Response to Reviewer 2:

Generally, I find the presented research decently described and the manuscript is well written. I have no major remarks.

1. Regarding bacterial species, please make clear in the introduction for which species Css54 has been tested so far and with what outcome.

Reply: We agree with the reviewer's comment. We have already described the previously reported antimicrobial activity of Css54 against *Staphylococcus aureus* and *Escherichia coli* (lines 68–69). Moreover, we described which strain was used to test the antimicrobial activity of Css54 in the Introduction section according to the reviewer's comment.

(After revision)

(Lines 70–73)

In this study, Css54 was tested for its antimicrobial activity against *L. monocytogenes*, *S. typhimurium*, *Streptococcus suis*, and *Campylobacter jejuni*, which cause zoonotic disease. We used melittin, an antimicrobial peptide found in bee venom. We confirmed that Css54 has antimicrobial activity similar to melittin.

Commented [E1]: Do you mean that this peptide has antimicrobial activity?

2. The statement "Css54 exhibited low cytotoxicity compared to melittin", appearing repeatedly in the manuscript, is a bit misleading as suggesting "low" Css54 toxicity. I surmise "lower" is what the authors had in mind.

Reply: Thank you for this comment. We have revised these sentences.

(After revision)

(Line 17)

the cytotoxicity and hemolytic activity of Css54 was lower than that of melittin isolated from bee venom.

(Line 127)

Css54 exhibited lower cytotoxicity than melittin.

(Line 438)

Css54 showed lower cytotoxicity than melittin.

3. Anyway, for the concentrations used, PK(15) cells exhibit c. 60% survival. Doesn't it make Css54 quite cytotoxic after all? In my opinion it requires detailed discussion in the manuscript.

Reply: Thank you for your comment. As the reviewer suggested, we have further explained this point in the Discussion section.

(After revision)

(Lines 254–259)

In a previous study, treatment with melittin showed no adverse effects on the stomach tissue, including on its function, and melittin was confirmed to have a protective effect against gastric inflammation and antitumor effects *in vivo* [39-41]. These reports indicate that Css54 can be developed as an effective treatment because of its lower toxicity than melittin. We confirmed that hemolytic activity towards sRBCs and cytotoxicity against pig kidney cells PK(15) was lower than that of melittin.

References

39. Abu-Zinadah, O.; Rahmy, T.; Alahmari, A.; Abdu, F. Effect of melittin on mice stomach. *Saudi J Biol Sci* **2014**, *21*, 99-108.
40. Rahmy, T.; Alahmari, A.; Abdu, F.; Abu-Zinadah, O. Protective effect of melittin against gastric inflammation in mice. *Life Sci J* **2013**, *10*.
41. Duffy, C.; Sorolla, A.; Wang, E.; Golden, E.; Woodward, E.; Davern, K.; Ho, D.; Johnstone, E.; Pfleger, K.; Redfern, A. Honeybee venom and melittin suppress growth factor receptor activation in her2-enriched and triple-negative breast cancer. *NPJ Prec Oncol* **2020**, *4*, 1-16.

4. Figure 6B and C: Why is there a 10 min lag before any change is observed? Could you clarify this in the figure description and manuscript text?

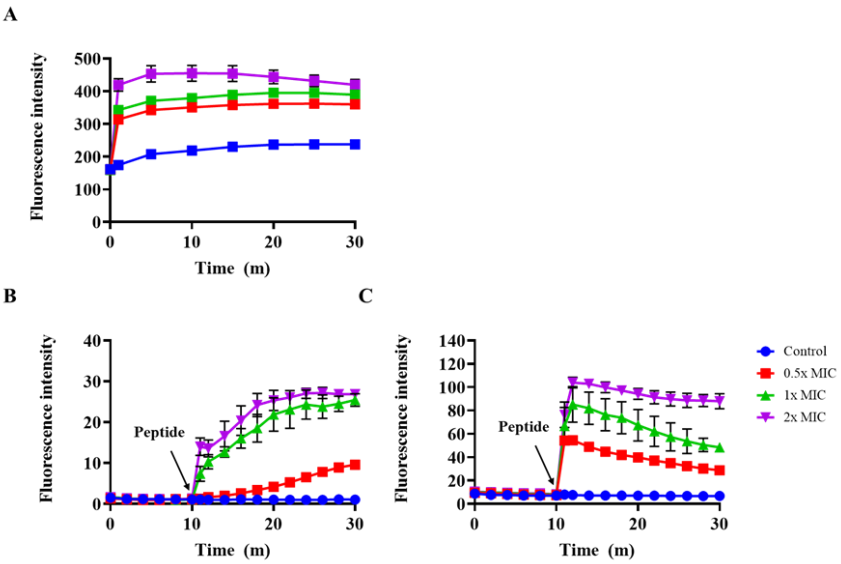
Reply: Thank you for this question. We confirmed that bacterial membrane depolarization occurred after treatment with Css54 using 3,3′-dipropylthiadicarbocyanine iodide $DiSC_3(5)$. $DiSC_3(5)$ accumulates in the lipid bilayer, causing self-quenching, and is released upon damage to the cytoplasmic membrane, resulting in increased fluorescence intensity. As mentioned in the Methods section, bacteria treated with $DiSC_3(5)$ were incubated for 1 h to stabilize the fluorescence intensity. The reason for adding Css54 after 10 min was to ensure that the fluorescence intensity had been stabilized. To address the reviewer's comment, we have revised Figure 6B and C.

References

1. Sun, Y.; Dong, W.; Sun, L.; Ma, L.; Shang, D. Insights into the membrane interaction mechanism and antibacterial properties of chensinin-1b. *Biomaterials* **2015**, *37*, 299-311.
2 Kim, M.K.; Kang, N.H.; Ko, S.J.; Park, J.; Park, E.; Shin, D.W.; Kim, S.H.; Lee, S.A.; Lee, J.I.; Lee, S.H. Antibacterial and antibiofilm activity and mode of action of magainin 2 against drug-resistant acinetobacter baumannii. *Int J Mol Sci* **2018**, *19*, 3041.

(After revision)

(Line 194)


A
Fluorescence intensity
500
400
300
200
100
0
0
10
20
30
Time (m)
B
C
Fluorescence intensity
40
30
20
10
0
Peptide
0
10
20
30
Time (m)
Fluorescence intensity
140
120
100
80
60
40
20
0
Peptide
0
10
20
30
Time (m)
Control
0.5x MIC
1x MIC
2x MIC