



RESEARCH PAPER

Screening and isolation of microbes from a Mud Community of Ischia Island Thermal Springs: preliminary analysis of a bioactive compound

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Keywords

Microbes • Thermal springs • Ischia island • Antimicrobial activity • Antibiofilm activity

Summary

Introduction. *Balneotherapy centers of Ischia island (Italy) offer treatments for different dermatological diseases (psoriasis, acne, atopic dermatitis) and upper respiratory tract infections. In this study, we integrated morphological and molecular approaches to give a focus on isolation and screening of extremophile bacteria from Ischia thermal mud for potential antimicrobial applications.*

Methods. *Samples were collected during 2019 at four sites. Some bacterial strains ATCC for antibacterial and antibiofilm activity were tested. After morphological characterization, screening for antagonistic isolates was made. The colonies isolated from thermal mud samples were submitted to molecular characterization. Susceptibility testing by dilution spotting was carried out*

and antibacterial efficacies of most active isolate were evaluated with a Minimal inhibition concentration assay. Biofilm formation, inhibition, eradication were examined. Statistical analyses were carried out utilizing Microsoft® Excel 2016/XLSTAT®-Pro.

Results. *We isolated a natural compound with antimicrobial and antibiofilm activities.*

Conclusions. *The results obtained in this study are discussed in the context of how hydrothermal systems are important environmental source of uncharted antimicrobial and antibiofilm compounds. In conclusion, to the most effective of our knowledge, this work presents the primary report on the preliminary investigation of thermophile microbial diversity and their antimicrobial and antibiofilm activities for future biotechnological interest.*

Introduction

The use of hot spring waters for health and recreation in Italy may be a traditional activity dating back to Roman times. This tradition continues today; thermal waters are used in the form of baths, inhalation, irrigation and mud therapy and are usually recommended as a therapeutic and prophylactic measure against many varieties of illnesses and toxicity [1, 2]. The immersion in mineral-medicinal water and the application of mud to improve the prognosis of various diseases are still unknown, but it is known that the neuroendocrine and immunological responses to these treatments lead to anti-inflammatory, analgesic and antioxidant effects [3, 4]. The chemical, physical and microbial characteristics of thermal waters can indirectly contribute to their curative effects [5].

Temperature, pH and concentration of salts can create suitable habitats for the multiplication of different bacteria: *Legionella*, *Pseudomonas*, *Mycobacteria*, protozoa, amoebae, algae and other microorganisms. They can become a danger to users if not properly controlled [6, 7]. Thermal muds are obtained by mixing clayey materials with thermo-mineral waters, enriched with organic materials [8]. They have different properties depending on the type of clay, the properties of the thermal water contained and the microorganisms present [9].

In Southern Italy, the Campania region is one of the richest regions in terms of thermal and mineral water resources. These waters have been used for therapeutic purposes since Roman times [10, 11].

This Mediterranean Island (originally called Pithacusae) was included in the first century AD Plinio's description of the interactions between earthquakes and water sources [12, 13]. The therapeutic effect of Ischia mud is said to its high content of mineral. It's been demonstrated that Ischia muds are useful in treating skin disorders and skin diseases like psoriasis, dermatitis [14].

This specific maturation procedure will be considered as an ancient biotechnological process. This maturation procedure allows a microbial community, mainly represented by cyanobacteria [15, 16], to grow on the mud surface. The microorganisms, associated in a polysaccharide matrix, produce a green biofilm which shows the correct maturation of the mud. Before using the mud for therapies, this can be mixed and put in tanks during which thermal water at nearly 60°C is present, so as to cut back the natural microbial load and maintain the fluidity of the merchandise. Some studies contributed to defining the properties of this mud, suggesting that its effectiveness isn't only thanks to the warmth and therefore the thermal water properties, but also to substances produced by cyanobacteria [17-19]. Cyanobacteria are

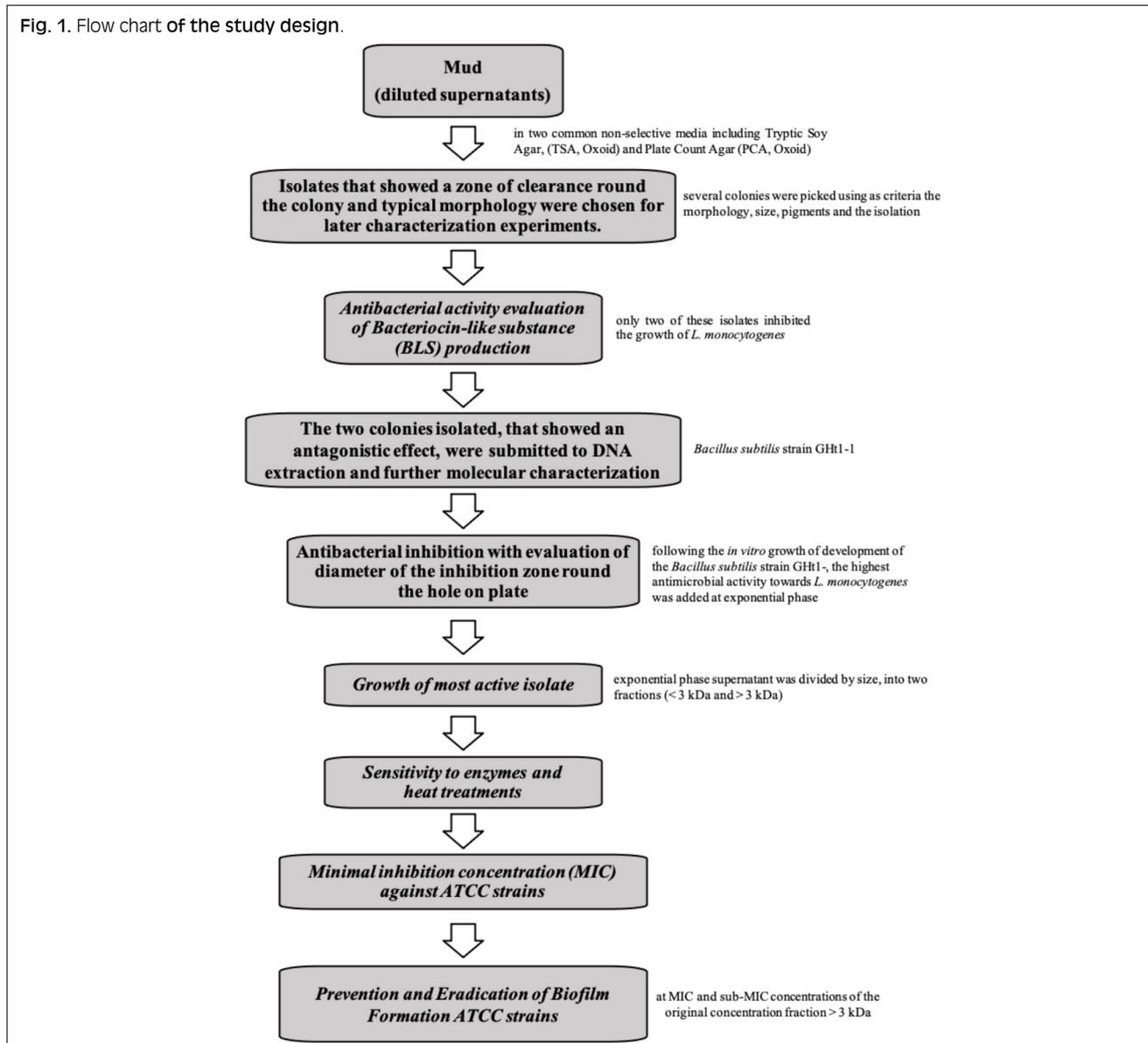
known to synthesize an outsized type of high-value bioactive compounds, including substances with UV protection, antiviral, antibacterial, and anticancer activities [20, 21]. Looking on the habitat, biofilms can include prokaryotic and eukaryotic microorganisms, like archaea, bacteria, cyanobacteria, microalgae, and fungi [22, 23]. Currently, interest dedicated to isolation and identification of bacterial strains from thermal springs. The target is that the exceptional and also the distinctive adaptation of those microorganisms under the influence of both heat and thermal stress. This special heat stability makes them prospective producers of high value thermostable bio-products and an important source for exploitation in new biotechnological developments. The tolerance of thermophilic microorganisms to thermal environments is usually attributed to exopolysaccharides (EPS). EPS are defined as high mass biopolymers that put together a considerable component of the extracellular polymers surrounding microbial cells membrane within the aquatic environment [24].

Ischia mud is comprised of specific minerals like argil and non-pathogenic microbes which produce more organic substances than other not of volcanic origin.

Despite the intense thermophilic environment, also the Ischia mud could be a unique ecosystem housing various microorganisms adapted to hot temperature that are growing interest because they might produce some biocatalysts that function under extreme conditions so useful in various industrial processes. In addition, as many antibiotics are now less effective for bacterial resistance, the demand for new stable and efficient molecules is increasing worldwide. The present study focuses on isolation and screening of extremophile bacteria from Ischia thermal mud for potential antimicrobial applications.

An integrated approach was went to understand the microbial diversity and to spot microorganisms which could grow at extreme temperature, using microbiological, morphological, and molecular identification so to bring new information on the extremophile bacteria in thermal environment (Fig. 1).

Fig. 1. Flow chart of the study design.



Methods

BACTERIAL STRAINS FOR ANTIBACTERIAL AND ANTIBIOFILM ACTIVITY TESTING

The following strains were utilized in this study: *Staphylococcus aureus* ATCC 6538, *Listeria monocytogenes* ATCC 7644, *Escherichia coli* ATCC 11229, *Pseudomonas aeruginosa* ATCC 9027. They were grown in Tryptone Soya Broth (TSB) at 37°C, for 16-18 h and maintained in Tryptone Soy Agar (TSA) containing glycerol at -80°C respectively. Overnight cultures of them were washed twice using sterile phosphate buffered saline (PBS) and standardized to 10⁶ cells/mL for next experiments.

SAMPLING COLLECTION

Samples were collected during winter and spring months 2019 at four sites distributed throughout the thermal springs of the north and south part of island. Samples, marked A, B, C, D, were chosen to vary the nature and location of the sampling.

Mud temperature and pH were measured by a transportable conductivity and pH meter.

Samples containing a combination of water and dust (four samples for every site) were collected approximately 10-15 cm below the surface, placed into sterile 50-mL polypropylene tubes, fixed using 5% formaldehyde, and transported to the laboratory at ambient temperature.

MORPHOLOGICAL CHARACTERIZATION

To identify the microorganisms in our collected samples, eight grams of every sample were homogenized using 10 mL NaCl. So as to be ready to culture the very best number of bacterial species, supernatants were diluted and plated in two common non-selective media including Tryptic Soy Agar, (TSA, Oxoid), Plate Count Agar (PCA, Oxoid), and so incubated at 37°C for 24-48 h, under aerobic conditions. Growth media were prepared using filtered water from Ischia Thermal spring to keep up original chemical composition of this environment. Then several colonies were picked using as criteria the morphology, size, pigments and the isolation. Isolates that showed a zone of clearance round the colony and typical morphology were chosen for later characterization experiments.

ANTIBACTERIAL ACTIVITY EVALUATION OF BACTERIOCIN-LIKE SUBSTANCE (BLS) PRODUCTION

Preliminary screening for antibacterial activity was performed by a method that allows the observation of antagonistic interactions among our strains and several target strains as reported previously by agar well diffusion assay [25].

Agar plates were previously spread with 100 mL of target microorganisms suspension, which corresponded to a 0.5 McFarland turbidity standard solution (10⁶⁻⁷ CFU/mL). With the help of a sterilized borer, 6 mm diameter wells were made in the agar plates, then 100 mL culture

of 25 selected colonies or/and cell-free supernatant of the isolates were poured into the wells and incubated for 8 h at 37°C, and zone of inhibition was measured for the antimicrobial activity. An isolated exhibiting a statistically larger inhibition zone was finally selected for further investigation. 5 µl of 25 mg/ml of Ampicillin (AMP) and 5 µl of sterile 0.9% NaCl were used as positive and negative control respectively.

MOLECULAR CHARACTERIZATION

The two colonies isolated from thermal mud samples, that showed an antagonistic effect, were submitted to DNA extraction and further molecular characterization. Colonies were recovered reconstituted with 70 µL Milli-Q Type 1 Ultrapure Water: extraction of DNA was obtained by denaturing samples at 98°C for 10 min and subsequent centrifugation (8,000 rpm for 5 min at 4°C) and supernatant recovery [26]. Amplification was meted out employing universal oligos, which are complementary to the V3-V6 16 S rRNA gene conserved regions [27]: V3f (5'-CCAGACTCCTACGGGAGGCAG-3') and V6r (5'-TCGATGCAACGCGAAGAA-3'). PCR reactions were performed with a Techne Prime PCR Thermal Cycler (TECHNE), using VWR Chemicals reagents, obtaining a final 55 µL volume: 5.5 µL PCR Key Buffer Tripton Free (Tris-HCl pH 8.5, KCl, 15 mM MgCl₂), 1.0 µL dNTP 12 µM, 0.44 µL forward and reverse oligos (50 µM), 0.5 µL Taq polimerase, 1.5 µL DNA template, and 47 µL sterile deionized water. Polymerase chain reaction conditions were the following: an initial denaturation stage (95°C for 2 min); 35 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 30 s; a final extension stage of 72°C for 5 min. Obtained amplified samples were run on a 1.5% agarose gel, disposing as a base pairs size reference a 100 bp DNA ladder, and stained using GelRed (BIOTIUM). PCR samples were delivered to an external service for the purification step and sequencing evaluations. Resulted sequences were identified through the comparison with sequences available on NCBI Sequence Database [28, 29].

IN VITRO GROWTH OF MOST ACTIVE ISOLATE

The identified isolate was used for further study. Bacteria were cultured in M9 medium at 30°C and 250 rpm. Until the late stationary phase, centrifuged and supernatant were filtered (0,22 µm Millipore, Bedford, MA, USA). After the supernatant was split up with a *cutoff spin column* of 3-kDa and 10-kDa, (Centricon, Millipore). The two fractions were tested for antibacterial inhibition with evaluation of diameter of the inhibition zone round the hole on plate.

SENSITIVITY TO ENZYMES AND HEAT TREATMENTS

The two aliquots of supernatants from *Bacillus* strain GHt1-1 were also screened for sensitivity to enzymes and heat treatments. For the results of proteinase K and trypsin activity, diluted supernatant was treated with 2.5 µL (100 µg/ml) 1 h at 42° and 37°C respectively. For temperature effect, supernatant was also heated at at 60°, 80° e 100°C for 15 min. All processed samples

were then tested for antimicrobial activity by using the well diffusion method.

MINIMAL INHIBITION CONCENTRATION (MIC)

The antibacterial efficacies of our supernatant were assessed with a MIC assay, in line with Clinical Laboratory Standards Institute guidelines (CLSI, 2006 M7-A6). Briefly, 10 mL of exponential bacterial culture grown in low-nutrient medium (M9 minimal medium Sigma-Aldrich) [30], was centrifuged and filtrated with a 0.22 μm filter (Millipore, Bedford, MA, USA). Supernatants were divided by size (cutoff spin column 3-kDa; Centricon, Millipore). Then, aliquots of the suspension higher 3 kDa were inoculated into each well of sterile, 96-well, polystyrene. Serial dilutions of sample were settled from a 50% (w/w) supernatant solution, leading to final concentrations of 50, 25, 12.5, 6.25, 3, 1.5%. After 18 h of incubation at 37°C, bacterial growth inhibition resolve by monitoring the optical density at 600 nm wavelength with a plate reader (SYNERGY H4 BioTek) [31]. The MIC was defined as the lowest concentration of supernatant capable of inhibiting the growth of each microorganisms. Positive control group composed of the microorganisms solution and liquid medium and negative control group composed of liquid medium only were used. All tests were executed in triplicate and repeated thrice.

BIOFILM FORMATION, INHIBITION, ERADICATION

The selected strains were examined for the ability to provide biofilm and categorized as active biofilm former within the polystyrene microplates using the *in vitro* biofilm model as previously described by Stepanović et al. [32]. Each well of a microplate was inoculated with 100 μL of PBS containing 1×10^7 cells/mL, incubated at 37°C to permit the adhesion of cells on the solid surface. The inhibition activity of supernatant on biofilm formation and the eradication activity against preformed biofilm were evaluated by using sub-MIC concentrations ranging from 6.25, 3, 1.5, 0.75% of the original concentration supernatant > 3 kDa.

For biofilm inhibition assay, 100 μL of bacteria in TSB were seeded into individual wells of microtiter plates with different concentrations of supernatant for 24 h. Data from dose-response experiments were represented because the percentage of inhibition compared to regulate. The MBIC50 corresponds to the concentration that will yield an inhibition of fifty of biofilm.

The effect on mature biofilms was evaluated adding the supernatant at different concentrations after 24 h. After other 24 h, biofilm was washed with PBS buffer to get rid of planktonic cells.

Biofilm growth was analyzed using the CV (crystal violet) assay. The remaining attached cells were heat-fixed at 60°C for 60 min and therefore the adherent biofilm layer was stained with 0.4% (w/v) bactericide for 25 min at 25°C. After washing with water, the stain sure to the biofilm was solubilized with ethanol (15-min incubation) and therefore the absorbance was measured

at 570 nm. The MBEC was calculated as percentage of eradication compared to regulate.

STATISTICAL ANALYSIS

Statistical analyses were carried out utilizing Microsoft® Excel 2016/XLSTAT®-Pro (version 7.2, Addinsoft, Inc., Brooklyn, NY, USA). Values are expressed because the mean \pm variance (\pm SD). The importance of the differences between different treatment groups was assessed by analysis of variance (ANOVA) with a 0.05 significance level. Moreover, the post-hoc analyses were kept on with Dunnet's test for MIC and Tukey's test for MBIC and MBEC.

Results

PHYSIOLOGICAL AND MICROBIOLOGICAL PROPERTIES OF THE SAMPLES

For each sites, we reported geographic location and chemical-physical characteristics as showed in Table I. Samples for physical, chemical and biological analyses were gathered from four pools at the hydrothermal Ischia stations at the end of the dry season and the beginning of the wet season (during April-November 2019).

The mud samples had pH values between 6.4 to 7.5 and relatively high temperature with the range 50.3°C to 67.8°C. Despite these particular conditions, the microorganisms developed well in diverse colonial morphology on growth media used. The isolates showed different types of colonies in shape, color and surface (yellow, white or creamy, circular, smooth or transparent colonies), with a diameter of 0.5-2 mm. The colonies were collected on the basis of the morphological difference and to be more detached on the culture medium than the others.

SCREENING FOR ANTAGONISTIC ISOLATES

Twenty-five isolates were chosen based on colony morphology and used in a screening for antagonistic isolates by agar plate assay. This screening was repeated for all considered ATCC strains. Only two of these isolates inhibited the growth of *L. monocytogenes* and they showed the same inhibition zone only for *L. monocytogenes* and no inhibition zones for the other bacteria strains tested (Tab. II).

IDENTIFICATION AND MOLECULAR CHARACTERIZATION

In this study, the two isolates, which inhibited *L. monocytogenes* ATCC 7644 were identified based on 16S rRNA analysis. Analysis of the 16S rRNA revealed that the two isolates were found to be very close to *Bacillus* species with identity > 99%. These two isolates were identified as the same strain: *Bacillus subtilis* strain GHt1-1 (Max Score: 1293; Total Score: 1293; Query Cover: 100%; E value: 0.0; Identity: 99.86%; Accession: GU434356.1). They were gram positive and catalase and oxidase positive.

Tab. I. Physicochemical and geographical characteristics of samples collected from Ischia island.

Samples	Spa area	Temperature (°C)	Conducibility (mS/cm ³)	pH	Geographical location
A	Ischia (NA)	50.8	10.82	7.07	Latitude: 40°43.00' N Longitude: 13°54.00' E
B	Ischia (NA)	50.3	45.8	6.40	Latitude: 40°44.3477' N Longitude: 13°57.02' E
C	Ischia (NA)	67.8	9.80	7.50	Latitude 40°43'8"40 N Longitude 13°55'54"48 E
D	Ischia (NA)	62.5	45.6	6.91	Latitude 40°32'48"84 N Longitude 13°33'35"28 E

Tab. II. Screening for antagonistic activity of mud isolates against *Staphylococcus aureus* ATCC 6538, *Listeria monocytogenes* 7644, *Escherichia coli* ATCC 11229, *Pseudomonas aeruginosa* ATCC 9027. G: Gentamicin; R: Resistant; I: Intermediate; S: Sensitive (Rasooli I, Owlia P. Chemoprevention by thyme oils of *Aspergillus parasiticus* growth and aflatoxin production. *Phytochemistry* 2005;66:2851-6. <https://doi.org/10.1016/j.phytochem.2005.09.029>).

Samples	<i>S. aureus</i> ATCC 6538	<i>L. monocytogenes</i> ATCC 7644	<i>P. aeruginosa</i> ATCC 9027	<i>E. coli</i> ATCC 11229
	Means ± SD (Ø mm)			
1	< 6.0	6.1 ± 2.2	< 6.0	< 6.0
2	< 6.0	< 6.0	< 6.0	< 6.0
3	8.5 ± 1.5	25.0 ± 1.0	< 6.0	< 6.0
4	< 6.0	< 6.0	< 6.0	< 6.0
5	< 6.0	< 6.0	< 6.0	< 6.0
6	< 6.0	< 6.0	< 6.0	< 6.0
7	< 6.0	6.8 ± 1.9	< 6.0	< 6.0
8	< 6.0	< 6.0	< 6.0	< 6.0
9	< 6.0	< 6.0	< 6.0	< 6.0
10	< 6.0	< 6.0	< 6.0	< 6.0
11	< 6.0	< 6.0	< 6.0	< 6.0
12	< 6.0	< 6.0	< 6.0	< 6.0
13	< 6.0	< 6.0	< 6.0	< 6.0
14	< 6.0	7.3 ± 3.1	< 6.0	< 6.0
15	< 6.0	< 6.0	< 6.0	< 6.0
16	< 6.0	< 6.0	< 6.0	< 6.0
17	< 6.0	< 6.0	< 6.0	< 6.0
18	6.7 ± 1.3	12.0 ± 2.3	< 6.0	< 6.0
19	< 6.0	6.9 ± 1.1	< 6.0	< 6.0
20	< 6.0	< 6.0	< 6.0	< 6.0
21	< 6.0	< 6.0	< 6.0	< 6.0
22	< 6.0	< 6.0	< 6.0	< 6.0
23	< 6.0	< 6.0	< 6.0	< 6.0
24	< 6.0	< 6.0	< 6.0	< 6.0
25	< 6.0	< 6.0	< 6.0	< 6.0
Gentamicin	S	S	S	S

Tab. III. Influence of the two fractions (> 3 kDa, < 3 kDa) on microbial cells growth. Values are expressed as the mean of three independent experiments ± Standard deviation; G= gentamicin.

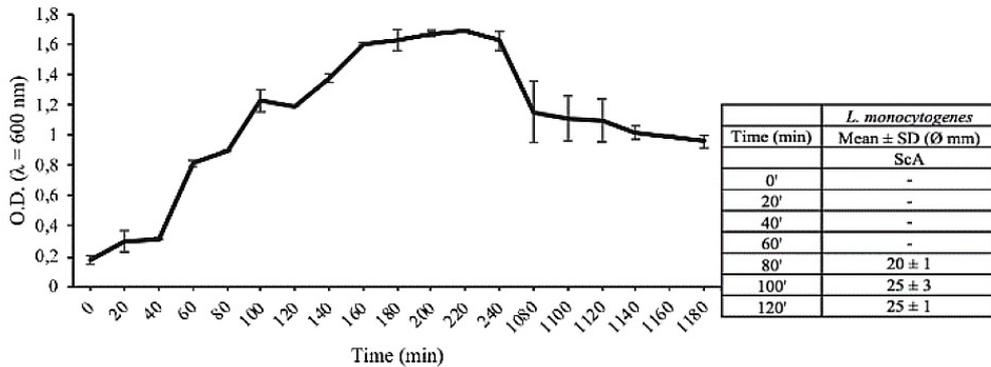
Strains	Mean±SD (Ø mm)	Control positive	G
	> 3 kDa	< 3 kDa	
<i>S. aureus</i> ATCC 6538	8.1 ± 2.2	< 6.0	S
<i>L. monocytogenes</i> ATCC 7644	25.0 ± 1.0	11.1 ± 2.3	S
<i>P. aeruginosa</i> ATCC 9027	< 6.0	< 6.0	S
<i>E. coli</i> ATCC 11229	< 6.0	< 6.0	S

Following the *in vitro* growth of development of the *Bacillus subtilis* strain GHt1-, the highest antimicrobial activity towards *L. monocytogenes* was added at exponential phase. (Figure 1). Indeed, inhibition zones diameter (mm) for *L. monocytogenes* was 20 ± 1 after 80', while it was 25 ± 3 and 25 ± 1, respectively after 100' and 120' (exponential growth phase). No activity was shown for the other tested strains [33] (Fig. 2).

Exponential phase supernatant was divided by size, into two fractions (< 3 kDa and > 3 kDa).

Only aliquots of the fraction higher 3 kDa showed growth inhibition against *L. monocytogenes* (Tab. III), forming an inhibition zones diameter of 25,0 ± 1,0 mm. The

Fig. 2. *In vitro* growth *Bacillus subtilis* strain GHt1-1 in M9 medium at 30°C as defined by measurement of the OD600. Data figure the means of three independent cultures ± standard deviation (SD).



Tab. IV. Effects of enzymes action and temperature on microbial growth. Values are expressed as the mean of three independent experiments ± Standard deviation.

	<i>L. monocytogenes</i> ATCC 7644
	> 3 kDa
	Mean±SD (Ø mm)
Control	25.0 ± 2.0
Trypsin	10.0 ± 1.1
Proteinase k	9.5 ± 2.8
60 °C	10.0 ± 1.5
80 °C	9.5 ± 1.2

SENSITIVITY TO ENZYMES AND HEAT TREATMENTS

Further to better characterize the > 3 kDa fraction, effects of proteinase K, trypsin and temperature action were evaluated. Table IV reports results showing the reduced persistence of inhibitory effects against *Listeria monocytogenes*.

MIC DETERMINATION

The active > 3 kDa fraction was tested for establishing the Minimal inhibitory concentration (MIC) against *S. aureus*, *L. monocytogenes*, *P. aeruginosa* and *E. coli* (Fig. 3). The absorbance of the negative control (TSB only) was found to 0.093 ± SD; therefore, based on this value, positive culture growth was considered when absorbance was found to > 0.2. The MIC of > 3 kDa fraction against *L. monocytogenes* was 6.25% of the original concentration. The MIC against *S. aureus* was (25%) of the original concentration. *P. aeruginosa* and *E. coli* growths were not inhibited at any tested fraction concentration.

fraction < 3 KDa formed an inhibition zones diameter of 11,1 ± 2,3 mm. For the other strains the inhibition zones diameter is always < 6 mm, indicating no growth inhibition activity of the suspension higher 3 KDa. Gentamicin, the antibiotic to which all tested strains are sensitive, was used as a positive control (S = diameter of inhibition zones > 23 mm).

Fig. 3. Bactericidal effect of > 3 kDa fraction on selected strains. The absorbance of negative control (only TSB) was found to 0.09 ± SD. Error bars represent standard deviation (n= 3). Statistical comparison between treated and untreated (control) groups were performed using one-way ANOVA (Dunnet * p < 0.05).

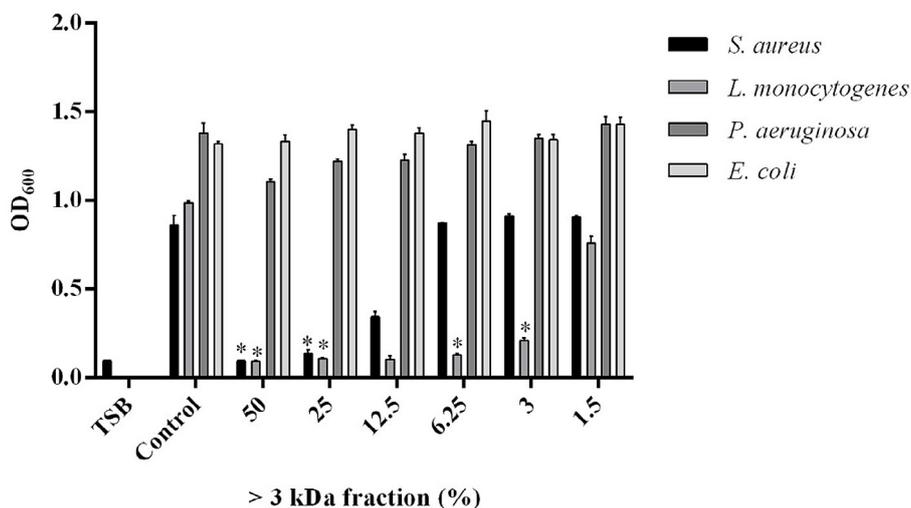
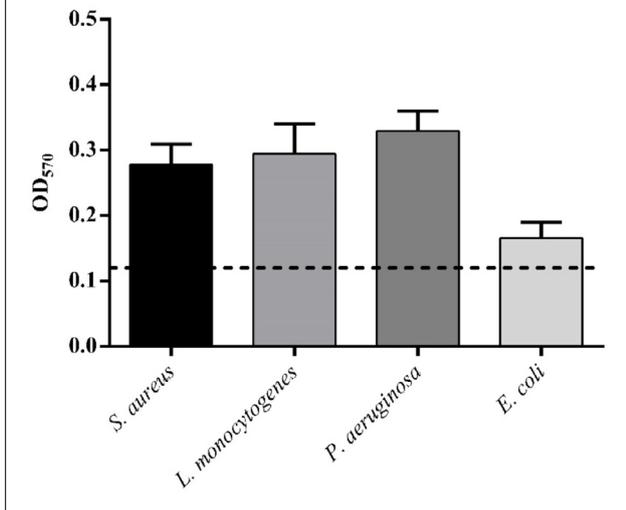


Fig. 4. Biofilm biomass quantitation of *S. aureus* ATCC6538, *L. monocytogenes* ATCC 7644, *P. aeruginosa* ATCC 9027, *E. coli* ATCC 11229, after 24 h. Biofilm formation evaluated by crystal violet light absorbance at 570 nm (optical density, OD₅₇₀). Dashed line represent the ODC. Negative (OD ≤ ODC), weakly adherent (ODc < OD ≤ 2 × ODc), moderately adherent (2 × ODc < OD ≤ 4 × ODc), strongly adherent (4 × ODc < OD). Results display the mean ± SD of three independent experiments.



PREVENTION AND ERADICATION OF BIOFILM FORMATION

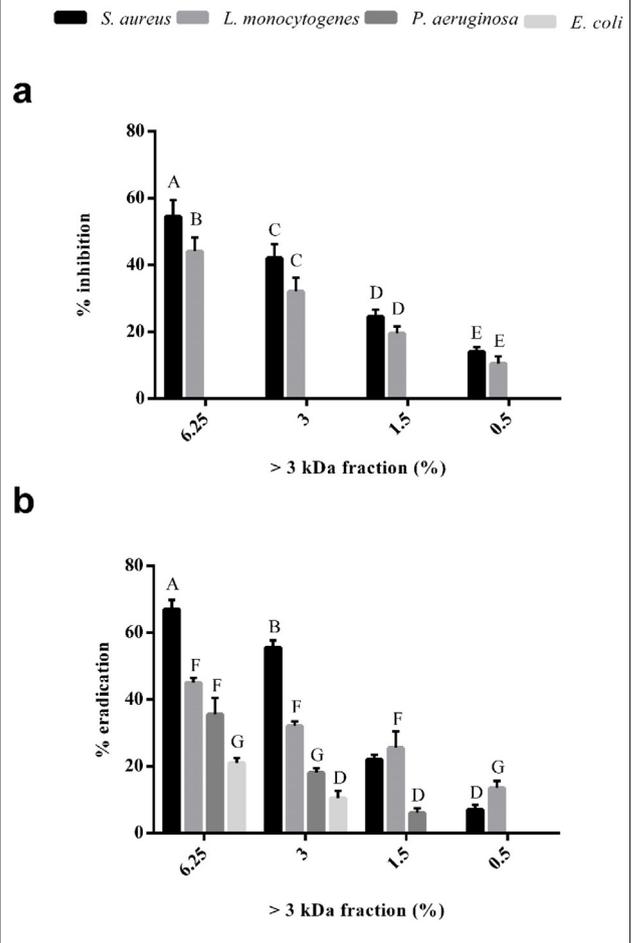
S. aureus, *L. monocytogenes*, *E. coli*, *P. aeruginosa* were tested for biofilm production. Figure 4 shows the total biomass of microbial biofilms after 24 h. The diagram highlights that the microbes form biofilms and they are classified as weakly biofilm-forming (*E. coli*), moderately biofilm-forming (*S. aureus*, *L. monocytogenes*) and strongly biofilm-forming (*P. aeruginosa*).

In Figure 5 (a) the minimal biofilm inhibitory concentration (MBIC) was reported as percentage of effects of different MIC and sub-MIC concentrations (0.75, 1.5, 3 and 6.25%) of the original concentration fraction > 3 kDa on biofilm formation of *S. aureus*, *L. monocytogenes*, *P. aeruginosa* and *E. coli*. The inhibition percentage of biofilm formation is appreciable only for *S. aureus* and *L. monocytogenes*. At MIC (6.25% of the original concentration fraction > 3 kDa) the biofilm formation of *L. monocytogenes* was inhibited by approximately 50%, while biofilm formation of *S. aureus* was inhibited at 58%. From the subsequent dilution the inhibition percentage dropped to values < 50% (45% inhibition biofilm *S. aureus* and 35% biofilm *L. monocytogenes* at ½ MIC), reducing to the greater dilutions gradually.

The inhibition percentage of *S. aureus* biofilm formation is always higher than that of *L. monocytogenes* biofilm.

In Figure 5 (b) the minimal biofilm eradication concentration (MBEC) was reported as percentage of dispersion of established mature biofilm of *S. aureus*, *L. monocytogenes*, *P. aeruginosa*, *E. coli* by MIC and sub-MIC concentrations (0.75, 1.5, 3 and 6.25%) of the original concentration supernatant > 3 kDa. At MIC all formed mature biofilms by the four tested strains were eradicated: the *S. aureus* and *L. monocytogenes* biofilms had higher eradication percentage (65 and 44%,

Fig. 5. (A) MBIC reported as percentage of effects of different concentrations of fraction > 3 kDa on biofilm formation of *S. aureus* ATCC6538, *L. monocytogenes* ATCC 7644, *P. aeruginosa* ATCC 9027, *E. coli* ATCC 11229. (B) MBEC reported as percentage of dispersion of established mature biofilm of *S. aureus* ATCC6538, *L. monocytogenes* ATCC 7644, *P. aeruginosa* ATCC 9027, *E. coli* ATCC 11229 by various concentrations of supernatant > 3 kDa. Values represent the means ± standard deviations. Data with different letters (A-G) are significantly different (Tukey's, $p < 0.05$).



respectively), compared to those of the *P. aeruginosa* and *E. coli* biofilms (32 and 20%, respectively). At ½ MIC only the *S. aureus* mature biofilm had an eradication percentage > 50%. At subsequent dilutions, the *L. monocytogenes* mature biofilm was more eradicated than that of *S. aureus*, although at percentages lower than 30%. The eradication of the *P. aeruginosa* and *E. coli* biofilms was not appreciable at the strongest dilutions.

Discussion

The growing interest in thermophilic microorganisms and their biotechnological applications is demonstrated by numerous studies on extremophilic microorganisms [34-40]. Environmental microbial populations link with one another forming complex, mixed communities. The natural habitats where thermophilic microorganisms are isolated change from terrestrial volcanic sites (including solfatara fields) with temperatures softly

above ambient temperature, to submarine hydrothermal systems (sediments, submarine volcanoes, fumaroles and vents) with temperatures above 300°C. Thermal environments also are inhabited by extremophilic microorganisms, important to check also for the beneficial properties that they will wear the visitors health [41]. Studies that specialize in microbial diversity of mud are increasing in numerous parts of the planet because the environmental microbial populations that interact with one another forming complex, mixed communities, even in extreme conditions, have a giant potential for ecological importance and biotechnological applications in aquaculture, bioremediation, agriculture, and energy production.

Hot springs adjacent to volcanic environments are generally acidic, but the pH is neutral or slightly alkaline in regions near limestone. Thermophiles might also live under harsh conditions involving extreme pH or high salt concentrations [42].

Previous studies have demonstrated that important differences exist in microbial communities among hot springs with various ranges of physicochemical parameters and discrete geographic locations and is strictly temperature that control the composition of sediment communities, mainly varying temperature in geothermal systems with degassing, mineral precipitation, evaporation, autotrophy and oxidation [43].

Microbial mats are found to develop in a very big selection of thermal habitats including hot springs, fumaroles, eruption vents, and on steaming round [44]. These microbial mats are rich, in organic substances with an ubiquitous communities which are tolerable to extreme conditions.

Hydrothermal systems are the best to know how microbial communities collaborate with different conditions. Hot springs are reported to be microbial-dominated ecosystems, sustaining an upscale microbial diversity and holding different microhabitats that constitute natural niches for thermophilic (> 50°C) and hyperthermophilic (> 80°C) microorganisms. However, characterizing the microorganisms of a natural matrix, like thermal mud, is incredibly difficult because of the vast phenotypic and genotypic heterogeneity. Therefore, a number of them aren't easily isolated by microbiological techniques.

The thermal area mud of Ischia island is widely employed in packs, masks, topical treatments for the body and face. Little is thought about its microbiological aspects. Therefore, this study was directed to extend our knowledge about extremophile bacteria from Ischia thermal mud for potential antimicrobial applications. Thermal mud samples from four thermal springs located within the Ischia island were used to isolate thermophilic microorganisms. These springs differed in temperature that adjust from 50-70°C and pH from 6,4 to 7,5. A complete of 25 thermophilic bacterial strains were chosen supported colony morphology and from a variety of antagonisms effect towards four ATCC strains. Only two isolates showed an antagonistic effect towards *L. monocytogenes*, in order that their

molecular identification established as *B. subtilis* GHt1-1. In fact, Bacilli could be a sporogenic, thermophilic bacterium that grows well in acid environments [45, 46] so this result's highly compatible with extreme environmental conditions. *Bacillus* sp. is gram-positive bacteria with a high level of additional cellular enzyme production capacity, which has grown its application in many industries.

To investigate the antimicrobial potential of the *B. subtilis* GHt1-1, previous selected bacteria were used. We found a bioactive metabolite produced during exponential phase of curve growth of *B. subtilis* GHt1-1 that in an exceedingly preliminary study showed maintaining its activity changing temperature and subjecting to enzymatic action. After its slitting up, the foremost active fraction resulted the one amongst > 3 kDa. This fraction exhibited an honest antimicrobial action towards *L. monocytogenes* reaching the worth of 6.25% of the first concentration as minimal inhibition concentration. Surprisingly, this molecule showed a decent antibiofilm action both for inhibition of formation and eradication of mature biofilm on all four bacteria Gram-positive and Gram-negative tested. The best result obtained was on the inhibition, but particularly on the eradication of the mature biofilm of *S. aureus*. This data can be important, especially in fields where the biofilm formation reduces or even cancels the disinfectants and antimicrobials action [47]. Biosurfactants are known to decrease the adhesion of pathogenic microorganisms to solid surfaces [48]. It's been hypothesized that biosurfactants manipulate the interactions between bacteria and surfaces [49]. Due to the capacity of growing in extreme conditions as stress generated in hot outpouring waters and therefore the presence of an antibiotic additive for the prevention of bacterial proliferation, these microorganisms could probably develop some modifications that allow the assembly of efficient protective bioproducts capable to combat opportunistic microorganisms.

Limitations of the study

In this research, the analysis of only one sample per location has been carried out, and this aspect can represent a limit of our study. As there are no similar published studies, we have been prompted to present these preliminary results to give a first information on the potential antimicrobial applications of the extremophilic bacteria of the Ischia thermal muds. However, to study in deep our observations, further studies have been planned in order to increase the number of examined samples and the locations of sampling.

Conclusions

In conclusion, to the most effective of our knowledge, this work presents the primary report on the preliminary

investigation of thermophile microbial diversity and their antimicrobial and antibiofilm activities for future biotechnological interest.

Our results suggest this molecule can be exploited as potential antimicrobial and/or anti-biofilm agents against microbial biofilm formation within new antibiotics production.

Moreover, further studies should be done to know the most metabolite to blame for the antibacterial effect, the composition and also the molecular mechanisms of this bacterial compound.

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Conflict of interest statement

The authors declare no conflict of interest.

Authors' contributions

Conceptualization, VDO and EG; methodology, VDO and EG; software, FC and RL; validation, MG and ODC; formal analysis, AM; investigation, VDO and EG; resources, MG; data curation, AM; writing, original draft preparation, VDO; writing, review and editing, EG; supervision, EG; project administration, MG. All authors have read and agreed to the published version of the manuscript.

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