Original Article

Characterization of Microflora Composition and Antimicrobial Activity of Algal Extracts from Italian Thermal Muds

Antonella Giorgio, Federica Carraturo, Francesco Aliberti, Salvatore De Bonis[1], Giovanni Libralato, Mario Morra[2], Marco Guida

Department of Biology, University of Naples Federico II, [1]Pausilya Therme di Donn'Anna, Naples, [2]ARPA Lazio Sezione Provinciale di Frosinone, Servizio Risorse Idriche e Naturali - Suolo, Rifiuti e Bonifiche, Frosinone, Italy

Abstract

Aim: Fine granular clay, the so-called "peloids," allowed to ripen for several months in contact with mineral thermal water and the organic substances derived from metabolic activities, represents the basis of thermal mud therapy. A complex microalgal community (*Cyanophyceae, Chlorophyceae* and *Bacillariophyceae*) is responsible for the therapeutic effects of thermal muds. Biological components of peloids produce bioactives, possessing anti-inflammatory, antirheumatic and antioxidant properties; for this reason, such matrix is widely used in thermal spas. The research reports results of a preliminary study aimed to characterize the microflora biodiversity of mature and nonmature thermal mud. Algal components were further extracted in order to test the antimicrobial activity of produced bioactive compounds. **Materials and Methods:** Microscopic, microbiological and molecular techniques were employed (DNA extraction, polymerase chain reaction and sequencing). For antimicrobial activity of algal extracts, Kirby–Bauer disck method was employed. **Results:** Results show a significant microfloral diversity in samples and a great number of taxa belonging to widely diffused genera such as *Leptolyngbya* sp., *Nostoc* sp., *Scenedesmus* sp., *Navicula* sp., and *Amphora* sp. Microbial communities indicate an absolute prevalence of a nonpathogenic flora, mostly composed of *Bacillaceae*. **Conclusion:** The association between the microbial and algal composition and the different maturation stages of thermal clay could represent an essential tool to identify markers of proper ripening. This ensures the best product quality and its beneficial properties. The extension of the study, characterizing the components of mud at different ripening times, consents the standardization of ripening process. Antimicrobial activity assay represents a preliminary step for subsequent analysis for the isolation of single component, employing analytical chemistry techniques, characterizing and identifying bioactive compounds of interest.

Keywords: Algae, antimicrobials, genotyping, molecular genetic, polymerase chain reaction

Introduction

Thermal muds have been widely employed since ancient times because of their beneficial effects.[1,2] Thermal muds are particularly recommended for cosmetic and medical use, treatment of cellulite, treating chronic rheumatic diseases, regeneration of the bones after fractures and as a therapy of chronic infections of bronchi, ear, nose and throat. Technically, muds (or peloids) are sludges derived from the mixture of a solid clay fraction with a thermo-mineral liquid component (cold or warm mineral and thermal water). Thermal water has different geothermic origins, whose action on underground water generates thermo-mineral water having different salinity and temperature values.

The specific properties of thermal muds depend on the mixing process: some examples include the water content, clay consistency and adhesiveness, thermal and exchange capacities, and cooling capabilities. The maturation process occurs in particular containers, where the inorganic, sterilized clay component and thermal water are combined. The mixture is generally kept in constant agitation, at a temperature of 60°C, ensuring constant luminous intensities. This procedure represents the critical stage in the development of the algal and microbial microflora, which will influence the subsequent clay maturation process.[3] The high variability in the chemical and physical parameters of the clays and the different thermal

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Address for correspondence: Dr. Federica Carraturo, Department of Biology, University of Naples Federico II, Via Cinthia Ed. 7, Naples, Italy.
E-mail: federica.carraturo@unina.it

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water is responsible for the thermal muds' high diversity of microorganisms, such as bacteria, protozoans, microalgae and cyanobacteria. .[4-6] The organic component, produced by the biological metabolism, can trigger mud maturation and confer specific healing properties.[7,8] In terms of biomass, cyanobacteria constitute the most abundant organisms able to colonize thermal muds. In addition, as pioneer species, they can form large-sized biofilms on the colonized surface. Scientific studies indeed demonstrated the connection between cyanobacteria and the production of several bioactive compounds, such as phycocyanins, flavonoids, unsaturated fatty acids and exopolysaccharides, which can be exploited in different ways, mostly for medical treatments.[9,10] Previous researches demonstrated the antioxidant activity of flavonoids and the anti-inflammatory and antitumoral properties of phycocyanins.[11] The aim of this study was to characterize, using morphological, microbiological and molecular techniques, the microalgal and microbial flora typical of thermal muds, which is responsible for ripen muds' healing properties. The experimented analytical approach is based on the evaluation of thermal muds' quality during the maturation process in order to establish the effectivity of established ripening times, disposing of two thermal muds, sampled at different maturation times. The analysis was first performed through the isolation and characterization of microalgae and natural microbial flora using morphological and microscopic methodologies. The same samples were then analyzed employing genomic techniques: thermal mud genomic DNA was extracted and amplified with specific primer by polymerase chain reaction (PCR) to highlight the differences between the several mud maturation times.

In order to analyze the antimicrobial activity of algal compounds against specific bacterial strains, an organic extraction protocol was employed.

The research has been conducted in collaboration with Pausilya Therme di Donn'Anna, thermal SPA (Naples, Regione Campania, Italy), in the framework of mining operation permits. A standardized protocol for the evaluation of thermal muds' ripening process has not been developed yet, as well as specific regulations were not defined. A system able to objectively indicate the characteristics of thermal muds, based on the microalgal biodiversity and abundance, would ensure the quantitative evaluation of mud maturation levels and therefore the quality of the mud, in terms of therapeutic efficacy and beneficial properties.

Materials and Methods

Sampling and laboratory cultivation

Mud samples were collected aseptically from tanks located in a thermal SPA of Naples. The two samples are indicated as "1-month mud," referred to thermal mud allowed to ripen for 1 month and "6-month mud," describing a sample allowed to ripen for 6 months. For every 20 days, from January to July 2016, we collected muds, disposing a totality of ten samples of each mud type.

Samples were stored at 4°C until they were processed in the laboratory (within 12 h). All mud samples were homogenized in sterile conditions and submitted for microbiological, microscopic and molecular analyses.

Analysis of microalgal community

An aliquot of 5 g from samples was weighed into sterile flasks and inoculated with 50 ml of specifying enrichment media, to allow microalgae growth. For the isolation of cyanobacteria, green algae and diatoms, Blue-Green 11,[12] Bold Basal,[13] and WC[14] media were used. The inoculated samples were incubated in a growth chamber at 30°C ± 2°C with a light intensity of 7.6 W/m2 (photosynthetically active radiation) and a dark/light cycle of 12/12. Three weeks after the beginning of incubation, microalgae biofilms were collected and transferred to new media, to avoid microalgal death. In order to isolate single microalgal species, an aliquot (100 µL) of algae mixture was plated, using sterile technique, onto the surface of a Petri plate containing agarized algal growth medium.[15] Inoculated plates were placed in the same condition of liquid culture until the growth of algal colonies. Colonies of different morphologies and color were collected and aseptically inoculated in the liquid medium. This procedure was repeated until achievement (the setup) of monoalgal cultures, which were used in the following laboratory analysis.

Microscopic analysis

Microscopic analysis of cyanobacteria and green algae, in living state, was made with an optical microscope, equipped with a digital image acquisition system (Leica DM4 B). Morphological features (such as chloroplast shape, cell dimension and colonies' structure) were observed at ×400, ×600 and ×1000 magnifications for the identification of genera and species. The identification of algae required standardized taxonomic literature.[16]

Samples for diatom analyses were cleaned with 30% hydrogen peroxide solution to remove organic material[17] before their mounting on microscope slides in a highly refractive medium (Naphrax Resine, Northern Biological Supplies Ltd., UK; refractive index=1.74). Diatom taxa were identified following the Krammer and Lange–Bertalot monographs,[18,19] as well from the Krammer[20] and Lange–Bertalot[21,22] monographs.

Total bacterial count isolation

Total bacterial count analysis was performed according to ISO 4833-1:2013, which includes pouring 1 mL from the thermal mud sample in plate count agar (PCA) (Oxoid, Thermo Fisher Scientific, USA). PCA plates were then incubated at 30°C ± 1°C for 72 h. The totality of colonies was counted. Different morphology colonies were subcultured in fresh PCA agar, reconstituted with 50 µL Milli-Q Type 1 ultrapure water and submitted for molecular analysis, in order to identify each bacterial strain.

Molecular analysis

Individually isolated microalgae cells and bacterial colonies were analyzed by molecular techniques too. DNA extraction

Table 1: Primers sets used for molecular assays, listed according to each target microorganism

Primer set	Primer	Sequence 5' → 3'	Target organism	Expected size of PCR product (bp)
Forward	V3_F	CCAGACTCCTACGGGAGGCAG	Bacteria	± 700 bp
Reverse	V6_R	TCGATGCAACGCGAAGAA		
Forward	Cya106_F	CGGACGGGTGAGTAACGCGTGA	Cyanophyceae	± 680 bp
Reverse	Cya783_Rev	GACTACAGGGGTATCTAATCCCT		
Forward	EuK1A_F	CTGGTTGATCCTGCCAG	Chlorophyceae	± 500 bp
Reverse	EUK516_Rev	ACCAGACTTGCCCTCC		
Forward	Baci_F	AGATTGCCCAGGCCTCTCG	Bacillariophyceae	± 500 bp
Reverse	Baci_Rev	CCATCGTAGTCTTAACCATAAAC		

procedure of Doyle and Doyle (1987) was performed.[23] Purity and quality of the extracted DNA were analyzed on a 0.8% agarose gel with 0.5% TAE buffer, stained with GelRed (GelRed Nucleic Acid Gel Stain, Biotium). DNA quantification was performed with spectrophotometer NanoDrop 2000 (Thermo Fisher Scientific). PCR amplification was carried out using four sets of primers [Table 1] specific for 16S rDNA for bacteria (Primer set 1), 16 rDNA for *Cyanophyceae* (Primer set 2), 18S rDNA for *Chlorophyceae* (Primer set 3) and 18S rDNA for *Bacillariophyceae* (Primer set 4).[24-26] All the PCR reactions were set up using a Prime Thermal Cycler (Techne) at a total volume of 50 µL containing 5 ng of DNA, 5 µL of 10X reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each primer and 2 U of Taq DNA polymerase (VWR Chemicals, Milan,Italy). The PCR amplifications were performed according to the bibliography indication.[24-27] An aliquot (5 µL) of PCR products was electrophoresed in 1.5% agarose gel at a voltage of 70 V for 1 h. Gels were stained with GelRed (GelRed Nucleic Acid Gel Stain, Biotium) and visualized under ultraviolet illuminator, using a 100 bp DNA ladder (DNA Molecular Weight ladders, Amresco) as size marker. Purified PCR products (polyethylene glycol precipitation protocol) were used as templates in sequencing reaction with the BigDye Terminator V3.1 (Applied Biosystems, USA) following manufacturer's procedure. Sequencing was performed using an ABI Prism 3100 (Applied Biosystems) and sequences were analyzed with Chromas Lite software, version 2.1.1. (Chromas Lite version 2.1, Technelysium; http://technelysium. com. au/?page_id=13, Technelysium Pty Ltd Unit 406 8 Cordelia St South Brisbane QLD 4101 Australia) and submitted for Blast analysis to taxonomic affiliation.

Algal material extraction

Algal cultures were collected after 15 days' growth and filtered through Whatman filter paper No. 2 (Whatman International Ltd., Maidstone, UK) for isolation of the cells from the culture medium. The algal communities extracted from the muds under analysis were cleaned up in tap water and rinsed several times in distilled water. The algal samples were allowed to dry in air and stored in 15 mL tubes at room temperature. The algal materials were soaked in different solvents within 50 mL tubes (3 g dry algae/25 mL solvent), kept on a shaker at 150 rpm at room temperature (30°C) for 24 h. In order to analyze the activity of all organic components, four solvents were used: chloroform, ethanol, hexane and methanol. The samples were then filtered by Whatman No. 1 filter paper: filtrates were dried under reduced pressure at 40°C, using a Rotavapor® R-300 (BUCHI). Dried pellets were then dissolved in 5 mL dimethylsulfoxide. Extracts were filtered using 0.2 µm Millipore filter and stored at −20°C until antimicrobial testing.[28,29]

Antimicrobial activity assay

The antimicrobial activity of mud algae extracts was evaluated against Gram-positive and Gram-negative bacteria. The organisms used in this study were ATCC® strains. The following five bacteria were used for antimicrobial assay: *Escherichia coli* ATCC® 8739™, *Klebsiella pneumoniae* ATCC® 13833™, *Pseudomonas aeruginosa* ATCC® 9027™, *Staphylococcus aureus* ATCC® 25923™ and *Streptococcus pyogenes* ATCC® 19615™.

Pathogenic bacteria were grown in Tryptone Soya Broth – OXOID, in 15 mL centrifuge tubes at 37°C, obtaining a 1×10^6 cells/mL concentration. Disc method as indicated by Bauer *et al*. was employed. Microorganisms, grown at a 10^6 cells/mL concentration, were plated, using a sterile swab, in Petri dishes containing Tryptone Soya Agar – OXOID. In the meanwhile, sterile discs of 6 mm diameter (antimicrobial susceptibility testing – OXOID) were soaked with 25 µl of the algal extracts (4 µg/µl), as for extracted samples, or with the used solvents, for negative controls: the prepared discs were added to the dishes spread with each of the five bacteria under analysis. Petri dishes were incubated for 24 h at 37°C and the toxic effects of algal bioactive compounds against microorganisms were determined by measuring the diameter of the halo developed around the discs. The experiments were performed in triplicate. Highly representative halos were those producing a halo with a 10 mm or higher diameter. For positive control, chloramphenicol was employed.

Results

Overall, seven taxa of green algae, two taxa of cyanobacteria and seven taxa of diatoms were observed in both samples [Table 2]. Regarding microalgal community, sample of muds allowed to ripen for 6 months (6-month mud) presented a higher biodiversity compared to the mud allowed to ripen for 1 month (1-month mud). The dominant groups were represented

Table 2: List of taxa isolated from the mud samples allowed to ripen for different times (1 month; 6-months)

1-Month Mud	6-Months Mud
BACTERIA	
Bacillus sp. Cohn, 1872	*Bacillus* sp. Cohn, 1872
Bacillus subtilis, (Ehrenberg 1835) Cohn, 1872	*Bacillus megaterium* de Bary, 1884
Bacillus amyloliquefaciens Priest *et al*., 1987	*Bacillus simplex* Priest *et al*., 1989 emend. Heyrman *et al*., 2005
Bacillus thuringiensis Berliner, 1915	*Bacillus subtilis* (Ehrenberg 1835) Cohn, 1872
Pseudomonas sp. Migula, 1894	*Bacillus licheniformis* (Weigmann 1898) Chester, 1901
	Bacillus thuringiensis Berliner, 1915
	Bacillus litoralis Yoon and Oh, 2005
	Paenibacillus sp.Ash *et al*., 1994
CYANOPHYCEAE	
Leptolyngbya sp. Anagnostidis et Komárek, 1988	*Leptolyngbya* sp. Anagnostidis et Komárek, 1988
Anabaena sp. Bory ex Bornet et Flahault, 1888	*Anabaena* sp. Bory ex Bornet et Flahault, 1888
	Nostoc sp. Vaucher ex Bornet et Flahault, 1888.
CHLOROPHYCEAE	
Chlorella sp. Beyerinck, 1890	*Chlorella* sp. Beyerinck, 1890
Scenedesmus sp.Meyen, 1829	*Coccomyxa* sp. Schmidle, 1901
Coccomyxa sp.Schmidle, 1901	*Scenedesmus* sp. Meyen, 1829
	Chlamydomonas sp.Ehrenberg, 1833
	Pseudococcomyxa simplex (Mainx) Fott, 1981
	Monodus sp. Chodat, 1913
BACILLARIOPHYCEAE	
Navicula cincta (Ehrenberg) Ralfs in Pritchard, 1861	*Amphora ovalis* (Kützing) Kützing, 1844
Cocconeis placentula Ehrenberg, 1838	*Surirella brebissonii* Krammer and Lange-Bertalot, 1987
Rhoicosphenia abbreviata (Agardh) Lange-Bertalot, 1980	*Rhoicosphenia abbreviata* (Agardh) Lange-Bertalot, 1980
	Nitzschia palea (Kützing) W. Smith, 1856
	Navicula cincta (Ehrenberg) Ralfs in Pritchard, 1861
	Cocconeis placentula Ehrenberg, 1838
	Gomphonema acuminatum Ehrenberg, 1832

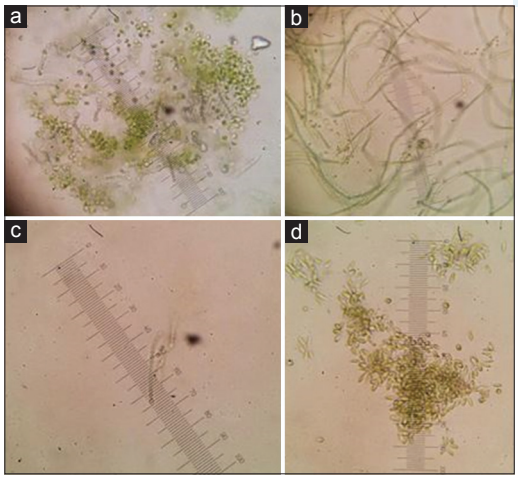

Figure 1: Microphotographs of the members of *Cyanophyceae* (a-c) and *Chlorophyceae* (d) isolated from mud samples

by *Chlorophyceae* (seven taxa) and *Bacillariophyceae* (seven taxa). Microscopic analysis based on the observations of morphological characteristics (size and shape of cells, chloroplast and the presence of wall ornamentation) have revealed the presence of some genera and species of microalgae and diatoms, such as *Leptolyngbya* sp., *Nostoc* sp., *Chlorella* sp., *Coccomyxa* sp., *Cocconeis placentula, Amphora* sp. And *Rhoicosphenia abbreviata* [Figures 1 and 2].

On light microscopy, *Leptolyngbya* is typified by cells organized into long, solitary, or coiled into clusters and filaments. Filaments are arcuated, waved, or intensely coiled, isopolar, thin and fine, with usually colorless facultative sheaths opened at the apical end. Cells are isodiametrical or longer than wide (up to several times), cylindrical, with homogeneous content and pale blue-green, grayish, olive-green, yellowish, or reddish. *Nostoc* cells, observed at microscope, appear cylindrical, spherical, or ovoid (barrel shaped) and are not shorter than broad. Cells are organized in trichomes and spherical or ovoid gelatinous thalli are often visible. *Chlorella* cells are spherical, subspherical, or ellipsoid and single or colony forming. A defining feature of the genus is the single and parietal chloroplast with a pyrenoid surrounded by starch grains. *Coccomyxa* cells are easily recognizable by green nonflagellated colonies and by laminated layers of secreted mucilage-surrounded cells.

The oxidation treatment, using hydrogen peroxide, proved to be valuable to morphological identification of diatoms. On light microscope, valves of *Cocconeis placentula* result elliptic to linear-elliptic and relatively flat, with a filiform rafe,

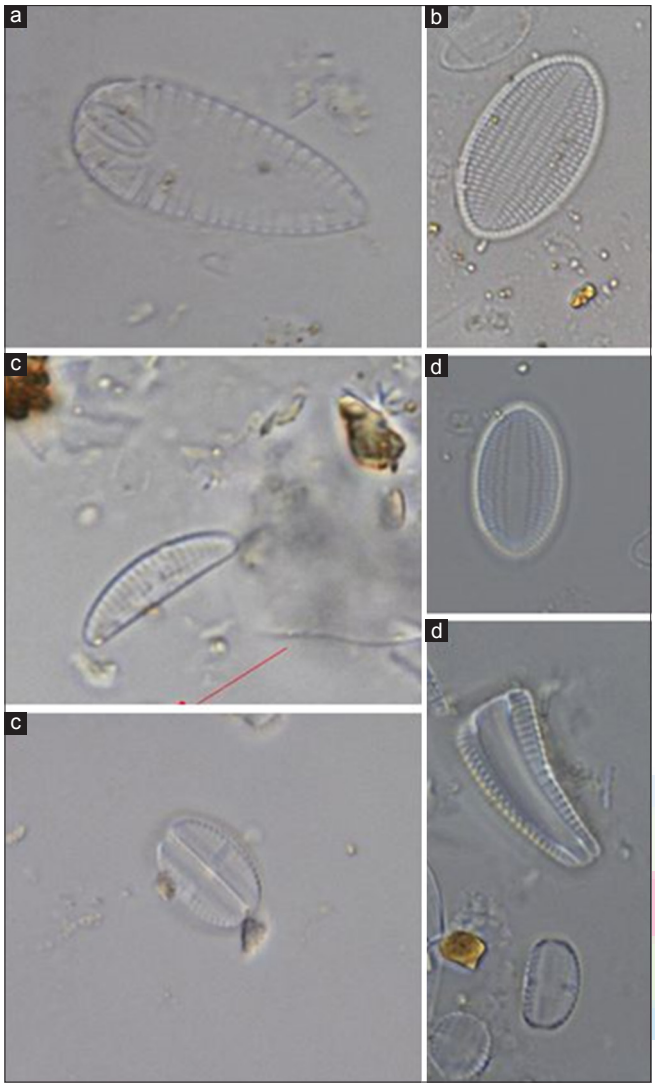

Figure 2: Microphotographs of the members of *Bacillariophyceae* isolated from mud samples. *Surirella brebissonii* (a), *Cocconeis placentula* (b), *Amphora ovalis* (c) and *Rhoicosphenia abbreviata* (d)

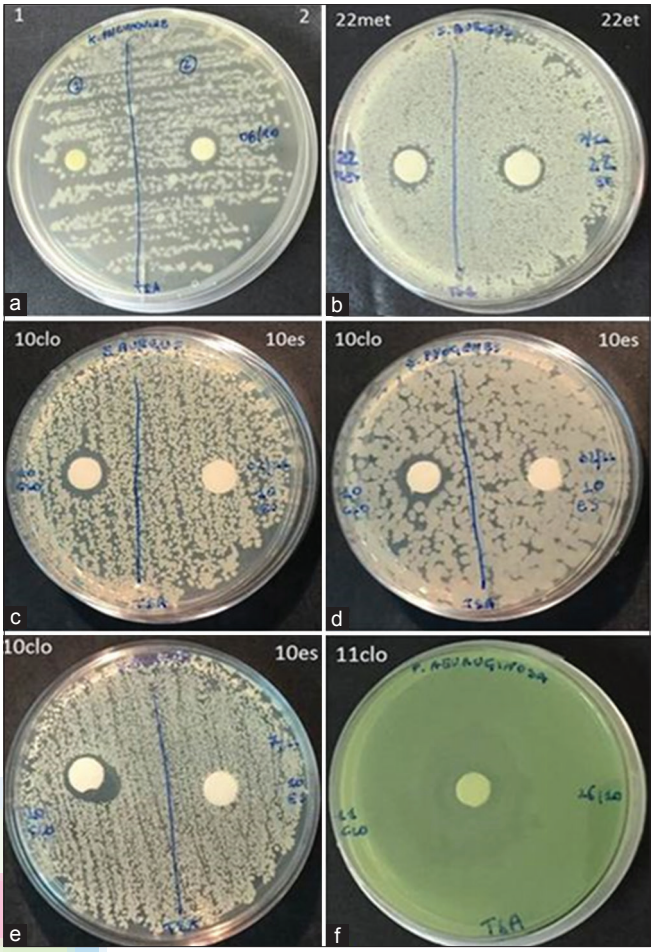

Figure 3: Antimicrobial assays of algal extracts. (a) Disc diffusion test of methanol algal extracts (1 = 1-month mud; 2 = 6-month-mud) on *Klebsiella pneumoniae*. (b) Disc diffusion test of methanol (22met) and ethanol (22et) algal extracts of 6-month-mud on *Staphylococcus aureus*. (c) Disc diffusion test of chloroform (10clo) and hexane (10es) algal extracts of 6-month-mud on *Staphylococcus aureus*. (d) Disc diffusion test of chloroform (10clo) and hexane (10es) algal extracts of 6-month mud on *Staphylococcus pyogenes*. (e) Disc diffusion test of chloroform (10clo) and hexane (10es) algal extracts of 6-month mud on *Escherichia coli*. (f) Disc diffusion test of chloroform algal extracts (11clo) of 6-month mud on *Pseudomonas aeruginosa*

a narrow axial area and a small circular or oval central area. Distinctive elements are striae radiate and the hyaline ring positioned close to valve margin. *Rhoicosphenia abbreviata* valves appear clavate or club shaped with rounded apices. Frustules, in girdle view, are distinctly arched and heteropolar. Amphora valves result lunate, presenting the dorsal margin much deeper than the ventral. The raphe toward ventral margin is sinuous to strongly arcuate. The dorsal margin sometimes has hyaline areas. Surirella valves appear, under microscope, heteropolar, with headpole rounded and footpole cuneately rounded. The valves are slightly concentrically undulate. The costae extend from the margins to the apical axis and fibulae are marginal.

Molecular assays allowed the characterization of thermal muds' microflora which confirmed the morphological outcomes. The most abundant benthic microalgae taxa, identified in both samples, are *Chlorella* sp., *Coccomyxa* sp., *Scenedesmus* sp., *Leptolyngbya* sp., *Anabaena* sp., *Cocconeis placentula, Rhoicosphenia abbreviata* and *Navicula cincta*. *Nostoc* sp., *Scenedesmus* sp., *Chlamydomonas* sp., *Pseudococcomyxa simplex*, *Monodus* sp., *Gomphonema acuminatum*, *Amphora ovalis* and *Nitzschia palea* were isolated exclusively from ripen mud.

Bacteria were characterized and selected for molecular analysis with respect to the morphology of the colonies. Isolated bacterial strains are listed in Table 2. As for microalgal communities, ripen mud presented a higher biodiversity in terms of genera and species isolated. Bacterial DNA characterization allowed the identification of 13 bacterial taxa. Among these, five were isolated from 1-month mud and eight from 6-month mud. An 85% isolation rate belongs to

Table 3: Antimicrobial activity of the extracts of 1-month-mud and 6-months-mud

Solvent employed	1 month mud	6 months mud
Ethanol		
Staphylococcus aureus	-	+
Streptococcus pyogenes	-	-
Klebsiella pneumoniae	-	-
Escherichia coli	-	-
Pseudomonas aeruginosa	-	-
Methanol		
Staphylococcus aureus	-	+
Streptococcus pyogenes	-	-
Klebsiella pneumoniae	++	++
Escherichia coli	-	-
Pseudomonas aeruginosa	-	-
Hexane		
Staphylococcus aureus	-	-
Streptococcus pyogenes	-	-
Klebsiella pneumoniae	-	-
Escherichia coli	-	-
Pseudomonas aeruginosa	-	-
Chloroform		
Staphylococcus aureus	-	++
Streptococcus pyogenes	-	++
Klebsiella pneumoniae	-	-
Escherichia coli	-	++
Pseudomonas aeruginosa	-	+++

A scoring system was generated in order to better explain the toxicity of algal bioactive molecules against bacterial inocula: "-", no activity (<6 mm); "+", low activity (6-8 mm halo); "++", good activity (8-10 mm halo); "+++", really good activity (>10 mm halo)

the Bacillaceae family, while one taxa of *Pseudomonas* sp. and one of *Paenibacillus* sp. were, respectively, identified from 1-month and 6-month mud. Comparing the isolates, *Bacillus sp., Bacillus subtilis* and *Bacillus thuringiensis* were isolated from both samples.

The bacterial isolates of the 1-month mud and of the 6-months mud, obtained using different solvents, have showed a different activity against the bacterial microorganisms under analysis [Figure 3]. Positive controls with chloramphenicol showed 20 mm halos using 4μg/μl concentrations and pouring the discs with 25 μl solutions (same as algal extracts).

For a better comprehension of the toxic effect on bacterial inocula, a scoring system was generated: whether no effect was observed, corresponding to a <6 mm halo, a "—" was used; when the zone of inhibition was between 6 and 8 mm, a "+" score was assigned, indicating a low inhibition effect; a good effect of the extract, with an 8–10 mm inhibition zone, was indicated with "++;" an inhibition zone >10 mm, indicating a really good effect, was classified as triple-positive symbol, "+++" [Table 3].

Analyzing the activity of the 1-month-mud extract, the methanol extraction evidenced antimicrobial activities only against Klebsiella pneumoniae [Figure 3a].

The extracts resulting from algal components of the 6-month-mud highlighted a various and different antimicrobial capability with the microbial strains tested. Extracts from chloroform protocol showed activity against all bacterial strains, excepting *Klebsiella pneumoniae*. In particular, the largest inhibition halo – <10 mm – was registered for *P. aeruginosa* [Figure 3f]. Methanol extraction allowed the evaluation of a good inhibition activity of the ripen mud algal extract against Staphylococcus aureus and Klebsiella pneumoniae, with inhibition halos comprised between 8 and 10 mm [Figure 3b]. The extractions performed using ethanol and hexane did not show microbial growth inhibition for both algal extracts (1-month-mud and 6-month-mud) against the microorganisms' inocula under analysis.

Discussion

The protocols adopted, based on microscopic and molecular analyses, were proven to be valuable for the identification and characterization of thermal muds' microflora and to associate the maturation process to the microalgal composition. Liquid and solid cultural methods, followed by morphological observations, employing optical microscope, allowed the identification of some of the isolated microalgal species belonging to the photosynthetic biomass, in particular, *Cyanophyceae*, *Chlorophyceae* and *Bacillariophyceae*. The molecular analysis confirmed the presence of the taxa which have been characterized at the microscope and consented the identification of microorganisms of difficult taxonomic classifications.

The analysis carried out on the muds allowed to ripen for 6 months evidenced a higher biological complexity. Considering that the evaluated samples have an environmental origin, during the maturation process, it is possible to observe the differentiation of the investigated consortia, expressed in terms of quantitative and/or qualitative variation of the biodiversity.

The results of the antimicrobial activities evidenced differences considering the extraction matrix (maturation levels) and the solvent type. Differences reside in the different extraction capabilities of the solvents; the different susceptibility of the analyzed strains toward the extracted metabolites (also depending on the varying virulence of the pathogenic bacteria).[30] Indeed, methanol is indicated for the extraction of polar molecules, such as proteins, while chloroform, with a lower polarity, consents the extraction of the grease component. The lack of efficacy of ethanol and hexane extraction could be linked to the rapid volatility of the two solvents or to the low antimicrobial activity of the extracted components, against the pathogens tested. It can be hypothesized to combine solvents in mixes in order to optimize the extraction process, widening the recovery of type of bioactive compounds.

The results indicate a higher concentration of metabolically active compounds, in continuous development because of the enrichment of the biological components produced by the thermal mud (bacteria, cyanobacteria, green algae and

diatoms). The isolation of *Cyanophyceae*, *Chlorophyceae* and *Bacillariophyceae*, overall nonpathogenic microorganisms, was confirmed by similar studies,[10,31] reporting that the metabolic activities of such microorganisms are responsible for the production of fatty acids, lipids and other macromolecules, having therapeutic properties. The microalgal diversity of the investigated thermal muds seems to be responsible for their beneficial activity. Some genera of *Chlorophyceae*, *Cyanophyceae and Bacillariophyceae, such as Nostoc sp., Anabaena sp., Leptolyngbya sp., Coccomyxa sp. and Chlorella* sp., isolated from the analyzed samples, are known to have important properties.

Among cyanobacteria, Leptolyngbya, Nostoc and Anabaena could exhibit bioactivity and produce interesting compounds and molecules capable of inducing cytotoxic effect in human cancer lines;[32,33] such microorganisms indeed have potential applications in pharmaceutics.[34] A study conducted by Gustafson *et al*. (1989) reported the isolation of anti-HIV molecules (active sulfoglico lipids) from strains of *Anabaena* sp. Some species of *Nostoc* sp., are proved to produce antiviral proteins and lipophilic extracts with antibacterial activity.[35] According to Takara et al., 1990 and Kravolec *et al*., 2007, *Chlorella* algal extract could have antitumoral and anti-immunostimulatory activities.[36] Polysaccharidic extract of some *Coccomyxa* strains has demonstrated efficacy against the influenza A virus infection.[37] Among diatoms, *Navicula* and *Amphora* were widely investigated for their antioxidant activities.[38]

The isolated microbial strain results confirm previous researches reporting Bacillaceae and Paenibacillaceae as the most frequently identified bacterial species from geothermal environments.[39] Bacillaceae, Gram-positive, aerobic and rod-shaped microorganisms[40] are frequently isolated from water and soils and generally do not represent a health risk for the consumers. Bacillaceae, indeed, have been employed for decades as probiotics and, because of their ability to germinate in the gastrointestinal tract, producing bacteriocins and antibiotics acting toward pathogens, they are widely employed for biotechnology applications. Some examples of the strains showing antimicrobial activity are *Bacillus cereus*, *Bacillus clausii*, *Bacillus subtilis* and *Bacillus licheniformis* (The last two species were both isolated from the thermal muds under evaluation).[41] *Bacillus* have been demonstrated to produce carotenoids, the most abundant pigment group in nature,[42,43] owning anticancer, anti-inflammatory and anti-oxidative properties. *Bacillus* spp., metabolism additionally enables the efficient production of purine nucleosides and vitamins. *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis* and *Bacillus megaterium* are largely exploited as producers of vitamins such as cobalamin, riboflavin, folic acid and biotin.[44-47]

Monitoring for pathogens considered fecal indicators which was moreover crucial to determine thermal mud safety, considering that the detection of such opportunistic pathogens in groundwater can represent an indication of contamination of surface waters. The isolation of fecal indicators in thermal waters employed for the treatment of pathologies involving the elderly, patients affected with various chronic conditions and, in general, immune-compromised individuals, constitutes a substantial health risk.[48] Strains having fecal origin, such as *Escherichia coli* or *Clostridium perfringens*, were not detected. One strain of *Pseudomonas* sp., a nonfecal microorganism, was isolated from the 1-month mud sample. The health risk connected to *Pseudomonas* genus is related to *P. aeruginosa* species, for which strict tolerance levels in case of isolation in bathwaters are allowed.[49] Hence, the isolation of a non-*P. aeruginosa* species does not represent a risk for the customers.

Conclusion

Based on the obtained results, further characterization of the bioactive compounds produced by the isolated microalgal species during the mud-ripening stages will confirm the effectiveness of the mud therapy. The available data reported the major microflora isolated from the 6-month-mud, not only in terms of abundance but also with respect to biodiversity and previous researches demonstrated the therapeutical efficacy of the microalgal flora isolated from the thermal clays under analysis: the combination of the results confirms the reliability of mud microorganism characterization to define ripening markers. Furthermore, the disposal of a method able to evaluate the maturation stages, through the identification of marker microorganisms, and to define the proper ripening times, could provide a precious contribution for the optimization of the maturation itself, aiming to the standardization of thermal muds' maturation process. Hence, thermal muds at the end of maturation process represent an important source of bioactive compounds, which can be employed as antimicrobials. Further studies, employing analytical chemistry techniques, will allow the identification of the active molecules responsible for the antibacterial activities.

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Conflicts of interest

There are no conflicts of interest.

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