In the Antimicrobial susceptibility section:

"strongly inhibited by physiological salt concentrations. In order to measure the antimicrobial activity of HNPs, they had to be incubated with cells in a low salt buffer as a separate initial step," Please add to this description. As currently there is a logical jump in that if HNPs are inhibited by physiological salt conditions, how are these antimicrobial activities determined with this VCC assay relevant to their biological activity in vivo? Therefore, if these results are not providing an understand of the peptide that is biologically relevant what is the advantage of this assay.
Has a physiologically relevant media been tested in this VCC assay instead of MHB? Has activity been monitored with the addition of serum or albumin?

In the MIC section:

" rich broth such as Mueller-Hinton broth (MHB)" is this the cation adjusted version? Cations can also have a big impact on the antimicrobial activity measured. Perhaps mentioning this effect would be worthwhile?

"because defensins must be incubated in a low salt buffer, not rich broth, in order to measure their activity." What is the difference in salt concentration between these two?

In VCC section:

"by simply adding an equal volume of twice-concentrated broth after the two hour incubation in the low salt buffer, as long as there is a way to determine how many cells survived at the end of the incubation period using batch cultures."
This sentence is not easy to understand, please rewrite and clarify.

"turbidity, or optical density, of the 96-well plate" it is not the 96-well plate that changes in optical density over time but the bacterial culture contained within. Change this wording to clarify.

" of the exponentially growing cells is known, then the number of cells originally present in the inoculum can be calculated. This inoculum"
Inoculum is not the right word to use here. Change to ….number of cells originally present in the *culture* can be calculated. This *starting number of cells*"

"is defined in the original VCC publication" please define it here as well

"as long as the activity of the agent is abrogated at the time twice-concentrated Mueller Hinton Broth is added" do you mean…. as long as the twice-concentrated Mueller Hinton Broth inactivates the antimicrobial activity of the agent?

"CFUv was held constant among the six strains tested so that the turbidity, and thus the amount of cell membrane, in each experiment was roughly equal. Because CFUv was

standardized to the CFU of *Escherichia coli* ATCC 25922, CFU, not CFUv, can be reported with this strain."
This does not belong in the general VCC description as it is specific for the experiments carried out that have not been introduced yet. This needs to be moved and incorporated below.

In the section: General laboratory procedure for use in VCC assays

"antimicrobial peptides are diluted on a 96-well plate (Costar 3595)"- "96-well plate" please define what type of plastic. Peptides will stick to different types of plastics effecting the amount of peptide available for killing bacteria. Tissue culture treated plates will also effect growth curves.

"Parafilm M six squares long by one half square" could this be something like the Breathe easy membrane instead?
(https://www.sigmaaldrich.com/catalog/product/sigma/z380059?lang=en&region=AU)

"threshold optical density of 0.02." How was this threshold determined? When would this change?

In the section: Quantitative growth kinetics

"It relates the kinetic time taken for the turbidity of a bacterial batch microbiological culture in a well of a 96-well microplate to reach a threshold difference in turbidity to a 10-fold dilution series of calibration growth curves." This definition seems verbose and meaning is lost because of it. Perhaps something like this instead- It relates the time taken for the turbidity of a bacterial culture in a well of a microplate to reach a threshold in relation to calibration growth curves.

Figure 1- Change the key in this figure to label the 6 replicates for each of the concentration of the calibration curves. Add more information to the legend to ensure this figure is stand alone. Information currently in legend regarding plating should be included as main text instead.

Figure one is not mentioned in the main text.

In the section: Bacteria studied in the history of VCC assays

"six strains of *E. coli*, *S. aureus*, *Bacillus cereus*, and *Enterobacter aerogenes*.[1] " Use the full names of the bacteria here.

In the section: Antimicrobial peptides studied in the history of VCC assays

" A conserved glycine in a beta bulge in HNP2 was replaced with a series of D-amino acids resulting in VCC activity **proportional** to side chain hydrophobicity and charge.[12] VCC showed that N-terminally acetylated and/or C-terminally amidated HNP2 activity is **proportional** to electrostatic charge.[13] VCC results were again **proportional** to charge for a

series of salt bridge-disrupting mutants, suggesting that the salt bridge is not required for HNP2 function.[14] VCC measured the importance of N-terminal natural and artificial pro segments of the propeptide HNP1, dramatically **altering** activity against *Escherichia coli* and *Staphylococcus aureus*.[15][16] " In this section the effect of these changes is not fully described. Define what is meant by proportional and altering? Do these changes result in a decrease on increase in antimicrobial activity?

In the section: Inoculum effect

"An inoculum effect has been previously described for many antimicrobial agents"
Provide references for this statement.

Change inoculum to the following underlined in this sentence. "such that the agent is less effective when more bacteria are added to the assay."

"Because the inoculum of bacteria was 20-fold greater in the VCC assay compared to the standardized traditional colony count protocol used, the difference could have been due to an inoculum effect, although the effect would have been the reverse of the inoculum effect normally seen with other antimicrobial agents, since the higher inoculum showed more activity. This possibility was investigated in a series of experiments mainly focusing on the defensin HNP1 and the bacterial strains *E. coli, S. aureus* and *B. cereus*."
This section currently reads as if the experiment detailed below should be a comparison of VCC assay and the traditional colony count in respect to a range of inoculum.

In the section: Example VCC experiment

"HNP1 was synthesized on an ABI 433A synthesizer using an optimized HBTU activation/DIEA in situ neutralization protocol developed by Kent and coworkers for Boc chemistry solid phase peptide synthesis (SPPS)"
What was the yield? How was this peptide purified? How was the peptide mass quantified? How did you ensure that you had successfully made and purified this peptide?

Why is this NaPiT buffer different from the one listed above in section "**General laboratory procedure for use in VCC assays?"** Define how the TSB enhances the defensing activity?

"to a cell concentration of $2x10^8$CFU/mL" how did you determine these cultures were at this CFU/mL? Do you know what absorbance equals a particular CFU/mL? If this is by absorbance to you check the CFU counts each time you perform the assay?

"and then the calibration dilutions were done in row G as described in the caption of Figure 2." The description of the calibration dilutions is better suited in the main text. Please remove from the figure legend and place here.

"Input controls were added to wells B1-B1" What volumes are added? What are the in the input controls?

“and then 100 µL of twice-concentrated MHB was added to all wells except row G.” Add a description of what is in row G and how this curve was prepared.

“Reading every 5 minutes and shaking 10s in a linear fashion before reading.” State the Absorbance that the plate is read at.

“Rows 36-134 of this spreadsheet were copied and pasted in rows 37-135 of spreadsheet "raw".” Add to this sentence to describe why this step was done.

In the section: Results: observation of the 96-well plate

“precipitates were visible as white specks” do you have images of these precipitates? What do you think makes up these precipitates? Is this to do with bacterial growth or the peptide falling out of solution? Does this happen with other peptides?

In the section: Results: calibration

“evenly spaced and parallel up to a change in optical density of about 0.3,” Explain why they lose this at this point?

“The calibration curve was linear with an R^2 value of 0.992.” Is there a figure for this?

Figure 3. Provide not just the well numbers but also the starting *E. coli* concentrations in these wells.

Results: calculation of virtual survival

“It is calculated according to Brewster.” Provide a brief summary of how this is performed.

“Virtual survival values are plotted in Figure 4.” Please provide description of these results and what they mean here.

Color for the different bacterial concentrations in figure 4 would make this figure easier to interpret.

Results and discussion: a pronounced inoculum effect

In figure 7 & 8, include the well number along with the HNP-1 concentration for all wells.

“These results unequivocally rule out the interpretation of the previously published experiments[2] comparing traditional colony count results with VCC results that would have attributed the difference to an inoculum effect.” A side by side comparison of the two assays would be required.

Replicate data and other strains

How much variation is there between experiments? This is a single biological replicate, that does not contain any technical replicates. This experiment needs to be repeated and replicate data shown.