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Diversity of the heterotrophic microbial populations for distinguishing natural mineral waters

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ABSTRACT

In the recent years the consumption of natural mineral waters has risen all over the world, becoming a usual alternative for tap water and other beverages. Natural mineral waters are complex environments containing a high diversity of autochthonous microbiota. The identification and characterization of this indigenous microbiota may help to detect changes occurring in the different steps of the bottling process and take preventive measures before the bottled water arrives to the consumer.

The aims of this study were to describe the bacterial heterotrophic populations in natural mineral waters with a cultivation-dependent method and determine whether their autochthonous microbiota were specific enough to be clearly distinguished from that of other natural mineral waters with a phenotypic-based method. For this purpose, water from three independent Spanish springs was sampled in two seasons (winter and summer) and heterotrophic aerobic bacterial strains were isolated at two temperatures (22 ± 2 °C and 36 ± 2 °C) on R2A agar. Isolates were phenotyped biochemically with Php-48 plates (Bactus AB, Sweden), and the indexes of diversity and similarity between populations were calculated. The 16S rRNA gene of the most representative strains of each biochemical cluster was sequenced for its identification. Finally, a ten-fold cross-validation method was assayed for the identification of the origin of a natural mineral water when phenotyping a set of isolates. High levels of diversity were found at all sites. One of the sources was found to present less diversity due to a confirmed contamination with *Pseudomonas aeruginosa*. The study of the similarities showed that growing temperatures and seasons caused significant differences in structures and composition at the sources. In addition, several bacterial species were isolated and identified, some of them rarely isolated in natural mineral waters, revealing the complexity and lack of knowledge of these ecosystems.

Consequently, the applied phenotypic methodology was found to be feasible for differential identification of microbiota in these environments. Moreover, the experimental model assayed was strong enough to identify the origin of a natural mineral water. It may thus be possible to confirm that the evaluation of diversity of heterotrophic aerobic bacterial populations could be applied to identify bottled water sources.

1. Introduction

The estimated *per capita* consumption of bottled water in the European Union rose from 43 l in 2003 to 105 l in 2008 (European Federation of Bottled Water (EFBW), 2010; Venieri et al., 2006). Various factors may have contributed to this increase: dissatisfaction with the odor and taste of tap water due to chlorine, greater consumer concern about the safety of tap water, or the use of bottled water as a substitute for other beverages (Abrahams et al., 2000; Doria, 2006; Papapetropoulou, 1998; Warburton, 2000). In addition, the successful promotion of bottled water as clean, pure, safe and especially suitable for infants may also have increased its consumption (Bharath et al., 2003; Misund et al., 1999).

Most bottled waters are taken from springs or ground waters. Natural mineral waters are not sterile environments, but complex ecosystems with a high phenotypic and genetic diversity of autochthonous bacteria (Rosenberg, 2003). The number of bacteria at the source point is generally low, although a wide range of genera have been reported, such as fluorescent and non-fluorescent *Pseudomonas*, *Acinetobacter*, *Stenotrophomonas*, *Alcaligenes*, *Comamonas*, *Acidovorax*, *Paucimonas*, *Brevundimonas*, *Cytophaga*, *Flavobacterium*, *Flexibacter*, *Arthrobacter* and *Corynebacterium*, among others (Bischofberger et al., 1990; Guillot and Leclerc, 1993; Manaia et al., 1990; Mavridou, 1992; Shwaller and Schmidt-Lorenz, 1980; Vachee et al., 1997).

The European Union directive states that natural mineral waters cannot be subjected to any disinfection treatment, including sterilization, pasteurization, or any other procedure to remove or destroy microorganisms. Therefore, natural mineral waters contain the original microbiota of the source (Anonymous, 2009). Furthermore, experimental and epidemiological data show that the indigenous bacteria found in natural mineral waters according to European standards have not been

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associated with any detectable human disease (Bohmer and Resch, 2000; Leclerc and Moreau, 2002; Payment et al., 1997).

Natural mineral waters complying with the European directive must not be contaminated with allochthonous organisms such as parasites, pathogens or indicators of fecal pollution. However, several studies have reported the occurrence of *E. coli*, protozoa, enteric viruses, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Campylobacter* and even *Vibrio cholerae* in bottled natural mineral waters (Beuret et al., 2002; Bharath et al., 2003; Blake et al., 1977; Evans et al., 2003; Gassilloud et al., 2003; Legnani et al., 1999; Mavridou et al., 1994; Obiri-Danso et al., 2003; Salazar et al., 1982; Schindler et al., 1995). In most cases, the source of these contaminants has been attributed to the bottling plant: pumps, pipes, caps and bottles (Warburton et al., 1986; Warburton, 1993). Nevertheless, the potential contamination of the sources with pathogenic microorganisms should not be underestimated. As a result, both water bottling plants and natural mineral waters have to be strictly controlled and monitored to prevent any contamination that may result in a health risk (Hrudey et al., 2006).

The identification and characterization of the indigenous microbiota may help operators to detect changes occurring in the source, during the bottling process, or in transportation or storage. They could then take preventive measures before the bottled water is released to sale. Moreover, the knowledge of the microbial composition and structure of the water before bottling could be used as a basis to track the water after bottling.

Various research groups have characterized the autochthonous microbiota of natural mineral waters under different approaches (Bischofberger et al., 1990; Gonzalez et al., 1987; Guillot and Leclerc, 1993; Guyard et al., 1999; Loy et al., 2005; Mavridou, 1992; Papapetropoulou, 1998; Shwaller and Schmidt-Lorenz, 1980; Venieri et al., 2006). It is widely known that cultivation-dependent methods recover only a fraction of the total heterotrophic bacteria present in a sample (Allen et al., 2004; Loy et al., 2005; Wang et al., 2009). Nevertheless, as new molecular methods are expensive, complex and they also suffer from certain biases (Jofre and Blanch, 2010), this study was based on an usual cultivation method using R2A agar, a specifically designed medium to isolate water-based bacteria (Reasoner and Geldreich, 1985) which is routinely used in water-bottling plants. This medium provides higher productivity and diversity than other media when using low incubation temperatures (20–28°) and long incubation times (5–7 days) (Massa et al., 1998).

Therefore, the aims of this study were to describe the bacterial heterotrophic populations in natural mineral waters with a cultivation-dependent method and determine whether their autochthonous microbiota were specific enough to be clearly distinguished from that of other natural mineral waters with a phenotypic-based method.

For this purpose, natural mineral waters from three Spanish springs used in different water-bottling plants were examined using five approaches: (1) the heterotrophic bacteria were isolated at two temperatures (22 ± 2 °C and 36 ± 2 °C) and seasons (winter and summer) and characterized phenotypically; (2) phenotypic groups of isolates were identified genetically, (3) phenotypical comparisons among populations and subpopulations were carried out; (4) when *P. aeruginosa* isolates were found, they were confirmed following standard procedures (Anonymous, 2006); and (5) a ten-fold cross-validation model was assayed to track the origin of the water.

2. Materials and methods

2.1. Sampling

Three geographically and climatically independent Spanish springs (sources A, B and C) were selected for this study. Eight water samples were taken at each source just before just before its entry into the bottling plant, four of them in summer and the other four in winter. The recorded temperatures in the spring waters differed less than 1 °C between seasons. All samples were collected with aseptic techniques, kept at 4 °C, and analyzed within 24 h.

Volumes of water between 20 mL and 100 mL were filtered through 0.44 μm nitrocellulose membranes (Millipore, USA). Half the filters were incubated on R2A plates (Oxoid, CM0906, England) at 22 ± 2 °C for 144 ± 2 h. The other half were incubated on R2A plates (Oxoid, CM0906, England) at 36 ± 2 °C for 144 ± 2 h. After incubation, a maximum of 50 strains from each spring and growing temperature were randomly isolated from the plates and subcultured on R2A plates (Oxoid, CM0906, England). This procedure was performed for each source and growing temperature in two seasons: winter and summer. Pure culture was confirmed by streak-plate technique on R2A plates (Oxoid, CM0906, England) at 22 ± 2 °C or 36 ± 2 °C for 144 ± 2 h and Gram staining. The number of strains isolated was assumed to be representative of the natural heterotrophic bacterial populations isolated (Bianchi and Bianchi, 1982). In parallel, all samples were tested for the presence of parasites, *Escherichia coli* and other coliforms, fecal streptococci, sporulated sulphite-reducing anaerobes and *Pseudomonas aeruginosa* as stated in the European Union directive (Anonymous, 2009).

2.2. Biochemical characterization

All isolates were phenotyped biochemically using PhP-48 microplates of the Phene-Plate System (Bactus AB, Sweden), following the manufacturer's instructions. The PhP-48 plates consist of 96-well microplates with dehydrated reagents, which were chosen to provide a high level of discrimination among species. Each plate allows the biochemical fingerprinting of two distinct isolates (48 biochemical tests per isolate). The biochemical fingerprinting procedure is described elsewhere (Kühn, 1985).

Cultures were prepared on R2A plates (Oxoid, CM0906, England) for 96 ± 2 h at 22 ± 2 °C or 36 ± 2 °C depending on the isolation temperature. Cell suspensions were prepared by harvesting the cultures in a solution of distilled water at 0.1% w/v proteose peptone (Difco, 211677, France) and 0.011% w/v bromothymol blue (Merck, 1030260005, Germany). Aliquots of 150 μl of bacterial suspension were added to each well. The inoculated microplates were incubated at 22 ± 2 °C or 36 ± 2 °C respectively. *Pseudomonas aeruginosa* NCTC 10332T was used as an internal control in all the experiments to confirm reproducibility. Growth in the wells was measured in a spectrophotometer at 620 nm with the iEMS Reader MF (Labsystems, Finland) at 16 h, 40 h and 64 h for the strains growing at 36 ± 2 °C, and at 16 h, 40 h, 64 h, 88 h and 122 h for the strains growing at 22 ± 2 °C. The biochemical profiles were calculated as described elsewhere (Kühn et al., 1991) using the software PhPWin® (Bactus AB, Sweden).

2.3. Phenotypic characterization and indexes of population diversity and similarity

The structure and composition of the populations were analyzed for each sample by cluster analysis on the basis of the biochemical PhP phenotypes of the isolates using the unweighted pair group method analysis (UPGMA). Isolates for each population were pooled to determine the main biochemical PhP phenotypes (main clusters) of bacterial populations. Clusters were defined by isolates showing a similarity index equal to or higher than 0.965. Isolates showing the highest minimum and the highest mean similarity to all other isolates belonging to the same PhP phenotype were selected as representative strains (Kühn et al., 1991) of phenotypic clusters for further identification by sequencing the gene of 16S rRNA.

Simpson's diversity index (Di) was used to calculate the diversity of bacterial populations and subpopulations (Hunter and Gaston, 1988). The Sp, a similarity population coefficient that measures the proportion of isolates that are identical in two samples was also calculated for each