

Supplementary Informations

Calcite moonmilk of microbial origin in the etruscan *Tomba degli Scudi* in Tarquinia, Italy

Angela Cirigliano^{1°}, Maria Cristina Tomassetti^{2°}, Marta Di Pietro¹, Francesco Mura³, Maria Lorella Maneschi⁴, Maria Donatella Gentili⁵, Barbara Cardazzo⁶, Chiara Arrighi⁷, Cristina Mazzoni¹, Rodolfo Negri¹ and Teresa Rinaldi^{1*}

¹ La Sapienza University of Rome, Dept. of Biology and Biotechnology “Charles Darwin”, Rome, 00185, Italy

² Freelance restorer, Via Flavia 16, 00062, Bracciano, Rome, Italy

³ Research Center For Nanotechnology Applied To The Engineering of Sapienza (CNIS) La Sapienza University of Rome, Rome, 00185, Italy

⁴ Archaeologist, Capo Delegazione FAI – Delegazione Viterbo, Via XX Settembre 56, 01016, Tarquinia, Italy

⁵ Archaeologist, Via de Carolis 135, 00136, Rome, Italy

⁶ Department of Comparative Biomedicine and Food Science, University of Padova, Viale dell’Università, 16, Legnaro, 35020, Padova, Italy

⁷ Freelance restorer, Via San Maria Mediatrice 10, 00165, Rome, Italy

* teresa.rinaldi@uniroma1.it

° These authors contributed equally to the work

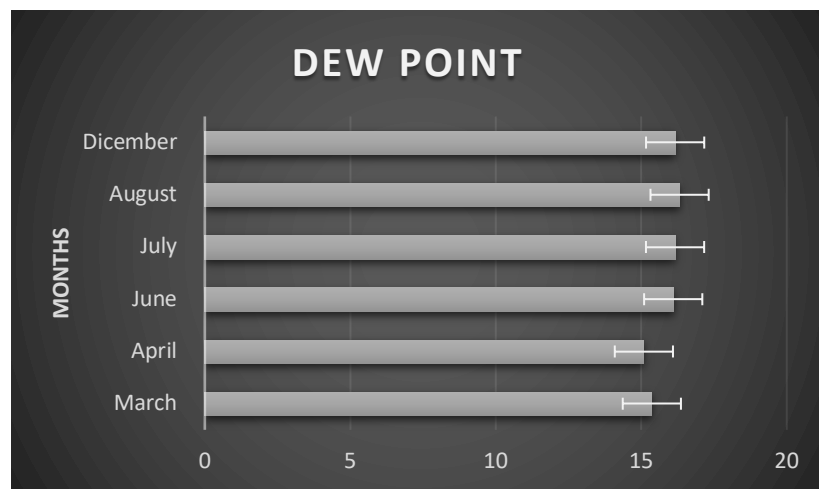
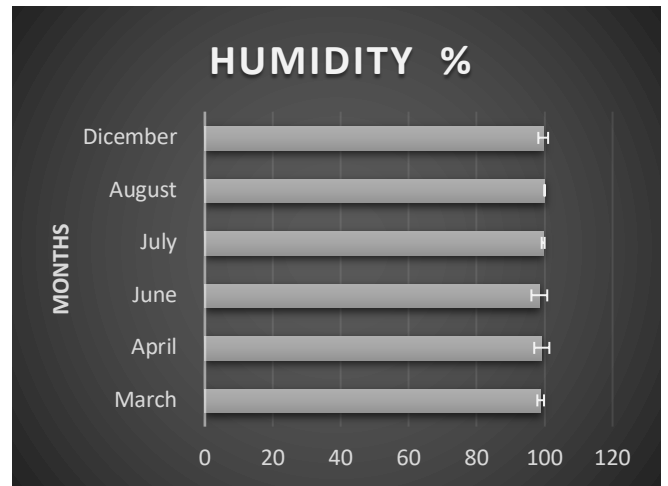
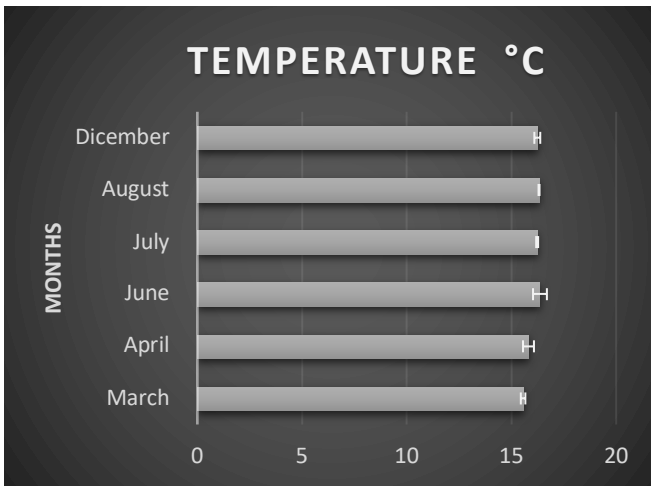
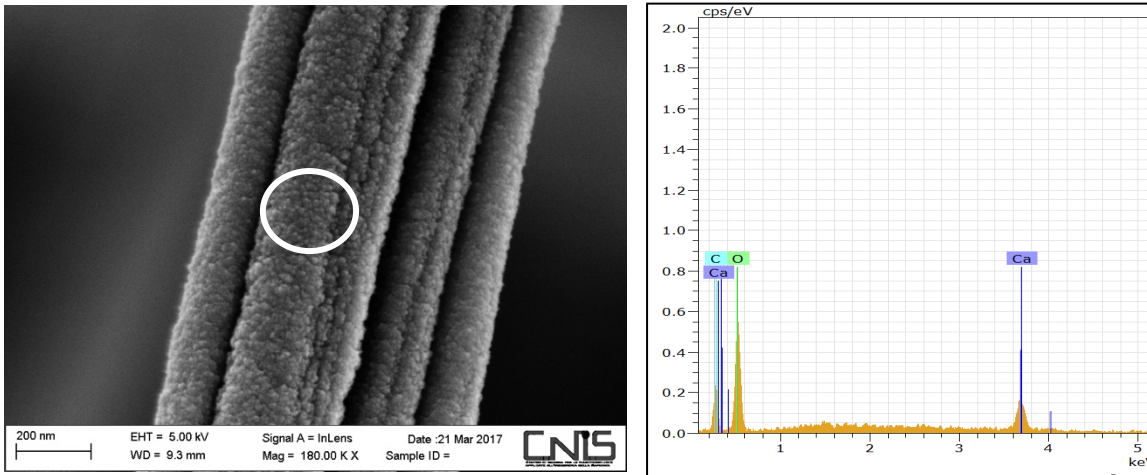


Figure S1. Temperature, humidity and dew point registered in the *Stanza degli Scudi* in the period March 2017 to December 2017.



| | El | AN | Series | norm. | C Atom. | C Error (1 Sigma) |
|--------|----|----------|--------|--------|---------|-------------------|
| | | | | [wt.%] | [at.%] | [wt.%] |
| ----- | | | | | | |
| O | 8 | K-series | 43.03 | 58.37 | 1.58 | |
| Ca | 20 | K-series | 48.45 | 26.24 | 0.58 | |
| C | 6 | K-series | 8.52 | 15.39 | 0.44 | |
| ----- | | | | | | |
| Total: | | | | 100.00 | 100.00 | |

Figure S2.
SEM analysis of nanofibers. The atomic composition of the deposit in Figure 3 suggests a calcium carbonate composition.

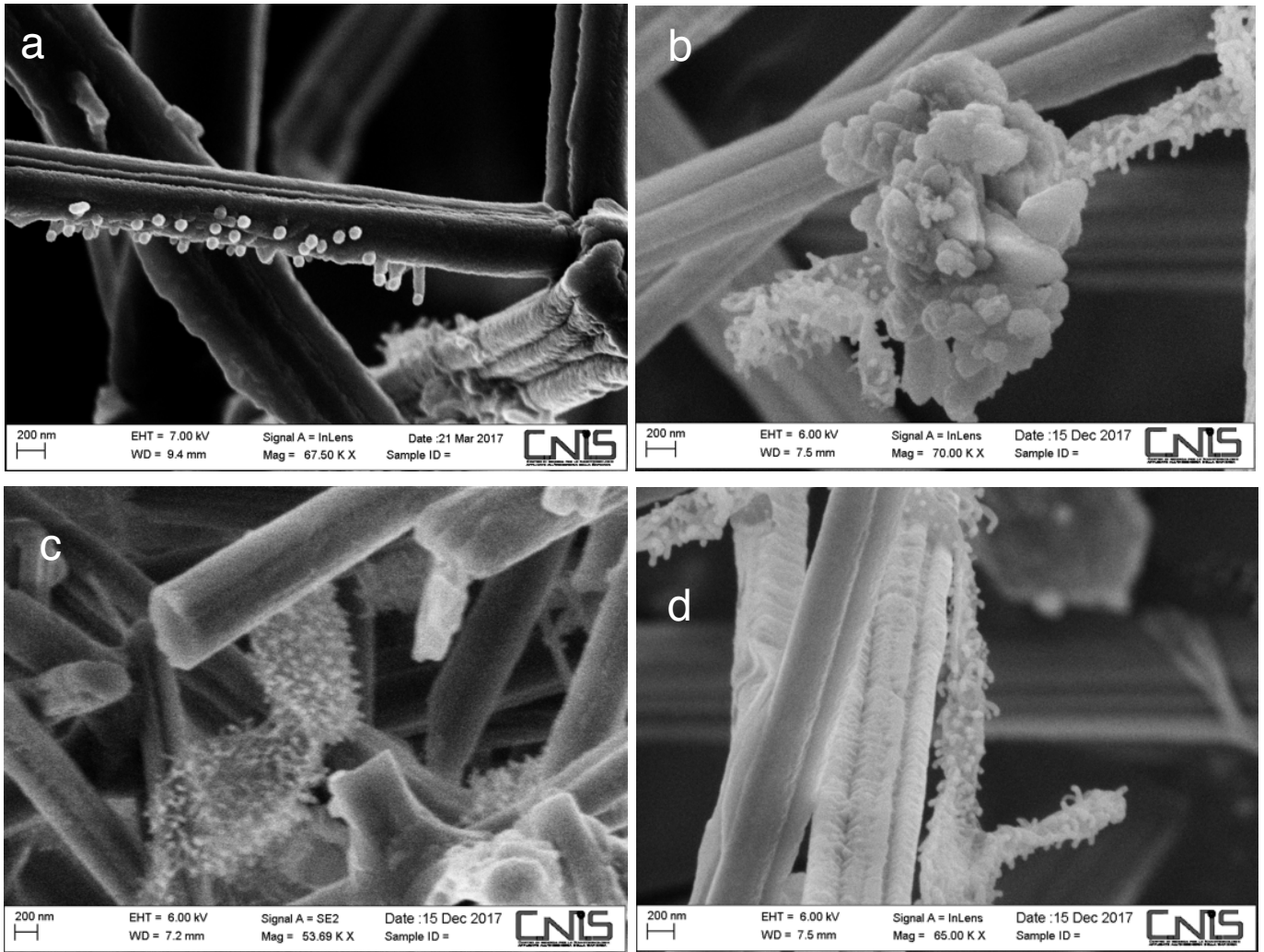


Figure S3.

SEM analysis of moonmilk isolated from the *Stanza degli Scudi*. (a) Magnification of the same microorganism of Fig. 3d and 3e wrapping a CaCO_3 nanofiber; the sample was taken in March 2017. (b, c and d) Magnification of the microorganism in Fig. 3 h sampled in December 2017.

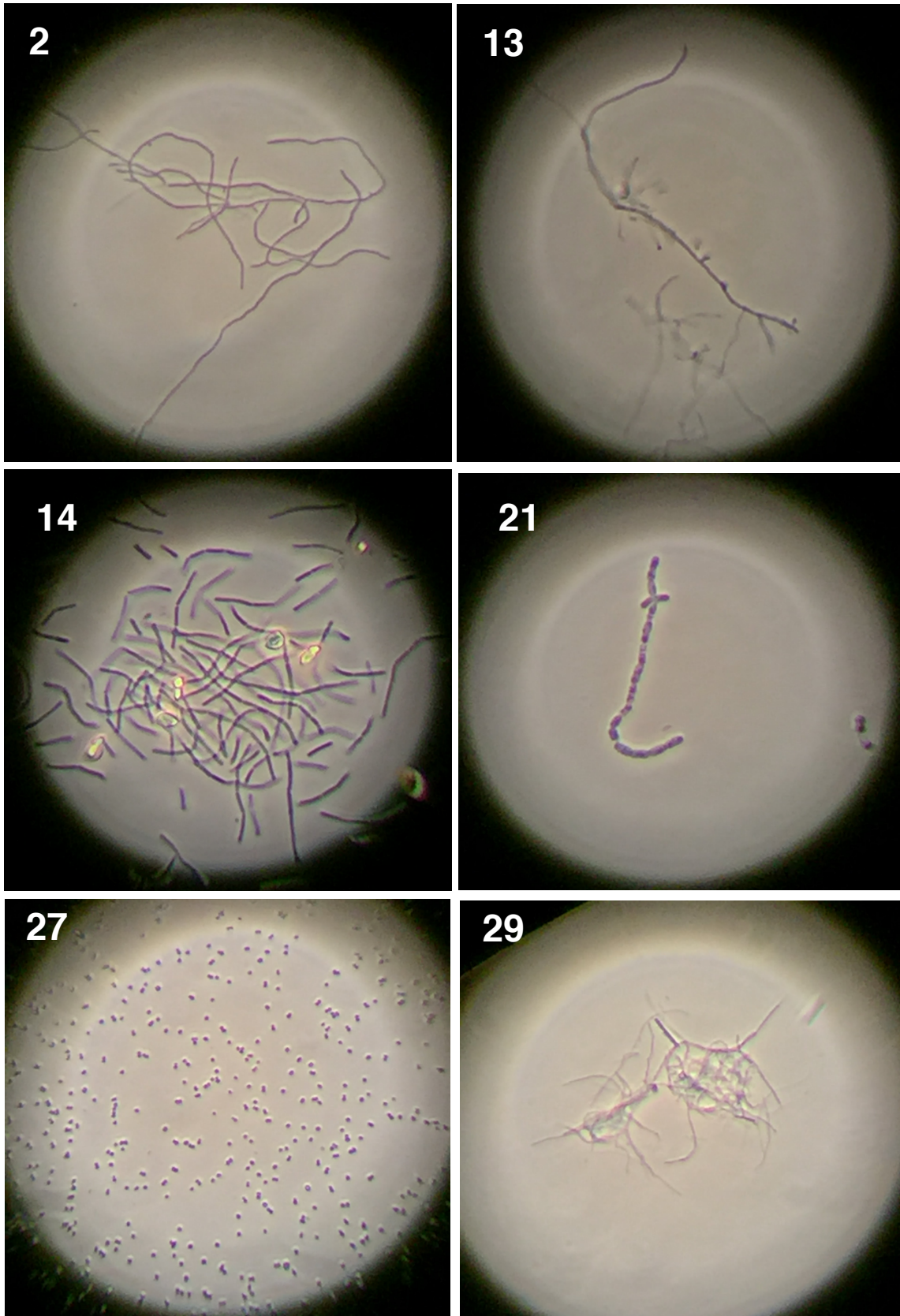


Figure S4. Examples of bacterial strains isolated from moonmilk in the *Stanza degli Scudi*. The strains were grown in complete medium and cultured at 18 °C. *Streptomyces* sp. TR2, *Streptomyces* 13, *Bacillus* 14, *Bacillus mycoides* 21, cocci 27 and *Streptomyces* 29. Pictures were taken using an Axio Star Zeiss microscope, magnification 40×.

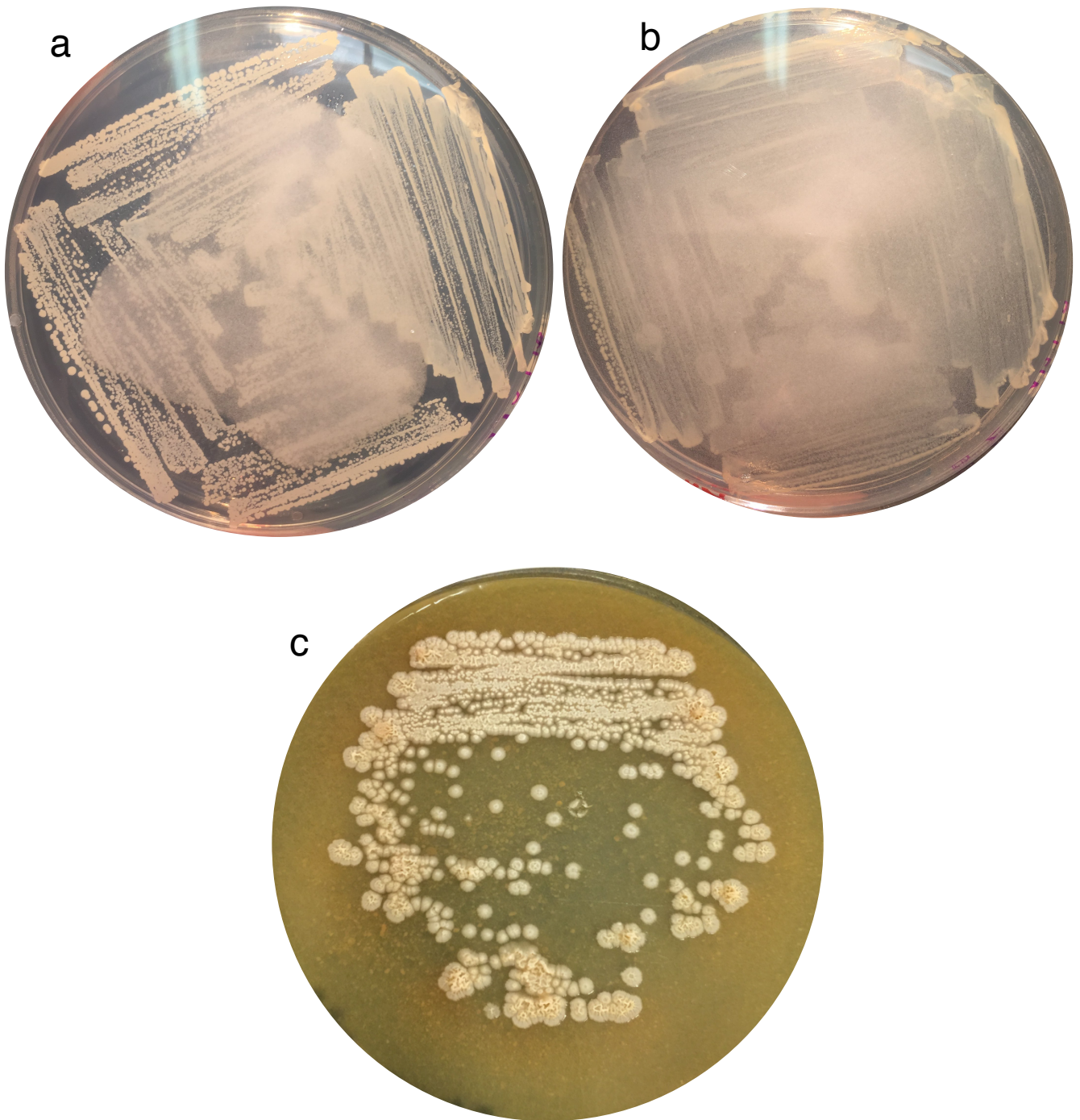


Figure S5.

Dissolution and deposition of calcium carbonate activity on plates of two strains isolated from moon-milk. a) Strain 27 was streaked on B4-C plates and during growth, the cells dissolved CaCO_3 , rendering the medium transparent. b) As a control, strain 14, a *Bacillus* sp., was not able to dissolve CaCO_3 , and the plate remained opaque. c) *Streptomyces* sp. TR2 was streaked on YPD supplemented with urea, and cells were observed directly with SEM (see Fig. 4f).

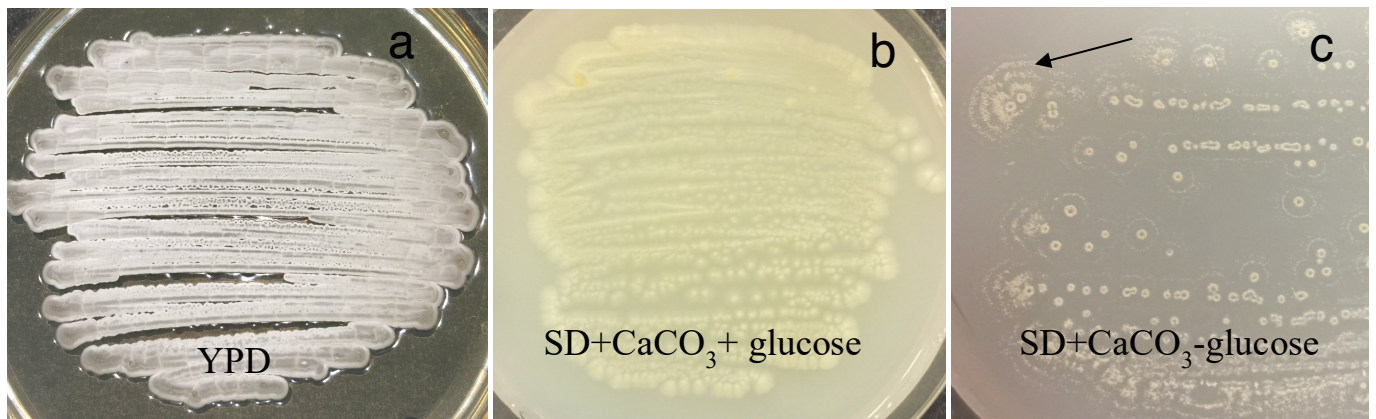


Figure S6.

Growth of *Streptomyces* colony 2 isolated from the *Stanza degli Scudi* in March 2017. (a) Complete medium YPD. (b) Synthetic medium supplemented with CaCO₃ and glucose. (c) Synthetic medium supplemented with CaCO₃ but without glucose. The black flag indicates where the SEM sample of Fig. 4 (d), (e), (f) was taken.

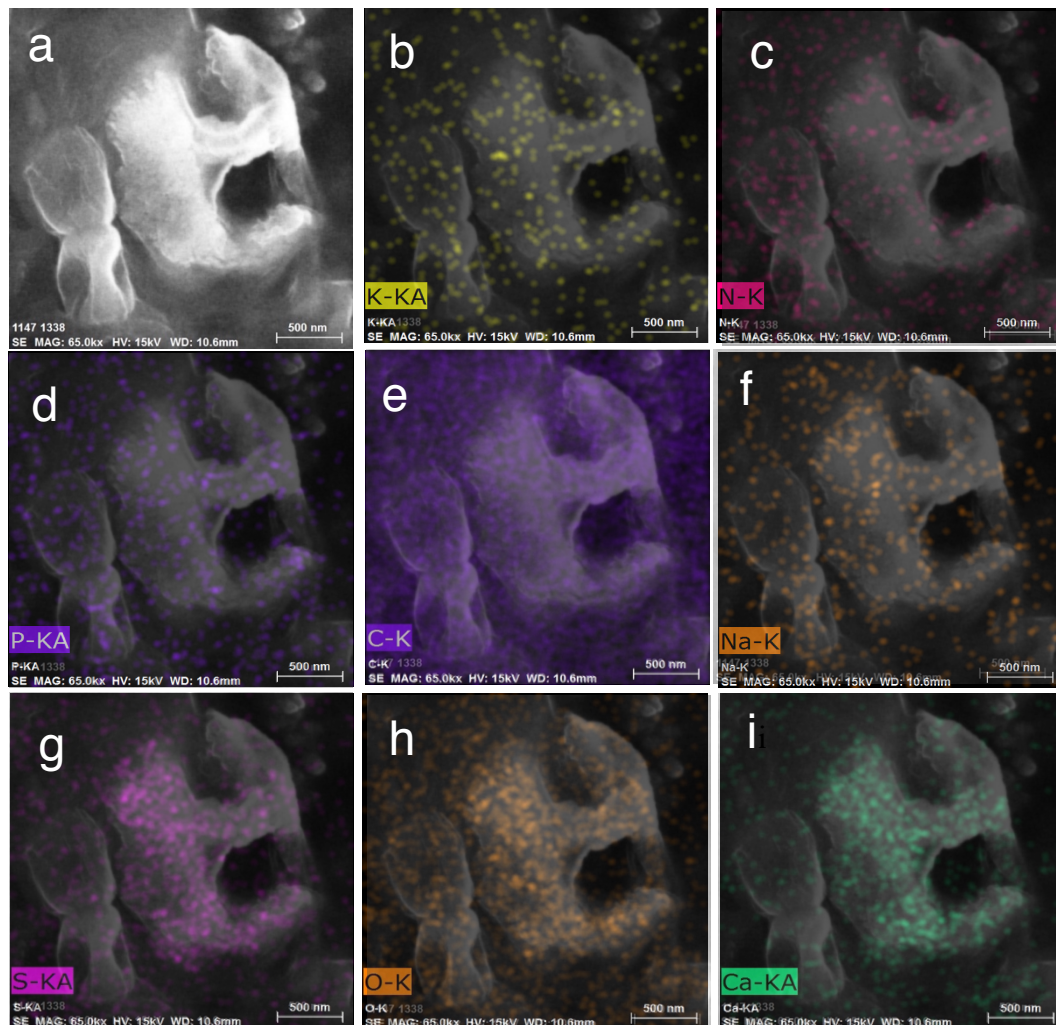
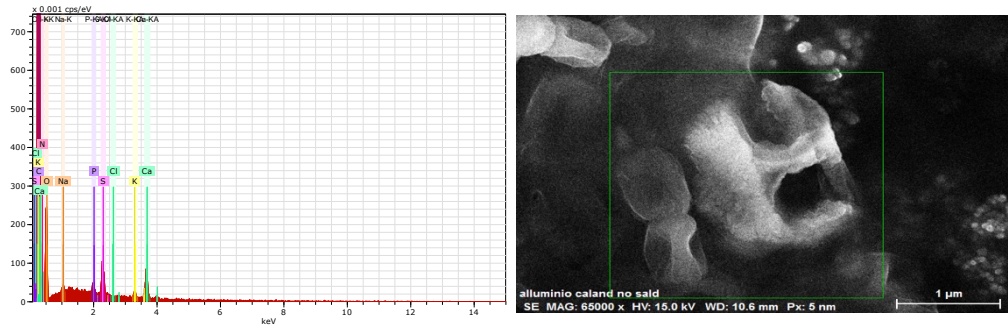
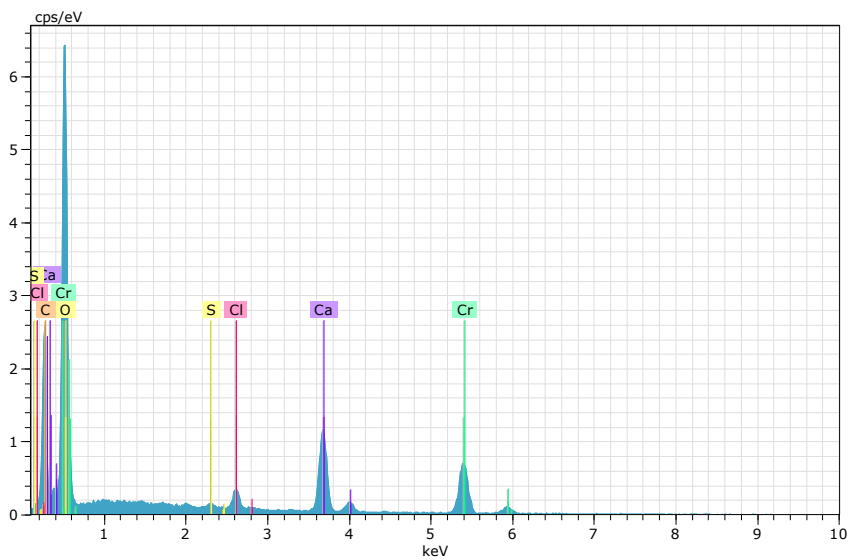
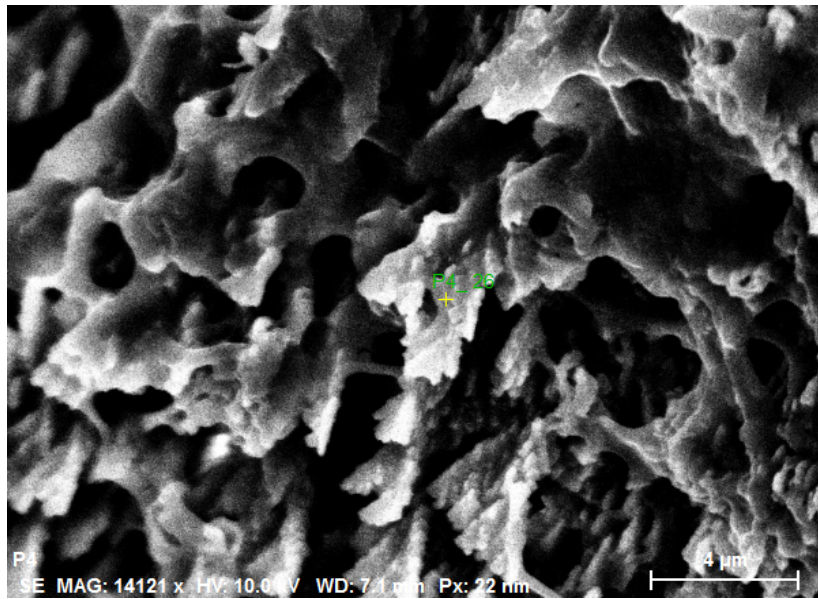


Figure S7.

SEM analysis of a pure culture of *Streptomyces 2* grown in SD medium without glucose. The atomic composition of the deposit in Figure 4e suggests a calcium sulfate composition (S5g-i).



Spectrum: P4_26

| El | AN | Series | unn. C [wt.%] | norm. C [wt.%] | Atom. C [at.%] | Error (1 Sigma) [wt.%] |
|--------|----|----------|------------------|-------------------|-------------------|---------------------------|
| O | 8 | K-series | 39.05 | 58.00 | 63.57 | 5.03 |
| C | 6 | K-series | 11.78 | 17.50 | 25.55 | 1.77 |
| Ca | 20 | K-series | 14.85 | 22.06 | 9.65 | 0.56 |
| Cl | 17 | K-series | 1.41 | 2.09 | 1.03 | 0.09 |
| S | 16 | K-series | 0.24 | 0.35 | 0.19 | 0.04 |
| Cr | 24 | K-series | 0.00 | 0.00 | 0.00 | 0.00 |
| Total: | | | 67.33 | 100.00 | 100.00 | |

Figure S8

SEM analysis of a pure culture of *Streptomyces 2* grown in YPD medium with urea. The atomic composition of the deposit in Figure 4f suggests a calcium carbonate composition.



Figure S9.

Pictures of the white deposit in the central atrium in the *Tomba degli Scudi* (a) General view of the central atrium before restoration. (b) The picture shows the left part (red arrow) of the western wall during restoration; in this part of the wall the white deposit covering the frescoes was soft. (c) The soft white deposit on the roof (red arrow) was not removed during restoration; it is still in place and corresponds to moonmilk. The pictures were taken directly with a video microscope. Photographer: Domenico Ventura.

Table S1. The closest relatives bacterial species of the colony 2 isolated in *Stanza degli Scudi*.

| Strain | Query cover | Identity | Max score | Total score | Accession number |
|--|-------------|----------|-----------|-------------|------------------|
| <i>Streptomyces</i> sp. MM122 16S ribosomal RNA gene, partial sequence | 100% | 99% | 1247 | 1247 | KU714879.1 |
| <i>Streptomyces</i> sp. strain KMS5 16S ribosomal RNA gene, partial sequence | 100% | 99% | 1247 | 1247 | KX950877.1 |
| <i>Streptomyces</i> sp. E1N444 16S ribosomal RNA gene, partial sequence | 100% | 99% | 1247 | 1247 | KX279644.1 |
| <i>Streptomyces</i> sp. E5N142 16S ribosomal RNA gene, partial sequence | 100% | 99% | 1247 | 1247 | KX279565.1 |
| <i>Streptomyces badius</i> strain DB-1 16S ribosomal RNA gene, partial sequence | 100% | 99% | 1247 | 1247 | KU886090.1 |
| <i>Streptomyces</i> sp. MOLA 1484 16S ribosomal RNA gene, partial sequence | 100% | 99% | 1247 | 1247 | KM273899.1 |
| <i>Streptomyces flavogriseus</i> strain Nr_2 16S ribosomal RNA gene, partial sequence | 100% | 99% | 1247 | 1247 | KT714128.1 |
| <i>Streptomyces flavogriseus</i> strain ACTK2 16S ribosomal RNA gene, partial sequence | 100% | 99% | 1247 | 1247 | KC990785.1 |

Table S2. Features defining *genus/species* in Western and Northern walls samples.

| Taxon | W % | N % |
|--|-----------|-----------|
| k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Nocardiodaceae;g__Actinopoly-morpha;s__ | 0,3678823 | 0 |
| k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Nocardiodaceae;g__Krib-bella;s__ | 0 | 0,2336085 |
| k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Pseudonocardiaceae;g__Pseu-donocardia;s__ | 5,6941779 | 3,4729793 |
| k__Bacteria;p__Chlamydiae;c__Chlamydiia;o__Chlamydiales;f__Parachlamydiaceae;g__Candidatus Pro-tochlamydia;s__ | 0 | 0,1401651 |
| k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Bacillus;s__muralis | 0 | 0,7475471 |
| k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Paenibacillaceae;g__Paenibacillus;__ | 0 | 0,7008254 |
| k__Bacteria;p__Nitrospirae;c__Nitrospira;o__Nitrospirales;f__Nitrospiraceae;g__Nitrospira;s__ | 0,6078055 | 2,1491979 |
| k__Bacteria;p__Planctomycetes;c__Planctomycetia;o__Gemmatales;f__Gemmataceae;g__Gemmata;s__ | 0,3039028 | 0,7942688 |
| k__Bacteria;p__Planctomycetes;c__Planctomycetia;o__Pirellulales;f__Pirellulaceae;g__Pirellula;s__ | 0 | 0,4983647 |
| k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae;g__Bradyrhi-zobium;s__ | 0,4158669 | 0,3737736 |
| k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Hy-phomicrobium;s__ | 2,9590531 | 2,4295281 |
| k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Pe-domicrobium;s__ | 0,5758157 | 0 |
| k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobi-aceae;g__Rhodoplanes;s__ | 0,5118362 | 1,8532939 |
| k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomona-daceae;g__Sphingopyxis;s__alaskensis | 0,1919386 | 0 |
| k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Me-thylibium;__ | 0,9916827 | 1,1991902 |
| k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Bdellovibrionales;f__Bdellovibri-onaceae;g__Bdellovibrio;s__ | 0 | 0,4672169 |
| k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales;f__Coxiellaceae;g__Aqui-cella;s__ | 0,1599488 | 0 |
| k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Sinobacter-aceae;g__Nevskia;s__ | 0,0799744 | 0 |
| k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Sinobacter-aceae;g__Steroidobacter;s__ | 1,0236724 | 0,7163993 |
| k__Bacteria;p__Verrucomicrobia;c__[Spartobacteria];o__[Chthoniobacterales];f__[Chthoniobacter-aceae];g__Candidatus Xiphinematobacter;s__ | 0 | 0,6852515 |
| % on the total | 13,883557 | 16,46161 |