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Peer Review File

The Potential Role of H2S in The Anti-infective Function of Antimicrobial Peptides

Corresponding Author: Dr Yipeng Wang

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)
In Wang et al., the authors detail how antimicrobial peptides (AMPs), primarily using LL-37, act on bacteria and macrophages to induce H_2S production in both, leading to bacterial death and immunosuppression in macrophages. In bacteria, they state that AMPs induce increased H_2S production that makes bacteria more susceptible to ROS, via inactivation of ROS-degrading enzymes, and use a number of inhibitors to suggest this. As for macrophages, they use inhibitors and siRNA to show that the increased H_2S production is mediated by upregulation of CSE upon AMP treatment, leading to reduced inflammatory cytokine levels. They also use a mouse infection model to support their conclusions.

While the authors have performed a large number of experiments to support their claims, there are two very major issues, in addition to several other concerns, that need to be addressed either experimentally or conceptually.

Major Points
1. Throughout the manuscript, the authors argue that H_2S induces oxidative damage, citing a few studies, despite a large body of literature suggesting that H_2S can act as a reducing agent. While it is true that H_2S might affect SOD and CAT activity, the paper they cite (Fu et al., 2018) used 0.5 mM Na_2S, a concentration far higher than what bacteria are likely to produce. Their own data suggesting inactivation of SOD and CAT are also weak, as they treated bacteria with LL-37 for one hour before measuring enzyme activity—during which time the bacteria could have simply died or stopped growing. This alone could explain the observed decrease.
The authors themselves acknowledge in the Discussion that inhibition of SOD and CAT is the only proposed mechanism by which H_2S could allow ROS to accumulate, and they also concede that H_2S can directly neutralize ROS.
Therefore, the authors must provide additional data to demonstrate that the levels of H_2S produced in response to LL-37 (e.g., 80–100 μM) can indeed cause oxidative damage. They should also clarify that this occurs after membrane disruption by LL-37. No strong evidence is presented that adding similar concentrations of H_2S donors induces oxidative damage. Alternatively, the authors could simply state that LL-37 treatment leads to increased H_2S production, which is detrimental to bacterial survival, without invoking ROS, provided the other concerns below are addressed. In this case, the strong claim regarding ROS in the abstract should be revised accordingly.

2. To argue that H_2S drives the enhanced bacterial killing by LL-37, the authors routinely use compound 7b—an H_2S scavenger previously reported by them—to rescue the effects of LL-37. However, there remains a simpler explanation: 7b may directly inactivate or interfere with LL-37. If no direct interaction data are available, the authors should at least perform two simple control experiments:
a) Test LL-37 with and without 7b in 3MST KO E. coli. Since LL-37 still kills 3MST KO bacteria, if 7b reduces this killing, then it would suggest that 7b is acting on LL-37, not on H_2S.
b) Add increasing concentrations of Na_2S and assess at what concentration 7b stops rescuing LL-37's bactericidal activity.

Other Points
1. The LL-37 concentrations used—relative to MIC—are not consistently reported for each experiment. This makes it very difficult to compare results across figures. These values should be clearly indicated throughout in an easy-to-read format.
2. The authors repeatedly describe SYTO9 as a live-cell-only dye. However, it is well established (e.g., https://www.thermofisher.com/us/en/home/life-science/cell-analysis/fluorophores/syto-9.html) that SYTO9 can also enter dead cells. How do the authors explain SYTO9-negative cells in some images, such as in Figures 2 and 3?

3. In Fig. 3C, the authors show lower H_2S production in LL-37+Asp and CRAMP+Asp compared to Asp alone. This is inconsistent with the claim that LL-37 induces H_2S production, which Asp is supposed to suppress. Can the authors explain this?
4. If the authors still maintain that ROS increases are mediated by H_2S, how do they explain that in Fig. 6D, 3MST KO E. coli still show elevated ROS upon LL-37 treatment?
5. Do the authors have any mechanistic insights into how AMPs might trigger pAkt activation?
6. Figure quality should be improved; several images, especially Fig. 4, are grainy and hard to interpret.
7. A consistent color scheme in Fig. 8 would greatly enhance clarity and readability.

Reviewer #2

(Remarks to the Author)
Summary:
This manuscript demonstrates a role of hydrogen sulfide (H2S) in mediating the anti-infective activities of antimicrobial peptides (AMPs). The study focuses on LL-37/CRAMP and extends the findings to other AMPs. The authors show that AMPs induce H2S production in both bacteria and macrophages, and that this contributes to both antimicrobial effects (via oxidative damage) and immunomodulatory functions.
The authors use a combination of bacterial genetics, enzyme inhibition, macrophage signaling assays, in vivo infection models, and AMP analyses to show these effects.

Major Comments:

1. Direct vs. H2S-Mediated Antimicrobial Effects: LL-37 is known to exert direct bactericidal activity through membrane disruption. It remains unclear how much of the observed effects can be specifically attributed to H2S and ROS. The use of a scrambled LL-37 peptide does not discriminate between direct membrane distrupting effects and H2S generation. Can the authors discriminate these two actions, eg by adding lower doses of LL-37 which are not directly antimicrobial? Will this still lead to H2S generation? The authors should add more experiments or at least discuss these possibilities.
2. Many possible sensors for H2S are described (Yang, M.; Zhou, Y.; Wang, K.; Luo, C.; Xie, M.; Shi, X.; Lin, X. Review of Chemical Sensors for Hydrogen Sulfide Detection in Organisms and Living Cells. Sensors 2023, 23, 3316.). How specific is probe L, and can the authors compare these results with another probe?
3. Fig. 2. Potential interactions between compound 7b and AMPs should be determined. The dose of 7b is not indicated in several panels. In 2G 5 uM LL-37 and 200 uM 7b was used. From the chemical structure (Sun et al., Nat Comm 2024) 7b looks hydrophobic and the question is then whether it directly affects AMP activity? Experiments using liposome based assays (PC/PE) could be utilised to directly assess membrane effects alone.
In the recent paper by Sun, J. et al., Nat Commun 2024, 7b seems not to affect bacterial growth alone, but sensitizes to antibiotics. Can the authors explain and discuss this in the light of their present data? In Fig. 2J, 7b alone increases IL-6 The concern here is that 7b at 100 uM has intrinsic effects. Can the authors provide an explanation? In the legend 5 mM LL-37/CRAMP is indicated, 5 uM? Overall, in I-J, direct interaction between AMP and 7b should be excluded.
4. Causality between H2S and ROS generation: While the manuscript presents data linking LL-37-induced H2S to increased ROS levels and bacterial killing, the directionality of this relationship should be explained. Time-course experiments or mechanistic studies would help establish whether H2S is upstream of ROS in this context. Alternatively, a clearer discussion of these limitations would be of benefit.
5. Gene upregulation studies in Fig. 4. Only one bacterial isolate is analysed. Addition of 2-3 other E. coli isolates or if possible other bacteria would strengthen this part. This would answer the question whether this upregulation can be generalised.
6. Specificity of ROS Detection: The ROS data rely heavily on the DCHF-DA probe, which has limitations. It would strengthen the manuscript to validate key findings using a more specific or complementary ROS detection method.
7. In general, pictures showing only colony formation (such as in 6E) should be backed up by counting CFUs.
8. For the in vivo experiments, all substances are injected ip. To avoid direct interactions between LL-37 and additions such as 7b, a different administration route for eg 7b, eg sc would demonstrate whether the effects related to H2S inhibition or not. As 7b is a small hydrophobic substance, it should distribute well. Alternatively, 7b could be given before injection of LL-37. Also, only the CMCC44102 is used. How about other E. coli such as ATCC 25922 or similar? The question is whether the H2S mechanism is common, and 7b has effects on other E. coli strains. Can the authors consider to generalise their findings?
9. Quantification of phagocytosis: The method used to assess macrophage phagocytosis relies on CFU counting after cell lysis, which may not distinguish between truly internalized bacteria and those adherent to the surface. This should be addressed.

Minor comments: At several places, there is a lack of specification of concentrations etc in the figures and legends.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

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This paper investigates the role of a gasotrasmitter hydrogen sulfide (H_2S) in the antimicrobial activity of AMPs across different animal species. The authors show that AMPs like LL-37 and its mouse homolog CRAMP stimulate H_2S production both in bacteria and macrophages, primarily through enzymes like 3MST and CSE. The generated H_2S contributes to bacterial killing via reactive oxygen species (ROS) and supports the immunomodulatory functions of AMPs. The study uncovers a previously unknown, conserved mechanism by which H_2S enhances the anti-infective effects of AMPs.

While hydrogen sulfide (H_2S) is generally recognized as a bacterial defense mechanism, this study tries to show that AMPs have evolved to exploit this gasotransmitter to enhance their antimicrobial activity. By inducing H_2S production, AMPs trigger oxidative stress and bacterial death, turning a typical bacterial survival strategy into a vulnerability.

The findings presented here are novel and interesting, particularly the concept that AMPs exploit bacterial H_2S production to exert their antimicrobial effects. However, it is notable that the authors have previously reported that targeting H_2S can sensitize bacteria to antibiotics (ref 29). Surprisingly, they do not cite their own earlier work in this context (Introduction line 77-79; Discussion line 525-532), instead only referencing studies from other groups. It would strengthen the manuscript to acknowledge and discuss their prior contributions to this area.

Overall the study is well conceived and the experiments are well designed. The description of the results is clear and the claims are in general supported by the results. But some clarification are needed, to valorise better the work done.

1) Fig. 1F would be clear if the authors included the staining of nuclei and an additional control with only peptide and the probe for H2S.
2) In Figure 2I and 2J, the authors attribute the anti-inflammatory effects of LL-37 to its modulation of H_2S production, based on experiments using compound 7b (an H_2S scavenger, Ref. 29). However, an alternative explanation should be considered: it is possible that LL-37 has a higher affinity for 7b than for LPS, which could reduce the availability of LL-37 to neutralize LPS rather than directly implicating H_2S modulation.
3) Furthermore, the cytokine data raise questions: in the control group, TNF-α levels are extremely high but decrease with the addition of 7b, whereas IL-6 levels behave oppositely (they are lower in the control and increase with 7b). The authors should clarify how they interpret these opposite trends and provide an explanation for this apparent discrepancy. Also, they should clarigy why the levels of cytokines are so high in the control?
4) Figure 5I, there is significant increase in phosphorylated Akt already at 1 h, not only at 8h. All the blots used for quantification should be provided in the source data.
5) GAPDH in figure 5C looks like form another blot, same for beta-actin in figure 5D.
6) How much lysate was loaded on the gel?
7) In Fig 6A and B, how do the authors explain the increase in ROS for the control of C. perfringers after 24 h?
8) In Fig. 8A, the schematic illustration shows that organs were collected after 3 days, but later in the Mat & Meth section it is written 24 h. Also, the schematic illustration shows that treatment was done after 2 h, while in the Mat & Meth section it is written 4 h. Please change the schematics accordingly.
9) Why 2 different concentrations of bacteria were used for survival and other assays?
10) In Fig. 8H it seems that there is a significant decrease in CFU independently of the bacterial strain.
11) For Fig. 1 and 9 (for macrophages) how do the authors explain that there is no dose-dependence?
12) The data suggest that AMPs stimulate H_2S production through two distinct pathways: 3MST in bacteria and CSE in macrophages. Could the authors clarify whether this difference reflects a broader, fundamental divergence between prokaryotic and eukaryotic systems, or if it might be specific to the particular bacterial strains and conditions used in this study?
13) I guess for Colony morphology assay LB agar was used, please clarify this in Mat & Meth.

Reviewer #4

(Remarks to the Author)

The manuscript described the effect of CRAMP and LL-37 on the H2S production in bacteria and in macrophages, and how the increased H2S production aids in the antimicrobial effect of these peptides (either directly on the bacteria or indirectly on the macrophage.
The authors have performed a very long list of experiments, which needs to be respected Some of the data seem to quite convincingly show data in favor of their hypothesis, but especially many of the in vitro experiments are less convincing because the effects are either relatively very small, are semi-quantitative or proper controls are lacking to show it is really the H2S that is involved. The authors also often neglect to add the exact concentrations of the AMPs used while this is needed to understand what the AMP does short term and long term in the in vitro experiments.
However, the in vivo infection experiment seems relatively convincing that there is a role of H2S, which is why I think the study could be good, after major revisions . I discuss my concerns in more detail below.

1) The effect of H2S induction in bacteria seems to be a long term effect of several hours. However, bacterial killing by AMPs is a fast process often within minutes. The authors should perform killing kinetics to determine how many bacteria are killed (at multiple concentrations of AMPs) after 5 min, 10 min. 2h. This is the basis for understanding many of the long-term experiments where H2S is involved because also at sub-MIC concentrations, up to 1 or 2 log decreases in viability of bacteria will be observed.

2) For all figures, please add in the figure legends what concentration of AMPs is used and how long incubation was performed for. Also different densities of bacteria are used in different experiments. Obviously higher CFU/ml would impact the killing and other effects of AMPs at every concentration. In addition, also in the methods many details regarding concentrations and incubation times and temperatures are missing, but are very important for the interpretation of the data. Please provide the missing details.

3) Fig. 2 and 3 shows really small effects of compound 7b and the Δ3MST on the antibacterial activity of CRAMP and LL-37. It is all about a few percent less killing or less PI staining or semi-quantitative colony forming, often shown in representative figures. This does not convince me that H2S plays a major role in bacterial killing by AMPs such as LL-37 and cannot convincingly explain the in vivo data later on in the manuscript. To properly address if H2S plays an important role the authors have to systematically test the effect of H2S on the antimicrobial mechanism of AMPs and this has to be done with the proper controls. So MIC and MBC values and kinetic killing studies should be determined for a large set of bacteria, including some of the non H2S producing ones in the presence and absence of compound 7B. In addition, besides LL-37 and CRAMP, a larger set of peptides that are supposed to increase H2S should be included, plus several antibiotics or other antimicrobial compounds that don't affect as H2S negative control. Similarly the set of AMPs should be properly tested (MIC, MBC Kinetic killing studies) against E.coli CMCC44102 and its Δ3MST variant. Only with these data you set a solid basis for showing in vitro that AMP-induced H2S production in bacteria adds to the antimicrobial mechanism of AMPs. Fig S16D shows what might look like a large increase in MIC based on the numbers, but in the setup used it means that only 1 well more showed growth after 24h. in theory if smaller steps are used for concentration increases, an MIC increase of for example 1.1-fold change would give the same observed result in Fig S16D.

4) The methods used to determine H2S production is very indirect by lead staining. And some of the differences are very small. For example in fig S3 showing the effect of LL-37 (and CRAMP) on H2S production of vibrio or E. coli. With n=3 it is very hard to imagine how these values can actually be statistically significant. And it is also questionable if that is actiually a biological relevant difference. Also by setting the control at 100% in every experiment you automatically have a paired statistical test and not an unpaired test.

5) also fig S3, as example of earlier point1: How long were the bacteria incubated with the lead strips in these figures? (the methods indicate multiple time points).

6) Scrambled LL-37 was tested in the bacterial H2S experiments which is a good control Was scrambled LL-37 also tested in the macrophage experiments? This should be included to show a real LL-37 specific effect.

7) Fig S7C: The scavenger does not affect growth. Officially correct because there is no significant difference with and without treatment. But for 50 and 200 uM the values are clearly lower than the control. Considering how much-smaller differences earlier mentioned in Fig S3 are apparently significant, I have serious problems with the blunt statement that the scavenger does not affect viability based on this data.
In addition: what are the short term toxic effects of compound 7B, the 24h shown might show effects of starting regrowth of RAW cells.

8) Fig 2C+D: (example of point 2) what concentration of CRAMP and LL-37 is used? Also ODmeasuremnt does not always represent viable bacteria (but could also just indicate color change). Bacteria after 12h incubation should be plated out on agar plates and enumerated to determine the real amount of viable bacteria.

9) Fig 2C+D: Why are the growth curves not extended for a longer period. Even for the WT the bacteria seem to not have reached stationary phase yet? Which is in itself very surprising if a regular growth medium is used, E. coli especially grows fast and reaches stationary phase in 5=6 hours . can the authors explain this ? And please show 24h curves and see if the proposed effect of H2S holds.

10) Fig2 LPS model: I always thought that it was well established that LL-37 neutralizes LPS (mainly) by direct binding and thereby preventing LPS from interacting with TLR4. Especially upon co-incubation of LPS with LL-37 (or CRAMP). How can pretreatment with compound 7b prevent that? This is an interesting observation obviously but very hard to explain.

11) Fig 2M: scratch assays are not usually done with macrophages. The authors should perform the scratch assay with (non-H2S producing) epithelial cells. If compound 7b has a similar effect it is not due to H2S.

12) Fig 6F: this is not really a quantitative assay?? Why not just count viable bacteria upon treatments?? Again the differences in Fig S17 are really small and no statistics are performed. Yet the authors imply that this is a real 'difference'.

13) Fig 8: This in vivo data seems very convincing unlike a lot of the in vitro data. But several questiond remain:
- Interestingly though, the total effect of LL-37/ H2S seems to be on the bacterial (H2S producing) level, and not the macrophages. This aspect needs to be further discussed.
-Is the AMP injected at exactly the same site as the bacterial burden??
-Is compound 7b and/or BT injected together with the AMP, and importantly could compound 7b/BT at these concentration physiologically affect LL-37 activity directly, preventing LL-37 from interacting with bacteria (and blocking killing) in the mouse. For example does 7b bind directly to LL-37 or can it induce aggregation of LL-37 in the peritoneal lavage fluid? This could explain the observed in vivo results.

-The addition of 7b only (without LL-37) significantly increases the bacterial burden in every organ. Do the authors think that this could be due to the effect on naturally produced H2S in macrophages (and their subsequent killing of E.coli) in these mice? This observation is interesting and should be discussed properly.

14) Methods paragraph 3.3 please describe more clearly how the experiment is performed. What for example is incubated for 10 min exactly?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
While the authors have performed multiple experiments and addressed some minor points reasonably well, the major issues remain unaddressed.

First major point: concerning how H_2S induces oxidative stress. The authors show that Na_2S can inhibit SOD and CAT activity; however, they used 100 μM of Na_2S, which is still not physiological. In the case of LL-37, only about 20-30 μM is observed in E. coli (Figure 1). Tests with lower doses of H_2S donors exhibiting the same effects are necessary to convincingly demonstrate the ROS-related mechanism. Furthermore, the authors have not specified the dose of LL-37 used in the revised experiments or shown whether it causes any cell death or growth defects by 12 hours, which could explain the data. Cell death via ROS can be self-sustaining and increase over time (see https://pnas.org/doi/abs/10.1073/pnas.1901730116).

Second major point: Although ITC can determine if there's an interaction between 7b and LL-37, the authors need to conduct straightforward experiments—testing LL-37 with and without 7b in 3MST knockout E. coli and assessing the effect of increasing Na_2S concentrations on bacterial survival, especially whether 7b can rescue LL-37's bactericidal activity at various dosages. The ITC data are also unclear: if the molar ratio of 7b to LL-37 is 1:10, why is the maximum ratio only 1.2? Moreover, in Figures 2G and 2H, a ratio of 1:40 is used. Another possibility is that 7b might interact with the bacterial membrane directly—helping bacteria tolerate LL-37. Performing the suggested experiments is critical to clarify these points.

Reviewer #2

(Remarks to the Author)
The authors have revised the manuscript and addressed my main concerns. They provide new experimental data which now includes: validation with an alternative H_2S probe, ITC assays excluding direct interaction of compound 7b with LL-37, time-course analysis of H_2S and ROS, and CFU quantification. They have also corrected and clarified methodological details. Moreover, they now discuss mechanistic claims where the evidence remains indirect, acknowledging limitations such as ROS probe specificity and strain variability.

Although I would still prefer to see some additional experiments—such as broader validation across different bacterial strains and further analysis of AMP activity at sub-MIC concentrations—the authors have clearly recognized these limitations and adjusted their claims accordingly.

Overall, my comments have been satisfactorily addressed.

Reviewer #3

(Remarks to the Author)
Reviewer Report:
I appreciate the revisions, and I see that the authors have addressed most of my previous concerns. However, two important issues remain:
1. The authors did not provide the original western blot images. For transparency and reproducibility, the images should be included (at least as supplementary material).

2. The normalization strategy is problematic. According to the manuscript, the same samples were run on two separate gels —one probed for the protein of interest and another for the housekeeping protein (GAPDH/actin). This approach is not valid, as normalization requires that the loading control (GAPDH/actin) be run on the same blot as the protein of interest. Otherwise, potential gel-to-gel variation cannot be accounted for. Furthermore, CSE and GAPDH or 3-MST and beta-actin are well separated in molecular weight and could have been probed on the same blot (e.g., by stripping and reproving or by using different antibody incubation steps).

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscript.

Reviewer #4

(Remarks to the Author)
The authors have revised the manuscript considerably and added multiple extra experiments to their already very large set of experimental data. I truly admire this input that the authors have provided in this revision. However, although the revision has taken away several of my earlier concerns there remains a true doubt in my mind about the actual importance of H2S in the mechanistic antimicrobial activity of LL-37. I highlight these below based on my earlier (reviewer 4) remarks:

Remark 1:
The fact that compound 7b affects the activity of LL-37 as early as 10 -30 would imply that H2S is not involved since sufficient H2S production hasn't started yet. And this, together with the very mild effects 7b seems to have on antimicrobial activity enforces my doubts on the importance of H2S. How do the authors explain the short term effects, in the light of their proposed antibacterial mechanism for LL-37?

Remark2: convincingly answered.
Remark 3: The authors acknowledge that the effect of H2S might be too subtle to be detected. But the role of (induced) H2S is the main message of the whole article and needs to be properly shown, also in vitro. Therefore, my suggested experiments are not as said by the authors 'beyond the primary focus' of the study. They are at the core of the study.
I understand that all suggested experiments are very time demanding, but MIC/MBC assays with smaller than 2 fold dilutions for a set of , let's say two H2S producing and 4 non-H2S producing bacteria should be easily doable.

Remark 4-12. Convincingly answered, happy with the additional data.

Remark 13: As indicated the in vivo data is convincing regarding the effect of 7b and LL-37 on infection but that it does not necessarily confirm the proposed mechanism. I understand that extra in vivo control experiments is very difficult to perform, however rereading the figure, I noticed that an infection with the delta3MST E.coli were performed but only the bacterial burden in kidney and peritoneal lavage fluid are shown. Surely, other data MUST have been collected for these infected mice, please show these data as they provide an important control experiment. How was weight and survival affected by LL=37 +/- 7b , and what was bacterial burden in other tissues ? These should be shown as they are an essential control experiment.
This could provide very important implications whether 7b acts on macrophages or bacteria. And the shown data are inclonclusive cause LL-37 affects the bacterial burden in the peritoneal fluid but not the kidney for the mutant E. coli.
And a small note on the ITC data: Indeed there seems to be no binding happening between 7b and LL-37. However, there is always a small chance that binding is based on hydrophobic interactions and therefor entropy-driven (and not enthalpy which is measured by ITC).

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have now done a lot more experiments to address our concerns which we appreciate.

For the first major point, the data the authors provided do suggest that H2S can lead to ROS and decreased SOD and CAT activity. However, it is very clear that to get the effects that LL-37 has, you need at least triple the H2S dose that is physiological, while the physiological amount has a much weaker effect. Thus, the authors must at least reduce their claims, especially the sentence in the abstract: "The generated H_2S further stimulates the accumulation of reactive oxygen species (ROS), leading to bacterial death by oxidative damage." Through the other experiments it does suggest that H2S is induced and the 7b and 3MST mutant experiments strongly suggest it plays a role, but data that death is by ROS is very weak.

For the second point, the data are somewhat convincing and do suggest that 7b mediates its effect via H2S scavenging. For the 3MST experiment, it is notable that the authors find much higher growth suppression when 7b is added with even 20 μM Na2S than with just LL-37 (3MST+LL-37 is much better than 3MST+LL-37+7b+20 μM Na2S) as shown in Fig R4A. Additionally, in Fig R4B the addition of 7b did not substantially improve the survival of 3MST+LL-37+20 μM Na2S. Thus, 7b is at best a moderate scavenger of H2S.

Two minor points. With all the additional experiments the paper is now very long with 30+ supplemental figures; this needs to be shortened. Also, what the authors refer to as "time-kill kinetics"/"bactericidal kinetics" are growth curves, so the language needs to be changed.

Reviewer #2

(Remarks to the Author)
Although I would still have preferred to see additional experiments—such as broader validation across multiple bacterial strains and further characterization of AMP activity at sub-MIC concentrations—the authors have acknowledged these limitations and have moderated their claims accordingly.

I do, however, share the concern raised by Reviewer 1 (point 2) regarding compound 7b and its potential effects. This remains an important issue that should ideally be addressed.

That said, in light of my previous assessment, I have no further formal comments, as my original concerns have been satisfactorily addressed.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #1 (Remarks to the Author):

Comments:

In Wang et al., the authors detail how antimicrobial peptides (AMPs), primarily using LL-37, act on bacteria and macrophages to induce H_2S production in both, leading to bacterial death and immunosuppression in macrophages. In bacteria, they state that AMPs induce increased H_2S production that makes bacteria more susceptible to ROS, via inactivation of ROS-degrading enzymes, and use a number of inhibitors to suggest this. As for macrophages, they use inhibitors and siRNA to show that the increased H_2S production is mediated by upregulation of CSE upon AMP treatment, leading to reduced inflammatory cytokine levels. They also use a mouse infection model to support their conclusions.

While the authors have performed a large number of experiments to support their claims, there are two very major issues, in addition to several other concerns, that need to be addressed either experimentally or conceptually.

Response: We sincerely thank the reviewer for their thorough evaluation and for recognizing the breadth of experiments we performed. We appreciate the constructive critique and have carefully addressed each of the major and minor concerns raised, either by conducting additional experiments or by providing further clarification in the revised manuscript. Below, we provide a point-by-point response to each specific issue.

Major Points

1. Throughout the manuscript, the authors argue that H_2S induces oxidative damage, citing a few studies, despite a large body of literature suggesting that H_2S can act as a reducing agent. While it is true that H_2S might affect SOD and CAT activity, the paper they cite (Fu et al., 2018) used 0.5 mM Na_2S, a concentration far higher than what bacteria are likely to produce. Their own data suggesting inactivation of SOD and CAT are also weak, as they treated bacteria with LL-37 for one hour before measuring enzyme activity—during which time the bacteria could have simply died or stopped growing. This alone could explain the observed decrease.

The authors themselves acknowledge in the Discussion that inhibition of SOD and CAT is the only proposed mechanism by which H_2S could allow ROS to accumulate, and they also concede that H_2S can directly neutralize ROS.

Therefore, the authors must provide additional data to demonstrate that the levels of H_2S produced in response to LL-37 (e.g., 80 – 100 μM) can indeed cause oxidative damage. They should also clarify that this occurs after membrane disruption by LL-37. No strong evidence is presented that adding similar concentrations of H_2S donors induces oxidative damage.

Alternatively, the authors could simply state that LL-37 treatment leads to increased H_2S production, which is detrimental to bacterial survival, without invoking ROS, provided the other concerns below are addressed. In this case, the strong claim regarding ROS in the abstract should be revised accordingly.

Response: We thank the reviewer for this thoughtful and important comment. We fully acknowledge the dual-dependent roles of H_2S in redox biology and appreciate the reviewer's concern regarding the concentration ranges and the interpretation of our SOD/CAT inhibition data.

First, we acknowledge that the literature supports both pro-oxidant and antioxidant roles of H_2S

depending on context and concentration. We agree with the reviewer that the concentrations of H_2S donors used in previous studies (e.g., Fu *et al*., 2018) are substantially higher than the levels likely to be produced endogenously in bacteria upon AMP treatment. We have clarified in the revised discussion that the physiological relevance of such concentrations is uncertain.

Revised discussion section (Manuscript-with tracks: Page:13, Line:536-548):

"Fu *et al*. reported that the bactericidal activity of hydrogen sulfide (H_2S) is mediated, at least in part, by oxidative damage. This effect was attributed to the inhibition of key antioxidant enzymes, including superoxide dismutase (SOD) and catalase (CAT), alongside the induction of stress response regulators such as SoxRS and OxyR[15]. Notably, their experiments employed a Na_2S concentration of 0.5 mM, which substantially exceeds the levels of H_2S that are typically produced by bacteria under physiological conditions. Therefore, while these findings provide mechanistic insights, the relevance of such high concentrations to the antimicrobial effects of bacterially derived H_2S remains to be firmly established and warrants further investigation."

Based on our minimum inhibitory concentration (MIC) assays, LL-37 exhibited an MIC of 33.4 μM against *Escherichia coli* CMCC44102 and *Clostridium perfringens*. When bacteria were exposed to LL-37 at concentrations of 80–100 μM, rapid bactericidal effects were observed. However, such high concentrations preclude the possibility of using LL-37 as a stimulus to induce bacterial H_2S production under experimental conditions. To address this limitation, we instead supplemented cultures with equivalent concentrations of the H_2S donor Na_2S, enabling us to assess oxidative damage in the bacteria and thereby test our hypothesis.

To strengthen our conclusions, we performed additional experiments using physiologically relevant concentrations of H_2S donors-Na_2S (100 μM) and assessed their direct effects on reactive oxygen species (ROS) levels, SOD and CAT activities, malondialdehyde (MDA) content in *Escherichia coli* CMCC44102.

As shown in **Figure R1** (**now presented in Supplementary Fig. S26**), treatment with Na_2S at 0 h did not induce reactive oxygen species (ROS) production. However, after 12 h, Na_2S treatment, while less potent than LL-37 alone, still led to a detectable increase in ROS levels. Notably, the combined application of Na_2S and LL-37 markedly enhanced ROS induction. Consistent with these findings, we observed in **Figure R2** (**now presented in Supplementary Fig. S27**) that Na_2S treatment inhibited the activities of superoxide dismutase (SOD) and catalase (CAT), with the inhibitory effect being significantly amplified in the presence of LL-37. Furthermore, as shown in **Figure R3** (**now presented in Supplementary Fig. S28**), Na_2S treatment resulted in elevated levels of malondialdehyde (MDA), a marker of lipid peroxidation, and this effect was substantially intensified when combined with LL-37.

Inserted results section (Manuscript-with tracks: Page:10, Line:431-435):

"To confirm that the H_2S response to LL-37 contributes to oxidative damage, we assessed oxidative stress in Escherichia coli using the H_2S donor Na_2S. Treatment with Na_2S induced ROS production (Fig. S26), suppressed SOD and CAT activities (Fig. S27), and increased MDA levels (Fig. S28). These findings further support the involvement of H_2S in LL-37-induced oxidative stress."

Collectively, these results demonstrate that supplementation with an H_2S donor induces oxidative damage, particularly when used in conjunction with LL-37.

Although our findings indicate that H_2S contributes to the oxidative damage elicited by LL-37, additional evidence is required to establish whether H_2S directly drives ROS production. In our revised manuscript, we have tempered our mechanistic interpretation and emphasize that LL-37-

induced H_2S production is associated with impaired bacterial survival to avoid overstatement regarding a direct role of H_2S in LL-37-induced oxidative damage.

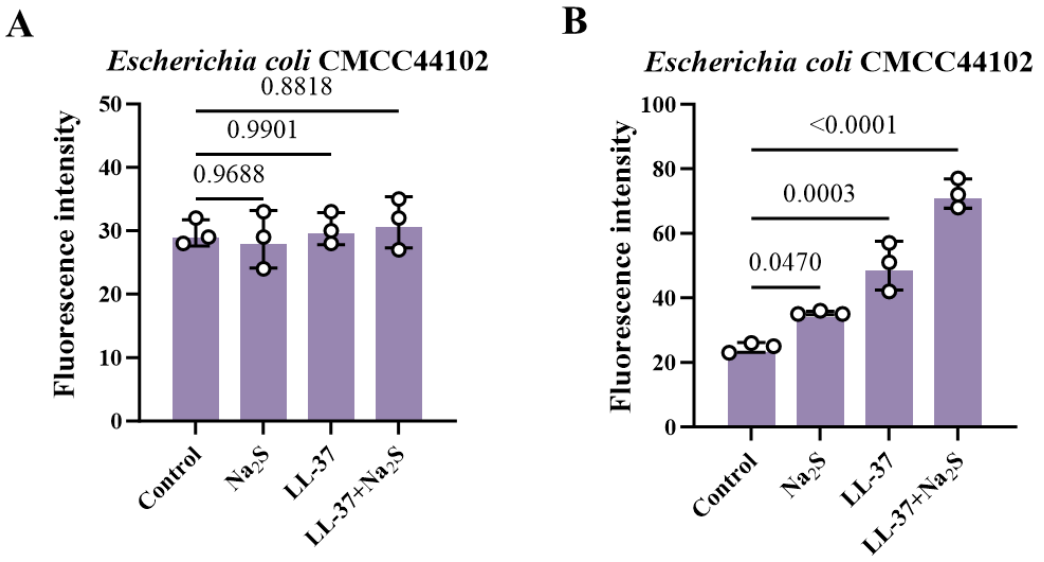

Fig. R1 Effect of Na_2S (100 μM) treatment on the levels of reactive oxygen species (ROS) at 0 h (**A**) and 12 h (**B**) in *Escherichia coli* CMCC44102 (1×10^8 CFU/mL) using DCHF-DA (5 μM) probe at absorbance of 488/525 nm. The results are expressed as mean ± S.D. n = 3 biological replicates. One-way ANOVA followed by Tukey's post hoc analysis. Source data are provided as a Source Data file.

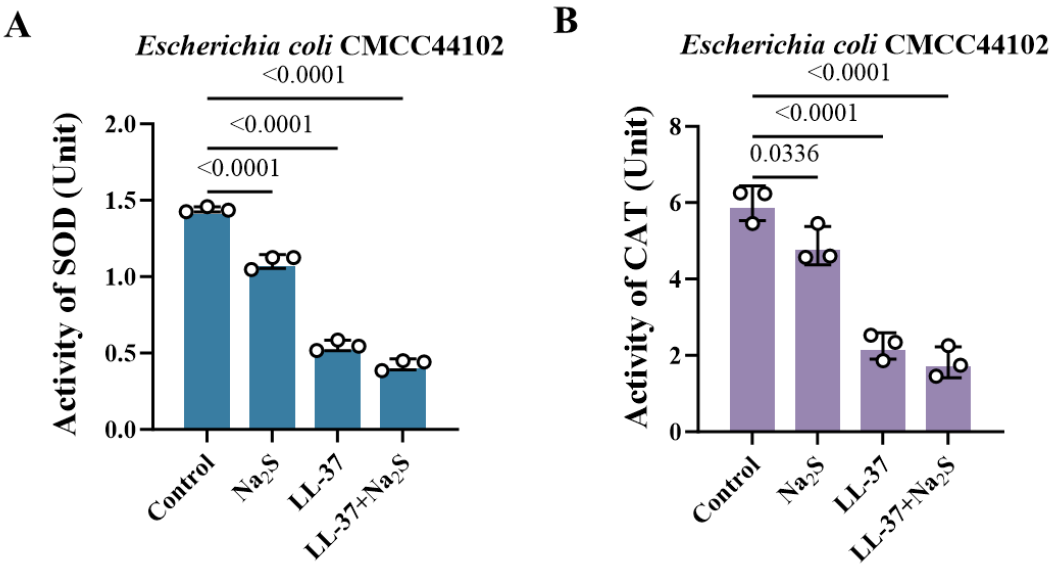

Fig. R2 Effect of Na_2S (100 μM) treatment on the enzymatic activities of superoxide dismutase (SOD) (**A**) and catalase (CAT) (**B**) in *Escherichia coli* CMCC44102 (3×10^7 CFU/mL). The results are expressed as mean ± S.D. n = 3 biological replicates. One-way ANOVA followed by Tukey's post hoc analysis. Source data are provided as a Source Data file.

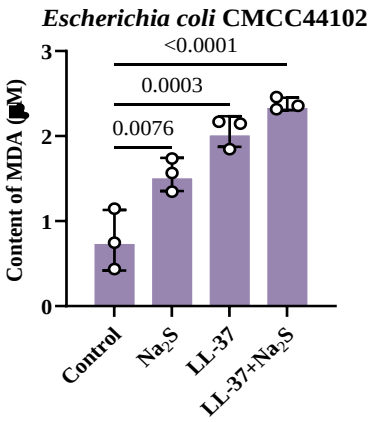

Fig. R3 Effect of Na_2S treatment on malondialdehyde (MDA) content in *Escherichia coli* CMCC44102 (3×10^7 CFU/mL). The results are expressed as mean ± S.D. n = 3 biological replicates. One-way ANOVA followed by Tukey's post hoc analysis. Source data are provided as a Source Data file.

2. To argue that H_2S drives the enhanced bacterial killing by LL-37, the authors routinely use compound 7b—an H_2S scavenger previously reported by them—to rescue the effects of LL-37. However, there remains a simpler explanation: 7b may directly inactivate or interfere with LL-37. If no direct interaction data are available, the authors should at least perform two simple control experiments:

a) Test LL-37 with and without 7b in 3MST KO E. coli. Since LL-37 still kills 3MST KO bacteria, if 7b reduces this killing, then it would suggest that 7b is acting on LL-37, not on H_2S.

b) Add increasing concentrations of Na_2S and assess at what concentration 7b stops rescuing LL-37's bactericidal activity.

Response: We appreciate the reviewer's thoughtful suggestion and fully agree that it is critical to exclude the possibility of a direct interaction between LL-37 and compound 7b. To address this concern, we conducted isothermal titration calorimetry (ITC) experiments to investigate potential binding between LL-37 and 7b under the experimental conditions used in our study.

As shown in **Fig. R4** (**now presented in Supplementary Fig. S12**), the ITC results revealed no detectable interaction between LL-37 and 7b. These findings indicate that compound 7b does not directly inactivate or interfere with LL-37's activity. We have updated the Methods section and figure legends to include details of the ITC experiments and the results.

Inserted results section (Manuscript-with tracks: Page:5, Line:207-209):

"Importantly, we confirmed that LL-37, as a representativeAMP, does not interact with compound 7b, ensuring that 7b does not interfere with or inactivate LL-37's activity (Fig. S12)."

Inserted methods section (Supplementary Materials with tracks: Page:11, Line:415-422):

"**11 Isothermal titration calorimetry (ITC) assay**

The binding affinity between LL-37 and 7b was measured by isothermal titration calorimetry (ITC) using a Malvern MicroCal PEAQ-ITC instrument (Malvern, USA) at 25°C. Experiments were performed in a buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl, and 5% DMSO. A solution of 7b was titrated into the sample cell containing LL-37 at a molar ratio of 10:1, with 13 injections performed following an initial 180 s equilibration period. Data were analyzed using Malvern MicroCal PEAQ-ITC Analysis Software."

We sincerely thank the reviewers for highlighting the potential interaction between 7b and LL-37. Clarifying this issue is essential for ensuring that the addition of 7b does not confound the interpretation of our experimental results.

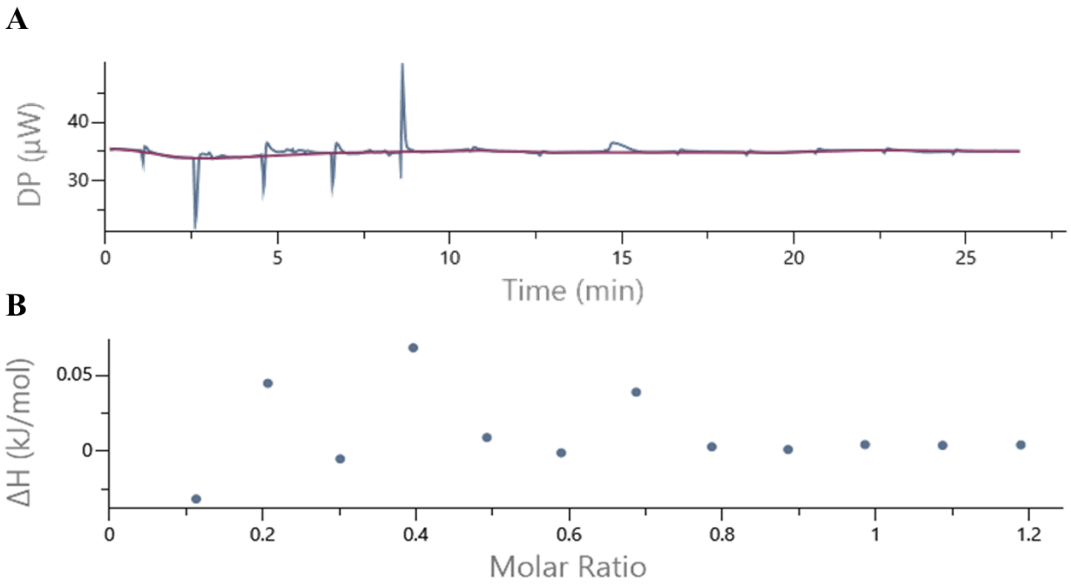

Fig. R4 The interaction between LL-37 and 7b determined by ITC assay, where 0.5 mM 7b was titrated into 0.05 mM LL-37 in HEPES buffer at 25°C.

Other Points

1. The LL-37 concentrations used—relative to MIC—are not consistently reported for each experiment. This makes it very difficult to compare results across figures. These values should be clearly indicated throughout in an easy-to-read format.

Response: We thank the reviewer for highlighting this important issue. We regret that the concentration of LL-37 relative to the MIC was not clearly stated, leading to potential confusion in data interpretation. We fully agree that consistent and transparent reporting of LL-37 concentrations is essential for clarity and reproducibility. In response, we have now clearly indicated LL-37 concentrations in the figure legends throughout the manuscript (e.g., Fig. 1A–D, Fig. 2C–F, Fig. 3C–D, G–H, I–J, M–N, Fig. 4A–B, Fig. 6A–D, G–H, I–K), and have provided detailed information in the corresponding *Materials and Methods* section. Once again, we thank the reviewer for this valuable feedback, which has helped improve the rigor of our manuscript.

2. The authors repeatedly describe SYTO9 as a live-cell-only dye. However, it is well established (e.g., https://www.thermofisher.com/us/en/home/life-science/cell-analysis/fluorophores/syto9.html) that SYTO9 can also enter dead cells. How do the authors explain SYTO9-negative cells in some images, such as in Figures 2 and 3?

Response: We thank the reviewer for pointing out this important clarification regarding SYTO9 staining. Based on the product link guide for SYTO9 dye provided, we have found that this dye is particularly suitable as a nuclear counterstain for both Gram-positive and Gram-negative bacteria, whether they are alive or dead.

We agree that SYTO9 is not exclusively a live-cell dye and can bind nucleic acids in both live and membrane-compromised (i.e., dead) cells, depending on the staining context and the presence of

counterstains such as propidium iodide (PI). In our original manuscript, the description of SYTO9 as a "live-cell-only dye" was imprecise, and we have now corrected this in the revised version.
(e.g., ***Results section, Manuscript-with tracks: Page 6, Line 228-231:*** "SYTO9 and PI—membrane-permeable dyes—are well suited for assessing bacterial viability. We therefore employed these dyes to quantify bacterial survival based on the relative intensities of green and red signals in confocal images."
Figure legend section: "The ratio of SYTO9 to PI fluorescence was used to distinguish live and dead bacterial cells.").
Specifically, we now refer to SYTO9 as a membrane-permeable nucleic acid dye that generally stains both live and dead bacteria, unless outcompeted by PI in viability assays. Regarding the SYTO9-negative cells observed in Figures 2 and 3: SYTO9 exhibits high affinity for DNA and labels all bacterial cells with green fluorescence. In contrast, PI selectively penetrates dead cells and, upon DNA binding, emits a stronger red fluorescence that dominates over SYTO9. This fluorescence shift is readily apparent in the merged images.
We are deeply grateful to the reviewer for this insightful comment, which prompted us to rectify a critical misstatement regarding the SYTO9 dye.

3. In Fig. 3C, the authors show lower H_2S production in LL-37+Asp and CRAMP+Asp compared to Asp alone. This is inconsistent with the claim that LL-37 induces H_2S production, which Asp is supposed to suppress. Can the authors explain this?
Response: We thank the reviewer for highlighting this apparent inconsistency and appreciate the opportunity to clarify.
First, in our screening of the major H_2S-producing enzymes in *Escherichia coli* CMCC44102, we found that inhibition of 3-mercaptopyruvate sulfurtransferase (3MST) by aspartic acid (Asp) had the most pronounced effect in reducing bacterial H_2S production. We acknowledge that in our experiments, H_2S production in the LL-37 + Asp and CRAMP + Asp groups was indeed lower than in the Asp-alone group.
This observation is consistent with the mechanism described by Shatalin *et al.* (*Science*. 2011), where 3MST catalyzes H_2S production through the intermediate 3-mercaptopyruvate (3-MP), which is synthesized by cysteine aminotransferase (CAT). Aspartic acid acts as a competitive substrate for CAT, thereby reducing the availability of 3-MP for 3MST and ultimately suppressing H_2S biosynthesis. Based on this mechanistic insight, we selected Asp as a functional inhibitor of the 3MST pathway in our study.
The observed reduction in H_2S production by *Escherichia coli* CMCC44102 upon treatment with LL-37/CRAMP + Asp, relative to Asp alone, can be explained as follows. As shown in the figure, aspartic acid inhibits 3-mercaptopyruvate sulfurtransferase (3MST)–mediated H_2S production by limiting the availability of 3-mercaptopyruvate (3MP), a critical substrate in this pathway. In our experiments, co-treatment with LL-37 or CRAMP further reduced H_2S levels compared to Asp alone. We speculate that LL-37/CRAMP increases the bacterial demand for sulfur metabolism, which exacerbates the suppression of cysteine aminotransferase (CAT)—the enzyme responsible for generating 3MP precursors—under Asp-mediated inhibition. Consequently, CAT activity remains constrained, preventing LL-37/CRAMP from rescuing 3MP production and resulting in even lower H_2S output than observed with Asp alone.
We thank the reviewer for highlighting this mechanistic detail and hope this explanation clarifies

the observed phenomenon

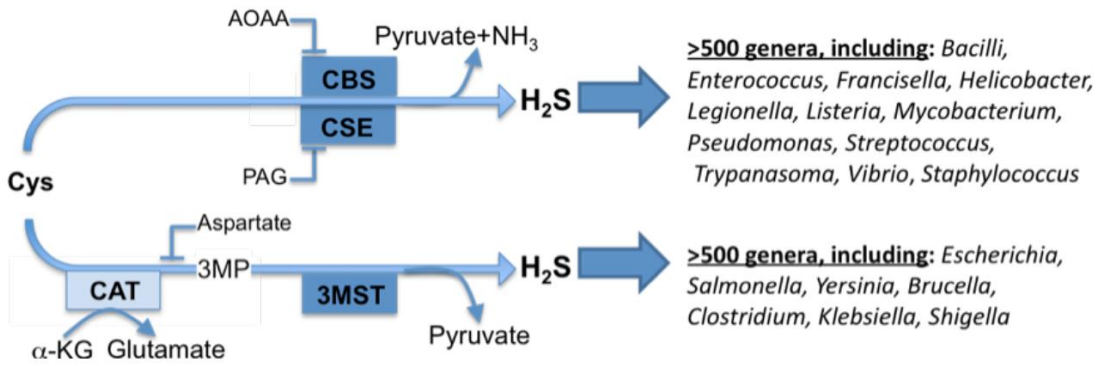

Fig. R5 Schematic representation of basic metabolic pathways of H_2S synthesis. (Shatalin *et al. Science*, 2011)

4. If the authors still maintain that ROS increases are mediated by H_2S, how do they explain that in Fig. 6D, 3MST KO E. coli still show elevated ROS upon LL-37 treatment?

Response: We thank the reviewer for this insightful observation. We agree that the increase in ROS observed in the 3MST knockout strain upon LL-37 treatment (Fig. 6D) requires further clarification, particularly in the context of our proposed H_2S-mediated mechanism.

As the reviewer notes, LL-37 treatment still induces some ROS accumulation in the 3MST KO strain, albeit to a significantly lower extent than in the wild-type background. We propose possible mechanisms underlying LL-37-induced ROS production in *3MST*-deficient *E. coli*.

1.Direct membrane damage by LL-37: leading to increased metabolic stress and leakage of redox-active components, which promotes ROS generation—this occurs in both WT and 3MST KO strains.

2. In wild-type cells, H_2S inhibits antioxidant enzymes such as SOD and CAT, thereby amplifying ROS accumulation. In contrast, in the *3MST* knockout strain, this H_2S-mediated suppression is absent, resulting in only a modest increase in ROS levels. This interpretation is consistent with our data in Fig. 6D, which shows a partial but not complete reduction in ROS levels in the knockout background. The residual ROS increase observed in 3MST-deficient *E. coli* following LL-37 treatment suggests that membrane perturbation itself contributes to oxidative stress, independent of H_2S. However, the attenuated ROS levels compared to wild-type cells support a modulatory role for 3MST-derived H_2S in oxidative damage.

We appreciate the reviewer's comment, which has helped us clarify the multifactorial mechanism of the LL-37 response.

5. Do the authors have any mechanistic insights into how AMPs might trigger pAkt activation?

Response: We thank the reviewer for this important question regarding the upstream signaling events that lead to pAkt activation upon AMPs treatment.

Activation of the Akt signaling pathway is known to regulate the expression of CSE (Xu Y, *et al. Pharmacol. Res.* 2014), a key enzyme in H_2S production, in macrophages. Based on this, we hypothesized that Akt signaling is involved in LL-37–induced immunomodulatory activity. This hypothesis is supported by our observations (Fig. 5I). While the precise mechanism remains to be fully elucidated, our data and prior reports suggest several plausible pathways:

1.Membrane perturbation leading to receptor activation:

AMPs such as LL-37 and CRAMP are known to interact with cell membranes, and in mammalian cells, LL-37 has been shown to activate membrane-bound receptors such as FPR2, EGFR, and P2X7, all of which can converge on PI3K-Akt signaling (Hariharan. *et al*. *J Biol Chem*. 2011).

2.Role of intracellular calcium and ATP signaling:

AMP-induced membrane permeabilization may lead to transient increases in intracellular Ca^{2+} or extracellular ATP release, both of which can activate Akt via P2 receptor pathways (Virgilio *et al*. *Nat Rev Cancer*. 2018).

3.Redox-mediated modulation:

Given that LL-37 induces mild oxidative stress, redox-sensitive signaling cascades could also play a role in Akt phosphorylation. ROS has been shown in some contexts to enhance Akt activity via PTEN inactivation or direct modulation of upstream kinases (Kma *et al*. *Biotechnol Appl Biochem*. 2021).

We acknowledge that the current data do not fully elucidate the mechanism by which AMPs activate the Akt signaling pathway. Nonetheless, this may represent a promising avenue for future investigation into the immunomodulatory functions of AMPs. We thank the reviewers for their valuable suggestions.

6. Figure quality should be improved; several images, especially Fig. 4, are grainy and hard to interpret.

Response: We thank the reviewer for this valuable feedback. We agree that image clarity is critical for accurate interpretation and presentation.

In response to this comment, we have replaced Figure 4 with high-resolution versions of the original images, ensuring improved contrast, sharpness, and resolution. We re-exported all panels directly from the raw image files at a higher DPI setting (600 dpi), using standardized brightness/contrast adjustments across conditions.

These improvements have been incorporated into the revised manuscript and figure files. We appreciate the reviewer's comment, which helped us enhance the visual quality and overall readability of the work.

7. A consistent color scheme in Fig. 8 would greatly enhance clarity and readability.

Response: We thank the reviewer for this helpful suggestion. We fully agree that a consistent and intuitive color scheme is important for visual clarity, especially in figure 8. In the revised version of Figure 8, we have implemented a unified color scheme throughout the schematic. We have also updated all figures in the revised manuscript and supplementary materials. This color logic is now consistent with the thematic colors used in earlier figures. We believe this revision has significantly improved the figure's clarity and appreciate the reviewer's suggestion in helping us enhance the visual communication of our results.

Reviewer #2 (Remarks to the Author):

Summary:

This manuscript demonstrates a role of hydrogen sulfide (H2S) in mediating the anti-infective activities of antimicrobial peptides (AMPs). The study focuses on LL-37/CRAMP and extends the findings to other AMPs. The authors show that AMPs induce H2S production in both bacteria and

macrophages, and that this contributes to both antimicrobial effects (via oxidative damage) and immunomodulatory functions.
The authors use a combination of bacterial genetics, enzyme inhibition, macrophage signaling assays, in vivo infection models, and AMP analyses to show these effects.
Response: We thank the reviewer for the thoughtful and constructive evaluation of our manuscript. We are pleased that the reviewer recognizes the novelty and significance of our findings regarding the role of hydrogen sulfide (H_2S) in mediating the anti-infective and immunomodulatory functions of antimicrobial peptides (AMPs), particularly LL-37 and CRAMP.

Major Comments:

1. Direct vs. H2S-Mediated Antimicrobial Effects: LL-37 is known to exert direct bactericidal activity through membrane disruption. It remains unclear how much of the observed effects can be specifically attributed to H2S and ROS. The use of a scrambled LL-37 peptide does not discriminate between direct membrane distrupting effects and H2S generation. Can the authors discriminate these two actions, eg by adding lower doses of LL-37 which are not directly antimicrobial? Will this still lead to H2S generation? The authors should add more experiments or at least discuss these possibilities.
Response: We thank the reviewer for raising this important point regarding the discrimination between direct bactericidal effects of LL-37 and those mediated by H_2S/ROS. We fully agree that LL-37's membrane-disrupting activity could contribute to the observed antimicrobial effects.
Currently, there is no direct experimental evidence to definitively distinguish whether LL-37 exerts its bactericidal effect through direct membrane disruption or via modulation of bacterial H_2S metabolism. In the revised manuscript, we have clarified in the *Results* section that the scrambled peptide control was primarily used to assess sequence specificity but is not sufficient to differentiate between these two mechanisms (see Table S2).
Notably, in the initial version of Fig. 9, we observed that AMPs from diverse animal sources promoted H_2S production even at sub-MIC concentrations (0.0625×, 0.125×, and 0.25×MIC). While this may provide indirect evidence that LL-37 facilitates bacterial killing through H_2S modulation rather than exclusively via membrane disruption, we agree that further mechanistic studies are needed to validate this hypothesis. We have therefore acknowledged in the revised manuscript the possibility of overlapping mechanisms, whereby both direct membrane damage and H_2S/ROS-mediated effects contribute to bacterial clearance.
We thank the reviewer for emphasizing this conceptual distinction, which has strengthened the mechanistic depth of our discussion and highlighted important directions for future work.

2. Many possible sensors for H2S are described (Yang, M.; Zhou, Y.; Wang, K.; Luo, C.; Xie, M.; Shi, X.; Lin, X. Review of Chemical Sensors for Hydrogen Sulfide Detection in Organisms and Living Cells. Sensors 2023, 23, 3316.). How specific is probe L, and can the authors compare these results with another probe?
Response: We thank the reviewer for this insightful and important comment regarding the specificity of H_2S detection in our study.
We acknowledge that numerous H_2S-sensitive probes have been developed, each with varying degrees of selectivity and sensitivity. Probe L, as used in our original experiments, is based on an

azide-reduction mechanism and is commonly employed for selective detection of free H_2S in live macrophages.

The synthesis of this probe is relatively straightforward, with readily available raw materials, high detection sensitivity (limit of detection as low as 24 nM), good stability, and minimal background fluorescence interference. Based on the references provided by the reviewer, we confirmed that this probe operates via a nucleophilic detection mechanism for H_2S. Upon reaction with H_2S, it generates a distinct fluorescent product, offering a clear and low-background signal.

To further validate the specificity of Probe L, we employed an alternative, widely used H_2S detection reagent—WSP-1—to assess H_2S production in LL-37/CRAMP-treated macrophages. As shown in **Fig. R6** (**now presented in Supplementary Fig. S11**), WSP-1 yielded results highly consistent with those obtained using Probe L, thereby supporting the specificity and reliability of our original observations.

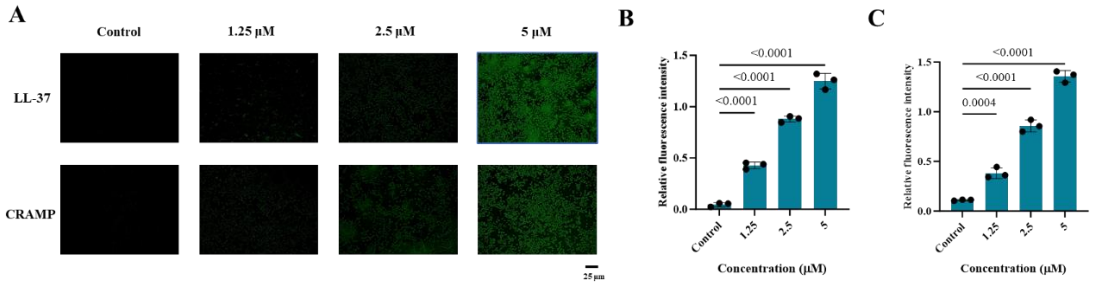

Fig.R6 Intracellular H_2S levels were assessed and visualized using the fluorescent H_2S probe WSP-1 under a fluorescent microscope (A) and the corresponding quantitative analysis (B-C). Scale bar: 25 μm. The results are expressed as mean ± S.D. n = 3 biological replicates. One-way ANOVA followed by Tukey's post hoc analysis. Source data are provided as a Source Data file.

Inserted results section (Manuscript-with tracks: Page: 5, Line:193-196):

"To validate the specificity of Probe L, we compared its performance with WSP-1. The consistent results obtained with both probes support the reliability of our detection approach and indicate that the observed signal predominantly reflects free H_2S (Fig. S11)."

We are grateful to the reviewer for prompting this important control, which has strengthened the methodological rigor of our study.

3. Fig. 2. Potential interactions between compound 7b and AMPs should be determined. The dose of 7b is not indicated in several panels. In 2G 5 uM LL-37 and 200 uM 7b was used. From the chemical structure (Sun et al., Nat Comm 2024) 7b looks hydrophobic and the question is then whether it directly affects AMP activity? Experiments using liposome based assays (PC/PE) could be utilised to directly assess membrane effects alone.

In the recent paper by Sun, J. et al., Nat Commun 2024, 7b seems not to affect bacterial growth alone, but sensitizes to antibiotics. Can the authors explain and discuss this in the light of their present data? In Fig. 2J, 7b alone increases IL-6 The concern here is that 7b at 100 uM has intrinsic effects. Can the authors provide an explanation? In the legend 5 mM LL-37/CRAMP is indicated, 5 uM? Overall, in I-J, direct interaction between AMP and 7b should be excluded.

Response: We thank the reviewer for these thoughtful and multifaceted comments. We address each point below:

To address this concern, we conducted isothermal titration calorimetry (ITC) experiments to investigate potential binding between LL-37 and 7b under the experimental conditions used in our study. As shown in **Fig. R4** (**now presented in Supplementary Fig. S12**), the ITC results revealed no detectable interaction between LL-37 and 7b. These findings indicate that compound 7b does not directly inactivate or interfere with LL-37's activity. We have updated the Methods section and figure legends to include details of the ITC experiments and the results.

Inserted results section (Manuscript-with tracks: Page:5, Line:207-209):

"Importantly, we confirmed that LL-37, as a representativeAMP, does not interact with compound 7b, ensuring that 7b does not interfere with or inactivate LL-37's activity (Fig. S12)."

Inserted methods section (Supplementary Materials with tracks: Page:11, Line:415-422):

"**11 Isothermal titration calorimetry (ITC) assay**

The binding affinity between LL-37 and 7b was measured by isothermal titration calorimetry (ITC) using a Malvern MicroCal PEAQ-ITC instrument (Malvern, USA) at 25 °C. Experiments were performed in a buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl, and 5% DMSO. A solution of 7b was titrated into the sample cell containing LL-37 at a molar ratio of 10:1, with 13 injections performed following an initial 180 s equilibration period. Data were analyzed using Malvern MicroCal PEAQ-ITC Analysis Software."

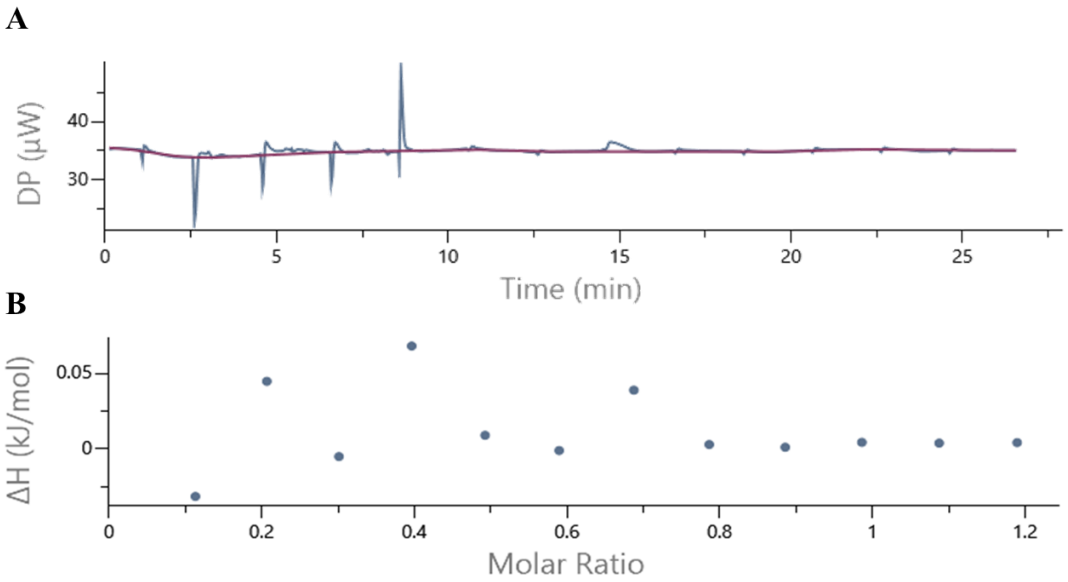

Fig. R4 The interaction between LL-37 and 7b determined by ITC assay, where 0.5 mM 7b was titrated into 0.05 mM LL-37 in HEPES buffer at 25°C.

We will ensure that the concentration of 7b is clearly indicated in the revised methods. In the study by Sun *et al*., (*Nat Commun*. 2024) the primary focus was on the synergistic antibacterial effects of compound 7b in combination with conventional antibiotics. In contrast, our work differs from theirs in several key aspects: (1) The antibacterial mechanisms of antibiotics and antimicrobial peptides (AMPs) are fundamentally different. Antibiotics typically act through single, well-defined targets, whereas AMPs exhibit multifaceted antibacterial activities, including membrane disruption and immunomodulation. Importantly, H_2S may play distinct roles depending on the type of antibacterial mechanism involved. (2) The bacterial species and their H_2S biosynthesis pathways also differ between the two studies. Sun *et al*. investigated Pseudomonas aeruginosa, while our work focuses on Escherichia coli. Notably, the enzymes responsible for H_2S production vary between these

species, potentially leading to differences in how H_2S modulation impacts bacterial physiology and susceptibility. (3) Our study further extends beyond antibacterial efficacy by exploring the roles of H_2S and ROS in the AMP-mediated bactericidal process, providing additional mechanistic insights into the interplay between these metabolic pathways and host-pathogen interactions.

Together, these distinctions highlight that our study addresses different scientific questions and mechanisms relative to the work of Sun *et al*.

In Fig. 2J, we agree that 7b at 100 μM appears to have intrinsic effects on IL-6 production. As 7b is a lipophilic compound, it may interact with cellular membranes or signaling pathways involved in cytokine induction. Such lipophilic properties could potentially influence membrane-associated receptors or modulate intracellular signaling cascades leading to IL-6 production. We have noted that the observed IL-6 induction by 7b alone may reflect its intrinsic immunomodulatory properties due to its physicochemical characteristics. Further mechanistic studies will be necessary to fully elucidate this effect.

We thank the reviewer for noticing this error. The correct concentration is 5 μM, not 5 mM. We apologize for this typographical mistake and have corrected it in the revised figure legend.

.As described in our responses to the previous comments, we have already addressed the possibility of interference between 7b and AMPs.

We thank the reviewer again for raising these important points, which have led us to clarify experimental details and strengthen the mechanistic interpretation of our findings.

4. Causality between H2S and ROS generation: While the manuscript presents data linking LL-37-induced H2S to increased ROS levels and bacterial killing, the directionality of this relationship should be explained. Time-course experiments or mechanistic studies would help establish whether H2S is upstream of ROS in this context. Alternatively, a clearer discussion of these limitations would be of benefit.

Response: We appreciate the reviewer's thoughtful comment regarding the causal relationship between H_2S and ROS in the context of LL-37-induced bacterial killing.

Although the initial version of the manuscript included speculative interpretations regarding the roles of H_2S and ROS in mediating the bactericidal effects of AMPs, these conclusions were not fully supported by direct experimental evidence and may have appeared overly definitive. We acknowledge the current limitations in establishing a causal relationship between LL-37–induced H_2S and ROS levels and their contributions to antimicrobial activity. In future studies, we aim to employ more targeted and rigorous approaches to elucidate this mechanistic link.

While our current data show that both H_2S and ROS levels increase following LL-37 treatment and that H_2S scavenging reduces ROS, we agree that definitive time course of this relationship is lacking. Therefore, we stimulated *Escherichia coli* CMCC44102 with LL-37 and monitored dynamic changes in H_2S and ROS levels over a 12-hour period. As shown in **Figure R7 (now presented in Supplementary Fig. S29**), in the wild-type (WT) group under normal growth conditions, both H_2S and ROS signals remained relatively stable. However, upon LL-37 treatment, we observed a marked increase in both H_2S and ROS levels. Notably, H_2S levels peaked within the first 6 hours and then rapidly declined, whereas ROS levels continued to rise throughout the 12-hour observation period. We speculate that the initial surge in ROS reflects a bacterial response to excessive H_2S production, which may disrupt redox homeostasis. The sustained increase in ROS at later stages could be attributed to ongoing ATP synthesis during bacterial survival efforts, leading to continued ROS

accumulation and amplification. While this experiment does not provide direct evidence for a causal or hierarchical relationship between H_2S and ROS regulation, it offers supportive data that informs our interpretation of their interplay during LL-37-induced stress responses.

Inserted results section (Manuscript-with tracks: Page: 10, Line: 435-440):

"To investigate the link between H_2S and ROS during AMP activity, we monitored LL-37-treated bacteria over 12 hours. The initial ROS surge likely reflects a response to excessive H_2S disrupting redox homeostasis, while the sustained ROS increase may result from ATP-driven accumulation (Fig. S29). These findings support a dynamic interplay between H_2S and ROS in LL-37's antibacterial mechanism."

We hope this transparent discussion addresses the reviewer's concern in the current version of the manuscript.

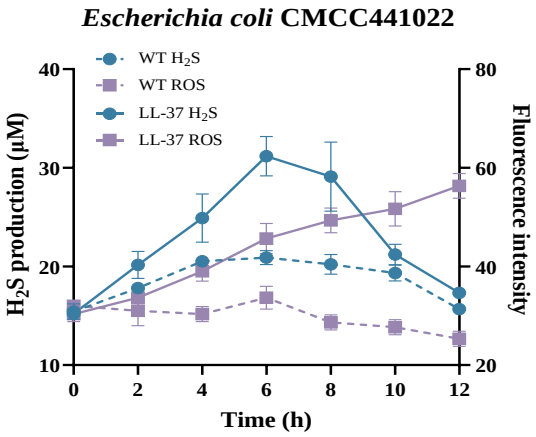

Fig.R7 The dynamics of H_2S and ROS levels in *Escherichia coli* CMCC44102 (1×10^8 CFU/mL) before and after treatment with LL-37 (0.5× MIC) over a 12-hour period.

5. Gene upregulation studies in Fig. 4. Only one bacterial isolate is analysed. Addition of 2-3 other E. coli isolates or if possible other bacteria would strengthen this part. This would answer the question whether this upregulation can be generalised.

Response: We appreciate the reviewer's comment and fully agree that only analyzing the limitations of a single bacterial isolate.

Shatalin *et al*. reported that the enzymes responsible for H_2S production vary across bacterial species (Fig. R5). Furthermore, as shown in Fig. S1, even within the same species, Escherichia coli CMCC44102 and E. coli ATCC25922 exhibit markedly different levels of H_2S production. Therefore, reproducing consistent results across strains is inherently challenging. Even among standard strains of the same bacterial species, H_2S production capacity can vary significantly. This variability arises from several factors, including genetic background, metabolic traits, and physiological adaptability. First, differences in the expression levels or regulatory mechanisms of sulfur metabolism–related genes (e.g., *cysK*, *phsABC*)—such as promoter mutations, deletions of regulatory elements, or plasmid variability—can lead to divergent enzymatic activities. Second, strains may differ in their capacity for substrate uptake and utilization, particularly for sulfur-containing compounds like cysteine and thiosulfate, which directly influence H_2S output. In addition, environmental conditions such as pH and redox state, as well as strain-specific physiological tolerance, may modulate H_2S phenotypes. Lastly, some standard strains may experience "phenotypic

silencing" of H_2S production due to long-term laboratory passaging, which should not be overlooked. Therefore, for the comparative transcriptomic analysis, we selected *Escherichia coli* CMCC44102 as the experimental strain, based on its observable H_2S induction phenotype. We did not include other *E. coli* isolates or strains, and thus do not generalize the observed gene upregulation to other backgrounds.

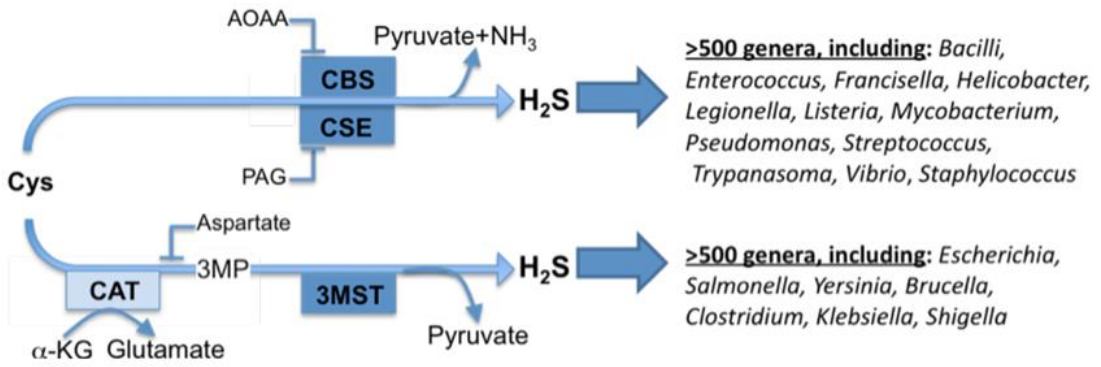

Fig. R5 Schematic representation of basic metabolic pathways of H_2S synthesis. (Shatalin *et al*. *Science*. 2011)

6. Specificity of ROS Detection: The ROS data rely heavily on the DCHF-DA probe, which has limitations. It would strengthen the manuscript to validate key findings using a more specific or complementary ROS detection method.

Response: We thank the reviewer for highlighting this important point regarding the specificity of ROS detection. We fully acknowledge the limitations of the DCFH-DA probe, including its susceptibility to non-specific oxidation and its limited subcellular resolution. DCFH-DA enters bacterial cells and is deacetylated by intracellular esterases to form DCFH, which is then oxidized by ROS to generate fluorescent DCF; the resulting fluorescence intensity reflects intracellular ROS levels.

Despite its limitations, DCFH-DA remains widely used for ROS detection in bacterial studies, as evidenced by its inclusion in several commercially available assay kits ("https://www.medchemexpress.cn/inhibitor-kit/ros-assay-kit.html", "https://www.biosharp.cn/index/product/details/language/cn/product_id/1156.html", "https://www.sigmaaldrich.cn/CN/zh/product/sigma/mak143?utm_campaign=LR2_083_Metabolism+Kits++China&utm_medium=cpc&utm_source=bing&utm_content=sigma%2Fmak143&utm_term=ros%E6%A3%80%E6%B5%8B%E8%AF%95%E5%89%82%E7%9B%92&msclkid=17845e7496761a848209364b2d108a15") and prior publications (e.g., Hong Y *et al*., *Nat Microbiol*. 2017). We recognize the need for more specific and robust ROS detection methods and will take this into account in our future studies to enhance the accuracy of ROS measurement.

Once again, we sincerely thank the reviewer for this valuable suggestion, which has helped improve the rigor of our manuscript.

7. In general, pictures showing only colony formation (such as in 6E) should be backed up by counting CFUs.

Response: We thank the reviewer for this important suggestion.

We adopted the approach described by Shatalin *et al.* (Science, 2021) to assess antibacterial activity by measuring colony formation efficiency.
To address this concern, we have now performed quantitative colony-forming unit (CFU) counts corresponding to the plate images shown in Fig. 6E.

The CFU data, **Fig.R8**, **now presented in Supplementary Fig. S23** confirm the trends observed in the qualitative images: LL-37 treatment significantly reduced bacterial survival, and this effect was partially reversed by co-treatment with the H_2S scavenger 7b.
Inserted results section (Manuscript-with tracks: Page:10 , Line:410):
"This finding is further supported by CFU counts (Fig. S23)."
We believe that these quantitative data not only validate the conclusions drawn from the colony images but also strengthen the statistical rigor of our findings. The figure legend and Results section have been updated accordingly to reflect these additions.
We appreciate the reviewer's feedback, which has led to a more robust and interpretable presentation of our antimicrobial assay data.

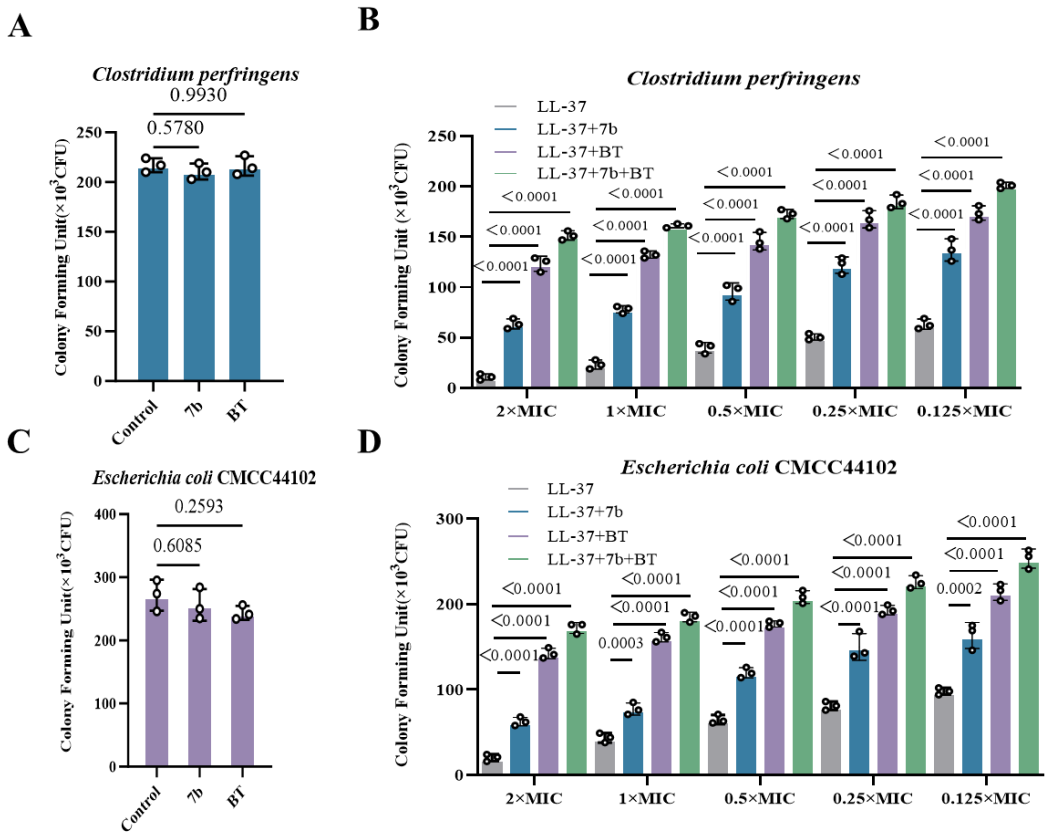

Fig.R8 Colony-forming units (CFUs) counting of (A-B) *Clostridium perfringens* and (C-D) *Escherichia coli* CMCC44102 (2×10^5 CFU/mL) with different treatments. **7b** as H_2S scavengers and bipyridine and thiourea (BT) as ROS scavengers. The results are expressed as mean ± S.D. n = 3 biological replicates. One-way ANOVA followed by Tukey's post hoc analysis. Source data are provided as a Source Data file.

8. For the in vivo experiments, all substances are injected ip. To avoid direct interactions between LL-37 and additions such as 7b, a different administration route for eg 7b, eg sc would demonstrate

whether the effects related to H2S inhibition or not. As 7b is a small hydrophobic substance, it should distribute well. Alternatively, 7b could be given before injection of LL-37. Also, only the CMCC44102 is used. How about other E. coli such as ATCC 25922 or similar? The question is whether the H2S mechanism is common, and 7b has effects on other E. coli strains. Can the authors consider to generalise their findings?

Response: We thank the reviewer for these thoughtful suggestions concerning the design and generalizability of our in vivo experiments.

As we have described in our previous responses and demonstrated in Fig. R4, LL-37 and 7b do not exhibit direct interactions that could confound their combined effects. We chose intraperitoneal (i.p.) administration for both agents to ensure consistent drug delivery and to maximize the effectiveness of 7b in depleting H_2S within the infected microenvironment. Using the same administration route also facilitates direct comparisons between treatment groups while minimizing potential variability introduced by differences in drug absorption, distribution, metabolism, and excretion associated with alternative routes (e.g., subcutaneous).

Regarding bacterial strains, our in vivo experiments were designed based on in vitro findings with *E. coli* CMCC44102, which exhibits robust H_2S production. As shown in Supplementary Fig. S1, the source enzymes and capacity for H_2S biosynthesis differ across *E. coli* strains. Therefore, our current findings cannot yet be generalized to strains with low or absent H_2S production, such as ATCC 25922. We have added a note in the revised manuscript emphasizing that the newly identified H_2S-mediated mechanism may primarily apply to bacteria with significant H_2S-producing capacity, and future studies should investigate its relevance in other *E. coli* strains and species.

We thank the reviewer again for these constructive recommendations, which have improved the clarity and robustness of our in vivo analyses.

9. Quantification of phagocytosis: The method used to assess macrophage phagocytosis relies on CFU counting after cell lysis, which may not distinguish between truly internalized bacteria and those adherent to the surface. This should be addressed.

Response: We thank the reviewer for raising this important point regarding the accuracy of our phagocytosis quantification.

To address the potential confounding effect of surface-adherent bacteria on CFU-based measurements, we refer to section 6.1 of the *Materials and Methods*, which provides a detailed description of the macrophage phagocytosis assay. “Finally, gentamicin (100 μg/mL) was added to kill the extracellular unphagocytosed bacteria, after washing with PBS, 1 mL of 0.03% Triton-X100 was added for lysis, and 50 μL of the lysate was placed on LB medium and incubated at 37℃ for 16 h.”

Furthermore, we noted that a similar experimental approach was employed in Wong’s study to distinguish between truly internalized bacteria and those merely adhering to the cell surface. This supports the validity of our approach in addressing this concern. (Wong *et al*., *Cell Host Microbe*, 2017). This is described in their *STAR ★Methods* section as follows.

“RAW 264.7 cells were seeded onto 12-well culture plates with Dulbecco’s Modified Eagle’s medium (DMEM; Sigma-Aldrich D5796) supplemented with 10% FBS and 100 U/mL penicillin-streptomycin. When ~70% confluency was reached, the cells were serum starved for 16 hr. *E. coli*^{Amp} was cultured to reach $OD_{600\ nm}$ of ~1.0 in LBA. Subsequently, the *E. coli*^{Amp} culture was spun down and resuspended in PBS and added to DMEM+10% FBS to make a

2% *E. coli*^{Amp} infection medium, with estimated multiplicity of infection (MOI) of ~20. The serum-starved macrophages were incubated with the infection medium at 37°C for 30 min, followed by washing with PBS containing 1 mM $CaCl_2$. One plate of the cells was taken for assessing the amount of phagocytosed *E. coli* immediately after washing. Another plate of cells was then cultured with DMEM+10% FBS containing 200 μg/mL gentamicin for the desired length of time. To release the viable *E. coli*^{Amp} inside the cells, the cells were incubated with 500 μL 0.1% saponin per well for 10 min on a rocking platform. 10 μL of the extract from each well was then spread onto LBA agar in a 10-cm petri dish, followed by the CFU quantification as described above."
We appreciate the reviewer's suggestion, which has led us to strengthen the rigor of our phagocytosis assay and confirm the bacteria internalization specificity of our findings.

Minor comments: At several places, there is a lack of specification of concentrations etc in the figures and legends.
Response: We thank the reviewer for highlighting this important point. In the revised manuscript, we have carefully reviewed all figure legends and ensured that the concentrations of all compounds (including peptides, inhibitors, and other reagents), as well as incubation times and other relevant experimental conditions, are clearly specified. We have also cross-checked the Methods section to ensure consistency and completeness of experimental details throughout the manuscript. These revisions will facilitate accurate interpretation and reproducibility of our findings.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.
Response: We appreciate the reviewer's contribution to the peer review process and commend *Nature Communications* for promoting transparency and recognition of Early Career Researchers through this initiative. We are grateful for the time and effort invested in the thoughtful evaluation of our manuscript.

This paper investigates the role of a gasotrasmitter hydrogen sulfide (H_2S) in the antimicrobial activity of AMPs across different animal species. The authors show that AMPs like LL-37 and its mouse homolog CRAMP stimulate H_2S production both in bacteria and macrophages, primarily through enzymes like 3MST and CSE. The generated H_2S contributes to bacterial killing via reactive oxygen species (ROS) and supports the immunomodulatory functions of AMPs. The study uncovers a previously unknown, conserved mechanism by which H_2S enhances the anti-infective effects of AMPs.

While hydrogen sulfide (H_2S) is generally recognized as a bacterial defense mechanism, this study tries to show that AMPs have evolved to exploit this gasotransmitter to enhance their antimicrobial activity. By inducing H_2S production, AMPs trigger oxidative stress and bacterial death, turning a typical bacterial survival strategy into a vulnerability.

The findings presented here are novel and interesting, particularly the concept that AMPs exploit

bacterial H_2S production to exert their antimicrobial effects. However, it is notable that the authors have previously reported that targeting H_2S can sensitize bacteria to antibiotics (ref 29). Surprisingly, they do not cite their own earlier work in this context (Introduction line 77-79; Discussion line 525-532), instead only referencing studies from other groups. It would strengthen the manuscript to acknowledge and discuss their prior contributions to this area.

Overall the study is well conceived and the experiments are well designed. The description of the results is clear and the claims are in general supported by the results. But some clarification are needed, to valorise better the work done.

Response: We sincerely thank the reviewer for this thoughtful comment and for highlighting the relevance of our earlier work. We agree that citing and discussing our prior findings would strengthen the context of the current study.

In our previous reports of reference 29 (Sun *et al*., *Nat Commun*. 2024), the primary focus was on the synergistic antibacterial effects of compound 7b in combination with conventional antibiotics. In contrast, our work differs from theirs in several key aspects: (1) The antibacterial mechanisms of antibiotics and antimicrobial peptides (AMPs) are fundamentally different. Antibiotics typically act through single, well-defined targets, whereas AMPs exhibit multifaceted antibacterial activities, including membrane disruption and immunomodulation. Importantly, H_2S may play distinct roles depending on the type of antibacterial mechanism involved. (2) The bacterial species and their H_2S biosynthesis pathways also differ between the two studies. Sun *et al*. investigated Pseudomonas aeruginosa, while our work focuses on *Escherichia coli*. Notably, the enzymes responsible for H_2S production vary between these species, potentially leading to differences in how H_2S modulation impacts bacterial physiology and susceptibility. (3) Our study further extends beyond antibacterial efficacy by exploring the roles of H_2S and ROS in the AMP-mediated bactericidal process, providing additional mechanistic insights into the interplay between these metabolic pathways and host-pathogen interactions. Together, these distinctions highlight that our study addresses different scientific questions and mechanisms relative to our previous work.

We hope these illustrations now clarify the continuity between our prior contributions and the novel mechanism uncovered here, namely that AMPs exploit bacterial H_2S production to enhance antimicrobial activity. We thank the reviewer again for this valuable suggestion, which has helped us to better position our study in the broader research landscape.

1) Fig. 1F would be clear if the authors included the staining of nuclei and an additional control with only peptide and the probe for H2S.

Response: We thank the reviewer for this helpful suggestion to improve the clarity of Fig. 1F. In Fig. 1F, our main focus was to compare the differences in H_2S signals between the control and the various treatment groups. The H_2S probe we employed is highly specific, which ensures the reliability of the observed signal. In addition, we further validated these results using the WSP-1 probe, which confirmed the specificity and robustness of the H_2S detection (**shown in Fig. R1, now presented in Supplementary Fig. S11**). These data provide strong evidence that the observed fluorescence originates from intracellular H_2S production induced by LL-37/CRAMP treatment.

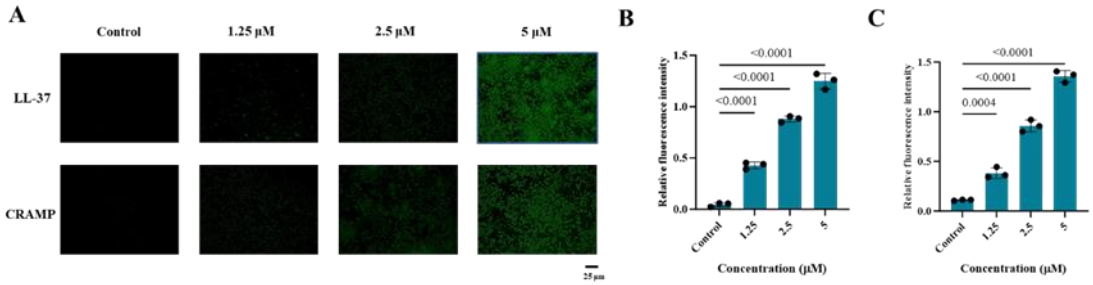

Fig. R1 Intracellular H_2S levels were assessed and visualized using the fluorescent H_2S probe WSP-1 under a fluorescent microscope (A) and the corresponding quantitative analysis (B-C). Scale bar: 25 μm. The results are expressed as mean ± S.D. n = 3 biological replicates. One-way ANOVA followed by Tukey's post hoc analysis. Source data are provided as a Source Data file.

We appreciate the reviewer's recommendation, which has helped us clarify the rationale for our experimental design.

2) In Figure 2I and 2J, the authors attribute the anti-inflammatory effects of LL-37 to its modulation of H_2S production, based on experiments using compound 7b (an H_2S scavenger, Ref. 29). However, an alternative explanation should be considered: it is possible that LL-37 has a higher affinity for 7b than for LPS, which could reduce the availability of LL-37 to neutralize LPS rather than directly implicating H_2S modulation.

Response: We thank the reviewer for raising this important alternative explanation. To clarify this concern, we conducted isothermal titration calorimetry (ITC) experiments to directly assess the potential interaction between LL-37 and compound 7b. The results demonstrated no detectable binding affinity between LL-37 and 7b, thereby excluding the possibility that 7b sequesters LL-37 and interferes with its interaction with LPS. As shown in **Fig. R2** (**now presented in Supplementary Fig. S12**), the ITC results revealed no detectable interaction between LL-37 and 7b. These findings indicate that compound 7b does not directly inactivate or interfere with LL-37's activity. These findings confirm that the observed anti-inflammatory effects of LL-37 in the presence of 7b are indeed attributable to H_2S modulation rather than to direct binding competition.

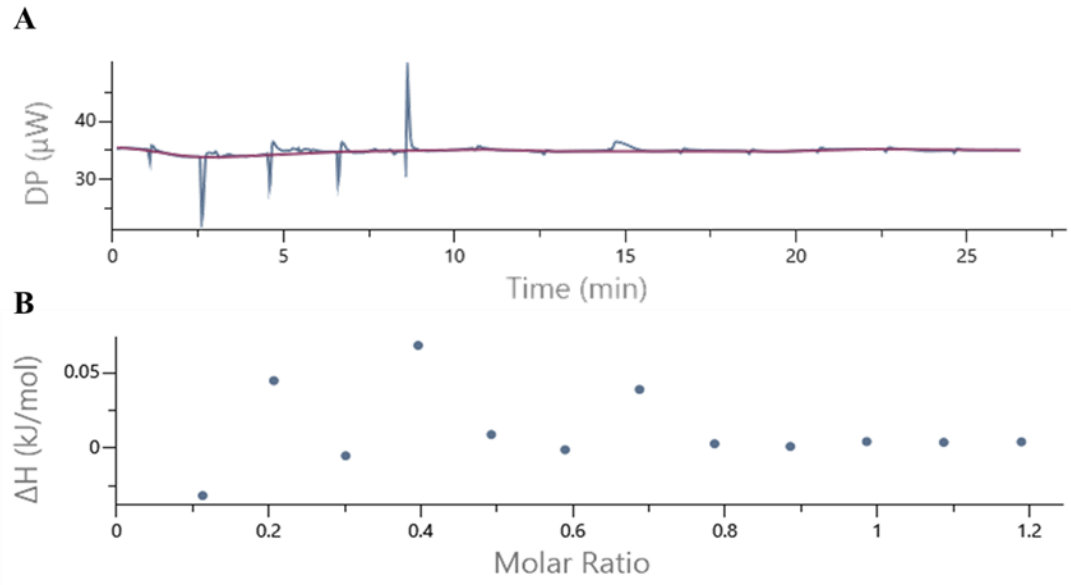

Fig. R2 The interaction between LL-37 and 7b determined by ITC assay, where 0.5 mM 7b was titrated into 0.05 mM LL-37 in HEPES buffer at 25°C.

We appreciate the reviewer's insightful comment, which prompted us to strengthen the mechanistic interpretation of our results.

3) Furthermore, the cytokine data raise questions: in the control group, TNF-α levels are extremely high but decrease with the addition of 7b, whereas IL-6 levels behave oppositely (they are lower in the control and increase with 7b). The authors should clarify how they interpret these opposite trends and provide an explanation for this apparent discrepancy. Also, they should clarify why the levels of cytokines are so high in the control?

Response: We appreciate the reviewer's thoughtful comment. Our study primarily focused on the role of H_2S in mediating the anti-inflammatory effects of LL-37/CRAMP under LPS-stimulated conditions, where the signaling context is well defined and reproducible. In this setting, the regulatory role of H_2S on cytokine expression can be more clearly interpreted.

In contrast, under non-LPS-stimulated conditions, cytokine levels can be influenced by multiple factors unrelated to our main mechanistic focus. For instance: 1) Basal activation of macrophages can lead to relatively high levels of TNF-α, reflecting the sensitivity of these cells to subtle environmental stimuli. 2) Differential regulation of TNF-α and IL-6: it is well documented that TNF-α is an early-response cytokine, whereas IL-6 expression is often sustained by distinct feedback pathways, which may explain the opposite trends observed. 3) Autocrine/paracrine signaling loops and variations in redox balance or metabolic activity can also modulate cytokine release independently of exogenous LPS stimulation. Taken together, the seemingly discrepant cytokine trends in the control group are likely due to the complex baseline regulatory mechanisms in macrophages, rather than a direct effect of compound 7b. To address this point, we have clarified that our mechanistic conclusions are drawn specifically from the LPS-stimulated model, while acknowledging that cytokine regulation under basal conditions may involve additional factors.

We appreciate the reviewer's insightful observation, which has helped us to more clearly interpret and present our cytokine data.

4) Figure 5I, there is significant increase in phosphorylated Akt already at 1 h, not only at 8h. All the blots used for quantification should be provided in the source data.

Response: We thank the reviewer for this valuable observation. We have carefully re-examined our quantitative results and revised the description in the Results section to reflect the significant increase in phosphorylated Akt already detectable at 1 h, in addition to the sustained elevation observed at 8 h.

Revised results section (Manuscript-with tracks: Page:9, Line:377):

"We observed that Akt phosphorylation was elevated after LL-37 treatment, with significant increases at 1 hour (Figure 5I),"

Furthermore, we have included all the original data for quantification in the source data file to ensure transparency and reproducibility.

We are grateful for the reviewer's suggestion, which has helped us improve both the accuracy and the completeness of data presentation.

5) GAPDH in figure 5C looks like form another blot, same for beta-actin in figure 5D.

Response: We thank the reviewer for this careful observation. We acknowledge that the loading controls (GAPDH in Fig. 5C and β-actin in Fig. 5D) were not obtained from the exact same gel as the target proteins. This was because the molecular weights of the target proteins were very close to those of the housekeeping proteins, making it technically difficult to probe both on the same blot without overlap. To address this, we ran the same batch of samples on duplicate gels under identical conditions, and probed one blot for the target protein and the other for the loading control.
We appreciate the reviewer's attention to detail, which has helped us improve the clarity and reproducibility of our data presentation.

6) How much lysate was loaded on the gel?

Response: We thank the reviewer for this question. For all Western blot experiments, we consistently loaded 30 μg of total protein lysate per lane, as determined by BCA protein assay. This information has now been explicitly stated in the Methods section to ensure clarity and reproducibility.
We appreciate the reviewer's attention to methodological detail, which has helped us further improve the transparency of our experimental description.

7) In Fig 6A and B, how do the authors explain the increase in ROS for the control of C. perfringers after 24 h?

Response: We thank the reviewer for this important question. We observed that in the untreated control group of C. perfringens, ROS levels gradually increased after prolonged incubation (24 h). This phenomenon is likely attributable to the natural progression of bacterial growth into the stationary phase, during which metabolic stress and nutrient limitation lead to endogenous ROS accumulation. Similar increases in ROS under extended culture conditions have been reported previously in anaerobic and facultative anaerobic bacteria.
We appreciate the reviewer's insightful comment, which has helped us provide a clearer explanation of our observations.

8) In Fig. 8A, the schematic illustration shows that organs were collected after 3 days, but later in the Mat & Meth section it is written 24 h. Also, the schematic illustration shows that treatment was done after 2 h, while in the Mat & Meth section it is written 4 h. Please change the schematics accordingly.

Response: We thank the reviewer for pointing out this inconsistency. The confusion was caused by imprecise labeling in the schematic illustration. In fact, the organs were collected at 24 h post-infection; the indication of "3 days" in Fig. 8A was only meant to show that body weight was monitored for three days. To avoid misunderstanding, we have now revised Fig. 8A accordingly. Regarding the timing of drug administration, treatment was indeed initiated 2 h after bacterial infection. We have accordingly revised the description in the Methods section.
(***Supplementary Materials with tracks-Page:11, line:388:*** "After 2 h, different administration groups were carried out by intraperitoneal injection."
We appreciate the reviewer's attention to detail, which has helped us improve the accuracy and consistency of data presentation.

9) Why 2 different concentrations of bacteria were used for survival and other assays?

Response: We thank the reviewer for raising this important point. Different bacterial concentrations were employed because the survival experiments (1×10^8 CFU/mL) required a more severe infection challenge in order to observe meaningful differences in mortality within an appropriate time frame, whereas the mechanistic and immunological assays (5×10^7 CFU/mL) were designed to monitor host responses under conditions that allow sufficient survival for sample collection and analysis. Thus, the two concentrations were chosen to balance biological relevance with experimental feasibility.
We appreciate the reviewer's insightful comment, which has enabled us to make the experimental design clearer for readers.

10) In Fig. 8H it seems that there is a significant decrease in CFU independently of the bacterial strain.

Response: We thank the reviewer for this thoughtful comment. In fact, the quantitative CFU analysis in Fig. 8H shows that LL-37 exerts a significantly stronger bactericidal effect against the wild-type *E. coli* CMCC44102 compared with the 3MST-deficient strain, with clear statistical differences. This indicates that although both strains exhibit CFU reduction, the extent of killing is strain-dependent and highlights the role of 3MST in mediating LL-37's antimicrobial activity.
We appreciate the reviewer's observation, which prompted us to refine the description and interpretation of the data.

11) For Fig. 1 and 9 (for macrophages) how do the authors explain that there is no dose-dependence?

Response: We thank the reviewer for this insightful question. In our experiments, the effects of LL-37 on H_2S production and macrophage responses reached a plateau within the concentration range tested. This likely reflects a saturation effect, where even low doses are sufficient to fully activate the relevant signaling pathways, and higher doses do not further enhance the response.

12) The data suggest that AMPs stimulate H_2S production through two distinct pathways: 3MST in bacteria and CSE in macrophages. Could the authors clarify whether this difference reflects a broader, fundamental divergence between prokaryotic and eukaryotic systems, or if it might be specific to the particular bacterial strains and conditions used in this study?

Response: We thank the reviewer for this insightful question. Our results indicate that AMPs stimulate H_2S production predominantly via 3MST in bacteria and via CSE in macrophages. In both prokaryotic and eukaryotic systems, H_2S can be produced through three main enzymatic pathways: cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3MST). However, the relative contribution of each enzyme varies substantially depending on the organism, cell type, and environmental context.
In bacteria, 3MST has been reported as a major H_2S-producing enzyme in several strains, but it is not the only possible pathway—different bacterial species may rely on distinct H_2S-generating enzymes depending on their genetic background and metabolic requirements. In contrast, in mammalian macrophages, CSE is often the dominant source of inducible H_2S production, although CBS and 3MST are also expressed and can contribute under certain conditions.
Shatalin *et al*. reported that the enzymes responsible for H_2S production vary across bacterial species (Fig. R3). Furthermore, as shown in Fig. S1, even within the same species, Escherichia coli CMCC44102 and E. coli ATCC25922 exhibit markedly different levels of H_2S production. Therefore, reproducing consistent results across strains is inherently challenging. Even among

standard strains of the same bacterial species, H_2S production capacity can vary significantly. This variability arises from several factors, including genetic background, metabolic traits, and physiological adaptability. First, differences in the expression levels or regulatory mechanisms of sulfur metabolism–related genes (e.g., *cysK*, *phsABC*)—such as promoter mutations, deletions of regulatory elements, or plasmid variability—can lead to divergent enzymatic activities. Second, strains may differ in their capacity for substrate uptake and utilization, particularly for sulfur-containing compounds like cysteine and thiosulfate, which directly influence H_2S output. In addition, environmental conditions such as pH and redox state, as well as strain-specific physiological tolerance, may modulate H_2S phenotypes. Lastly, some standard strains may experience "phenotypic silencing" of H_2S production due to long-term laboratory passaging, which should not be overlooked. Therefore, the differences observed in our study are not indicative of a strict, fundamental divergence between prokaryotic and eukaryotic systems, but rather reflect the context-dependent predominance of specific H_2S-producing enzymes. We appreciate the reviewer's thoughtful comment, which has allowed us to refine the interpretation and scope of our findings.

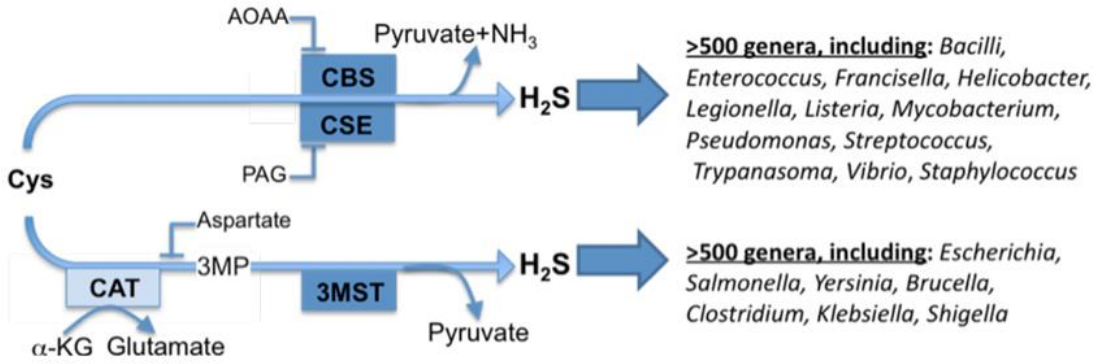

Fig. R3 Schematic representation of basic metabolic pathways of H_2S synthesis. (Shatalin *et al*. *Science*. 2011)

13) I guess for Colony morphology assay LB agar was used, please clarify this in Mat & Meth.

Response: We thank the reviewer for pointing this out. Indeed, LB agar was used for the colony morphology assay. We have mentioned this information explicitly in the Methods section (***Supplementary Materials with tracks-Page:5, line:128-130:*** "Subsequently, 5 µL of each sample were spotted onto **LB medium** under different monitoring times and incubated at 37°C for 16 h.") to ensure accuracy and reproducibility. We appreciate the reviewer's careful reading, which has helped us improve the completeness of our methodological description.

Reviewer #4 (Remarks to the Author):

The manuscript described the effect of CRAMP and LL-37 on the H2S production in bacteria and in macrophages, and how the increased H2S production aids in the antimicrobial effect of these peptides (either directly on the bacteria or indirectly on the macrophage.

The authors have performed a very long list of experiments, which needs to be respected Some of the data seem to quite convincingly show data in favor of their hypothesis, but especially many of

the in vitro experiments are less convincing because the effects are either relatively very small, are semi-quantitative or proper controls are lacking to show it is really the H2S that is involved. The authors also often neglect to add the exact concentrations of the AMPs used while this is needed to understand what the AMP does short term and long term in the in vitro experiments.
However, the in vivo infection experiment seems relatively convincing that there is a role of H2S, which is why I think the study could be good, after major revisions . I discuss my concerns in more detail below.

Response: We sincerely thank the reviewer for the thoughtful summary and constructive critique of our manuscript. We appreciate the recognition of the breadth of experimental work conducted and are pleased that the in vivo infection data were found to convincingly support a role for H_2S in the antimicrobial action of LL-37 and CRAMP. In response to the reviewer's broader concerns:

In vitro data and quantitative rigor:

We acknowledge that some of the in vitro effects appeared modest or lacked quantitative robustness in the original submission. To address this, we have added additional replicates and statistical analyses to reinforce reproducibility. Introduced new control experiments (e.g., scrambled peptide controls, and dose-response studies). We have also supplemented semi-quantitative assays with quantitative methods, including CFU counts and time-course analyses using complementary approaches.

Clarification of H_2S involvement:

We recognize the need for clearer mechanistic evidence directly linking H_2S to the observed phenotypes. In the revised manuscript, we now provide alternative H_2S probes to validate specificity. and new phasing experiments to assess H_2S induction and ROS accumulation over time.

Reporting of AMP concentrations:

We thank the reviewer for highlighting this omission. We have now systematically revised all figure legends, main text, and supplementary materials to explicitly include the exact concentrations and exposure times for LL-37, CRAMP, and related compounds. The relationship of AMP concentration to MIC in each assay context (e.g., Fig. 1A–D, Fig. 2C–F, Fig. 3C–D, G–H, I–J, M–N, Fig. 4A–B, Fig. 6A–D, G–H, I–K).

We believe these revisions significantly enhance the clarity, mechanistic support, and overall rigor of the manuscript. We are grateful for the reviewer's helpful comments, which guided several key improvements in our revised work.

1) The effect of H2S induction in bacteria seems to be a long term effect of several hours. However, bacterial killing by AMPs is a fast process often within minutes. The authors should perform killing kinetics to determine how many bacteria are killed (at multiple concentrations of AMPs) after 5 min, 10 min. 2h. This is the basis for understanding many of the long-term experiments where H2S is involved because also at sub-MIC concentrations, up to 1 or 2 log decreases in viability of bacteria will be observed.

Response: We thank the reviewer for this insightful and important comment. We agree that establishing the kinetics of AMP-mediated bacterial killing is essential to distinguish direct antimicrobial effects from downstream consequences such as H_2S induction.

To address this, we have performed bacterial killing kinetics assays using LL-37 at multiple concentrations (0.5× and 1×MIC), assessing CFU counts at 5, 10, 30, 60, 120 and 180 min post-treatment. These new data are presented in Fig. R9.

Our results (**Fig.R9**, **now presented in Supplementary Fig. S16**) show that at 1× MIC, LL-37 markedly inhibits bacterial growth, with strong bactericidal activity evident even at 180 minutes. At 0.5× MIC, the antimicrobial effect of LL-37 begins to wane after 60 minutes. Notably, in the co-treatment group with 7b, a significant reduction in antimicrobial activity is observed as early as 30 minutes, indicating that H_2S may contribute to modulating the bactericidal effect of LL-37 during the early stages of treatment. This inhibitory effect persists and remains detectable at 180 minutes. These findings provide important insights into the temporal involvement of H_2S in the antimicrobial mechanisms of LL-37.

We have incorporated this new kinetic data into the revised manuscirpt and clarified that direct killing and H_2S-mediated mechanisms operate on distinct timescales, likely reflecting complementary modes of AMP action.

Inserted results section (Manuscript-with tracks: Page:6, Line:234-237):

"To further investigate the role of H_2S in LL-37's antibacterial mechanism, we conducted bactericidal kinetics studies. The results showed that H_2S modulated LL-37's activity early in treatment, with this effect persisting up to 180 minutes (Fig. S16)."

We are grateful for the reviewer's suggestion, which helped clarify the temporal dynamics of AMP activity and H_2S involvement

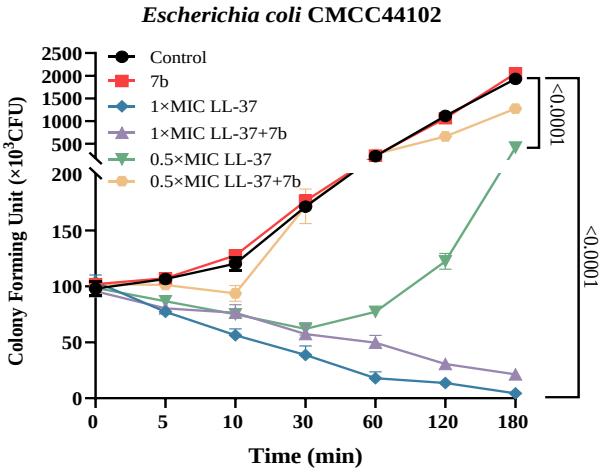

Fig.R9. Bactericidal kinetics of LL-37 (1×MIC, 0.5×MIC) against *Escherichia coli* CMCC44102 (1×10^5 CFU/mL) with or without 7b co-treatment. The results are expressed as mean ± S.D. n = 3 biological replicates. One-way ANOVA followed by Tukey's post hoc analysis. Source data are provided as a Source Data file.

2) For all figures, please add in the figure legends what concentration of AMPs is used and how long incubation was performed for. Also different densities of bacteria are used in different experiments. Obviously higher CFU/ml would impact the killing and other effects of AMPs at every concentration. In addition, also in the methods many details regarding concentrations and incubation times and temperatures are missing, but are very important for the interpretation of the data. Please provide the missing details.

Response: We thank the reviewer for emphasizing the importance of precise experimental detail. We sincerely apologize for the lack of clarity in the figure legends and Methods section. In response, we have thoroughly revised the figure legends to include details. Additionally, we have updated the Materials and Methods section to provide comprehensive information on experimental conditions,

including precise concentrations, incubation times, temperatures, and CFU/ml used in each assay. These changes ensure the reproducibility and proper interpretation of our data

3) Fig. 2 and 3 shows really small effects of compound 7b and the Δ3MST on the antibacterial activity of CRAMP and LL-37. It is all about a few percent less killing or less PI staining or semi-quantitative colony forming, often shown in representative figures. This does not convince me that H2S plays a major role in bacterial killing by AMPs such as LL-37 and cannot convincingly explain the in vivo data later on in the manuscript. To properly address if H2S plays an important role the authors have to systematically test the effect of H2S on the antimicrobial mechanism of AMPs and this has to be done with the proper controls. So MIC and MBC values and kinetic killing studies should be determined for a large set of bacteria, including some of the non H2S producing ones in the presence and absence of compound 7B. In addition, besides LL-37 and CRAMP, a larger set of peptides that are supposed to increase H2S should be included, plus several antibiotics or other antimicrobial compounds that don't affect as H2S negative control. Similarly the set of AMPs should be properly tested (MIC, MBC Kinetic killing studies) against E.coli CMCC44102 and its Δ 3MST variant. Only with these data you set a solid basis for showing in vitro that AMP-induced H2S production in bacteria adds to the antimicrobial mechanism of AMPs. Fig S16D shows what might look like a large increase in MIC based on the numbers, but in the setup used it means that only 1 well more showed growth after 24h. in theory if smaller steps are used for concentration increases, an MIC increase of for example 1.1-fold change would give the same observed result in Fig S16D.

Response: We thank the reviewer for this critical and valuable assessment. We acknowledge that some of the effects of 7b and Δ3MST deletion on AMP-mediated bacterial killing observed in Figs. 2 and 3 are modest in magnitude. However, even these relatively small changes (e.g., a few percent reductions in PI staining and colony formation) are consistent across independent replicates and reach statistical significance, suggesting a reproducible biological effect.

We fully agree that a systematic analysis involving a larger set of bacterial strains (including non-H_2S producers), multiple antimicrobial peptides, and antibiotics as negative controls would provide a more detailed understanding of the role of H_2S in modulating antimicrobial efficacy. However, we respectfully note that performing such an extensive series of experiments—including MIC and MBC determinations, time-kill kinetics across a broad bacterial panel, and testing additional peptides and antibiotics—would require substantial time and resources beyond the scope of the current study. Our present work was designed as a mechanistic proof-of-concept focused on LL-37 and CRAMP, supported by in vivo data using a well-characterized H_2S-producing *E. coli* strain (CMCC44102). To address the reviewer's concern, we have revised the manuscript to explicitly acknowledge this limitation. We now emphasize that while our findings support a contributory role for H_2S in modulating AMP activity in H_2S-producing bacteria, future studies involving a wider range of bacteria and antimicrobial agents will be essential to determine the generality of this mechanism and to rigorously establish its specificity.

We thank the reviewer for this thoughtful suggestion and for highlighting the need for rigorous MIC, MBC, and kinetic killing studies using *E. coli* CMCC44102 and its Δ3MST variant. We fully agree that such systematic data would strengthen the mechanistic evidence for AMP-induced H_2S production contributing to antimicrobial activity. However, as noted in our current work, LL-37 itself exhibits relatively limited antibacterial activity against the wild-type *E. coli* CMCC44102

strain (see Fig. S22D), making it challenging to detect clear and consistent differences in MIC or MBC values between the wild-type and Δ3MST backgrounds. Furthermore, since H_2S appears to act as a modulatory factor that only partially contributes to the killing activity of AMPs, the magnitude of its effect may be too subtle to produce robust shifts in MIC/MBC values, particularly given the inherent resolution limitations of standard twofold dilution assays.
We acknowledge the reviewer's point regarding Fig. S22D and have clarified in the revised figure legend that the apparent MIC increase reflects growth in only a single additional well under the assay conditions, which may overestimate the biological significance of this change. To address this limitation, we have also stated that future studies with finer concentration gradients and alternative quantitative approaches will be necessary to fully elucidate the contribution of H_2S to AMP-mediated killing.

4) The methods used to determine H2S production is very indirect by lead staining. And some of the differences are very small. For example in fig S3 showing the effect of LL-37 (and CRAMP) on H2S production of vibrio or E. coli. With n=3 it is very hard to imagine how these values can actually be statistically significant. And it is also questionable if that is actiually a biological relevant difference. Also by setting the control at 100% in every experiment you automatically have a paired statistical test and not an unpaired test.
Response: We thank the reviewer for raising these important points regarding the methodology and statistical analysis. We agree with the reviewer that the lead acetate test paper method provides an indirect measure of bacterial H_2S production, particularly in terms of sensitivity and specificity. However, it remains a classic and widely recognized technique for detecting bacterial H_2S, and has been employed in two *Science* publications (e.g., Shatalin *et al.*, *Science* 2011, 2021). To address this limitation and provide more robust evidence for LL-37/CRAMP-induced H_2S production, we also applied the methylene blue assay (Fig. 1C–D), which offers a more quantitative and sensitive characterization of H_2S levels.
We would like to clarify that Fig. S3 represents a relative quantification of PbS staining intensity on lead acetate strips, intended to complement the primary data in Fig. 1. Regarding the statistical analysis, we have now explicitly indicated in the revised figure legend that an unpaired t-test was performed for this dataset.

5) also fig S3, as example of earlier point1: How long were the bacteria incubated with the lead strips in these figures? (the methods indicate multiple time points).
Response: We appreciate the reviewer's careful attention to experimental details. We recognize that our Methods section currently lists multiple time points for lead strip exposure, which may have caused confusion. We acknowledge that we did not clearly indicate the incubation time in the figure legend, and we apologize for this oversight. Fig. S3 presents the quantitative results of H_2S production by bacteria induced by LL-37/CRAMP, corresponding to the data shown in Fig. 1. In this experiment, the bacteria were incubated with the lead acetate strips for 4 hours. We have revised the figure legend in the updated manuscript to include this information for clarity. We will clarify the exact incubation durations used in each experiment within the figure legend section.

6) Scrambled LL-37 was tested in the bacterial H2S experiments which is a good control Was scrambled LL-37 also tested in the macrophage experiments? This should be included to show a

real LL-37 specific effect.

Response: We thank the reviewer for this valuable suggestion. To address this, we have conducted additional macrophage experiments using scrambled LL-37. The results, now included in **Fig. R10** (**now presented in Supplementary Fig. S10**), demonstrate that scrambled LL-37 does not induce H_2S production in RAW264.7 macrophages, in contrast to native LL-37. These findings confirm that the observed effects on macrophages are specific to LL-37.

Inserted results section (Manuscript-with tracks: Page:5, Line:190-193):

"We also observed that the scrambled LL-37 peptide failed to induce H_2S production in macrophages, indicating that the stimulatory effect of LL-37 on H_2S synthesis is sequence-specific (Fig. S10)."

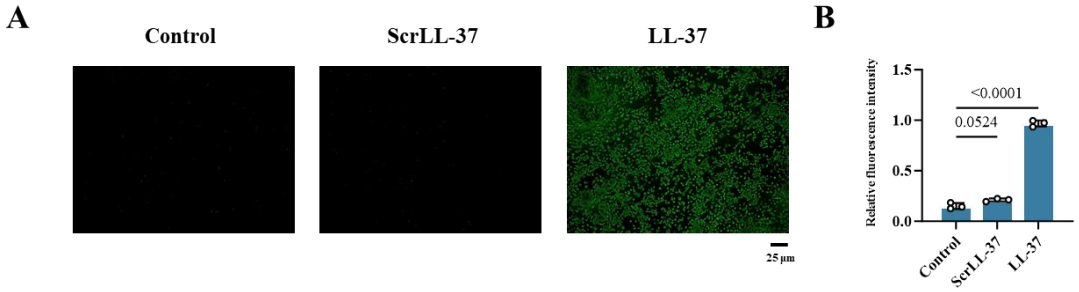

Fig.R10 Extracellular H_2S level was assessed and visualized by a fluorescent H_2S probe under a fluorescent microscope (A) and the corresponding quantitative analysis (B). Scale bar: 25 μm. The results are expressed as mean ± S.D. n = 3 biological replicates. One-way ANOVA followed by Tukey's post hoc analysis. Source data are provided as a Source Data file.

7) Fig S7C: The scavenger does not affect growth. Officially correct because there is no significant difference with and without treatment. But for 50 and 200 uM the values are clearly lower than the control. Considering how much-smaller differences earlier mentioned in Fig S3 are apparently significant, I have serious problems with the blunt statement that the scavenger does not affect viability based on this data.

In addition: what are the short term toxic effects of compound 7B, the 24h shown might show effects of starting regrowth of RAW cells.

Response: We thank the reviewer for highlighting this important point. To address these concerns, we have repeated the experiments assessing the effects of compound 7b (at 50 μM, 100 μM and 200 μM) on bacterial growth and RAW264.7 macrophage viability. The new data (now presented in Supplementary Fig. S7C) confirm that treatment with 7b at these concentrations does not result in statistically significant differences in bacterial growth compared to untreated controls.

In the current study, we evaluated the short-term (4 h) effects of 7b on RAW264.7 cell viability and observed no significant cytotoxicity at the concentrations tested. These findings suggest that 7b does not exert appreciable toxic effects on RAW cells within this timeframe. However, we acknowledge that we did not assess the potential effects of 7b on RAW cell viability beyond 4 h, including at the 24 h time point. As such, we cannot fully exclude the possibility that changes in cell growth or recovery might occur during longer exposures.

We have updated the manuscript section to reflect these additional results. We are grateful to the reviewer for this valuable suggestion, which has allowed us to strengthen the experimental evidence supporting our conclusions.

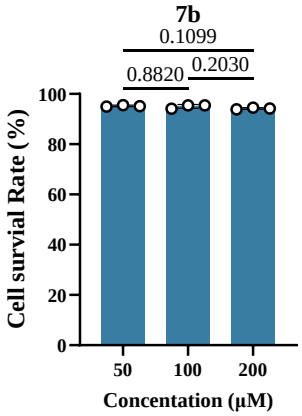

Fig. S7C MTT assay showed the cell viability of RAW264.7 macrophages treated with various concentrations of H_2S scavenger **7b** (50, 100, 200 μM) for 24 h. The results are expressed as mean ± S.D. n = 3 biological replicates. One-way ANOVA followed by Tukey's post hoc analysis. Source data are provided as a Source Data file.

8) Fig 2C+D: (example of point 2) what concentration of CRAMP and LL-37 is used? Also ODmeasuremnt does not always represent viable bacteria (but could also just indicate color change). Bacteria after 12h incubation should be plated out on agar plates and enumerated to determine the real amount of viable bacteria.

Response: We appreciate the reviewer's thoughtful suggestion regarding the limitations of OD measurements. In Fig. 2C and 2D, the concentrations of CRAMP and LL-37 used were 0.5×MIC each, as now indicated in the revised figure legends for clarity.

We fully agree with the reviewer that OD_{600} measurements may not always accurately reflect viable bacterial counts, as they do not discriminate between live and dead cells or account for potential colorimetric interferences. To address this, we have performed additional experiments in which bacteria were incubated for 12 hours under the same experimental conditions, followed by plating on agar to quantify viable bacterial counts (CFUs). These results (**Fig.R11**, **now presented in Supplementary Fig. S13**) are consistent with the OD measurements and further support the conclusion that LL-37 and compound 7b influence bacterial viability in an H_2S-dependent manner.

Inserted results section (Manuscript-with tracks: Page: 5, Line: 218-222):

"To address the limitation of the OD_{600} method in directly reflecting viable bacterial numbers, we quantified colony-forming units (CFUs) after 12 hours of incubation. These results further support that LL-37/CRAMP exert antibacterial activity, at least in part, by inducing H_2S production (Fig. S13)."

We are grateful to the reviewer for suggesting this important addition, which has strengthened the robustness of our findings.

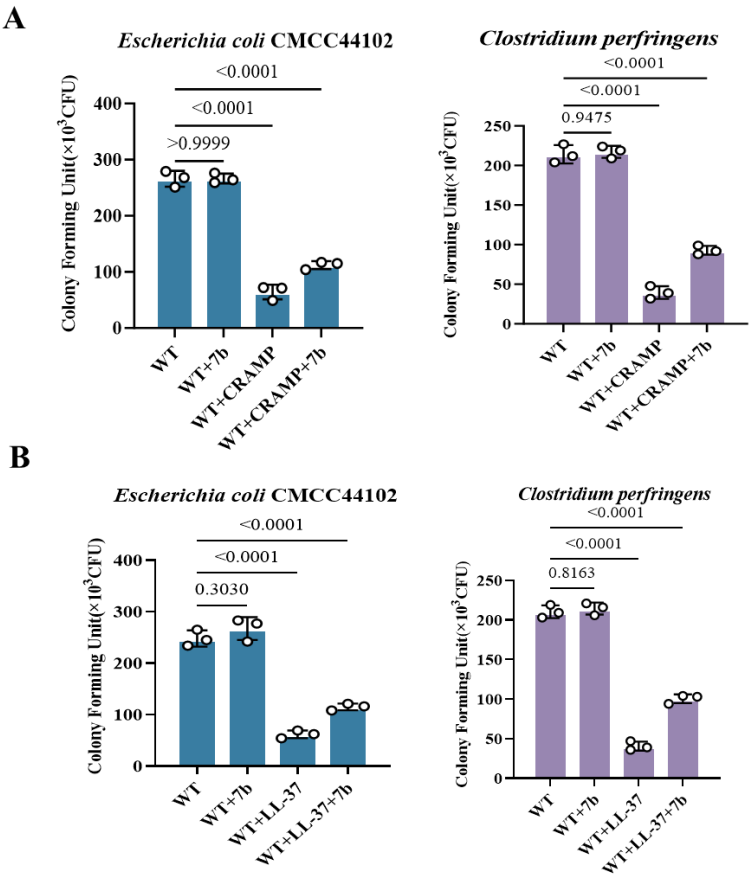

Fig. R11 Colony-forming units (CFUs) counting of *Clostridium perfringens* and *Escherichia coli* CMCC44102 in the presence of CRAMP (A) and LL-37 (B) with different treatments after 12h incubation. The results are expressed as mean ± S.D. n = 3 biological replicates. One-way ANOVA followed by Tukey's post hoc analysis. Source data are provided as a Source Data file.

9) Fig 2C+D: Why are the growth curves not extended for a longer period. Even for the WT the bacteria seem to not have reached stationary phase yet? Which is in itself very surprising if a regular growth medium is used, E. coli especially grows fast and reaches stationary phase in 5=6 hours . can the authors explain this ? And please show 24h curves and see if the proposed effect of H2S holds.

Response: We thank the reviewer for this important observation and suggestion. As requested, we have extended the bacterial growth curve experiments for the wild-type *E. coli* strain to 24 hours. The additional data (**Fig. R12**, **now presented in Supplementary Fig. S14**) show that the bacteria eventually enter stationary phase within this extended timeframe. Importantly, the effects of LL-37 and compound 7b on bacterial growth remain evident throughout the 24-hour period, supporting our conclusion that H_2S contributes to the modulation of bacterial viability.

As shown in the revised data, we observed that the bacterial growth curve reached a plateau at approximately 12 h. This extended growth phase compared to the typical 5–6 h stationary phase may be attributable to the use of diluted culture conditions in our assay system, which likely resulted in slower bacterial proliferation.

Inserted results section (Manuscript-with tracks: Page:6, Line:222-224):

"When the monitoring period was extended to 24 hours and bacterial growth reached the stationary

phase, LL-37 remained effective in inhibiting *Escherichia coli* CMCC44102 and *Clostridium perfringens* (Fig. S14)."

We greatly appreciate the reviewer's insight, which has prompted us to strengthen our experimental timeline and provide additional context for the observed bacterial growth patterns.

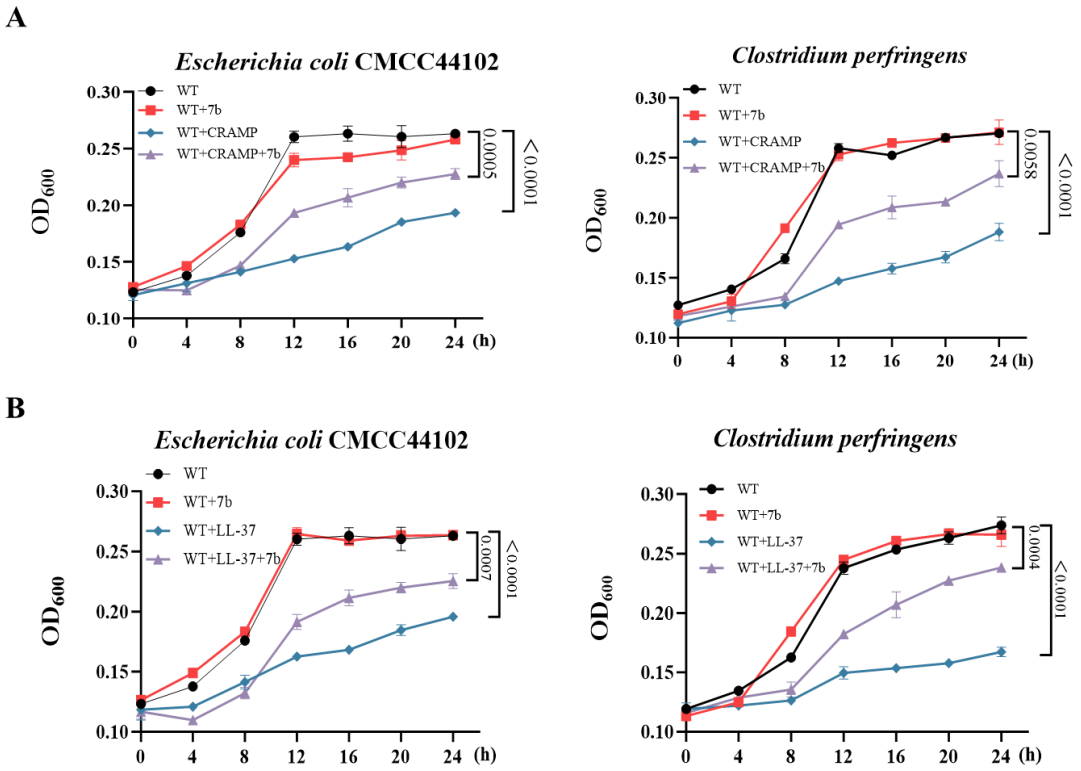

Fig.R12 Representative growth curves of *Escherichia coli* CMCC44102 and *Clostridium perfringens* (1×10^8 CFU/mL) in the presence of CRAMP (A) and LL-37 (B) (0.5×MIC) in 24 h. Data are expressed as the mean values of optical density at a wavelength of 600 nm (OD_{600}). The results are expressed as mean ± S.D. n = 3 biological replicates. One-way ANOVA followed by Tukey's post hoc analysis. Source data are provided as a Source Data file.

10) Fig2 LPS model: I always thought that it was well established that LL-37 neutralizes LPS (mainly) by direct binding and thereby preventing LPS from interacting with TLR4. Especially upon co-incubation of LPS with LL-37 (or CRAMP). How can pretreatment with compound 7b prevent that? This is an interesting observation obviously but very hard to explain.

Response: We thank the reviewer for carefully noting this point and for highlighting the well-established mechanism by which LL-37 neutralizes LPS through direct binding. We acknowledge that the description of our experimental design in the Methods section and figure legends was unclear and may have led to this confusion.

In our experiments, compound 7b, LL-37 (or CRAMP), and LPS were co-incubated with the cells simultaneously, rather than applying 7b as a pretreatment prior to the addition of LPS and LL-37. Therefore, the observed effects of 7b are not due to any interference with LL-37's ability to bind LPS, but rather are likely attributable to its role in scavenging endogenous H_2S and modulating downstream macrophage signaling pathways.

We have now revised the Methods section and the figure legend for Fig. 2 I-J accordingly to clarify

the actual experimental procedure.

Revised figure legend section (Manuscript-with tracks: Page:10, Line:414-415):

"RAW264.7 macrophages were treated with different groups and incubated for 6 hours. The concentrations of 7b, LPS, and LL-37/CRAMP were 100 μM, 100 ng/mL, and 5 μM, respectively."

We greatly appreciate the reviewer's observation, which has allowed us to correct this oversight and improve the clarity of our manuscript.

11) Fig 2M: scratch assays are not usually done with macrophages. The authors should perform the scratch assay with (non- H2S producing) epithelial cells. If compound 7b has a similar effect it is not due to H2S.

Response: We thank the reviewer for raising this important point regarding the suitability of the scratch assay model. We agree that scratch assays are conventionally performed using epithelial or fibroblast cell types, which are more directly representative of wound healing processes.

In our study, we utilized RAW264.7 macrophages as a model system to examine cell migration within the context of infection and inflammatory responses. While macrophages are inherently migratory and play crucial roles in resolving inflammation and contributing to tissue repair, we acknowledge that this model does not fully capture the behavior of non-H_2S-producing epithelial cells, which are classically employed in wound healing assays. Our intent in Fig. 2L–M was to provide an initial indication of how LL-37 and H_2S modulation might influence macrophage migration, rather than to model wound closure per se.

We concur that performing comparable assays in non-H_2S-producing epithelial cells would be valuable for determining whether the observed effects of compound 7b are specific to H_2S-producing systems or reflect broader influences on cell motility. However, such experiments would involve a substantially different experimental design and are beyond the primary focus of the current study, which centers on the crosstalk between antimicrobial peptides, bacterial H_2S production, and macrophage function.

In light of this, we have not added additional scratch assays in epithelial cells in the current revision. Instead, we have revised the Discussion section to explicitly acknowledge this limitation and to note that future studies could investigate whether 7b's effects on cell migration extend to other cell types involved in wound healing. Furthermore, we have amended the figure legend and main text to clarify the preliminary and exploratory nature of the macrophage scratch assay and to avoid overinterpretation of these findings.

We greatly appreciate the reviewer's thoughtful comment, which has allowed us to better define the scope of our work and to direct future research toward more appropriate mechanistic systems.

12) Fig 6F: this is not really a quantitative assay?? Why not just count viable bacteria upon treatments?? Again the differences in Fig S17 are really small and no statistics are performed. Yet the authors imply that this is a real 'difference'.

Response: We thank the reviewer for this important observation. We agree that the method used in Fig. 6F (plate imaging of colony formation) is semi-quantitative at best and should not be interpreted as a robust measure of bacterial viability. In response, we have supplemented our data with quantitative colony counting assays to directly measure viable bacteria upon treatment. The results (**Fig. R13**, **now presented in Supplementary Fig. S24**) are consistent with the trends observed in Fig. 6F, supporting our original conclusion.

Inserted results section (Manuscript-with tracks: Page:10, Line:414-415):

"The quantitative colony counting results were consistent with the observed trend (Fig. S24)."

We would like to clarify that the fluorescence-based assay in **Fig. S17** (**now presented in Supplementary Fig. S25**) was intended to provide a relative measure of bacterial fluorescence rather than an absolute quantification of viable cells. We state explicitly that only one experimental dataset was available for **Fig. S17** (**now presented in Supplementary Fig. S25**), which precluded statistical analysis. This limitation is now acknowledged in the revised manuscript.

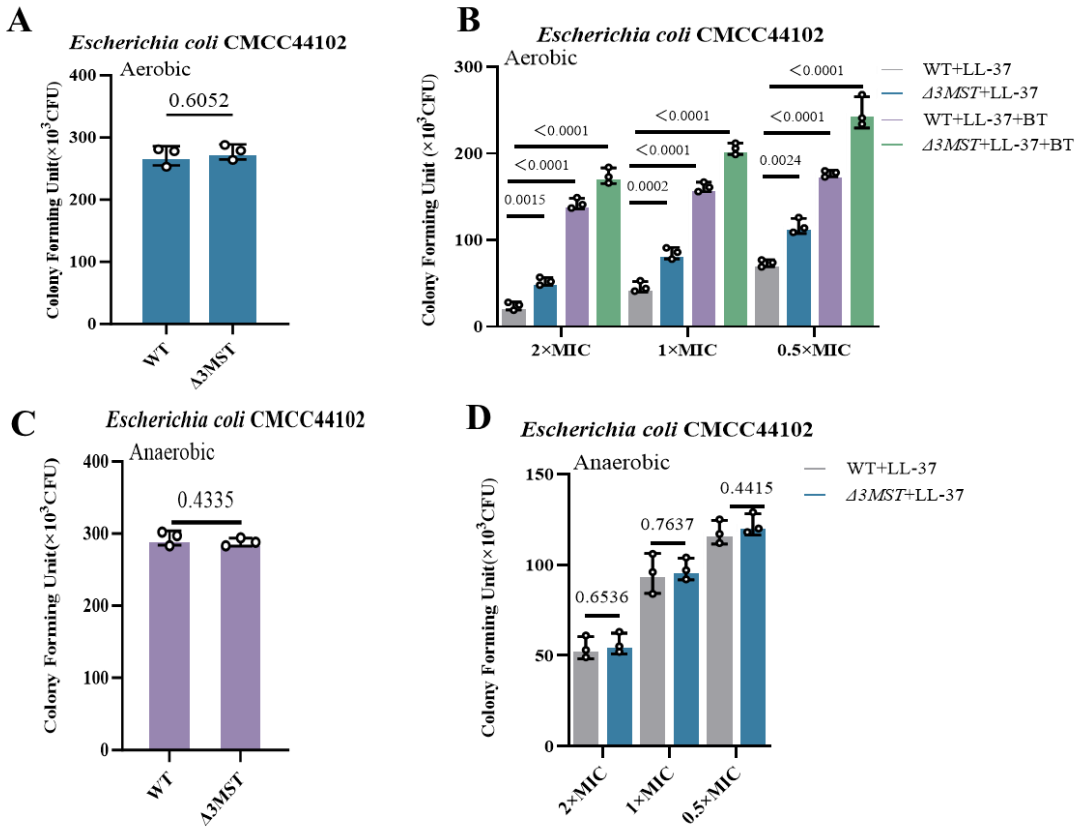

Fig.R13 Colony-forming units (CFUs) counting of the wild-type (WT) and *Δ3MST* (2×10^5 CFU/mL) treated with LL-37 under aerobic (A-B) and anaerobic conditions (C-D). The results are expressed as mean ± S.D. n = 3 biological replicates. One-way ANOVA followed by Tukey's post hoc analysis. Source data are provided as a Source Data file.

13) Fig 8: This in vivo data seems very convincing unlike a lot of the in vitro data. But several questiond remain:

- Interestingly though, the total effect of LL-37/ H2S seems to be on the bacterial (H2S producing) level, and not the macrophages. This aspect needs to be further discussed.

-Is the AMP injected at exactly the same site as the bacterial burden??

-Is compound 7b and/or BT injected together with the AMP, and importantly could compound 7b/BT at these concentration physiologically affect LL-37 activity directly, preventing LL-37 from interacting with bacteria (and blocking killing) in the mouse. For example does 7b bind directly to LL-37 or can it induce aggregation of LL-37 in the peritoneal lavage fluid? This could explain the observed in vivo results.

-The addition of 7b only (without LL-37) significantly increases the bacterial burden in every organ. Do the authors think that this could be due to the effect on naturally produced H2S in macrophages (and their subsequent killing of E.coli) in these mice? This observation is interesting and should be discussed properly.

Response: We thank the reviewer for highlighting the strength of the *in vivo* data and for raising several insightful and important questions that help to clarify the underlying mechanisms.

Discussion of the Effect of LL-37–Induced H_2S on Macrophage Responses:

Indeed, while our in vitro data clearly show that LL-37 induces H_2S production in both bacteria and macrophages, the in vivo results presented in Fig. 8 suggest that the predominant contribution to bacterial clearance and host protection is more likely mediated through the modulation of bacterial H_2S metabolism. Specifically, we propose that in the context of infection, LL-37–induced H_2S production in macrophages may exert more subtle immunomodulatory or tissue-protective effects that are not readily captured in the short-term peritonitis model employed in this study.

Although our current data do not directly explore the role of macrophages in peritonitis progression, existing literature underscores their critical involvement in host defense. For instance, Luo *et al.* reported that macrophage-derived acetylcholine contributes to the resolution of murine peritonitis (*Proc. Natl. Acad. Sci.* 2024). In parallel, Büscher *et al.* demonstrated that macrophages and recruited neutrophils cooperate within omental milky spots to sequester and eliminate *E. coli*, and that impairment of this local immune response accelerates sepsis progression (*Nat. Commun.* 2016). Although these studies do not directly implicate H_2S signaling, they highlight the importance of macrophage function in peritoneal infections. In light of these findings, we aim to further investigate the role of LL-37–induced H_2S production in macrophages, particularly in terms of its potential to modulate inflammation or enhance bacterial clearance in vivo.

To this end, we are planning follow-up studies using conditional knockout models and cell-specific delivery systems to dissect the respective contributions of bacterial versus host-derived H_2S in LL-37–mediated immune responses. These future investigations will help clarify the mechanistic basis of AMP–H_2S crosstalk within specific cell populations and their roles in orchestrating host defense against infection.

On the site and timing of injections:

In our *in vivo* infection model, both the antimicrobial peptide (AMP) and the bacterial suspension were administered via intraperitoneal (i.p.) injection into the same anatomical compartment. Therefore, the AMP was delivered directly to the site of bacterial challenge, allowing for immediate interaction between LL-37 and the infecting bacteria within the peritoneal cavity.

This clarification supports the interpretation that the observed effects reflect direct in situ interactions between LL-37 and bacteria, potentially modulated by local H_2S dynamics.

Potential for direct interaction between 7b and LL-37:

In our *in vivo* experiments, compound 7b and BT were administered intraperitoneally together with LL-37, which raised the possibility of direct interaction between these agents. To directly address this concern, we performed isothermal titration calorimetry (ITC) to evaluate the potential interaction between LL-37 and compound 7b under physiological buffer conditions. The ITC results showed no detectable binding between LL-37 and 7b (**Fig. R4, now presented in Supplementary Fig. S12**), indicating that compound 7b does not physically associate with LL-37 nor alter its

structure or bioavailability. These findings strongly support the interpretation that the effects observed in vivo are not due to a direct interference of 7b with LL-37, but rather reflect the functional consequences of H_2S scavenging.
The ITC data have been included in the revised manuscript as Supplementary Fig. S12, and we have added corresponding descriptions in both the Results and Discussion sections to clarify this point.

Effect of 7b alone increasing bacterial burden:
We agree with the reviewer that this may be attributable, at least in part, to the scavenging of endogenously produced H_2S from host immune cells—particularly macrophages—which are known to produce H_2S via the CSE pathway during infection and inflammation. The most likely explanation is that 7b suppresses endogenous H_2S production in host cells, particularly macrophages, thereby impairing their antibacterial responses. This interpretation is consistent with our macrophage experiments showing that inhibition of H_2S production reduces ROS generation and bacterial clearance. We have now expanded the Discussion section to explore this possibility more explicitly. Specifically, we highlight the potential role of macrophage-derived H_2S as a contributor to antibacterial defense in vivo, consistent with prior reports implicating H_2S in macrophage bactericidal activity and modulation of inflammatory signaling. The dampening of this endogenous H_2S axis by systemic 7b administration may weaken innate immune responses and reduce bacterial clearance, even in the absence of exogenous LL-37. We will expand the discussion to highlight this hypothesis and its implications for host-pathogen interactions, and plan to explore this further in future studies using macrophage-specific 3-MST knockdown mice.
This observation also reinforces the notion that both bacterial and host-derived H_2S may operate in parallel or complementary ways to influence infection outcomes. We thank the reviewer again for pointing out this important and interesting finding, which has helped strengthen the biological interpretation of our in vivo data.

14) Methods paragraph 3.3 please describe more clearly how the experiment is performed. What for example is incubated for 10 min exactly?
Response: We thank the reviewer for pointing out the ambiguity in the description of Methods section 3.3. In the revised manuscript, we have clarified the procedure by specifying the exact components involved in the 10-minute incubation. We have updated the description of method 3.3 in supplementary materials.
Inserted methods 3.3 section (Supplementary Materials with tracks: Page:5, Line:140-155):
"Representative OD_{600} growth curves were obtained to evaluate the impact of AMP-induced H_2S production on bacterial proliferation. *E. coli* cultures were first diluted to 1×10^8 CFU/mL in LB medium supplemented with 500 μM L-cysteine. Different treatment groups—PBS (control), 7b/Asp, LL-37/CRAMP, and LL-37/CRAMP+7b/Asp—were then added to the bacterial suspension. The mixtures were incubated at 37°C with shaking at 200 rpm to allow initial interaction between the compounds and bacteria. After 10 minutes of incubation under these conditions, 50 μL of each mixture was transferred, in triplicate, into individual wells of a 96-well plate (50 μL per well). The plates were subsequently incubated at 37°C in a microplate reader, and bacterial growth was monitored by measuring OD_{600} values at 0, 2, 4, 6, 8, 10, and 12 hours. To further examine the role of H_2S in AMP-mediated antibacterial activity, a parallel growth curve analysis was performed using both wild-type (WT) and *Δ3MST E. coli* strains. The experimental groups were as follows: (1) WT,

(2) *Δ3MST*, (3) *Δ3MST*+Na_2S, (4) WT+LL-37/CRAMP, (5) *Δ3MST*+LL-37/CRAMP, and (6) *Δ3MST*+LL-37/CRAMP+Na_2S. OD_{600} measurements were recorded at the same time points to assess bacterial growth dynamics under each condition."

Reviewer #1

First major point: concerning how H_2S induces oxidative stress. The authors show that Na_2S can inhibit SOD and CAT activity; however, they used 100 μM of Na_2S, which is still not physiological. In the case of LL-37, only about 20-30 μM is observed in E. coli (Figure 1). Tests with lower doses of H_2S donors exhibiting the same effects are necessary to convincingly demonstrate the ROS-related mechanism. Furthermore, the authors have not specified the dose of LL-37 used in the revised experiments or shown whether it causes any cell death or growth defects by 12 hours, which could explain the data. Cell death via ROS can be self-sustaining and increase over time (see https://pnas.org/doi/abs/10.1073/pnas.1901730116).

Response:

We thank the reviewer for this important comment regarding the physiological relevance of the H_2S concentrations used to support the proposed ROS-related mechanism and for highlighting the need to clarify LL-37 dosing and long-term effects.

To address the concern regarding Na_2S concentrations, we performed additional dose-dependent experiments using lower concentrations of the H_2S donor Na_2S (10, 20, 40, and 80 μM), encompassing the estimated intracellular H_2S levels induced by LL-37 (~20–30 μM; **Fig. R1**) (**now presented in Supplementary Fig. S30C-D**). Under these conditions, Na_2S inhibited SOD and CAT activities in a concentration-dependent manner, including within the physiologically relevant range. Consistently, intracellular ROS measurements revealed a parallel, dose-dependent increase in ROS levels (**Fig. R2**) (**now presented in Supplementary Fig. S29C-D**). These results demonstrate that biologically relevant H_2S concentrations are sufficient to suppress antioxidant enzyme activity and perturb bacterial redox homeostasis.

We have also clarified the exact LL-37 concentrations used in all revised experiments in the Methods section and corresponding figure legends, including their relation to strain-specific MIC values (e.g., 0.5× MIC), to ensure transparency and reproducibility.

To determine whether LL-37 at sub-MIC concentrations induces sustained cell death or transient growth inhibition., we performed 12-hour colony-forming unit (CFU) assays following 0.5×MIC LL-37 treatment (**Fig. R3**) (**now presented in Supplementary Fig. S34**). These results show that LL-37 treatment leads to a significant and sustained inhibition of bacterial growth during prolonged observation, confirming its antimicrobial activity under the conditions used. These analyses provide a temporal framework for interpreting ROS measurements and indicate that the observed oxidative stress cannot be solely attributed to late-stage, self-amplifying cell death effects.

Taken together, these additional experiments strengthen the mechanistic conclusion that LL-37/CRAMP-induced H_2S contributes directly to oxidative stress by impairing bacterial antioxidant defenses.

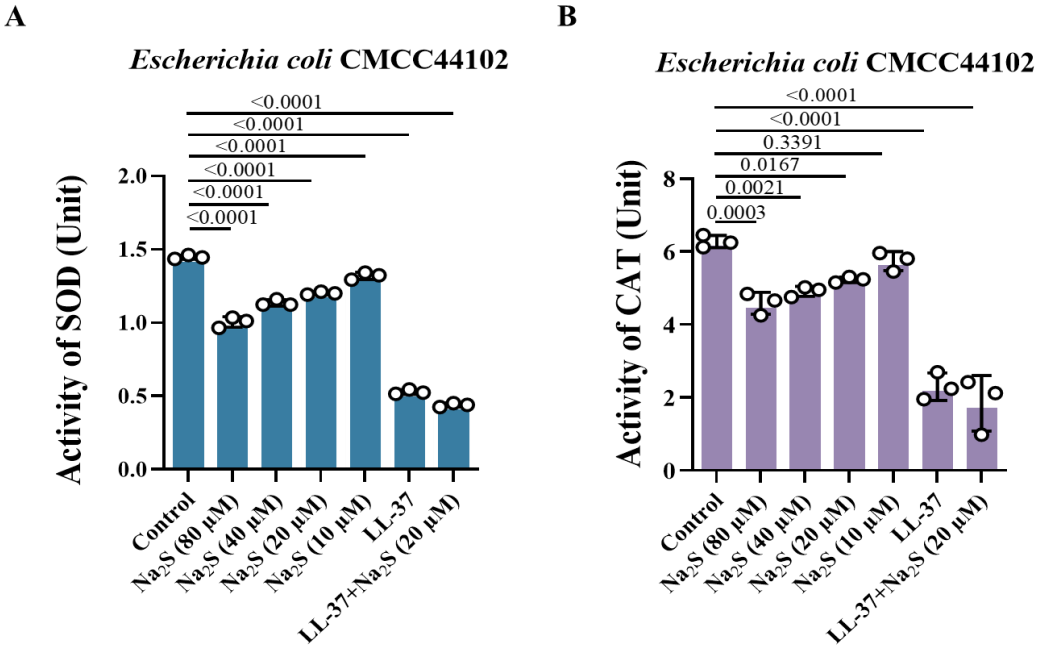

Fig.R1 Effect of Na_2S treatment (80 μM, 40 μM, 20 μM, 10 μM) on the enzymatic activities of superoxide dismutase (SOD) (A) and catalase (CAT) (B) in *Escherichia coli* CMCC44102 (3×10^7 CFU/mL). The results are expressed as mean ± S.D. n = 3 biological replicates. One-way ANOVA followed by Tukey's post hoc analysis. Source data are provided as a Source Data file.

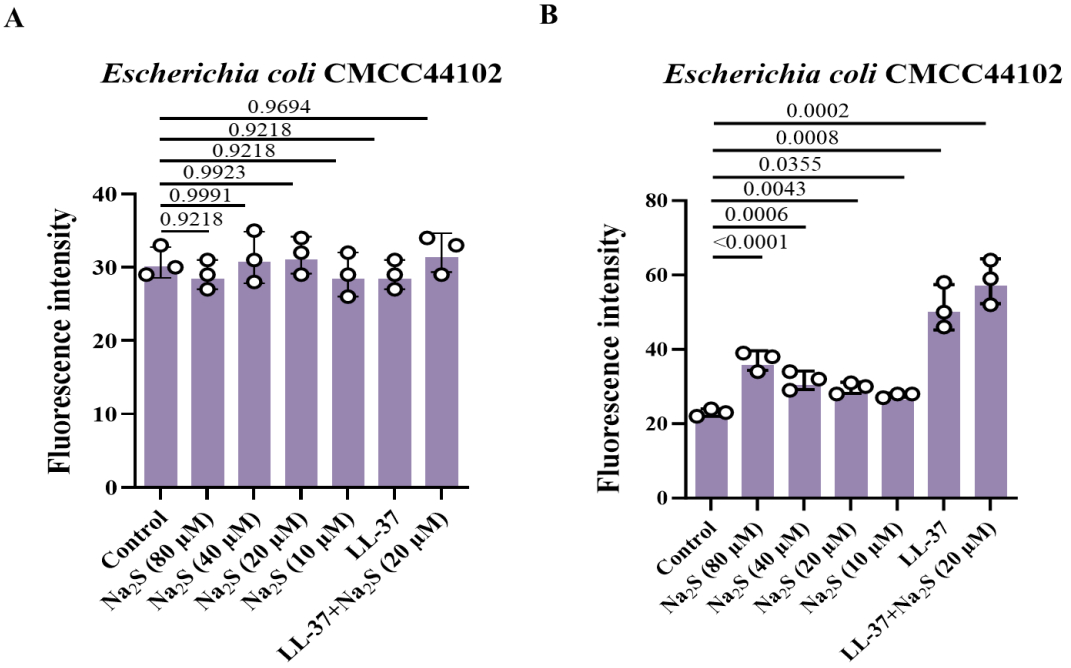

Fig.R2 Effect of Na_2S treatment (80 μM, 40 μM, 20 μM, 10 μM) on the levels of reactive oxygen species (ROS) at 0 h (A) and 12 h (B) in *Escherichia coli* CMCC44102 (1×10^8 CFU/mL) using DCHF-DA (5 μM) probe at absorbance of 488/525 nm. The results are expressed as mean ± S.D. n = 3 biological replicates. One-way ANOVA followed by Tukey's post hoc analysis. Source data are provided as a Source Data file.

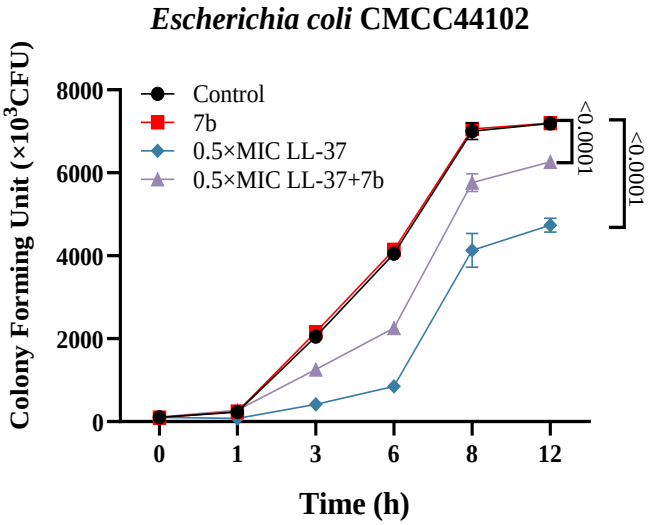

Fig.R3 Bactericidal kinetics of LL-37 (0.5×MIC) against *Escherichia coli* CMCC44102 (1×10^5 CFU/mL) with or without 7b co-treatment. The results are expressed as mean ± S.D. n = 3 biological replicates. One-way ANOVA followed by Tukey's post hoc analysis. Source data are provided as a Source Data file.

Second major point: Although ITC can determine if there's an interaction between 7b and LL-37, the authors need to conduct straightforward experiments—testing LL-37 with and without 7b in 3MST knockout E. coli and assessing the effect of increasing Na_2S concentrations on bacterial survival, especially whether 7b can rescue LL-37's bactericidal activity at various dosages. The ITC data are also unclear: if the molar ratio of 7b to LL-37 is 1:10, why is the maximum ratio only 1.2? Moreover, in Figures 2G and 2H, a ratio of 1:40 is used. Another possibility is that 7b might interact with the bacterial membrane directly—helping bacteria tolerate LL-37. Performing the suggested experiments is critical to clarify these points.

Response:

We thank the reviewer for these constructive suggestions and fully agree that functional validation in a genetically defined background is essential to distinguish H_2S-dependent regulation from alternative mechanisms such as direct peptide interaction or membrane effects.

To directly assess whether 7b modulates LL-37 bactericidal activity through an H_2S-dependent mechanism, we performed colony-forming unit (CFU) assays using the 3MST knockout *E. coli* strain. We first evaluated LL-37–mediated bacterial killing in the presence of increasing concentrations of exogenous Na_2S over a 12-hour time course. Na_2S supplementation restored the antibacterial activity of LL-37 against the 3MST-deficient strain in a concentration-dependent manner (**Fig R4A**) (**now presented in Supplementary Fig. S32A**). Furthermore, under a fixed Na_2S concentration (intracellular H_2S levels induced by LL-37), LL-37 bactericidal efficacy remained recoverable even in the presence of 7b during prolonged incubation (**Fig R4B**) (**now presented in Supplementary Fig. S32B**). These findings demonstrate that exogenous H_2S functionally compensates for the loss of endogenous 3MST-derived H_2S and rescues LL-37–mediated bacterial killing despite 7b treatment, providing direct evidence that H_2S is the key mediator of this regulatory effect.

We also addressed the reviewer's concerns regarding the ITC molar ratio design. Additional

ITC experiments were conducted using a substantially expanded range of molar ratios, extending up to a 1:80 molar excess of 7b relative to LL-37. Across all tested conditions, no detectable heat changes indicative of direct binding was observed (**Fig R5**) (**now presented in Supplementary Fig. S12D**). These expanded analyses clarify that the absence of measurable interaction is not attributable to insufficient stoichiometric coverage and support the conclusion that 7b does not physically associate with LL-37.

To further examine the alternative possibility that 7b directly interacts with bacterial membranes, we performed high-performance liquid chromatography (HPLC) analyses to monitor 7b stability and recovery following incubation with bacterial cells. The chromatographic peak area corresponding to 7b remained largely unchanged across all incubation time points (**Fig R6**) (**now presented in Supplementary Fig. S13**), indicating that 7b was neither significantly depleted from the medium nor sequestered by bacterial cells. These results do not support membrane binding or adsorption as a major mechanism.

Taken together, the added evidence indicates that 7b modulates LL-37 antibacterial activity indirectly through H_2S-related pathways rather than via direct peptide binding or membrane interaction, thereby addressing the reviewer's concerns with definitive functional evidence.

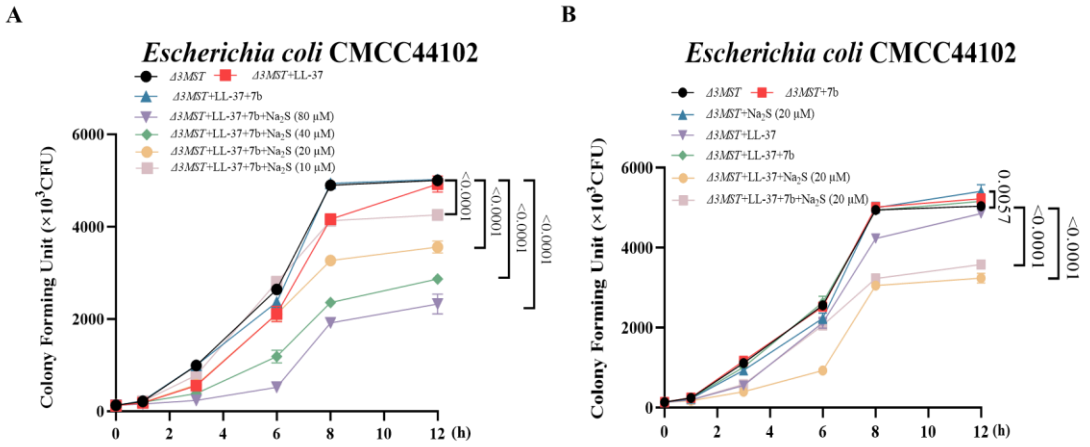

Fig.R4 (A) Time-kill kinetics of LL-37 (0.5×MIC) in combination with 7b and Na_2S at different concentrations (80, 40, 20, and 10 μM) against the 3MST knockout strain of Escherichia coli CMCC44102. (B) Time-kill kinetics of LL-37 (0.5×MIC) in combination with 7b and Na_2S (20 μM) against the 3MST knockout strain of Escherichia coli CMCC44102. The results are expressed as mean ± S.D. n = 3 biological replicates. One-way ANOVA followed by Tukey's post hoc analysis. Source data are provided as a Source Data file.

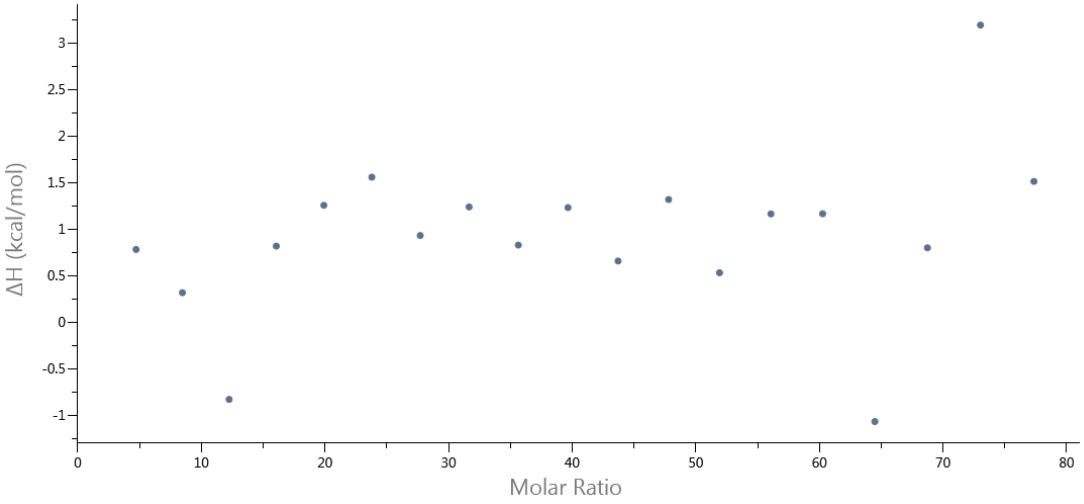

Fig.R5 The interaction between LL-37 and 7b determined by ITC assay, where 7b was titrated into LL-37 with various concentrations in HEPES buffer at 25°C.

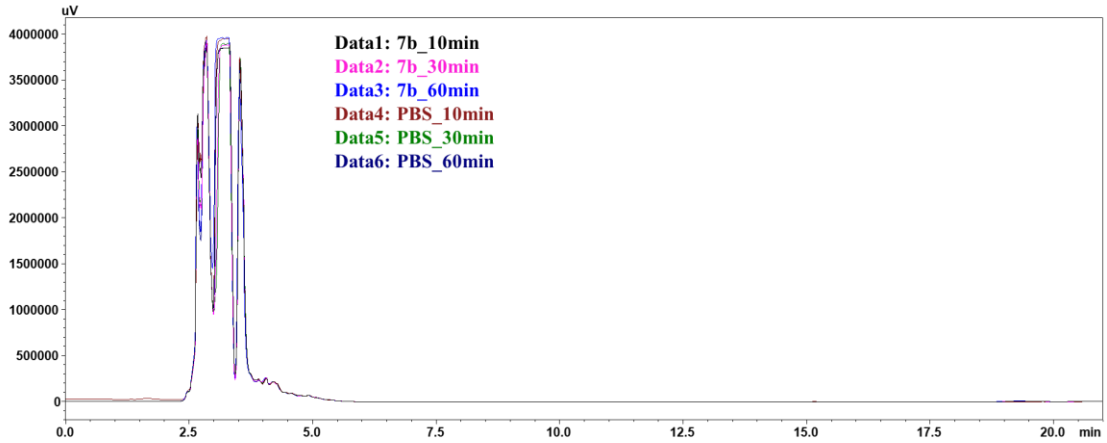

Fig.R6 High-performance liquid chromatography (HPLC) chromatograms of lysates from *Escherichia coli* CMCC44102 incubated with or without 7b for 10, 30, and 60 min at wavelength of 254 nm.

Reviewer #2

Although I would still prefer to see some additional experiments—such as broader validation across different bacterial strains and further analysis of AMP activity at sub-MIC concentrations:

Response:

We thank the reviewer for this helpful suggestion regarding broader strain validation and evaluation of AMP activity at sub-MIC concentrations.

Broader validation across bacterial strains.

We would like to clarify that such validation is already included in the current manuscript. Specifically, Figs. 1C–D present the antibacterial effects of LL-37 and CRAMP across multiple bacterial strains, demonstrating consistent activity profiles. In addition, Fig. S4 further expands this analysis by including four additional clinical isolates, thereby extending the strain coverage and strengthening the generalizability of the findings.

AMP activity at sub-MIC concentrations.

In line with the reviewer's suggestion, AMP activity below MIC levels has also been evaluated.

These data are presented in Figs. 9A – C, where LL-37 and CRAMP were systematically tested under sub-MIC conditions, demonstrating measurable biological effects within this concentration range.

We appreciate this valuable comment, which provides an opportunity to better highlight these datasets. In the revised manuscript, we have clarified and emphasized the corresponding descriptions in both the Results and Discussion sections to improve visibility and avoid potential ambiguity.

Reviewer #3

1. The authors did not provide the original western blot images. For transparency and reproducibility, the images should be included (at least as supplementary material).

2. The normalization strategy is problematic. According to the manuscript, the same samples were run on two separate gels—one probed for the protein of interest and another for the housekeeping protein (GAPDH/actin). This approach is not valid, as normalization requires that the loading control (GAPDH/actin) be run on the same blot as the protein of interest. Otherwise, potential gel-to-gel variation cannot be accounted for. Furthermore, CSE and GAPDH or 3-MST and beta-actin are well separated in molecular weight and could have been probed on the same blot (e.g., by stripping and reproving or by using different antibody incubation steps).

Response:

We thank the reviewer for emphasizing the importance of transparency, reproducibility, and rigorous normalization in the western blot analyses.

Provision of original blot images.

In response, we have now provided the original, uncropped western blot images corresponding to all relevant experiments (**Fig R7**) (**now presented in Supplementary Fig. S25**). The raw blot data from three independent biological replicates have been included as supplementary material in the revised submission to ensure full transparency and reproducibility.

Correction of the normalization strategy.

We fully agree that accurate normalization requires the protein of interest and the corresponding loading control to be detected on the same blot to account for potential gel-to-gel variability. Accordingly, we have revised the experimental design and repeated the relevant western blot experiments by probing the target proteins (CSE or 3-MST) together with their respective loading controls (GAPDH or β-actin) on the same membrane, using stripping/re-probing or sequential antibody incubation where appropriate (**Fig R8**) (**now presented in Fig. 5A-D**).

The updated representative blots and corresponding quantitative analyses have now replaced the original data in the revised figures. These modifications ensure proper normalization and substantially improve the rigor and reliability of the presented results.

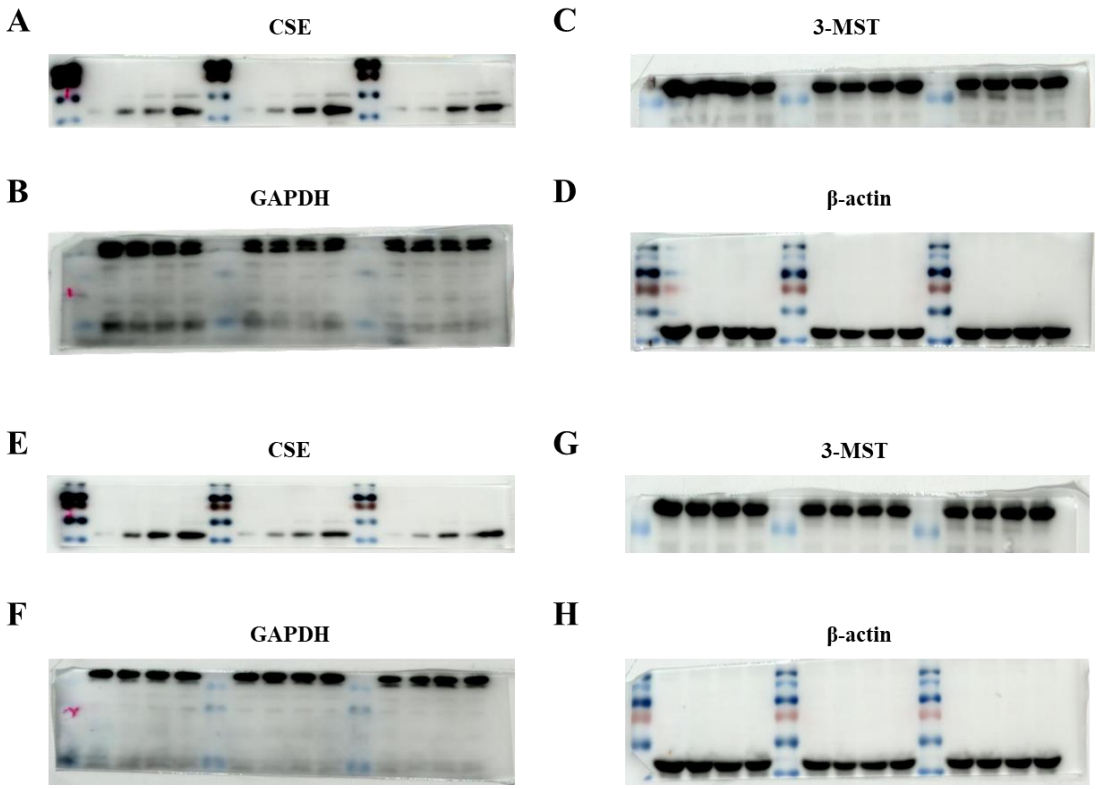

Fig.R7 Dose- and time-dependent effects of LL-37 on the protein expressions of H_2S-producing enzymes in macrophages. (A–B) Original western blot images showing CSE and GAPDH protein levels in RAW264.7 cells treated with LL-37 (1.25, 2.5, and 5 μM) for 24 h. (C–D) Original western blot images showing 3-MST and β-actin protein levels in RAW264.7 cells treated with LL-37 (1.25, 2.5, and 5 μM) for 24 h. (E–F) Original western blot images showing CSE and GAPDH protein levels in RAW264.7 cells treated with 5 μM LL-37 for 12, 24, and 36 h. (G–H) Original western blot images showing 3-MST and β-actin protein levels in RAW264.7 cells treated with 5 μM LL-37 for 12, 24, and 36 h.

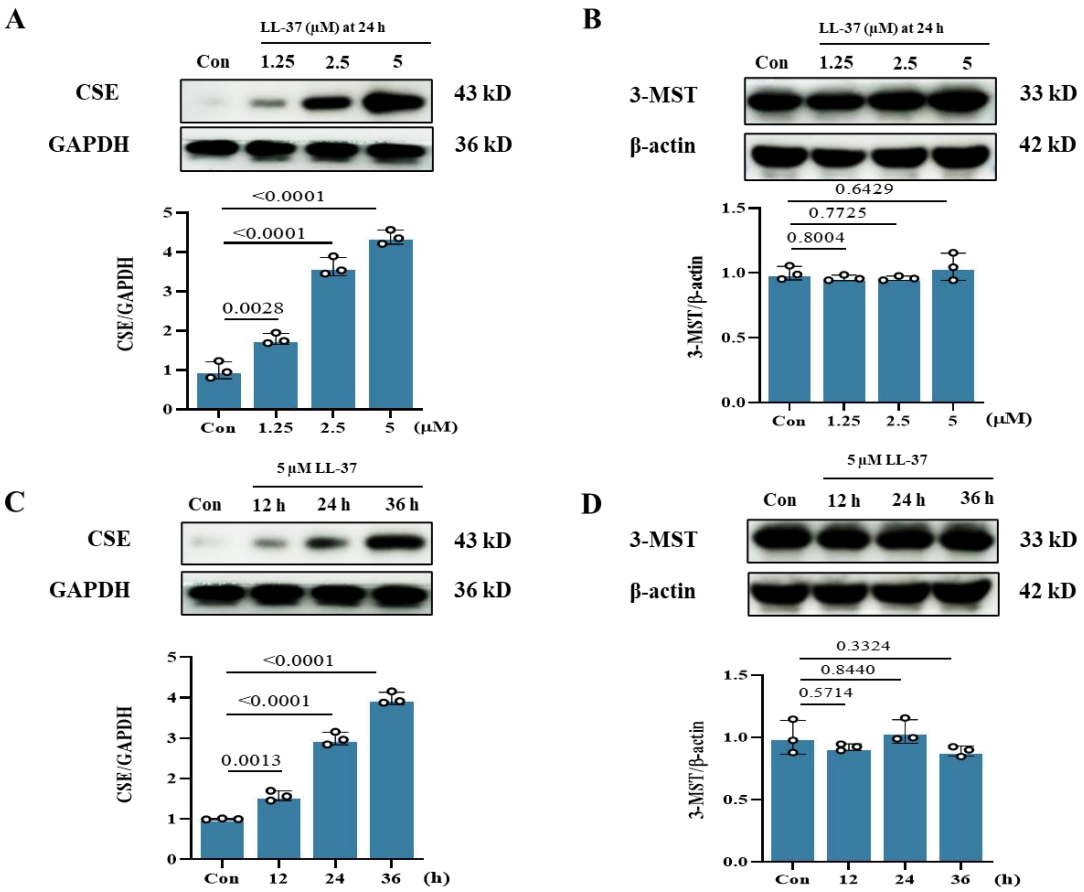

Fig.R8 Dose- and time-dependent effects of LL-37 on the protein expressions of H_2S-producing enzymes in macrophages. (A-D) RAW264.7 macrophages were treated with LL-37 (1.25, 2.5 and 5 μM) for 24 h or 5 μM for 12, 24, 36 h on CSE protein and 3-MST protein assessed by western blot analysis. The results are expressed as mean ± S.D. n = 3 biological replicates. One-way ANOVA followed by Tukey's post hoc analysis. Source data are provided as a Source Data file.

Reviewer #4

Remark 1:

The fact that compound 7b affects the activity of LL-37 as early as 10 -30 would imply that H2S is not involved since sufficient H2S production hasn't started yet. And this, together with the very mild effects 7b seems to have on antimicrobial activity enforces my doubts on the importance of H2S. How do the authors explain the short term effects, in the light of their proposed antibacterial mechanism for LL-37?

Response:

We thank the reviewer for this thoughtful comment regarding the temporal relationship between the effects of compound 7b and the proposed H_2S-dependent mechanism.

Upon careful re-examination of our time-course data, we found no evidence that 7b alters LL-37 antibacterial activity during early incubation periods. We note that the "10–30" mentioned in the comment may refer to minutes; accordingly, we carefully analyzed the 10–30 min time window. As shown in Fig. 2C–D, bacterial survival curves indicate that the presence of 7b does not measurably influence LL-37–mediated killing at these early time points.

Instead, the modulatory effects of 7b become apparent only during prolonged incubation, when

H_2S accumulation is detectable and sufficient to affect bacterial redox homeostasis. This time-dependent pattern is consistent with our proposed mechanism in which 7b influences LL-37 activity indirectly through regulation of H_2S-related pathways rather than via an immediate or direct antibacterial effect. We hope this clarification resolves the reviewer's concern regarding the short-term observations.

Remark 3:

The authors acknowledge that the effect of H2S might be too subtle to be detected. But the role of (induced) H2S is the main message of the whole article and needs to be properly shown, also in vitro. Therefore, my suggested experiments are not as said by the authors 'beyond the primary focus' of the study. They are at the core of the study.

I understand that all suggested experiments are very time demanding, but MIC/MBC assays with smaller than 2 fold dilutions for a set of , let's say two H2S producing and 4 non-H2S producing bacteria should be easily doable.

Response:

We sincerely thank the reviewer for this important clarification and fully agree that rigorously demonstrating the functional role of H_2S is central to the main message of this study. We apologize for the lack of clarity in our previous response.

In response to the reviewer's suggestion, we performed additional MIC and MBC assays using dilution intervals smaller than two-fold to increase measurement resolution and quantitative reliability. Moreover, the analysis was expanded to a broader bacterial panel comprising two representative H_2S-producing strains and four non–H_2S-producing strains tested under identical experimental conditions.

The refined susceptibility data consistently show that LL-37/CRAMP exhibits significantly greater antibacterial potency against H_2S-producing bacteria than against non–H_2S-producing species. This pattern was observed across both MIC and MBC measurements (**Fig R9**) (**now presented in Supplementary Fig. S20**). Together, these in vitro results provide direct functional evidence that endogenous H_2S production enhances bacterial susceptibility to LL-37/CRAMP, thereby substantiating the central conclusion that H_2S is an active and mechanistically relevant mediator of LL-37/CRAMP antibacterial activity.

A

Bacteria	Peptides			
	CRAMP		LL-37	
	(μM)	(μg/mL)	(μM)	(μg/mL)
H_2S producing bacteria				
Enterococcus faecalis ATCC29212	38.7	150.0	33.4	150.0
Vibrio alginolyticus	19.4	75.0	16.7	75.0
Non-H_2S producing bacteria				
Staphylococcus aureus	77.4	300.0	66.8	300.0
Methicillinresistant Staphylococcus aureus	77.4	300.0	66.8	300.0
Acinetobacter baumannii 19606	77.4	300.0	66.8	300.0
Pseudomonas aeruginosa CMCC10104	77.4	300.0	66.8	300.0

B

Bacteria	Peptides			
	CRAMP		LL-37	
	(μM)	(μg/mL)	(μM)	(μg/mL)
H_2S producing bacteria				
Enterococcus faecalis ATCC29212	77.4	300.0	66.8	300.0
Vibrio alginolyticus	38.7	150.0	33.4	150.0
Non-H_2S producing bacteria				
Staphylococcus aureus	ND	ND	ND	ND
Methicillinresistant Staphylococcus aureus	ND	ND	ND	ND
Acinetobacter baumannii 19606	ND	ND	ND	ND
Pseudomonas aeruginosa CMCC10104	ND	ND	ND	ND

Fig.R9 (A-B) The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of LL-37/CRAMP against two H_2S-producing and four non-H_2S-producing bacteria. ND means "None detected at the concentration of 300 μg/mL".

Remark 13: As indicated the in vivo data is convincing regarding the effect of 7b and LL-37 on infection but that it does not necessarily confirm the proposed mechanism. I understand that extra

in vivo control experiments is very difficult to perform, however rereading the figure, I noticed that an infection with the delta3MST E.coli were performed but only the bacterial burden in kidney and peritoneal lavage fluid are shown. Surely, other data MUST have been collected for these infected mice, please show these data as they provide an important control experiment. How was weight and survival affected by LL=37 +/- 7b , and what was bacterial burden in other tissues? These should be shown as they are an essential control experiment.
This could provide very important implications whether 7b acts on macrophages or bacteria. And the shown data are inclonclusive cause LL-37 affects the bacterial burden in the peritoneal fluid but not the kidney for the mutant E. coli.

And a small note on the ITC data: Indeed there seems to be no binding happening between 7b and LL-37. However, there is always a small chance that binding is based on hydrophobic interactions and therefor entropy-driven (and not enthalpy which is measured by ITC).

Response:

We sincerely thank the reviewer for these insightful comments regarding the interpretation of the in vivo data and for emphasizing the importance of additional control measurements.

To address this concern, we have now expanded our in vivo analyses in the murine peritonitis model established using both wild-type and *Δ3MST E. coli* strains. Specifically, under LL-37 treatment with or without 7b, we systematically assessed: (i) bacterial burdens in peritoneal lavage fluid and major organs, (ii) longitudinal body weight changes, and (iii) survival rates. These newly added datasets are presented in the revised figures.

We replicated the results shown in the original Fig. 8H – I. The expanded analyses show that LL-37 significantly reduces bacterial loads in both visceral organs and peritoneal lavage fluid. Importantly, the therapeutic efficacy of LL-37 against infections caused by the wild-type strain is consistently greater than that observed with the *Δ3MST* strain (**Fig R10**) (**now presented in Supplementary Fig. S38**). These findings indicate that bacterial H_2S production contributes to the *in vivo* anti-infective activity of LL-37.

Regarding the role of 7b, our data support its primary function as an H_2S scavenger. While 7b modulates the antibacterial outcome in vivo, the current evidence does not allow us to definitively distinguish whether its effects are mediated through actions on bacteria, host cells, or both. We therefore agree that the precise cellular target of 7b requires further dedicated investigation. We appreciate the reviewer's comment, which has helped us present these results more rigorously and transparently.

Concerning the reviewer's note on the ITC measurements, we appreciate the important point that entropy-driven hydrophobic interactions may not generate detectable enthalpy changes. To address this possibility, we performed additional ITC experiments across multiple temperatures (15°C, 25°C, and 37°C) and tested a broader range of 7b:LL-37 molar ratios. These results demonstrate that the absence of measurable interaction between 7b and LL-37 is consistent under varying thermodynamic conditions, including those that would favor entropy-driven binding (**Fig R11**) (**now presented in Supplementary Fig. S12C-E**). Together, these additional experiments strengthen the mechanistic interpretation of the in vivo findings while ensuring a rigorous and balanced presentation of the data.

To directly assess whether 7b modulates LL-37 bactericidal activity through an H_2S-dependent mechanism, we performed colony-forming unit (CFU) assays using the 3MST knockout *E. coli* strain. We first evaluated LL-37–mediated bacterial killing in the presence of increasing

concentrations of exogenous Na_2S over a 12-hour time course. Na_2S supplementation restored the antibacterial activity of LL-37 against the 3MST-deficient strain in a concentration-dependent manner (**Fig R12A**) (**now presented in Supplementary Fig. S32A**). Furthermore, under a fixed Na_2S concentration (intracellular H_2S levels induced by LL-37), LL-37 bactericidal efficacy remained recoverable even in the presence of 7b during prolonged incubation (**Fig R12B**) (**now presented in Supplementary Fig. S32B**). These findings demonstrate that exogenous H_2S functionally compensates for the loss of endogenous 3MST-derived H_2S and rescues LL-37–mediated bacterial killing despite 7b treatment, providing direct evidence that H_2S is the key mediator of this regulatory effect.

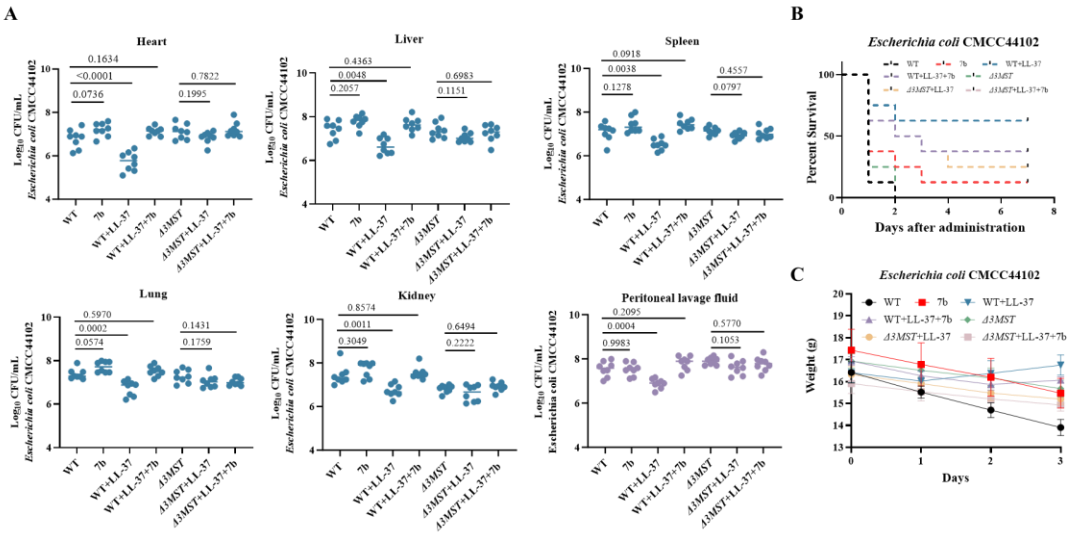

Fig.R10 (A) Bacterial burden in heart, liver, spleen, lung, kidney and peritoneal lavage fluid of peritonitis mouse with administration with LL-37 (10 mg/kg), **7b** (60 mg/kg) 2 h after intraperitoneal injection of the wild-type (WT) and *Δ3MST Escherichia coli* CMCC44102 (5×10^7 CFU). (B-C) Survival and weight changes of mice in different administration groups after intraperitoneal injection of the wild-type (WT) and *Δ3MST Escherichia coli* CMCC44102. The results are expressed as mean ± S.D. n = 8 biological replicates. One-way ANOVA followed by Tukey's post hoc analysis. Source data are provided as a Source Data file.

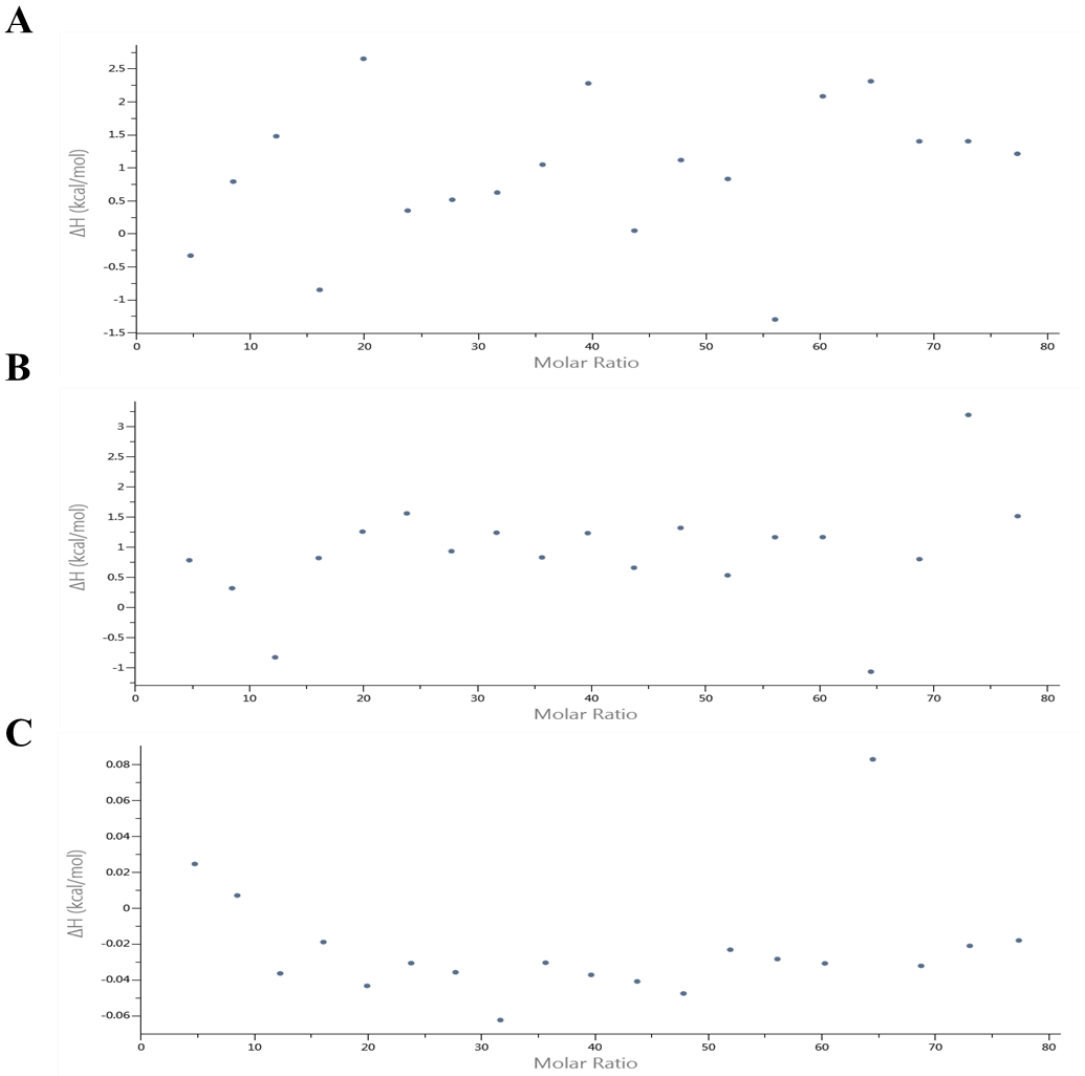

Fig.R11 (A-C) The interaction between LL-37 and 7b determined by ITC assay, where 7b was titrated into LL-37 with various concentrations in HEPES buffer at 15°C, 25°C, 37°C.

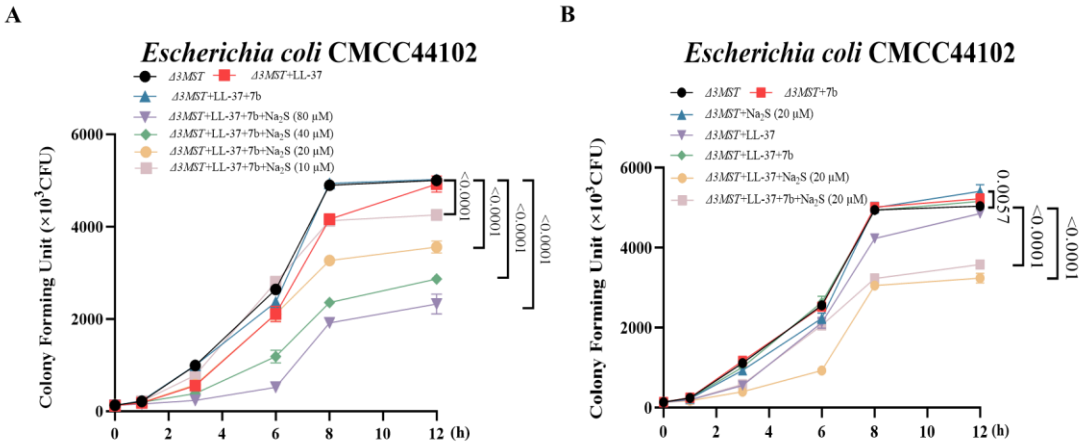

Fig.R12 (A) Time-kill kinetics of LL-37 (0.5× MIC) in combination with 7b and Na_2S at different concentrations (80, 40, 20, and 10 μM) against the 3MST knockout strain of Escherichia

coli CMCC44102. (B) Time-kill kinetics of LL-37 (0.5× MIC) in combination with 7b and Na_2S (20 μM) against the 3MST knockout strain of Escherichia coli CMCC44102. The results are expressed as mean ± S.D. n = 3 biological replicates. One-way ANOVA followed by Tukey's post hoc analysis. Source data are provided as a Source Data file.

Reviewer #1 (Remarks to the Author):

The authors have now done a lot more experiments to address our concerns which we appreciate.

For the first major point, the data the authors provided do suggest that H2S can lead to ROS and decreased SOD and CAT activity. However, it is very clear that to get the effects that LL-37 has, you need at least triple the H2S dose that is physiological, while the physiological amount has a much weaker effect. Thus, the authors must at least reduce their claims, especially the sentence in the abstract: "The generated H_2S further stimulates the accumulation of reactive oxygen species (ROS), leading to bacterial death by oxidative damage." Through the other experiments it does suggest that H2S is induced and the 7b and 3MST mutant experiments strongly suggest it plays a role, but data that death is by ROS is very weak.

Response:

We thank the reviewer for this thoughtful and important comment. We agree that our previous wording overstated the current evidence linking H_2S-induced ROS accumulation directly to bacterial death. While our additional experiments support a functional role for H_2S in promoting oxidative stress, we acknowledge that the ROS-related effects observed at physiologically relevant H_2S concentrations are more modest than those induced by LL-37 treatment itself. We therefore agree that the present data are insufficient to conclusively establish ROS-mediated oxidative damage as the primary mechanism of bacterial killing.

In response to the reviewer's suggestion, we have revised the relevant statements throughout the manuscript to more accurately reflect the strength of the evidence. Specifically, we have modified the sentence in the Abstract from: ***"The generated H_2S further participates in the accumulation of reactive oxygen species (ROS), leading to bacterial death by oxidative damage."*** to a more cautious interpretation indicating that H_2S contributes to ROS accumulation and redox imbalance during LL-37 treatment, without directly attributing bacterial death solely to oxidative damage.

Importantly, our combined genetic, pharmacological, and rescue experiments—including the effects of 7b treatment and the *Δ3MST* strain—continue to support the conclusion that endogenous H_2S functionally contributes to LL-37 antibacterial activity. We have therefore revised the manuscript to ensure that the mechanistic interpretation remains fully aligned with the experimental evidence and appropriately balanced throughout.

For the second point, the data are somewhat convincing and do suggest that 7b mediates its effect via H2S scavenging. For the 3MST experiment, it is notable that the authors find much higher growth suppression when 7b is added with even 20 μM Na2S than with just LL-37 (3MST+LL-37 is much better than 3MST+LL-37+7b+20 μM Na2S) as shown in Fig R4A. Additionally, in Fig R4B the addition of 7b did not substantially improve the survival of 3MST+LL-37+20 μM Na2S. Thus, 7b is at best a moderate scavenger of H2S.

Response:

We thank the reviewer for this careful evaluation of the revised data and for the

balanced interpretation of the role of 7b. We agree that the current results suggest that 7b functions as a moderate rather than complete H_2S scavenger under the experimental conditions used.

As noted by the reviewer, exogenous supplementation with 20 μM Na_2S was able to substantially restore LL-37-mediated antibacterial activity in the *Δ3MST* strain, even in the presence of 7b, indicating that 7b does not fully neutralize H_2S at this concentration. Similarly, in Fig. R4B, the addition of 7b only partially affected the survival phenotype observed in the presence of exogenous Na_2S. These observations are indeed consistent with a partial or moderate scavenging effect.

Importantly, however, the overall trend of the rescue experiments remains consistent with the conclusion that H_2S contributes functionally to LL-37 antibacterial activity. Specifically, the ability of exogenous H_2S to restore bacterial killing in the *Δ3MST* background, together with the attenuating effects of 7b, supports the interpretation that modulation of H_2S availability influences the antibacterial outcome.

In response to the reviewer's comment, we have revised the relevant text in the Results and Discussion to avoid overstating the scavenging capacity of 7b and to more accurately describe its effects as partial under the tested conditions. We believe these revisions provide a more balanced and mechanistically appropriate interpretation of the data.

Two minor points. With all the additional experiments the paper is now very long with 30+ supplemental figures; this needs to be shortened. Also, what the authors refer to as "time-kill kinetics"/"bactericidal kinetics" are growth curves, so the language needs to be changed.

Response:

We thank the reviewer for these helpful suggestions regarding the presentation and terminology of the revised manuscript.

In response to the reviewer's concern about the length of the Supplementary Information, we have carefully streamlined the supporting data and reduced the number of supplementary figures from 40 to 28 by consolidating related results where appropriate. We believe that the revised organization improves the clarity and readability of the manuscript while retaining all data necessary to support the study's conclusions.

In addition, we agree that the terms "time-kill kinetics" and "bactericidal kinetics" were not fully appropriate for the experiments presented. We have therefore revised the terminology throughout the manuscript and now consistently refer to these data as "growth curves" as appropriate.

Reviewer #2 (Remarks to the Author):

Although I would still have preferred to see additional experiments—such as broader validation across multiple bacterial strains and further characterization of AMP activity at sub-MIC concentrations—the authors have acknowledged these limitations and have moderated their claims accordingly.

I do, however, share the concern raised by Reviewer 1 (point 2) regarding

compound 7b and its potential effects. This remains an important issue that should ideally be addressed.

That said, in light of my previous assessment, I have no further formal comments, as my original concerns have been satisfactorily addressed.

Response:

We sincerely thank the reviewer for the careful re-evaluation of our revised manuscript and for acknowledging that the major concerns raised in the previous round have been satisfactorily addressed.

Regarding the reviewer's suggestion for broader validation across bacterial strains and further characterization under sub-MIC conditions, we would like to clarify that these analyses have now been further emphasized and integrated into the revised manuscript. Specifically, we expanded the validation across multiple bacterial species, including both H_2S-producing and non–H_2S-producing strains, and consistently observed greater susceptibility of H_2S-producing bacteria to LL-37/CRAMP. In addition, we included further analyses of AMP activity under sub-MIC conditions, which support the functional relevance of H_2S during prolonged bacterial growth inhibition.

We also appreciate the reviewer's continued attention to the potential effects of compound 7b. In response to this concern, we performed additional experiments to exclude direct interference of 7b with LL-37 antibacterial activity. Expanded ITC analyses across multiple molar ratios and temperatures revealed no detectable interaction between 7b and LL-37, while HPLC-based analyses did not support direct association of 7b with bacterial cells or membranes. Together, these results support the interpretation that the effects of 7b are mediated primarily through modulation of H_2S availability rather than through direct interference with LL-37 activity.

We are grateful for the reviewer's constructive evaluation and thoughtful comments, which have substantially improved the rigor and clarity of the manuscript.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response:

We thank the reviewer and the co-reviewer for the careful evaluation of our manuscript and for the constructive comments provided throughout the review process. We also appreciate the opportunity to benefit from the Nature Communications co-review initiative supporting early-career researcher training in peer review. The thoughtful suggestions and critical feedback have substantially improved the rigor, clarity, and overall quality of the revised manuscript.

Reviewer #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in

peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response:

We thank the reviewer and co-reviewer for their careful assessment of our manuscript and for the constructive feedback provided throughout the review process. We also appreciate the Nature Communications initiative supporting the involvement and recognition of Early Career Researchers in peer review. Their thoughtful comments have helped us substantially improve the rigor, clarity, and presentation of the revised manuscript.