>Pseudomonas fluorescens</i>	<i>Flavobacterium succinicans</i>
	C	<i>Pseudomonas veronii</i>	<i>Pseudomonas alcaligenes</i>
		<i>Rhodoferax saidenbachensis</i>	<i>Pseudomonas fluorescens</i>
		<i>Stenotrophomonas maltophilia</i>	<i>Pseudoxanthomonas mexicana</i>
			<i>Pseudomonas veronii</i>
			<i>Rhodoferax saidenbachensis</i>
			<i>Sphingopyxis ginsengisoli</i>
F		<i>Sphingopyxis soli</i>	
		<i>Stenotrophomonas maltophilia</i>	
		<i>Acidovorax radialis</i>	
		<i>Brevundimonas mediterranea</i>	
		<i>Acidovorax radialis</i>	
Sparkling	A	-	-
	D	-	-
	E	-	-
		<i>Delftia acidovorans</i>	<i>Delftia acidovorans</i>
		<i>Delftia lacustris</i>	<i>Delftia lacustris</i>
		<i>Pantoea agglomerans</i>	<i>Pantoea agglomerans</i>
		<i>Sphingomonas aquatilis</i>	<i>Stenotrophomonas maltophilia</i>
		<i>Acidovorax delafieldii</i>	<i>Acidovorax delafieldii</i>
		<i>Pseudomonas extremaustralis</i>	<i>Pseudomonas extremaustralis</i>
	<i>Pseudomonas veronii</i>	<i>Pseudomonas veronii</i>	
	<i>Stenotrophomonas maltophilia</i>	<i>Stenotrophomonas maltophilia</i>	

The results evidence that the presence of genus *Acidovorax* sp., followed by *Pseudomonas* sp., according to the employed methodologies, seems to be dominant in flat water samples during the winter season. The species *Rhodoferax saidenbachensis* in sample B was isolated both in the winter and spring seasons. In sample C, *Acidovorax radialis*, *Delftia acidovorans*, *Delftia lacustris* and *Pantoea agglomerans* were isolated in both seasons; in sample F, *Acidovorax delafieldii*, *Pseudomonas extremaustralis*, *Pseudomonas veronii* and *Stenotrophomonas maltophilia* were detected both in winter and spring seasons. For the shelf-life experiments, no bacteria were identified in naturally sparkling water, since samples were free of microorganisms. Based on the classification of pathogens and

opportunistic pathogens available from the bibliography (Tables S2 and S3 in Supplementary Materials [24–46]), in sample B, *Brevundimonas vesicularis* [31], *Pseudomonas fluorescens*, *Pseudomonas alcaligenes* and *Stenotrophomonas maltophilia* were identified, and the latter was described as a common colonizer of water and soil [42]; in sample C, *Pantoea agglomerans* [47] was characterized; in sample F, *Stenotrophomonas maltophilia*, a common inhabitant of water [27], was present; in samples A, D and E, no microorganisms were isolated. The data obtained show that samples C and F have fairly stable microbial facies between the two seasons, unlike sample B, for which greater variation in microbial species is observed. These results are not attributable to quite similar chemical–physical characteristics assessed on the day of purchase, and it is, therefore, conceivable that these parameters will also be influenced by the way bottled mineral waters are conserved and that they, in turn, could influence microbial facies.

3.4. Next-Generation Sequencing Analysis

The next-generation sequencing analysis performed on the six mineral bottled water brands at the beginning of the storage evaluation allowed characterizing the microbial composition of specimens under analysis, discriminating between the six bottled water samples. In total, 591,042 sequencing reads were generated by 16S rRNA sequencing of the six mineral bottled water brands analyzed. After removing the low quality, chimeric and non-bacterial reads and 418,624 bacterial 16S rRNA sequencing reads with high quality (70.82% of the total reads) were obtained for data analysis. The Shannon diversity index, indicating common measures of bacterial diversity between the analyzed bottled water samples, and highlighting information regarding the microbial community composition, was greater in sparkling waters compared to flat waters: Figure S1 in Supplementary Materials describes the high microbial diversity in the sparkling waters group (on the left), additionally evidenced by the low mean value. On the other hand, the flat water group data (on the right) highlight a lower diversity between the samples, which may be translated into a lower microbial diversity, and therefore a higher OTUs similarity. In the sparkling water samples, the average is lower than flat water samples: the diversity is brought by sample A, reporting a higher OTUs count, compared to the samples from the sparkling water group (D and E). Furthermore, the two groups of samples (flat and sparkling water) evidence a diversity that overlaps between 2.8 and 3.2.

Each of the six bottled water brands holds different microbial communities at Phylum, Class, Genus and OTU levels, as shown in the barplot (Figure 2 and Figure S2–S4 in Supplementary Materials).

The highest abundance at Phylum level was observed in A water (sparkling), where Proteobacteria (60%), Actinobacteria (20%) and Bacteroidetes (10%) were the most prevalent Phyla. The highest richness at Phylum level was instead evidenced in B water (flat), where strains from Proteobacteria (93%), Bacteroidetes (4%), Patascibacteria, Omnitrophicaeota, Acidobacteria, Elusimicrobia, Verrucomicrobia and Fibrobacteres Phyla (all <1%) were characterized (Figure 2 and Figures S2–S4 in Supplementary Materials).



Figure 2. Community structure of the mineral water brands at Phylum (a), Order (b) and Genus (c) levels according to 16s rRNA sequencing analysis. Results are expressed as the relative abundance of reads. (Refer to the web version of the paper and to Figures S2–S4 in Supplementary Materials for the interpretation of colors and references in the legend.)

The dominant microorganisms, in flat waters, at Genus level, were *Polaromonas* (54%), *Parvibaculum* (13.8%), *Perlucidibaca* (9.7%) and *Rhodoferax* (5.6%) in sample B; *Rhodoferax* (53%), *Pseudorhodobacter* (22%), *Acidovorax* (5.8%) and strains from Solimonadaceae Family (5.3%) in sample C; and *Curvibacter* (49%), Xanthobacteraceae (10%), *Hyphomicrobium* (10%), Nocardioideae (8%) in sample F. In sparkling waters, the most present Genera were strains from the Burkholderiaceae Family (37%), *Solirubrobacter* (7.6%), *Rhodocytophaga* (7.4%) and *Pseudomonas* (5.3%) in sample A; *Phreatobacter* (64%) and *Methylothermobacter* (27.2%) germs from the Burkholderiaceae Family (5.8%) in sample D; and *Sulfuritalea* (67.7%) strains from the Burkholderiaceae Family (17.3%), *Caulobacter* (1.6%), *Bacillus* (1%) in sample E. Several unclassified bacteria were isolated (Figure 2 and Figures S2–S4 in Supplementary Materials). It is, however, important to note that 16S rRNA gene amplicon sequencing covers DNA from both active and dead, inactive cells, and it may be possible that a bacterial viability assessment would have allowed the detection of a lower variety of microorganisms. Further studies will consider the implementation of the technologies in order to consent to a more targeted characterization.

3.4.1. Comparison between Sanger Sequencing of Cultivated Bacteria and NGS Results

Many genera detected by MiSeq Illumina analysis were not detected by Sanger sequencing, with the exception of *Acidovorax* sp., *Aquabacterium* sp., *Pseudomonas* sp., *Flavobacterium* sp. and *Rhodoferax* sp. in sample B; and *Acidovorax* sp., *Delftia* sp. (from Burkholderiaceae Family) and *Sphingomomas* sp. (Sphingobacteriales) in sample C. In sample F, no sequences were common between NGS and Sanger sequencing analysis. Since no viable bacteria were isolated from samples A, D and E for Sanger sequencing, the comparison with NGS was not possible. Beyond NGS higher sensitivity, with respect to cultural methods, followed by the Sanger sequencing approach, it can be hypothesized that the majority of OTUs detected using next-generation sequencing techniques were not recovered employing cultural methods because microorganisms may not be viable. Hence, besides the microbial composition of water sources could be mainly the same, the presence of viable strains detected employing Sanger sequencing could be linked to contamination at the production plant level. Therefore, in terms of water safety, the Sanger sequencing should still complement NGS techniques: alternatively, for practical applications, molecular methods that are able to detect pathogens more quickly, compared to standard cultural methodologies based on Sanger sequencing outcomes, may represent an essential tool for periodic water source monitoring, pointing at microbial risk analysis. In addition, the differences between the two methods could be linked to the use of different primers pairs, together with the final amplicon length [9].

3.4.2. Comparison with Similar Studies

The comparison with similar studies, aimed at the evaluation of mineral waters at the source or at bottling plant level, resulted useful to detect common genera in the water samples examined. For example, in the research conducted by Sala-Comorera et al. [9] in 2019, the genus *Polaromonas* resulted ubiquitous in being detected in more than 50% of bottled mineral water samples: genus *Polaromonas* was detected in the present study in sample B (54%) and C (3%). Strains from *Acidovorax* (samples C and E), *Bacillus* (in A, D and F), *Novosphingobium* (in B, C, D, E and F), *Pseudomonas* (in A, B, D and E), *Rhizobium* (in A, C and F), *Staphylococcus* (in A, B, E and F) and other genera were detected in similar studies [7–9,23,48]. Furthermore, strains of *Strenotrophomonas* and *Sulfuritalea* genera, isolated employing cultural methods but not with the NGS approach, were also typical of mineral waters analyzed in current studies [48]. Brumfield et al. (2020) recently demonstrated that Actinobacteria and Proteobacteria are the dominant bacterial phyla detected in bottled water. The majority of Betaproteobacteria was identified in sparkling natural mineral bottled water and Gammaproteobacteria in non-mineral bottled water. Burkholderiales were prevalent in sparkling natural mineral bottled water and Alteromonadales and Enterobacteriales in diverse non-mineral bottled water. *Propionibacterium* was observed in

both sparkling natural mineral and non-mineral bottled water [49]. Lesaulnier et al. (2017) analyzed the microbiota of 12 natural mineral water samples from six European countries. They identified less than 10 species-level OTUs dominating the bacterial communities: particularly, members of the Betaproteobacteria genus, such as *Curvibacter*, *Aquabacterium* and *Polaromonas* (Comamonadaceae), which represent ubiquitous heterotrophic aerobes in most bottled waters [22]. Lately, Sala-Comorera et al. (2020) identified Gammaproteobacteria in bottled mineral water with *Acidovorax* the dominant genera in 3 out of 7 brands analyzed, each one featured by highly diverse and characteristic bacterial communities. Proteobacteria resulted as the dominant Phylum identified with NGS (>99% of the reads). The 85% of the isolated bacteria detected with cultural method also belong to Proteobacteria Phylum, followed by Actinobacteria, Firmicutes and Bacteroidetes [50].

3.4.3. Definition of Fingerprints for Water Traceability

The essential advantage of conducting this research lies in the possibility to define fingerprints for each bottled water brand, with the aim of being able to use this method to establish water traceability identifying early variation in the microbial facies that could be reflected in the emergence of potentially pathogenic bacteria. The results allowed demonstrating that the six bottled water brands analyzed only hold one OTU in common at the family level (Burkholderiaceae). Furthermore, at the genus level, sample A can be detected employing 14 marker OTUs, exclusively isolated in the specific water (e.g., *Solirubrobacter* and *Rhodocytophaga*), as much as the other brands: respectively, 27 unique OTUs from sample B (e.g., *Parvibaculum* and *Cavicella*), 5 unique OTUs from sample C (e.g., *Pseudorhodobacter* and *Acidovorax*), 6 unique OTUs from sample D (e.g., *Phreatobacter* and *Tunicibacter*), 5 unique OTUs from sample E (e.g., *Sulfuritalea* and *Ferribacterium*) and 15 unique OTUs from sample F (e.g., *Hyphomicrobium*) were detected. The outcomes, therefore, support the differential classification, and thus the traceability, of the brands under analysis, based on the proportions between the detected OTUs. Fingerprinting analyses are surely expensive, and at the current technology development status, it could be difficult to implement such an approach for mineral bottled water distributing companies: however, yearly characterizations supported with less expensive molecular techniques targeting new indicators could be more affordable.

3.5. Metagenomic Analysis

Principal Component Analysis

A multivariate analysis employing Principal Component Analysis of MiSeq Illumina fingerprints associated with metadata, such as pH and conductivity, revealed differences between mineral water brands (Figure 3): axes F1 and F2 explained 76.56% and 20.51% of the variance, respectively. The two groups of samples (flat versus sparkling waters) are positioned in opposite quarters of the figure. Samples D and E are the closest to free CO₂, conductivity and total dissolved solids parameters, while samples B, C and F are closer to pH parameters. Samples B, C and F (flat waters) and sample A (sparkling) are directed towards the Shannon Index line.

The principal component analysis demonstrated the existence of distinct bacterial communities depending on the water brands evaluated. A, D and E water brands (sparkling) are distinct from the other brands (flat waters), whereas flat waters share similarities, as well as sparkling samples (Figure 3). Flat waters (B, C and F) hold high microbial diversity, while A has mid diversity; D and E samples had low diversity instead.

Values of pH were similar for flat waters (B, C and F), compared to differences evidenced for sparkling, whereas D and E had similar values, although different from A. Free carbon dioxide values are, of course, higher for sparkling waters, in spite of flat waters. Conductivity and total dissolved solids concentrations are highly comparable in D and E, and lower in sample A (whose chemical parameters concentrations are intermediate between those of the other samples); conductivity values in flat water brands are, on average, three times less concentrated than sparkling samples, while total dissolved solids

results report values which are 5–6 times higher in sparkling waters, in contrast with flat waters.

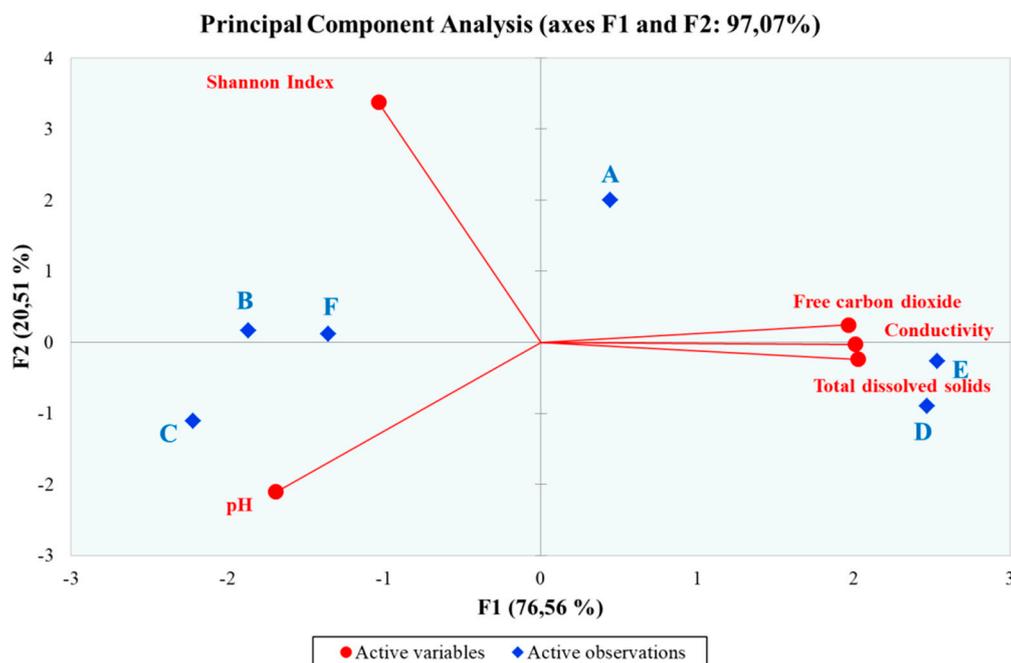


Figure 3. Principal components analysis (PCA) with the MiSeq Illumina fingerprints. F1 and F2 are shown on x and y axes, respectively. For a better comprehension of Figure 3, samples were colored in blue and parameters considered in the analysis in red. Whereas the samples are positioned in different or opposite quarters with respect to the parameters and are therefore far from the parameters lines, lower values of the considered parameters were registered. If samples registered higher values for specific parameters, they are positioned closer to the respective physical–chemical indicator.

The advantage of exploiting microbial fingerprints in the evaluation of mineral bottled waters risk analysis lies in the possibility of detecting potential pathogens in the water before the bottled mineral waters reach the distribution phase. In Europe, nonetheless, with the recent upgrade of the Drinking Water Directive in 2020, with the release of Directive (EU) 2020/2184 [51], the microbiological risk assessment for drinking water was not considered a priority with respect to chemical risk analysis (microplastics, endocrine disruptors and lead were added as additional parameters for water safety analysis assessment). The obtained results, analyzing higher water volumes and employing molecular microbiology methods, suggest the importance of complementing standard cultural protocols with more advanced techniques, supporting the identification of emerging pathogens in bottled drinking water.

4. Conclusions

The present study reports the results of preliminary research aimed to identify the diversity in the microbial composition of bottled natural mineral water brands, based on the physical–chemical composition and the storage conditions, additionally comparing Sanger and NGS approaches. The research also pointed at suggesting practical applications for the implementation of microbial risk analysis for mineral bottled water distribution plants, with the possibility to identify pathogens from changes in microbial composition. The shelf-life investigation was essential to analyze the trends of microbial loads in time and the evaluations performed for thirty days in two seasons and two different storage conditions (constant and variable temperatures) provided an essential tool for the definition of the best shelf-life conditions. The employment of a modified cultural method to better concentrate microorganisms allowed to enhance bacterial recovery. Recent advances in molecular characterization allowed for the generation of typical profiles for each bottled water, together with markers, bringing out the possibility to differentiate bottled mineral

water brands based on their specific and unique microbial composition strictly related to the peculiar physical–chemical composition. However, further studies on different batches of the bottled water brands analyzed will be required in order to confirm the repeatability of the fingerprints methodology. The suggested analytical approach may also be employed for the improvement of groundwater quality and traceability analysis: the systematic monitoring of the microbial facies could indeed consent to the identification of pathogens, whose development may alter water sources, resulting in serious risks for human health. Nevertheless, it is important to specify that, even if NGS analysis represents a fundamental approach for microbial diversity and bottled water safety, it is not able to differentiate between viable and non-viable microorganisms. The employed methodology was based at a lab-scale level, and of course, foresees further evaluations, especially on-field applications, to verify the on-site feasibility of the method. Future evaluations will be focused on the exclusive analysis of viable bacteria fractions to set up reliable fingerprints as a reference for official and internal periodic monitoring aimed to verify both the quality and safety of mineral bottled water. As an example, NGS analysis, improved with the characterization of viable microorganisms, would be essential for the identification of potentially pathogenic genera, whose research is not foreseen by current laws. Multiplex molecular tests, using primer sets selective for the newly identified genera, may be implemented as complementary methods for health risks prevention. The lack of upgrades in the microbiological risk assessment (MRA) of bottled mineral water in the recently released European Drinking Water Directive (Directive (EU) 2020/2184) brings to the urgent need of proposing the update of available microbiological methods for the detection of pathogenic microorganisms in bottled drinking water. Indeed, the characterization of specific microbial fingerprints for different bottled water brands may ensure improvements in the MRA, preventing the onset of waterborne outbreaks. Both authorities in charge of official controls and bottled water plants managers may benefit from the implementation of upgraded methodologies, hypothetically consisting in the periodic (seasonal evaluations, i.e., four times per year) fingerprint analysis of the bottled water sources, supporting the standard cultural methods in the detection of emerging pathogens: fingerprint analysis may constitute an early-warning system, enabling the online detection of potential pathogens in the sources, thus consenting the prompt application of risk mitigation and prevention measures before batches reach the market. Hence, the analysis of shelf life complemented with the evaluation of the differences in the microbiota indeed opens to the possibility of monitoring the quality of bottled water along with the storage, aiming to preserve consumers' health.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/w13202824/s1>: Table S1. Results of physical–chemical parameters not shown in the Piper diagram (Figure 1), expressed in the respective unit of measure for the six mineral bottled water brands examined in the study; Figure S1. Changes in the Shannon index of diversity based on the number of 16S rRNA Illumina reads; Figure S2. Community structure of the mineral water brands at Phylum level according to 16s rRNA sequencing analysis. Results are expressed as the relative abundance of reads; Figure S3. Community structure of the mineral water brands at Order level according to 16s rRNA sequencing analysis. Results are expressed as the relative abundance of reads; Figure S4. Community structure of the mineral water brands at Genus level according to 16s rRNA sequencing analysis. Results are expressed as the relative abundance of reads; Table S1. Results of physical–chemical parameters not shown in the Piper diagram (Figure 1), expressed in the respective unit of measure, for the six mineral bottled water brands examined in the study; Table S2. Microorganisms identified with Sanger sequencing in samples B, C and F from shelf-life evaluation in the winter season and isolated both in sample storage at variable temperature (VT) and at constant temperature (CT). (AE: aerobe; AN: anaerobe; FA: facultative aerobe; T: growth temperature; M: mesophilic; P: Proteobacteria.) In the “Main sources of isolation” column, whereas germs were previously isolated from mineral waters, it was evidenced in bold; Table S3. Microorganisms identified with Sanger sequencing in samples B, C and F from shelf-life evaluation in the spring season and isolated both in sample storage at variable temperature (VT) and at constant temperature

(CT). (AE: aerobe; AN: anaerobe; FA: facultative aerobe; T: growth temperature; M: mesophilic; B: Bacteroidetes; P: Proteobacteria.) In the “Main sources of isolation” column, whereas germs were previously isolated from mineral waters, it was evidenced in bold.

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