nature portfolio

Peer Review File

A bovine pulmosphere model and multiomics reveal early host response signature in Tuberculosis

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This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Communications Biology.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript describes the characterization of a novel 3D pulmosphere model for M. tuberculosis infection in bovine systems. The model focuses on BTB pathogenesis and host responses during early-stage of infection. Although interesting data are included in the manuscript, the validation of some parameters are missing and should be included.

Major remarks:
- L132-133: Characterization of the spheres was only done via LC-MS. Is there a reason why a general method for characterization like immunohistochemistry for specific cell markers was not used? Are you sure that your method was sufficient for this characterization?
- The model is not validated in the sense that when a new pulmosphere is produced the same cells and ECM are found and in the same levels. In other words, if you produce a new pulmosphere will you have the same composition?
- L242: Were the transcriptome data validated by profiling secretion of inflammatory mediators via Luminex assay?

Minor remarks:

- L100-101: The last sentence of the introduction is a too strong statement: "…., this study enhances our understanding of TB pathogenesis, contributing to the global goal of TB eradication through the discovery of improved prevention and control strategies."
- L111: What is a "TC" plate?
- Figure 1: Scale bars are almost not visible.
- General comment: check the spelling/grammar. For example, L154: "are integral part lung basement membrane"
- L196: it is stated that "The BCG strain exhibited restrained replication within the pulmospheres". Is this evidenced by CFU enumeration or based on fluorescent signal?
- L200 (Fig3B): Was cellular necrosis in microspheres evaluated?
- L219-222: Was bacterial load also evaluated?

Reviewer #2

(Remarks to the Author)
The manuscript by Bhaskar et al. introduces a new 3D pulmosphere model to study the early host response to Mycobacterium tuberculosis infection in bovine systems. This model, which addresses significant limitations of traditional 2D cell culture systems, could revolutionize the field by more accurately replicating the multicellular complexity and extracellular matrix components of the lungs. This advancement is a crucial step towards understanding the host-pathogen interactions at

the cellular level, potentially leading to more targeted and effective tuberculosis control strategies, particularly in the agriculture sector. The manuscript is well-organized, with a clear and logical progression from methods through results and discussions. It contributes valuable insights to the field of infectious disease, specifically the study of tuberculosis, and has the potential to shape future research directions and drug discovery in a significant way.
While the manuscript by Bhaskar et al. presents a promising new 3D pulmosphere model, it's important to acknowledge that there are a few fundamental questions about this model. Addressing these concerns will not only strengthen the robustness of this new 3D model but also demonstrate the author's commitment to thorough and rigorous research.

1. It would be beneficial to include a validation section in which the expression levels of key markers identified in the transcriptomic and proteomic analysis are verified using real-time PCR.

2. Implement and describe the use of viability assays to evaluate the live and dead status of cells within the pulmospheres at various time points during the experiment. This will contribute to understanding cell dynamics in response to TB infection in the 3D model and differences between virulent and avirulent infections.

3. Determine the hypoxia status in the pulmospheres over the experimental conditions. This could reveal critical insights into how hypoxia influences TB pathogenesis and host responses in a 3D environment.

4. Additionally, host defense mechanisms functional within the 3D pulmospheres, such as ROS or NOS activity, may be analyzed, providing deeper insight into the efficacy of cellular responses in more physiologically relevant models.

Reviewer #3

(Remarks to the Author)
Dey et al describe the development of in vitro 3D pulmosphere derived from bovine lung tissues with the primary objective to establish a model that better recapitulates bovine TB and help elucidate (using a multi-omics approach) the host responses during the early phases of infection infected with either vaccine strains of M. bovis BCG or the H37Rv strain of M. tuberculosis sensu stricto.

Their study builds on previously published developments of 3D culture systems as part of a broader effort to reduce the reliance on animal models by providing more accurate in vitro models that better represent the physiological and biochemical characteristics of bovine (and other animal) lungs, particularly in the context of understanding host-pathogen interactions. This is a virtuous goal.

The authors provide -omic evidence that the 3D pulmosphere model constructed with bovine primary lung cells results in a three-dimensional structure that mimics the in vivo pulmonary environment with diverse cell types including macrophages, epithelial cells, fibroblasts, and alveolar cells, as well as evidence for extracellular matrix (ECM). The authors next apply transcriptome and proteome analyses to M. bovis BCG or M. tuberculosis H37Rv-infected pulmospheres, revealing differentially expressed genes and clusters that may help inform our understanding the molecular mechanisms governing host-pathogen interactions during TB in bovines. Based on this evidence, the authors suggest that the 3D pulmoshere represents “… a robust and reproducible ex vivo disease model” that “recapitulates bovine TB and elucidates the fundamental signaling pathways and pivotal genes/proteins implicated in host-pathogen interaction during the early phase of TB”.

While the overall goals are virtuous and the pulmonosphere model appears promising, there are several major weaknesses in the study design and interpretation that detract from the study and the robustness and relevance of the findings and conclusions.

1. There are considerable concerns regarding the robustness and reproducibility of the model that need to be clarified and better described. Important details regarding the source of the lung tissue, including species (Bos taurus? Bos indicus?), disease status (since bTB is endemic in India, were these confirmed IGRA negative animals from negative herds?), and other similarly essential details were not provided. More importantly, the number of individual animals used to derive the pulmospheres was not provided, and there is no evidence presented to show whether there were individual animal differences in host response? In the absence of this information, neither the robustness nor the reproducibility of the findings can be ascertained, and the utility of the model may be considerably reduced. The authors are encouraged to provide this information and also to discuss.

2. Perhaps of much greater concern, the relevance of “challenge” strains used for model validation and comparative analyses calls into question the premise and the rationale for the study design. In particular, the use of H37Rv as the strain used to represent “virulent” MTBC in the model system is both curious and unfortunate given the long understood and well-documented attenuation of this strain in the bovine system. See, for instance - https://pubmed.ncbi.nlm.nih.gov/29343690/ or https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2795854/). Hence, since H37Rv is not expected to be substantially attenuated and not cause much, if any, disease in cattle, the relevance of using this strain and lineage as a proxy for “virulent” MTBC in the context of a bovine model is neither well premised nor rationalized.

Moreover, since the stated goal of the study was to establish a system that replicates and enables a better understanding of the early pathogenesis of bovine TB, it would be expected to validate the model with M. bovis (or perhaps of greater relevance to India, M. orygis) instead.

Finally, given the many genetic and phenotypic differences between M. tuberculosis lineage 4 to which H37Rv belongs and M. bovis even outside of the attenuating RD1 region, the comparative analysis of H37Rv with M. bovis BCG is difficult to interpret in context and limits conclusions, if any, that might be inferred from the observed differences in transcriptional profiles.

Hence, the use of H37Rv and BCG considerably limit the interpretability of the results or assessment of the ability / utility of the model system to faithfully recapitulate bovine TB and the signaling pathways and genes/proteins involved in host-pathogen interaction during the early phase of TB, and the authors are encouraged to address this major shortcoming.

3. Given the focus on early responses, the use of a single 24h post infection time point seems reasonable. However, as presented in Figure 1B, for the 1d post-infection time point, despite the inoculation of 1:10 MOI of challenge strains, there are already apparently visible differences in BCG versus H37Rv signals. Thus, in the failure to include verified viable bacterial burden cell counts at that time point to better normalize or interpret results in context and without a time-course investigation of difference in expression, it is not clear whether the observed differences in transcriptional profiles result from underlying differences in the pathogens / their apparent virulence, or whether these are simply artefacts of dose or time-course related differences. This seems key since without this “normalization” or longitudinal analyses, it is difficult to interpret the observations in relevant context.

Finally, in general, the manuscript appears overly long with excessive hand-waving in the Discussion section (and too numerous citations) and could do with considerable trimming and copy-editing.

Taken together, while the authors report presents a promising step forward in the development of 3D pulmosphere models derived from bovine lung tissues, aiming to enhance the understanding of the pathogenesis of bTB through multi-omics approaches, it is encumbered by several critical limitations that need addressing to both assess the robustness of the model and to better validate and understand it’s utility in bTB and TB research.

I do hope that the authors are able to address the study limitations as noted above, since the pulmosphere model may represent a needed advance in the field and it would be good to see published.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have responded to all my comments and have even included additional information, such as the validation of the pulmospheres. The revised manuscript has improved significantly and I have no further comments.

Reviewer #2

(Remarks to the Author)
The response to comments, and additional data is satisfactory. No comments.

Authors response to the reviewer's comments on the manuscript (COMMSBIO-24-2254-T) by Bhaskar et al. "A pulmosphere model and multiomics reveal early host response signature in Tuberculosis".

The authors are thankful and appreciative of the thorough review and extremely valuable comments raised by the reviewer. The point-to-point responses to the reviewer's comments are presented below.

Reviewer's comments are in '*italics*' and Author's response are in '**bold**' font.

Reviewer #1:

***General observation*:** *The manuscript describes the characterization of a novel 3D pulmosphere model for M. tuberculosis infection in bovine systems. The model focuses on BTB pathogenesis and host responses during early-stage of infection. Although interesting data are included in the manuscript, the validation of some parameters are missing and should be included.*

Major remarks:

Comment 1*: L132-133: Characterization of the spheres was only done via LC-MS. Is there a reason why a general method for characterization like immunohistochemistry for specific cell markers was not used? Are you sure that your method was sufficient for this characterization?*

Author's response: **We appreciate the reviewer's insightful comment and suggestion to include additional characterization methods such as immunohistochemistry (IHC). Our choice of Liquid Chromatography-Mass Spectrometry (LC-MS) was guided by its capability to provide detailed proteomic profiles, allowing simultaneous identification and quantification of multiple cell-specific proteins and peptides in a single run. While IHC is an established method for detecting specific cell markers, it is often limited to a small number of proteins, depending on the availability and quality of antibodies. For bovine cell type identification, LC-MS offers a robust alternative, particularly given the limited availability of validated antibodies for bovine cells. LC-MS circumvents these limitations as it does not rely on antibodies, enabling unbiased and comprehensive molecular analysis. Its high sensitivity, specificity, and multiplexing capabilities make it an invaluable tool for the detailed characterization of our pulmospheres.**

Comment 2*: The model is not validated in the sense that when a new pulmosphere is produced the same cells and ECM are found and in the same levels. In other words, if you produce a new pulmosphere will you have the same composition?*

Author's response: We appreciate the reviewer for raising this important point regarding the reproducibility and consistency of our pulmosphere model. Indeed, to address this concern, we conducted additional analyses to validate the reproducibility of the model. Specifically, the RNA-Seq data that were used to evaluate the composition of extracellular matrix (ECM) components and cell types, we analysed the variation of data across 3 biological replicates, the origin of which were three different cow lungs derived cells. These analyses confirmed that there are no significant variations in cell

populations and ECM components across 3 independent sets of pulmospheres. We have included this additional data as a Supplementary Fig S4 in the revised manuscript. Moreover, our proteomic data obtained via LC-MS corroborate the RNA-Seq findings, further supporting the reproducibility of the model at both transcriptomic and proteomic levels. Together, these results suggest that our pulmosphere model demonstrates a reasonable degree of reproducibility and can produce spheres with consistent composition in terms of ECM and cell types across biological replicates.

Comment 3*: L242: Were the transcriptome data validated by profiling secretion of inflammatory mediators via Luminex assay?*

Author's response: We appreciate the reviewer for this insightful comment. While we did not use a Luminex assay, we validated the transcriptomic data using two complementary approaches. First, we performed real-time PCR analysis to validate the expression of key genes shortlisted as a signatures of early *M. tuberculosis* infection. The real time PCR data confirmed the patterns observed in the RNA-Seq data. This new data is now included in the revised manuscript as Fig 11D and additional subsection in the results, materials and methods have also been included in the revised manuscript. Second, the proteomics data obtained via LC-MS further supported the consistency of inflammatory mediator profiles at the protein level, aligning with the transcriptomic findings. These validation approaches provide additional evidence of the reliability of our transcriptomic data.

<u>Minor remarks:</u>

Comment 4: L100-101: The last sentence of the introduction is a too strong statement: "…., this study enhances our understanding of TB pathogenesis, contributing to the global goal of TB eradication through the discovery of improved prevention and control strategies."

Author's response: We appreciate the reviewer's feedback regarding the strength of the concluding statement in our introduction. Upon reflection, we agree that the assertion may have been overly ambitious. We acknowledge that while our study contributes valuable insights into TB pathogenesis, it may not singularly lead to the discovery of improved prevention and control strategies on a global scale. We have now revised the last sentence of the introduction to better reflect the findings and implications of our study as: "..., this study adds to our understanding of TB pathogenesis, potentially informing efforts aimed at developing effective prevention and control strategies."

Comment 5:- L111: What is a "TC" plate?

Author's response: Thank you for bringing this to our attention. Apologies for oversight TC plate means Tissue culture plate and it is now modified in the revised manuscript accordingly.

Comment 5:- Figure 1: Scale bars are almost not visible.

Author's response: Thank you for bringing this to our attention. We apologize for the oversight and understand the importance of clearly visible scale bars in our figures. In

the revised manuscript, we have included a revised figure ensuring that the scale bars are clearly visible.

- *General comment 6: check the spelling/grammar. For example, L154: "are integral part lung basement membrane"*

Author's response: Thank you for bringing this to our attention. To rectify the oversight, the revised manuscript is thoroughly checked for spelling/grammar and corrected as appropriate.

Comment 7- L196: it is stated that "The BCG strain exhibited restrained replication within the pulmospheres". Is this evidenced by CFU enumeration or based on fluorescent signal?

Author's response: In this study, the bacterial growth was monitored via measurement of total fluorescent signal from each pulmospheres infected with fluorescent reported strains of bacteria. This allowed us to monitor bacterial replication kinetics longitudinally on each infected pulmospheres. A strong correlation of the fluorescence levels to the number of reporter bacteria were confirmed and published in our previous work (Kumar et. al. 2024. https://doi.org/10.3389/fimmu.2023.1199092).

Comment 8- L200 (Fig3B): Was cellular necrosis in microspheres evaluated?

Author's Response: We thank the reviewer for this important query. While cellular necrosis was not directly evaluated in this study, we have now performed extensive additional experiments to characterize the pulmosphere cellular status, such as cell viability via live/dead assay, reactive oxygen species (ROS) levels, and hypoxia levels. These additional data are now included in the revised manuscript as Fig 2 A-F, and related sections in the results, discussion, and materials and methods are also added.

Comment 8- L219-222: Was bacterial load also evaluated?

Author's Response: As mentioned in response to Comment 7 above, bacterial load was not directly evaluated by CFU plating in this study, instead bacterial total fluorescence was measured in each pulmosphere and used as a surrogate to indicate the bacterial load. This approach allowed for a more precise and dynamic evaluation of bacterial replication longitudinally on the same sample using a fluorescence microscope capable of capturing three dimensional sphere images and provide total fluorescence data.

Reviewer #2:

General observation: The manuscript by Bhaskar et al. introduces a new 3D pulmosphere model to study the early host response to Mycobacterium tuberculosis infection in bovine systems. This model, which addresses significant limitations of traditional 2D cell culture systems, could revolutionize the field by more accurately replicating the multicellular complexity and extracellular matrix components of the lungs. This advancement is a crucial step towards understanding the host-pathogen interactions at the cellular level, potentially leading to more targeted and effective tuberculosis control strategies, particularly in the agriculture sector. The manuscript is well-organized, with a clear and logical progression

from methods through results and discussions. It contributes valuable insights to the field of infectious disease, specifically the study of tuberculosis, and has the potential to shape future research directions and drug discovery in a significant way.

While the manuscript by Bhaskar et al. presents a promising new 3D pulmosphere model, it's important to acknowledge that there are a few fundamental questions about this model. Addressing these concerns will not only strengthen the robustness of this new 3D model but also demonstrate the author's commitment to thorough and rigorous research.

Author's response: We appreciate the reviewer's general observation about the significance and implication of this research especially in the area of tuberculosis. And we have also performed extensive additional experiments to addressed all of the concerns raised by the reviewer.

Comment 1: It would be beneficial to include a validation section in which the expression levels of key markers identified in the transcriptomic and proteomic analysis are verified using real-time PCR.

Author's response: We appreciate the reviewer's valuable suggestion to include a validation section using real-time PCR to verify the expression levels of key markers identified in our transcriptomic and proteomic analyses. We agree that this additional validation would strengthen the robustness of our findings. Accordingly, we have now included the real-time PCR validation data of the key markers in the revised manuscript (Fig 11D) to provide further confirmation of our results.

Comment 2: Implement and describe the use of viability assays to evaluate the live and dead status of cells within the pulmospheres at various time points during the experiment. This will contribute to understanding cell dynamics in response to TB infection in the 3D model and differences between virulent and avirulent infections.

Author's response: We appreciate the reviewer's insightful suggestion to include viability assays to evaluate the live and dead status of cells within the pulmospheres. We agree that such assays would contribute valuable information on cellular live/dead-state dynamics within the pulmosphere. We have now performed live/dead assays on the pulmospheres over a 21-day period, using CFSE to stain live cells and propidium iodide for dead cells. These assays provided us with information on the viability of cells in the pulmosphere model under baseline conditions. The new data is included in the revised manuscript as Fig 2 A-C and additional text is also included in relevant sections.

Comment 3: Determine the hypoxia status in the pulmospheres over the experimental conditions. This could reveal critical insights into how hypoxia influences TB pathogenesis and host responses in a 3D environment.

Author's response: We appreciate the reviewer's suggestion to investigate the hypoxia status within our pulmospheres. Understanding the role of hypoxia in TB pathogenesis and host responses is indeed critical, particularly in a 3D environment that closely mimics *in vivo* conditions. We have now performed additional assays on the pulmospheres over a 21-day period to measure hypoxia and ROS levels. These assays provided us with information on the hypoxia and ROS levels in the pulmosphere model

under baseline conditions. The new data is included in the revised manuscript as Fig 2 D-F and additional text is also included in relevant sections.

Comment 4: Additionally, host defense mechanisms functional within the 3D pulmospheres, such as ROS or NOS activity, may be analyzed, providing deeper insight into the efficacy of cellular responses in more physiologically relevant models.

Author's response: We appreciate the reviewer's insightful suggestion to analyze host defence mechanisms such as reactive oxygen species (ROS) or nitric oxide synthase (NOS) activity within the 3D pulmospheres. As mentioned in response to comment 4 above, in addition to measuring hypoxia, we have now performed additional assays on the pulmospheres over a 21-day period to measure ROS levels. The new data is included in the revised manuscript as Fig 2D-F and additional text is also included in relevant sections.

Reviewer #3:

General observation: Dey et al describe the development of in vitro 3D pulmosphere derived from bovine lung tissues with the primary objective to establish a model that better recapitulates bovine TB and help elucidate (using a multi-omics approach) the host responses during the early phases of infection infected with either vaccine strains of M. bovis BCG or the H37Rv strain of M. tuberculosis sensu stricto.

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While the overall goals are virtuous and the pulmonosphere model appears promising, there are several major weaknesses in the study design and interpretation that detract from the study and the robustness and relevance of the findings and conclusions.

Comment 1: There are considerable concerns regarding the robustness and reproducibility of the model that need to be clarified and better described. Important details regarding the

source of the lung tissue, including species (Bos taurus? Bos indicus?), disease status (since bTB is endemic in India, were these confirmed IGRA negative animals from negative herds?), and other similarly essential details were not provided. More importantly, the number of individual animals used to derive the pulmospheres was not provided, and there is no evidence presented to show whether there were individual animal differences in host response? In the absence of this information, neither the robustness nor the reproducibility of the findings can be ascertained, and the utility of the model may be reduced. The authors are encouraged to provide this information and also to discuss.

Author's response: We appreciate the reviewer for raising this important point regarding the reproducibility and consistency of our pulmosphere model. We appreciate the opportunity to clarify and strengthen the manuscript by addressing these concerns in details in the revised manuscript as outlined below:

The lung tissues used for deriving the 3D pulmospheres were obtained from crossbred Sahiwal × Holstein Friesian cattle (Bos indicus × Bos taurus). These animals were sourced from a government-approved slaughterhouse, where they underwent thorough clinical and post-mortem inspection by certified veterinarians. To ensure the inclusion of only mycobacteria-naive and healthy animals, post-mortem lung tissue samples were carefully inspected for any pathological lesions. Additionally, a section of the same lung tissue was subjected to PCR-based evaluation for *Mycobacterium tuberculosis* Complex (MTBC) pathogens and non-tuberculous mycobacteria (NTM). Only tissues that tested negative for all mycobacterial DNA were included in the study. Pulmospheres were derived from three animals per experimental group. From each animal, at least 10 pulmospheres were included for analysis in each group. This experimental design allowed us to capture biological replicates and assess individual animal variations in host responses. Additionally, to address concerns regarding reproducibility, we conducted additional analyses to validate the consistency of the pulmosphere model. Specifically, the RNA-Seq data used to evaluate the composition of extracellular matrix (ECM) components and cell types were analyzed for variation across three biological replicates derived from three different cow lungs. These analyses confirmed no significant variations in cell populations or ECM components across the independent sets of pulmospheres. We have included this additional data as a new Supplementary Fig S4 in the revised manuscript. This information underscores the utility of the 3D pulmosphere model as a robust and reproducible system. These updates have now been incorporated into the revised manuscript, and we hope they comprehensively address the reviewer's concerns regarding the robustness, reproducibility, and utility of the model.

Comment 2: Perhaps of much greater concern, the relevance of "challenge" strains used for model validation and comparative analyses calls into question the premise and the rationale for the study design. In particular, the use of H37Rv as the strain used to represent "virulent" MTBC in the model system is both curious and unfortunate given the long understood and well-documented attenuation of this strain in the bovine system. See, for instance - https://pubmed.ncbi.nlm.nih.gov/29343690/ or https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2795854/). Hence, since H37Rv is not

expected to be substantially attenuated and not cause much, if any, disease in cattle, the relevance of using this strain and lineage as a proxy for "virulent" MTBC in the context of a bovine model is neither well premised nor rationalized.

Moreover, since the stated goal of the study was to establish a system that replicates and enables a better understanding of the early pathogenesis of bovine TB, it would be expected to validate the model with M. bovis (or perhaps of greater relevance to India, M. orygis) instead.

Finally, given the many genetic and phenotypic differences between M. tuberculosis lineage 4 to which H37Rv belongs and M. bovis even outside of the attenuating RD1 region, the comparative analysis of H37Rv with M. bovis BCG is difficult to interpret in context and limits conclusions, if any, that might be inferred from the observed differences in transcriptional profiles.

Hence, the use of H37Rv and BCG considerably limit the interpretability of the results or assessment of the ability / utility of the model system to faithfully recapitulate bovine TB and the signaling pathways and genes/proteins involved in host-pathogen interaction during the early phase of TB, and the authors are encouraged to address this major shortcoming.

Author's response: We thank the reviewer for raising this critical concern regarding the selection of bacterial strains used for infection experiments and the relevance of these choices to the study's objectives. We have carefully considered the points raised and have taken steps to address this major shortcoming in the revised manuscript.

While we concur that *M. tuberculosis* H37Rv has lower infectivity in the bovine system compared to *M. bovis*, we respectfully disagree with the assertion that H37Rv is not a virulent strain. A wealth of studies document clinical infections caused by *M. tuberculosis* in cattle and other animal species globally, emphasizing its role as a pathogen with significant cross-species transmission capability. The inclusion of M. tuberculosis H37Rv as a model strain in our study provides important insights into host-pathogen interactions, particularly in understanding the host response to a pathogen with well-characterized virulence determinants. The use of *M. bovis* BCG as an attenuated strain allows for a direct comparison of host responses between a pathogenic strain (H37Rv) and an attenuated strain lacking the RD1 region and other virulence determinants. This comparison provides valuable information about the differential host responses elicited by virulent and attenuated MTBC strains, aiding in the identification of early infection markers.

We fully agree with the reviewer that validation with more relevant MTBC species such as *M. bovis* and *M. orygis* is critical for establishing the broader utility of the model, particularly in the Indian context wherein an increase in the reporting of *M. orygis* infection in cattle has been on the rise. To address this, we performed additional validation experiments using virulent *M. bovis* AN5 and *M. orygis* NIAB1 strains (a bovine pulmonary TB clinical isolate). These experiments validated the transcriptional gene signature identified during *M. tuberculosis* infection by assessing the expression of key genes using real-time PCR following infection of pulmospheres with these pathogens. This consistency underscores the robustness and relevance of the gene signature identified in our study. These new validation data have been included in the

revised manuscript as Fig 11D, significantly enhancing the interpretability and utility of the 3D pulmosphere system as a model for studying early host-pathogen interactions in bovine TB.

Comment 3. Given the focus on early responses, the use of a single 24h post infection time point seems reasonable. However, as presented in Figure 1B, for the 1d post-infection time point, despite the inoculation of 1:10 MOI of challenge strains, there are already apparently visible differences in BCG versus H37Rv signals. Thus, in the failure to include verified viable bacterial burden cell counts at that time point to better normalize or interpret results in context and without a time-course investigation of difference in expression, it is not clear whether the observed differences in transcriptional profiles result from underlying differences in the pathogens / their apparent virulence, or whether these are simply artefacts of dose or time-course related differences. This seems key since without this "normalization" or longitudinal analyses, it is difficult to interpret the observations in relevant context.

Author's response: We thank the reviewer for highlighting the importance of normalizing bacterial burdens and considering longitudinal analyses to better interpret transcriptional differences observed in our study.

We acknowledge the challenge of ensuring equal bacterial numbers within the pulmospheres at 24 hours post-infection, which the reviewer correctly pointed out. Despite inoculating with the same multiplicity of infection (MOI) of 1:10 for both M. tuberculosis H37Rv and M. bovis BCG, achieving identical bacterial burdens at Day 1 is inherently difficult due to the known differences in infectivity and intracellular multiplication between these strains. Specifically, *M. bovis* BCG, being an attenuated vaccine strain, demonstrates compromised infectivity and intracellular growth compared to the virulent M. tuberculosis H37Rv. This phenomenon is well documented in tuberculosis research and is a limitation for direct normalization at later time points. Consequently, for the current study, normalization was based on the equal number of bacterial inoculum at the time of infection (MOI 1:10), which is a standard approach for comparative analyses of host responses. To address concerns about the relevance of observed transcriptional differences, we have now performed additional validation experiments with *M. bovis* AN5 and *M. orygis* NIAB1, two pathogenic strains more relevant in the bovine context. The transcriptional gene signatures identified during *M. tuberculosis* infection were validated using real-time PCR analysis of pulmospheres infected with these strains and included in the revised manuscript as Fig 11D. The data confirmed consistent early infection gene signatures across *M. tuberculosis* H37Rv, *M. bovis* AN5, and *M. orygis* NIAB1, supporting the robustness of our findings despite the limitations related to bacterial burden normalization.

We agree with the reviewer that a longitudinal investigation of host responses to infection would yield additional valuable insights. This remains a priority for future research and subsequent projects focused on expanding the utility of the 3D pulmosphere model. However, such analyses are beyond the scope of the present study, which is primarily focused on establishing and characterizing the model as a proof-of-concept. We have included this discussion and the new validation data in the revised

manuscript, which we hope addresses the reviewer's concerns. We are grateful for this constructive feedback, which has allowed us to strengthen our study further.

General comment: Finally, in general, the manuscript appears overly long with excessive hand-waving in the Discussion section (and too numerous citations) and could do with considerable trimming and copy-editing.

Taken together, while the authors report presents a promising step forward in the development of 3D pulmosphere models derived from bovine lung tissues, aiming to enhance the understanding of the pathogenesis of bTB through multi-omics approaches, it is encumbered by several critical limitations that need addressing to both assess the robustness of the model and to better validate and understand it's utility in bTB and TB research. I do hope that the authors are able to address the study limitations as noted above, since the pulmosphere model may represent a needed advance in the field and it would be good to see published.

Author's response: We sincerely thank the reviewer for their constructive feedback and for recognizing the potential of our study as a needed advance in the field of bovine TB research. We greatly appreciate the acknowledgment of the promise held by the 3D pulmosphere model and the encouragement to see this work published.

In response to the reviewer's comments, we have made substantial revisions to the manuscript to streamline the Discussion section, ensuring that it is more concise and focused. Excessive citations and redundant content have been removed to improve readability and coherence. Number of citation in the revised manuscript is reduced to 71 from 99. The revised manuscript now emphasizes the key findings and their implications while avoiding unnecessary elaboration.

We believe that the revised manuscript now effectively addresses the reviewer's concerns and provides a robust foundation for the 3D pulmosphere model as a tool for TB research in general and bovine TB research in specific. We are grateful for the reviewer's insights and encouragement, which have been instrumental in strengthening the manuscript and ensuring its relevance to the field.