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RESEARCH

Characterization by 16S Amplicon Sequencing of Bacterial Communities Overall and During the Maturation Process of Peloids in Two Spas of an Italian Thermal Complex

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Abstract

Peloids are made by mixing clay materials with thermo-mineral waters, enriched with organic substances from microorganisms during maturation. Their beneficial properties may depend on clay minerals, water characteristics, and microbial components, although strong evidence is lacking. Next Generation Sequencing (NGS) allows a comprehensive approach to studying the entire microbial community, including cultivable and uncultivable bacteria. Our study aims to characterize, by NGS, the bacterial community overall and during the maturation process of thermal muds in two spas (A-B) of an Italian thermal complex. Peloids were produced from sulfurous-bromine-iodine thermal water and clay material: natural mud for spa A and sterile clay for spa B. Thermal waters and peloids at different maturation stages (2/4/6 months) were analyzed for microbiome characterization by 16S amplicon sequencing. Biodiversity profiles showed a low level of similarity between peloids and water used for their maturation. Peloids from spa A showed greater microbial richness than those from spa B, suggesting that natural mud with an existing bacterial community leads to greater biodiversity than sterile clay. Genera involved in sulfur metabolism were prevalent in both spas, as expected considering peloids matured in sulfide-rich water. For all three maturation stages, the prevalent genera were *Thiobacillus* and *Pelobacter* in spa A and *Thiobacillus*, *Thauera*, *Pelobacter*, and *Desulfuromonas* in spa B. Richness and diversity indices showed that the community seemed to stabilize after 2–4 months. The 16S amplicon sequencing to study bacterial communities enables the identification of a biological signature that characterizes a specific thermal matrix, defining its therapeutic and cosmetic properties. The bacterial composition of peloids is affected by the thermal water and the type of clay material used in their formulation and maturation.

Keywords Thermal waters · Microbiome · Next generation sequencing · Bacterial community · Mud maturation · Biodiversity

Introduction

Thermal muds are complex matrices derived from a long mixing of clayey materials with thermo-mineral waters, accompanied by organic materials produced by the biological-metabolic activity of microorganisms growing during maturation [1]. The maturation process consists of the interaction of these components in terms of physicochemical reactions and biological and biochemical processes performed by microorganisms and microalgae growing during the different maturation stages [2].

Peloids used for mud therapy usually derive from natural in situ resources, but they can also be prepared by maturation of tailored clayey raw materials mixed with thermo-mineral

Stefania Paduano and Isabella Marchesi contributed equally to this work and shared the first authorship.

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waters in order to avoid the depletion of the natural resources [1].

The clay minerals are used in pharmaceutical formulations, spas, and esthetic medicine for their beneficial effects on human health. Peloids have a stimulatory, antiphlogistic, and analgesic action. They are mainly used to alleviate the pain of chronic rheumatic inflammation and are also recommended to treat dermatological diseases and disorders following vasculopathies [2, 3]. The peculiar healing properties of thermal muds depend on the characteristics of clay minerals, the physical and chemical properties of thermal water, the growth of different microorganisms during the maturation process, and their metabolic products [1].

In a recent review, Carretero [4] collected many publications on the mineralogy, chemistry, physical and physicochemical properties of thermal muds. On the other hand, few papers studying microorganisms inhabiting peloids have been found in the literature [5–7]. Most authors described the development of microalgae, diatoms, and thermophilic blue-green algae (Cyanobacteria) in peloids, which are all part of the so-called "bioglea." The presence of these microorganisms in some thermal muds has been related to their therapeutic effect, due to their connection with different organic compounds (sulfoglycolipids, galactolipids, phospholipids, fatty acids, etc.), with anti-inflammatory properties [8–11].

However, very few studies investigated the characterization and evolution of the microbial community of peloids, although the need for new information has been underlined in the literature [6, 12–14].

Most environmental microorganisms are difficult to cultivate under laboratory conditions, thus limiting our knowledge of the entire bacterial community [15]. In the last decades, the use of next generation sequencing (NGS) and bioinformatics tools has allowed a more extensive approach to characterizing the entire microbial community, detecting culturable and unculturable microorganisms by sequencing segments of their DNA [16–20]. Nevertheless, the characterization of the microbiome over the different stages of thermal mud maturation is scarcely investigated [12, 13, 21].

In this context, our study aims to characterize using 16S amplicon sequencing the bacterial community overall and during the maturation process of thermal muds produced in two spas of the same Italian thermal complex.

Materials and Methods

Sampling

This investigation was conducted in a thermal complex in Sirmione on Lake Garda (Northern Italy). As previously described in our pilot study [21], the Sirmione thermal water is classified as hyperthermal water because of its temperature of 69 °C at the Boiola spring. Due to its chemical composition, it is defined as sulfurous-bromine-iodine thermal water. In this complex, thermal water from drilling wells and from the spring is blended into a mixing plant and then distributed to spas through a complex system of insulated pipes, which maintain its chemical characteristics unaltered. Spas (A and B) represent the extremes of this thermal aqueduct, as spa A is located approximately 500 m and spa B 5 km from the mixing plant. In both spas, thermal water is used for therapeutic and cosmetic purposes in the form of baths, inhalation, irrigation, and peloids. Table 1 reports the chemical composition of Sirmione thermal water during this study.

Peloids are prepared in situ in open-air pools by the maturation of natural mud (spa A, see Fig. 1a) or clayey virgin materials mainly composed of smectite (spa B, see Fig. 1b) mixed with thermal water. This commercial clay is mainly composed of smectite and contains SiO_2 and Al_2O_3 as principal chemical compounds, as previously reported [21].

During two sampling sessions (July 2019 and February 2020), peloids at different stages of maturation (2, 4, and 6 months) were collected into sterile 50-ml tubes after mixing the mud from the surface up to 10–20 cm below. During both sessions, we took two samples one after the other (duplicates) for each sampling point. Figure 2 shows the scheme of the peloid sampling plan. Limited to spa B, two samples of dried virgin clay were also collected during the first sampling session.

A total of 64 peloid samples, including duplicates, were collected. Thirty-two samples (16 per spa: 4 samples at 2 maturation months, 4 at 4 months, and 8 at 6 months) were taken during each sampling session.

In addition, water samples were collected in duplicate from the pipe feeding the pools where peloids mature, for a total of 8 samples, 4 for each sampling session. Figure 1 shows water sampling sites for each spa. Water samples (2 L) were collected in glass bottles filled up to the top and closed with caps and under-caps in order to prevent the dispersion of H_2S. At the same time as sampling, the water temperature was measured with a digital thermometer.

DNA Extraction

DNA was extracted from all mud samples (0.5 g), using the FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH, USA). Water samples were concentrated by membrane filtration (0.22-µm polycarbonate membrane, extraction DNA kit, Minerva Biolabs, Germany). The filter membrane was stored in a Petri plate at − 20 °C. The filter membrane was turned upside down onto an incubation dish filled with 2 mL of lysis buffer (Minerva BioLabs, Germany) and then incubated at 37 °C for 30 min. Subsequently, the lysis solution was transferred into an incubation tube with 0.1 mg of

Table 1 Chemical analysis of Sirmione thermal water in both spas (data from annual monitoring by environmental protection agency of Emilia-Romagna region—ARPAE—years 2019 and 2020)

Chemical parameters	Measure unit	Sirmione thermal water			
		Year 2019		Year 2020	
		SPA A	SPA B	SPA A	SPA B
pH	/	6.7	7.0	6.8	6.8
Conductivity at 20 °C	µS/cm	3870	3830	3835	3819
Fixed residue at 180 °C	mg/L	2500	2490	2570	2516
Oxidability	mg/L O_2	10.4	15.4	14.2	18.0
Silica (SiO_2)	mg/L	55.5	72.0	65.5	57.3
Bicarbonate (HCO_3^-)	mg/L	318	322	322	317
Chloride (Cl^-)	mg/L	1120	1110	1150	1257
Sulfate (SO_4^{--})	mg/L	156	151	132	151
Sodium (Na^+)	mg/L	650	600	625	623
Potassium (K^+)	mg/L	62.2	57.5	58.0	55.5
Calcium (Ca^{++})	mg/L	196	182	182	177
Magnesium (Mg^{++})	mg/L	31.8	33.0	34.6	33.4
Ammonium (NH_4^+)	mg/L	2.00	1.90	1.96	1.90
Hydrogen sulfide (H_2S)	mg/L	13.6	12.0	14.7	10.1
Bromide (Br^-)	mg/L	5.6	5.2	5.1	5.5
Iodide (I^-)	mg/L	0.5	1.9	0.7	0.7
Fluoride (F^-)	mg/L	4.2	4.9	3.6	4.4
Nitrate (NO_3^-)	mg/L	< 1.0	< 1.0	< 1.0	< 1.0
Nitrite (NO_2^-)	mg/L	< 0.002	< 0.002	< 0.002	< 0.002

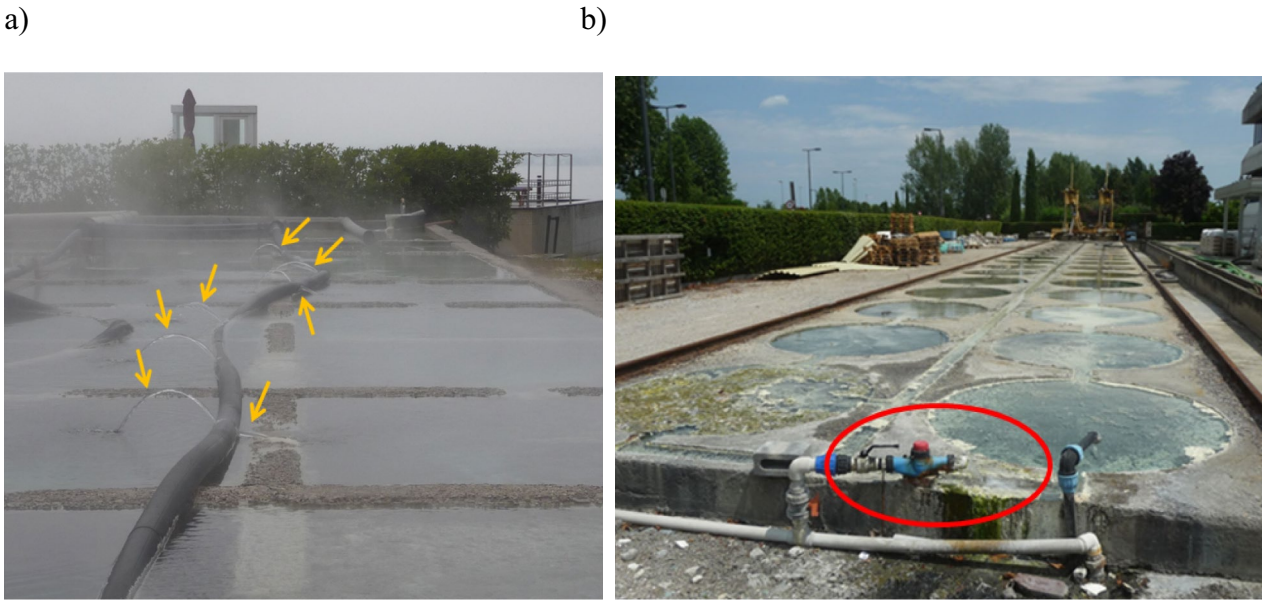


Fig. 1 Open-air pools for mud maturation in spa A (**a**) and in spa B (**b**). The yellow arrows and the red circle indicate the points from which thermal water flows and feeds the pools

glass beads (Sigma-Aldrich, USA) and incubated at 56 °C for 15 min after vortexing for 1 min. DNA purification was performed using Aqua screen Fast Extract Kit according to the manufacturer's protocol (Minerva BioLabs, Germany).

16S rRNA Gene Sequencing and Analysis

Libraries were prepared using the MiSeq Reagent Kit Preparation Guide (Illumina, San Diego, CA, USA). Sequencing

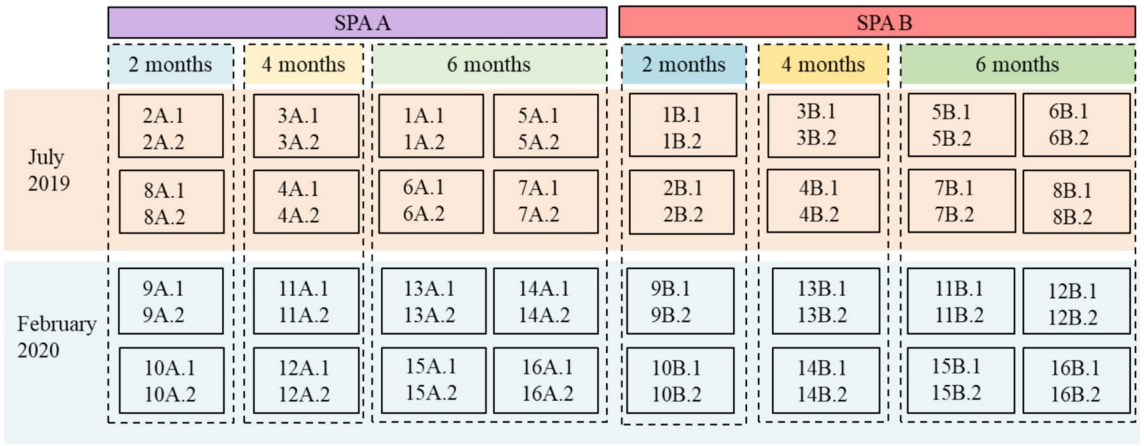


Fig. 2 Scheme of the peloids sampling plan. Each rectangle indicates the sampling pool. The letter (**A**, **B**) identifies the spa; the two duplicates for each sample are named .1 and .2

(2 × 250 bp paired-end) was performed on MiSeq sequencer (version 1.0.1 (Illumina®, San Diego, CA, USA). Raw sequence data was processed using an in-house pipeline which was built on the Galaxy platform and incorporated various software tools to evaluate the quality of the raw sequence data (e.g., FastQC, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). All data sets were rigorously screened to remove low-quality reads (short reads < 200 nt, zero-ambiguous sequences). Demultiplexing was performed to remove PhiX sequences and sort sequences; moreover, to minimize sequencing errors and ensure sequence quality, the reads were trimmed based on the sequence quality score using Btrim [22]. OTUs (operational taxonomic units) were clustered at a 97% similarity level, and final OTUs were generated based on the clustering results. Taxonomic annotation of individual OTUs was based on representative sequences using RDP's 16S Classifier 2.5. Observed OTUs were defined as observed species. The sequence reads were analyzed, also, in the cloud environment BaseSpace through the 16S Metagenomics app (version 1.0.1 (Illumina®, San Diego, CA, USA)): the taxonomic database used was the Illumina-curated version (May 2013 release of the Greengenes Consortium Database [23]).

Data Analysis

Relative abundances of community members were determined with rarefied data and summarized at each taxonomic level. Alpha and beta diversity were calculated using EstimateS software at a level of 97% sequence similarity. Regarding alpha diversity, microbial richness was computed based on the number of OTUs observed. Biodiversity was estimated through the Shannon and evenness index at the genus level [24]. Morisita Horn index (beta diversity) was calculated in order to evaluate the similarity between duplicates [25–27]. The index ranges from 0 (no similarity) to 1 (complete similarity). Principal components analysis (PCA) was performed using the METAGENassist platform [28] in order to investigate the dissimilarity between groups. Moreover, statistical significance of dissimilarity was assessed using ANOSIM (ANalysis Of SIMilarities), and it was run to assess significant differences in the relative abundance of OTUs on different samples [29]. For assessing sequencing depth, alpha rarefaction plots were done in Mothur (version 1.31.1, www.mothur.org) and R (version 3.1.3, www.R-project.org) using packages "ggplot2" and "vegan" [30].

We reported mean and standard deviation (SD) or range for continuous variables. Comparison for biodiversity indices was performed by *t*-test and one-way analysis of variance (ANOVA) with the Bonferroni test. All statistical analyses were performed with the software Stata v18.0 (StataCorp, College Station, TX, USA, 2023).

Results

A total of 72 samples were analyzed (8 water samples and 64 peloid samples) from two spas of the same thermal complex. In addition, the microbiological analysis of virgin clay samples used to prepare the peloids in spa B was performed. However, the extracted DNA concentration was < 1 ng/μL, and therefore, these samples were not considered in the subsequent analyses.

Rarefaction curves for each sample reached a stable plateau (Figure S1). Separately amplified and barcoded duplicates of samples were sequenced to verify the reproducibility of the procedure. Morisita Horn's analyses of duplicates demonstrated high levels of community similarity at the genus level, with an average equal to 0.96 ± 0.05 and values

higher than 0.90 for 89% of duplicates (n. 32 couples) and values between 0.78 and 0.89 for the remaining 11% (n. 4 couples).

A total of 15,104,694 sequence reads were generated; thus, the reads for each sample after passing through filters ranged between 59,435 and 435,042.

Table 2 shows the values of temperature, operational taxonomic units (OTUs), Shannon, and evenness indices for water samples. No significant differences were observed for waters between spas. The microbiological similarity of water samples between the spas and from the same spa emerged from the PCA, as shown in Fig. 3. The PCA demonstrates the clustering of 16 rRNA sequences based on matrix, water and peloid, and spa, A and B. The ANOSIM result also indicated dissimilarity between water and peloid samples within the same spa ($R = 0.85$, $p = 0.001$).

Focusing on the thermal muds, the PCA shows the similarity of samples from the same spa and the dissimilarity between the spas.

The biodiversity indices revealed significant differences between the two spas ($p < 0.05$). The mean value of peloid OTUs in spa A was 1643 (range, 230–2501) and in spa B 1360 (range, 911–1839). Shannon and evenness indices' means were higher in peloids collected in spa A compared to peloids from spa B, as shown in Fig. 4.

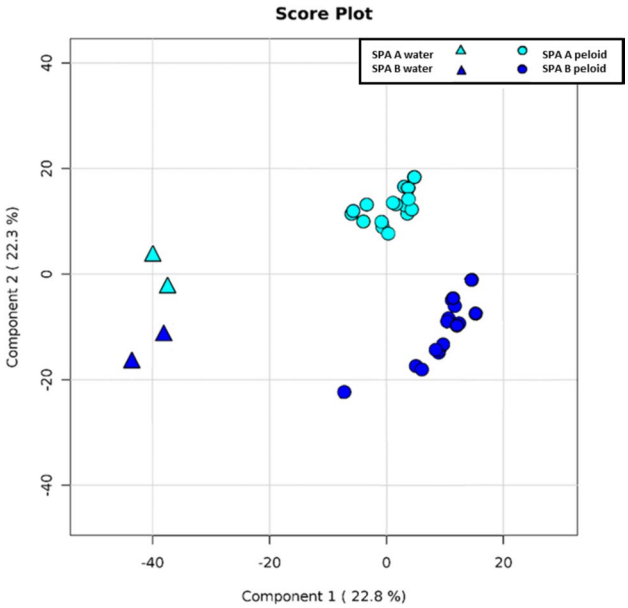


Fig. 3 Principal component analysis (PCA) of robust Aitchison distance values between samples. Data are plotted following the genus-level classification. The variance is explained for 22.8% and 22.3%, respectively, for components 1 and 2. Light blue is for spa A, and dark blue is for spa B. Triangle is for water samples, and circle is for peloids. Each triangle and each circle are the average of the two duplicates

Bacterial Community Composition

Pseudomonadota (mean ± SD 96.92% ± 0.27%) was the predominant phylum in water samples. The genera with the highest relative abundance were *Thiofaba* (mean ± SD 51.20% ± 5.19%) and *Sulfuricurvum* (18.96% ± 15.71%).

Figure 5 shows the predominant phyla of peloid samples clustered for spa. In spa A, the prevalent phyla were *Pseudomonadota* (mean ± SD 52.64% ± 9.66%), *Bacillota* (12.47% ± 3.50%), and *Bacteroidota* (5.47% ± 2.45%). In spa B, the predominant phyla were *Pseudomonadota* (74.68% ± 5.8%), *Bacillota* (5.32% ± 1.5%), and *Actinomycetota* (4.02% ± 1.00%).

The genera distribution in peloid samples is illustrated in Fig. 6. In spa A, the genera with a relevant abundance > 5% were *Thiobacillus* (mean ± SD 14.58% ± 4.89%) and *Pelobacter* (6.94% ± 4.96%). In spa B, the predominant genera (> 5%) were *Thiobacillus* (23.96% ± 4.61%), *Thauera* (13.37% ± 4.85%), *Pelobacter* (8.47% ± 2.38%), and *Desulfuromonas* (7.41% ± 2.66). Moreover, ANOSIM was calculated by comparing the two pools of peloids (spa A *vs* spa B). The result indicated a higher dissimilarity between peloid samples from different spas than those within the same spa ($R = 0.932$, $p = 0.001$).

Figures S2 and S3 show the phyla and genera distribution in both water and peloid samples.

Maturation Phases

The sequence data were further analyzed in order to investigate the differences at the genus level between the spas in the three different maturation stages (2, 4, and 6 months) and between stages within each spa. Table 3 shows OTUs, Shannon, and evenness indices according to stage and spa. In stage 1, significant differences were observed between

Table 2 Temperature and summary of NGS analysis after quality assessment step of sequences for water samples collected at the entry of the pools where peloids mature in spa A (WA) and spa B (WB)

ID	*t* (°C), mean (min–max)	OTUs, mean (min–max)	Shannon index, mean (min–max)	Evenness index, mean (min–max)
WA ($n = 4$)	39.3 (38.1–40.5)	597 (533–670)	1.509 (1.217–1.824)	0.244 (0.189–0.304)
WB ($n = 4$)	42.3 (34.9–49.7)	542 (288–844)	0.837 (0.349–1.334)	0.153 (0.051–0.248)

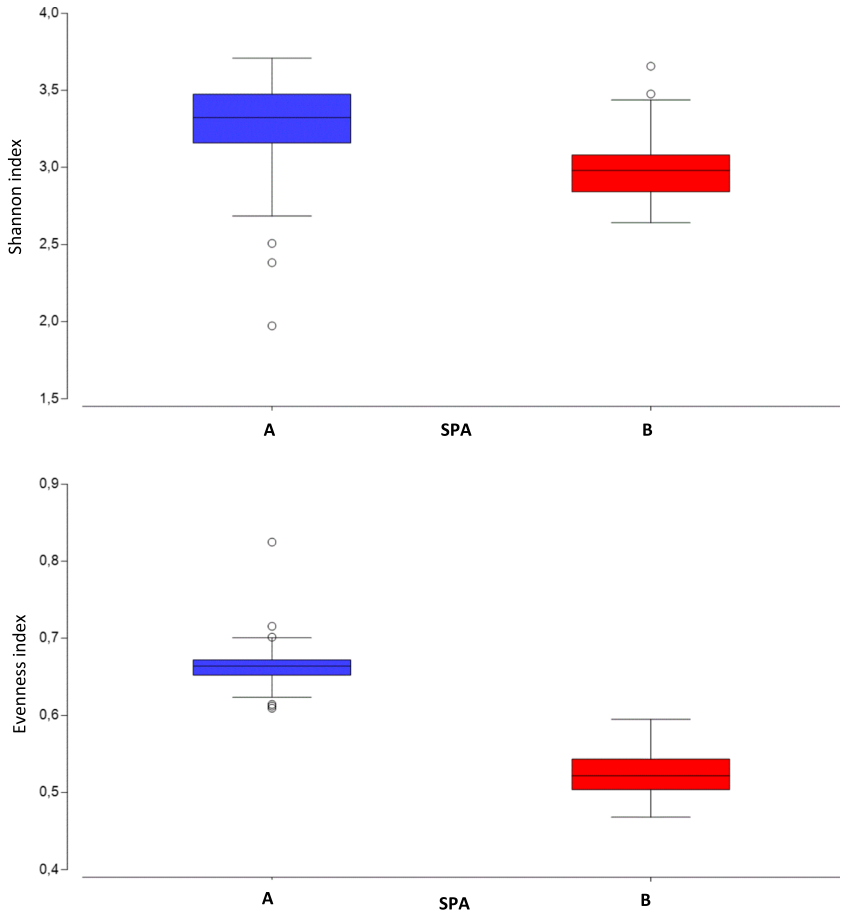


Fig. 4 Shannon and evenness indices of peloid samples according to the spa

spa A and spa B for all indices: OTUs ($p = 0.002$), Shannon index ($p < 0.001$), and evenness index ($p < 0.001$). In stages 2 and 3, only the evenness index differed significantly between spa A and spa B ($p < 0.001$ for both stages). No significant differences in biodiversity indices were observed between the thermal muds collected at different phases in each spa. However, a decreasing trend was highlighted in OTUs and the Shannon index during the maturation process in spa A.

The subgroup analysis displayed a lower similarity between the spas in phase 1 (Table 3), and the ANOSIM also showed a significant dissimilarity ($R = 1$, $p = 0.001$) in this phase. The two-way ANOSIM revealed a significant reduction in variance during maturation ($R = 0.698$, $p < 0.001$) between the two spas.

The microbiological similarity of the peloids at the same maturation phase emerged from the PCA (Fig. 7). The pools by stage appear more separated in spa A compared to B, where they tend to overlap.

The bacterial community profiles were quite similar during the maturation process in the two spas, as shown in Figs. 8 and 9. In spa A, the prevalent genera (> 5%) for all three phases were *Thiobacillus* (13.61%, 15.35%, and 14.67%, respectively) and *Pelobacter* (9.10%, 6.56%, and 6.19%, respectively), as shown in Fig. 8. Moreover, in spa A, the number of genera with a relative abundance above 1% increased comparing phase 1 with phases 2 and 3. In all three maturation stages, the same four genera predominated (> 5%) in spa B (Fig. 9): *Thiobacillus* (22.45%, 24.73, and 24.34), *Thauera* (12.46%, 13.29%, and 13.87%), *Pelobacter* (10.02%, 7.29%, and 8.28%) and *Desulfuromonas* (7.98%, 6.45%, and 7.61%).

Discussion

In this study, we characterized by 16S amplicon sequencing the bacterial community of thermal muds produced in two spas of the same Italian thermal complex. Also, we investigated its evolution during the maturation process. The results revealed differences in the microbiome composition between the spas due to the different initial matrices: natural mud for spa A and sterile clayey virgin materials mainly composed of smectite for spa B, mixed with the same sulfurous-bromine-iodine thermal water. The microbiome of the thermal muds was dominated by typical bacteria primarily involved in the sulfur cycle and lipids synthesis. The biodiversity in all samples was quite similar, regardless of the maturation stage and the predominant genera were almost the same.

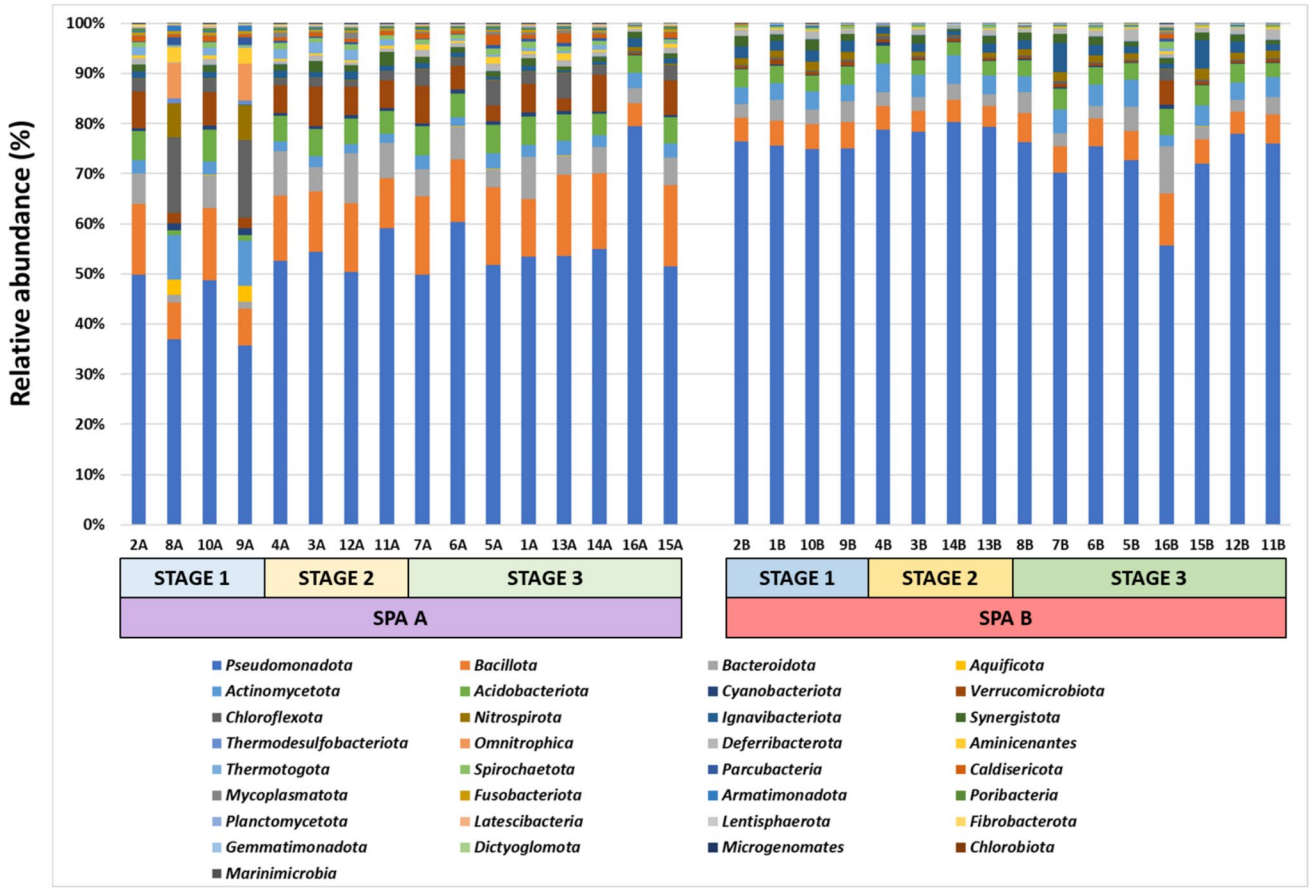


Fig. 5 Distribution of phyla in peloid samples according to spa. Each column is the average of the two duplicates. The samples are arranged according to the stage of peloid maturation and numbered in ascending order from 1 to 16, followed by "A" for spa A and "B" for spa B

Considering the results obtained with the principal components analysis, the samples were clustered according to the matrix (water or peloid) without significant differences for water between spas. In contrast, a lower level of similarity between water and peloids was found in both spas, in line with the study of Pesciaroli et al. [13]. These authors studied the bacterial population that developed during the maturation process of bentonite with water rich in calcium magnesium sulfate under controlled laboratory conditions. They found great differences in the microbial composition of the peloids and thermal water used for their preparation. Indeed, mud maturation is an extremely complex process involving not only the clay/mud/mineral water interactions but also biological and biochemical factors related to the growth of microorganisms and their metabolic products, as well as the habitat where the clay/mud is left to mature [1, 2].

The results from the analysis of the peloids showed significant differences in the biodiversity indices between the two spas. Specifically, the mean Shannon and evenness indices were higher in peloid samples collected from spa A compared to those from spa B. Bearing in mind that the clay used in spa B for thermal mud preparation is sterile, this observation suggests that in spa A, the use of natural mud, which already contains an initial bacterial community, may result in greater biodiversity than peloid obtained by maturing sterile clay in the same thermal water (spa B). Indeed, in the Euganean District, the development of green microbial mats on mature muds, which is a peculiarity of Euganean peloids, has been linked to the use of the local "virgin clay" and the application of a specific maturation process [6, 10]. Tolomio et al. [14] investigated mud maturation comparing the natural substrate with commercial ones (such as bentonite). They showed a strong impairment of the green biofilm growth in the second case. The biodiversity of microbial communities varies significantly between natural and non-natural matrices, with different implications for ecosystem health and environmental sustainability. Natural matrices tend to support greater microbial biodiversity as reported by other authors [14, 31]. In contrast, artificial or non-natural matrices often present reduced biodiversity, with negative consequences for ecosystem health and functionality [32]. These findings are consistent with our observations, suggesting that greater naturalness of environmental matrices can promote higher variability and diversity of the microbiome.

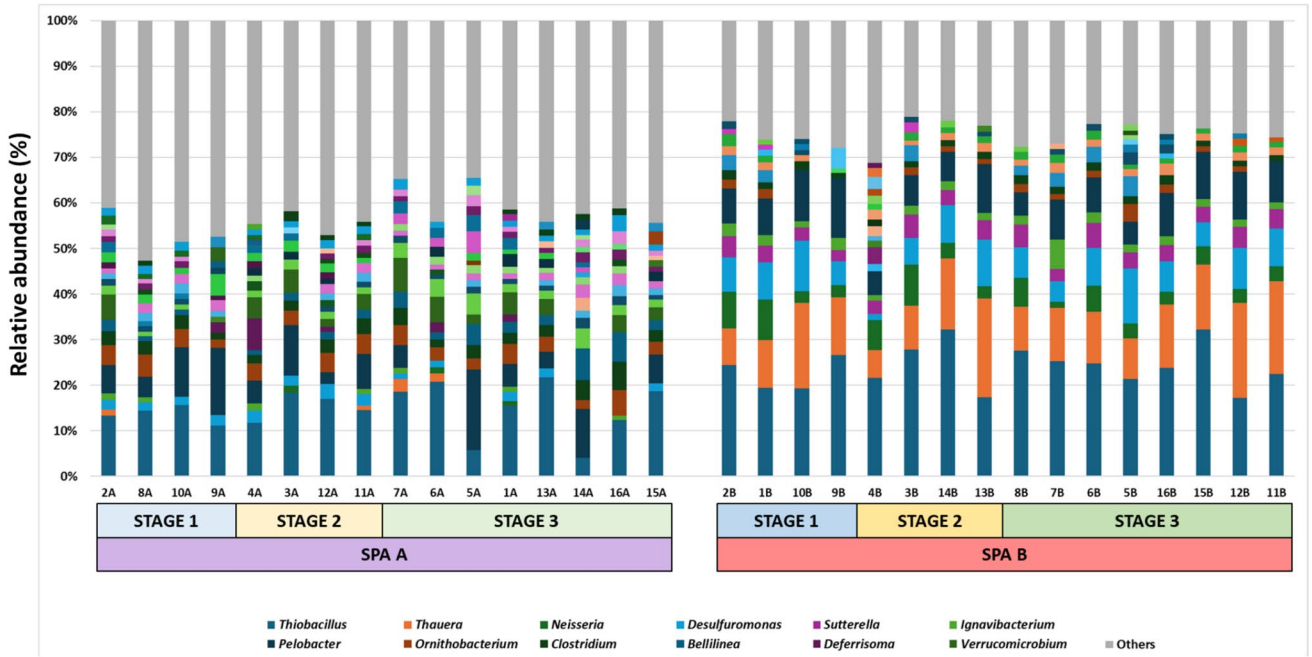


Fig. 6 Distribution of genera in peloid samples according to the spa. Each column is the average of the two duplicates. The samples are arranged according to the stage of peloid maturation and numbered in ascending order from 1 to 16, followed by "A" for spa A and "B" for spa B. Genera with relative abundance < 1% are listed in "others." The legend includes genera with relative abundance > 5%

Table 3 The number of OTUs, Shannon index, and evenness index was calculated for each maturation stage in the two spas. *In stage 1, significant differences between spa A and spa B for all indices: OTUs ($p = 0.002$), Shannon index ($p < 0.001$), and evenness index ($p < 0.001$). **In stages 2 and 3, only the evenness index ($p < 0.001$)

Spa	Stage	OTUs, mean (min–max)	Shannon index, mean (min–max)	Evenness index, mean (min–max)
A	1 (2 months), $n = 4$	1931 (1511–2501)*	3.420 (3.134–3.708)*	0.665 (0.634–0.701)*
	2 (4 months), $n = 4$	1622 (230–2102)	3.295 (2.383–3.636)	0.680 (0.612–0.825)**
	3 (6 months), $n = 8$	1509 (1100–2040)	3.114 (1.973–3.599)	0.657 (0.610–0.715)**
B	1 (2 months), $n = 4$	1261 (911–1407)*	2.898 (2.640–3.096)*	0.529 (0.507–0.551)*
	2 (4 months), $n = 4$	1377 (1058–1839)	3.080 (2.807–3.656)	0.520 (0.468–0.595)**
	3 (6 months), $n = 8$	1400 (1017–1734)	2.981 (2.769–3.190)	0.521 (0.477–0.552)**

The presence of an initial bacterial community in the natural mud used in spa A, combined with the unique properties of thermal water, could create a richer and more varied habitat compared to the sterile substrate employed in spa B. This indicates that natural and less altered conditions can enhance microbiological diversity, highlighting the importance of preserving the natural characteristics of the materials used.

At the phylum level, water and peloids showed similar bacterial profiles due to the prevalence of *Pseudomonadota* (synonym *Proteobacteria*) which was the major phylum in all samples, with percentages ranging from 97 to 75% and 52% of sequences in water, peloids from spa B and peloids from spa A, respectively. This phylum was predominant in water that feeds the pools for peloid maturation, but its relative abundance was reduced in favor of other phyla appearing in peloids like *Bacillota* (synonym *Firmicutes*), the second dominant in both spas, *Bacteroidota* (synonym *Bacteroidetes*) in spa A or *Actinomycetota* (synonym *Actinobacteria*) in spa B. These phyla are described as the common bacterial groups in water and sediment from various terrestrial hot springs around the world [33]. *Pseudomonadota* and *Bacillota* were among the dominant phyla also in the water samples collected along the water network both from the spring and wells to inlet points of both spas and within spa B during our pilot study [21].

At the genus level, the bacterial community characterizing the water that feeds the pools for peloid maturation was dominated by chemolithoautotrophic sulfur-oxidizing bacteria belonging to *Thiofaba* and *Sulfuricurvum* genera, as expected considering that it is sulfurous water. *Thiofaba*

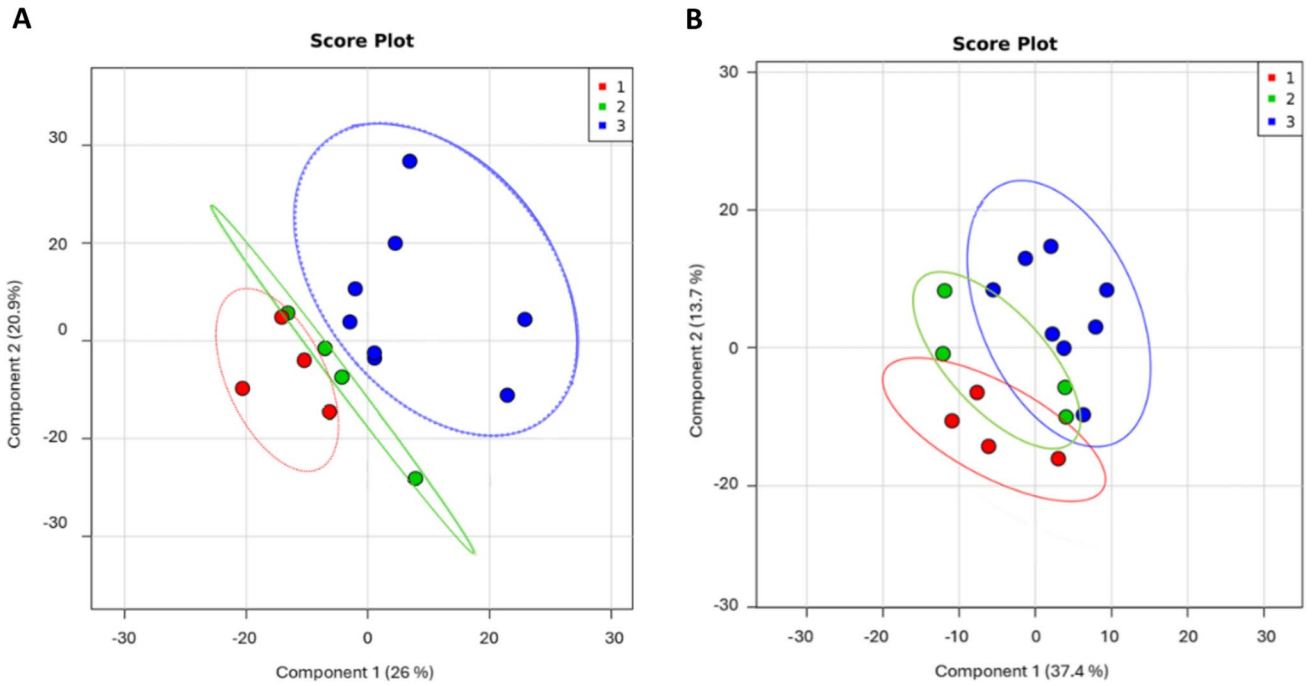


Fig. 7 Principal component analysis of the normalized relative abundance of peloid samples (the duplicates' results are averaged) according to the maturation stage, collected from spa A (**A**) and spa B (**B**). Data are plotted at the genus-level classification. The variance is explained for 26% and 20.9%, respectively, for components 1 and 2 in spa A and 37.4% and 13.7%, respectively, for components 1 and 2 in spa B. Stages 1, 2, and 3 correspond to 2, 4, and 6 months, respectively

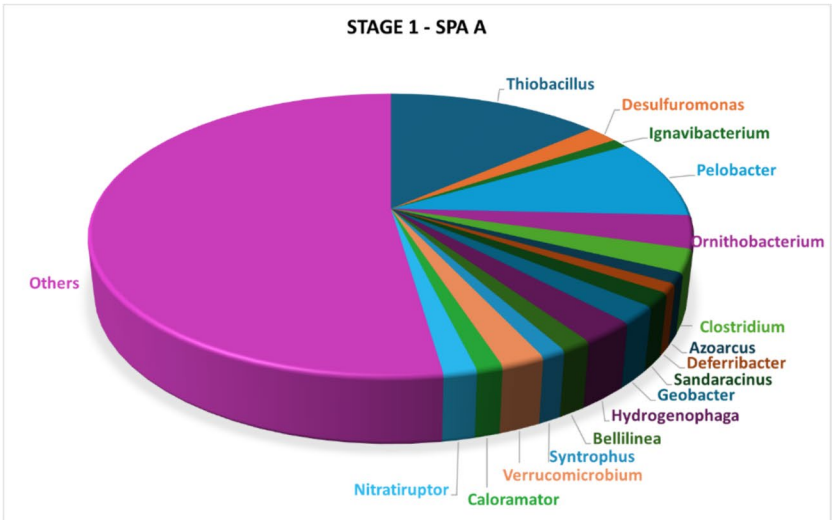
spp. are aerobic bacteria commonly found worldwide in hot sulfur springs [34–36] and geothermal water environments [37, 38]. The monotypic *Sulfuricurvum* genus includes bacteria capable of thriving under microaerobic and anaerobic conditions in sulfide-rich subsurface and groundwater environments [37, 39–42].

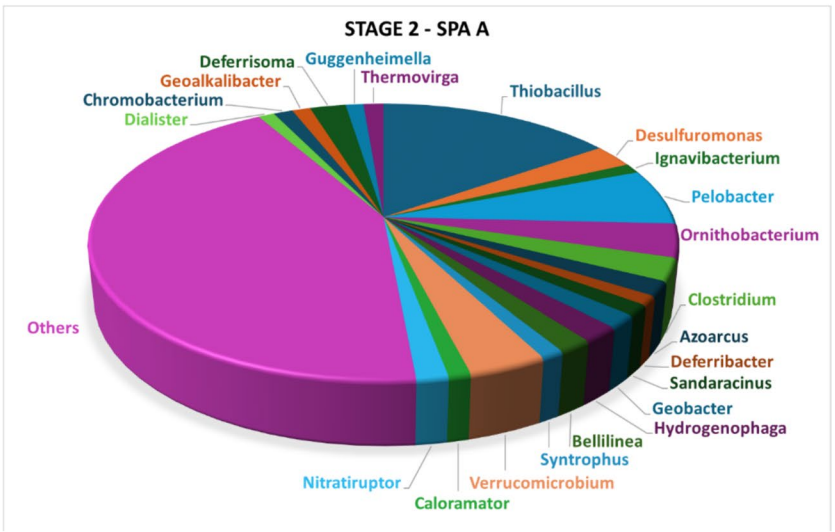
Bacterial genera involved in the sulfur cycle were also found in peloids, as expected considering that they mature in sulfide-rich water. Sulfur-oxidizing *Thiobacillus* genus was found prevalent in both spas, according to our pilot study [21]. Other research reported *Thiobacillus* in peloids utilized in spa treatments in Lithuania [7] and in healing mud sediments from Croatian hot sulfidic springs [43]. Bacteria belonging to *Desulfuromonas* genus, most represented in spa B, are known to live in freshwater sediments and to reduce elemental sulfur to sulfide [44]. These sulfur-reducer bacteria were also observed in sediments from saline environments [45, 46].

Another dominant genus in the peloids of both spas was *Pelobacter*, a bacterium involved in the lipid synthesis, which had already been found in the mud samples collected in the spa B during the pilot study [21]. Sun and colleagues [47] developed genome-scale metabolic models for *Pelobacter carbinolicus* and *Pelobacter propionicus*, first isolated from marine muds and from freshwater sediments and sludge, respectively. They demonstrated that reactions for metabolism of lipids, including fatty acids and glycerophospholipid, are among the most abundant in both *Pelobacter* models. Therefore, the finding of *Pelobacter* in our peloids is a noteworthy result as it is known that the anti-rheumatic action of peloids is related to the anti-inflammatory properties of their lipid fraction, such as fatty acids and phospholipid [2, 48], sulfoglycolipds [49, 50], and galactolipids [51, 52]. However, we acknowledge that this is a purely speculative consideration since the detection of *Pelobacter* was not accompanied by the determination of the lipid content in the mud samples.

In the peloids from spa B, *Thauera* was confirmed among the prevalent genera, according to our previous study [21]. *Thauera* genus consists of denitrifying bacteria isolated from various natural habitats such as hot springs [53–55], freshwater [56, 57], and coastal sediments [58]. Moreover, it is widely used for biotechnological applications due to its adaptable metabolism and ability to grow in several environmental conditions [59, 60]. *Thauera* is known for detoxifying the environment by removing aromatic compounds [61], and its presence might improve the medicinal properties of mud.

Considering the maturation phases, the differences in terms of biodiversity between the two types of thermal mud were clearly visible only in the first phase. In the subsequent maturation phases, the differences became smaller





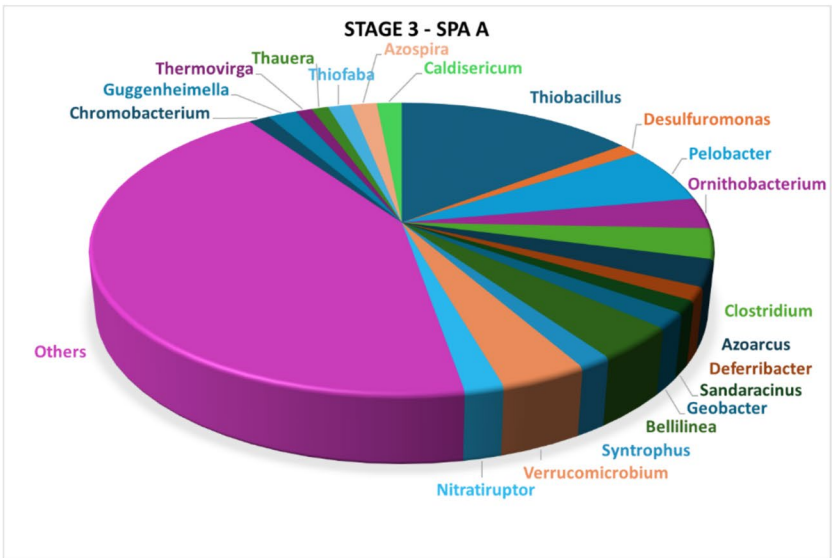


Fig. 8 Distribution of genera in peloid samples collected from spa A, clustering for the three maturation stages (pools). Genera with relative abundance < 1% are listed in "others". Stages 1, 2, and 3 correspond to 2, 4, and 6 months, respectively

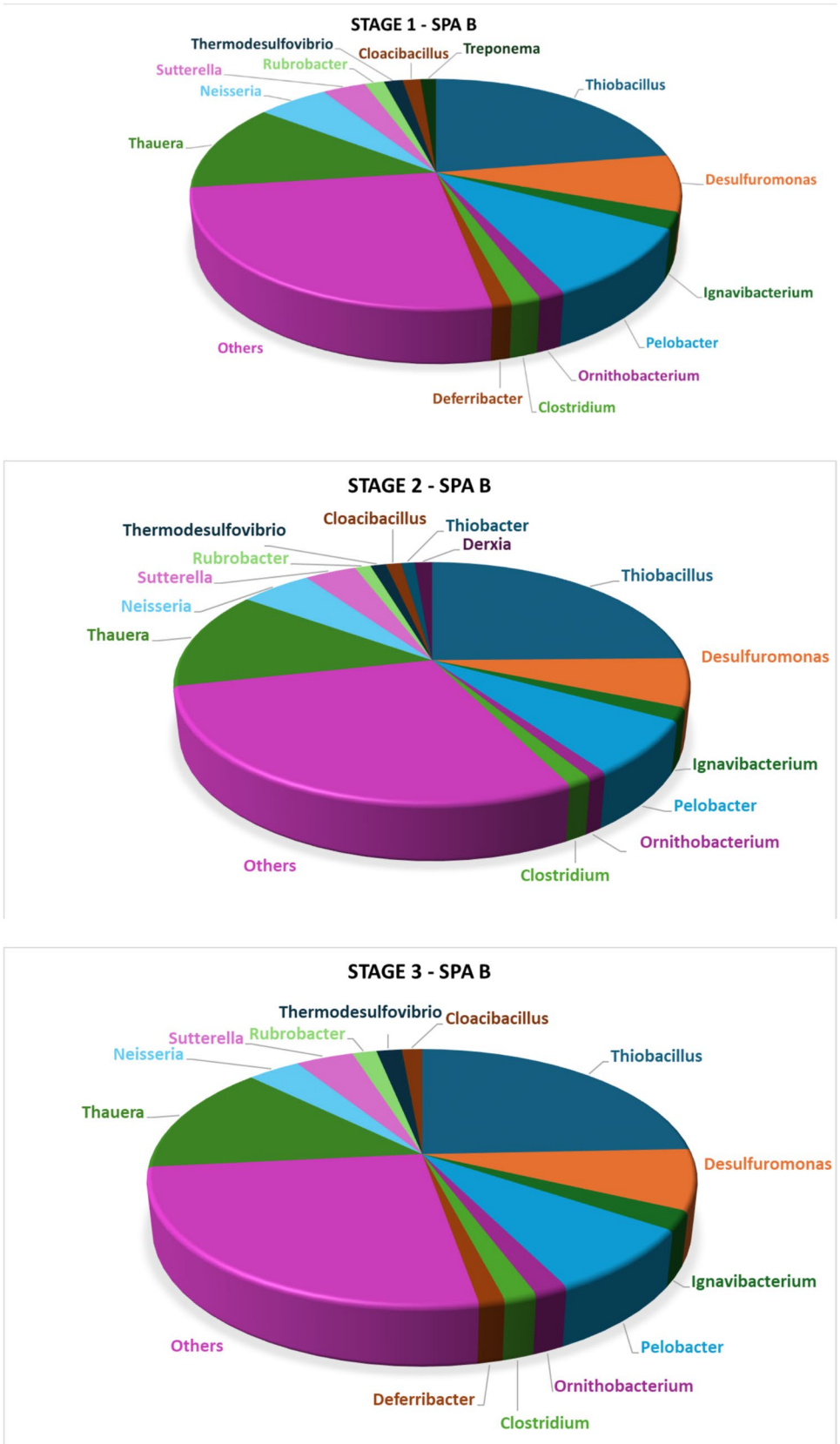


Fig. 9 Distribution of genera in peloid samples collected from spa B, clustering for the three maturation stages (pools). Genera with relative abundance < 1% are listed in "others". Stages 1, 2, and 3 correspond to 2, 4, and 6 months, respectively

due to a slight decrease in biodiversity (OTUs and Shannon index) in the peloids from spa A. Both spas confirmed the predominant genera along the maturation process, with minimal variation between the phases. These modifications were more evident in spa A due to an increase of genera between 1 and 5%. However, a significant difference remained between the two spas at all stages regarding equity in the genera distribution (evenness index), highlighting a higher degree of genera homogeneity in the peloid produced from natural mud than from sterile virgin clay.

Examining the results obtained with PCA according to maturation stages, the pools by phase appear to be more separated in spa A than in B, where they tend to overlap. A possible explanation could be that there is less use of thermal mud in spa A, resulting in more defined stages.

Substantial changes could occur in the early stages of maturation, leading to a relatively stable microbial profile in the later stages as highlighted by Pesciaroli et al. [13]. Under controlled laboratory conditions, they concluded that major changes occurred during the early phases of maturation since the bacterial community was different compared to thermal water, while in all peloid samples (2/3/6 months of maturation), the community was almost the same according to our study.

Currently, mud maturation processes are still often not standardized, and consequently, the reactions characterizing peloid maturation could not be reproducible [1]. Centini et al. [2] emphasize the need to establish standards for thermal mud processing methods to assess their suitability for therapeutic or cosmetic applications. Other authors report the need for protocols to certify the quality and suitability of thermal muds [1, 12, 62]. The Euganean District is one of the few to have a regional rule (BUR, 2015) that codifies the traditional maturing procedures of Euganean mud and must be followed by thermal structures in order to obtain the "Mature Mud AOC" certification [10, 63]. In light of our study, we propose to define guidelines and protocols based on the peculiar chemical and physical characteristics and microbiological components of each type of thermal water and clay/mud involved in the maturing process. For this reason and given our results and the scarcity of studies currently available, it would be necessary to continue our study by characterizing the bacterial community in the early stages of maturation (within the first 2 months) and determining together the inorganic and organic composition of peloids, particularly the lipid fraction.

As natural muds such as lake mud are expected to become scarce in the future, and because of the current widespread use of thermal mud, it would also be important to define specific protocols for the maturation process when thermal mud is produced from sterile virgin clay or other alternative materials and thermal water. The protection of natural mud may circumvent the shortcomings of artificial peloid management, including the reduction of biodiversity [10].

The main limitations of our study are the sample size and the lack of determination of chemical and physical parameters of each peloid sample to evaluate a possible relationship with the microbiological data. Nevertheless, it should be emphasized that the study's strength lies in the fact that it is one of the few studies to evaluate the bacterial community along the peloid maturation process under real conditions. Furthermore, the high level of similarity between duplicates is evidence of the reproducibility of our procedure.

In conclusion, the use of 16S amplicon sequencing in studying the bacterial communities of thermal waters and peloids enables the identification of a biological signature that characterizes a specific thermal matrix, defining its therapeutic and cosmetic properties. Despite the slight fluctuations in genera variety and relative abundance during maturation, our data highlight the presence of a typical bacterial community, mainly composed of sulfur-cycling bacteria, which could be considered the biological signature of this thermal mud. The bacterial composition of peloids appears to be affected by both the thermal water and the type of clay material used in its formulation and maturation. Furthermore, in the studied spas, thermal mud seems to achieve stability in the composition of its bacterial community after 2–4 months of maturation.

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Author Contribution S.P., I.M., and A. B. conceptualized the study; S.P. and I.M. designed the methodology; S.P. and F.V. performed data analysis; S.P., I.M., F.V. and V.R.S. managed data curation; S.P., I.M., M.C.F. and A.B. wrote the original draft; S.P., I.M., F.V. and A.B. reviewed and edited the manuscript; I.M. and G.F. collected the samples; V.R.S. and A.B. supervised the research activity. All authors read and approved the final manuscript.

Data Availability The raw sequencing data have been submitted to NCBI Sequence Read Archive (SRA) with the BioProjects and SRA accession number: PRJNA1134674. Data will be made available on request.

Declarations

Competing Interests The authors declare no competing interests.

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