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BIOGENICITY AND CHARACTERIZATION OF MOONMILK IN THE GROTTA NERA (MAJELLA NATIONAL PARK, ABRUZZI, CENTRAL ITALY)

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Abstract: Observations and hypotheses on the possible influence of unidentified calcifying bacteria on moonmilk speleothem formation in the Grotta Nera are reported for the first time. The Majella Massif hosts a complex karst system of several caves; the accessible Grotta Nera is the most interesting one. Despite its name, the cave is characterized by particularly abundant ivory-white deposits of moonmilk. Two samples of moonmilk were analyzed to determine the geochemistry, fabric, depositional setting, and extent of biogenicity. For this, we combined geochemical, scanning electron microscopic, microbiological, and *in vitro* precipitation studies. X-ray diffraction of the moonmilk deposits gave clear evidence for the presence of calcite. Scanning electron microscopy showed that moonmilk in the Grotta Nera consists of a network of calcite fibers oriented in all directions, resembling a felted mat. The cultivation on specific medium of moonmilk and drip-water samples showed the presence of fungi, actinomycetes, and other bacteria, but the dominant cultivable microorganisms were bacteria, which produced significant crystallization. Examination of Gram-stained smears taken from the fifteen different colony types showed that the majority (66.7%) of the bacterial isolates were Gram-negative. Single small rods and rod chains were the most common bacteria isolated from the Grotta Nera. None of the molds isolated from the Grotta Nera samples were able to precipitate $CaCO_3$ crystals, suggesting a major bacterial contribution to moonmilk deposition in the cave. Bacteria were capable of precipitating $CaCO_3$ on B-4 solid medium at 15 (cave temperature), 22, and 32 °C. The calcifying bacteria isolated from the Grotta Nera showed a greater capability to solubilize $CaCO_3$ than those associated with consolidated stalactites sampled from previously studied caves. The electron microscopy and microbiological evidences, together with the geochemistry and environmental data, allowed us to postulate the biogenic nature of the moonmilk in the Grotta Nera Cave.

Introduction

Moonmilk is a whitish material described as soft and pasty, resembling cream-cheese, when wet, and crumbly and powdery, like chalk, when dry (Fisher, 1988; Hill and Forti, 1997; Northup and Lavoie, 2001; Cañaveras et al., 2006). On aging, the moonmilk becomes dry and more rigid and compact, but the external morphology stays unchanged (Gradziñsky et al., 1997). It is a microcrystalline aggregate, typically found on the ceilings, floors, and walls of carbonate caves and on speleothems. Moonmilk deposits have been reported in numerous caves worldwide, in a variety of different countries and in climates from alpine to tropical (Onac and Ghergari, 1993; Hill and Forti, 1997; Chirienco, 2002; Lacelle et al., 2004; Ford and Williams, 2007; Blyth and Frisia, 2008; Richter et al., 2008; Curry et al., 2009). Frequently, moonmilk is the only speleothem present in cold, high-altitude or high-latitude caves, where massive calcite speleothems such as stalagmites do not form (Onac and Ghergari, 1993; Hill and Forti, 1997; Borsato et al., 2000; Lacelle et al., 2004). Moonmilk is composed of water and small crystals of minerals such as $CaCO_3$ polymorphs (calcite, aragonite, vaterite), monohydrocalcite ($CaCO_3 \cdot H_2O$), magnesite ($MgCO_3$), hydromagnesite ($Mg_5(OH)_2(CO_3)_4 \cdot 4H_2O$), dolomite ($CaMg(CO_3)_2$), nesquehonite ($MgCO_3 \cdot 3H_2O$), huntite ($Mg_3Ca(CO_3)_4$), and gypsum ($CaSO_4 \cdot 2H_2O$) (Onac and Ghergari, 1993; Hill and Forti, 1997; Northup and Lavoie, 2001; Lacelle et al., 2004). This array of minerals relates to various host lithologies (Gradziñski et al., 1997) and water chemistries associated with each cave.

About 95% of moonmilk deposits are carbonatic, and its most common type is calcite moonmilk with greater than 90% calcite in its solid phase (Fisher, 1992, 1993). The water content of active moonmilk varies considerably. Under its hydrated phase, its water content ranges from 40 to 70% by weight (Hill and Forti, 1997; Lacelle et al., 2004). According to Istvan et al. (1995), the water retaining

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capacity of active moonmilk can be attributed to its porous network of calcite fibers.

The morphology of moonmilk is as varied as its composition (Curry et al., 2009). Microscopically, the most diagnostic calcitic moonmilk feature is needle-shaped or fibrous crystal morphology that appears as unstructured aggregates of micrometer- to nanometer-sized crystals with no apparent preferred orientation (Onac and Ghergari, 1993; Gradziñski et al., 1997; Hill and Forti, 1997; Borsato et al., 2000; Cañaveras et al., 2006). Moonmilk that is biologically active also contains significant amounts of cells, filaments, and apparent biofilms (Gradziñski et al., 1997; Boston et al., 2001; Curry et al., 2009). The origin of moonmilk has long been discussed, and many hypotheses for its genesis have been proposed since its first description was made by Nicolas Langh in 1708 (Bernasconi, 1976). Biotic and abiotic processes have been postulated (Gradziñsky et al., 1997; Borsato et al., 2000; Forti, 2001; Northup and Lavoie, 2001). Some evidences of microbial activities related with calcite moonmilk deposits have been reported (Bertouille, 1972; James et al., 1982; Callot et al., 1985; Gradziñski et al., 1997; Barton and Northup, 2007; Braissant et al., 2012). Moonmilk is the cave deposit most commonly associated with biogenic calcite precipitation, either by direct precipitation by microorganisms (fungi, algae, bacteria, and archaea) (Castanier et al., 1999; Barton and Northup, 2007; Ercole et al., 2012) or by passive precipitation in which microorganisms themselves act as nucleation surfaces on which minerals precipitate (Jones and Kahle, 1993; Blyth and Frisia, 2008). There is an ongoing debate about the extent of the microbial role in the formation of moonmilk. Some researchers have claimed to have identified microbial structures associated with the crystal matrix, including calcified cells and filaments (Gradziñsky et al., 1997; Cañaveras et al., 2006), whereas others, citing the lack of unequivocal evidence of bioprecipitation, have attributed moonmilk formation to predominantly inorganic processes (Bernasconi, 1961; Mélon and Bourguignon, 1962; Gèze, 1976; Onac and Ghergari, 1993; Hill and Forti, 1997; Moore and Sullivan, 1997; Borsato et al., 2000). In addition to being precipitated inorganically or by microbes, moonmilk can also be formed by speleothem weathering (Sweeting, 1973; Hill and Forti, 1997). This process is also thought to be biologically mediated; for example, it can result from biochemical corrosion of bedrock by organic acids produced by microrganisms (Caumartin and Renault, 1958). More recently, moonmilk has been attributed to a combination of both physico-chemical and biogenic processes (Onac and Ghergari, 1993; Basillais, 1997).

To determine the role of calcifying bacteria as geological agents in the genesis of moonmilk speleothems from the Grotta Nera in the Abruzzi Region of Italy, a combination of studies have been carried out: microscopic, microbiological, *in vitro* precipitation, and geochemical investigations. Because DNA analysis of bacteria provides no information on the metabolism, the physiology, the ecology, the biochemistry, or the geomicrobiology of a strain, laboratory-based culture experiments and geochemical techniques were used to determine their ability to alter the chemistry of their microenvironment and produce biominerals. This paper reports the results of a preliminary identification and characterization of microorganisms isolated from moonmilk and drip-water samples from the Grotta Nera, Gram staining and cell morphology analysis by light microscopy, chemical analysis by X-ray diffraction and scanning electron miscroscopy of moonmilk and calcium carbonate crystals obtained *in vitro* in the presence of bacterial isolates, and *in vitro* solubilization tests of calcium carbonate by the calcifying bacteria. The results provide additional arguments for the significant role of bacteria as geochemical agents.

Site and Environmental Parameters

Grotta Nera (129A in Catasto Grotte Abruzzo, source of these data)
Elevation: m 1380 a.s.l.
Total length: 110 m Vertical extent: 10 m
Maps: Carta d'Italia Istituto Geografico Militare (1:25000) sheet 147 III NE (Pennapiedimonte), geological map of Abruzzi (1:100,000) (Vezzani and Ghisetti, 1993)

The Grotta Nera is not only one of the most famous and peculiar caves in the Abruzzi region in central Italy (Fig. 1), but it also has the most impressive examples of moonmilk speleothems that have been described in Italy (Forti and Rossi, 2003). It is located in the heart of the Majella National Park, in the Feudo Ugni Natural Reserve, a dense mixed-deciduous forest characterized by large seasonal temperature variations and relatively high precipitation. This park was established in part to protect this peculiar high-altitude karst landscape. In this area, surface karst features have been affected by important signs of glacial erosion. The Majella Massif hosts a complex karst system of several caves (Burri, 2003), some of which are show caves, the most famous of which is the Grotta del Cavallone.

Access to the Grotta Nera has been strictly regulated for many years and is only allowed for scientific purposes to preserve the peculiar concretions for which the cave is famous. The cave is characterized by a wide entrance (Fig. 1). This opens directly on the cliff and is accessible by a rocky route that is made easier by a few cut steps and a safety wire rope. This trail leads into a large room with its floor completely covered by collapsed material, including angular boulders, some of them covered by the remains of broken stalactites (Figs. 1 and 2). This first large space leads on to a narrow passage that is lined with stalagmite columns and protected by a gate. This is the entrance to the main room of the cave, where its peculiar speleothems are concentrated (Figs. 3 and 4a,b,c). In this inner, bell-shaped room, extensive ivory-white deposits of moonmilk as

Figure 1. Grotta Nera entrance from inside. In the foreground a fallen big boulder clutters the passage. In the inset, the red dot shows the approximate location of the cave.

stalactites, stalagmites, and cave pearls cover the whole cavity (Fig. 4). Really impressive stalactites hang from the 10-m high ceiling and show a peculiar shape characterized by an apex diameter that is larger than that of the root and a development not vertical (Fig. 3). These beautiful rounded stalactites are concentrated mostly in a small portion of the room (Fig. 2b) and are huge, with an average length of about 1.5 m. Their size, and consequently their weight, play a key role in their general development. In fact, in the center of the room there is a buildup of broken or fallen stalactites that have been re-cemented by flows of coating material (Fig. 4g). Numerous active gours with growing pisolites inside are also present on the flowstone floor (Fig. 4i).

These peculiar moonmilk formations were described by Forti and Rossi (2003) as *trays* whose development was inferred to be related to strong evaporation caused by complex air flows within the room (Forti and Rossi, 2003; Savini, 2004). Trays are flat-bottomed speleothems that end in a flat, horizontal surface (Hill and Forti, 1986). The mechanism of their formation is still not well understood. Martini (1986) was the first to speculate on the abiotic origin of carbonate (calcite-aragonite) trays. Calaforra and Forti (1994) have proposed an abiotic mechanism for the growth of gypsum trays. Biogenic trays were found in the submarine cave Lu Lampiùne, one of the most complex and largest caves in the Salento coast of southeastern Italy. Those structures hang abundantly from the roof and lateral walls of the cave, where they show a slanted, non-vertical orientation (Onorato et al., 2003). The trays in the Grotta Nera (Fig. 3) are the biggest so far described in Italy; they have an asymmetric enlargement at the bottom and are similar to a tongue (Forti and Rossi, 2003).

As clearly exposed in the geological map of the cave (Figs. 2, 5), the development of the cave was strongly controlled by several variously oriented faults. One of these faults controls the shape of the room where the moonmilk trays grow and where a clear fault plane is outcropping

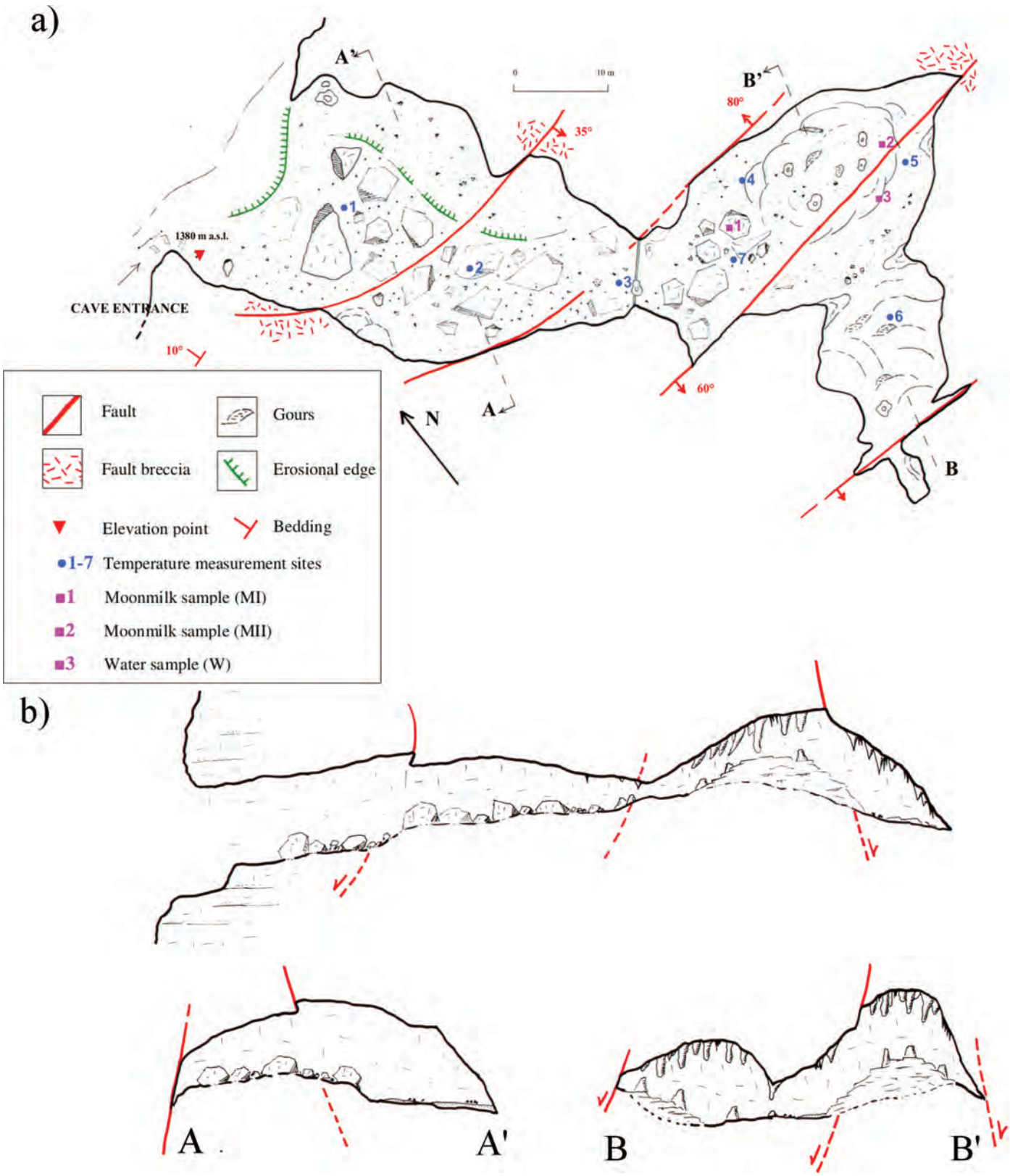

Figure 2. Map and geology of Grotta Nera (Abruzzi, central Italy). Based on a first survey in 1969 by E. Burri, E. Bevilacqua, and G. Di Iorio drawn by E. Burri and a second survey in 2005 by G. Di Prinzio and G. Ferrini drawn by G. Ferrini.

Figure 3. Moonmilk stalactites (trays) hanging from the ceiling of the inner room of the Grotta Nera. Note the peculiar shape and the development differing from the vertical axis.

(Fig. 2b); several plant roots are leaning along the fault plane, showing that the ceiling is relatively thin.

From a speleogenesis point of view, the cavity can be seen as a relict gallery undergoing an important gravity reshaping, especially in the great entrance antechamber; here the big boulders cluttering the passage could be related to collapse linked to the last Holocene glacial period. To date, no water flows or water-transported deposits have been reported in the cave; only a moderate dripping in the inner portion has been noted.

A preliminary survey of air-temperature distribution was carried out to highlight the relationship between the cave and the outside temperatures, measure any thermal gradients inside the cave and their influence on air movement in the cave, and the cave's microclimate. In late

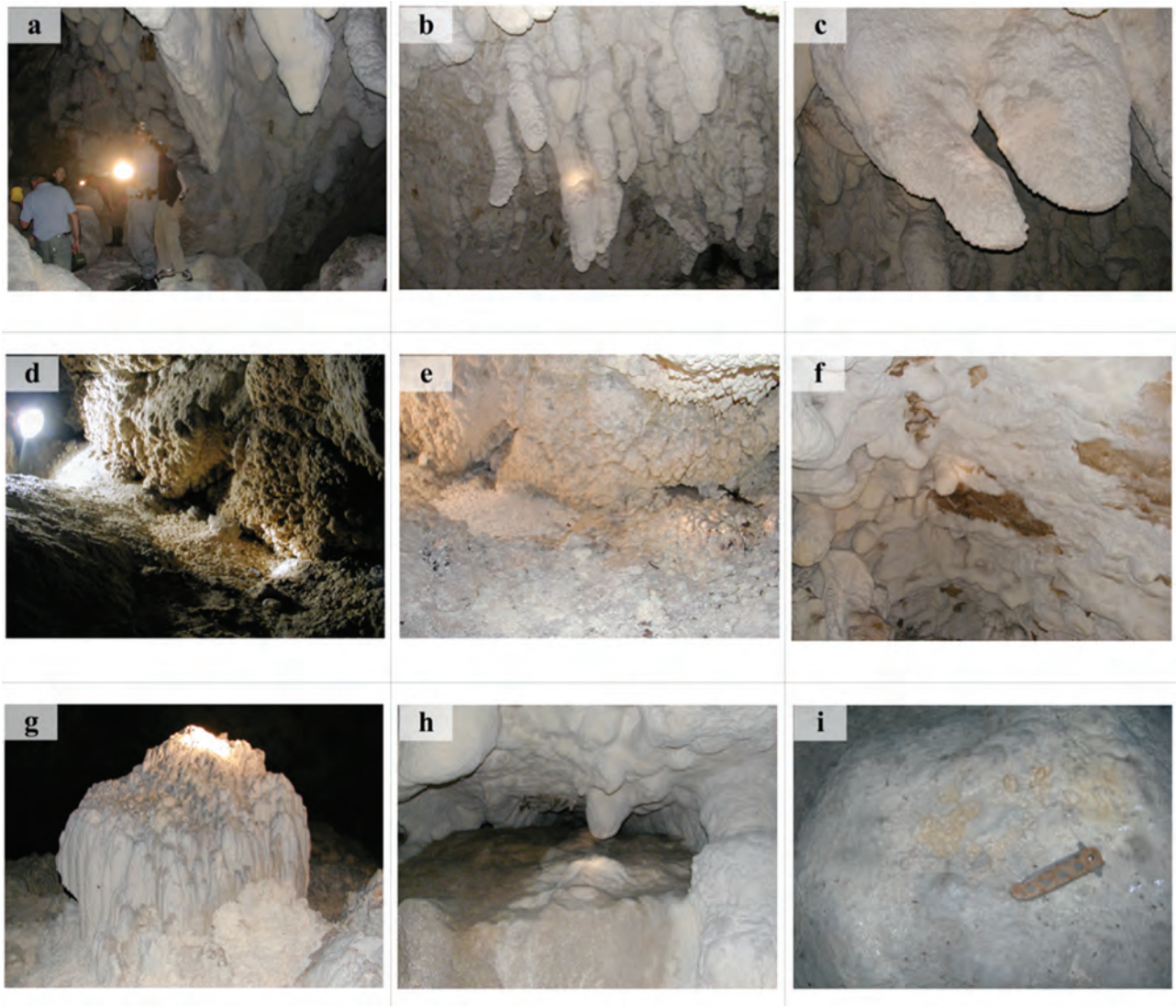

Figure 4. Moonmilk decorating the ceiling (a, b, c), the walls (d, e, f) and the floor (g, h, i) of the inner room of Grotta Nera. Note the huge moonmilk stalactites with an average height of about 1.5 m (a), round and smooth in their form (a, b, c), with a non-vertical development (b,c); a mound formed by fallen stalactites (g); the presence of a bat (h); the presence of pisolites (i).

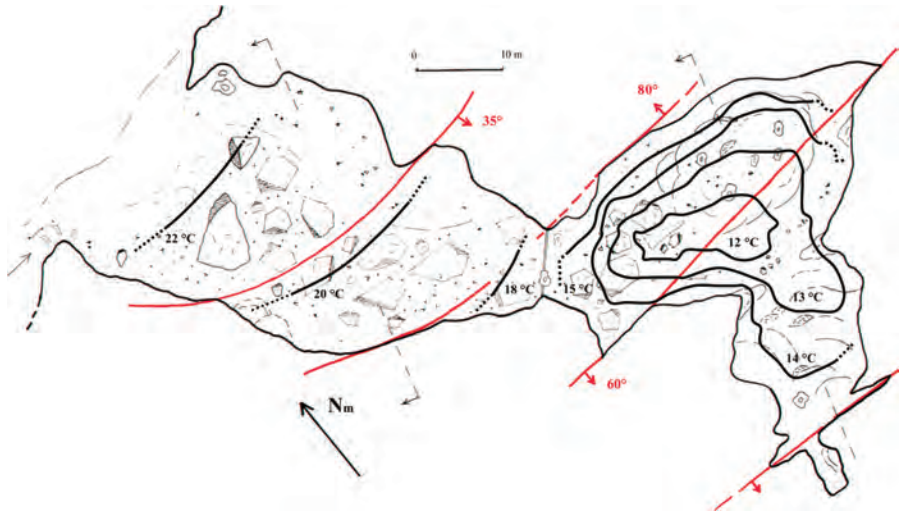

Figure 5. Temperature distribution in Grotta Nera in late autumn.

spring, early summer, and autumn the air temperature was monitored in seven sites, three in the first room and four in the inner one (Fig. 2a). The collected data showed that the air temperature inside the large entrance room is strongly affected by the climatic conditions outside, which are characterized by strong seasonal variability. The situation is quite different for the inner room. Regardless of the season, a temperature pattern showed relatively small differences across the inner room. It was noted that for most of the year the temperatures recorded (an average of 15 °C) were higher than those on the outside. The high temperature measured in the inner room could be due to a low or lack of water circulation during the last period of the cave's evolution and to the geometry of the cave. The Grotta Nera is an ascending cavity with a single input, the entrance being in the lower part of the cave, suggesting that its inner room may behave as a hot trap where air is trapped during the winter (Crammer, 1899). During the summer in a hot trap, the air, which is colder than the outside atmosphere, flows downward, whereas in the winter, the cave air, which is warmer than the outside atmosphere, remains in equilibrium, and there is only a limited circulation at the entrance (Crammer, 1899).

Airflows within the inner room of the Grotta Nera were assessed by smoke movement, and by the three-dimensional pattern of temperatures (Fig. 5) at varying distances in the cave and at varying heights above the cave floor. Circulation inside the inner room seems not to be linked to changes in barometric pressure, which has no influence on the insignificant total volume of the cave, but it might possibly be due to an internal convective flow generated by temperature gradients and by exchanges with external air. During the summer, hot air comes in from outside and flows into the inner room, thereby activating a circulation. During the cold season, cold air penetrates in from the exterior through the thin ceiling and generates the same type of air circulation.

In conclusion, the inner room has its own microclimate that, in general, is typical of the hot trap with the peculiarities that are caused by the presence of a very thin layer of upper soil so that the internal circulation in this room of the cave is not limited to the entrance, according to Crammer (1899) but is a more complex circulation (Forti and Rossi, 2003).

Materials and Methods

Sample Location and Sampling

The studied samples were collected in the inner room of the Grotta Nera (Fig. 2a). Two moonmilk samples (MI and MII) were collected respectively from site 1, located 74 m from the entrance, and site 2, located in a more distant portion of the cave (96 m); a sample of percolating water (W) was taken in a sterile tube at site 3. Moonmilk samples were taken aseptically in sterile tubes from small stalactites approximately 15 to 20 cm in length and 3 to 4 cm in diameter. MI was a portion of a white solid stalactite without an internal feeding tube. MII was taken from a stalactite, colored brown due to plant roots, with a large internal tube. The moonmilk and percolating water samples were stored at room temperature (18 °C) for about 18 hours before microbiological analysis was carried out.

Moonmilk Physicochemical Analyses

Moonmilk samples were analyzed by X-ray diffraction and X-ray fluorescence. X-ray diffraction was used to determine the minerals in the moonmilk deposits and the crystals deposited *in vitro*. Measurements were made by using a two-circle θ/2θ diffractometer with a Cu radiation source, secondary graphite monochromator, and scintillation detector (Seifert MZ IV). The supply voltage of the X-ray tube was set at 50 kV and 30 mA. The 2θ-scan range was between 22 and 50°; each scan was done at steps of

0.05°. Depending on the sample density, a counting time between one and ten seconds per step was selected. The crystalline phases were identified using database cards from the International Center for Diffraction Data.

X-ray fluorescence spectroscopy is a non-destructive analysis technique from which it is possible to obtain the elemental composition of a sample through the study of fluorescence radiation. We performed fluorescence spectroscopy on both samples of moonmilk taken in the Grotta Nera at sites 1 and 2 utilizing a SPECTRO (mod. XEPOS 2000) spectrophotometer. Four grams of moonmilk powder were ground down to < 100 μm, well homogenized with 0.9 g of Hoechst wax, and then pressed with 12 tons to a 32-mm pellet.

Isolation and Characterization of Cultivable Heterotrophic Calcifying Bacteria

To isolate the cultivable heterotrophic bacterial microflora that is associated with the moonmilk samples from the Grotta Nera, 10 g of each solid sample was ground to a powder using a sterile mortar and pestle. This powder was then suspended in 90 mL of saline solution (0.9% NaCl). Triplicate B-4 agar plates (Boquet et al., 1973) were inoculated with moonmilk sample dilutions ranging from 10^{-1} to 10^{-6}. The drip-water sample was similarly plated, undiluted or diluted to 10^{-5}. Solid B4 medium was composed (per liter) of 2.5-g calcium acetate, 4.0-g yeast extract, 10.0-g glucose, and 18.0-g agar. The final pH was adjusted, after sterilization, to 8.0 using NaOH. The inoculated plates were incubated at 22 °C, a higher temperature than in the cave from where the samples were obtained, for four weeks in order to isolate slowly growing strains. Previous studies have demonstrated that colonies from cave samples grow very slowly at cave temperature and that the diversity of the cultivable genera observed was similar whether the bacteria were grown at 13 °C (cave temperature) or at 28 °C (Groth et al., 2001; Laiz et al., 2003). Individual colonies were selected and purified by streaking on B-4 agar. The relative abundance of each isolate, with respect to the total cultivable bacterial microflora, was determined by direct counts on B-4 agar plates. For short-term preservation, the isolates were streaked on B-4 agar slants and stored at 4 °C, but for long-term maintenance, pure calcifying isolates were stored in liquid nitrogen at −196 °C. Cell and aggregate morphology was studied under a light microscope (Leitz-Biomed), and Gram-staining was performed with the Color Gram 2 kit (bioMérieux, Marcy-l'Etoile, France).

Calcium Carbonate Precipitation and Dissolution by Calcifying Bacteria

We assessed the calcite production of isolates by culturing them on B-4 agar plates, as described above. The bacterial isolates were spread in triplicate on the surface of agar plates and were then incubated aerobically at 15, 22, or 32 °C. For up to 30 days after inoculation, to follow crystal production, all plates were examined daily under a light microscope (Leitz-Biomed). With respect to negative controls, we checked for the presence of crystals in a sterile medium and in a medium inoculated with autoclaved bacteria.

Since it has been established that microbially mediated reactions can generate considerable amounts of H^+ ions that can dissolve the cave walls or the speleothems and that moonmilk can also be formed by speleothem decay (Sweeting, 1973; Hill and Forti, 1997), the ability of calcifying bacteria to dissolve calcium carbonate was also tested. Calcifying isolates were grown on Deveze-Bruni medium (pH 6.8) containing 0.14 or 2.5% $CaCO_3$ at 15 °C (Normal Commision, 1990). Carbonate solubilization after 7, 15, and 30 days was quantified by measuring the diameter of the clear halo that surrounded each colony in response to decreased pH (Martino et al., 1992).

SEM Analysis

Morphological characteristics were studied by scanning electron microscopy. Cultured solid media samples were dried at 37 °C; agar medium was cut into flat blocks, gold-shadowed, and observed with a Philips scanning electron microscope XL30CP.

For crystallite-poor samples, the method of Rivadeneyra et al. (1998) was applied. The crystals produced by cultured bacteria were removed from the medium by cutting out agar blocks and placing them in boiling water until the agar dissolved. The supernatants were decanted and the sediment was resuspended and washed in distilled water until the crystals were free of impurities. The washed crystals were air-dried at 37 °C and then used for SEM analysis. To observe the inner portion of the bioliths, crystals were first powdered by using a mortar and pestle.

Results and Discussion

Moonmilk Physicochemical Analyses

X-ray diffraction revealed that both samples of moonmilk taken at the Grotta Nera consist of a single mineral phase of calcium carbonate, i.e., calcite. On the other hand, according to previous literature, moonmilk is usually composed of calcite (Fisher, 1992, 1993), even though the presence of other carbonates, as well as sulfates and phosphates in moonmilk, has been reported by several authors (Onac and Ghergari, 1993; Hill and Forti, 1997; Moore and Sullivan, 1997; Borsato et al., 2000; Lacelle et al., 2004). X-ray fluorescence analysis showed that the moonmilk consists primarily of CaO (60.87% at site 1, sample MI, and 60.18% at site 2, sample MII), while other constitutive elements such as MgO and Al_2O_3 never reached 1% (Table 1).

Microbial Cultures from Moonmilk and Dripping Water

The cultures from both moonmilk deposits and dripping water yielded a visible growth for common soil

Table 1. Chemical contents of moonmilk from site 1 (sample MI) and site 2 (sample MII) in Grotta Nera.

Mineral Constituent	Sites	
	Site 1	Site 2
CaO	60.87	60.18
MgO	< 0.83	< 0.83
Al_2O_3	< 0.19	< 0.19
P_2O_5	0.18	0.19
SiO_2	0.05	0.06
SO_3	0.05	0.04
Fe_2O_3	0.01	0.02
MnO	0.01	0.01
K_2O	< 0.01	< 0.01

microflora groups (fungi, bacteria, and actinomycetes bacteria) (Fig. 6), but the dominant cultivable microorganisms were bacteria that caused significant crystallization. A wide range of microbes, particularly bacteria and streptomycetes bacteria, but also fungi, algae, and protozoa, can also be cultured from moonmilk, often in very high densities (Northup et al., 2000).

By using the traditional cultivation techniques, an abundant cultivable heterotrophic bacterial microflora from the moonmilk samples MI (2.1×10^4 colony-forming units per gram of dry weight) and MII (6.0×10^4 cfu/g d.w.) was isolated, suggesting that bacteria presence was probably not accidental. A similar bacterial density has been reported by Baskar et al. (2011) for moonmilk deposits from Krem Mawmluh Cave, India. A similar bacterial density for the calcareous speleothems from the Stiffe and Cervo Caves and also for an unusual newly described calcite speleothem from Grave Grubbo Cave were reported in our previous studies (Cacchio et al., 2003, 2004, 2012). The higher bacterial density in sample MII may be related to a greater presence of organic matter released from plant roots and to a greater water content. The active moonmilk samples taken from the Grotta Nera had a water content of 74% (MI) and 78% (MII) in summer.

Based on the cell and colony morphologies of the isolates (Table 2), it was concluded that the sample MI contained nine cultivable microbial strains (eight bacterial isolates numbered from M1 to M8 and one mold), while the moonmilk sample named MII contained eight cultivable microbial strains (four bacterial isolates numbered from M9 to M12 and four molds). The cultivable heterotrophic bacterial density found in the sample of percolating water, was of two orders of magnitude lower than that found in moonmilk (4.1×10^2 cfu/mL). However, the dripping water contained a considerable number of bacteria, and they could not have filtered through the thin layer of rock that covers the cave, due to the fact that the Grotta Nera is in a mixed-deciduous forest. From this sample four strains were isolated, three bacterial strains named from W1 to W3 and one mold.

Relative Abundance and Preliminary Characterization of Bacterial Isolates

The most abundant strains were the following: isolate M8 from the moonmilk sample MII, isolate M9 from the moonmilk sample MII, and the W2 strain from the dripwater sample. These bacterial strains represented 40.00%, 73.12%, and 68.05% of their samples' respective bacterial populations. The strains M2 and M3 represented 22.90%

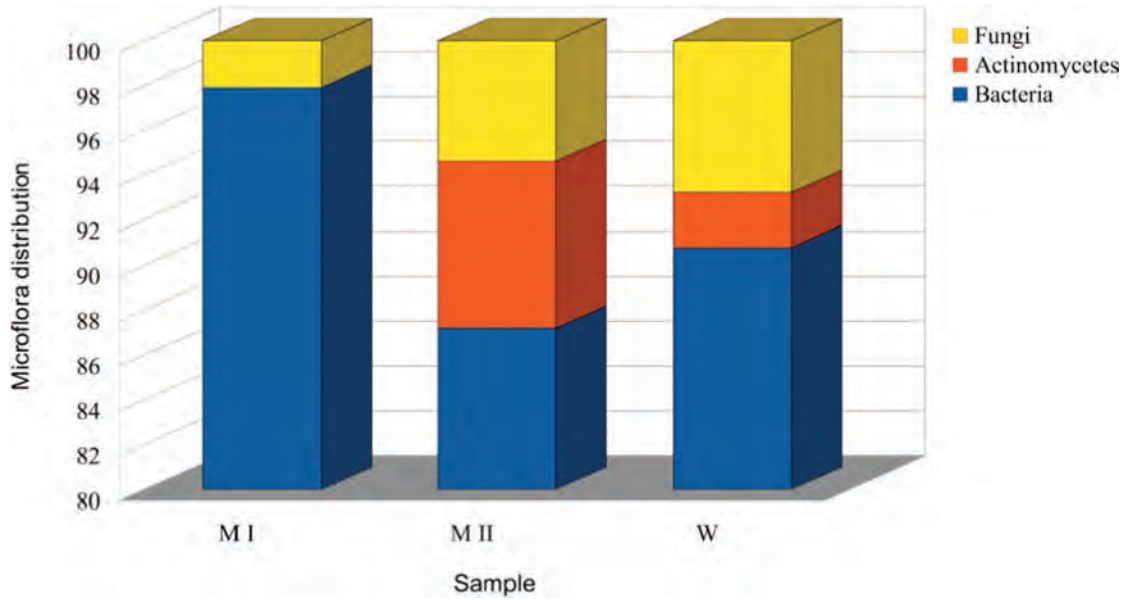

Figure 6. Distribution of actinomycetes, other bacteria, and fungi (percentage of strains) in the cultivable heterotrophic microflora from moonmilk (MI and MII) and percolating water (W) samples collected from Grotta Nera.

Table 2. Bacterial strains isolated from moonmilk and water samples collected from Grotta Nera, Abruzzi, Italy, with their colony and cell morphologies, Gram result, and relative abundances.

Isolate	Colony Morphology	Cell Morphology	Gram	Relative Abundance
MI Moonmilk Sample				
M1	Medium creamy	Rod chain	G−	2.13
M2	Medium creamy	Single rod	G−	22.90
M3	Medium white	Single rod	G−	17.89
M4	Punctiform creamy	Single rod	G−	2.18
M5	Big creamy	Single rod	G−	4.26
M6	Big white	Rod chain	G+	4.26
M7	Medium yellowish	Single rod	G−	6.38
M8	Medium white	Single rod	G−	40.00
MII Moonmilk Sample				
M9	Punctiform pink	Rod chain	G−	73.12
M10	Medium black	Actinom.	G+	7.42
M11	Small creamy	Rod chain	G−	14.13
M12	Big creamy	Rod chain	G+	5.33
Water Sample				
W1	Punctiform creamy	Single rod	G+	29.51
W2	Small yellowish	Single rod	G−	68.05
W3	Punctiform white	Actinom.	G+	2.44

and 17.89% of the MI moonmilk bacterial population, respectively. The strain M11 represented 14.13% of the MII sample, while the strain W1 represented 29.51% of the bacterial population in the percolating water sample. The contributions of the remaining isolates to the overall respective bacterial population ranged from 2.13% to 7.42%.

The actinomycetes component did not exceed 7.42% of the total bacterial population in the MII moonmilk sample, taken in the presence of plant roots, and 2.44% in the drip-water sample. In sample MI from the fully moonmilk speleothem, the actinomycetes component was absent (Table 2). Actinomycetes are common in caves, where their growth is related not only to particular environmental conditions, such as temperature ranging from 10 to 15 °C and high relative humidity, but also to the input of refractory organic matter in dripping water (Groth and Saiz-Jimenez, 1999). Dissolved organic matter from soil, which is the origin of the organic carbon found in dripping waters, contains aliphatic organic acids and phenolic compounds produced by lignocellulose degradation (Guggenberg et al., 1994; Saiz-Jimenez and Hermosin, 1999). Both lignocellulose and humic materials are almost selectively degraded by actinomycetes, which are well known for their ability to grow on very poor media and to use recalcitrant organic matter (Crawford et al., 1983; McCarthy, 1987; Groth and Saiz-Jimenez, 1999). The findings of this research not only confirm the importance of temperature and relative humidity to determine actinomycetes colonization and long-term growth on cave surfaces, but also underlines the role of recalcitrant organic matter that, according to our results, seems to be the main determining factor. In fact, the proportion of actinomycetes among the bacterial microflora is higher in the sample MII, which receives organic matter from percolating water and plant roots, than in the drip-water sample, and they disappear entirely in the stalactite of moonmilk that does not receive organic matter from dripping water and root exudates.

These results are interesting, but may not reflect the actual microbial activity taking place in the speleothem because cultivation techniques are thought to greatly underestimate microbial diversity due to the non-culturability of the large majority of microorganisms (Dojka et al., 2000). In this study, single small rods and rod chains predominate in the strains isolated (Table 2). An examination of Gram-stained smears taken from the fifteen different colony types from moonmilk deposits and drip-water showed that the majority (66.7%) of the isolated strains were Gram-negative (Table 2). The relative abundances of each isolate with respect to the total cultivable bacterial microflora showed that (Fig. 7 and Table 2) the most abundant strains (M2 and M8 strains in the MI sample, M9 and M11 strains in the MII sample) in both the studied moonmilk samples were Gram-negative, as previously found by some authors elsewhere (Danielli and Edington, 1983; Mulec et al., 2002). The Grotta Nera is located in a mixed-deciduous forest, and it is known from previous literature that Gram-negative bacteria tend to be more abundant in rhizosphere soil compared to the bulk soil (Schlegel, 1993; Steer and Harris, 2000): Gram-negative bacteria biomass increases when rapidly decomposable

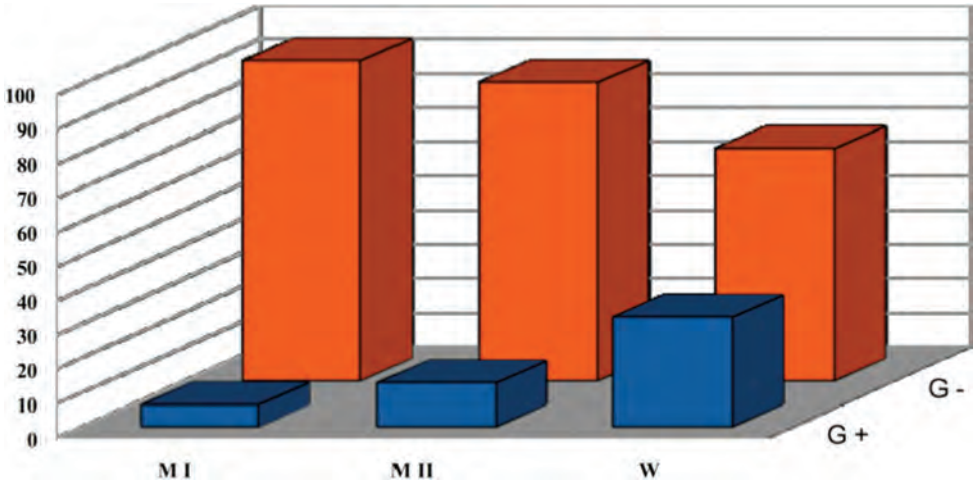

Figure 7. Gram-negative/Gram-positive proportions in the cultivable heterotrophic bacterial microflora of moonmilk (M I and M II) and percolating water (W) samples collected from Grotta Nera, by relative abundance (see Table 2).

carbon compounds, such as sugars and organic and amino acids, are available (Marilley and Aragno, 1999), whereas higher proportions of Gram-positive bacteria are usually found in resource limited areas (Atlas and Bartha, 1998; Kourtev et al., 2002). In fact, the ratio of Gram-negative to Gram-positive bacteria in moonmilk samples MI (95.74%) and MII (87.25%) was significantly higher than in previously studied consolidated calcareous speleothem and soil samples (Cacchio et al., 2003, 2004, 2012) from which nearly all strains isolated were Gram-positive. A high proportion (68.05%) of Gram-negative bacteria was also found in the percolating water sample. In general, cultivable bacterial communities from groundwater have low proportions of Gram-positive bacteria (Laiz et al., 1999). No attempts were made to identify the various bacterial species present, as attention was directed on the processes of carbonate deposition rather than on taxonomy.

In Vitro Bacterial Precipitation and Dissolution of Calcium Carbonate

Since all the molds isolated from moonmilk and water samples were incapable of precipitating $CaCO_3$ crystals, this suggests a major participation of bacteria in the biomineralization processes involved in the moonmilk stalactite formation. Some previous studies have suggested fungi as the major participants in the process (Callot et al., 1985), although more recent investigations have proposed bacteria as the major inducers of carbonate deposition forming moonmilk in caves (Cañaveras et al., 2006; Barton and Northup, 2007; Portillo and Gonzales, 2011). Bacterial $CaCO_3$ precipitation on B-4 solid medium occurred at all the temperatures tested, 15 °C (cave temperature), 22 °C, and 32 °C. Under laboratory conditions, it was found that all of the bacterial isolates associated with the hollow stalactite (sample MII) and the drip-water sample were capable of forming crystalline calcium carbonate. This confirms the hypothesis that in appropriate conditions, especially in carbonate-rich environments such as limestone caves, many bacteria can form calcium carbonate crystals (Boquet et al., 1973; Cacchio et al., 2003, 2004, 2012). Not all of the bacterial strains isolated from the solid moonmilk stalactite (sample MI) were calcifying; the high relative abundance (40%) of strain M8, which was not capable of precipitating crystals, is consistent with the less intense calcification of this stalactite. This is consistent with our previous hypothesis (Cacchio et al., 2004), according to which drip-water may select calcifying bacteria, and hence the intensity of calcifying activity is greater in younger stalactites. It is worth noting that in tubular stalactites, calcification also takes place in the inner surface, giving rise to filling of the space (Moore and Sullivan, 1997). By using a knock-out in the *cha*A calcium antiporter protein, Banks et al. (2010) have suggested that calcium toxicity provides both the physiological basis and selection pressure for the calcification phenotype. All the Gram-positive strains isolated from the Grotta Nera were capable of depositing crystals on B4 agar plates at all the studied temperatures. The comparison of the percentages of calcifying bacteria isolated from the previously studied caves (Cacchio et al., 2003, 2004, 2012) has shown that the temperature factor plays a key role in the extent of calcium carbonate deposition by bacteria. In fact, in Stiffe and Cervo Caves, the temperature ranges from 10 to 12 °C, whereas in Grave Grubbo Cave, located in south Italy, where the range is 14 to 15 °C the biogenic activity is, as in the Grotta Nera, much more impressive. Hence, it can be suggested that 15 °C is the threshold of two biologically different calcification environments.

Carbonate precipitation by calcifying isolates at 22 and 32 °C started with some delay compared to that at 15 °C. All the calcifying bacteria began to precipitate carbonate after three days at 15 °C, after one week at 22 °C, and after

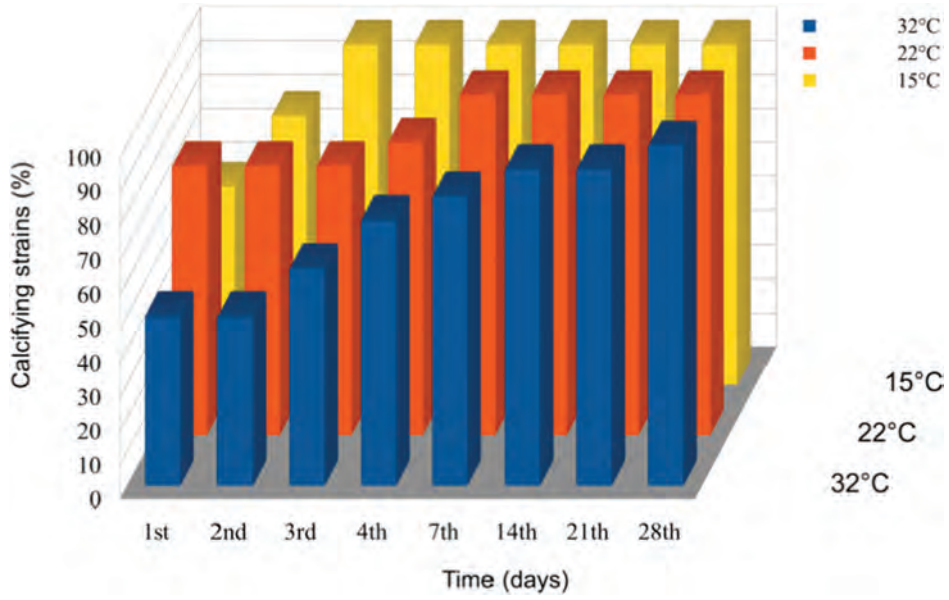

Figure 8. Relationship between percentage of calcifying strains showing production of calcite by the indicated duration of culturing on B-4 agar cultures at 15 °C, 22 °C, and 32 °C. Note non-linear X axis.

four weeks at 32 °C (Fig. 8). In our previous studies, it was found that calcification took place more quickly at 32 °C than at lower temperatures, including the temperature of the studied caves (Cacchio et al., 2003, 2004, 2012). In fact, the bacteria isolated from moonmilk deposits are much more rapid in the process of calcification in the cave than the strains isolated from cave speleothems not consisting of moonmilk (Cacchio et al., 2003, 2004, 2012). To explain this significant difference, a working hypothesis is adopted that microbes inhabiting the Grotta Nera have been exposed to a longer period of evolution at a constant temperature of 15 °C, hence strengthening their calcification capability at such a temperature. In the other studied caves, which have running water, the temperature tends to change throughout the year according to the discharge changes and periods of drought.

The calcite nature of the crystals deposited on B4 agar plates was confirmed by X-ray diffraction analysis. No carbonate precipitation took place in the controls since metabolic activity is necessary for precipitation.

The corrosion behavior of the calcifying isolates was also studied showing that 13% of the calcifying bacteria isolated from the MII moonmilk speleothem solubilized calcium carbonate when grown on agar plates containing 0.14% and 2.5% $CaCO_3$ after two weeks at 15 °C. This percentage increased to 32% for the calcifying bacteria isolated from the dripping water. With respect to the solubilization activity of the calcifying bacteria associated with the MI moonmilk speleothem, this was at least 32% after one week at 15 °C. However, the calcifying bacteria isolated from the Grotta Nera Cave showed a greater ability to dissolve calcium carbonate than those associated with consolidated stalactites sampled from previously studied caves (Cacchio et al. 2003, 2004 and 2012).

SEM Analysis

Scanning electron micrographs of the crystals deposited on B-4 agar plates revealed significant amounts of bacterial cells on the inner surface of the crystals (Fig. 9a,b), calcified bacterial cells (Fig. 9e,f) and their imprints (Fig. 10c,d,e,f); newly formed (Fig. 9a,b,c,d) or calcified biofilms (Fig. 9e,f) cementing the carbonate grains; and the presence of crystals that vary in size and shape (Fig. 10a,b). SEM studies of the extensive moonmilk deposits in the Grotta Nera revealed that they were mainly composed of a network of fiber calcite crystals and filaments (Fig. 10g,h). The results of SEM examination point toward a significant bacterial influence in the genesis of moonmilk in the Grotta Nera.

Conclusions

Caves are extreme and specialized habitats for terrestrial life that sometimes contain moonmilk. There are many caves around the world with impressive amounts of moonmilk (Onac and Farcas, 1992; Chirienco, 2002). In Italy, calcite moonmilk is found in many caves in the Italian Alps (Borsato et al., 2000). The Cesare Battisti Cave contains moonmilk deposits up to 0.5 m thick and 120 m long that developed under several seepages (Borsato et al., 2000) and a moonmilk flowstone on the wall of the Cripta Chamber that has developed to a thickness of 40 to 50 cm. In the Bus del Toni Cave, there are extensive curtains of moonmilk (Miorandi and Borsato, 2005)

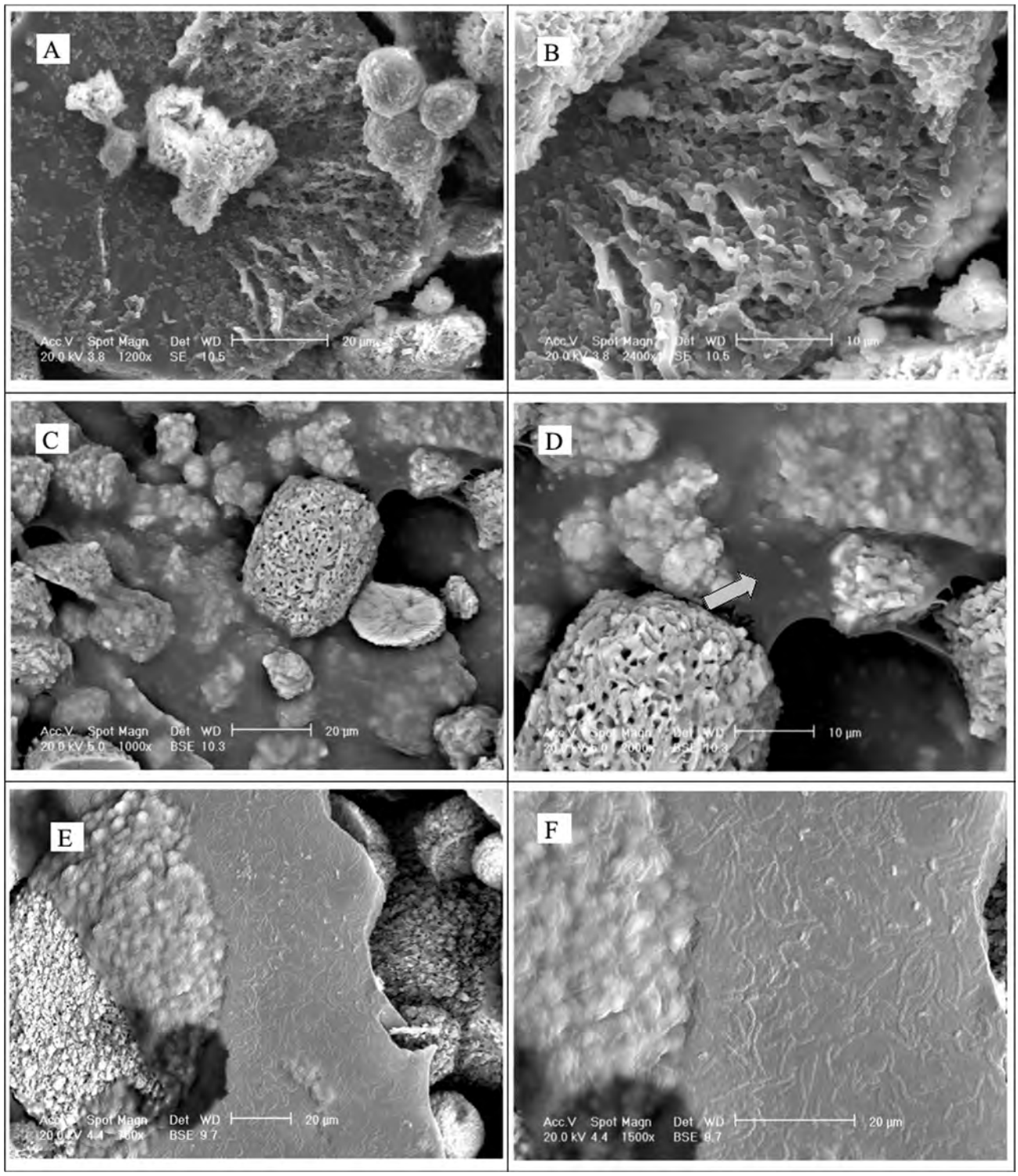

Figure 9. Scanning electron micrographs: (a) Calcifying bacterial cells isolated from moonmilk collected from Grotta Nera of the unidentified strain M5 on the inner surface of a $CaCO_3$ crystal precipitated on B-4 agar, after 30 days at 22 °C; scale bar 20 µm. (b) Higher magnification view of (a); scale bar 10 µm. Observe the significant number of bacterial cells and the presence of biofilm. (c) Bacterial cells included in a biofilm that bridges calcite crystals deposited on B-4 agar plates after 30 days of incubation at 32 °C of the strain W1 isolated from a sample of drip-water collected in Grotta Nera; scale bar 20 µm. (d) Higher magnification view of (c); arrow points to some of the cells. Scale bar 10 µm. (e) Cemented biofilms that incorporate the calcifying cells of the M9 strain isolated from moonmilk collected in Grotta Nera, observed after 30 days of incubation on B-4 agar at 32 °C; scale bar 20 µm. (f) Higher magnification view of (e); scale bar 20 µm. Observe the significant thickness of the calcium carbonate layer produced by M9, the most abundant strain in the MII moonmilk sample; compare with (c).

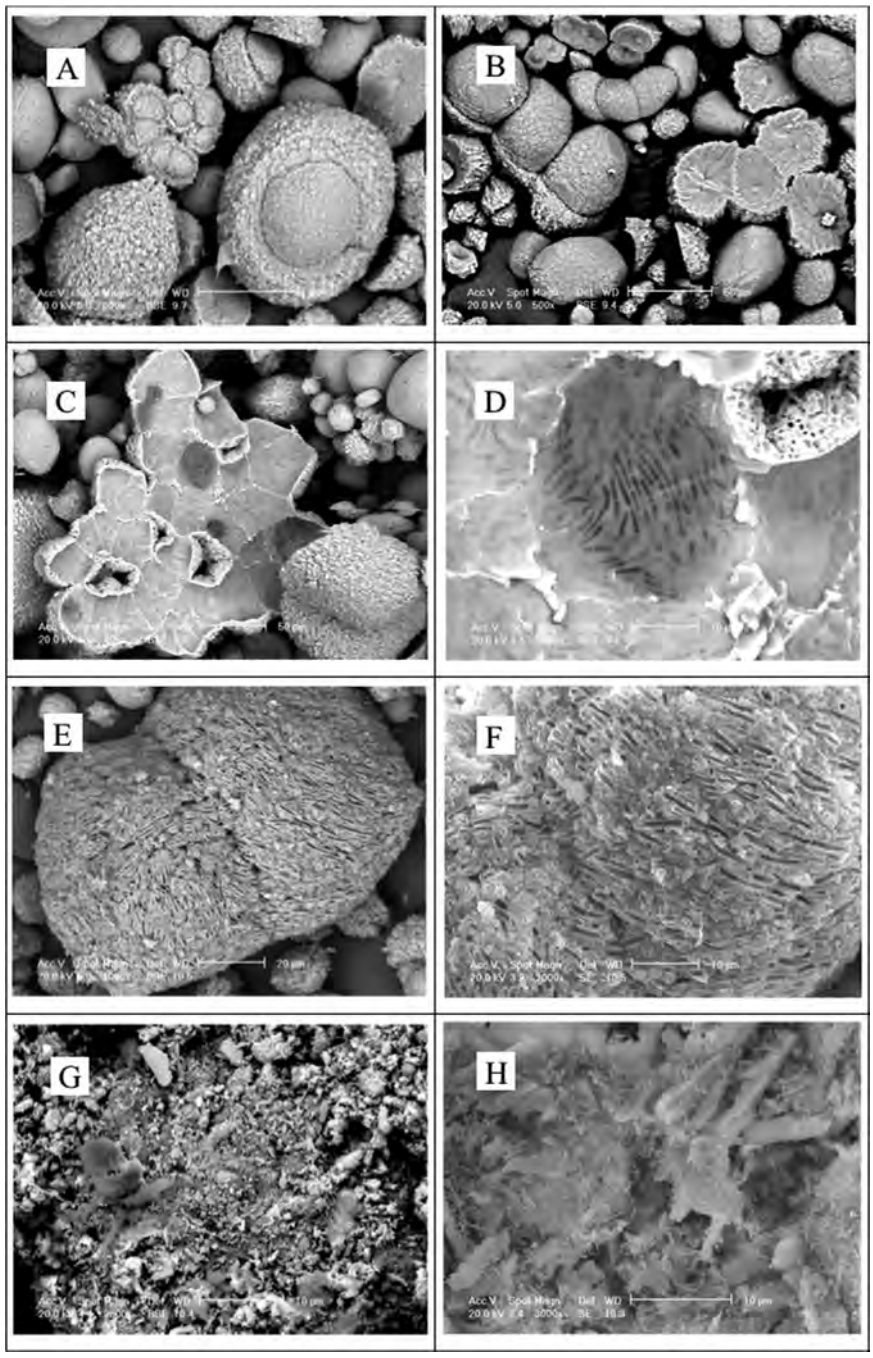

Figure 10. Scanning electron micrographs of $CaCO_3$ deposited on B4 agar plates by the strain M9, isolated from moonmilk sample MII collected in Grotta Nera. Spherulite crystals and calcite aggregates; (a) scale bar 50 μm, (b) scale bar 50 μm. Bacterial imprints in the inner portion of the crystals; (c) scale bar 50 μm, (d) scale bar 10 μm, (e) scale bar 20 μm, (f) scale bar 10 μm. Calcite moonmilk fiber; (g) scale bar 10 μm, (h) scale bar 10 μm.

In the Grotta Nera, moonmilk entirely covers the walls, the ceiling, and the floor of the room, generating exceptional examples of decorations, including stalactites, stalagmites, and cave pearls. Even though in Italy it is not common to find extensive moonmilk deposits, the extraordinary scientific importance of this cave is mainly linked to the presence of unusual speleothems classified as *trays* whose size has no equal in other caves in Italy (Forti and Rossi, 2003).

Our preliminary study of cultivable microbial populations in two samples of moonmilk and one of dripping water from Grotta Nera was carried out to estimate the concentration of colony-forming units; no identification of genera and species was provided. This study revealed high

cultivable heterotrophic population densities and a diverse microbial community, including filamentous bacteria, associated with the moonmilk. Moonmilk and drip-water bacterial communities tend to have low proportions of Gram-positive bacteria and are mainly composed of Gram-negative single rods and rod chains. This can be ascribed to the fact that Gram-positive bacteria are likely to be more successful in resource-limited areas, suggesting that the association of moonmilk with Gram-negative bacteria is at least partially driven by the characteristics of the soil and overlying vegetation. Most of the bacteria isolated have the ability to precipitate calcite crystals when cultured using B-4 agar, and due to their metabolic activity this probably happens also in natural habitats. In carbonate caves, the metabolism of microorganisms can alter their microenvironment, if products of microbial activity result in a pH increase in the environment. An increase in metabolic end products, such as carbonate ions, can increase precipitation of calcium carbonate in caves (Braissant et al., 2002; Barton and Northup, 2007; Portillo et al., 2009). The different morphologies of the precipitates formed by the different calcifying isolates confirmed that crystal morphology was species-specific, and this suggests that the bacteria play a major role in the precipitation process. When examined by scanning electron microscope, the Grotta Nera moonmilk samples exhibited a felted mat of fibers; X-ray diffraction analysis of the speleothems gave clear evidence for calcite.

The electron microscopic and microbiological evidences, together with the geochemistry and environmental data obtained in this study, indicate that moonmilk from the Grotta Nera stalactites is of biogenic origin. It is therefore possible to infer that there are two different bacterial contributions to the biogenic moonmilk hosted in the Grotta Nera, active precipitation of moonmilk by bacteria and bacterial biochemical corrosion of the bedrock by organic acid, as suggested by the presence of non-calcitic mineral inclusions into the moonmilk and by the solubilization activity of the calcifying bacterial strains.

The extensive presence of biogenic moonmilk in Grotta Nera may be related to the peculiar $CaCO_3$ precipitation environment, i.e., its middle elevation, mixed-deciduous vegetation cover, and local microclimate. Therefore a conclusion of this study is that microbial activity at a constant and optimum temperature appears to be a key factor promoting calcite precipitation and moonmilk formation in Grotta Nera.

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