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Unravelling the composition of tap and mineral water microbiota: divergences between next-generation sequencing techniques and culture-based methods

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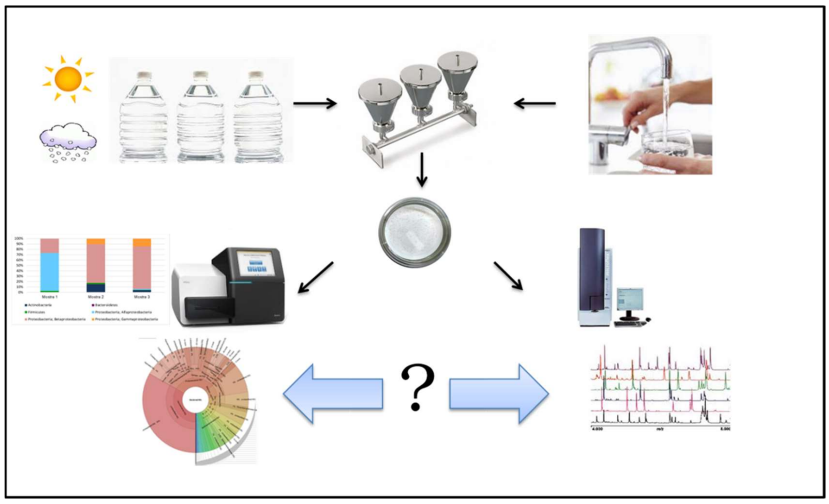
Graphical abstract



Highlights (3 to 5 bullets 85 characters)

- Tap water from different sources differs in their microbial community composition.
- Each mineral water brand has its own microbial community composition.
- Heterotrophic bacteria counts depend on the season and mineral water brand.
- MALDI-TOF MS and NGS are complimentary to study the microbiota of drinking water
- Important taxonomic differences between both approaches were detected.

Abstract

Drinking water is a complex and highly unexplored microbial environment with a high diversity, involving mainly bacteria at different metabolic states. The aim of this work was to characterize the bacterial communities from two main drinking water sources used in modern societies, tap water and mineral bottled water. A total of 11 tap water samples from different locations and distribution networks and 10 bottled natural mineral water brands were analysed using two different approaches that target different bacteria in different physiological states: a) MALDI-TOF mass spectrometry (MS), to characterize the cultivable heterotrophic communities, often referred to as HPC in the regulations, and b) Illumina amplicon sequencing for the non-cultivable. Cultivable heterotrophic bacteria were isolated in WPCA (ISO) agar at 22 ± 2 °C for 72 h and 2,042 isolates identified using matrix-assisted laser desorption/ionization time of flight mass-spectrometry (MALDI-TOF MS). The Bruker Daltonics Library and a previously customized library (Drinking Water Library, DWL) related to water were used as reference databases. Important differences were observed between mineral and tap water with a general dominance of Alphaproteobacteria, mainly of *Blastomonas* genus in tap water and Gammaproteobacteria in mineral water. The bacterial communities present in the different water brands were highly diverse and characteristic of each one. Moreover, the season in which the water was commercialized also affected the species distribution, with some of them being identified in only one season. For the non-cultivable fraction, DNA was extracted from 6 l of water and Illumina 16S rRNA sequencing of the v4 region performed. Among the cultivable bacteria, the phylum Proteobacteria was the most abundant (around 85% of the isolates), followed by Actinobacteria, Firmicutes and Bacteroidetes. Proteobacteria was also the most abundant phylum detected with Illumina sequencing (>99% of the reads). Differences between both methods were observed at the different taxonomic levels especially at genus level. Therefore, both approaches should be regarded as complimentary for understanding the microbiota of mineral water environments. MALDI-TOF mass spectrometry could be a promising method for routine water analysis for the rapid identification of heterotrophic bacteria for bottling industry management.

Keywords: Mineral water, Microbiota, MALDI-TOF mass spectrometry, Illumina, 16S rRNA sequencing

Significance and Impact of the Study: Methods used here demonstrate the complementarity of MALDI-TOF MS and NGS to assess the diversity of bacterial communities in water intended for human consumption that can be used routinely in the water industry for water quality management.

1. Introduction

The access to safe drinking water is a human right although more than two thousand million people still do not have access to it (UNICEF and WHO, 2019). Two of the most important sources of drinking water in high income countries include tap water and bottled natural mineral water (referred to as mineral water from now on). Both waters have different origin and characteristics and are subjected to different water quality regulations. In Europe, the quality of tap water is regulated by the Drinking Water Directive 98/83/EC, which is currently under revision, whereas bottled natural mineral water is regulated by Directive 2009/54/EC. Tap water is usually treated water produced by drinking water treatment plants (DWTP) to comply with the regulations that arrives to homes through a distribution network. Meanwhile, natural mineral waters are originated from protected underground water sources which differ from ordinary drinking water by their purity at source and their constant level of minerals and are intended for direct consumption without any disinfection or chemical treatments. In both regulations, from a public health point of view, microbial water quality monitoring relies on the detection and quantification of bacterial faecal indicators to guaranty the absence of pathogens and the enumeration of heterotrophic bacteria (HPC).

Heterotrophic bacteria are those microorganisms requiring organic carbon for their growth. Although different methodologies for assessing the HPC are available, a standardized method exists based on mass inoculation of the sample with yeast extract agar and incubation at a given temperature and stipulated time (ISO, 1999). The maximum number of HPC allowed for treated water at the DWTP is 100 per millilitre at 20 to 22 °C in 72 hours and HPC shall not suffer any changes in their concentration during distribution. In the case of mineral water, HPC is analysed within 12 hours and must comply the same limits as for treated water but it also includes a limit of 20 HPC per millilitre at 37 °C in 24 hours. At the marketing stage the HPC may only be that resulting from the normal increase in the bacterial count which it had at source.

The incorporation of the HPC in the different regulations responds to different reasons. First of all, they are good indicators of regrowth and biofilm formation through the different steps of the water treatment process and distribution networks and pipelines. Second, HPC englobes a wide range of bacterial genera, among which some opportunistic pathogens such as *Aeromonas*, *Klebsiella* and *Pseudomonas* which are typically recovered by traditional HPC methods stated in the regulations and may have consequences from a public health of view perspective, especially for the elderly and immunocompromised people. Nonetheless, their clinical significance has been questioned (Allen et al., 2004). Last but not least, the main reason for its incorporation in the regulations, is that a high number of heterotrophic microorganisms may interfere with the enumeration of the faecal indicator microorganisms (Edberg et al., 2000).

Nevertheless, it should be noted that bacteria recovered by these methods represent a minor proportion of the actual bacterial diversity of the water samples since the majority of the bacteria present in water are non-cultivable (in many cases <1%) (França et al., 2014; Loy et al., 2005). Among culture independent methods for bacterial enumeration, microscopy enumeration using vital fluorescence dyes, such as syto 9 and propidium ioidide, which allows differentiation between live and dead cells, has shown a difference of up to 2 log units with respect to traditional HPC counts (Sala-Comorera et al., 2019), whereas flow cytometer-based cell counts have reported a difference up to 4 log units (Lautenschlager et al., 2013). Therefore, the current regulations underestimate the real number of microorganisms and thus any risk assessment based on HPC counts suffers from several limitations. Moreover, in the case of mineral water, HPC must be determined 12 h after bottling. However, an increase in the bacterial counts after bottling with a peak 1-3 weeks after bottling has been reported (Diduch et al., 2016; Falcone-Dias and Farache Filho, 2013; Sala-Comorera et al., 2019; Urmeneta et al., 2000), probably due to water oxygenation and temperature rise together with the release of organic matter from the bottles (Warburton et al., 1998; Warburton, 2000).

In spite of the abundance, the characterisation of the microbial populations in drinking water is also important for water managers, since it may allow the detection of impairment of the water treatments and/or in distribution pipelines or bottling plants. In regards to routine monitoring, a rapid, easy, affordable and if possible high-throughput method is required. Among cultivable methods, the use of MALDI-TOF mass spectrometry has shown great promise for the rapid characterization of bacteria isolated from drinking water treatment plants (Laura Sala-Comorera et al., 2016b) with equivalence or superior resolution power than other identification methods such as Sanger 16S rRNA sequencing of bacterial isolates or biochemical based identification such as API strips (Biomérieux). On the other hand, the advent of massive sequencing techniques has facilitated enormously the study of the non-cultivable fraction of the microorganisms due to the high-throughput power of these methods at a reasonable price. The studies performed so far have shown that water bacteria are largely dominated by Proteobacteria, followed by Actinobacteria, Bacteroidetes and Firmicutes (Tamames et al., 2010). However, 16S rRNA amplicon sequencing by NGS techniques also suffers from some shortcomings such as the bias due to the selected primers or platforms or the limitation to detect low abundant species, within a theoretical limit of detection of 0.01% abundance in a typical sequencing deep of 10,000 reads.

The aim of this study was to compare two rapid methods for the characterization of the cultivable and non-cultivable microbial communities present in two types of drinking water, tap water and natural mineral bottled water. To this end, 11 tap waters of different distribution systems and locations and 10 non-carbonated mineral water brands were assessed. Mineral water brands were analysed one month after bottling in two different periods of the year to assess the effect of temperature on the composition of bacterial communities in the bottles at the retailers'. In order to characterize the cultivable bacteria, matrix-assisted laser desorption/ionization time of flight mass-spectrometry (MALDI-TOF MS) was used for the rapid identification of waterborne bacteria using the custom database "Drinking Water Library" made in a previous study for Bruker Daltonics instrumentation (Galofré et al., 2019). Non-culturable communities were assessed by

V4 16S rRNA massive sequencing using Illumina Miseq sequencing platform. The bacterial communities in the different water matrices are reported.

2. Material and methods

2.1. Samples and sampling conditions

Eleven taps including 2 public fountains and 9 household taps were randomly selected from a variety of locations and distribution systems (Table S1). In order to guaranty that the samples were representative of water consumed, six litres of water were directly collected from the faucet without cleaning the faucet by directly flushing the water into the sterile bottles. Sterile sodium thiosulfate was added to the samples immediately after sampling, so as to neutralize chlorine from treated water. Samples were transported to the laboratory at 4 ºC for further analysis.

In the case of mineral water analysis, ten commercial bottled natural mineral water brands were selected for this study. Samples consisted of 1.5 litre polyethylene terephthalate (PET) recently bottled mineral water, purchased from local retailers. Samples were taken to the laboratory and stored in the dark at 22 ± 2 ℃ and analysed one month after bottling. Three bottles per batch were analysed. Additionally, three brands (M01, M02, and M03) were selected to assess the variation in the microbial populations between different seasons and batches. In this case, two batches per brand were taken during the summer season and two batches more during the winter season. Each batch of each brand was analysed in triplicate by selecting and analysing 3 independent bottles of each batch.

2.2. Heterotrophic bacteria enumeration

For heterotrophic enumeration (HPC), sample volumes ranging between 100 µl and 100 ml were filtered through 0.22 µm pore size nitrocellulose membrane filters (Millipore, Germany). Those samples with volumes less than 10 ml were filtered together with 10 ml of sterile Ringer 1/4. Filters were incubated on Water Plate Count Agar ISO (Oxoid, Spain) supplemented with 0.5 g/L 2,3,5-triphenyltetrazolium chloride for 72 hours at 22 ± 2 ℃ and red colonies were enumerated. All the samples were analysed in triplicate.

184

185 **2.3. Bacterial community characterization**

186 **2.3.1. Matrix-Assisted Laser Desorption/Ionization time of flight mass-spectrometry**

187 **(MALDI-TOF MS)**

188 Around 50 isolates per sample were selected when possible and subcultured for MALDI-TOF MS
189 identification. Analysis was performed by the extended procedure using 70% formic acid as
190 recommended by the manufacturer and α-cian-4-hydroxycinnamic acid as matrix (Bruker
191 Daltonics protocol). As a whole, a total of 1,222 isolates were analysed and the obtained spectra
192 screened against the Bruker Daltonics bacteria database and the customized Drinking Water
193 Library (Galofré et al., 2019). If the obtained score was ≥ 2.000 the isolate was identified with
194 high probability at genus level and possibly species level, whereas a score ≥ 2.300 was indicative
195 or probable identification at species level. A score < 1.700 indicated that the isolate could not be
196 detected with the used databases. Whereas a score ≥ 1.700 and < 2.000, the isolate was identified
197 probably at genus level.

198

199 **2.3.2. Sequencing of 16S rRNA gene V4 region by next generation sequencing**

200 Six litres of water were filtered from each sample through 0.22 µm pore size polycarbonate
201 membrane filters (Millipore, German). DNA was extracted from the filters applying mechanical
202 disruption through bead beating and phenol/chloroform purification using a modification of a
203 previous protocol (Griffiths et al., 2000) as previously described (Sala-Comorera et al., 2019). A
204 negative control was performed for each DNA extraction batch. A positive control (commercial
205 mock microbial community Zymmobiomics, Zymmo Research) was included for the amplicon
206 sequencing. Illumina sequencing of samples was performed in a single run using the Illumina
207 MiSeq platform at Research Technology Support Facility of Michigan State University
208 (Michigan, USA). Amplicon libraries of the V4 hypervariable region of the 16S rRNA gene were
209 prepared using the primers 515f and 806r described previously with corresponding adaptors,
210 following the protocol elaborated by (Kozich et al., 2013). Sequencing was performed in 2 x 250
211 bp paired end format using a MiSeq v2 reagent cartridge following the manufacturer's instructions

(Illumina MiSeq, USA). Base calling was done by Illumina Real Time Analysis (RTA) v1.18.54 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.19.1.

2.3.4. Bioinformatic analysis

Sequences were processed to sequence variants (SV) using the default parameters of the Dada2 workflow (Callahan et al., 2016). Reverse reads were trimmed to 160 bp as recommended previously to improve downstream processing of the reads (Callahan et al., 2016) and a maximum of 2 errors per read were allowed (maxEE=2). This parameter has been shown to be a better filter than simply averaging quality scores (Edgar and Flyvbjerg, 2015). Taxonomic classifications were assigned to the SV using the reference SILVA database v132. Phyloseq package (R library) was used for further processing the SV. In order to diminish the presence of false negative sequences derived from sequencing reagents the decontam R package was used (R library).

2.4. Statistical analyses

The limit of detection was used for the negative samples in the HPC calculations. The Kruskal – Wallis test was used to assess for differences in HPC between samples and stations. R studio was used to assess for difference in mineral water brands and seasons. This test was also used to assess differences between SVs and taxonomic classifications of the different mineral water groups, because the data did not present a normal distribution. An analysis of differential proportions was also performed to detect the most and least differentially present genera using a binomial test of proportions in R, adjusting the p-value (“fdr” method) for multiple hypothesis testing.

3. Results

3.1. Cultivable heterotrophic bacteria counts

3.1.1 Tap water

All the tap water samples (n=11) were positive for the presence of HPC. Heterotrophic plate counts ranged between 0.47 $\log_{10}$ CFU/100 ml and 4.40 $\log_{10}$ CFU/100 ml units (geometric mean= 2.21; SD=1.05) (**Fig1a**). Samples T01 to T04, which were a mix of drinking water originated from river and groundwater treated at a drinking water treatment plant (DWTP group), and shared the distribution network, showed highly similar values although the site of collection was located up to 30 km apart. Notably, samples T06 to T08, representing chlorinated water from piped wells (PW group), displayed the highest HPC. It has to be noted that sample T07 surpassed the limit of 100 CFU/ml for HPC indicated in the national regulations. Two samples (T10 and T11) taken from two neighbour countries representing chlorinated water from piped wells showed HPC median values between DWTP and PW groups. On the contrary, the lowest HPC were detected in a sample from a distribution network of chlorinated spring water taken from a public fountain (T09).

3.1.2 Mineral bottled water

A total of 7 water brands out of 10 were positive for HPC (limit of detection 1 CFU/ 100 ml) one month after bottling. Positive bottled mineral water brands showed highly heterogeneous values ranging from 0.22 $\log_{10}$ CFU/100 ml to 5.62 $\log_{10}$ CFU/100 ml units, with a geometric mean of 2.57 (SD=1.65) (**Fig1b**). No statistically significant differences were found between tap and mineral water HPC.

In order to know the variation of HPC in the mineral water consumed along the year, three water brands (M01-M03) were selected to assess the differences in HPC with respect to the bottling season (summer or winter, which reflected differences in the air temperature). Seasonal differences were found for water brands M01 and M02 (Kruskal-Wallis test $p < 0.05$) (**Fig. 2**). In water M01 counts ranged from around 5.5 $\log_{10}$ CFU/100 ml in summer to less than 0 $\log_{10}$

CFU/100 ml (limit of detection) in winter (mean around 2 log_{10} CFU/100 ml units). Meanwhile, in water M02 summer samples surpassed in around 1 log_{10} units winter samples. In this water HPCs ranged between 3 and less than 0 log_{10} CFU/100 ml units. Water brand M03 was highly stable in both seasons with a HPC average of around 1.5 log_{10} CFU/100 ml units.

3.2. Identification of bacterial isolates by MALDI-TOF MS

In order to characterize the bacterial communities represented in the HPC around 50 colonies of each sample were further selected for MALDI-TOF MS identification using the Bruker Daltonics and the Drinking Water Library, specifically constructed for the Bruker Daltonics MALDI-TOF analyser. The structure of bacterial communities was different according to the water sample origin. Shannon index varied between 0 and 1.33 for tap water and between 0 and 1.68 for mineral water samples (**Table 1**). Concerning the beta-diversity, there were statistically significant differences between bacterial communities structure in mineral and tap water (PERMANOVA, p= 0.01199) as shown in the clustering of the samples according to the Bray-Curtis distance (**Fig. 3)**. All the tap water clustered together with the exception of a sample from a piped well (T06) and a sample from France (T11), also from a piped well, that clustered with the main mineral water cluster. M06 community structure was completely different from the other mineral water samples.

3.2.1 Tap water isolates characterisation

A total of 495 colonies were selected from the different tap water samples and analysed by MALDI-TOF MS. The number of isolates of each sample is shown in **Fig. 4a**. Thirty-one colonies of sample T05 were removed from the study since they were further confirmed as fungi. In 7 out of 11 samples the dominant genus was *Blastomonas.* These samples corresponded to treated water originated from a mixture of chlorinated piped wells and river water (samples T02, T03, T04 and T05) or piped wells (T06, T08 and T10, the latter being from outside of Spain). In the case of sample T01 it was dominated by *Brevundimonas*, whereas sample T07 was dominated by

285 *Pseudomonas* and *Citrobacter*. Sample T11, that was taken from France and was also from a
286 chlorinated piped well source was dominated by *Acidovorax*. In the case of the only sample that
287 was from a chlorinated spring source (sample T09) it was dominated by *Bacillus* and
288 *Acinetobacter* although only 7 isolates were analysed. At phylum level Proteobacteria was
289 dominant in all of the samples with the exception of this latter sample that dominated by
290 Firmicutes (**Fig. S1**). Actinobacteria was also detected in minor amount in three samples. At class
291 level, Alphaproteobacteria (order Sphingomonadales and Caulobacterales) dominated in tap
292 water samples (**Fig. S2**, **Fig. S3**).

293

294 *3.2.2 Bottled mineral water isolates characterisation*

295 In the case of mineral water, a total of 1,578 colonies were isolated for further characterization.
296 The number of isolates of each sample is shown in **Fig. 4b**.

297 With the exception of three samples the bacterial community composition of each mineral water
298 brand was highly diverse and had its own microbial composition, although some genera were
299 dominating. Accordingly, *Acidivorax* dominated in 3 out of 7 samples (M03, M04 and M10),
300 whereas sample M01 was dominated by *Rhizobium*, *Acidovorax*, *Polaromonas* and *Caulobacter*.
301 Sample M02 was dominated by *Aquabacterium*. Samples M06 and M07 by *Variovorax* and
302 *Bosea*, respectively. Concerning the non-dominating species, some of the genera were shared
303 among the different water brands such as *Acinetobacter*, *Bacillus, Brevundimonas* ,
304 *Dermacoccus*, *Phyllobacterium*, *Moraxella*, *Micrococcus*, *Rhodococcus*, and *Staphylococcus*
305 but in general each water brand had each own community. At phylum level the majority of the
306 water brands were dominated by Proteobacteria followed by Actinobacteria and in minor
307 proportion Bacteroidetes (**Fig. S1**). The most abundant class was Gammaproteobacteria (order
308 Betaproteobacteriales) (**Fig. S2**, **Fig. S3**), with *Bulkhorderiaceae* being the most frequenly
309 detected family (**Fig. S4).**

Three of the samples were further assessed for differences in the composition of the communities according to the bottling season. A shown in **Fig. 5** the communities were in samples M2 and M3 were more diverse in summer compared to winter, with some genera appearing only in summer such as *Hydrogenophaga*, *Phyllobacterium, Caulobacter*, *Bosea* and *Acinetobacter,* among others, or *Pantoea* in winter. Meanwhile, others such as *Acidovorax*, *Aquabacterium*, *Variovorax***,** *Polaromonas, Bacillus* and *Rhizobium* were detected in both seasonal samplings taking all the samples together. If each water brand is separately considered, sample M01 only shared *Rhizobium*, sample M02, *Aquabacterium*, and sample M03 *Acidovorax* and *Variovorax* between both seasons.

3.3. Identification of bacterial communities by next-generation sequencing

Cultivable bacteria identification provides information on bacteria that are metabolically active, but represent a minor part of the overall bacterial communities. Therefore, in order to characterise the bacterial communities of the water, bacterial communities were further analysed by 16S rRNA amplicon massive sequencing (16S rRNA-NGS). DNA concentration extracted from tap water samples (6 l) was below LOD of Qbit analyser (<0.4 ng/µl) and therefore these samples were not included in the analyses. Concerning the mineral water samples only those brands with seasonal replicates, M01, M02 and M03 were analysed. It has to be noted that two batches of sample M01 (1 summer and 1 winter) were removed from the study since they mostly clustered together with the negative control. A total of 2,355,700 reads were obtained in total after filtering, with samples ranging between 87,372 and 228,310 reads. The reads were classified into a total of 544 SV that were distributed as follows: 278, 270 and 252 for M01, M02 and M03 mineral water brands, respectively.

The phylum Proteobacteria (class Gammaproteobacteria, order Betaproteobacteriales, family *Bulkhorderiaceae*) was the most abundant in all the 3 water brands analysed (**Fig. S6**). The most abundant genus was *Acidovorax* in sample M01 and *Polaromonas* in samples M02 and M03. As shown in **Fig 6** the dominant species was shifted in 16S rRNA-NGS compared to MALDI-TOF

MS results for all the three mineral water brands analysed, from *Rhizobium* to *Acidovorax*, *Aquabacterium* to *Polaromonas*, *Acidovorax* to *Polaromonas* in samples M01, M02 and M03, respectively. In spite of the differences between the genera found in the different water brands a total of 79 SVs were shared between them with the genera *Polaromonas*, *Acidovorax*, *Caenimonas*, the *Rhizobium* complex, and *Afipia* being the most frequently sequenced genera with more than 100 reads each (**Fig. S5**).

Concerning the alpha-diversity (Shannon index), in general lower values were observed with 16S rRNA-NGS (0.96, 1.79 and 1.20, for samples M01, M02 and M03 respectively) compared to MALDI-TOF MS results (1.54, 1.70, and 1.50, respectively).

In the case of 16S rRNA-NGS, as in the case of HPC characterisation seasonal differences were also observed. A higher diversity was observed in the summer with the exception of sample M01 in which the reverse was observed, with Shannon indices of 0.63, 1.74 and 1.35 in the summer season compared to 1.17, 1.19 and 0.81 in the winter season for M01, M02 and M03, respectively,

Some genera were detected in both seasons, some of which had also been previously detected in the HPC-MALDI-TOF MS in both seasonal samplings such as *Acidovorax*, *Rhizobacterium* complex, *Polaromonas*, *Aquabacterium* (**Fig. 7**) (see **Fig. S5,** for further details). Meanwhile, other genera were detected in only one season such as *Caulobacter*, *Hydrogenophaga*, and *Brevundimonas*, while other had not even been detected in the HPC such as *Sphingopyxis*, *Caenimonas*, *Afipia*, *Perlucidibaca* or *Reyranella*. On the other hand, the genera *Pantoea*, *Pedobacter*, *Rhodococcus* and *Variovorax,* detected by HPC analyses were not detected by 16S rRNA-NGS.

4. Discussion

In this work, the microbiome of water has been investigated, including tap and bottled natural mineral water, at the consumers interface. Each water has its own microbial diversity which often shifts from source after treatment and/or distribution (Hoefel et al., 2005; Sala-Comorera et al., 2017). In the case of tap water, from a microbiological point of view, two basic procedures are used to control the microbial stability during distribution. The first one, which is the most extended, is based on the utilization of a disinfectant with residual activity, such as chlorine-based disinfectants. The second one is based on the limitation of the available carbon for regrowth. The different treatments, which impose different selective pressures, shape the microbial populations that survive and therefore, arrive to consumers. On the contrary, natural mineral water according to the EU directive cannot be subjected to any disinfection treatment that alters its microbiome.

All the analysed tap waters with exception of tap water T07 complied with the national regulations, which stipulate a limit for HPC in treated water of 10^4 CFU/100 ml. In fact, in this tap water showed a completely different community structure compared to the other tap waters with a dominance of *Pseudomonas*, followed by *Citrobacter* and *Bacillus*. It has to be noted that water from this distribution network enters a deposit of 4 m^3 before reaching the tap, and therefore regrowth of certain microorganisms can occur. In fact *Pseudomonas* is a ubiquitous bacterium with opportunistic representants such as *Pseudomonas aeruginosa*, which is able to form biofilms. It has been isolated from soils, and water with high potential to acquire antibiotic resistance genes (Kormas et al., 2010; Laura Sala-Comorera et al., 2016a; Sala-Comorera et al., 2017; Tokajian et al., 2005). On the other hand, *Citrobacter* is also a ubiquitous bacterium but has rarely been associated with illness although is also able to form biofilms. Notably, differences in HPC of up to 1 $\log_{10}$ units were detected in sample T04 compared to samples T03 and T02, although they were part of the same distribution network, suggesting that regrowth at any part of the network had occurred. Stagnation in drinking water distribution network has shown to increase in 4 to 580-fold the HPC values in household taps (Lautenschlager et al., 2014).

389 Concerning the tap water microbiome, the dominant bacterial genus in 7 out of the 11 analysed
390 tap waters was *Blastomonas* independently of the water distribution network and source (river
391 water or groundwater). This genus was also the dominant in a sample taken from outside Spain
392 (Andorra, T10). This genus has been previously detected in drinking water (Revetta et al., 2010;
393 Tokajian et al., 2005) but has been rarely involved in pathogenesis. On the other hand, in samples
394 T01, T09 and T11 presented a unique dominant species different from the others, being
395 *Brevundimonas*, *Bacillus* and *Acidovorax* the dominant genera, respectively. All the sources of
396 these tap waters were different, a big distribution network in sample T01 and small size
397 distribution network for samples T09 and T11 with groundwater as water source in the two latter.
398 It has to be noted that *Acidovorax* was also the dominant genus in 3 out of 7 mineral water brands.
399 All these genera have been previously been detected in drinking water environments (Falcone-
400 Dias et al., 2012; Sala-Comorera et al., 2019; Tokajian et al., 2005). Whereas *Brevundimonas* and
401 *Bacillus* have opportunistic pathogenic representants (Logan NA, 1999; Ryan and Pembroke,
402 2018), *Acidovorax* has broad catabolic abilities and participate in organic and inorganic nutrient
403 cycling in nature (Navarro-Noya et al., 2013). All of them have been previously reported in
404 drinking water (Tokajian et al., 2005)with *Bacillus* being the most frequently detected genus in
405 chlorine-treated drinking water in a previous work (Sala-Comorera et al., 2017). The obtained
406 results differ from a large study of HPC in Paris distribution network recently conducted (Perrin
407 et al., 2019). Contrary to the results obtained in the present study, in Paris drinking water
408 distribution network, the heterotrophic bacteria were dominated by *Mycobacteria* and
409 *Sphingomonadaceae* family members. In any case, the microbial diversity of tap water depends
410 largely on the process of water treatment (Li et al., 2017), which also depends largely on the initial
411 quality of the source water from a microbiological but also from a chemical point of view, and
412 the possibility of regrowth of the microorganisms during distribution and in the case of mineral.
413

414 In the case of natural mineral water, a variation in the HPC was observed between the different
415 water brands but also between the different sampling campaigns. It has to be noted that the

samples were analysed one month after bottling and although there is no regulation about HPC counts during shelf life, the concentration was highly diverse ranging from <1/100 ml to more than 10^5 CFU/100 ml, being higher in 2 out of the 3 brands in the high temperature sampling campaigns.

A total of 19 genera were only detected in mineral water, including *Aquabacterium*, Bosea, *Brevundimonas*, *Caulobacter*, *Chryseobacterium*, *Dermacoccus*, *Herminiimonas*, *Hydrogenophaga*, *Kytococcus*, *Limnobacter, Methylibium*, *Phyllobacterium*, *Moraxella*, *Nocardia*, *Pedobacter*, *Polaromonas*, *Rhizobium*, and *Variovorax.* However, all of them with the exception of *Dermacoccus, Kytococcus*, *Limnobacter, Methylibium, Phyllobacterium*, and *Polaromonas* have been detected both in mineral and drinking water (Casanovas-Massana and Blanch, 2012; Kalmbach et al., 2000; Loy et al., 2005; Otterholt and Charnock, 2011; Sala-Comorera et al., 2019, 2017; Tokajian et al., 2005). *Dermacoccus* is a member of human skin microbiota and, therefore, could have reached water during manipulation, whereas *Kytococcus* has been isolated in aerosol samples and indoor air samples and blood cultures of a patient with endocarditis (Asif et al., 2018; Kämpfer et al., 2009; Weil et al., 2002). *Limnobacter* and *Methylibium* have only been reported in mineral water: *Limnobacter* is a chemolithoheterotrophic, thiosulfateoxidizing bacteria whereas *Methylibium* is a facultative methylotroph (Otterholt and Charnock, 2011) and *Phyllobacterium* is a nitrogen-fixin bacteria that associated to plants (Lambert et al., 1990). *Polaromonas* is a psychrophilic chemoorganotrophic bacterium that has only been associated to mineral water and Antarctic marine (Sala-Comorera et al., 2019).

Concerning the bacterial diversity of the mineral water, a lower diversity index was observed in samples analysed by 16S rRNA-NGS analysis compared to HPC MALDI-TOF MS. This can be explained by the dominance of certain species that may hinder the other genus when analysed by NGS.

Members of *Alphaproteobacteria* dominated the population in the different tap water analysed and suggested the resilience of this class in low nutrient concentrations environments such as tap

water. Whereas *Gammaproteobacteria* predominated in mineral water, *Gammaproteobacteria* is selectively competent for easily assimilating carbon that could be released during the bottling process (Pinhassi and Berman, 2003).

With the exception of one tap water sample, Proteobacteria was the most abundant phylum detected in both tap and bottled mineral water analysed by HPC MALDI-TOF MS and 16S rRNA-NGS, confirming previous studies (Pinto et al., 2012; L. Sala-Comorera et al., 2016; Shi et al., 2013). Nevertheless, significant differences in the bacterial communities at lower taxonomic affiliations such as genus have been identified. Moreover, both methods showed differences in the biodiversity index. The two different methods used in this study have their own forthcomings and limitations; whereas HPC allows identification of viable cells, its use restricts the study of the heterotrophs that can grow at a certain temperature and incubation time in a selected medium. Commonly reported HPC values vary from <0.02 to 10^4CFU/ml, while direct microscopy counts range between 10^4 to 10^7 cells/ml (Hoefel et al., 2005; Sala-Comorera et al., 2019). Nevertheless, identification of the isolates by MALDI-TOF MS is a rapid and affordable technology for many laboratories and allows the possibility to use a custom database such as the DWL used in our study (Galofré, B., Vilaró, C., Fernández, S., Baquero, D., Blanco, S. G., Blanch, A. R., ... & Ruvira, 2019). This allows the water companies a rapid method for tracking their HPC for assessing biofilm formation or regrowth of certain microorganisms, i.e microbial stability during distribution. However, they have been shown inaccurate in different studies. For example, in some studies, conventional HPC counts were not able to detect changes in the microbial numbers and microbial composition of drinking water (Lautenschlager et al., 2013, 2010; Pepper et al., 2004). Therefore, additional information provided by non-culture based techniques such as 16S rRNA-NGS should not be dismissed for water monitoring. The main advantage of this technique is the ability for high throughput analysis, but the main limitation is the inability of distinguish between live and died microorganisms. Another major drawback is the needed time for analysis and the difficulty in stablishing a standardized procedure. Culture-based and culture independent techniques are complimentary and may be used as the basis for further analyses of the bacterial

populations for the water companies. For example, culturing conditions can be optimized for the cultivation of the taxa that were not recovered in the HPC analyses to study in more detail these populations.

A seasonal heterogeneity in the microbial communities has also been identified, with some differences in the dominant genus. Natural mineral waters are often referred to as conservative systems with a minimal variation in their physic chemical characteristics (Casanovas-Massana and Blanch, 2012), although some temporal hydrological variations in aquifers have also been reported (Farnleitner et al., 2005). Moreover, they are a dynamic ecosystem throughout their shelf life as we previously demonstrated (Sala-Comorera et al., 2019). Besides this temporal variation, in this study a seasonal variation in the microbial communities has been demonstrated: mineral water consumed in different seasons differs in their microbial composition independently of the aging time (time after bottling). Concerning the observed variation, it could originate from changes in the microbial dynamics inside the bottle related to the increase in temperature and available nutrient resources. Nevertheless, it could also originate from differences in the microbial communities forming part of the water distribution pipes or during bottling (Jayasekara et al., 1999; Liu et al., 2014). For example, biofilm forming bacteria in the water pipes could differ in different environmental conditions or after different cleaning practices in different moments of the year. Moreover, changes could also arise from the different temperatures at which the samples were kept in the supermarket or during transportation. The objective of this study was far from assessing these influences, but to assess the variation of the mineral water microbial populations that are ingested by consumers under real conditions in two different periods of the year. It has to be noted that temporal changes in the microbial communities have also been reported in drinking water treatment plants (Pinto et al., 2014). Nevertheless, the public health relevance of the changes in the water microbiome is not known and warrants further investigation, especially considering the relevance of opportunistic pathogens and/or the possibility of these bacteria to carry antibiotic resistance genes that could be transmitted to other bacteria inside the human body. Understanding the water microbiome dynamics and microbial ecology is essential to improve

water management practices during water production and to improve water quality regulations to warrant public health.

Declarations of interest

The authors have no conflict of interests to declare

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Figures and tables

Figure captions

Fig. 1. Box plots showing the heterotrophic plate counts in a) tap water and b) bottled mineral water. Black line represents the median value of the samples. Lower and upper boxes represent 2nd and 3rd quartile. Dots represent statistically significant outliers. The detection limit was used for counts lower than the detection limit (1 UFC/100 ml).

Fig 2. Seasonal differences in HPC in 3 selected brands of bottled mineral water.

Fig.3. Clustering of the bacterial isolates of the different samples according to Bray Curtis distance.

Fig.4. Identification of bacterial isolates by MALDI-TOF mass spectrometry from a) tap water, and b) bottled mineral water. Number of isolates indicated at the top of the bars.

Fig. 5. Seasonal distribution of the cultivable bacterial communities in mineral water.

Fig. 6. Comparison of the bacterial communities identified by HPC-MALDI-TOF MS and 16S rRNA-NGS analysis of mineral bottled water

Fig. 7. Seasonal distribution of the non-cultivable bacterial communities in mineral water.