Peer Review File

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Reviewer A

This paper investigates the transcriptomic response to Stenotrophomonas maltophilia lung infection in mice. The authors have established a reproducible mouse model and investigated in a time course experiment the gene expression after infction. The work described seems solid but there are several points to be considered to improve the manuscript.

In general the figures should be improved: most of them are unreadable and have too many panels.

In particular:

-Figure 1: the bar joining the two curves is confusing as it is, please remove it or modify. Please also expand the histopathological description of the lungs, at least in the figure legend.
Response: Thanks for your good suggestion. The figure 1 had been revised. And the histopathological description had been added in the figure 1 legend.

-Figure 2: panel A (PCA) is not readable. Panels B and C could be easily removed and substituted with a table reporting the number of up and down-regulated genes at each time point. The figure in panel D is partially cut, please change the legend removing "SMA_" and only leaving Nhpi,
Response: Thanks for your good suggestion. According to reviewer's suggestion, the figure 2 had been revised.

-Figure 3 is again barely readable. In panel A the x axis should only report the time points (0, 4, 12 etc), the authors should also explain the "membership" parameter, also the y axis should be normalized expression (I believe).
Response: Thanks for your good suggestion for improving the quality of manuscript. The figure 3 had been revised. And the "membership" was explained in the figure 3 legend.

Figure 4: it is irrelevant to show the Dynamic Tree Cut. The heatmap is unreadable, it could be reduced to show only the significant correlations.
Response: Thanks for your good suggestion. The figure 4 had been revised. Dynamic Tree Cut represents the initial division of modules based on correlation, and merged colors represents the result of merging similar modules. In the report (Front Cell Infect Microbiol. 2022 Apr

27;12:833080), the Dynamic Tree Cut was still showed in the results. In our opinion, the Dynamic Tree Cut was needed.

Figure 6: panel A is again not readable, are data presented here the same as in panels B and C? In this case redundancy should be avoided and panel A deleted. Also it is not clear what "abundance" parameter represents.
Response: Thanks. The panel A showed abundance of 36 immune cell types in all samples. A similar panel was reported in the reference (Front Cell Infect Microbiol. 2022 Apr 27;12:833080). The meaning of “abundance” was added in the figure 6 legend.

lines 331-340: the authors should probably try to analyze the shared up and down regulated genes separately. A simple enrichment analysis of the DEGs by time point is missing.
Response: Thanks for your good suggestion. There was a total of 1,747 common DEGs for all time points. In 1747 common DEGs, 1078 DEGs were up regulated and 635 DEGs were down regulated. The result was showed in the figure 2D and E. A total of 3,165 upregulated DEGs and 2,231 downregulated DEGs were detected at 4 hpi, 2,798 upregulated DEGs and 2,774 downregulated DEGs were detected at 12 hpi, 2,423 upregulated DEGs and 2,398 downregulated DEGs were detected at 24 hpi, 2,601 upregulated DEGs and 2,877 downregulated DEGs were detected at 48 hpi, and 2,512 upregulated DEGs and 2,468 downregulated DEGs were detected at 72 hpi. The enrichment analysis was represented in the page 11.

line 357: the behaviour of cluster 3 seems different from 5 and 7
Response: Thanks. The sentence had been revised in the page 11.

lines 408-420: the layers should be better explained also here
Response: Thanks. The Immune Cell Abundance Identifier uses a hierarchical strategy that divides cells into three levels based on cell lineage to ensure accuracy in the prediction of cell types and their subtypes. B cell, NK cell, and T cell are three of the lymphoid lineage cells in layer 1, and macrophage, DC, monocyte, and granulocyte are four of the myeloid lineage cells. Cells in layer 3 are subtypes of CD4 T and CD8 T cells, while cells in layer 2 are subtypes of cells in layer 1 (Bioinformatics. 2022 Jan 12;38(3):785-791). The related contents were added in the page 13.

Finally, in my opinion, there is no need to cross-validate RNAseq with qPCR, which is inherently less sensitive and accurate, I would delete or move to the supplementary materials the paragraphs and figure 7.
Response: Thanks. In our opinion, it is better to validate the results of the RNA-Seq data by qPCR. In some reports (DOI: 10.3389/fcimb.2022.833080), the validation of the RNA-Seq data

by qPCR was performed.

In the discussion the authors should compare their results with other transcriptomic analyses performed in mice infected with other bacteria. Many parts could be shortened, e.g. lines 433-440 summarizing the results. lines 449-455, where the authors found the same results using two different analysis methods. I found that many references in the discussion are not really appropriate, e.g. refs 28 and 29. Lines 465-466: "For energy to be supplied to the body, cardiac metabolism is essential" does not make much sense and should be deleted, the same for line 492 "The response to the host may be bidirectional"
Response: Thanks. The ref 28 and 29 had been replaced by new refs in the discussion (page 15). The line 465-466 and 492 had been deleted in the discussion (page 15 and 16).

Also the idea that the pathways identified in the study could be a therapeutic target, despite being tempting, is probably not worth to mention, since the authors identified conserved inflammatory pathways which are probably redundant.
Response: Thanks. Some sentences had been deleted in the discussion (page 16).

Other points:

line 36: invasive and fatal infections
Response: Thanks. We had revised the sentence in the abstract (page 2).

lines 39-40: delete "and to identify..."
Response: Thanks. We had deleted the sentence in the page 2, line 39.

line 49: predicted abundance
Response: Thanks. We had revised in the page 2, line 49.

lines 55-56: delete "which may suggest..."
Response: Thanks. We had deleted the sentence in the page 2, line 55.

lines 79-83: this is not much relevant for this study
Response: Thanks. We had deleted the sentence in the page 3, line 79-83.

line 94: adhesion to
Response: Thanks. We had revised in the page 3, line 94.

line 102: it is really rare that any pathogen has a really specific signature of infection

Response: Thanks. We had revised in the page 4.

lines 121-123: plant biology and cancer research are not relevant here
Response: Thanks. We had revised in the page 3, line 123.

line 133: what does "stored in our lab" mean?
Response: Thanks. Here, "stored in our lab" was meant that the used SMA strain K279a in the study was kept or cryopreservated in our lab.

lines 134-135: the protocol of growth is not clear: the cultures were incubated ON and then diluted in fresh medium? please use rcf not rpm
Response: Thanks. The protocol had been revised in the page 5.

lines 136-142: The protocol for inoculum preparation is not very clear. The authors state that the pellet was resuspended in 1 ml of PBS up to an OD of 1 corresponding to 5x10^8 CFU/ml, but then they use 50 ul as an inoculum, please clarify. It is also not clear what the 24 hrs of inversion are for.
Response: Thanks. The protocol had been revised in the page 5.

line 142: Why did the authors use male mice?
Response: Thanks. In the study, the male mice were used avoiding the effects of estrogen.

line 155: please specify the supplier of the anesthesia machine
Response: Thanks. We had revised in the page 5, line 155.

line 163: please specify the number of mice/group here
Response: Thanks. We had revised in the page 5, line 165.

lines 166-168: "the goups were named..." is not relevant and can be deleted
Response: Thanks. We had deleted the sentence in the page 6, line 168.

lines 184-195: please report the method for freezing and storing lungs; please specify the kit used for library prep, please specify the ratio between DNA and beads (v:v); please add details on adapter ligation, flow cell type and cycles used
Response: Thanks. The methods for freezing and storing lungs had revised in the page 6. Construction of the library and sequencing were carried out by Novogene Company (Beijing, China). Because the ratio between DNA and beads (v:v) and adapter ligation are the business secrets of the company. Novogene Company can’t provide the information for us. 15 cycles of PCR were performed. It was added in the page 7.

lines 201-202: the pocess of "ceaning" reads is not clear

Response: Thanks. To ensure the quality and reliability of data analysis, it is necessary to screen the original data. The containing adapter, reads containing N base and low-quality reads (the base number of Qphred<=5 accounts for more than 50% of the entire read length in reads) were removed. The image data measured by the high-throughput sequencer are converted into sequence data (reads) by CASAVA base recognition. Raw data (raw reads) of fastq format were firstly processed through in-house perl scripts. In this step, clean data (clean reads) were obtained by removing reads from raw data. At the same time, Q20, Q30 and GC content the clean data were calculated. All the downstream analyses were based on the clean data with high quality.

line 204: which is the genome website used?

Response: Thanks. Here, the genome (mm10) was used.

lines 208-209: the use of FPKM is quite "old-fashioned"; how was PC performed?

Response: Thanks. FPKM is currently the most commonly used method for estimating gene expression levels. In the latest report (Proc Natl Acad Sci U S A. 2023 Apr 18; 120(16): e2213512120), the FPKM was still used.

line 215: the DESeq2 version seems pretty old (probably 5 years)

Response: Thanks. The DESeq2 version seems pretty old, but it was still used in Novogene Company (Beijing, China).

line 235: WGCNA is a computational technique

Response: Thanks. We had revised in the page 8, line 236.

lines 294-295: please rephrase "survival events" is not really sound

Response: Thanks. We had revised in the page 9, line 296.

line 296 "demise"?

Response: Thanks. We had revised in the page 9, line 297.

line 297: please specify the number of deaths out of the total number of inoculated mice

Response: Thanks. We had stated clearly the number of deaths in page 10.

line 299: please specify the number of deaths

Response: Thanks. We had stated clearly the number of deaths in page 10.

line 319: please specify the number of libraries/group
Response: Thanks. The number of libraries was 22, page 10.

lines 320-323: this info is not much important, can be synthetisez and probably does not deserve a table in the main text
Response: Thanks. The sentence had been deleted in the page 10.

lines 323-324: please delete the sentence "All libraries..."
Response: Thanks. We had deleted the sentence in the page 10, line 323-324.

lines 325-327: the authors should try to describe PCA results, I would at least specify the different clustering of time 0 compared to post infection
Response: Thanks. PBS group and post infection group samples were dispersed over various locations, according to the PCA scores plot. The samples were scattered between groups, and samples within groups were clustered, indicating good duplication within groups and significant differences between groups.

line 346: I would avoid the term "discovered"
Response: Thanks. We had revised in the page 11, line 347.

Table 3, data at 72 hpi are missing, I would also show the put the lfc column before the adjusted p-value
Response: Thanks. The date at 72 hpi were not missed in the table 3.

line 375: the authors say "these ten genes" about cluster 6, while the cluster contains 3008 genes
Response: Thanks. The gene numbers had been revised in the page 11 and 12.

Reviewer B

Stenotrophomonas maltophilia (SMA) is an important gram-negative multidrug-resistant bacterial pathogen that is widely distributed in the environment and has an effect on human health around the world. In the manuscript “Time-course transcriptome analysis of lungs from mice infected with inhaled aerosolized Stenotrophomonas maltophilia”, authors performed RNA sequencing (RNA-seq) of lung tissues from mice with pulmonary SMA infection over time via aerosolized intratracheal (i.t.) inhalation to investigate transcription profile changes in SMA-infected lungs and to identify genes or other valuable research targets.

Couple questions are required to be answered before it will be accepted.

(1) In the study, why to focus on SMA? Please state in the introduction.

Response: Thanks for your good suggestions. Stenotrophomonas maltophilia (SMA) is an important gram-negative multidrug-resistant bacterial pathogen that is widely distributed in the environment and has an effect on human health around the world. It becomes increasingly challenging to treat SMA infection effectively. Novel therapeutic and preventive approaches targeting SMA infection are still lacking. In our previous research (BMC Infect Dis. 2018 Jul 27;18(1):347), outer membrane proteins (Omps) from SMA were isolated and potential vaccine candidates of Omps against SMA were identified. These related contents were added in the introduction, page 3.

(2) What were the associations between COVID-19 and SMA infection? Please state in the introduction.

Response: Thanks for your good suggestions. SMA are Gram-negative bacilli involved in nosocomial infections, notably hospitalacquired pneumonia (HAP) in the intensive care unit (ICU) setting. Bacterial superinfection occurs in approximately 20% of coronavirus disease of 2019 (COVID-19) patients within two days of intubation and can progress to ventilator-associated pneumonia (VAP). A latest research (Sci Rep. 2023 Feb 28;13(1):3392) reported that there was a significant association between COVID-19 and SMA infection. But the Reviewer A considered that the related contents were not much relevant for this study. We accept the suggest of Reviewer A. So, the related contents had not been supplemented in the introduction.

(3) How to identify the mouse model of acute lethal SMA pneumonia was successfully established? Please state in the methods.

Response: Thanks. Mice model were successfully established by inoculated via the aerosolized intratracheal route with SMA (ref 16). In our lab, the technique of mice model of SMA pneumonia is a routine technique. The observation of H&E staining from infected mice was consistent to the report (Infect Immun. 2010 Jun;78(6):2466-76.). And the pathological characteristics of SMA mice model were detected by H&E staining in our pre-experiment.

(4) What were the roles of endoplasmic reticulum stress (ERS) in the infection with SMA? Please supplement in the discussion.

Response: Thanks. Endoplasmic reticulum stress (ERS) is defined as the accumulation of unfolded or misfolded proteins in the ER and subsequently triggers the unfolded protein response. Recently, studies have demonstrated that ERS is involved in infected pneumonia. And, it is definitely confirmed that ER stress has emerged as a novel autophagy inducer. Bacterial infection could activate ER stress and autophagy in alveolar epithelial cells. In the study, the expression of genes associated with ERS and autophagy increased at 24 hpi. So, the

ERS played an important role in the infected pneumonia. The related contents were added in the discussion, in page 15.

(5) This is the first study to investigate the pulmonary transcriptional response to SMA infection. How about the further study plan? Please state in the discussion.

Response: Thanks for good suggestion. The pulmonary time-course transcriptional response to SMA infection was firstly investigated in the study. The research may shed light on the molecular mechanisms underlying the pathogenesis of SMA pneumonia. The expression levels of Cxcl10, Cd14, Gbp5, Cxcr2, Tnip1, Zc3h12a, Egr1, Sell and Gbp2 were high and previously unreported in SMA pneumonia, and they may be important targets for future studies. The innate immune response to acute bacterial infections heavily relies on myeloid cells, such as NK cells and macrophages. The roles of infiltrated NK cells and mechanism of recruitment of NK cells in protecting host is worth of further study. The related contents were added in the discussion, in page 17.

(6) What were the functions of neutrophils infiltration in the SMA pneumonia? Please state in the discussion.

Response: Thanks for good suggestion. In response to bacterial infection, innate immune cells, such as neutrophils, monocytes, and macrophages become activated during sepsis, resulting in an inflammatory response and recruitment of immune cells into tissues. The infiltrated neutrophil was a double edge sword. When neutrophils are overrecruited and activated following severe infection, the exaggerated inflammatory response can lead to severe tissue damage and result in organ failure. Our future research plan is to study the specific roles of neutrophils infiltration in the SMA pneumonia. The description of neutrophils infiltration in the SMA pneumonia was showed in discussion, in page 15.

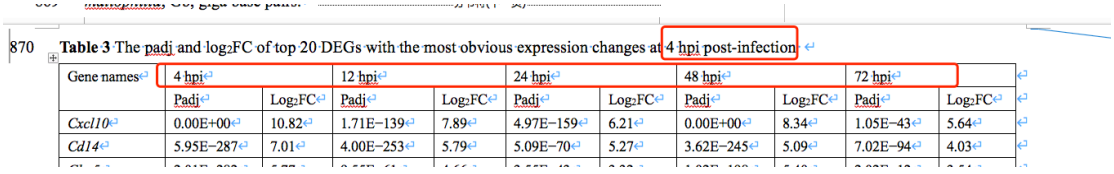
(7) How to obtain the datasets presented in this study? Please supplement in the text.

Response: Thanks for good suggestion for improving the quality of manuscript. Data availability statement was added in page 17.

Reviewer C

1. Table 3

The legend does not seem to match the table. Please check.



870 **Table 3** The padj and log_2FC of top 20 DEGs with the most obvious expression changes at 4 hpi post-infection

Gene names	4 hpi		12 hpi		24 hpi		48 hpi		72 hpi	
	Padj	Log_2FC	Padj	Log_2FC	Padj	Log_2FC	Padj	Log_2FC	Padj	Log_2FC
Cxcl10	0.00E+00	10.82	1.71E−139	7.89	4.97E−159	6.21	0.00E+00	8.34	1.05E−43	5.64
Cd14	5.95E−287	7.01	4.00E−253	5.79	5.09E−70	5.27	3.62E−245	5.09	7.02E−94	4.03

Response: thanks. We had revised.

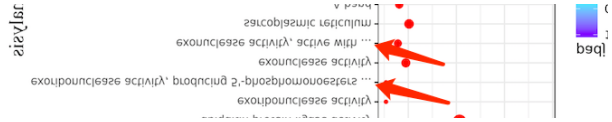
2. Figure 3

There seems to be no “PBS” in Figure 3, while it was explained in the legend. Please check and revise.

Response: thanks. We had deleted the full-name of “PBS” in the figure 3 legend.

3. Figure 5A & Figure 5B

Some information is missing. Please check and revise.



Response: thanks. We had revised the figure 5.

4. Figure 6

4.1 Please explain “ns” in the legend.

Response: thanks. We had supplemented the explanation of “ns” in the figure 6 legend.

4.2 There seems to be no “Tgd” in Figure 6, while it was explained in the legend. Please check and revise.

Response: thanks. Actually, the “γδT” is gamma delta T cell. We had revised in the figure 6 legend.

5. There are two reference lists in the manuscript. Please check and delete the unnecessary one.

Response: thanks. We had deleted the reference in the end.