Dominance of Arcobacter in the white filaments from the thermal sulfidic spring of Fetida Cave (Apulia, southern Italy)

Dominance of *Arcobacter* in the white filaments from the thermal sulfidic spring of Fetida Cave (Apulia, southern Italy)

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Abstract

The thermal spring of Fetida Cave, a still active sulfuric acid cave opening at ~~the~~ sea level and located in Santa Cesarea Terme, southeastern Salento (Apulia region, Southern Italy) hosts abundant floating white filaments. The white filaments were mainly composed of sulfur crystals surrounded by microbial mass of the phyla *Epsilonbacteraeota*, *Proteobacteria*, *Bacteroidetes*, and *Patescibacteria*. The most abundant genus in the white filaments collected

from the waters in the innermost part of the cave dominated by sulfidic exhalations was *Arcobacter*. This abundance can be related to the higher concentration of sulfide dissolved in water, and low oxygen and pH values. Conversely, lower *Arcobacter* abundances were obtained in the filaments collected in the entrance and middle part of the cave, where sulfidic water mixes with seawater, as the cave is subjected to tides and the mixing of fresh (continental) with marine water. The geochemical analysis of water ~~geochemistry~~ and atmospheric gases confirmed these environmental constraints. In fact, higher concentrations of H_2S in the air and water were recorded closest to the spring upwelling in the innermost part of the cave, and the lower ones near the cave entrance. The metabolic versatility of *Arcobacter* might provide a competitive advantage in the colonization of water bodies characterized by high sulfide, low oxygen, and dynamic fluid movement.

Keywords: Microbial diversity, sulfuric acid speleogenesis, microbial filaments, sulfur, cave atmosphere, water geochemistry

1. Introduction

Hypogene sulfuric acid speleogenesis (SAS) caves are underground karst systems formed in carbonate areas where acidic fluids, derived from the interactions with deep-seated sulfates and/or sulfides, rise through deeply rooted geological structures (Audra et al., 2009). In particular, SAS caves form when host rock dissolution is mainly related to the interaction with the sulfuric acid produced by the oxidation of hydrogen sulfide. Around 25% of the known worldwide SAS systems are located in Italy, especially along the Italian Apennine Chain, and some of them are still in active conditions (D'Angeli et al., 2019a).

Within SAS caves, hydrogen sulfide provides a rich energy source for chemolithotrophic microorganisms, which support chemosynthetic primary production for the growth of heterotrophic organisms (Jones et al., 2008; Bizic et al., 2020). In association with this, SAS caves typically host conspicuous microbial biofilms and mats that are visible on the walls, ceilings, and in the

water. Those covering the cave walls and ceilings have variable morphologies and colors in the form of viscous snottites and vermiculations (Jones et al., 2010; D'Angeli et al., 2019b). In the water, that has a milky appearance (due to elemental sulfur), the biofilms are in the form of white filaments that are typically visible as either rock-attached streamers or sediment surface biofilms. These two morphologies were first referred to as feathery biofilms and cotton biofilms, respectively, by Macalady et al. (2007) in Frasassi Cave. Analogous white filaments were observed and described in a series of other SAS caves in Acquasanta Terme, Capo Palinuro, Monte Sellaro, Cassano allo Ionio, and Santa Cesarea Terme (D'Angeli et al., 2019a,b) and also in Romania (Bizic et al., 2020).

The microbiology and composition of white filaments was previously studied, but limited to a few caves and springs. The caves with major research efforts were Frasassi (Macalady et al., 2006, 2008; Engel, 2007), Movile (Hutchens et al., 2004; Chen et al., 2009; Kumaresan et al., 2014; Bizic et al., 2020), and Lower Kane (Engel et al., 2003, 2004, 2010). A few individual reports on other caves and springs can be found in the literature (Mattison et al., 1998; Engel et al., 2001; Elshahed et al., 2003; Barton and Luiszer, 2005; Reigstad et al., 2011; Rossmassler et al., 2012). Among these, the microbiology of the water streamers has been the most extensively studied through molecular methods (16S rRNA clone library) but also through microscopy and culture-based experiments (Hose and Pisarowicz, 1999; Engel et al., 2004; Hamilton et al., 2015). Sulfur-oxidizing microorganisms belonging to *Gamma*-, *Beta*- and *Epsilonproteobacteria*, reclassified as *Epsilonbacteraeota* (Waite et al., 2017) dominate the water streamer microbial communities. Additionally, members of *Deltaproteobacteria*, associated with sulfur-reduction processes were identified in lower abundance (Macalady et al., 2006). Among the different environmental factors possibly affecting these biofilms, the water flow (shear stress) and the ratio sulfide/oxygen were reported to be the major ones influencing white filament morphologies (i.e. long rock-attached streamers or shorter sediment biofilm) and microbial diversity (Macalady et al., 2008).

The present work is focused on the analysis of white filaments from Fetida Cave, a still active sulfuric acid cave opening at ~~the~~ sea level and located in Santa Cesarea Terme, southeastern Salento (Apulia region, Southern Italy)

(D'Angeli et al., 2019b; 2021) (Fig. 1). The cave hosts abundant microbial biofilms on cave walls and ceiling as vermiculations and gypsum moonmilk. A previous study described the mineralogy, geochemistry, and microbial diversity associated with the different biofilms found in Fetida Cave (D'Angeli et al., 2019b).

The microbial communities featuring Fetida Cave water are characterized by the presence of abundant floating white filaments and are related with the constant mixing between the thermal sulfidic fluids, rising from below, and seawater entering from outside that leads to variable contents of dissolved H_2S along the cave (D'Angeli et al., 2021). The purpose of the present work is to extend the knowledge on the microbial communities composing white filaments in this unique SAS cave environment. In this regard, gaseous composition of the cave atmosphere and water geochemistry as well as morphological (through FESEM) and microbiological (using Illumina sequencing of 16S rRNA gene) analyses were performed to get deep into the diversity of the white floating filaments in Fetida Cave (Fig. 1) and the possible predicted metabolic functions associated with their development.

2. Methods

2.1. Field sampling

Microbiological and air sampling was performed in different sites inside the cave (Fig. 1). In particular, four microbiological samples were collected in sites FC1, FC4, FC6, and FC7, moving from the innermost part of the cave towards the coastline. D'Angeli et al. (2019b) described three different typologies of water filaments based on their location, *i.e.*, floating on the water surface (named F-float), sedimented on the bottom of the water (F-sed), and attached to the cave rocks (F-stream). The four samples analyzed in this study are different from those described by D'Angeli et al. (2019) but belong to the F-float category described in this previous paper. Microbial sampling for this paper was done during a field trip in June 2017 (summer conditions with calm sea), while the samples analyzed in the study by D'Angeli et al. (2019) were collected in October 2015 and December 2017 (during winter, when marine conditions were

rougher). The samples were collected using sterile scalpels, stored in sterile tubes at 4°C until arrival at the laboratory. Five replicas from each sampling point were taken for molecular biology as well as for field emission scanning electron microscopy (FESEM). The samples for molecular biology were preserved in Lifeguard preservation solution (Qiagen, Hilden, Germany) and then held at -80ºC until analysis, whereas samples for microscopy were fixed in-situ with 2.5% glutaraldehyde in 0.1M cacodylate-buffer (pH 7.4).

Gas samples were taken into the cave in sites P1, P2, P3, P4 (Fig. 1, Table A1 in Appendix A - Supplementary data), and outside to take external air and soil in several locations, both in June 2017 and in May 2018. They were collected using a handheld pump and 1 L Tedlar bags (Fig. 1). For each sample two replicas were taken, filling only 2/3 of the entire bag to avoid bag damage and/or explosion during transport. Immediately after the collection, 12 Tedlar bags (for the two fieldworks in June 2017 and May 2018) were stored in a rigid luggage and analyzed within 48 hours at the stable isotopes laboratories of Museo Nacional de Ciencias Naturales in Madrid and University of Almeria. CO_2 and CH_4 molar fractions and $\delta^{13}C$ in both gases were measured with a CRDS spectrometer (G2201-i analyser, Picarro Inc., USA) with a precision of 200 ppb (±0.05 of reading) and 10 ppb (±0.05 of reading) for $^{12}CO_2$ and $^{13}CO_2$, respectively, resulting in a precision better than 0.16 ‰ for $\delta^{13}C$-CO_2 after 5 min of analysis. The measurements of methane isotopologues ($^{12}CH_4$ and $^{13}CH_4$) reached a precision of 5 ppb (±0.05 of reading) and 1 ppb (±0.05 of reading) for $^{12}CH_4$ and $^{13}CH_4$, respectively. The precision for $\delta^{13}C$-CH_4 was better than 1.15 ‰ after 5 min of analysis. $\delta^{13}C$-isotope values were referenced to the Vienna PeeDee Belemnite (V-PDB). Three in-house standards with certified CO_2 and CH_4 concentrations and known $\delta^{13}C$ values for each gas were processed to verify the proper functioning of the CRDS analyzer. Fernández-Cortès et al. (2018) reported further details on the methodological procedures and quality results. Spot measurements of CO_2 concentration and levels of other key gases (H_2S and O_2) were also taken in the same locations for air sampling using handheld devices (XP200, Lufft) and a multigas monitor (MX6 iBrid, Industrial Scientific), respectively.

In addition, during June 2017 and May 2018 field trips, water samples (SCC1, SCB1, SCAfen, SCA1) were collected in the same location of

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161 microbiological samplings (Fig. 1, Table 1A in Appendix A - Supplementary
162 data). The results of a comprehensive water monitoring campaign that began in
163 October 2015 and ended in November 2018 have been published in the
164 framework of a hydrogeological-geochemical study (D'Angeli et al., 2021).
165 Nevertheless, in this work, we will consider only the results obtained during the
166 microbiological and gas sampling (i.e. June 2017 and May 2018). Water
167 parameters such as temperature (T °C), pH, electrical conductivity (EC), and
168 total dissolved solids (TDS) were measured using a multiparametric probe
169 Hanna HI991001 (relative accuracies at 25 °C: ±0.5 °C, ±0.02 pH, ±2%
170 EC/TDS). Two replicas of water were collected in each sampling site in 250-ml
171 HDPE bottles, and one was acidified with 65% HNO_3, and stored at 4°C until
172 analysis at the laboratory of Politecnico di Torino.
173 The δ^2H and $\delta^{18}O$ in a third replica of water collected during May 2018 were
174 measured simultaneously at University of Almeria by cavity ringdown
175 spectroscopy (CRDS) by a L2140-i Picarro water isotope analyzer interfaced
176 with an A0211 high-precision vaporizer (Picarro Inc., USA), coupled with a
177 Picarro micro-combustion module (MCM®) to remove combustible organic
178 compounds from water samples. Each sample was injected 10 times into the
179 vaporizer, which was heated to 110 °C. Memory effects from previous samples
180 were avoided by rejecting the first three analyses, so values for the final 7
181 injections were averaged with a typical in-sample precision (±1σ) of ±0.04‰ for
182 $\delta^{18}O$ and ±0.18‰ for δ^2H. The results were normalized against V-SMOW by
183 analyzing internal standards before and after each set of twenty samples and
184 are given as per mil (‰). ^{13}C measurements of the dissolved inorganic carbon
185 in water samples ($\delta^{13}C$-DIC) were also obtained by using the same G2201-i
186 analyzer coupled with an Automate FX sample preparation device. NBS-18
187 (Carbonatite) and IAEA-603 (calcite) were used to calibrate the AutoMate-
188 CRDS system and referring the results to the Vienna Pee Dee Belemnite (V-
189 PDB). Three replicates per sample were analyzed together with two internal
190 standards run before and after the water sample set, reaching an average
191 precision (±1σ) of ±0.10 ‰.
192
193 *2.2. Field emission scanning electron microscopy with Energy Dispersive X-ray*
194 *Spectroscopy (FESEM-EDS)*

The morphology of the white filaments was studied by FESEM using a FEI Teneo FESEM (FEI Company, Eindhoven, The Netherlands) with secondary electron detection mode and an acceleration voltage of 5 kV for high resolution images and 10 kV for elemental microanalysis. Before FESEM observations, the fixed samples were washed in cacodylate-buffer, post-fixed in 1% osmium tetroxide and dehydrated by serial dilutions in ethanol and acetone. Subsequently, samples were dried in a Leica EM CPD300 critical point dryer at 34.5 °C and then sputter coated with a gold thin film, as described by De la Torre Noetzel et al. (2018).

2.3. DNA extraction, sequencing and functional prediction

Genomic DNA extraction was carried out using the FastDNA SPIN Kit for Soil (MP Biomedicals, Illkirch, France). DNA quantification was measured by means of a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA) and the DNA concentration values of FC1, FC4, FC6, and FC7 samples were 22.1, 217.6, 22.0 and 31.5 ng/μL, respectively.

High-throughput sequencing of extracted DNA was performed by Macrogen (Seoul, Korea). We targeted the V3-V4 hypervariable regions of the 16S rRNA gene using the primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3'). PCR amplification reaction per sample consisted of 5 μL of each primer (1μM), 12.5 μL of 2x KAPA HiFi HotStart ReadyMix (Roche) and 2.5 μL of DNA template (5 ng/μL), for a total of 25 μL/sample. The PCR program was run as follows: primary denaturation at 95°C for 3 min, followed of 30 cycles beginning with a denaturalization step at 95°C for 30 s, a second step of annealing at 56°C for 30 s and a third step of elongation at 72°C for 1 min. The PCR program concluded with an elongation step at 72°C for 5 min. Illumina MiSeq platform was used for 2 × 250 paired-end sequencing, following the Illumina protocol for library Nextera XT Index Kit preparation.

Raw data were checked for quality using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Amplicon Sequence Variant (ASV)-based analyses were conducted with the QIIME2 platform (Bolyen et al., 2019). First, DADA2 (Callahan et al., 2016) filtered the

raw data according to the quality, generating an amplicon sequence variant (ASV) table. Afterwards, taxonomic assignment was implemented using the feature-classifier classify-sklearn (Bokulich et al., 2018) and the SILVA database 132 version (Quast et al., 2013). Finally, alpha diversity analysis was carried out for evenness and richness measurement of microbial communities using the following metrics: Chao1, Shannon and Simpson indices, as well as Pielou's evenness measure (Chao, 1984; Shannon and Weaver, 1949; Simpson, 1949; Pielou, 1966).

Taxonomic distribution and relative abundance of the microbial community were depicted in heat-maps using R package *gplots* (Warnes et al., 2015). Samples were arranged through dendrograms based on the taxonomic abundance and representativeness. Prediction of functionality in the microbial community based on 16S rRNA gene data was carried out using FAPROTAX (Louca et al., 2016).

3. Results and discussion

3.1. Gaseous composition of the cave atmosphere and water geochemistry

The values of measured CO_2 and CH_4 concentrations and their stable isotopic compositions ($\delta^{13}C$-CO_2 and $\delta^{13}C$-CH_4) in Fetida Cave environment, above-cave soil, and local exterior atmosphere are shown in Table 1. The mean concentrations of CO_2 and CH_4 in the local external atmosphere were 437.4 ± 17.3 ppmv and 2.00 ± 0.02 ppmv, respectively. CO_2 concentration of cave air varied within a narrow range between 460 and 650 ppmv, i.e. it usually ranges between 100 and 200 ppmv higher than the local atmospheric background outside. CH_4 concentration of cave air (2.13 ± 0.09 ppmv, on average) is slightly higher than those recorded for the local external atmosphere, but with maximum values around 2 - 2.2 ppmv in the inner locations closer to the acid springs. H_2S concentrations in the air of Fetida Cave ranged from 0.8 to 2.4 ppmv, with the highest concentrations closest to the acid springs (P2 and P1) and the lower concentrations at the entrance and middle cave locations (P3 and P4), due to an efficient exchange and mixing with H_2S-free outdoor atmosphere.

At these cave-air levels of H_2S, the spectral lines of the CRDS spectrometer avoid the strongly absorbing H_2S spectral lines and, therefore, the measurable

effect on the reported CO_2 concentrations can be considered negligible. The changes in the $^{12}CO_2$ and $^{13}CO_2$ with an addition of H_2S ranging from 0.8 to 2.4 ppm would be 0.5–0.75 % and less than 7%, respectively, according to Malowany et al. (2015). In the case of a well-ventilated atmosphere as Fetida Cave, with CO_2 contents slightly higher than local exterior atmosphere and $^{13}CO_2$ roughly being 1% of the $^{12}CO_2$ molar fraction, these percentages entail a total variation for CO_2 concentration of only 4.1 ppm, on average (±3.7 ppm for $^{12}CO_2$ and ±0.4 ppm for $^{13}CO_2$). The small interferences of H_2S on CO_2 measurements by CRDS were confirmed through the field CO_2 measurements obtained with the handheld XP200 logger equipped with a NDIR probe.

However, the H_2S spectral lines, that partially overlap with some spectral features of the CO_2 used in the CRDS system, turns into some relative changes on $^{12}CO_2$ with respect to $^{13}CO_2$ that provokes a remarkable interference on the CO_2 isotopic measurements (Malowany et al., 2015). Thus, H_2S in air samples from Fetida Cave is enough to influence measurements by an unreal increase in the $^{12}CO_2$ concentration and a more significant decrease in the $^{13}CO_2$ concentration, resulting in quite depleted $\delta^{13}C\text{-}CO_2$ values. Consequently, the carbon isotopic values for CO_2 of cave air were not reported in Table 1.

Fortunately, the H_2S levels of Fetida Cave have a negligible interference on the CRDS-reported $\delta^{13}C\text{-}CH_4$ for cave air samples, similar to what reported in Malowany et al. (2015) described for standard gases in laboratory measurements, allowing for comparison of results of both concentration and carbon isotopic signal for this gas. Despite the fact that CH_4 concentrations of cave air are slightly higher than those recorded for the local atmosphere, the $\delta^{13}C\text{-}CH_4$ varies within a narrow range between -45.1 ‰ and -47.5 ‰ and is not linearly related to the inverse CH_4 concentration. These $\delta^{13}C\text{-}CH_4$ values evidence that CH_4 in cave air is not locally generated by biogenic processes since if this would be the case it should be significantly enriched in ^{12}C relative to the external atmosphere. Methane present in cave air, however, is mainly sourced by the atmospheric CH_4 from ventilation through the sole entrance, i.e. bypassing the soil zone. This suggests that the subterranean atmosphere is greatly diluted by inputs from the outside atmosphere, which is also corroborated by low CO_2 concentrations and O_2 levels close to 21%.

Soil-CO_2 concentrations show a remarkable difference between the two field surveys (Table 1) ~~that~~ depending on the prevailing soil microbial respiration rate each time, which, in turn, is mainly controlled by soil temperature and moisture. However, in all samples nearly constant $\delta^{13}C$-CO_2 values (-19.73 ± 0.98 ‰, on average) prevail being relatively heavier than the expected composition range for CO_2 derived from C3 organic matter. These $\delta^{13}C$-CO_2 values of soil air likely the result~~s~~ of the upward diffusion of CO_2 to the open atmosphere and the effects of a kinetic fractionation on the residual CO_2 from the soil zone. Contrary to what has been observed in the cave-air CH_4, an intense microbial oxidation of CH_4 in soil provokes the residual methane to become isotopically depleted in ^{12}C, a fact that was particularly evident in the first air sampling campaign.

Overall, the gaseous composition of the external soil denotes its evident disconnection from the cave atmosphere. Consequently, the isotopic composition of CO_2 and CH_4 in cave air confirms a prevailing gas exchange pathway with the atmospheric background source, ruling out both the biogenic CH_4 and soil-derived CO_2 sources or a remarkable deep-sourced input for both gases.

In general, the collected waters show mean temperature higher than 20°C, and pH ranges between 6.89 (in the innermost portion -SCC1 and SCB1- of the cave which is influenced by rising sulfuric acid fluids) and 7.38 (in the sampling sites close to the entrance of the cave). The higher concentration of [HS^-] dissolved in water was observed in the innermost sampling sites SCB1 and SCC1 (Table 2). In addition, SCB1 showed the most mineralized waters with higher values of temperature and lower value of pH. Detailed information on the water geochemistry can be found in D'Angeli et al. (2021).

The stable isotope analyses of water from Fetida Cave revealed δ^2H values that ranged from −4 to +7 ‰, approximately, with an average of +2.85 ‰, and $\delta^{18}O$ values from −0.5 to +1.1‰, with an average of +0.39 ‰ (Table 3). The isotopic composition of water samples from the thermal spring of Fetida Cave, reported by Santaloia et al. (2016), was 0.60 and 0.05 ‰ for δ^2H and $\delta^{18}O$, respectively. This single previous data practically coincides with the average isotopic composition of the water sampled in the end passage of the cave

(SCC1-FC1 and SCB1-FC4 sampling locations), where the main inputs of deep acid water are observed.

The isotopic data pairs of thermal water from Fetida Cave define a Local Water Line (LWL) (Fig. 2), which is also aligned with the only isotopic data for local meteoric water, as the ones reported by Santaloia et al. (2016) for a cold borehole located in the cave's water recharge area. The isotopic data for local meteoric water roughly matches the average isotopic composition of precipitation in the area, according to data for St. Maria di Leuca reported by Longinelli and Selmo (2003). Here, the LWL is fitted with a slope of 5.83 and deuterium excess of 0.29. This linear function is almost equal to the LWL fitted by Santaloia et al. (2016) (slope: 5.68 and deuterium excess: -0.69), but which tends to positive $\delta^{18}O$ with respect to the GMWL (Clark and Fritz, 1997) and the "regional" MWL for Southern Italy (Longinelli and Selmo, 2003).

Both the thermal water of Fetida Cave and the previous spring water reported by Santaloia et al. (2016) have $\delta^{2}H$ and $\delta^{18}O$ values along a line between the meteoric samples (cold well) and ~~the~~ seawater, suggesting a mixing of fresh, thermal and marine waters. The low deuterium excess of samples depends on the contribution of seawater, similar to what has been described for other sulfide-bearing waters due to the variable contribution of saline formation waters (Toscani et al., 2001). The positive $\delta^{18}O$-shift with respect to the GMWL also indicates the prevailing influence of a mixing process with seawater. Similar precipitation-seawater mixing lines have been isotopically defined to assess some other coastal processes as groundwater discharge in a hypersaline lagoon (Rocha et al., 2015) or the precise identification of river plumes within the Great Barrier Reef (Munksgaard et al., 2012).

There is a spatial gradient in the mixing process with seawater, in this way the $\delta^{18}O$-$\delta^{2}H$ data pairs of cave water are closer to those of meteoric water as we move away from the coastline (Fig. 2 and Table 3). As a reference, Santaloia et al. (2016) reported a mean isotopic composition of water from nearby non-thermal water boreholes as -32.34±1.86, -5.57±0.15 and -12.43±1.27 for $\delta^{2}H$, $\delta^{18}O$ and $\delta^{13}C$-DIC, respectively (Table 3). The relative position of the data pairs on the precipitation-seawater mixing lines will vary over time and this will be determined by the tides controlling the sea level in each specific sampling period, as well as the degree of meteoric water recharge

at that time (previous rainfall). The δ^{13}C-DIC values also show a gradient with distance from the shoreline, with more negative values in the innermost areas of the cave (< -2.5 ‰ in SCC1-FC1 and SCB1-FC4 locations - Table 3) indicating an input of seepage water that is, in any case, distinguishable from seawater. The more negative δ^{13}C-DIC values coincide with areas with more abundance of bacteria suggesting processes of biomediated CO_2-fixation.

An enrichment in hydrogen isotopes based on the ^{2}H exchange between H-bearing species (H_2O–H_2S) has been previously reported in shallow groundwater mixed with H_2S enriched gases under volcanic and hydrothermal settings (Chiodini et al., 2000; Hsu and Yeh, 2020). In contrast, our current data set for Fetida Cave waters shows no evidence of an isotopic exchange of water with the hydrogen in H_2S linked to rising sulfuric acid fluids. This effect would lead to an increase in the δ^2H content of the water and, graphically, the isotopic data pairs would lie along straight lines parallel to the ordinate axis (δ^2H-axis), a behavior that is not observed in Figure 2.

3.2. Morphology of Fetida Cave white filaments

Filamentous morphologies were observed in the four samples of Fetida Cave, as depicted in Figure 3. A dense net of partially corroded filaments embedded in extracellular polymeric substances is shown in Figure 3A. Cells resembling *Arcobacter* morphology were evidenced in this figure. The structures and morphologies are similar to those previously reported by D'Angeli et al. (2019b) for filaments collected in this cave. These authors reported that the damaged structures can be associated with the constant exposure to rising acidic sulfidic water. Mineral grains were attached and/or entrapped by the filaments (Figs. 3B and D). D'Angeli et al. (2019b) concluded that the grains were sulfur crystals based on EDS spectra. The detailed morphology of the dense tangled masses of septate filaments observed in Figure 3C closely resemble those reported by Bauermeister et al. (2012) and Flot et al. (2014) for *Thiothrix* filaments.

3.3. Microbial community composition

Sequence quality control and construction of feature table using QIIME2 and DADA2 resulted in a total 180,528 features or sequences for the four samples, clustering in a total of 5,209 amplicon sequence variants (ASVs). The mean length of sequences was 453.6 bp, with a range oscillating between 274–468 bp length. The most representative sample was FC6, with 62,628 features, whereas FC4 presented only 30,826. Besides, the number of ASVs found in the four samples varied in the 704–1,902 interval, where FC6 was observed to have the higher value and FC4 the sample with the lower number. Samples FC6 and FC1 presented 1,662 and 941 ASVs, respectively.

Summarizing the structure of the bacterial communities, assessing the number of taxonomic groups (richness), and the distribution of abundances of these groups (evenness) leads to a better understanding of the bacterial ecology. Bacterial community composition was measured through Shannon, Simpson and Chao indices, and Simpson's and Pielou´s evenness. Thus, resulted values from alpha-diversity analysis (Table A2 in Appendix A - Supplementary data) showed the sample FC6 both more diverse and even, followed by FC7, since the values in every analyzed metric were differentially higher in these samples than in FC1 and FC4.

Table A3 (Appendix A - Supplementary data) shows that the microbial communities of the white filaments from Fetida Cave were almost totally composed of *Bacteria*, ranging from 97.9% (in FC6) up to 99.8% (in FC1). *Archaea* exhibited a low percentage ranging from 0.2% (in FC1) to 2.1% (in FC6) and were composed exclusively of *Woesearchaeia*. This distribution is similar to those reported for other caves (Itcus et al., 2018; Jurado et al., 2020).

Engel (2007) compared the 16S rRNA gene sequences retrieved from microbial mats from six active sulfidic caves (including Frasassi, Movile and Lower Kane caves) and revealed a diverse range of microorganisms among which the phyla *Epsilonbacteraeota*, *Proteobacteria*, and *Bacteroidetes*, were identified in all the caves. This agrees with our data on Fetida Cave (Table A4 in Appendix A - Supplementary data), in which the microbial mats resulted to be mainly composed of these three phyla with total relative abundances varying between 57.2 and 82.7%. Moreover, if the phylum *Patescibacteria* is also considered, the four samples under analysis reached between 72.6 and 88.2% of the total microbial community composition. This indicates that the microbial

mats populating the acidic sulfidic waters have a remarkable homogeneity in phyla distribution in different geographical settings. Less abundant phyla were *Planctomycetes*, *Spirochaetes*, and *Chloroflexi*, which were also detected in the caves studied by Engel (2007).

The phyla distribution presented in Table A4 (Appendix A - Supplementary data) is similar to Engel's (2007) findings. In fact, five additional bacterial phyla rarely exceed 1% of relative abundance in at least one of the white filaments investigated: *Actinobacteria, Calditrichaeota, Deferribacteres, Firmicutes,* and *Lentisphaerae*.

In the sulfidic caves studied by Engel (2007) the archaeal phylum *Euryarchaeota* (class *Methanomicrobia*) was identified, however, in the filaments from Fetida Cave *Nanoarchaeaeota* (class *Woesearchaeia*) was present, although the relative abundance of *Archaea* in Fetida Cave was scarce. The very low contribution of *Archaea* must be related with unfavorable environmental conditions. Patin et al. (2014) sampled a West Florida Shelf blue hole and found that *Woesearchaeia* comprised up to 40% of the water column community below the oxycline around 100 m, which also featured elevated sulfide levels. The metabolic pathways and potential biogeochemical roles of *Woesearchaeia* likely include a strict anaerobic lifestyle and possible syntrophy with a sulfate-reducing gammaproteobacterial clade (Castelle and Banfield, 2018).

Figure 4 shows the heat-map of the bacterial classes occurring in the white filaments. These data were roughly in accordance with those of D'Angeli et al. (2019b) on Fetida Cave microbial communities, in which a high abundance of members related to sulfur metabolism and belonging to *Gammaproteobacteria, Deltaproteobacteria*, and *Epsilonbacteraeota* were found in white filaments. D'Angeli et al. (2019b) investigated floating and sedimented white filaments that were generally dominated by *Gammaproteobacteria* (12–34%), followed by *Deltaproteobacteria* (8–15%), *Alphaproteobacteria* (4–10%), and *Epsilonbacteraeota* (3–12%). Archaeal sequences accounted for a maximum of 6% and were mainly affiliated with the phylum *Woesearchaeota*.

Conversely, in our samples, the phylum *Epsilonbacteraeota* (12.9–53.7) dominated the filaments, followed by *Proteobacteria* (18.0–39.4%). Among *Proteobacteria*, *Gammaproteobacteria* (10.0–16.4%), *Deltaproteobacteria* (5.9–

15.9), and *Alphaproteobacteria* (2.2–7.7%) were the most abundant classes. *Woesearchaeota* ranged between 0.2 and 2.1%. The different phyla and classes distribution can be attributed, among other reasons, to the diverse environmental conditions and the period of sampling (2015 *vs* 2017) as well as to different methodological procedures, including the use of different primer pairs in D'Angeli et al. (2019b) that might provide slightly different but complementary results (Wasimuddin et al., 2020).

The most abundant class was *Campylobacteria*, with relative abundances of 44.0 and 53.7% in samples FC1 and FC4, respectively, that decreased (12.9 and 15.3%) in FC6 and FC7. With relative abundances above 15% appeared *Gammaproteobacteria* (F1 and F7) and *Deltaproteobacteria* (FC6 and FC7). The abundances of *Bacteroidia* ranged between 10.4 and 12.3% in FC4, FC6, and FC7. *Gracilibacteria* was the class with abundances above 5% (FC6 and FC7). Other classes reached abundances below 5%.

Figure 5 depicts the heat-map of the genera found in the microbial mats. The genus *Arcobacter* attained 35.3% of relative abundance in FC1 and 45.8% in FC4. Relative abundances above 5% were found for the genera *Arcobacter* in FC6, *Sulfurimonas* in FC7, and *Halothiobacillus* in FC1, whereas the genera *Arcobacter* in FC7, *Saprospira* in FC4, *Sulfurimonas* in FC1, FC4, and FC6, *Sulfurovum* in FC1, FC4, and FC7, *Thioflexothrix* in FC4, *Candidatus* Thiobios in FC7, and *Spirochaeta* in FC4 were identified between 5 and 2%. In the same range, unidentified members of the family *Rhodobacteraceae* and order *Campylobacterales* were found in FC1, of the class WS6 (*Dojkabacteria*) in FC6 and a gammaproteobacterium in FC7. Genera with relative abundances around 1% were *Thermomarinilinea* in FC1, FC4, and FC6, *Sulfurovum* in FC6, *Desulfocapsa* in FC6 and FC7, MSBL7 in FC6, *Peredibacter* in FC7, and *Hydrogenovibrio* in FC1. In addition, a number of uncultured genera and families were unevenly distributed among the four samples. It must be noticed that the genus *Beggiatoa* was only retrieved in sample FC7, with a low relative abundance (0.7%) and *Thiothrix* reached even lower abundances (0.1-0.2%) in samples FC1, FC6 and FC7.

The high abundance of *Arcobacter* (45.8% in FC4 and 35.3% in FC1) is a novelty respect to previous studies on SAS caves (Macalady et al. 2006; D'Auria et al., 2018). This abundance in the two samples taken in the innermost

part of the cave can be related to the higher concentration of [HS^-] dissolved in water and lower pH values.

Recently, Talà et al. (2021) studied the prokaryotic communities in Zinzulùsa, a submerged coastal cave at 6 km from Fetida Cave. In samples from submerged black crusts on the walls and bottom sediments (depths 1.9 m to 2.4 m) *Arcobacter* were lower than 0.01% and 0.07%, respectively. No filaments were reported. Anaerobic, sulfate-reducing bacteria (mainly *Thermodesulfovibrio* and *Fervidobacterium*) dominated the black crusts, but they were absent in Fetida Cave. This seems to indicate that Zinzulùsa anchialine waters were not favorable for the growth of *Arcobacter*.

Sievert et al. (2007) stated that *Candidatus* Arcobacter sulfidicus tolerates higher concentrations of H_2S and grows at very low oxygen concentrations, which allows an efficient competition with other sulfur-oxidizing bacteria. This bacterium produces sulfur filament mats in high sulfidic waters (Wirsen et al. 2002). The sulfur-oxidizer genus *Arcobacter* includes free-living species with the ability to fix CO_2, and grow chemolithotrophically via sulfur-oxidation linked to denitrification. He et al. (2020) reported that *Arcobacter* showed a relative abundance of 24.1% in the oligotrophic, anoxic, and sulfidic bottom layer (100 m) of Sansha Yongle Blue Hole, China. Therefore, the metabolic versatility of *Arcobacter* might provide a competitive advantage in the colonization of oligotrophic environments characterized by high sulfide, low oxygen, and dynamic fluid movement.

In a 60 m deep sulfide-rich groundwater, Deja-Sikora et al. (2019) found that the representations of *Beta*- and *Deltaproteobacteria* were small, while the *Epsilonbacteraeota* genera *Sulfurimonas, Sulfurovum*, and *Arcobacter* were very abundant (nearly 77%). Conversely, in waters collected from greater depths (148–300 m), the dominance of *Betaproteobacteria* and sulfate/sulfur-reducing *Deltaproteobacteria* was evident. The authors correlated the shift in microbial communities to depth, and changing nitrogen and oxygen contents. Macalady et al. (2008) indicated that high sulfide to oxygen ratio (> 150) promoted the intensive growth of *Epsilonbacteraeota* (e.g., *Sulfurovum* and *Arcobacter* among others) in the sulfidic water of the Frasassi Cave. Hotaling et al. (2019) found that the microbiome of H_2S-rich stream waters in southern Mexico was composed of *Acidithiobacillus, Sulfuricurvum, Sulfurimonas,*

Thiomonas, and *Arcobacter*, where *Arcobacter* reached a relative abundance of 2.1%.

According to Macalady et al. (2006) in Frasassi Cave white filaments are dominated by filamentous *Gammaproteobacteria* with *Beggiatoa*-like (cottony) or *Thiothrix*-like (feathery) cell morphologies and abundant sulfur inclusions. *Beggiatoa*-related clones were present in both biofilm types (cottony and feathery) and formed a monophyletic clade within the *Thiotrichaceae*. This clade accounted for almost half of the total sequences retrieved from the cottony biofilm. *Betaproteobacteria* and *Epsilonbacteraeota* related to *Thiobacillus*, *Arcobacter*, and other sulfur-oxidizing groups were retrieved in clone libraries but constituted a small fraction of the biomass in both biofilm types. In Fetida Cave, *Beggiatoa* was only found in sample FC7 with a minority abundance (0.7%) and *Thiothrix* was even lower (0.1–02%) in three samples (FC1, FC6, and FC7).

D'Angeli et al. (2019b) found *Arcobacter* in different samples of white filaments inside the cave, either floating or sedimented, but the higher relative abundance was 2.1% in a white filament at the bottom of the water stream close to the location of the rising H_2S-rich fluids. Outside the cave, the presence of *Arcobacter* in white filaments is missing or insignificant (relative abundance 0.2%). The authors suggested that this can be due to the selection imposed on the microbial diversity by the peculiar physico-chemical characteristics of the water inside the cave with a higher concentration of H_2S, slower water flow and absence of light.

Arcobacter was also found in Monte Conca, a Sicilian gypsum cave with an active sulfidic spring in the inner part of the cave. Other important genera were *Sulfurovum, Sulfurimonas,* and *Thiovirga*. The spring generates a small pool where in summer, sulfur-oxidizing bacteria reached up to 95%, and in winter represented 13.6% of the total population. *Arcobacter* relative abundances were 2.5 to 4.6% in summer and absent or insignificant in winter (0–0.2%) (Davis et al., 2020). These changes in abundances denote seasonal variations.

Apart from *Arcobacter*, other sulfur-oxidizing genera identified in the white filaments were *Sulfurimonas, Halothiobacillus, Thiothrix, Thioflexothrix, Thiomicrospira, Sulfurovum*, *Hydrogenovibrio*, *Candidatus* Thiobios, and *Beggiatoa*. These have been found in many of the investigated sulfide waters

(Brigmon et al., 2003; Engel, 2007; Boden et al., 2012; Rossmassler et al., 2012; Han and Perner, 2015; Fomenkov et al., 2017; Jiang et al., 2017; Deja-Sikora et al., 2019; Davis et al., 2020).

Villanueva Alvarez (2005) studied the occurrence and bacterial succession in sulfur-rich blooms in the Ebro delta river. She reported that the bacterial succession started with a dominance of *Beggiatoa*, followed by a *Spirillum*-bloom composed of a high population of *Arcobacter* and after the dominance of *Spirillum*-like cells a spirochaetal bloom was observed in which *Halothiobacillus* and *Thiomicrospira* were detected. The succession was related to the occupation of microniches at different oxygen and sulfide concentrations. These could be the factors inducing the abundant population of *Arcobacter* in Fetida Cave.

Sulfur-reducing bacteria, such as *Desulfocapsa* were also present in the filaments from Fetida Cave, which indicates that microorganisms mediate both sulfur oxidation and sulfur reduction within the white filaments and stream. *Desulfocapsa* has been previously found in Lower Kane and Frasassi caves (Engel, 2007). Other sulfur-reducing bacteria were the lineage MSBL7 (Häusler et al., 2014; Nigro et al., 2020) and *Spirochaetes* (Berlanga et al., 2008; Dubinina et al., 2011).

Within the *Patescibacteria*, Wrighton et al. (2016) identified RubisCO genes, with a central role in CO_2 fixation in members of the *Dojkabacteria* (WS6) and *Parcubacteria* (*Candidatus* Moranbacteria). *Candidatus* Moranbacteria are relatively abundant in groundwaters (Probst et al., 2018). Other *Patescibacteria* (*Gracilibacteria*) have limited metabolism and were predicted to be symbionts, possibly episymbionts (Sieber et al., 2019).

The anaerobic ammonium-oxidating SM1A02 lineage (*Planctomycetes*) was found in an anaerobic biological reactor, using ammonium and sulfate as the substrate to start sulfate-reducing ammonium oxidation (Zhang et al., 2019). Other anaerobic bacteria include *Thermomarinilinea* (*Chloroflexi*) first isolated from a submarine hot spring (Nunoura et al., 2013).

The identification of the genus *Peredibacte*r and an uncultured bacterium from the family *Micavibrionaceae* is remarkable, which points to a well-established trophic chain in the cave. These bacteria belong to the *Bdellovibrio*-and-like organisms (BALOs), composed of *Bdellovibrionaceae*, *Bacteriovorax*,

Peredibacter, and *Micavibrio*, and are widespread obligatory predators of other Gram-negative bacteria, including cyanobacteria (Davidov et al., 2006; Cai et al., 2014). In addition, *Saprospira*-like organisms have been reported to behave as predators of bacteria and algae (McIlroy and Nielsen, 2014).

3.4. 16S rRNA gene-based metabolic inference

The most relevant ecological roles of microbial communities in Fetida Cave were analyzed by FAPROTAX. Almost 25% of ASVs were assigned to at least one group of the 43 found in the analysis; the remaining percentage could not be assigned to any group (Fig. 6). This database is not exhaustive; therefore, only small proportions of ASVs were assigned.

The major predicted ecological functions were chemoheterotrophy (7.6–26.6%), respiration of sulfur compounds (8.5–18.8%), dark oxidation of sulfur compounds (5.8–15.8%), and fermentation (3.3–14.7%). Chemoheterotrophy was predicted to be greater in FC4, in which *Proteobacteria* (*Delta*- and *Gammaproteobacteria*), and *Bacteroidetes* were the most abundant involved phyla. In addition, the abundance of sequences assigned to the respiration of sulfur compounds and sulfate respiration was higher in FC6 and, slightly, in FC7. However, FC6 was the sample with the fewest predicted sequences within the dark oxidation of sulfur compounds group. Respiration of sulfur compounds was mainly assigned to the order *Desulfobacteral*es while dark oxidation of sulfur compounds was related to the genera *Sulfurimonas, Halothiobacillus*, and *Beggiatoa.* In a similar way, the abundance of *Desulfobacteral*es was related to the higher abundance of sulfite respiration and thiosulfate respiration predicted functions in FC6 and FC7, but there was no linkage with the sulfur respiration. FAPROTAX predictions also identified *Halothiobacillus* as the main taxon assigned to dark thiosulfate oxidation, dark sulfide oxidation and dark sulfur oxidation. Dark oxidation of sulfur compounds was not predicted by the FAPROTAX analysis for the genus *Arcobacter*, probably due to the absence of metabolic information on *Arcobacter* in the database.

Wirsen et al. (2002) related *Candidatus* Arcobacter sulfidicus, a chemolithoautotrophic sulfur oxidizer, to CO_2 fixation and filamentous sulfur formation. This bacterium has the capacity to fix carbon via the reductive

630 tricarboxylic acid cycle (Hügler et al., 2005). More recently, Noguerola et al.
631 (2015) reported that a member of the genus *Arcobacter* fixes CO_2 in the dark in
632 the sulfidic redoxcline of a meromictic karstic lake, via the same cycle, and
633 Evans et al. (2018) revealed that *Arcobacte*r, associated with filamentous sulfur
634 material, have the ability to fix CO_2.
635 Some previous studies on laboratory-grown strains of sulfur-oxidizing
636 bacteria described a ^{13}C depletion in the fixed medium with respect to the
637 ambient source of CO_2 and bicarbonates (Ruby et al., 1987). Turning the
638 spotlight once more on our δ^{13}C-DIC dataset (Table 3), an important difference
639 in δ^{13}C-DIC (around +8 ‰) is noticeable between the meteoric water and
640 thermal water from Fetida Cave, particularly at the cave locations with greater
641 evidence of water recharge from the upper vadose zone. These locations
642 correspond to the innermost points of the cave, where the mixing with seawater
643 is lower and a greater abundance of white filaments was also detected.
644 Therefore, the *Arcobacter* from Fetida Cave might likely be involved in the ^{12}C
645 fixation from the bicarbonates dissolved in the mixture of meteoric water
646 (seepage water) and seawater, and this would cause a carbon isotopic
647 fractionation in the bicarbonates resulting in higher δ^{13}C-DIC values in relation
648 to the typical isotopic values of bicarbonates in the seepage water.
649 Fermentation was predicted in FC4 associated to the genus *Spirochaeta.*
650 The implication of *Spirochaeta* in the sulfur cycle was not predicted by
651 FAPROTAX; however, the existence of sulfide-oxidizing and sulfur-reducing
652 bacteria within the genus *Spirochaeta* has been described by Dubinina et al.
653 (2011) in sulfide-rich water from a saline spring. Deja-Sikora et al. (2019)
654 reported the coexistence of sulfur-oxidizing and sulfur-reducing bacteria in
655 Polish sulfidic waters. In addition, many of the genera they found were the same
656 as those retrieved from Fetida Cave. A similar coexistence was found in marine
657 sediments (Ihara et al., 2019).
658 In light of the data shown, *Arcobacter* appears as a sulfur-oxidizing
659 microorganism in coastal seawater that produces filaments trapping sulfur
660 crystals and exhibits nitrogen fixation in concurrence with carbon dioxide
661 fixation, as suggested by the water carbon isotope values.

The high variability in the composition of the white filaments, at the lower taxonomic levels, even in the same cave in different sampling periods, is due to changes in the water hydrochemistry and hydrodynamics, subjected to tidal water level fluctuation and the variable mixing of fresh continental with marine water.

Similar fluctuations were observed in Polish sulfide-rich mineral waters, since the composition of the bacterial communities strongly varied across the samples. However, most of the bacteria participating in the sulfur cycle were common in all sulfidic waters (Deja-Sikora et al., 2019). This was also found in Fetida Cave, where a certain homogeneity in phyla distribution and abundance can occur at higher taxonomic levels, due to the selection imposed by well-defined environmental conditions: the sulfide-rich water, and the bacterial groups participating in the sulfur cycle that were common to sulfidic waters.

CRediT authorship contribution statement

Valme Jurado, Ilenia D'Angeli, Tamara Martin-Pozas, Martina Cappelletti, Daniele Ghezzi, Jose Luis Gonzalez-Pimentel, Soledad Cuezva and Ana Zelia Miller: Investigation. Angel Fernández-Cortès: Investigation, writing - original draft. Jo De Waele: Conceptualization, review & editing, supervision. Sergio Sanchez-Moral: Conceptualization, Writing - original draft, Funding acquisition. Cesareo Saiz-Jimenez: Conceptualization, Supervision, Project administration, Funding acquisition, Writing – original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the Spanish Ministry of Economy and Competitiveness through project CGL2016-75590-P with ERDF funds and PID2019-110603RB-I00/ AEI/10.13039/501100011033. The whole Santa

694 Cesarea Terme Spa and Sergio Pispico are thanked for making Fetida Cave
695 accessible for the sampling campaigns and the research. Thanks to Rosangela
696 Addesso and Orlando Lacarbonara (the photographer) who were present during
697 the field trips. Ilenia D'Angeli thanks Mario Parise for the support in collecting
698 gas and water samples in May 2018. Thanks to Bartolomeo Vigna and Adriano
699 Fiorucci for water analysis performed at the Politecnico of Turin.
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FIGURE LEGENDS

Figure 1. The planimetry of Fetida Cave and the sampling locations (Image G) are shown on the right. Microbial (FC1, FC4, FC6, FC7) and water (SCA1, SCAfen, SCB1, SCC1) samples are reported in yellow, whereas gas samples are shown in red. The H_2S spring is indicated with a black line and corresponds to the SCB1-FC4 sampling location. The collected white floating filaments and their respective habitats (Images from A to F) are visible on the left. AE means anthropic entrance, whereas NE natural entrance.

Figure 2. Binary δ^2H–$\delta^{18}O$ diagram for thermal water samples from Fetida Cave, compared with the isotopic composition of meteoric and thermal spring, in accordance to Santaloia et al. (2016), and local rainfall. The local water meteoric line (black dashed line) is plotted in relation to the water meteoric line for southern Italy (Longinelli and Selmo, 2003) and the GMWL (Clark and Fritz, 1997).

Figure 3. White filaments from Fetida Cave. A: Sample FC1. Damaged filaments, extracellular polymeric substances and *Arcobacter*-like cells (in the center). B and C: Net of filaments from sample FC6. Magnification of C shows *Thiothrix*-like filaments. D: Filaments from sample FC7 and associated mineral grains of sulfur.

Figure 4. Heat-map analysis of Fetida Cave samples with taxonomic identifications of *Bacteria* at class level. The classes are described in the right column and their respective abundances included in the boxes. Colored left bar groups the classification at phylum level.

Figure 5. Heat-map analysis of Fetida Cave samples with taxonomic identifications of *Bacteria* at family/genus level. The families/genera are described in the right column and their respective abundances included in the boxes. Colored left bar groups the classification at order level.

Figure 6. FAPROTAX analysis of white filaments from Fetida Cave with the predicted ecological functions based on 16S rRNA genes (Y axis). The size of the cycles indicates the relative abundance of the assigned ASVs