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

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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 karsts 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 CO_2, 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.

120 The cave length is 270 m. The thickness of the cover (host-rock and soil) in most of the
121 galleries varies between 4 and 10 m (see Fig. 1). A poorly differentiated anthroposoil
122 with little development (30-70 cm) cover the cave. It is a silicate-based soil on which
123 develops a plant cover (mainly pasture), which results in a carbon-rich upper level
124 (above 10 cm with 10-15% organic carbon) (Cuezva et al., 2011).
125
126 The hydrological dynamics in this cave are attributable exclusively to the rainwater that
127 seeps directly into the cave through different strata. Speleothems are relatively rare.
128
129 In addition to calcite moonmilk deposits, isotopic data shown in this article are
130 from samples of stalactites, stalagmites, flowstones and recent 'soda-straw' stalactites
131 (Fig. 2). Other types of speleothems have been recognized and described in Altamira
132 such as hydromagnesite moonmilk deposits (Cañaveras et al., 1999) and calcite
133 coralloids and aragonite frostwork speleothems (Sanchez-Moral et al., 1999).
134
135 Moonmilk deposits were sampled from different passages in Altamira Cave. These
136 deposits developed on different types of materials (Fig. 1), specifically on porous
137 stalagmitic flowstones (Big Hall) and concrete walls (Hall of the Walls).
138
139 On the surface of both substrates, in contact with moonmilk deposits, generally, there is
140 a thin layer rich in clays (or a clay-enriched zone) (phyllosilicates: 10-12%; calcite: 30-
141 50%; dolomite: 5-15%; quartz: 15-25%; K-feldspar: 0-15%). The isotopic
142 composition of carbonates in this material has also been analyzed.
143

144 These deposits, which are mainly composed of clays, terrigenous grains and carbonate
145 grains, are interpreted as insoluble residues from bedrock dissolution, related to
146 percolation of soil-derived material and/or as the result of cave condensation-corrosion
147 processes (Sanchez-Moral et al., 1999). The development of cave condensation-
148 corrosion residues have also been attributed to organic biokarst phenomena
149 (Cunningham et al., 1995).
150
151 Different types of carbonate deposits were collected with emphasis on different stages
152 of accumulation from incipient moonmilk formation to consolidated calcium carbonate
153 deposits (Table 1). The different stages of moonmilk deposits were considered as
154 previously described (Cañaveras et al., 2006). Sampling was carried out using sterilized
155 scalpels and tubes. The distribution of the sampled locations in the studied cave are
156 shown in Fig. 1. Microscopic observations, mineralogical analysis, and isotopic analysis
157 were performed immediately after arrival to the laboratory. Samples for the
158 quantification of microbial DNA and RNA and respirometric measurements were
159 processed *in situ* in the cave immediately upon collection.
160
161 **2.2 Microscopical and mineralogical analysis**. Carbonates deposits from Altamira
162 Cave were observed on an Environmental Scanning Electron Microscope (ESEM) with
163 a FEI INSPECT and a secondary electron detector (Oxford Instruments Analytical-
164 INCA, Madrid, Spain). The semi-quantitative mineral composition was determined
165 using X-ray diffraction on a Philips PW-1710 (Madrid, Spain).
166
167 **2.3 Isotopic analysis**. Different types of carbonate deposits throughout Altamira
168 Cave were analyzed for their stable ($\delta^{18}O$ and $\delta^{13}C$) isotopic characterization. The

169 $^{18}O/^{16}O$ and $^{13}C/^{12}C$ 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_3PO_4). The analytical reproducibility for both $\delta^{18}O$ and $\delta^{13}C$ is ±
173 0.10‰.
174
175 **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

194 data were normalized by the dry weight of the sample in grams. Statistical analyses for
195 the significance of the difference between the RNA/DNA ratios were carried out using
196 single classification ANOVA as described by Sokal and Rohlf (1995).
197
198 **2.5 *In situ* respiratory activity.** The aerobic respiratory activity by the bacterial
199 communities in samples both showing and lacking calcite deposits was assessed by
200 nanorespirometry. The procedure has been described in detail by Nielsen et al. (2007).
201 Briefly, each sample (on average 40 mg) was placed in the bottom of a glass cuvette (3
202 mm inner diameter) and filled with 100 µl sterile distilled water. As glass is completely
203 impermeable to oxygen, oxygen was only supplied by molecular diffusion from the
204 atmosphere into the water column above the sample in the glass well. Oxygen
205 consumption by the sample was monitored using an oxygen microelectrode with a
206 sensor tip diameter of 20 µm (Unisense, Århus, Denmark) and determining the
207 concentration gradient through the water column in the cuvette. The sensor was attached
208 to a motorized micromanipulator system controlled by computer software (Unisense).
209 At about two hours, the gradient reached a steady state in our experiments and the depth
210 profile of oxygen concentration followed a linear gradient (Nielsen et al., 2007).
211 Samples were analyzed *in situ* and incubated in the cave. Controls lacking sample
212 showed constant oxygen concentration along the depth profile. The slope of oxygen
213 concentration was used to provide relative estimates of oxygen consumption (Nielsen et
214 al., 2007) because it is a result of oxygen consumption by respiratory activity in the
215 sample and the oxygen flux towards the bottom of the cuvette (Crank, 1997). Slopes
216 were normalized by sample dry weight. Statistical analyses for the significance of the
217 difference between the slopes of oxygen consumption were performed by comparison of
218 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 monocrystalline 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.

244 However, compact host rock samples exhibited values ($\delta^{13}C$= -1.3), above those
245 observed for hard speleothems.
246
247 Quantifications of microbial DNA and RNA have been reported as indicators of
248 abundance and metabolic activity of microbes in the environment, respectively (Molin
249 and Givskov, 1999; Portillo and Gonzalez, 2009b). In Altamira Cave, the initial stages
250 of moonmilk formation showed high ratios of RNA/DNA (Table 2) similar to those
251 found in clay-rich substrates showing no calcification. For increasing calcite deposition,
252 the RNA/DNA ratios significantly decreased ($P<0.05$; Table 1) indicating a decrease of
253 metabolic activity per cell.
254
255 Aerobic respiratory activity in samples showing no visible precipitation of calcium
256 carbonate and consolidated carbonate deposits was assessed by nanorespirometry in
257 order to confirm that accumulation of calcite leads to the inhibition of bacterial activity.
258 The results showed that bacterial respiratory activity in calcite deposits was much lower
259 ($P<0.001$) than in samples lacking carbonate precipitates (Fig. 5). On average, the slope
260 of oxygen concentration during the nanorespirometric experiments in calcitic deposits
261 (average slope ± standard deviation: -0.0101 ± 0.0003 $\mu M\ O_2\ g^{-1}$ sample) only
262 represented 9.0% of the slope determined in samples lacking carbonate deposits
263 (average slope ± standard deviation: -0.1118 ± 0.0030 $\mu M\ O_2\ g^{-1}$ sample).
264
265 **4 Discussion**
266
267 Morphological evidence (Cañaveras et al., 2006) suggested that moonmilk formation
268 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 $\delta^{18}O$ values from moonmilk does not suggest isotopic equilibrium precipitation in the calcite-water system (O'Neil et al., 1969). Posible carbon sources responsible for the carbon signature within moonmilk deposits are bedrock dissolution, atmospheric CO_2 and biogenic soil CO_2. Nevertheless, low $\delta^{13}C$ 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 $\delta^{13}C$ 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 $\delta^{18}O$ and $\delta^{13}C$ 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.

294 The stable isotope composition of moonmilk deposits ($\delta^{18}O$ -4.9 to -9.1‰ and $\delta^{13}C$ -9.5
295 to -14.5‰) indicates significant incorporation of organically-derived CO_2 and probably
296 a biological influence on calcite crystals. Concerning the $\delta^{13}C$ values, the possible
297 reservoirs of carbon in speleothems are the inorganic carbon from dissolved Cretaceous
298 marine limestones and dolostones (host rock); the CO_2 produced in the soil by
299 respiration of organic material, with $\delta^{13}C$ values ranging from −15 to −25‰ (average -
300 21‰); and the carbon from atmospheric CO_2, normally with $\delta^{13}C$ values close to −7‰
301 (Sanchez-Moral et al., 2010). Clay samples in Altamira Cave, which show the presence
302 of an active microbial community (Gonzalez et al., 2006; Portillo et al., 2008; Portillo
303 and Gonzalez, 2009a) and accumulate the highest nutrient concentrations in the cave
304 (Portillo et al. 2009), contained fractions of $\delta^{18}O$ and $\delta^{13}C$ similar to moonmilk.
305
306 The $^{13}C/^{12}C$ analyses for air, water and rock/speleothem in Altamira Cave confirm the
307 importance of the external soil as the CO_2 source for the underground system and the
308 effect of ventilation on system equilibrium, in agreement with Sanchez-Moral et al.
309 (2010). The $\delta^{13}C$ of the water (averaging -12.6‰) varies according to the degree of
310 interaction with the host rock ($\delta^{13}C$ calcite: 2‰, $\delta^{13}C$ dolomite: 3‰). In this study, the
311 speleothems had values ($\delta^{13}C$ -4 to -12.5‰) that are congruent with their precipitation
312 in isotopic equilibrium with the interior of the cave (discrimination coefficient of 0.4‰
313 at 14°C, Labonne et al. 2002).
314
315 As the moonmilk carbon isotopic signature is significantly lower than estimated
316 by calculating equilibrium we infer the possibility that microorganisms play a role in the
317 CO_2 depletion and, directly or indirectly, in the isotopic composition of moonmilks.
318 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* monitorized 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 (Braissant 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/CO_2 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

394 microenvironment, carbonate/CO_2 saturation, and isotopic analysis of carbonates. An
395 important consequence from this study is that bacteria actively participate in carbonate
396 precipitation and this biotic process is not only a result of an indirect influence of
397 bacteria on their environment (i.e., altering the pH). In addition, carbonate deposition is
398 not limited to microbially-induced processes. At increasing carbonate accumulation and
399 through bacterial deactivation, the abiotic processes take over to continue the
400 precipitation of calcite forming consolidated speleothems.
401
402 **5 Conclusions**
403
404 The participation of microorganisms in the formation of carbonate deposits in caves has
405 been a matter of debate. In this study, different methodologies were used to reveal the
406 actual role of microorganisms in the formation of calcium carbonate depositions in
407 Altamira Cave . Microbial activity induces carbonate precipitation in the early stages of
408 deposition. As carbonate accumulates, a progressive microbial deactivation occurs.
409 Microorganisms play a minimum role in the growth of consolidated speleothems. This
410 study contributes to the understanding of the effect of biotic and abiotic processes on
411 the deposition of carbonates and speleothem formation in caves, showing an agreement
412 between the major current trends existing in cave geobiology.
413
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415
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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, moonmilkdeposits 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).

589 Figure 1
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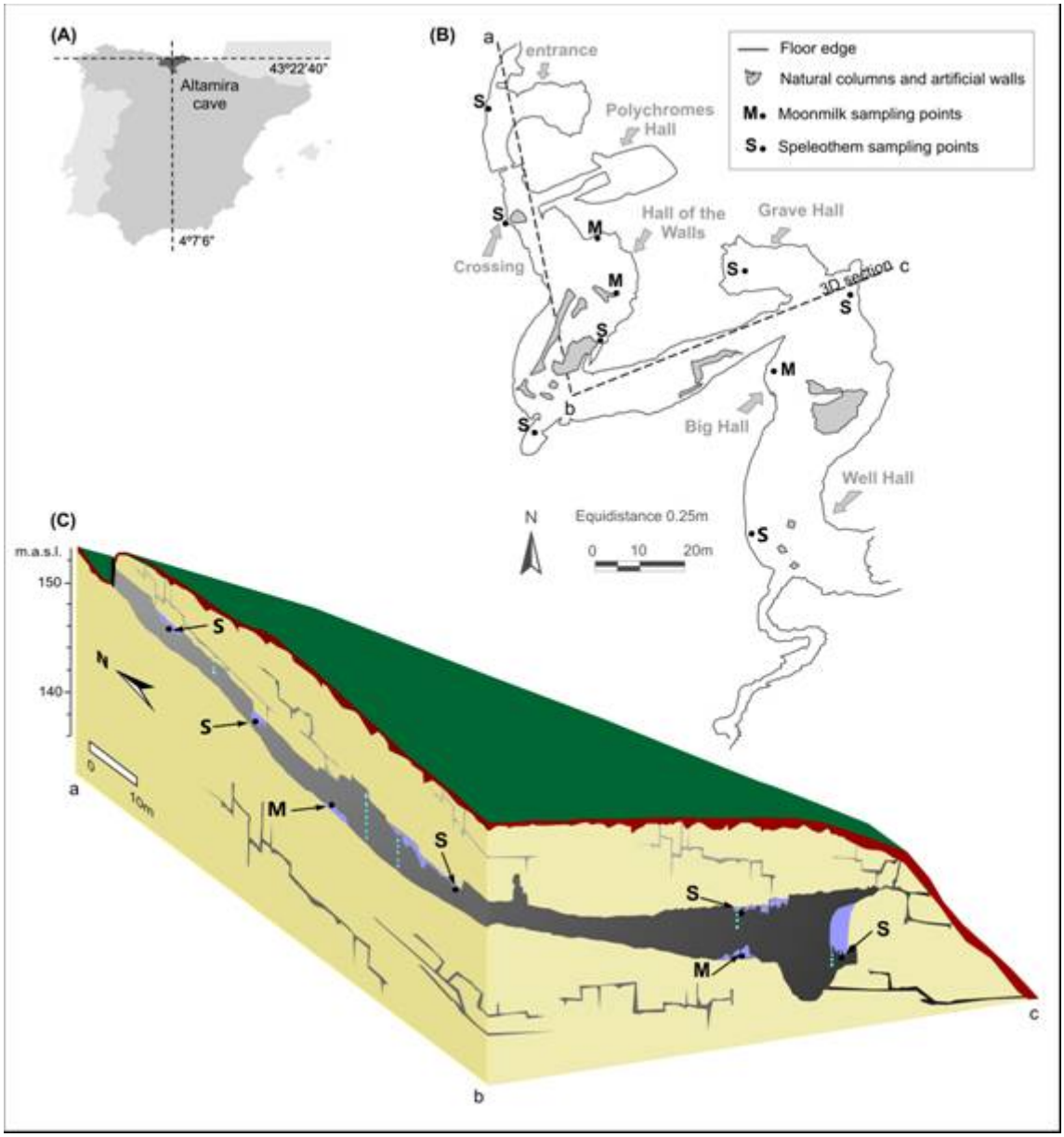

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595 Figure 2
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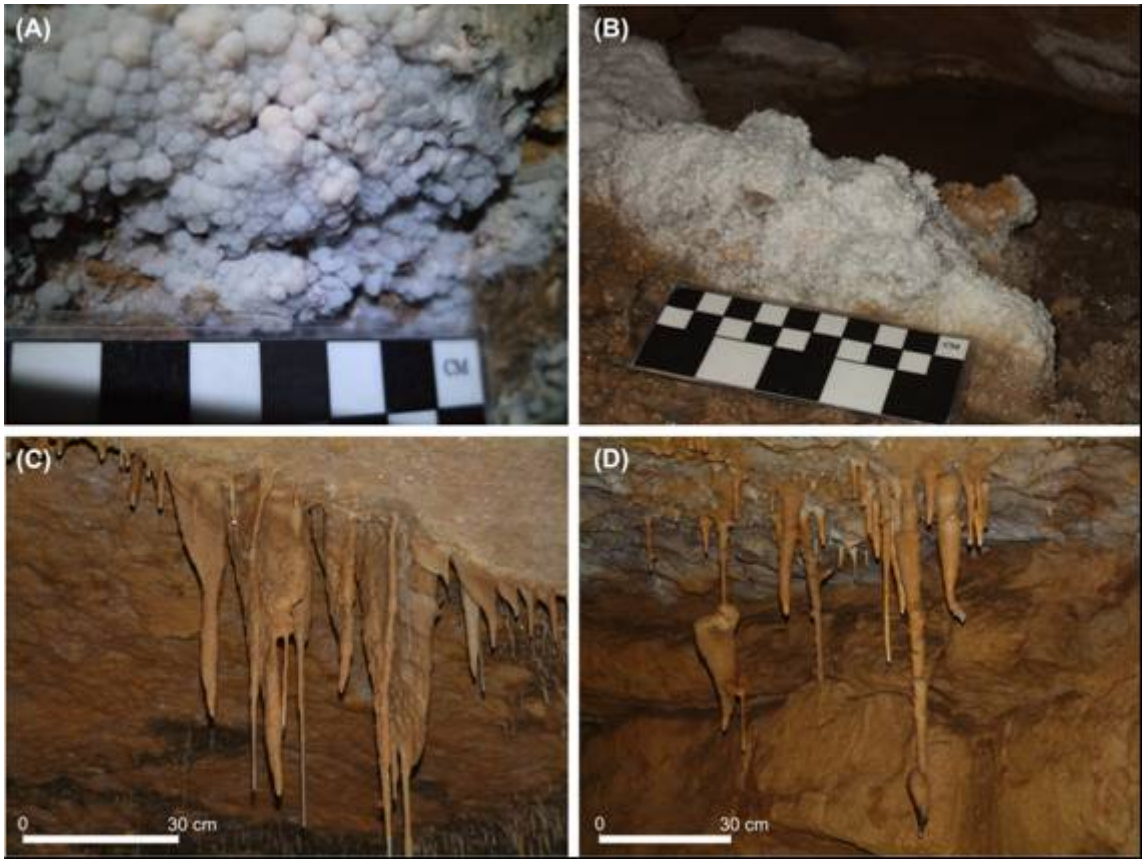

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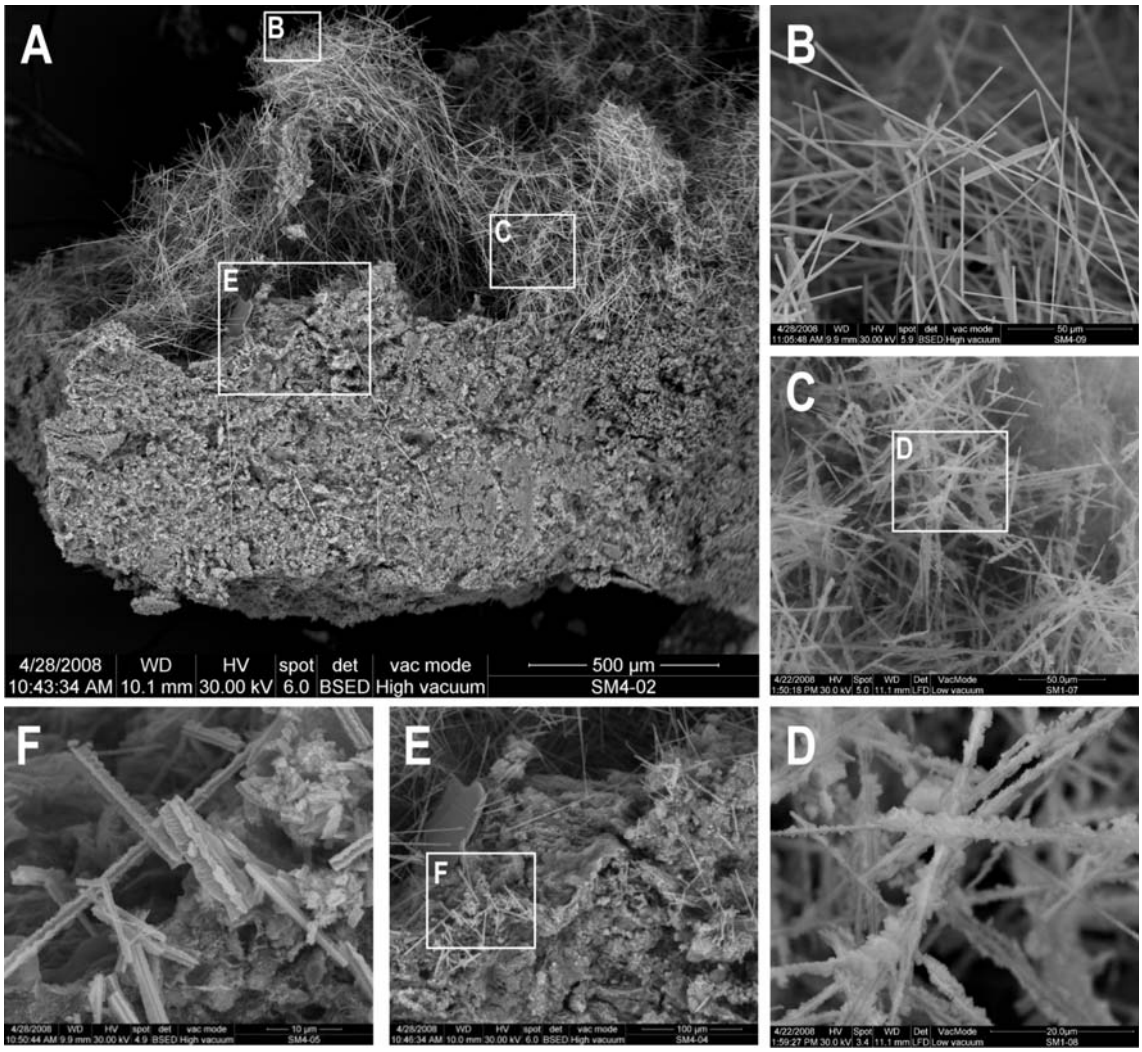
Figure 3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608 Figure 4
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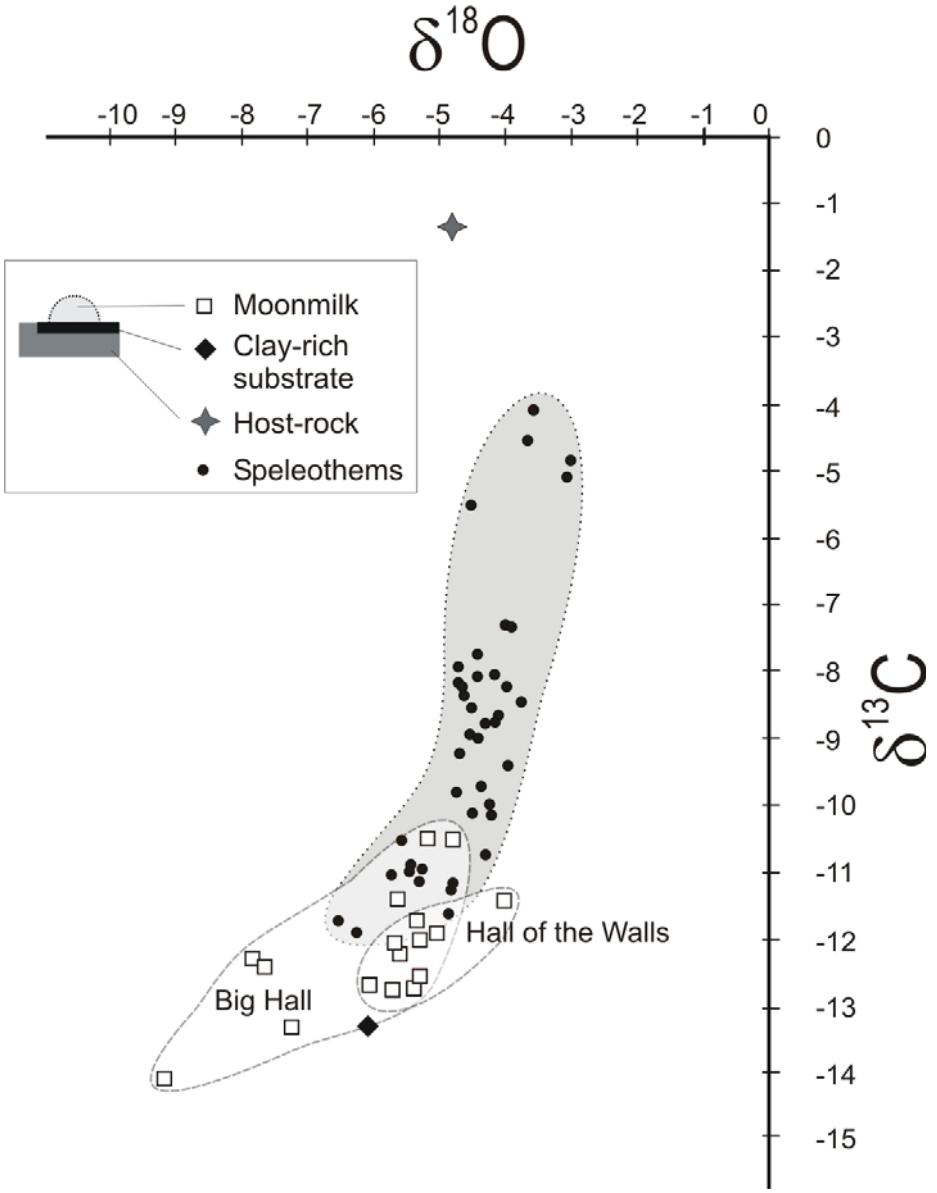

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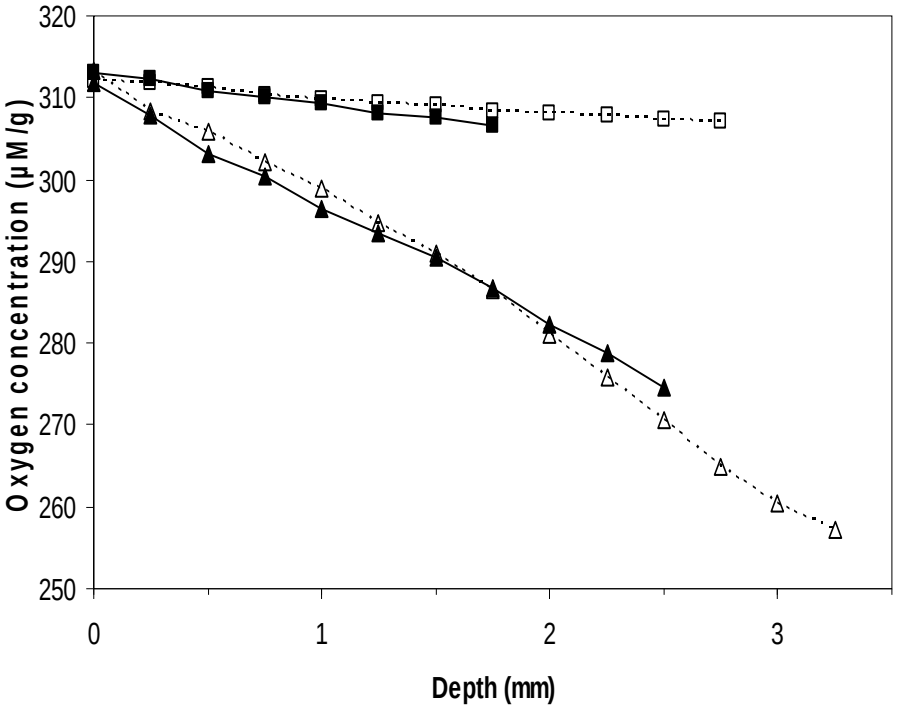
Figure 5


320
310
300
290
280
270
260
250
Oxygen concentration (µM/g)
0
1
2
3
Depth (mm)

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642 **Table 1.** Composition of moonmilk deposits and the substrates where they formed

643 in Altamira Cave.

644

Hall	type	calcite	dolomite	Terrigenous	clays
Big Hall	Moonmilk	90-95%	0-5%	5-10%	
	Host rock (flowstone)	95-98%		2-5%	
Hall of the Walls	Moonmilk	98-100%		0-2%	
	Host rock (concrete)	50-60%		20-30%	15-20%
	Clay-rich substrate	30-50%	5-15%	15-40%	10-15%

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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.

Sample type	Average RNA/DNA
Moonmilk at an incipient stage	1.325 (0.230)
Moonmilk at an intermediate stage	0.883 (0.097)*
Moonmilk at an advanced stage	0.743 (0.226)*
Clay-rich substrate	1.447 (0.212)