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Research Paper

Study of bacterial community structure and diversity during the maturation process of a therapeutic peloid

Chiara Pesciaroli [a,*], César Viseras [b,c], Carola Aguzzi [b], Belén Rodelas [a,d], Jesús González-López [a,d]

[a] Institute of Water Research, University of Granada, 18003 Granada, Spain
[b] Department of Pharmacy and Pharmaceutical Technology, School of Pharmacy, University of Granada, 18071, Granada, Spain
[c] Andalusian Institute of Earth Sciences, University of Granada-CSIC, 18004, Armilla, Granada, Spain
[d] Department of Microbiology, School of Pharmacy, University of Granada, 18071 Granada, Spain

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ABSTRACT

The presence of bacteria in peloids is essential for the development of their therapeutic properties. Therefore, the study of bacterial community composition in peloids and its changes along the maturation process is of paramount importance. For this purpose, samples of thermal waters and peloids at different stages of maturation were collected and the MiSeq Illumina sequencing platform was employed to obtain information on bacterial diversity and community structure. Richness and diversity indices showed that a clear change during the first period of maturation occurred, while, after two months, the community seemed to stabilize. The same results were obtained based on the different OTU present. A phylogenetic analysis was performed and taxonomic affiliations for sequences with abundance higher than 1% and important to determine differences among samples were obtained. Relative abundance of bacterial community composition was also investigated. Analysis indicated a dominance of α- and β-*Proteobacteria* in water samples, while γ-*Proteobacteria* prevailed in peloids. Again, biodiversity profiles showed a low level of similarity between water and peloids, while in all peloid samples the populations were almost the same. Results demonstrate that the bacterial community in peloids changed mostly on the early stages of maturation and it reached stability after two months.

1. Introduction

The practice of using natural substances such as herbs, animal parts, inorganic materials and minerals for medical therapies has a long historical background in the medical tradition (De Vos, 2010). In particular, clay muds have always been well known for their healing properties and their use is remarkably remote: it is reported that muds were employed by *Homo erectus* and *Homo neanderthalensis* to cure wounds (Reinbacher, 1999; Carretero et al., 2006).

Clays and clay muds are still largely employed in modern medical therapy for the treatment of skin pathologies and gastrointestinal disorders, among others (Carretero and Pozo, 2009, 2010; Sánchez-Espejo et al., 2014). They are also widely used in the pharmaceutical and cosmetic industries as main ingredients in many health care products (Viseras et al., 2007; López-Galindo et al., 2007; Silva et al., 2011).

In the twentieth century, the use of clay muds in traditional European medicine focused mainly on the therapeutic practice of pelotherapy (Veniale et al., 2007; Tateo et al., 2009; Gomes et al., 2013). In this context, clays are employed in the formulation of peloids, thermal muds obtained by mixing clayey materials with thermo-mineral waters (Veniale et al., 2004, Sánchez-Espejo et al., 2014), which are used in many spas as part of balnotherapy due to their rheological and thermal characteristics, among others, that present several beneficial effects in particular on rheumatoid-arthritic diseases and dermatological pathologies (Quintela et al., 2012; Carretero et al., 2013; Verhagen et al., 2015).

Even if the term peloid has evolved during the past century, the most recent definition describes it as "a maturated mud or muddy dispersion with healing and/or cosmetic properties, composed of a complex mixture of fine-grained natural materials of geologic and/or biologic origin, mineral water or sea water, and common organic compounds from biological metabolic activity" (Gomes et al., 2013).

The characteristics of peloids vary mainly due to factors such as composition and granulometry of the clay, geochemistry of the water used for the mixture, mixing and maturation of thermal muds (Veniale et al., 2004). Among these factors, the maturation process is of paramount importance for the therapeutic properties of peloids (Gomes et al., 2013). The time in which this process develops can range from a few months to years (Fernández-González et al., 2013). Maturation induces several modifications on clay physical characteristics such as its plasticity, capacity of absorption, cooling index and grain size (Veniale, 1999; Sánchez et al., 2002; Veniale et al., 2004; Gámiz et al., 2009). One of the main modifications occurring during the maturation time is the

* Corresponding author at: Institute of Water Research, Calle Ramón y Cajal, 4, 18003 Granada, Spain.
E-mail address: chiara@ugr.es (C. Pesciaroli).

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developing of microorganisms that are responsible for important biological and biochemical processes that lead to the enrichment of peloids in organic compounds (Veniale et al., 2007; Suárez et al., 2011; Centini et al., 2015). It has been postulated that the microorganisms' growth improves the quality of peloids, which are enriched with therapeutically active compounds due to the secretion of metabolic products (Veniale et al., 2007; Quintela et al., 2012; Gomes et al., 2013).

As for the microbiota present in peloids, it is reported that, during maturation, peloids are progressively colonized by thermophilic microorganisms, mainly Cyanobacteria, green algae and diatoms (Andreoli and Rascio, 1975; Tolomio et al., 2002, 2004; Quintela et al., 2012). However, very little is known about the microbial changes that take place during the maturation process and, so far, no molecular-based studies have been carried out to assess the real composition of the developing bacterial population.

In this context, the main purpose of the present work was to gain information on the bacterial community developed during the maturation of a therapeutic mud. To reach this goal, muds were prepared in the laboratories of the Andalusian Institute of Earth Sciences by dispersing a commercial clay in thermal water from Graena Spa (Granada, Spain) and samples were collected at different maturation stages. Cultivation independent methods and next generation sequencing techniques were used to compare bacterial diversity and community structure in peloids at different stages of maturation and to determine the microbiological changes that occurred during the maturation process.

2. Material and methods

2.1. Peloid preparation

To prepare peloids, Innogel LBB clay was purchased from Aplicaciones Especiales del Vallés S.L. (Granollers, Spain). As reported in a previous work (Sánchez-Espejo et al., 2014), the clay was composed of smectite (80% w/w) and kaolinite (12% w/w), quartz (7% w/w) and gypsum (2% w/w). As for the trace elements, it contained acceptable amounts of As (1.8 ppm) and Pb (8.1 ppm) according to the limits for these elements of the most relevant normative (Sánchez-Espejo et al., 2014). Peloids were prepared following a methodology previously optimized (Sánchez-Espejo et al., 2014). Briefly, 1:4 (w/w) clay/water muds were prepared in 250 L tanks by using a mechanical stirrer and stored at room temperature for six months. The tanks were kept covered but unsealed, to avoid contamination but permitting contact with the environment. Weekly, the content of the tanks was stirred using a mechanical stirrer of appropriate size for the total volume, ensuring homogeneous mixing. Humidity and temperature in the laboratory were checked and monitored during the study to guarantee their maintenance within acceptable limits (no more than 60% relative humidity and 15°–25°C).

The water used to prepare the peloids was taken from the pipeline in the thermal spring, carried in plastic containers and used within 12 h.

2.2. Collection of samples

Sampling was carried out between February 2014 and July 2014. Peloid samples were taken from the tanks after 2, 3 and 6 months of maturation (samples L2, L3 and L6 respectively). Before each sampling, the clay/water mixture was stirred to obtain a well homogenized sample (Tateo et al., 2010). Mixture was gathered using 1.5 L sterile plastic containers and each sample was collected in triplicate to assure analysis reliability (Centini et al., 2015). Besides peloids, thermal water was also collected directly from the thermal spring (sample SW) and from the pipeline (sample PW). Since the clay used to prepare peloids was bacteriologically sterile, these two samples were considered as the starting communities.

2.3. DNA extraction

Two different methods were used for DNA extraction, depending on the sample.

For peloid samples, due to the reduced bacterial levels expected, a previous concentration process was carried out. The total volume of each sample (1.5 L) was transferred in amounts of 30 mL in several 50 mL Falcon tubes containing 10 mL of sterile saline solution (0.9% NaCl) and then sonicated for 15 min to allow cell detachment from the clayey matrix. Subsequently, tubes were centrifuged during 10 min at 800 rpm to precipitate clay particles. The supernatant, containing a dispersion of cells and fine clay, was transferred to 2 mL microtubes and further centrifuged at 14,500 rpm for 2 min. Pellets obtained after these treatments were merged in 2 mL microtubes and processed for DNA extraction using the FastDNA®SPIN Kit for soil (MP Biomedicals, Santa Ana, California, USA) as reported by Reboleiro-Rivas et al. (2016).

For water samples, a total volume of 8 to 10 L was vacuum-filtered on sterile membranes (0.22 μm, Millipore, USA). As reported by Pesciaroli et al. (2015), membranes were placed in sterile water and sonicated during 15 min to promote cell detachment. After sonication, membranes were triturated grossly with a sterile pipette tip and stirred by vortex. The dispersion obtained was used to extract DNA with the MasterPure® purification Kit (Epicentre Biotechnologies, USA) according to the manufacturer's instructions.

DNA obtained by the two methods was resuspended in 100 μL of DES (DNase/Pyrogen-Free Water). For each sample, three extractions were performed and, after quantification, the three DNA products were pooled together in an equimolar solution to obtain a reliable representation of the sample community. The DNA obtained was maintained at −20°C and used for sequencing by using the MiSeq Illumina platform.

2.4. Microbiological analysis

In a previous work (Sánchez-Espejo et al., 2014), microbiological tests were carried out on the same type of clay used in this study indicating that it fulfills the required absence of pathogenic microorganisms such as *Pseudomonas* and Enterobacteria. However, in order to confirm that results in the present work, clays were further investigated to verify the absence of *Pseudomonas* and also the sterility of the samples. For this purpose, 200 g of clay were dispersed in sterile saline solution and concentrated, as reported in Section 2.3 for peloids, and then used to inoculate plates containing King A agar, King B agar (Pharmafaster SA, Barcelona, Spain) and TSA (Trypticase Soy Agar, BD, USA). Cultures were maintained at 36 ± 2°C for five days and examined daily to detect the presence of *Pseudomonas aeruginosa* and *Pseudomonas* spp. in King A and King B respectively, as reported by Casanovas-Massana et al. (2010). TSA media were incubated at 28°C for 3 days and then examined for the presence or absence of colonies. Observation of greenish pigmentation on King A and fluorescence on King B during the five days of observation were considered evidence of the presence of *P. aeruginosa* and *Pseudomonas* spp., respectively.

2.5. Analysis of bacterial community using the MiSeq Illumina sequencing platform

The MiSeq Illumina sequencing method was used to study the bacterial communities present in peloids and water samples. The sequencing was carried out by the service of Research and Testing Laboratory (Lubbock, Texas, USA) using the primers 28F-519R of the bacterial 16S rRNA gene (28F: 5′-GAGTTTGATCNTGGCTCAG-3′, 519R: 5′-GTNTTACNGCGGCKGCTG-3′) used by Reboleiro-Rivas et al. (2016). Reads were trimmed and denoising was performed using the USEARCH algorithm. Chimera checking was carried out on the selected OTU using the UCHIME chimera detection software executed in de novo mode. The resulting sequences were then clustered into OTU using the UPARSE

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