Reply to Reviewer #1 comments **on the egusphere-2022-1457 manuscript**

*Dear Reviewer, thank you for your review and constructive suggestions on our manuscript "Biogenic calcium carbonate as evidence for life " (manuscript ID: **egusphere-2022-1457**). These comments are very valuable and helpful for us to revise and improve the manuscript. Below you will find answers to comments made in your reviews. The manuscript has been revised in accordance with your suggestions.*
Yours sincerely

This manuscript has an admirable goal- to determine whether or not certain forms of calcium carbonate constitute a biosignature. This is certainly of interest to the broad geobiologic and astrobiologic community (as outlined in the introduction), and I was intrigued by the sampling site and methods used. However there are some things that should be addressed before publication can proceed. Overall, I am very interested in the results of the paper, but framed such as they are currently, I find them inconclusive. Comments are as follows:

Lines 49-51: The Nutman et al. structures are highly controversial, the subject of great debate, and not at all accepted currently as definitive biogenic 'stromatolites'. They should not be presented so certainly.

We thank the referee for this comment, the text was changed accordingly and adding the reference of Alwood et al 2006 (Stromatolite reef from the Early Archaean era of Australia).

Lines 56-57: are the terms 'microbialite' and 'stromatolite' used interchangeably?

Not exactly, we used microbialite to indicate carbonate rocks of microbial origin, while stromatolite follows the definition of Riding 2011." Stromatolites are macroscopically layered authigenic microbial sediments with or without interlayered abiogenic precipitates". Thus, we added "stromatolite (sensu Riding 2011)".

Because the decline in stromatolite form diversity started in the Mesoproterozoic, with a large crash happening prior to the Neoproterozoic-Cambrian boundary, not in the Cambrian period (see papers by Frantz et al 2015; Awramik and Sprinkle, 1999).

Thank you for this comment, we modified the text to clarify we are not talking about stromatolite form diversity, we are explaining that the microbial carbonate factory decreased because of the increase and the expansion of biologically controlled carbonate production operated by organisms which strictly regulate their calcification (sensu Pomar 2020). Thus, not only stromatolite, but in general the production of carbonate induced or influenced by microbes decreases because of the increase of organisms with carbonate skeletons.

Also, many of the Archean forms are dubious in their biogenicity (Grotzinger and Knoll, 1999; Mclauglin et al, 2007).

Thank you for the comment; however, biogenic stromatolites are abundantly recorded (Alwood et al 2006, 2009, 2014, Schopfs 2011).

This seems to be my main general problem with the manuscript- the assumption is that all carbonates are biogenic instead of acknowledging that these forms are ultimately ambiguous.

In line 45 we wrote "Carbonate rocks on Earth are of abiogenic or biogenic origin". In the following lines 60-87 we discussed this point. We thank the referee for raising this point and the text was changed. However, recently, increasing evidence was found that many abiotic carbonates are contrarily of biogenic origin. For example, the ooid carbonate grains were considered for long time of abiotic origin. Recently Diaz and Eberli (2019) demonstrated that ooids are of microbial origin, considerably reducing the fraction abiotic carbonate production.

Certainly, if we are saying that carbonates on Mars mean that there was once life on Mars, the drive is on us to prove beyond a shadow of a doubt that the structures here on Earth are/were all biogenetic. It cannot just be assumed that they are.

Thank you for the comment, we clarified the text to express a different concept: (line 77-80= Carbonate rocks, being mostly of biogenic origin, could be considered in this perspective, but even if specific analyses could discriminate between biotic and abiotic carbonate rocks (Blanco et al., 2013), the attempt to unequivocally distinguish between carbonates of biogenic or abiogenic origin remains vacuous, especially in a search for evidence of life on other planets). Our only aim is to provide evidence that the formation of calcium carbonate nanofibers are biologically controlled/driven and, potentially, could represent a biosignature.

Lines 77-78: same problem as above- has it been conclusively proven that most carbonates have biogenic influence? The null hypotheses should be that these structures are abiogenic. Without that, the logic ends up being circular, and it is argued that these structures must be biogenic because carbonate structures are biogenic.

As above, in the line 75-80 we are explaining that "the mineral phases resulting from abiogenic or biogenic activity are indistinguishable, and the identification of irrefutable biosignature, evidence of past or present life, is still lacking. The attempt to unequivocally distinguish between carbonates of biogenic or abiogenic origin remains vacuous".
We are not concluding that carbonates are only biogenic, and, we apology if the text gave the idea we wanted to conclude that carbonate are only biogenic, we have now clarified this point.

Line 86: The authors rightly acknowledge that direct evidence for biogenicity is lacking. This should be the main point. Many research *assume* biogenicity, without direct evidence. The utility of this paper is an attempt to provide a direct link. Reframing the subject around this point would be more impactful.

Thank you for this comment, we have updated the text accordingly. Focusing on the bio related specific $CaCO_3$ structure, and not the $CaCO_3$ itself should solve this point.

Lines 93- 95: I am not familiar with the previous work on the subject, but why does containing an active microbial population mean the forms are conclusively biogenic? Microbes may just be living in the structure. Just because I live in a building does not mean I built it (see arguments in Grotzinger and Knoll, 1999, Petryshyn et al. 2021). It is nearly impossible to find a surface on Earth that does not contain an active microbial community.

We totally agree on this point, and we have clarified the text to report that in this case, the absence of karst features, such as abundant dissolution and reprecipitation processes with development of carbonate concretions and meteoric cements, is indicative that inorganic calcium carbonate precipitation from interstitial meteoric waters does not occur in the bedrock *where the tombs are carved. Moreover, the area of interest in this study insists on a high flat relief, in which the infiltrated water mostly derives from rainfall, without any groundwater circulation. Such evidence, along with the diffuse presence of microorganisms in the carbonate nanofibers deposit supports that the moonmilk origin could be controlled or driven by microbial metabolism. Finally, the Phila already analysed in other karst caves all over the world are very similar to the Phila reported from our studies, and this study could suggest a possible natural selection of specific microorganisms adapted to rocks rich in calcium carbonate.*

Line 94: is there any mechanism known by which abiogenic calcite nanofibers are precipitated?

We have added in the text a paragraph concerning hypothesis on the origin of the fibers, as an example, a paper was published claiming the abiogenic origin of calcite fibers: Jones, B., & Peng, X. (2014). This paper states: "that the calcite fibers found in the spring deposits at Shiqiang were a late diagenetic product that ***probably*** *formed through abiogenic precipitation". In this case evidence is reported that spring waters led to the formation of the fibers. By the way, there is no demonstration that these fibers are not biogenic, there are not microbiological experiments in this paper but only SEM analysis. Indeed, in fig 14F an entombment is showed, and many microorganisms are also present in SEM pictures. It should also be noted that the SEM analysis is not suitable for microbe observations because the largest part of microorganisms is destroyed by the vacuum of the SEM chamber. That's the reason why only filamentous microorganisms such as fungi and actinomyces or their spores are frequently observed; below an example is Figure 3, Moonmilk deposit in the Etruscan Stanza degli Scudi (Cirigliano et al 2018).*

Methods are good, sound, and well-described. I don't see a 16S plot of results in the figures or supplemental files. This would be interesting to look at.

Experimental 16S data have been added to the supplementary materials.

Lines 182-195: It is very interesting that the moonmilk is found within the walls as well. Is it possible that it was deposited there previously, and then covered by a new layer? Or is it possible that endolithic bacteria have burrowed into the wall, causing retrograde neomorphism and creating void space that could later be filled?

Data describe the extant microbial community and do not provide information about the previous history of the stones. Concerning the walls of the tombs, we have already published observations about the thickness of the moonmilk patina, comparing different tombs. Thera are differences in moonmilk thickness from calcarenite (thicker) and sandstone (thinner). This result correlates with higher calcium content in calcarenite than in sandstone. The interpretation is that microorganisms in calcarenite must cope with higher calcium content potentially detrimental for them, inducing a thick monnmilk deposition (Cirigliano et al 2021a). Thus, in this manuscript we present, for the first time, a comparison of the endolytic community of the rocks with the respective moonmilk community. We show in the manuscript that they are statistically very similar, hence the importance to deeply investigate in the future the microbial-rock interaction.

A scan of the 16S data might reveal the presence of endoliths, and would be helpful in interpretation of the SEM and petrographic analysis.

In this work we show that both the internal (endolytic) and the moonmilk forming external microbial community are statistically the same. Macroscopic and microcroscopic observations along with SEM analysis revealed that the moonmilk concretions developed inside the calcarenite in the vuggy and moldic porosity and in intergranular and moldic pores of the hybrid sandstone bedrock (Fig. 2., 3 and Supplement Fig 2-4), (Fig. 2b), regardless of where the samples were collected, outdoor or indoor. As shown in Supplem. Fig 5, moonmilk was also found in the void space at the contact between a fossil bivalve and the host calcarenite (Supplementary Fig. 5). Although dissolution effect induced by bacterial communities on the carbonate rock substrate cannot be excluded, the high porosity of the rocks is surely a peculiar character of the rocks hosting the moonmilk and, it is the physical chemical features of the substrate (i.e. the high value of open porosity and the carbonate composition) that promote the microbial colonization and the moonmilk deposition.

Line 208-214: This point, and SI Figure 9, are incredibly intriguing. However, with the evidence given, it may just be that the urea in solution fostered the precipitation of the calcium carbonate, as it is known to do, and that this nucleation centered around the colony because the charged surface is a good template for precipitation. Ideally, in order for this to be conclusive, I would like the authors to consider a labeled bicarbonate uptake experiment. If the labeled bicarbonate is incorporated into the calcite crystals, it would conclusively show that microbial metabolism is driving precipitation. Otherwise, this remains intriguing but inconclusive.

Thank you for the comment, the experiment with labeled bicarbonate will be considered for future experiments, by the way, since moonmilk cannot be reproduced in laboratory conditions, again this will be an indirect evidence. The result presented in supplementary 9 reports a carbonatogenic strain able to activate metabolic pathways promoting calcium carbonate precipitation. We tested hundreds of bacterial strains in the laboratory, and only a small percentage promotes calcium carbonate precipitation. Thus, many bacterial colonies just grow on YPD, urea and $CaCl_2$ without promoting calcium carbonate precipitation. Our evidence is that bacteria are centers of nucleation for nanofibers formation, they do not control the mineralization process directly, but induce the precipitation of calcium carbonate by changing the chemistry of the environment as a consequence of their metabolic activity and also serving as nuclei for crystallization. The mechanism consists of pH increase, direct effect of the negatively charged bacterial surface, presence of a metabolic ureolytic process. The three causes are intimately connected and are not untangled in the present analysis. Finally, our data and published data (Banerjee, S., & Joshi, S. R. (2014). Ultrastructural analysis of calcite crystal patterns formed by biofilm bacteria associated with cave speleothems. Journal of Microscopy and Ultrastructure, 2(4), 217-223.), showed that inactivated (killed) cells were unable to precipitate CaCO3 in laboratory, suggesting that cells need to be metabolically active for calcification and that cell structure alone is not sufficient to promote bioprecipitation.

Line 226: This should be noted as 16S SSU rRNA analysis, not 16S RNA.

Thank you, the text was changed as suggested.

Line 230-235: I agree with the authors, no habitat should be considered 'extreme' or a 'refuge'- microbes like it there!

Thank you for this comment, we consider this point very important.

Comment on egusphere-2022-1457', Anonymous Referee #2,

*Dear Reviewer, thank you for your review and constructive suggestions on our manuscript "Biogenic calcium carbonate as evidence for life " (manuscript ID: **egusphere-2022-1457**). These comments are very valuable and helpful for us to revise and improve the manuscript. Below you will find answers to comments made in your reviews. The manuscript has been revised in accordance with your suggestions.*
Yours sincerely

General Comments:

The manuscript has two goals: 1) to provide the first description of moonmilk and associated microfossils within rock cavities, and 2) to provide evidence for the biogenicity of moonmilk.

Both goals fit within BG's scope. The first goal is attained well, with certain areas that could be opened up for further discussion noted in the Specific Comments below. However, the second, larger goal to assess moonmilk biogenicity does not have enough evidence to support the paper's conclusions.

The first argument for biogenicity discusses microbial cells entombed in moonmilk crystals, as shown in Fig. 4 and Sup. Figs. 6-9. The pictures are beautiful, but do not conclusively prove that microbial metabolisms were the source of crystallization. In short, mineral precipitation on microbial cells can either occur 1) as microbial metabolisms influence local chemistry, or 2) due to external abiotic chemical conditions, just like a stick becomes encrusted with minerals in a hot spring (ex: Dupraz et al., 2009, Petryshyn et al., 2021, Geobiology).

Thanks for comment. The reviewer quoted some examples (Dupraz et al., 2009, Petryshyn et al., 2021) in which it is clear that the physio-chemical process for abiogenic carbonate precipitation is clearly driven by CO_2 degassing. In the Petryshyn case history, there is a spring, and the abiotic calcium carbonate precipitation takes place close to it. On the contrary, the investigated moonmilk developed in caves and rocks which are located in a flat elevated area, in the shallow vadose zone in which infiltrated water mostly derives from rainfall. Evidence of water circulation is generally lacking. On the other hand, if it was present, it should have left a diagenetic footprint producing meteoric mosaic cement between pores with clear (limpid) inclusion-free crystals.

For example, silica frequently precipitates on microbial cells in supersaturated conditions without any influence from metabolisms (Benning et al., 2004, GCA). The entombed cells are biosignatures, but the silica itself is not. Similarly, the microbial cells entombed in moonmilk are biosignatures, but unless the manuscript can show that moonmilk only occurs in the presence of biology, moonmilk itself cannot be a biosignature.

Thank you for this comment, indeed, to produce moonmilk, not only the negatively charged bacterial surface is needed, but also the specific metabolic pathway that is activated to cope with high calcium content is required. The biogenic $CaCO_3$ precipitation is widely studied for several technological applications (as an example, we studied this phenomenon in laboratory for bioconsolidation in the field of Cultural Heritage, but it is studied also for self-healing cement, sand consolidation etc.). We have not mentioned this point in the text to avoid confusion to the readers, here we are reporting a natural phenomenon. Thus, the mechanism of biogenic precipitation is well studied, the most common (and fast) metabolism for $CaCO_3$ precipitation is the ureolytic metabolic pathway. This process involves the production of carbamate (NH_2COOH) by urea hydrolysis, which spontaneously hydrolyses to form ammonia (NH_3) and carbonic acid (H_2CO_3). These products react with water to form carbonate (CO_3^{2}), ammonium ions (NH_4^+) and hydroxyl ions (OH^-), finally resulting in an increase of pH. In an alkaline environment, the presence of calcium ions and the bacterial cells, as nucleation site, allows the precipitation of calcium carbonate. Thus, it is not the presence of the bacterium alone, as a structure, that provides evidence of precipitation, but also high pH, metabolism, and negative charged membranes. In laboratory conditions (suppl Fig. 9), if urea is present, the mechanism is stimulated/accelerated.

The second argument for biogenicity involves the similarity of microbial communities within rocks and on their surfaces. While interesting, similarities between the two communities is not enough evidence to support biogenicity. For example, silica precipitation can preserve the same cyanobacteria on sinter surfaces and interiors (Phoenix et al., 2006, Geobiology). Supersaturated environments, whether hydrothermal or cavern fluids, will entomb similar microbes equally regardless of location.

In the area we studied, there is no evidence of cavern fluids or hydrothermal fluids in the investigated tombs. Actually, the absence of karst features such as abundant dissolution and reprecipitation processes with development of carbonate concretions and meteoric cements indicates that inorganic calcium carbonate precipitation from infiltrated meteoric waters does not occurs in the bedrock where the tombs are carved.

In short, the manuscript describes an interesting new location to search for microfossils and unique mineral precipitation, but the discussion of biogenicity either needs to be removed, or supported by new datasets.

We hope the answers to comments above clarified the point that not only the physical presence of microorganisms promotes the nanofibers formation, but also the active metabolism. Of course, nobody knows how these nanofibers elongated (Cirigliano et al 2018), and this point deserves more scientific research in the future and it is not the goal of this paper. In the future, the unique possibility to compare the moonmilk from 13 tombs could provide inspiration for future experiments.

I agree with the previous reviewer that labeled carbon isotope experiments would be a great way to test if metabolisms truly cause carbonate precipitation (e.g., Wilmeth et al., 2018, Front. In Microb.)- however, these methods are potentially too costly and/or time intensive for the scope of this study. Another alternative dataset would be to show that groundwater inside and around the tomb is undersaturated with respect to calcium carbonate, and could only precipitate with the assistance of microbial communities.

Thank you for the comments, we will continue this research and we will take this point into account.

Specific Comments:

Lines 37, 45, 66, 75-81: Like the previous reviewer, I agree that the introduction should shift away from the idea that carbonates are usually biogenic, especially since several types of abiotic carbonates are mentioned. Instead, the authors can make a simple change throughout the introduction: "many biogenic deposits are made from carbonate minerals". There is a subtle difference between "carbonate minerals are usually biogenic" and "many biogenic deposits are made from carbonate minerals". I think the second idea is closer to the point the authors make in Lines 77-81, that it's difficult to tell biogenic from abiogenic, which I definitely agree with.

Thank you, the text was changed in some parts as suggested.

Lines 49-51: The Nutman samples are highly controversial and can be removed from the discussion without damaging the arguments made.

This part of text was changed and the reference to Nutman et al 2016 structures was removed.

Lines 82-93: This specific section on moonmilk should be expanded to discuss research history and the debate of biogenic vs. abiotic formation. For example, Borsato et al., 2000 concludes that moonmilk is abiotic, an idea which is not mentioned in the current manuscript. At this stage, the reader would conclude that all previous moonmilk literature assumes biogenicity. A more detailed discussion would provide more context for the evidence presented.

Thank you for the comment, the text was revised. The paper Borsato et al., 2000 have already been cited, it is an important paper. Indeed, in their SEM images there are a lot of structures (figs 4-6) that could be microbial calcifications (a microanalysis is not presented in the paper, it is impossible to know if the fibers are calcium carbonate) and the authors stated in fig5: "These filaments are commonly composed of nanofibers, micrite grains, and biogenic (?) filamentous material.". Thus, there is not a clear demonstration of a-biogenicity origin.

Lines 96-97, 216: I'm not sure what is meant by a "geological process", can you please be more specific?

Thank you, we changed this part of the text for clarification.

I have no issues with the methods and materials- this is a very interesting sampling location, worth further research!

Thank you for the comment, indeed we are asking permission to take new samples to perform new experiments (also following the comments to this manuscript) and to get the physical parameters inside and outside the tombs for a long period of time.

Lines 183-199: Since this is the first description of moonmilk within rocks, a broader comparison with other moonmilk textures would be very helpful. Are there any similarities or differences between moonmilk inside the rocks vs. outside, or in other moonmilk locations?

Macroscopic and microscopic observations along with SEM analysis revealed that the nanofibers have developed inside the calcarenite in the vuggy and moldic porosity and in intergranular and moldic pores of the hybrid sandstone bedrock (Fig. 2., 3 and Supplement Fig 2-4), (Fig. 2b), regardless the samples were collected outdoors or indoors. As shown in Supplem. Fig 5, the calcium carbonate nanofibers was also found in the void space at the contact between a fossil bivalve and the host calcarenite (Supplementary Fig. 5). Although dissolution effect induced by bacterial communities on the carbonate rock substrate cannot be excluded, the high porosity of the rocks is surely a peculiar character of the rocks hosting the moonmilk and also the physical chemical features of the substrate (i.e. the high value of open porosity and the carbonate composition) that promote the microbial colonization and the moonmilk formation.

Lines 204-214: See General Comments. Entombment alone is not enough to prove biogenicity-minerals can precipitate on cells without direct metabolic influence. The manuscript needs to show that moonmilk can only be made in the presence of microbes.

From a geological point of view, the entombment due to inorganic precipitation of calcium carbonate may occur in environments with circulation of supersaturated fluids (i.e springs, hot springs, cascades, lacustrine areas). The area analysed here corresponds to a high flat relief, in which the infiltrated water mostly derives from rainfall, without any groundwater input.

From a biological point of view, by now, it is impossible to reproduce moonmilk in laboratory conditions. By the way, we can report that we have experience in calcium carbonate production in the laboratory, both from pure bacterial culture (an example is in suppl fig. 9) and from rock microbial cultures. We have evidence that the grinded calcarenite, with its entire microbial community, in a medium containing urea and CaCl2 easily and fast produced pure calcite. On the contrary, pure carbonatogenic bacterial cultures produce vaterite (Benedetti et al. 2023 submitted to J. of Cultural Heritage). It's clear that the field of microbial-rock interaction studies is still in its infancy. Of course, as already highlighted microbes are everywhere, by the way, all over the world, moonmilk always hosts very similar microbial communities that again, indirectly, suggest the intimate link microbes-rocks. This is the logical limit of the concept of the moonmilk biogenesis and the limit of its potential value as a beacon for life.

Lines 210-214: If there have been any hypotheses regarding how microbes produce nanofibers, even if currently untested, they should be discussed here. I agree with the authors that precipitation experiments are best performed using the entire microbial community and not single cultures alone. If it's possible to perform labeled isotope experiments or moonmilk saturation experiments on biofilms, that would be very interesting data. See also lines 82-93-alternative hypotheses regarding abiotic moonmilk formation should be discussed here as well.

Thank you, we clarified these points in the introduction. As stated above, we have evidence that the grinded calcarenite, with its entire microbial community, in a medium containing urea and CaCl2 easily and fast produced pure calcite. On the contrary, pure carbonatogenic bacterial cultures produce vaterite (Benedetti et al. 2023 submitted to J. of Cultural Heritage). Suggestions will be taken into account because we are planning experiments to try to understand the origin of nanofibres, a very complex point, that is beyond the scope of this paper.