Author’s response to the referees
Reviever 1

Suggestions for revision or reasons for rejection

(visible to the public if the article is accepted and published)

This revised manuscript investigates the formation of calcite nanofibers (moonmilk) in the Monterozzi Necropolis in Central Italy. It is an intriguing field site with beautiful microfabrics. I appreciate the large amount of work the authors did in order to address reviewer comments on a previous version. The topic of the biogenicity of carbonates is of wide interest to geo/astrobiologists, and the complexities of the issues are well stated in the introduction. There are a few issues here and there (outlined below), but after minor revision, I believe this manuscript is suitable for publication.

For instance, the term ‘bacterial biomineralization’ or just ‘biomineralization’ is used throughout the paper, especially in section 3.3, enough so that a definition of the term would be useful, since I assume the authors do not mean direct biomineralization in the way shells and bones are produced, but more of indirect biomineralization wherein microbial metabolisms influence the surrounding geochemistry and foster precipitation. Dupraz et al. 2009 (Earth Science Reviews) defined ‘organomineralization’ for this class of precipitation. Overall, a definition of what the authors mean by the term is necessary up front (or they can go with the Dupraz classification).
Thank you for this comment, we added the term Organomineralization sensu Dupraz et al 2009

My biggest concern is the treatment of the laboratory results that being by line 304. It is unclear to me what ‘in plates’ refers to- is this is reference solely to the previous work, or did the authors do some of their own experiments? If their own, the methodology should be included. In line 325 it seems this might be referencing another paper- however I am not sure. Since it is a good line of evidence for microbial involvement in precipitation, which is the main goal of the manuscript, it would be useful if the experimental conditions were described in a bit more detail.

Thank you for this comment, the same point has been raised by the second referee, we have performed a new experiment, not present in the paper under review, in supplementary fig 10, showing that the microbial community of the calcarenite precipitates high amount of calcium carbonate and we updated the Methods section.

Line 44: might be better to state that carbonates form in/from environments most likely to contain life, rather than assuming biogenicity.
Line 57: use “biogenic” rather than ‘biotic’, since organisms are not directly making the carbonate (eg, through biomineralization)
Thank you, we changed biotic with biogenic in the text.

Lines 330-335: a breakdown of the specific metabolic processes happening here would be useful. What is the overall metabolism producing a rise in pH? Is the rise in pH actually due to bicarbonate/carbonate production, and how is the presumed saturation index impacted by the metabolism? How is the presence of a metabolic ureolytic process confirmed? Phylum level diversity won’t necessarily shine any light on whether or not he microbes in the sample are capable of these metabolic processes, but there are a high proportion of Firmicutes (or Bacillota), which are known to contain several members (such as those in Lysinibacillus groups) that have been shown to have extremely high urease activity and therefore greatly enhance carbonate precipitation. It should be pointed out that this phylum is present and fairly abundant.

we update the text following these suggestions, we have also detailed that the strain of the supplementary figure 9 is indeed a Lysinbacillus species.

The pictures are beautiful, especially in the supplemental files. The fibers entombing bacteria – wonderful!
Thank you!

Report #2

Submitted on 23 Jun 2023
Anonymous referee #2

Suggestions for revision or reasons for rejection

(visible to the public if the article is accepted and published)

(modified from Response to Authors)
The authors have addressed many of the questions from the previous round of reviews.Overall, I think the authors have begun to strengthen their case for the biogenicity of moonmilk in Tarquinia, but there are still three major areas of revision that need to happen before publication. In short, the argument for biogenicity is divided into three parts- environment, cultures, and RNA.

First, the authors note that meteoric waters in the tombs should be undersaturated with respect to calcite, generally fostering dissolution. I broadly agree with this point, but it should be noted that meteoric waters dissolving calcite will gradually increase in saturation, re-precipitating crystals elsewhere without the need for life. The authors discuss these potentials, but the text contains several contradicting statements about the presence/absence of carbonate dissolution and abiotic precipitation of cements.

Thank you, we fixed this contradiction in the text. We showed that the dissolution process is dominant and can make available Ca2+ for microbial metabolism.

Second, the text compares culturing experiments between living and sterilized bedrock- this is the strongest evidence for biogenic moonmilk in the paper. However, this argument relies on data from an unpublished paper. Furthermore, the culturing experiments from this research are missing from the Methods section, and need an expanded discussion to compare lab conditions with in-situ environments.

The referee is right, a new experiment, never published before and not present in the paper under review, has been added, in supplementary fig 10, showing that the microbial community of the calcarenite promotes the precipitation of high amount of calcium carbonate. When the calcarenite is sterilized, there is no calcium carbonate production. We also updated the Methods section.

Finally, the text asserts that similar microbial communities in bedrock and moonmilk is a sign of biogenicity. There are many abiotic scenarios where similar microbial communities can be preserved in different parts of the same rock. In short, the similarity alone cannot be a biosignature.
Following also the comments below, we modified the text.

1) Section 3.1
In my first comments, I mentioned that minerals can entomb cells without requiring microbial metabolisms, especially in supersaturated environments. The authors responded that the tombs are in the vadose zone, that there’s no carbonate-rich groundwater source nearby, and that any water in the system is likely meteoric. I agree with the authors on all these points. The authors added these points in Lines 265-280, and they strengthen the manuscript.

However, the processes of carbonate dissolution and precipitation in the tomb walls need to be clarified. At the moment, there are a few contradicting statements about dissolution and precipitation in the bedrock. For example, in Lines 249-250 describing Macco facies, "In the inner walls of the intergranular voids microsparite cement precipitation and/or recrystallization often occurs, due to diagenetic processes of dissolution."
This sounds reasonable to me- the vadose zone often shows signs of dissolution followed by precipitation, as previously undersaturated waters become concentrated in Ca and CO3. However, in Lines 267-9 describing the general bedrock:
"Noteworthily, the characteristics of bedrock porosity indicate that the dissolution processes prevail on those of inorganic carbonate precipitation; indeed, meteoric cements as well as speleothems in the largest cavities are absent. Moreover, the bedrock where the tombs are carved is located in the shallow vadose zone (few meters below the surface) and it is barely susceptible to dissolution-reprecipitation processes,…"

The reviewer is right, we deleted 'reprecipitation' because it can be misleading as reprecipitation can give the idea of development of concretions and abundant cements and this is not the case.

In short, Section 3.1 says that the bedrock 1) contains evidence for dissolution and cement precipitation, 2) mostly evidence for dissolution with no cement precipitation, and 3) barely any dissolution or precipitation.

Thank you for this comment, we deleted precipitation.

These contradictions either need to be corrected, or if the authors are talking about different locations, that information needs to be clarified.
Here's why the discussion of dissolution and precipitation is important. A convincing argument for biogenic moonmilk requires: 1) a source of calcium, provided from calcite dissolution somewhere in the vadose zone, and 2) undersaturated meteoric waters which are less likely to precipitate calcite, requiring microbial metabolisms to foster precipitation. The authors address these points (end of 3.1), but there are two major questions left to address:
If the bedrock has no evidence for calcite dissolution, where is the calcium coming from to fuel biogenic moonmilk formation? On the other hand, if abiotic cements are present elsewhere in the bedrock, indicating periods of supersaturation, why does moonmilk need to be biogenic?
For publication to continue, the authors should:
1: clarify their interpretations on patterns of precipitation and dissolution in the bedrockis it an environment that contains both dissolution and precip? Is it dominated by dissolution? Or are both dissolution and precip limited?
The porosity type (mainly vuggy and and moldic), the absence of mosaic cements (or other meteroric cements) points toward a dominance of dissolution processes instead of precipitation. On the other hand the main components are represented by coralline algae constituted by high magnesium calcite that is a metastable mineral phase of calcite. The original cabonate phase upon exposure to meteoric water will dissolve partially, increasing the availability of Ca2+ for microbial metabolism and mediated carbonate precipitation.
2: once the authors have a consistent interpretation of precip/dissolution, clearly describe how such groundwaters create conditions where only microbes can make moonmilk (the

authors start to do this in Lines 278-279).
Please, see the previous comment.
3: (Optional) If the authors have any data on local groundwater, especially pH, Ca, and estimates for calcite saturation, that would greatly help build their case for a subsurface environment where only microbes could make moonmilk. This data could be from the authors themselves, or collected from the literature.

We try to sample the groundwater in the rocks, but unfortunately the rock was dry because the summer weather conditions, but we will consider this comment for future experiments.

2) Section 3.3- cultures
The strongest evidence for biogenic moonmilk at Tarquinia is presented in Line 329: "Indeed, under laboratory conditions, we have evidence that the grinded calcarenite, with its entire microbial community, when present in a medium containing urea and CaCl2 produced calcite. In the same conditions, calcium carbonate is not produced with sterile (autoclaved) grinded calcarenite (Benedetti et al. 2023)."
This is exactly the type of experiment needed to show the biogenicity of carbonate precipitation.
However, I have a few notes about the culture experiments and references.
First, Benedetti et al., 2023 is still in review. I don't know Biogeosciences' citation policies, but many journals require such references to be cited as (Benedetti et al. in review). More importantly, if this crucial data is rejected by the scientific community, then the argument for moonmilk biogenicity in this paper becomes much weaker.
This paper provides its' own culture experiments, as mentioned in Lines 304-329 and shown in Supp. Fig. 9. The methods for these experiments must be described in the Methods section- my apologies for missing that note last time. How much CaCl2 was used? How much urea? What was the temperature and carbonate saturation of the experiments? Most importantly, were sterilized experiments run during the research for this paper (not the unpublished work)? If so, sterilized comparisons would greatly help convince an audience that moonmilk is biogenic.
We added a new experiment, never published before, demonstrating that the microbial community is able to induce calcium carbonate precipitation.
The paper also needs to describe differences between the culturing experiments and the natural conditions in the bedrock. While it is impossible to exactly replicate the tomb environment inside a culture, the differences must still be addressed in the discussion. For example- do the authors think that Ca and urea are abundant in the bedrock environment? Especially important: What was the saturation of calcite in these experiments, and how does that relate to potential saturation states in the bedrock?
On a similar note, the authors provide a nice description of urea hydrolysis in the author reply. They mentioned leaving it out of the manuscript to avoid confusion, but I think this metabolism should be included in the discussion. Otherwise, the reader is missing crucial information on a potential process behind moonmilk formation.
Thank you, we clarified this point in the text.
For publication to continue, the authors should:
1: Describe culturing experiments in the Methods.
2: Change Benedetti references to "in review".
3: Provide extra discussion on the differences between culturing experiment conditions and conditions in the tombs- especially for chemical conditions (temperature, Ca, urea, calcite saturation).

4: Include a description of urea hydrolysis in the discussion. The best location is probably alongside the discussion of culturing experiments.

Thank you, for the above suggestions, the text was updated.

3) Section 3.3- RNA

The paper asserts that similar 16S RNA in bedrock and moonmilk are evidence for moonmilk biogenicity. While the results are interesting, and belong in the paper, such similarities alone are not enough to determine biogenicity, for one main reason.

There are other scenarios where moonmilk and bedrock communities are similar, but do not require biogenic moonmilk. For example, if the same microbes inhabited the entire porous bedrock sample before moonmilk formation, and then moonmilk formed abiotically through environmental change, both locations should have the same microbial populations. Therefore, similar populations can not distinguish biogenic vs abiogenic moonmilk.

When I brought this idea up in the previous round of reviews, the authors responded that the environment was unlikely to precipitate calcite abiotically. However, my issue here is not with environmental saturation- I've already addressed that topic. My issue is with the claim that similar RNA data can be used as evidence for biogenic moonmilk, as stated in Line 346:

These results show that in moonmilk and rocks the microbial composition is similar, irrespective of rock type (calcarenite and hybrid sandstone) or the environment where the samples were collected (outdoor or indoor). It should be noted that 16S SSU rRNA analysis does not provide information about metabolic activity, thus these data do not identify microorganisms that are active in CaCO3 deposition, but the overall data demonstrate that the endolytic community of the rocks is promoting moonmilk deposition.

In short, if the response to the question "Are similar communities in moonmilk and bedrock a biosignature?" is "Yes, but only because the environment cannot precipitate moonmilk", then the biosignature is not the RNA itself, but the presence of moonmilk in an undersaturated environment.

For publication to continue, the authors should:

1: Keep the RNA data and keep the comparisons of similar communities in the bedrock and moonmilk. However, any sentences that mention the RNA itself as evidence for biogenic moonmilk need to be removed (Lines 338-339, 350-352).

We followed the suggestion of the referee, and we updated the text accordingly.