The role of microorganisms in the formation of calcitic moonmilk deposits and speleothems in Altamira Cave

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Running title: Moonmilk originates from bacterial colonizations
Abstract

Bacteria are able to induce carbonate precipitation although the participation of microbial or chemical processes in speleothem formation remains a matter of debate. In this study, the origin of carbonate depositions such as moonmilk, an unconsolidated microcrystalline formation with high water content, and the consolidation of carbonate precipitates into hard speleothems were analyzed. The utilized methods included measurements of the composition of stable isotopes in these precipitates, fluorimetric determinations of RNA/DNA ratios and respirometric estimations in Altamira Cave. Results from isotope composition showed increases of the $\delta^{18}O$ and $\delta^{13}C$ ratios from moonmilk in the very first stages of formation towards large speleothems. Estimates of RNA/DNA ratios suggested an inactivation of microorganisms from incipient moonmilk towards consolidated deposits of calcium carbonate. Respiratory activity of microorganisms also showed a significant decrease in samples with accumulated calcite. These results suggest that bacterial activity induces the conditions required for calcium carbonate precipitation, initiating the first stages of deposition. Progressive accumulation of carbonate leads towards a less favorable environment for the development of bacteria. On consolidated speleothems, the importance of bacteria in carbonate deposition decreases and chemical processes gain importance in the deposition of carbonates.
1 Introduction

Although the role of bacteria in most biogeochemical cycles is critical for the functioning of natural systems (Whitman et al., 1998; Madigan et al., 2003), the importance of chemical versus biological processes is often poorly understood. One example is the formation of carbonate precipitates in caves. While recently geomicrobiologists are proposing a critical role for microorganisms, either bacteria or fungi (Verecchia and Verecchia, 1994; Cañaveras et al., 2001), other scientists attribute the speleothems formation to abiotic geochemical processes (Hill and Forti, 1997; Ehrlich, 1998; Borsato et al., 2000). Independently of its origin, carbonate precipitation in underground systems constitutes an important factor shaping karst systems.

Calcium carbonate deposition in cave environments is a complex phenomenon (Catanier et al., 1999; Barton and Northup, 2007) that is strongly dependent on pH. Generally, pH values of 8 and above are required for carbonate to precipitate (Butler, 1982; Morse and Mackenzie, 1990) forming calcite or other calcium carbonate mineral. Caves usually exhibit levels above the minimum required of other critical factors that favor calcium carbonate precipitation (Butler, 1982; Ehrlich, 1998; Castanier et al., 1999), such as, elevated partial pressure of CO₂, and elevated concentrations of carbonates and calcium ions (Butler, 1982; Ehrlich, 1998; Kowalski et al., 2008). One of the major problems to accepting a completely chemical process of carbonate precipitation is the general assumption that a nucleation site is required to initiate that deposition (Pentecost and Bauld, 1988; Phoenix and Konhauser, 2008).
Biologically-induced carbonate deposition has frequently been reported (Boque et al., 1973; Douglas and Beveridge, 1998; Barton et al., 2001; Cañaveras et al., 2001; Forti, 2001). While this process was initially attributed mainly to fungi (Verrecchia and Verrecchia, 1994), recent studies confirm that generally bacteria are the major players in the induction of carbonate precipitation in caves (Cañaveras et al., 2001, 2006; Portillo et al., 2009). Diverse forms of crystallizations have been reported; for example, moonmilk which is an unconsolidated microcrystalline cave deposit with a high water content. Moonmilk represents a soft, wet, plastic, fine-grained speleothem, which has been suggested to be of microbial origin (Forti, 2001; Cañaveras et al., 2006; Curry et al., 2009). In contrast, moonmilk formation has also been described as an abiotic process (Borsato et al., 2000). A model of moonmilk and speleothem formation was previously described based on observations from microphotographs and petrological analysis (Cañaveras et al., 2006). These authors describe in detail the different stages of moonmilk formation as a result of the distinctive structures and fabrics formed during the process. According to Cañaveras et al. (2006), the process of moonmilk formation follows a progressive accumulation of bacterially-induced calcite fibers. The process is initiated by the formation of thin-fiber calcite crystals (microbial colonization phase) which progressively accumulate leading to the breakdown of these needles (microstructural breakdown phase). The accumulation of carbonate fibers and further consolidation result in an advanced formation stage (crusting phase) (Cañaveras et al., 2006). The major drawbacks on the biological perspective are the scarce knowledge available on how and when microorganisms form those precipitates and the microbial role in these formations. At this respect, molecular and physiological studies have shown that many different bacteria can lead to carbonate precipitates under laboratory conditions (Boquet et al., 1973; Barabesi et al., 2007). For instance, the consumption of
specific nutrients has been shown to represent a critical determinant in the induction of carbonate precipitates by bacteria (Portillo et al., 2009).

This study aims to estimate the role of microbiological processes on calcium carbonate deposition leading to speleothem formation in caves. The origin of calcitic speleothems was analyzed by microscopy, petrochemical analysis and isotope analysis, and the role and fate of bacteria was studied through RNA and DNA quantification and nanorespirometry with the objective of solving the current controversy on the chemical versus microbiological processes leading to speleothem formation.

2 Materials and Methods

2.1 Sample site and collection. This study was performed at Altamira Cave (Cantabria, Spain). This cave has been previously described from the geological, microenvironmental (Sanchez-Moral et al., 1999), geomicrobiological (Cuezva et al., 2009) and microbiological (Portillo et al., 2008; Portillo and Gonzalez, 2009a) perspectives. Altamira Cave is placed in the vadose zone of a tabular polygenic karst system which was developed on a small calcareous hill (158-m.a.s.l.) composed of a succession of sub-horizontal decimeter-thick beds of Cretaceous fossiliferous limestones that are partly dolomitized (Sanchez et al. 2007; Cuezva et al., 2009). Thin (2±10 cm thick) marly and clayey layers are interbedded among the calcareous beds (Sanchez-Moral et al., 1999; Cuezva et al., 2007; Sanchez et al., 2007). Altamira Cave is situated at a depth of 3-22m (averaging 8 m) below the surface (Cuezva et al., 2011). The cavity has a sole entrance, situated at 152 m.a.s.l. The cave features a main passage (Fig. 1) whose height varies from 2 to 12 m and whose width ranges from 6 to 20 m.
The cave length is 270 m. The thickness of the cover (host-rock and soil) in most of the galleries varies between 4 and 10 m (see Fig. 1). A poorly differentiated anthroposol with little development (30-70 cm) cover the cave. It is a silicate-based soil on which develops a plant cover (mainly pasture), which results in a carbon-rich upper level (above 10 cm with 10-15% organic carbon) (Cuezva et al., 2011).

The hydrological dynamics in this cave are attributable exclusively to the rainwater that seeps directly into the cave through different strata. Speleothems are relatively rare.

In addition to calcite moonmilk deposits, isotopic data shown in this article are from samples of stalactites, stalagmites, flowstones and recent ‘soda-straw’ stalactites (Fig. 2). Other types of speleothems have been recognized and described in Altamira such as hydromagnesite moonmilk deposits (Cañaveras et al., 1999) and calcite coralloids and aragonite frostwork speleothems (Sanchez-Moral et al., 1999).

Moonmilk deposits were sampled from different passages in Altamira Cave. These deposits developed on different types of materials (Fig. 1), specifically on porous stalagmitic flowstones (Big Hall) and concrete walls (Hall of the Walls).

On the surface of both substrates, in contact with moonmilk deposits, generally, there is a thin layer rich in clays (or a clay-enriched zone) (phyllosilicates: 10-12%; calcite: 30-50%; dolomite: 5-15%; quartz: 15-25%; K-feldspar: 0-15%). The isotopic composition of carbonates in this material has also been analyzed.
These deposits, which are mainly composed of clays, terrigenous grains and carbonate grains, are interpreted as insoluble residues from bedrock dissolution, related to percolation of soil-derived material and/or as the result of cave condensation-corrosion processes (Sanchez-Moral et al., 1999). The development of cave condensation-corrosion residues have also been attributed to organic biokarst phenomena (Cunningham et al., 1995).

Different types of carbonate deposits were collected with emphasis on different stages of accumulation from incipient moonmilk formation to consolidated calcium carbonate deposits (Table 1). The different stages of moonmilk deposits were considered as previously described (Cañaveras et al., 2006). Sampling was carried out using sterilized scalpels and tubes. The distribution of the sampled locations in the studied cave are shown in Fig. 1. Microscopic observations, mineralogical analysis, and isotopic analysis were performed immediately after arrival to the laboratory. Samples for the quantification of microbial DNA and RNA and respirometric measurements were processed in situ in the cave immediately upon collection.

2.2 Microscopical and mineralogical analysis. Carbonates deposits from Altamira Cave were observed on an Environmental Scanning Electron Microscope (ESEM) with a FEI INSPECT and a secondary electron detector (Oxford Instruments Analytical-INCA, Madrid, Spain). The semi-quantitative mineral composition was determined using X-ray diffraction on a Philips PW-1710 (Madrid, Spain).

2.3 Isotopic analysis. Different types of carbonate deposits throughout Altamira Cave were analyzed for their stable ($\delta^{18}$O and $\delta^{13}$C) isotopic characterization. The
169 $^{18}\text{O}/^{16}\text{O}$ and $^{13}\text{C}/^{12}\text{C}$ ratios of calcite were determined using a Dual-Inlet type VG SIRA-
170 II isotope mass spectrometer at the Stable Isotopes Laboratory of the University of
171 Salamanca (Spain) on CO$_2$ gas released by the powdered calcite reacting with 100%
172 phosphoric acid (H$_3$PO$_4$). The analytical reproducibility for both $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ is ±
173 0.10‰.

174

2.4 DNA and RNA determinations. In situ determinations of DNA and RNA were
176 performed fluorometrically as previously described (Portillo and Gonzalez 2009b).
177 DNA- and RNA-specific fluorescent dyes Quant-iT PicoGreen dsDNA and Quant-iT
178 RNA, respectively (Invitrogen, Carlsbag, California), were used throughout this study.
179 Independent triplicate analyses were carried out from each sample. Briefly, the
180 procedure consisted of the suspension of the samples in a buffer (10 mM Tris-HCl, 1
181 mM ethylenediaminetetraacetic acid [EDTA], and 1% sodium dodecyl sulfate [SDS],
182 pH 8.0) and mixed by vortexing. Ten microliters of the liquid phase was transferred to
183 190 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and mixed. Ten
184 microliters of this suspension was mixed with an equal volume of 2x dye working
185 solution (1:200 dilution of commercial stock), which was prepared as suggested by the
186 manufacturers. This procedure includes a dilution step to remove most particles and
187 reduce the concentration of SDS (0.005% SDS final concentration) previous to
188 fluorescence measurements. After 15 min of reaction time, the solutions were
189 transferred to a fluorometer cuvette and fluorescence was measured. A Modulus
190 fluorometer (Turner Inc., Sunnyvale, CA) was used during this study, following the
191 recommended excitation and emission wavelength settings. Controls without sample
192 and controls without dye were carried out. The RNA/DNA ratio is used as an indicator
193 of metabolic activity per cell, which is independent of microbial abundance. Fluorescent
data were normalized by the dry weight of the sample in grams. Statistical analyses for
the significance of the difference between the RNA/DNA ratios were carried out using
single classification ANOVA as described by Sokal and Rohlf (1995).

2.5 *In situ* respiratory activity. The aerobic respiratory activity by the bacterial
communities in samples both showing and lacking calcite deposits was assessed by
nanorespirometry. The procedure has been described in detail by Nielsen et al. (2007).
Briefly, each sample (on average 40 mg) was placed in the bottom of a glass cuvette (3
mm inner diameter) and filled with 100 µl sterile distilled water. As glass is completely
impermeable to oxygen, oxygen was only supplied by molecular diffusion from the
atmosphere into the water column above the sample in the glass well. Oxygen
consumption by the sample was monitored using an oxygen microelectrode with a
sensor tip diameter of 20 µm (Unisense, Århus, Denmark) and determining the
concentration gradient through the water column in the cuvette. The sensor was attached
to a motorized micromanipulator system controlled by computer software (Unisense).
At about two hours, the gradient reached a steady state in our experiments and the depth
profile of oxygen concentration followed a linear gradient (Nielsen et al., 2007).
Samples were analyzed *in situ* and incubated in the cave. Controls lacking sample
showed constant oxygen concentration along the depth profile. The slope of oxygen
concentration was used to provide relative estimates of oxygen consumption (Nielsen et
al., 2007) because it is a result of oxygen consumption by respiratory activity in the
sample and the oxygen flux towards the bottom of the cuvette (Crank, 1997). Slopes
were normalized by sample dry weight. Statistical analyses for the significance of the
difference between the slopes of oxygen consumption were performed by comparison of
the regression coefficients according to Sokal and Rohlf (1995).
3 Results

Moonmilk and other carbonate deposits from Altamira Cave collected at different points in the cave (Fig. 1) were analyzed by X-ray diffraction and showed values above 90% calcite (Table 1). Detailed views of different types of the analyzed carbonate precipitates, including moonmilk and consolidated speleothems are shown in Fig. 2. In addition, Fig. 3 shows ESEM microphotographs of moonmilk in Altamira Cave. ESEM analysis of well developed moonmilk deposits revealed that calcite moonmilk showed a three layered structure (Fig. 3A): an upper/external layer, composed of smooth monocristalline rods (Fig. 3B); an intermediate layer, mainly composed of serrated needle-fibers (Figs. 3C, 3D); and a lower/inner layer composed of overgrown fiber crystals (Figs. 3E, 3F). These structures correspond to the three major stages of formation previously described (Cañaveras et al. 2006).

In order to determine the biotic or abiotic origin of carbonate deposits, isotopic analysis estimates were performed from natural samples. These analyses discriminate between different speleothems. Fig. 4 shows a summary of the results for the isotopic signature of moonmilk and a variety of speleothems. The $\delta^{13}C$ values of moonmilk are similar throughout the cave, ranging from -9.5 to -14.5‰. These values are isotopically lighter compared to most speleothems (stalactites and stalagmites) present in Altamira Cave galleries ($\delta^{13}C$ -4 to -12.5‰; on average $\delta^{13}C = -9.1‰). With respect to $\delta^{18}O$, moonmilk samples showed values between -4.9 and -9.1‰, and large and hard speleothems exhibited values ranging from -3.0 to -6.7‰. Clay samples exhibited fractions of $\delta^{18}O$ and $\delta^{13}C$ within the range observed for incipient moonmilk samples.
However, compact host rock samples exhibited values ($\delta^{13}C = -1.3$), above those observed for hard speleothems.

Quantifications of microbial DNA and RNA have been reported as indicators of abundance and metabolic activity of microbes in the environment, respectively (Molin and Givskov, 1999; Portillo and Gonzalez, 2009b). In Altamira Cave, the initial stages of moonmilk formation showed high ratios of RNA/DNA (Table 2) similar to those found in clay-rich substrates showing no calcification. For increasing calcite deposition, the RNA/DNA ratios significantly decreased ($P<0.05$; Table 1) indicating a decrease of metabolic activity per cell.

Aerobic respiratory activity in samples showing no visible precipitation of calcium carbonate and consolidated carbonate deposits was assessed by nanorespirometry in order to confirm that accumulation of calcite leads to the inhibition of bacterial activity. The results showed that bacterial respiratory activity in calcite deposits was much lower ($P<0.001$) than in samples lacking carbonate precipitates (Fig. 5). On average, the slope of oxygen concentration during the nanorespirometric experiments in calcitic deposits (average slope ± standard deviation: $-0.0101 \pm 0.0003$ µM O$_2$ g$^{-1}$ sample) only represented 9.0% of the slope determined in samples lacking carbonate deposits (average slope ± standard deviation: $-0.1118 \pm 0.0030$ µM O$_2$ g$^{-1}$ sample).

4 Discussion

Morphological evidence (Cañaveras et al., 2006) suggested that moonmilk formation starts with an initial phase through the microbial colonization of rock surfaces; incipient
moonmilk consists of long and thin needles in association with microorganisms. A second or intermediate phase includes overgrowth on needle surface, microstructural breakdown, and accumulation of collapsed fibers resulting in a more densely-packed structure. In a more evolved phase, polycrystals composed of stacked tabular rhombohedra form an internal microhabitat which favours physico-chemical precipitation. An ongoing deposition and further crystallization lead to a layered structure with consolidated calcium carbonate precipitates.

The wide range of the δ\(^{18}\)O values from moonmilk does not suggest isotopic equilibrium precipitation in the calcite-water system (O’Neil et al., 1969). Possible carbon sources responsible for the carbon signature within moonmilk deposits are bedrock dissolution, atmospheric CO\(_2\) and biogenic soil CO\(_2\). Nevertheless, low δ\(^{13}\)C values of moonmilk deposits could be related to a fractionation effect due to microbial activity (Romanek et al., 1992). As previously indicated (Romanek et al. 1992), cave calcite precipitates with δ\(^{13}\)C lower than -13‰ are incongruent with their precipitation in isotopic equilibrium from the present cave water (both fast and slow dripping water) in any season of the year.

Co-variation between δ\(^{18}\)O and δ\(^{13}\)C also indicates that calcite precipitation forming moonmilk occurred under non-equilibrium conditions suggesting the influence of microbial activity and the generation of microenvironments acting as nucleation sites (Hendy, 1971). Moonmilk from different galleries in the cave can be differentiated by their isotopic signal, indicating that both water chemistry and the hydrologic setting also influence the isotopic composition.
The stable isotope composition of moonmilk deposits ($\delta^{18}$O -4.9 to -9.1‰ and $\delta^{13}$C -9.5 to -14.5‰) indicates significant incorporation of organically-derived CO$_2$ and probably a biological influence on calcite crystals. Concerning the $\delta^{13}$C values, the possible reservoirs of carbon in speleothems are the inorganic carbon from dissolved Cretaceous marine limestones and dolostones (host rock); the CO$_2$ produced in the soil by respiration of organic material, with $\delta^{13}$C values ranging from $-15$ to $-25$‰ (average $-21$‰); and the carbon from atmospheric CO$_2$, normally with $\delta^{13}$C values close to $-7$‰ (Sanchez-Moral et al., 2010). Clay samples in Altamira Cave, which show the presence of an active microbial community (Gonzalez et al., 2006; Portillo et al., 2008; Portillo and Gonzalez, 2009a) and accumulate the highest nutrient concentrations in the cave (Portillo et al. 2009), contained fractions of $\delta^{18}$O and $\delta^{13}$C similar to moonmilk.

The $^{13}$C/$^{12}$C analyses for air, water and rock/speleothem in Altamira Cave confirm the importance of the external soil as the CO$_2$ source for the underground system and the effect of ventilation on system equilibrium, in agreement with Sanchez-Moral et al. (2010). The $\delta^{13}$C of the water (averaging $-12.6$‰) varies according to the degree of interaction with the host rock ($\delta^{13}$C calcite: 2‰, $\delta^{13}$C dolomite: 3‰). In this study, the speleothems had values ($\delta^{13}$C -4 to -12.5‰) that are congruent with their precipitation in isotopic equilibrium with the interior of the cave (discrimination coefficient of 0.4‰ at 14°C, Labonne et al. 2002).

As the moonmilk carbon isotopic signature is significantly lower than estimated by calculating equilibrium we infer the possibility that microorganisms play a role in the CO$_2$ depletion and, directly or indirectly, in the isotopic composition of moonmilks. Using the temperature-dependent oxygen isotope fractionation equation for calcite-
water (Friedman and O’Neil, 1977) the calculated isotopic range for calcite precipitation in equilibrium is higher than the $\delta^{18}O$ range for moonmilk, indicating that water from which moonmilk precipitated was enriched in the light $^{16}O$ isotope compared to karstic water possibly due to biological activity. This fact could indicate evaporation-condensation processes related to seasonal variations in microclimatic parameters of the cave, including air temperature and humidity (Lacelle et al., 2004).

Microclimate in Altamira cave has been monitored in situ monitored for several years (Sanchez-Moral et al., 1999). The cyclic variation of cave air CO$_2$ concentration indicates that Altamira Cave behaves as a CO$_2$ reservoir or source in different seasons (Sanchez-Moral et al., 2010). Relative humidity in Altamira Cave is saturated (>99%) and during the summer season, when exterior air temperature is higher than cave air temperature, as well as water content, the entry of external warm and wet air causes condensation in the cave galleries.

Nevertheless, the oxygen isotopic signature of moonmilk was also influenced by biological processes and microenvironments around the nucleation sites (Coletta et al., 2001). Both physicochemical and biological processes appear to be occurring and even overprinting each other (Lacelle et al., 2004; Blyth and Frisia, 2008; Curry et al., 2009). Measurements of microbial activity, both RNA/DNA ratios and respirometry, indicated an inhibition of this activity dependent on increasing accumulation of calcium carbonate. When calcite accumulation is significant and consolidated deposits of calcite start to be formed, microorganisms (mainly bacteria) find themselves in an unfavorable, nutrient poor environment leading to decreasing activity. Evidence from microbial
activity as well as isotopic analyses confirms a progressive deactivation of microbial
cells during the accumulation of calcite deposits.

A switch from microbially-induced precipitation to abiotic carbonate deposition is
deduced from the above results. Data from petrographical observations, isotopic
analysis, biomolecular determinations, and bacterial respiratory estimates confirm that
both biotic and abiotic factors affect carbonate moonmilk and speleothem formation in
caves. These data suggest that initial carbonate precipitation is mainly governed by the
activity of microorganisms which induce deposition. However, a progressive
accumulation of carbonate leads to an entrapment of bacteria in the mineral. Previous
work (Barton et al., 2001) has reported the entrapment of bacteria in carbonate deposits,
and microphotographs of cavities formed by the entombment of bacteria in calcitic
speleothems have been shown (Barton and Northup, 2007). If microorganisms are
inhibited when enclosed in the calcite deposits, further growth of these speleothems is
mainly a consequence of abiotic processes. The theory of a combination of microbial
and chemical processes leading to the formation of consolidated speleothems is in
agreement with previous observations from both the microbiological and chemical
perspectives. The abiotic precipitation of calcium carbonate has been suggested as
requiring an initial step for a nucleation site to be formed (Pentecost and Bauld, 1988;
Phoenix and Konhauser 2008). Although the term nucleation site is a relatively
ambiguous expression, the fact appears to be that a microbial initiation step is required
and originates moonmilk formations. In these initial accumulations, carbonate fibers
continue to deposit through a microbially-induced process. As the amount of carbonate
increases, microbial metabolism starts to be inhibited. At that time, the conditions that
favor carbonate crystal deposition have been generated and speleothem growth can
continue abiotically in the absence of further participation, or with minimal
contribution, of microbial activity.

The isotopic signature of moonmilk is consistent with microbial activity and implies
that microorganisms participate actively in the formation of carbonate precipitates.
Bacterial metabolism can produce changes in the surrounding environment pH and the
magnitude of this process depends on the metabolized nutrients (Braisant et al., 2002;
Portillo et al., 2009). Increasing pH values can create suitable conditions for carbonate
precipitation assuming a scenario with saturation of calcium and carbonate ions (Butler,
1982; Portillo and Gonzalez, 2010). However, for a discrimination of carbon isotopes to
occur, bacterial metabolism must participate directly in the selection and saturation of
carbonate/CO₂ during the biomineralization process leading to calcium carbonate
formations. Numerous studies have reported on the precipitation of calcium carbonate
by bacterial isolates (Boquet et al., 1973; Rivadeneyra et al., 1994; Cañaveras et al.,
2001). Nevertheless, the molecular and biochemical processes involved in carbonate
precipitation have been scarcely studied (Hammes and Verstraete, 2002; Barabesi et al.,
2007). The molecular mechanisms for carbonate biomineralization proposed in *Bacillus
subtilis* (Barabesi et al., 2007) suggest the implication of genes related to the β-
oxidation pathway. Thus, the incorporation or removal of acetyl groups through β-
oxidation during fatty acid metabolism in bacteria can play a critical role on the isotopic
fractionation occurring during microbially induced carbonate precipitation. In this
respect, Portillo et al. (2009) have recently highlighted the importance of acetate
metabolism in carbonate precipitation by bacteria in Altamira Cave. This indicates that
the bacterial mineralization of the simplest organic compounds (i.e., acetate) during
aerobic metabolism has a direct influence on alkalinization of the bacterial
microenvironment, carbonate/CO$_2$ saturation, and isotopic analysis of carbonates. An important consequence from this study is that bacteria actively participate in carbonate precipitation and this biotic process is not only a result of an indirect influence of bacteria on their environment (i.e., altering the pH). In addition, carbonate deposition is not limited to microbially-induced processes. At increasing carbonate accumulation and through bacterial deactivation, the abiotic processes take over to continue the precipitation of calcite forming consolidated speleothems.

5 Conclusions

The participation of microorganisms in the formation of carbonate deposits in caves has been a matter of debate. In this study, different methodologies were used to reveal the actual role of microorganisms in the formation of calcium carbonate depositions in Altamira Cave. Microbial activity induces carbonate precipitation in the early stages of deposition. As carbonate accumulates, a progressive microbial deactivation occurs. Microorganisms play a minimum role in the growth of consolidated speleothems. This study contributes to the understanding of the effect of biotic and abiotic processes on the deposition of carbonates and speleothem formation in caves, showing an agreement between the major current trends existing in cave geobiology.

6 Acknowledgements

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7 References


Figure legends

**Figure 1.** Map of Altamira Cave with the location of collected samples. A, Moonmilk deposits partially covering the ground of the Big Hall. B, White nodular moonmilk deposits developed on an artificial wall at the Hall of the Walls.

**Figure 2.** Photographs showing different carbonate formations analyzed in this study. A, moonmilk deposits partially covering the ground of the Big Hall. B, white nodular moonmilk deposits developed on an artificial wall at the Hall of the Walls. C and D, consolidated speleothems analyzed during this study showing examples of recent ‘soda-straw’ stalactites.

**Figure 3.** ESEM microphotographs of the microstructural organization of moonmilk deposits. The three major stages of moonmilk formation (according to Cañaveras et al., 2006) are shown (A). The early stages of moonmilk formation (microbial colonization phase) are represented by thin fiber calcite crystals induced by microbial activity (B). A second stage (microstructural breakdown phase) is represented by thicking fibers and the breakdown of a large number of them (C, D). In the most advanced stage (crusting phase), the carbonate needles compact forming consolidated depositions (E, F).

**Figure 4.** Carbon and oxygen isotope composition of moonmilk samples compared to ancient speleothems and host-rock in Altamira cave.
Figure 5. Oxygen profiles during nanorespirometry measurements as a result of *in situ* bacterial activity. The curves correspond to calcitic formations (squares) and clay-rich substrate lacking visible carbonate precipitates (triangles). The slopes of the oxygen consumption vs. depth in the experimental cuvette were proportional to the aerobic respiratory activity in the studied samples. White and black symbols represent different samples collected from the same area. Significant differences (P<0.001) are shown between the slopes for clay-rich substrate lacking carbonate precipitates (triangles) and consolidated calcitic formations (squares).
Figure 1
Figure 2

(A) 

(B) 

(C) 

(D)
Figure 3
Figure 4

δ¹⁸O

-10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0

-1 -2 -3 -4 -5 -6 -7 -8 -9 -10 -11 -12 -13 -14 -15

Moonmilk
Clay-rich substrate
Host-rock
Speleothems

Hall of the Walls
Big Hall
Figure 5
Table 1. Composition of moonmilk deposits and the substrates where they formed in Altamira Cave.

<table>
<thead>
<tr>
<th>Hall of the Walls</th>
<th>type</th>
<th>calcite</th>
<th>dolomite</th>
<th>Terrigenous</th>
<th>clays</th>
</tr>
</thead>
<tbody>
<tr>
<td>Big Hall</td>
<td>Moonmilk</td>
<td>90-95%</td>
<td>0-5%</td>
<td>5-10%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Host rock (flowstone)</td>
<td>95-98%</td>
<td></td>
<td>2-5%</td>
<td></td>
</tr>
<tr>
<td>Hall of the Walls</td>
<td>Moonmilk</td>
<td>98-100%</td>
<td></td>
<td>0-2%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Host rock (concrete)</td>
<td>50-60%</td>
<td></td>
<td>20-30%</td>
<td>15-20%</td>
</tr>
<tr>
<td></td>
<td>Clay-rich substrate</td>
<td>30-50%</td>
<td>5-15%</td>
<td>15-40%</td>
<td>10-15%</td>
</tr>
</tbody>
</table>
Table 2. RNA/DNA ratios estimated for different stages of moonmilk formation and clay-rich substrate. Six samples from each stage were analyzed. Standard deviations are shown in brackets. No significant differences were observed between carbonate deposit-free, clay-rich substrate and early moonmilk stages. Significant differences (*; P<0.05) were observed between incipient stages of moonmilk formation and the advanced phases of carbonate deposition.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moonmilk at an incipient stage</td>
<td>1.325 (0.230)</td>
</tr>
<tr>
<td>Moonmilk at an intermediate stage</td>
<td>0.883 (0.097)*</td>
</tr>
<tr>
<td>Moonmilk at an advanced stage</td>
<td>0.743 (0.226)*</td>
</tr>
<tr>
<td>Clay-rich substrate</td>
<td>1.447 (0.212)</td>
</tr>
</tbody>
</table>