Dear reviewers,

Many thanks for the careful revision of our manuscript and the overall positive evaluation. We acknowledge and appreciate your comments that helped us to improve the manuscript. Please find a point-to-point response to your comments below.

Reviewer #1:

Manuscript Summary:

Metabolic profiling to determine bactericidal or bacteriostatic effects of new natural products using isothermal microcalorimetry

The authors describe a new method to be used for determination of mode of action of antimicrobial compounds. The method uses isothermal microcalorimetry with calScreener allowing a high-throughput analysis with low volumes. The paper is well organized and clearly written. My recommendation is accept with minor modifications.

Overall comments:

Define better which mode of action you want to measure. Mode of action could also be interpreted as activity against e.g. cell wall synthesis or protein synthesis. It should be mentioned which media and conditions were used to create the representative results.

The presented method can be used to get first insights into the mode-of-action (MoA) of new antibiotics regarding major pathways being inhibited. This application is however still under investigation. Here, we only look at mechanisms by differentiating between cidal and static effects. This is highlighted in the title and abstract. We would like to keep some general comments on the possibility to use IMC for more in-depth MoA analyses but this was not part of the presented study. Media and conditions used to generate the representative results have been added to the protocol section.

Find below my comments to the sections:

Introduction
Line 99-102: may also comment on the production of EPS that can lead to higher OD values than for the actual cell count. Also, IMC detects growth earlier than OD measurements.

Thanks for this comment. In a separate study, we are currently evaluating the formation of biofilms of pathogens that heavily interact with surfaces and the effects of antibiotics on pre-formed biofilms. However, these experiments have not yet been elaborated and in the presented study, we focussed on antibacterial effects assuming that the initial *A. baumannii* population mostly consists of planktonic cells in the setup we used. We currently lack data for a concise evaluation of the dependency of e.g. OD vs. EPS formation vs. heat release reads. Since we did not present data on the correlation of OD vs. IMC, in terms of detecting bacterial growth, we did not further comment on this in the manuscript.

Protocol

Line 148: Why you need 2 passages after streaking out on the plate? Is there a difference in the measurements if used less or more passages? In phenotypic measurements, the amount of passages can lead to a slightly different profile.

In the first culturing step, we streak out the cryopreserved culture on CASO agar plates to obtain single colonies. For the actual experiment, we obtain most reproducible results starting from an overnight culture in liquid medium and then diluting this culture to the desired target concentration (CFU/mL). With a liquid overnight culture, it is easier for us to ensure that we have the same starting number of bacterial cells between experiments and thus, low plate-to-plate variations. Experiments can be set up as well by inoculating the assay culture directly from single colonies on solid media, however, in our hands, the CFU count variability was slightly higher compared to the presented protocol. The major difference that we observe when having minor variations in the starting bacterial cell number is the lag phase duration.

Line 153-155: Although OD is an easy way to adjust the density, is it valuable for any bacteria? See my comment above for EPS production. However, it can be valid if a basic calibration is done (e.g. one time measurement OD and cfu). On the other hand, if you use OD for determination of the inoculum, skip the correlation with cfu.

This is true. We typically perform concise studies on the correlation of OD to CFU/mL for all microbes that we use. This has been also done for the *A. baumannii* strain by measuring OD (following the culturing protocol as described in the manuscript), plating of serial dilutions, and colony counting. E.g. starting from an OD_{600} of 1, which equals in this case ~ $5x10^8$ CFU/mL we stepwise dilute the culture for starting the experiment by factor 1000 to finally achieve $5x10^5$ CFU/mL, which is recommend for IMC. We added a note to the manuscript.

Line 162-165: Are no growth controls used? Meaning wells without antimicrobial compound?

Thanks for this comment. Indeed, growth controls are used and we added this information to the protocol section.

Line 262: May define what low heat flow means

Low heat flow in the beginning stages of the experimental run means heat emission is equal or above zero, but not higher than 10 µW. This has been added to the protocol section.

Line 260-267: May rewrite it since it seems that the description of baseline definition is repeated

We carefully checked the manuscript for repetitions and we could not identify sections that might be skipped in the protocol.

Line 273-274: You may want to mention that it is a web-based application

The analysis of data is performed with the web-based application mentioned in step 7.6 (given along with the link). The initiation and stopping of experiment is done on software installed on the computer connected to the instrument.

Line 276-285: Modify it as above to avoid repetitions (mention the figures only once)

We are not sure how to implement this. Each figure panel is referred only once in the text.

Representative results

Why did you not test directly at the MIC and may one level above?

To ensure the correct determination of bacteriocidal and bacteriostatic effects (and possible dual MoAs), we typically assess a broader range of concentrations. However, MIC data is generated in parallel.

Line 309-311: Why was the MIC not already determined using calScreener and directly?

In our standard workflow, we determine MIC using protocols recommended by EUCAST as this is already widely accepted. However, we do compare microbroth dilution to IMC-based MIC and data correlate well. We did not comment on this in the manuscript as the focus of the presented method was the differentiation of cidal vs. static MoA.

Line 336-340: Why did you not do this experiment?

The displayed results supported the published cidal mechanism of ciprofloxacin. Indeed, we are refining (and extending) our data sets with reference antibiotics. Here, we think it is of interest to also display an experiment that should undergo further optimization (adapting the assay concentration range) to further improve the experimental outcome.

Line 341-345: May add how many replicates you did

All tests were done in triplicate and we added this info to the manuscript.

Line 398-407: In the same manner as rifampicin or with a lower effect?

We did not do a direct comparison of individual data sets but we analyzed each antibiotic separately to conclude in cidal/static effects. However, in the presented case, the effect was similar to rifampicin but more pronounced.

Discussion
Line 423-425: It may also increases the MIC

Yes. We added a comment and a reference.

Line 449-450: Rewrite the sentence on solid media

This has been done.

Major Concerns:

none

Minor Concerns:

The protocol might need to be adapted for other microorganisms than those used.

This point is well taken and we agree. Indeed, we test different microorganisms in different media using different concentrations of antimicrobial compounds to achieve fully informative results.

Reviewer #2:

Manuscript Summary:

This manuscript introduces the isothermal microcalorimetry for primary screening of candidates with potential bactericidal or bacteriostatic activity on target cells. This is an interesting work, and the description of the protocol is complete. However, because the assayed samples are some purified compounds, the applications of this protocol will be limited. Based on the policy of this journal, I recommend acceptance of this manuscript for the publication.

Major Concerns:

The authors establish this protocol based on that if the compounds affected the growth of target cells, the IMC value will change, which could be easily detected by a isothermal microcalorimeter. My major concern is that, if the assayed samples are not purified in advance, i.e. assaying on a mixture, then how about the rate for false positives?

The described method is applicable to purified compounds. We do work with microbial crude extracts as well but so far, we did not apply IMC to determine antimicrobial activities of such samples. We fully agree that this application might be challenging as crude extracts are very complex and they typically contain multiple natural product classes, which might lead to results that are not easy to interprete if the assay system and/or readout is complex. We routinely assess crude mixtures in microbroth dilution assays. Only if actives are purified and structurally characterized, we continue with in-depth profiling, thereby also applying IMC. It will be interesting to see which additional data can be retrieved for extracts when switching from typical microbroth assays to IMC. However, we believe that only well characterized (i.e. fully dereplicated) extracts should be used in such a pilot study.

With kind regards,

Jennifer Herrmann (on behalf of the authors)