nature portfolio

Peer Review File

Positive Intervention of Distinct Peptides in Clostridoides difficile Infection in a Mouse Model

Corresponding Author: Ms Yuan Wu

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Key Results

• Peptides from E. japonicus and G. max may alter goblet cell numbers, inflammatory response, cell death and cell turn over responses and the microbial diversity of the gut following CDI. Convincing data is present for the cell death/turnover and microbial diversity. However, there are many flaws and inconsistencies with the remaining data.

Conclusions
• Peptides from E. japonicus and G. max may be a promising adjunct therapy to aid in repair during CDI

Concerns/Issues/Originality

• There is a spelling error in the title and remaining manuscript – Clostridioides difficile is spelled in correctly and is missing an “i” in Clostridioides
• Line 62 is incorrect. C. difficile has only been names Clostridioides difficile since ~2016/2017. Hall and O’Toole first described C. difficile and named it difficilis
• Lines 73-74: More recent data from the CDC is available and should be used as these numbers do not reflect the recent incidence.
• Lines 216-218: Missing punctuation and issues with the number of animals (Forty listed, but numbers add up to 48).
• Line 222: blank group should be normal group.
• Line 226: change standing hair and curling up to – piloerection and huddled as these are more inline with standard descriptions of mouse phenotypes.
• Lines 229-233: the description of the treatment groups is very unclear. Please amend this.
• Line 234: I am confused to when or how much spiperone is administered?
• Line 237: Cervical dislocation? Not spinal detachment
• Line 249: aliquots should be sections or regions.
• Line 263: Please rephrase “closed with 3% H2O2”.
• Line 277: Repair should be replaced with retrieval, and sealed should be replaced with blocked, to match standard procedures.
• Line 287: remove the phrase “from the refrigerator”.
• Lines 332-341: This section needs to be reworked. The inconsistency in describing the samples creates confusion (E. japonicus protein vs anchovy peptide). I am also unsure what you mean by “…dominated by 3–5 of them”. Is this meant to be tri and pentapeptides? Peptide is also used interchangeably to describe the E. japonicus and G. max protein, making it hard to follow this section
• Line 361: rephrase confused and listless. These are not terms typically used to describe mice or C. difficile infected mice in particular.
• Lines 358-367: This section is very hard to follow. Please rephrase this section.
• Line 366: Is the “model group” an infected untreated group? This is very unclear, as model groups is used earlier to describe all infected mice.
• Figure 2B: weight loss appears to occur before CDI established (based on the timeline). Is that correct? Additionally, weights should be presented as % weight loss to account for the variation in starting weights. As it stands the Ti group appears to have a higher starting weight, which is skewing the weight change and may not be that different from the other groups.
• Figure 2C and D: I take issue with the images and numbers provided. It is very clear from the images that different regions of the gut have been examined, and this will change the number of goblet cells counted. For instance, the control, model and

vanc group appear to be distal colon section, with a uniform crypt architecture, whereas the Ti and Da images represent the proximal colon, where distinctive mucosal folds are seen. Imaging and counting these different regions will lead to a biased result as there are natural differences in the architecture of the gut regions and number of goblet cells within these regions. These images should be all taken/represent one area of the colon (proximal, mid or distal) and counts should be presented for a single region alone.
• Figure 2E: My same concerns for Figure 2C/D apply here. The images presented represent different regions of the colon that have significant changes in their architecture naturally. Additionally, images should be presented in the same orientation and as a uniform layer, as the current depiction of the tissues make it difficult to determine the level of damage/repair.
• Lines 370-378: The representative images in figure 2 do not depict the differences described in text. The images are all in different orientations, represent different regions of the gut (see comments above regarding colon regions) and appear to mistake mucosal folds and changes in tissue plane when sectioning for changes in pathology.
• Lines 379-389: As described above, I take issue with the way this has been analysed, as the images presented represent different gut regions, where goblet cell numbers and state (pH) are known to change. If these changes are true, please provide images from the same region (proximal, mid or distal colon).
• Line 384: What do you mean by recovery of the mucous area? Did you measure the mucous layer? To do this, tissues need to be fixed in Carnoy's to preserve the mucous integrity due to its solubility in water containing fixatives.
• Figure 2F: What do the letters represent above the bars? There is no indication of the significance levels in the figure legend.
• Lines 432-444: Was the protein level of ZO-1 examined in the tissues? While gene expression may be elevated, tissue repair and connections between the cells may not be repaired?
• General comment on figures: the "normal" group is labelled very inconsistently throughout the figures – mostly as control, sometimes as N. This is hard to then match to the text that refers to them as normal for the majority of the manuscript.
• Line 554: This is the first mention of cup cells throughout the manuscript. Where is there evidence that the peptides promote their growth? Additionally, are cups cells even present within the colon? Or only in the small intestine?
• General comment on discussion: The paper focusses highly on CDI, yet the discussion has one paragraph on the results of the C. difficile work, and do so in such a superficial way that the reasoning for using this model is completely unclear. This needs more work to show why this model was used, and the significance of the work to the CDI field.
• General comment on figure legends: No useful detail has been provided here (sample size, significant etc.). This needs to be fixed so the reviewers can understand what they are trying to interpret.

Reviewer #2

(Remarks to the Author)
General comments:
Overall, the study is good, and well intentioned. It looks into the impacts of various short peptides as alternatives or adjuvants to antibiotics in gut. Technically, apart from a few things, the study was well conducted. The only gap was the authors have not shown the confirmation of CDI being established in mouse gut. Although an assay can help, the microbiome sequencing can help to at least qualitatively establish this. Just the external symptoms are not enough to claim a successful CDI establishment.

Another issue I have is that there is no ethics statement provided in the whole document. If ethics approval was successfully sought before the study, the approval number, and approving agency must be indicated in the study.

Finally, the study although good on technicalities, does not read well, particularly for people who are not very well aware with the mass spectrometry or genomics jargons. As a person working in multi-omics, I understand a good amount of these terms, even if some technical phrases are used incorrectly. However, the same does not apply to readers who are not protein or genome specialists. I have indicated the issues in the Specific Comments below for the authors to refer.

The authors need to also remove a good amount of filler words and sentences. I have indicated some below. These do not add to the story, and increase the word count unnecessarily. The document needs a good proofreading before it can be accepted for publication. English is not my first language, so as a reader, I don't expect a flowery language. A simple write-up is good enough to carry the story. However, use of slangs (e.g., tinfoil) increases the difficulty of reading.

Overall, the manuscript needs a major revision before it can be accepted.

Specific comments:
1. Whenever indicating an equipment, please indicate (Model, Company, City, State/Province, Country) on first mention. Same for the chemicals, particularly specialist chemicals (internal standards, standard peptides, calibrants etc.).

2. Lines 157-162: Please provide the HPLC protocol in detail. In current form, it cannot be replicated. Which machine was used? Was the solvent used in isocratic or in gradient manner? If gradient, what was the gradient used? If the overall addition takes too much of space, the protocol can be added to the Supplementary materials. Alternatively, a previous study can be referred to, if the current protocol derives from that study with no or minor modifications.

3. Lines 18-186: The solevnt mixtures indicated are a bit confusing. Do the authors mean Water + 0.1% formic acid (Solvent A) and Acetonitrile + 0.1% formic acid (Solvent B)? Also, the gradient can be simplified as: "The gradient was set as 0 - 2 min. (A = 5%), 27 min. (A = 10%), 37 min. (A = 25%), 39 min. (A = 80%), 42 min. (A = 80%), 43 min. (85%). The column was reconditioned with 5% A from 43 - 50 min."

4. Lines 191 - 200: The terms 'Primary mass spectrometry' and 'Secondary mass spectrometry', are incorrect. This sentence can be restructured as, "Resolution for mass spectrometery were kept at 70,000 (MS1 level) and 17,500 (MS2 level)." Also, the phrase 'number of strongest ions' is confusing. Do the authors mean, 'ions with the highest intensities'? If this is the case, were there 20 top ions that were shortlisted? Overall, this paragraph needs to be re-written for better clarity.

5. Lines 203-204: Same as above. Some of this needs to be re-written as the technical phrases used are incorrect. For example, the correct sentence for Line 203 would be 'Thresholds for Precursor mass tolerance and fragment tolerance were kept at 10 ppm and 0.02 Da, respectively'. If the authors are worried about the plagiarism being an issue, please note that there is a limit on how many ways these type of (technical) sentences can be written without a hazard of repetition. I would not worry about the plagiarism in the highly technical parts such as this paragraph.

6. Lines 205-207: The database entry for Glycine max needs to be correctly written. For excample, it can be written as, 'the dataset was assessed against proteomic database of Glycine max (NCBI id: 123456789) and Clupeiformes (NCBI id: 123456789). For example, the proteome id for Glycine max on Uniprot is UP000008827. What would be the equivalent id of Glycine max on NCBI that authors have used?

7. Lines 224-227: The weight decrease should be part of Results section, not this section.

Line 231: What do the terms 'Ti' and 'Da' refer to? This must be clearly defined.

Line 234: When was spiperone fed to mice? It is not indicated in earlier lines.

Line 237: The correct term for 'killing' is 'euthanized' in the context of animal trials.

Line 246: Does the terrm 'tinfoil' refers to 'aluminium foil'? If yes, it must be used as such.

Line 262: What is the full form of TUNEL? This must be indicated somewhere in the paragraph, if not in the heading of this subsection.

Line 334: This sentence is unnecessary, and can be deleted.

Line 335: Which other 2 protein products? Please clarify.

Line 345-347: Please support this statement that lower molecular weight is always indicative of better absorption effect. please cite the relevant studies which have indicated this relation.

Line 351: ideal amino acid pattern defined by WHO. Please cite the relevant document

Line 361: Do the symptoms 'confused and listless' indicate successful infection, clinically? Please cite the supporting documents/reports previously published. Was any diagnostic study done to assess the successful infection? If yes, indicate the outputs of that study here.

Line 379: Please provide citation for this statement.

Line 403-414: Please indicate the Figure number. For example, the fuorescence intensity is showed in Figure 3C.

Lines 415-421: Caspase 2 and 3 increased more in Ti and Caspase 8 and 10 increased more in Da. What would this infer?

Line 429-430: What would be the previous immunofluorescence staining results? Is it a previously reported study? If yes, please cite. Same for lines 442-444.

Lines 490-491: This sentence does not add to the story. Can be deleted.

Line 512: Do the authors mean 'petides above 11 amino acid lengths"?

Reviewer #3

(Remarks to the Author)
In the study by Li et al., authors analyzed and compared peptides from Engraulis japonicus and Glycine max and investigated the potential effect of oral administration in an animal model of CDI. They reported a reduction of intestinal inflammation and apoptosis, and improved repair of the intestinal barrier by promoting colonic epithelial cells proliferation. Observed beneficial effects were associated to a partial restoration of bacterial diversity, as well as an increase of beneficial bacteria abundances and reduced the proportion of harmful bacteria. The results presented could have a significant impact for the scientific community since peptides have a significant biological activity, but there is no identification of active peptides in the study which is very limiting. Moreover, improvements in the discussion section and in the Figure descriptions could help to understand the message.

Major comments:

Most figures are difficult to understand without the main text. Figure captions have to be more descriptive (including abbreviations meaning, statistics, …).

Line 328 - Composition analysis of the peptides: I do not understand what is the aim of this analysis since no correlation between peptides structures and biological activity is presented in the paper. Authors should fractionate peptides to identify the biologically active compounds in their extract, this would strengthen the conclusions.

l.451: how can you affirm that that C. difficile infection reduced the number and diversity of bacteria in the cecum while you used antibiotics and the effects of antibiotics on bacterial diversity is similar to model group (Figure 4B) ? Please explain or revise.

Discussion: has to be revised. Line 521 to 541: data presented in this paragraph of the discussion does not bring valuable input to discuss the results, while the results obtained on CDI model are not discussed in the Discussion section. Please use data from literature to discuss the results presented.

Minor comments:

The term “protein peptide” is used several times in the main text (l.36 for example) but does not make sense to my opinion. Is it “peptides and/or proteins”, “protein-derived peptides” ? Please revise.

The nomenclature for bacterial phyla has changed and are often wrong in the paper. Please revise.

Line 391: Figure 2, panel F has to be mentioned.

Line 503-504: The sentence “To reveal the relationship between the differences in their peptide compositions and their bioactivities” does not make sense. Please revise.

Figure 4B: what is group P? please revise/explain

Supp Figures should appear after main figures.

Author Rebuttal letter:

The authorâs response to these comments can be found at the end of this file.

Version 3:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
General Overview and Comments:

There are many formatting and grammatical errors throughout the manuscript that need to be addressed. These kinds of errors are careless and time consuming for reviewers to address, especially given that this is the second time we have addressed these issues. It’s extremely disappointing to see many of the same errors highlighted before, and issues with copying and pasting reviewer comments of text from other sources. This is unprofessional and unacceptable.

Engraulis japonicus and E. japonicus are not consistently used. Once defined at the beginning of the document, E. japonicus should be used thereafter.

Concerns/Issues/Originality

• There is a repetition error in the abstract (lines 36-37)
• As discussed in my previous review C. difficile has been an important pathogen for several decades. Please remove "… and, in recent years, has been identified as a common hospital pathogen." From lines 61-62.
• As discussed in my previous review Hall and O'Toole named C. difficile "difficilis". Please fix this again on line 63
• I take issue with the statements on lines 66-68, which suggest that C. difficile induces enough dysbiosis to promote infection. This is simply not correct, and a long history of literature supports that the most common dysbiosis in via antibiotic off target effects. Indeed many people have tried to induce infection without antibiotics without success. Please remove this.
• Line 101: please define what mouse AD is.
• Lines 101-102: The following "…induced AD mouse model showed various ameliorative effects and a mitigating effect on oxidative and inflammatory stress." Does not make sense. What effects occurred?
• Lines 124-125: repetition error FITC(Fluorescein Isothiocyanate)
• Lines 124-125: "BS also inhibited FITC(Fluorescein Isothiocyanate) -dextran(Fluorescein Isothiocyanate) permeability, which is important for protecting intestinal mucosa health" this is confusing and needs rephrasing. BS protected from gut permeability which is important for health, and this was shown using FITC dextran.
• Line 126: "It is currently an economical and common protein product" This does not make sense. Please rephrase. Do you mean its cheap to produce and readily available?
• Line 140-141: "Water was used to extract the proteins, neutral protease, and alkaline protease complex enzyme." – this does not make sense, please rephrase.
• Line 223: What CFU was the C. difficile preparation. This is important!
• Line 226: Please fix to remove "standing hair and curling up to" – this is copied directly from my revision and is quite disappointing to see.
• I take issue with the CFU quantification in line 228 to 232, as the rebuttal document suggests that colonisation was confirmed by plating? Which method was used?
• Lines 233-240: When and how were saline, vanc and Ti given? There is not mention of the mode of delivery for these compounds, which you do describe for Da.
• Line 253: aluminium is spelled incorrectly
• Line 260: As per my previous revision, what does closed with 3%H2O2 mean?
• Lines 264-265: Repetition of scoring method, which still needs a reference!
• Line 273: Fix this, tunel should not be defined this way, but rather in parentheses. Furthermore the way this leads into the rest of the paragraph does not make sense. It is disappointing to see the has been a lot of carelessness with the revisions.
• Line 345: fix "dominated by 3-5 peptides" to tri and pentapeptides to match the remainder of the document.
• Line 364: "As shown in Figure 2A, on 1-12thof the experiment,…" do you mean days 1 to 12 of the experiment?
• Line 365: "on day 13th" should be day 13 or the 13th day of the experiment
• Lines 365- 367: ". On day 13th, oral administration of C. difficile to mice. C. difficile was quantified by selective culture counting of faeces collected from mice on the first day of infection, …" There are many issues here! What amount of C. difficile was administered? Was C. difficile truly quantified by counting? The remaining review comments and methods suggests qPCR was used?
• Lines 369-373: Again many issues here. Frequency and delivery mode are not clear, the other controls have not been described etc. This needs significant work.
• Lines 529-540: There are so many different errors in this section that need to be addressed.
o Grammatical errors
o Errors in how CDI is established (while surface proteins may contribute to dysbiosis this is not how CDI is established, and many years of literature supports the idea that antibiotics facilitate the majority of dysbiotic events that induce CDI)
o Clostridium is referenced, which suggests this may be copied from elsewhere
o There are repeated sentences on lines 532-534
o There are effective treatments for CDI, they just perpetuate disease and AMR. This needs to be rephrased

Suggestions

• Based on the number of corrections with the work I would like to reject this manuscript still. There are many sections that are carelessly addressed, which is frustrating to see.

Reviewer #2

(Remarks to the Author)
The manuscript looks significantly better now, after the revisions. From my understanding, the manuscript is ready to be accepted after these issues are addressed.

Just some minor corrections needed
Line 40 and elsewhere: The correct term is UPLC-MS/MS
Line 167 and elsewhere: The term 35 °C is with spaces in some part, and without spaces in others. The correct way is to put it as 35°C (no spaces)

Line 259: Do the authors mean cm2 here?

Reviewer #3

(Remarks to the Author)
Thank you to the authors for carrefully revising their manuscript, which as been strongly improved. Authors have replied to my major concerns. I still have some minor comments:

Figure 2F caption: Please replace pro-inflammatory factors by anti-inflammatory factors regarding IL-4 and IL-10.

Line 529: Please correct "Clostridioides dificileinfection"

Author Rebuttal letter:

The authorâs response to these comments can be found at the end of this file.

Version 5:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
General Overview and Comments:

There are still many formatting and grammatical errors throughout the manuscript that need to be addressed. These errors appear to have come from copying requests across to the document or from the LetPub website. Besides these most of the issues in the previous version have been addressed.

Concerns/Issues/Originality
• Line 39: grammar issue and CDI is a different font
• Line 65: formatting issues
• Line 68: Virulent megacolon is not a disease – CDI leads to toxic megacolon please fix this
• Line 72/73: change are to were, as the cases were from 2021
• Lines 101/103: formatting issues
• Line 130: remove parentheses
• Line 206: formatting issue
• Line 221: remove with
• Lines 230/231: based on the remainder of the manuscript faeces should be spelled feces
• Line 233: as above, colonised should therefore be colonized
• Lines 239/240: remove with and replace of with at
• Line 270: change 3% H2O2 treatment on to “3% H2O2 treated colon sections”
• Line 299: formatting issue
• Lines 339/340: “The total number of peptides in G. max were 7666. The total number of peptides in E. japonicus G. max products were 1325, and in E. japonicus the total peptides were dominated by tri and pentapeptides.” Can you please clarify if the 1325 products are meant to be shared between the two peptide preparations? I am a little confused by this section
• Line 348: fix G. ma
• Lines 362-365: faeces should be feces based on the remaining document conventions
• Line 369: remove with and replace with “at 2 weeks post treatment”
• Line 371: replace HE with H&E
• Line 380/381: “and a large number of inflammatory cells infiltration” change to “a high amount of inflammatory cell influx was seen…”
• Line 389: C. difficile is missing a period
• Line 465: remove an
• Line 511: change is carried out to was carried out. Capitalise We
• Lines 528-533: this needs significant rewording. Change as follows: “C. difficile infection (CDI) establishes in a host, following dysbiosis of the intestinal microbiota, which often occurs following the heavy use of antibiotics to treat unrelated infections, leading to the proliferation of C. difficile within the colon 5. C. difficile’s toxins TcdA and TcdB, can destroy intestinal cells, change the cytoskeleton and release inflammatory factors， which results in the clinical symptoms associated with C. difficile infection27.”
•
Suggestions

• There are still a number of errors that need to be addressed for this manuscript, however, once addressed, the manuscript would be fit for publishing.

Reviewer #3

(Remarks to the Author)
Thank you to the authors for carrefully revising their manuscript. One of my concern has not been revised:
Figure 2F caption: Please replace pro-inflammatory factors by anti-inflammatory factors regarding IL-4 and IL-10.

Author Rebuttal letter:

Reviewer comments:

Reviewer #1 (Comments for the Author):

General Overview and Comments:

There are still many formatting and grammatical errors throughout the manuscript that

need to be addressed. These errors appear to have come from copying requests across

to the document or from the LetPub website. Besides these most of the issues in the

previous version have been addressed.

Dear reviewer, Thank you very much for your careful review, and we will continue to

revise carefully in response to the issues you have raised.

Concerns/Issues/Originality

â¢ Line 39: grammar issue and CDI is a different font

A: Line 39. It has been corrected.

â¢ Line 65: formatting issues

A: Line 84. It has been corrected.

â¢ Line 68: Virulent megacolon is not a disease â CDI leads to toxic megacolon please

fix this

A: Line 87. It has been corrected.

â¢ Line 72/73: change are to were, as the cases were from 2021

A: Line 90-91. It has been corrected.

â¢ Lines 101/103: formatting issues

A: It has been corrected.

â¢ Line 130: remove parentheses

A: Line 149. It has been removed.

â¢ Line 206: formatting issue
A: It has been corrected.

â¢ Line 221: remove with

A: Line 240. It has been removed.

â¢ Lines 230/231: based on the remainder of the manuscript faeces should be spelled

feces

A: It has been corrected.

â¢ Line 233: as above, colonised should therefore be colonized

A: Lines 252/384.Thanks. It has been corrected.

â¢ Lines 239/240: remove with and replace of with at

A: It has been corrected.

â¢ Line 270: change 3% H2O2 treatment on to â3% H2O2 treated colon sectionsâ

A: Line 289. It has been corrected.

â¢ Line 299: formatting issue

A: It has been corrected.

â¢ Lines 339/340: âThe total number of peptides in G. max were 7666. The total number of peptides in E. japonicus G. max products were 1325, and in E. japonicus the total peptides were dominated by tri and pentapeptides.â Can you please clarify if the 1325 products are meant to be shared between the two peptide preparations? I am a little confused by this section

A: Yes, the 1325 products are meant to be shared between the two peptide preparations.

â¢ Line 348: fix G. ma

A: Lines 367.Thanks. It has been corrected.

â¢ Lines 362-365: faeces should be feces based on the remaining document conventions

A: Thanks. It has been corrected.

â¢ Line 369: remove with and replace with âat 2 weeks post treatmentâ

A: Lines 388. It has been corrected.

â¢ Line 371: replace HE with H&E

A: Lines 390. It has been corrected.

â¢ Line 380/381: âand a large number of inflammatory cells infiltrationâ change to âa high amount of inflammatory cell influx was seenâ¦â

A: Lines 399-400. It has been corrected.

â¢ Line 389: C. difficile is missing a period

A: Lines 408. It has been corrected.

â¢ Line 465: remove an

A: Line 484. It has been removed.

â¢ Line 511: change is carried out to was carried out. Capitalise We

A: Line 530. It has been corrected.

â¢ Lines 528-533: this needs significant rewording. Change as follows: âC. difficile

infection (CDI) establishes in a host, following dysbiosis of the intestinal microbiota,

which often occurs following the heavy use of antibiotics to treat unrelated infections,

leading to the proliferation of C. difficile within the colon 5. C. difficileâs toxins TcdA

and TcdB, can destroy intestinal cells, change the cytoskeleton and release

inflammatory factorsï¼ which results in the clinical symptoms associated with C.

difficile infection27.â

A: Line 547-552. It has been corrected.
Reviewer #3 (Remarks to the Author):

Thank you to the authors for carrefully revising their manuscript. One of my concern

has not been revised:

Figure 2F caption: Please replace pro-inflammatory factors by anti-inflammatory

factors regarding IL-4 and IL-10.

A: Line 746. It has been corrected.

Reviewer comments:

Reviewer #1 (Comments for the Author):

Peptides from E. japonicus and G. max may alter goblet cell numbers, inflammatory response, cell death and cell turn over responses and the microbial diversity of the gut following CDI. Convincing data is present for the cell death/turnover and microbial diversity. However, there are many flaws and inconsistencies with the remaining data.

A: We are very sorry for the inconsistency due to unclear description and other reasons, we have carried out a careful check, for the issues you raised have been carefully revised, the specific changes are as follows.

Some specific comments are as follows:

There is a spelling error in the title and remaining manuscript – *Clostridioides difficile* is spelled in correctly and is missing an "i" in *Clostridioides*.

A: Thanks. The word has been re-worded.

Line 62 is incorrect. C. difficile has only been names *Clostridioides difficile* since ~2016/2017. Hall and O' Toole first described C. difficile and named it difficilis

A: Thank you, we have made changes according to your comments.

Lines 73-74: More recent data from the CDC is available and should be used as these numbers do not reflect the recent incidence.

A: What you are proposing is excellent and we have revised it with the latest data published on the CDC website.

Lines 216-218: Missing punctuation and issues with the number of animals (Forty listed, but numbers add up to 48).

A: Thank you for your advice. We've fixed the missing punctuation,, and the number of experimental animals was 40.

Line 222: blank group should be normal group.

A: Thanks, We have uniformly changed to Normal group..

Line 226: change standing hair and curling up to – piloerection and huddled as these are more in line with standard descriptions of mouse phenotypes.

A: It has been corrected.

Lines 229-233: the description of the treatment groups is very unclear. Please amend this.

A: A more specific description is as follows: *Clostridioides difficile* infection group (n=32) was randomly divided into 4 groups: model group (Model group), vancomycin group (Van group), E. japonicus group (Ti group) and G. max group (Da group), with 8 animals in each group. The treatment methods for each group were as follows. The normal and model groups were given with saline of 200 mg/kg bw for the mice. The Van group was given vancomycin of 100 mg/kg bw. The Ti group was given with E. japonicus peptide of 400 mg/kg bw. In the Da group, 400 mg/kg bw of G. max peptide was dosed by given once daily for two consecutive weeks.

Line 234: I am confused to when or how much spiperone is administered?

A: I'm sorry, carelessness in our revisions led to confusion in the use of words, this should be “cefoperazone”, not “spiperone”. Cefoperazone is used on 1-10th at a dose of 100 mg/kg of body weight daily.

Line 237: Cervical dislocation? Not spinal detachment

A: It has been changed.

Line 249: aliquots should be sections or regions.

A: It has been changed.

Line 263: Please rephrase “closed with 3% H2O2”.

A: Thanks, it's been revised.

Line 277: Repair should be replaced with retrieval, and sealed should be replaced with blocked, to match standard procedures.

A: Thank you for your suggestions. It has been corrected.

Line 287: remove the phrase “from the refrigerator”.

A: It has been removed.

Lines 332-341: This section needs to be reworked. The inconsistency in describing the samples creates confusion (E. japonicus protein vs anchovy peptide). I am also unsure what you mean by “…dominated by 3–5 of them”. Is this meant to be tri and pentapeptides? Peptide is also used interchangeably to describe the E. japonicus and G. max protein, making it hard to follow this section

A: "E. japonicus protein and anchovy peptide" has been amended to "Ti protein". “…dominated by 3–5 of them” is meant to be tri and pentapeptides. It has been uniformly described in terms of Ti and Da protein.

Line 361: rephrase confused and listless. These are not terms typically used to describe mice or C. difficile infected mice in particular.

A: It has been changed.

Lines 358-367: This section is very hard to follow. Please rephrase this section.

A: As shown in Figure 2A, on 1-12th of the experiment, mice in other groups except the normal group were given cefoperazone and clindamycin. On day 13th, oral administration of C. difficile to mice. C. difficile was quantified by selective culture counting of faeces collected from mice on the first day of infection, and the logarithmic value of the amount of colonisation was 7.13±0.26 (per gram of faeces), which indicated that C. difficile had successfully colonised the faeces. Then therapeutic intervention of different peptide products was carried out . Ti group and Da group were given 400 mg/kg bw Ti peptide and Da peptide solution, respectively. At the end of the trial with 2 weeks, diarrhea gradually stopped, stools became soft, and weight gradually recovered in the treated group compared with the model group.

Line 366: Is the “model group” an infected untreated group? This is very unclear, as model groups is used earlier to describe all infected mice.

A: All infected mice have been described as the " *Clostridioides difficile* infection group " and the group with no later intervention as the "model group".

Figure 2B: weight loss appears to occur before CDI established (based on the timeline). Is that correct? Additionally, weights should be presented as % weight loss to account for the variation in starting weights. As it stands the Ti group appears to have a higher starting weight, which is skewing the weight change and may not be that different from the other groups.

A: Yes, the initial use of cefoperazone also resulted in a certain weight loss in mice, but there was no significant difference compared to the normal group. The specific body weight graph is as follows, with a sharp drop in body weight from day 14 in the *Clostridioides difficile* infection group of mice. After infection with *Clostridioides difficile*, mice lost 15.7% of their body weight.

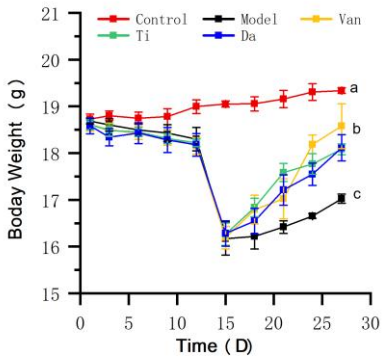
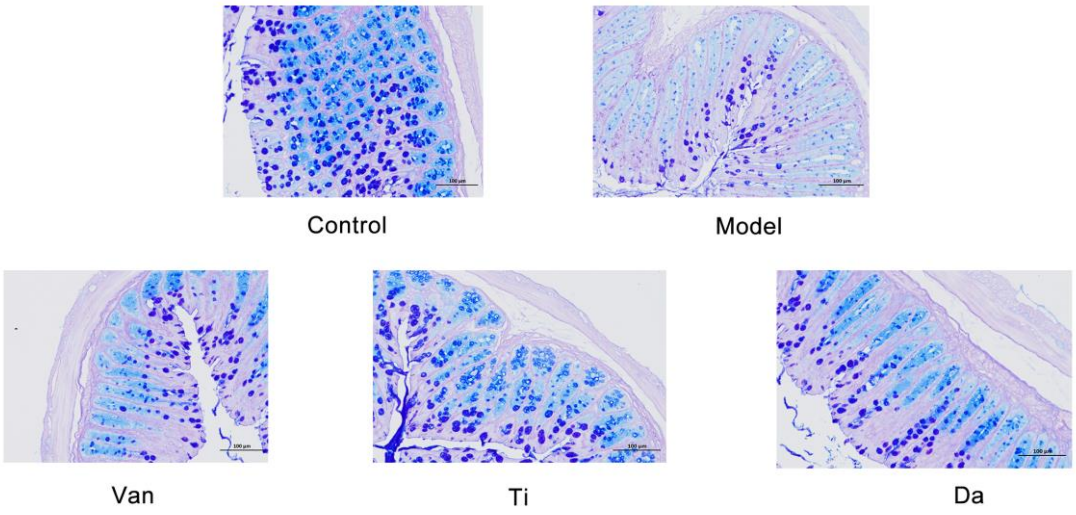


Figure 2C and D: I take issue with the images and numbers provided. It is very clear from the images that different regions of the gut have been examined, and this will change the number of goblet cells counted. For instance, the control, model and van group appear to be distal colon section, with a uniform crypt architecture, whereas the Ti and Da images represent the proximal colon, where distinctive mucosal folds are seen. Imaging and counting these different regions will lead to a biased result as there are natural differences in the architecture of the gut regions and number of goblet cells within these regions. These images should be all taken/represent one area of the colon (proximal, mid or distal) and counts should be presented for a single region alone.

A: The same region of the distal colon section has been selected for presentation as shown below:



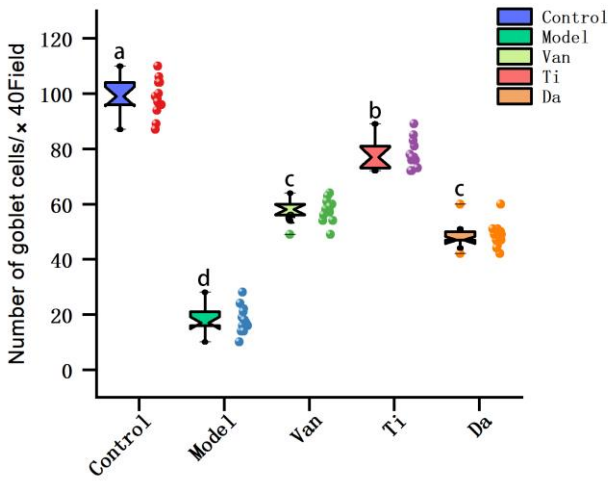
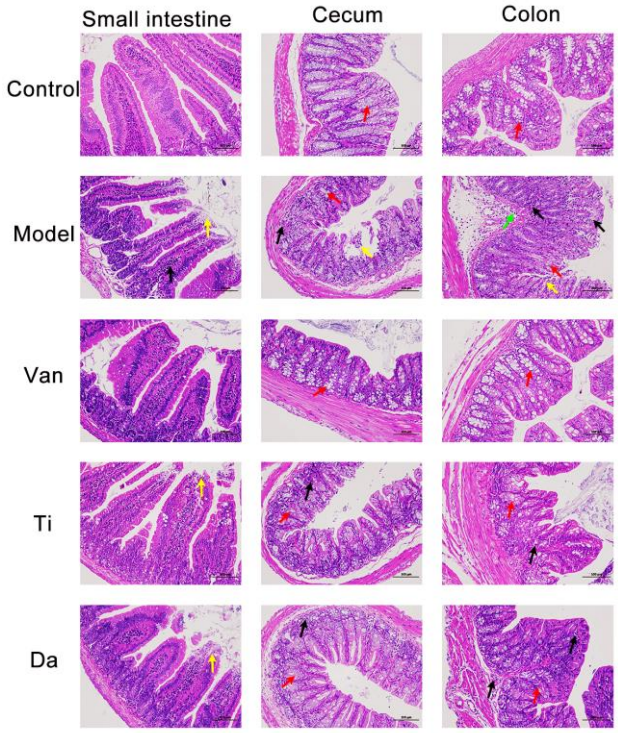


Figure 2E: My same concerns for Figure 2C/D apply here. The images presented represent different regions of the colon that have significant changes in their architecture naturally. Additionally, images should be presented in the same orientation and as a uniform layer, as the current depiction of the tissues make it difficult to determine the level of damage/repair.

A: Apologies for the inconsistency in the selection of images in our manuscript. We have re-selected HE sections of mouse small intestine from each experimental groups. cecum as well as colon and marked the different categories of damage with different coloured arrows, please check.



Lines 370-378: The representative images in figure 2 do not depict the differences described in text. The images are all in different orientations, represent different regions of the gut (see comments above regarding colon regions) and appear to mistake mucosal folds and changes in tissue plane when sectioning for changes in pathology.

A: Thanks, the specific modifications are as above.

Lines 379-389: As described above, I take issue with the way this has been analysed, as the images presented represent different gut regions, where goblet cell numbers and state (pH) are known to change. If these changes are true, please provide images from the same region (proximal, mid or distal colon).

A: It has been corrected.

Line 384: What do you mean by recovery of the mucous area? Did you measure the mucous layer? To do this, tissues need to be fixed in Carnoy's to preserve the mucous integrity due to its solubility in water containing fixatives.

A: I am very sorry, but the mucus layer was not tested, only the goblet cell count was performed, which has been revised. The main function of goblet cells is to secrete and produce mucin, which in turn produces a mucus layer to maintain the integrity of the intestinal barrier[1], so although we did not specifically measure the thickness of the mucus layer, the integrity of the intestinal mucosa can also be assessed to some extent by counting the number of goblet cells.

[1] Yang S, Yu M. Role of Goblet Cells in Intestinal Barrier and Mucosal Immunity. J Inflamm Res. 2021 Jul 13;14:3171-3183.

Figure 2F: What do the letters represent above the bars? There is no indication of the significance levels in the figure legend.

A: Different letters represent significant differences between the two ($p<0.05$), which have been specifically labelled in the "Figure Legend". The following literature and I used the same method of difference analysis:

Zhu Y , Chen Y , Li Q .Preparation, characterization, and anti-Helicobacter pylori activity of Bi~(3+)-Hericium erinaceus polysaccharide complex[J].Carbohydrate Polymers: Scientific and Technological Aspects of Industrially Important Polysaccharides, 2014.

Lines 432-444: Was the protein level of ZO-1 examined in the tissues? While gene expression may be elevated, tissue repair and connections between the cells may not be repaired?

A: We are sorry to say that we have only done the transcriptional level of ZO-1, through the results, we found that the expression level of ZO-1 was significantly increased after the peptide intervention, which indicates to some extent that the tight junction of the epithelial cells is enhanced.

General comment on figures: the "normal" group is labelled very inconsistently throughout the figures – mostly as control, sometimes as N. This is hard to then match to the text that refers to them as normal for the majority of the manuscript.

A: Thanks. Verification revealed the use of "N" in figure 4B, which has now been changed to "Control".

Line 554: This is the first mention of goblet cells throughout the manuscript. Where is there evidence that the peptides promote their growth? Additionally, are goblet cells

even present within the colon? Or only in the small intestine?

A: As mentioned above, the primary function of the goblet cells is to secrete and produce mucin, which in turn forms a mucus layer to maintain the integrity of the intestinal barrier. In this study, the difference in the number of goblet cells between different groups was analysed by AB–PAS staining, by which it was hypothesised that a high number of goblet cells indicated a strong protective effect on the intestinal barrier. We've changed "promote their growth" to “maintain the number of goblet cells ” Goblet cells are found in the small intestine and colon, and we have mainly studied changes in the number of goblet cells in the colon.[1]

Gipson IK. Goblet cells of the conjunctiva: A review of recent findings. Prog Retin Eye Res. 2016 Sep;54:49-63. doi: 10.1016/j.preteyeres.2016.04.005.

General comment on discussion: The paper focusses highly on CDI, yet the discussion has one paragraph on the results of the C. difficile work, and do so in such a superficial way that the reasoning for using this model is completely unclear. This needs more work to show why this model was used, and the significance of the work to the CDI field.

A: Thank you very much for your comments. On the one hand, CDI, as a common intestinal disease, leads to intestinal flora disorder as well as intestinal barrier damage and intestinal inflammation. Traditional antibiotic therapy has a high recurrence rate, and in recent years, some new natural therapeutic methods have become a research hotspot, including probiotics and fucoidan, etc. On the other hand, peptides, as a small molecule, have been shown to play a role in repairing the intestinal mucosal barrier and restoring the disordered intestinal flora in the treatment of enterocolitis, such as IBD, etc. However, the study of peptides on CDI it limited, so, the present study was conducted to investigate whether peptide, as a natural small molecule, also has its efficacy on CDI.

Therefore, in the discussion of the results, we again focus on the pathogenic mechanism of CDI, as well as the therapeutic preventive effects of some related natural substances similar to peptides on CDI, and briefly describe the role of peptides in the treatment of other types of enterocolitis, which is used to better illustrate the reasons why we chose the CDI model and why we chose the peptides for the study.

General comment on figure legends: No useful detail has been provided here (sample size, significant etc.). This needs to be fixed so the reviewers can understand what they are trying to interpret.

A: Thank you. It has been corrected.

Reviewer #2 (Comments for the Author):

Overall, the study is good, and well intentioned. It looks into the impacts of various short peptides as alternatives or adjuvants to antibiotics in gut. Technically, apart from a few things, the study was well conducted. The only gap was the authors have not shown the confirmation of CDI being established in mouse gut. Although an assay can help, the microbiome sequencing can help to at least qualitatively establish this. Just the external symptoms are not enough to claim a successful CDI establishment.

A: Thank you for your advice. The successful answer to this model is recognized in the literature as the detection of toxin producing *Clostridioides difficile i*n diarrhea feces. In addition, 16s diversity analysis can also support the successful establishment of CDI infection models.

So we detect the *Clostridioides difficile* in faeces by real-time fluorescence quantitative PCR in this study. The results of the experiment showed that the level of *Clostridioides difficile* in the faeces of mice in the model group was 9.12±0.47 (log_{10}CFU/g)， , indicating that *Clostridioides difficile* had successfully colonised the mice and the CDI model was successfully established.

[1] Wang, Zhang ,Chen. Sequence variation in tcdA and tcdB of Clostridium difficile: ST37 with truncated tcda is a potential epidemic strain in China[J].[2024-01-25].

[2] Chu Qiongfang , Li Xianping, Hua Yuting, Song Liqiong, Xiao Yuchun, Huang Yuanming, Zhu Siyi, Ren Zhihong. Detection of Clostridium difficile with TaqMan-based quantitative RT-PCR[J]. Disease Surveillance, 2018, 33(5): 417-422.

Another issue I have is that there is no ethics statement provided in the whole document. If ethics approval was successfully sought before the study, the approval number, and approving agency must be indicated in the study.

A: This animal experiment was ethically reviewed by the Specialised Committee on Scientific Ethics of Ocean University of China before it was conducted, and the experiment was carried out in strict accordance with the relevant norms and standards.

Finally, the study although good on technicalities, does not read well, particularly for people who are not very well aware with the mass spectrometry or genomics jargons. As a person working in multi-omics, I understand a good amount of these terms, even if some technical phrases are used incorrectly. However, the same does not apply to readers who are not protein or genome specialists. I have indicated the issues in the

Specific Comments below for the authors to refer.

A: Thank you very much for your suggestion, we have double-checked and corrected the terminology used in the mass spectra, please check!

The authors need to also remove a good amount of filler words and sentences. I have indicated some below. These do not add to the story, and increase the word count unnecessarily. The document needs a good proofreading before it can be accepted for publication. English is not my first language, so as a reader, I don't expect a flowery language.

A: Thankfully, we've removed a lot of unnecessary filler words and sentences to make the sentences more concise and powerful. In addition, we sent it to a professional company for language modification.

A simple write-up is good enough to carry the story. However, use of slangs (e.g., tinfoil) increases the difficulty of reading.

A: These have been corrected.

Specific comments:

1. Whenever indicating an equipment, please indicate (Model, Company, City, State/Province, Country) on first mention. Same for the chemicals, particularly specialist chemicals (internal standards, standard peptides, calibrants etc.).

A: Thank you for your advice. It has been detailed in the experimental material.

2. Lines 157-162: Please provide the HPLC protocol in detail. In current form, it cannot be replicated. Which machine was used? Was the solvent used in isocratic or in gradient manner? If gradient, what was the gradient used? If the overall addition takes too much of space, the protocol can be added to the Supplementary materials. Alternatively, a previous study can be referred to, if the current protocol derives from that study with no or minor modifications.

A: Thanks. We refer mainly to this literature for the determination of peptide molecular weights: Chen T, Hou H, Li J. Protective effect of gelatin and gelatin hydrolysate from salmon skin on UV irradiation-induced photoaging of mice skin. 2016, 15(004):711-718.

3. Lines 18-186: The solevnt mixtures indicated are a bit confusing. Do the authors mean Water + 0.1% formic acid (Solvent A) and Acetonitrile + 0.1% formic acid (Solvent B)? Also, the gradient can be simplified as: "The gradient was set as 0 - 2 min. (A = 5%), 27 min. (A =10%), 37 min. (A = 25%), 39 min. (A = 80%), 42 min. (A = 80%), 43 min. (85%). The column was reconditioned with 5% A from 43 - 50 min."

A: Thank you for your suggestions. Yes, Your description is correct. It has been

corrected.

4. Lines 191 - 200: The terms 'Primary mass spectrometry' and 'Secondary mass spectrometry', are incorrect. This sentence can be restructured as, "Resolution for mass spectrometery were kept at 70,000 (MS1 level) and 17,500 (MS2 level)." Also, the phrase 'number of strongest ions' is confusing. Do the authors mean, 'ions with the highest intensities'? If this is the case, were there 20 top ions that were shortlisted? Overall, this paragraph needs to be re-written for better clarity.

A: Thank you very much. Your description is correct .You must be an expert in mass spectrometry and I am just a beginner in this field and English is not my first language, so I have problems with terminology. I have taken your advice and made some corrections. The "number of strongest ions" here it is changed to: " number of loop count monitored in each acquisition cycle at 20 "

5. Lines 203-204: Same as above. Some of this needs to be re-written as the technical phrases used are incorrect. For example, the correct sentence for Line 203 would be 'Thresholds for Precursor mass tolerance and fragment tolerance were kept at 10 ppm and 0.02 Da, respectively'. If the authors are worried about the plagiarism being an issue, please note that there is a limit on how many ways these type of (technical) sentences can be written without a hazard of repetition. I would not worry about the plagiarism in the highly technical parts such as this paragraph.

A: Thanks, it's been revised.

6. Lines 205-207: The database entry for Glycine max needs to be correctly written. For excample, it can be written as, 'the dataset was assessed against proteomic database of Glycine max (NCBI id: 123456789) and Clupeiformes (NCBI id: 123456789). For example, the proteome id for Glycine max on Uniprot is UP000008827. What would be the equivalent id of Glycine max on NCBI that authors have used?

A: The dataset was assessed against proteomic database of Glycine max (NCBI id: 3874) and Clupeiformes (NCBI id: 32446). The proteome id for Glycine max on Uniprot is UP000008827.

7. Lines 224-227: The weight decrease should be part of Results section, not this section.

A: Thank you. It has been removed to the Results part.

Line 231: What do the terms 'Ti' and 'Da' refer to? This must be clearly defined.

A:"Ti" stands for "E. japonicus" and "Da" stands for "G. max", using the initials in

Hanyu Pinyin. And the full name is mentioned on page 5, line 139

Line 234: When was spiperone fed to mice? It is not indicated in earlier lines.

A: I'm sorry, carelessness in our revisions led to confusion in the use of words, this should be “cefoperazone”, not “spiperone”. Cefoperazone is used on 1-10th at a dose of 100 mg/kg of body weight daily.

Line 237: The correct term for 'killing' is 'euthanized' in the context of animal trials.

A: Thanks, it's been revised.

Line 246: Does the terrm 'tinfoil' refers to 'aluminium foil'? If yes, it must be used as such.

A: Yes, the terrm 'tinfoil' refers to 'aluminium foil'.It has been changed.

Line 262: What is the full form of TUNEL? This must be indicated somewhere in the paragraph, if not in the heading of this subsection.

A: The full name "TUNEL" is "Terminal Deoxynucleotidyl Transferase mediated dUTP Nick-End Labeling", which has been added.

Line 334: This sentence is unnecessary, and can be deleted.

A: Thank you, this sentence has been deleted.

Line 335: Which other 2 protein products? Please clarify.

A: The other two protein products refer to the Ti product and the Da peptide product mentioned above.

Line 345-347: Please support this statement that lower molecular weight is always indicative of better absorption effect. please cite the relevant studies which have indicated this relation.

A: Sorry, it was a problem with my description. After further review of the information, I found that it is not the case that the lower the molecular weight, the better the absorption of the peptide. From the absorption point of view, peptides less than 1,000, there is almost no obstacle to absorption, can be unimpeded in the intercellular flow. Therefore, the best molecular weight distribution of small molecule peptide supplements with really good effects is between 200-800, so as to meet the body's needs in various aspects.

Pérez-Gregorio R, Soares S, Mateus N, de Freitas V. Bioactive Peptides and Dietary Polyphenols: Two Sides of the Same Coin. Molecules. 2020 Jul 29;25(15):3443. doi: 10.3390/molecules25153443.

Line 351: ideal amino acid pattern defined by WHO. Please cite the relevant document

A: Thanks, it's been revised.

[1] Nations, Food Agriculture Organization Ofunited , et al. "Protein and amino acid requirements in human nutrition: report of a joint FAO/WHO/UNU expert consultation.####Protein quality evaluation: report of the Joint FAO/WHO Expert Consultation, Bethesda, Md. USA, 4-8 December 1989. " (2007).

Line 361: Do the symptoms 'confused and listless' indicate successful infection, clinically? Please cite the supporting documents/reports previously published. Was any diagnostic study done to assess the successful infection? If yes, indicate the outputs of that study here.

A: To demonstrate the success of the *Clostridioides difficile* model, the results have been supplemented by the detection of *Clostridioides difficile* in faeces by real-time fluorescence quantitative PCR in this study. The results of the experiment showed that the level of *Clostridioides difficile* in the faeces of mice in the model group was 9.12±0.47 (log_{10}CFU/g)，, indicating that *Clostridioides difficile* had successfully colonised the mice and the CDI model was successfully established.

Line 379: Please provide citation for this statement.

A: Thanks, it's been revised.

Birchenough GMH, Johansson ME, Gustafsson JK, Bergström JH, Hansson GC. New developments in goblet cell mucus secretion and function. Mucosal Immunology 2015; 8:712–9.

Line 403-414: Please indicate the Figure number. For example, the fuorescence intensity is showed in Figure 3C.

A: It has been changed.

Lines 415-421: Caspase 2 and 3 increased more in Ti and Caspase 8 and 10 increased more in Da. What would this infer?

A: Firstly, as a marker of apoptosis, Caspase can reflect the expression level of epithelial cell apoptosis. Our results show that both Ti and Da can reduce their expression level instead of increasing as you mentioned. Secondly, Caspase-2, caspase-3, caspase-8, and caspase-10 are different apoptotic factors, and the results showed that the Ti and Da groups differed in their inhibitory effects on these four apoptotic factors. The mRNA relative expression levels of caspase-2 and caspase-8 of mice in the Ti group significantly decreased ($P < 0.05$). By contrast, the mRNA relative expression levels of caspase-2, caspase-3, and caspase-8 of mice in the Da

group significantly decreased ($P < 0.05$).

Line 429-430: What would be the previous immunofluorescence staining results? Is it a previously reported study? If yes, please cite. Same for lines 442-444

A:I apologize for not being clear enough, the previous immunofluorescence staining results refer to the apoptosis (Tunel) and cell proliferation (PCNA) staining results above

Lines 490-491: This sentence does not add to the story. Can be deleted.

A:Thanks. It has been removed.

Line 512: Do the authors mean 'petides above 11 amino acid lengths"?

A:Yes, it has been changed.

Reviewer #3 (Comments for the Author):

In the study by Li et al., authors analyzed and compared peptides from Engraulis japonicus and Glycine max and investigated the potential effect of oral administration in an animal model of CDI. They reported a reduction of intestinal inflammation and apoptosis, and improved repair of the intestinal barrier by promoting colonic epithelial cells proliferation. Observed beneficial effects were associated to a partial restoration of bacterial diversity, as well as an increase of beneficial bacteria abundances and reduced the proportion of harmful bacteria. The results presented could have a significant impact for the scientific community since peptides have a significant biological activity, but there is no identification of active peptides in the study which is very limiting. Moreover, improvements in the discussion section and in the Figure descriptions could help to understand the message.

A: Thank you very much for your comments, this study mainly focuses on the analysis of the peptide composition of the two hybrid peptide products. The two were found to have different roles in interfering with CDI through the activity validation. And the next plan is to exactly identify the peptide-binding database of them to determine which peptide segments play a role in them. Moreover, we have carefully revised the the discussion section and in the Figure descriptions to facilitate better understanding.

Major comments:

Most figures are difficult to understand without the main text. Figure captions have to

be more descriptive (including abbreviations meaning, statistics, ⋯).

A:We apologize for the lack of details in the information, which is supplemented in Figure Legend, including abbreviations meaning , statistics, etc.

Line 328 - Composition analysis of the peptides: I do not understand what is the aim of this analysis since no correlation between peptides structures and biological activity is presented in the paper. Authors should fractionate peptides to identify the biologically active compounds in their extract, this would strengthen the conclusions.

A: Thank you for your suggestions. Currently, peptide products are all mixed peptides, and the molecular weight per unit of liquid phase detection is below 1,000, but the peptide composition is unclear, and the differences in the composition and activity of these peptides from different protein sources are not clear too. So we need to differentiate mixed peptide products and to analyze the difference of mixed peptide products.

Line451: how can you affirm that that C. difficile infection reduced the number and diversity of bacteria in the cecum while you used antibiotics and the effects of antibiotics on bacterial diversity is similar to model group (Figure 4B) ? Please explain or revise.

A: The antibiotics cefoperazone as well as clindamycin were usedin this study in order to firstly disrupt the intestinal flora and create conditions for C. difficile to colonize the intestinal tract. Previous studies conducted on the effect of antibiotics on the abundance and diversity of the intestinal flora showed a lesser decrease in abundance and diversity than after gavage of C. difficile. A comparison of the results is shown in the following table:

Sample	Chao1	Shannon
normal group	1511.63 ± 277.98^{a}	1732.4 ± 238.93^{a}
Antibiotic group	847.9 ± 106.0^{b}	914.3 ± 248.9^{b}
model group	289.23 ± 32.25^{c}	252.38 ± 28.94^{c}

[1] Luo J , Wang Z , Fan B ,et al.A comparative study of different fucoidan on the cefoperazone-induced gut microbiota disturbance and intestinal inammation[J].Food & Function, 2021.

Discussion: has to be revised. Line 521 to 541: data presented in this paragraph of the discussion does not bring valuable input to discuss the results, while the results obtained on CDI model are not discussed in the Discussion section. Please use data

from literature to discuss the results presented.

A: Thank you very much for your comments. We had carefully revised the discussion part. Details of results on CDI model was added in the discussion part.

On the one hand, CDI, as a common intestinal disease, leads to intestinal flora disorder as well as intestinal barrier damage and intestinal inflammation. Traditional antibiotic therapy has a high recurrence rate, and in recent years, some new natural therapeutic methods have become a research hotspot, including probiotics and fucoidan, etc.

On the other hand, peptides, as a small molecule, have been shown to play a role in repairing the intestinal mucosal barrier and restoring the disordered intestinal flora in the treatment of enterocolitis, such as IBD, etc., and there is a gap in the research on CDI. The present study was conducted to investigate whether peptide, as a natural small molecule, also has its efficacy.

Therefore, in the discussion of the results, we again focus on the pathogenic mechanism of CDI, as well as the therapeutic preventive effects of some related natural substances similar to peptides on CDI, and briefly describe the role of peptides in the treatment of other types of enterocolitis, which is used to better illustrate the reasons why we chose the CDI model and why we chose the peptides for the study.

Minor comments:

The term “protein peptide” is used several times in the main text (l.36 for example) but does not make sense to my opinion. Is it “peptides and/or proteins”, “protein-derived peptides” ? Please revise.

A: Thanks, it's been revised to "protein-derived peptides".

The nomenclature for bacterial phyla has changed and are often wrong in the paper. Please revise.

A: We are very sorry, but we have changed to the latest nomenclature of bacterial phyla, e.g. *Bacteroidetes*, *Verrucomicrobia*, *Actinobacteria*, etc. The specific URL for reference is below:

http://cctcc.whu.edu.cn/portal/dictionary/detail.html?id=21163

Line 391: Figure 2, panel F has to be mentioned.

A: It has been changed.

Line 503-504: The sentence “To reveal the relationship between the differences in their peptide compositions and their bioactivities” does not make sense. Please

revise.

A: Our peptide analysis is done to clarify the chemistry and structure of the peptides used, although it is not known which peptide plays a role, as the peptide products consumed nowadays are mixed peptides.

Figure 4B: what is group P? please revise/explain Supp Figures should appear after main figures.

A: P group refers to the Van group. The positive drug used in this study is vancomycin, which is a relatively common drug for the treatment of Clostridioides difficile infection. In addition, we have revised the location of the Supp Figure.

Reviewer comments:

Reviewer #1 (Comments for the Author):

General Overview and Comments:

There are many formatting and grammatical errors throughout the manuscript that need to be addressed. These kinds of errors are careless and time consuming for reviewers to address, especially given that this is the second time we have addressed these issues. It's extremely disappointing to see many of the same errors highlighted before, and issues with copying and pasting reviewer comments of text from other sources. This is unprofessional and unacceptable.

Dear reviewers, I'm very sorry. After receiving your modifications again, I have made a deep reflection, and it is indeed due to my own improper attitude that I have let you down and delayed your very valuable time. I have put forward your comments for a careful view of the article appeared in the grammatical and formatting errors throughout the check, I hope that you can take time out of your busy schedule to give me another opportunity, thank you very much.

Engraulis japonicus and *E. japonicus* are not consistently used. Once defined at the beginning of the document, *E. japonicus* should be used thereafter.

A: Line 41, etc. I am very sorry, we have uniformly changed it to " *E. japonicus* "

Concerns/Issues/Originality

• There is a repetition error in the abstract (lines 36-37)

A: Line 36. It has been corrected.

• As discussed in my previous review C. difficile has been an important pathogen for

several decades. Please remove "… and, in recent years, has been identified as a common hospital pathogen." From lines 61-62.

A: Line 60. I'm very sorry, I didn't quite understand your meaning when you first mentioned it. It has been removed.

• As discussed in my previous review Hall and O'Toole named C. difficile "difficilis". Please fix this again on line 63

A: Line 62. It has been corrected.

I take issue with the statements on lines 66-68, which suggest that C. difficile induces enough dysbiosis to promote infection. This is simply not correct, and a long history of literature supports that the most common dysbiosis in via antibiotic off target effects. Indeed many people have tried to induce infection without antibiotics without success. Please remove this.

A: Line 65. Thank you for your suggestion. After careful literature search, it was found that this is indeed the case and has been removed.

• Line 101: please define what mouse AD is.

A: Line 99. AD stands for Alzheimer's disease.

• Lines 101-102: The following "…induced AD mouse model showed various ameliorative effects and a mitigating effect on oxidative and inflammatory stress." Does not make sense. What effects occurred?

A: Line 100. Different concentrations of PAYCS (PAYCS-L and PAYCS-H) in a scopolamine-induced AD (Alzheimer's disease) mouse model showed different concentrations of PAYCS (PAYCS-L and PAYCS-H) improve oxidative stress by significantly inhibiting the expression of oxidative indices such as MDA (malonaldehyde) and SOD（Superoxide Dismutase）, and alleviate inflammation by reducing the expression of pro-inflammatory factors TNF-α（tumor necrosis factor)

and IL-1β（Interleukin-1 beta）.

AD is used in this paper primarily to illustrate the memory-improving activity of anchovy peptides.

• Lines 124-125: repetition error FITC(Fluorescein Isothiocyanate)

A: Line 127. It has been corrected.

• Lines 124-125: “BS also inhibited FITC(Fluorescein Isothiocyanate) -dextran(Fluorescein Isothiocyanate) permeability, which is important for protecting intestinal mucosa health” this is confusing and needs rephrasing. BS protected from gut permeability which is important for health, and this was shown using FITC dextran.

A: Line 127. It has been changed to **“**The leakage of FITC (Fluorescein Isothiocyanate) dextran was evaluated to analyze intestinal permeability, and it was found that BS significantly reduced the leakage of FITC glucan into the blood, indicating that BS intervention effectively enhanced intestinal barrier function.”

• Line 126: “It is currently an economical and common protein product” This does not make sense. Please rephrase. Do you mean its cheap to produce and readily available?

A: Line 130. What I mean is that soy peptides are currently the most commonly used economic peptides in the market for health products.

• Line 140-141: “Water was used to extract the proteins, neutral protease, and alkaline protease complex enzyme.” – this does not make sense, please rephrase.

A: Line 145. It has been modified to: " Neutral protease and alkaline protease were used for complex enzymatic hydrolysis."

• Line 223: What CFU was the C. difficile preparation. This is important!

A: Line 230. The concentration of *C. difficile* used is 1×10^6 CFU/mL

• Line 226: Please fix to remove “standing hair and curling up to” – this is copied

directly from my revision and is quite disappointing to see.

A: Line 230. Very sorry, after reviewing the literature, it has been modified to " The weight and state of mice were observed, and the weights of mice in the model group decreased, accompanied by diarrhea , piloerection and huddled."

• I take issue with the CFU quantification in line 228 to 232, as the rebuttal document suggests that colonisation was confirmed by plating? Which method was used?

A: Specific *C. difficile* quantification methods are as follows:

1. The standard curve for *C. difficile* was firstly performed.

   (1) *C. difficile* plate count: *C. difficile* was recovered by inoculation on BHI blood plates, placed in an anaerobic jar and incubated in a 37°C CO_2 incubator for 48 h. Single colonies were picked with an inoculation loop and transferred to new BHI blood plates for substitution under the same conditions. Dip a sterile cotton swab into *C. difficile*, suspend the bacteria in sterile PBS and adjust the turbidity of the liquid to 3.5 MCF then dilute it 10 times, the concentration of the liquid is about 10^5 CFU/mL at this time. Take 1 mL of the bacterial solution for doubling dilution, plate counting and anaerobic incubation for viable bacteria counting. Plate counts of undiluted *C. difficile* samples yielded a samples concentration of 1.00×10^5CFU/mL, resulting in a diluted concentration of 1.00×10^5 CFU/mL~1.00CFU/mL.

   (2) Standard Curve Plotting: Bacterial DNA in *C. difficile* was extracted and then subjected to gradient dilution. The DNA was extracted from different concentrations of *C. difficile* bacterial fluids and then subjected to real-time fluorescence quantitative PCR, with the Ct value as the horizontal coordinate and $\log_{10}$CFU as the vertical coordinate to draw the calibration curve.

Table *C. difficile* concentration and its corresponding Ct value

	1.00×10^6	1.00×10^5	1.00×10^4	1.00×10^3	1.00×10^2
Sample	23.38	27.93	30.95	34.29	37.74
Replication1	24.34	28.39	31.51	35.07	38.13
Replication2	23.72	28.14	30.69	34.77	37.46
Average	23.81	28.15	31.05	34.71	37.77

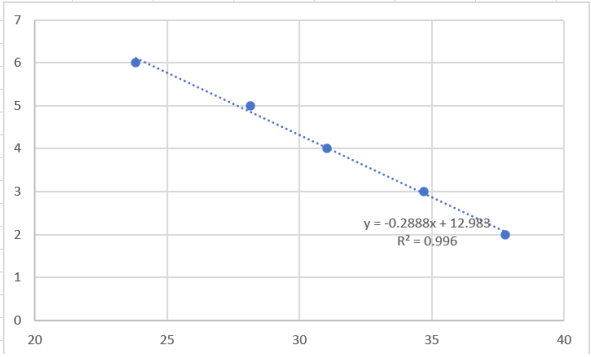


Fig. Standard curve for *C. difficile*

2. At the end of the experiment, mouse faeces were collected, bacterial DNA was extracted, and the content of *C. difficile* in mouse faeces was calculated according to the standard curve. CFU=（$10^{-02888X+13.983}$）/2m×100.

[1] Chu Qiongfang , Li Xianping, Hua Yuting, Song Liqiong, Xiao Yuchun, Huang Yuanming, Zhu Siyi, Ren Zhihong. Detection of *Clostridium difficile* with TaqMan-based quantitative RT-PCR[J]. Disease Surveillance, 2018, 33(5): 417-422.

• Lines 233-240: When and how were saline, vanc and Ti given? There is not mention of the mode of delivery for these compounds, which you do describe for Da.

A: Line 246. The above test substances were administered orally once a day for two consecutive weeks.

• Line 253: aluminium is spelled incorrectly

A: Line 260. Thanks, It has been corrected.

• Line 260: As per my previous revision, what does closed with 3% H_2O_2 mean?

A: In DAB colour development, the results are easily affected by endogenous peroxidase and must be inactivated with hydrogen peroxide. The purpose of using 3% H_2O_2 is to reduce the activity of endogenous peroxidase.

• Lines 264-265: Repetition of scoring method, which still needs a reference!

A: Thanks, It has been corrected.

• Line 273: Fix this, tunel should not be defined this way, but rather in parentheses. Furthermore the way this leads into the rest of the paragraph does not make sense. It is disappointing to see the has been a lot of carelessness with the revisions.

A: Line 276. Thanks, It has been corrected. TUNEL (Terminal Deoxynucleotidyl Transferase mediated dUTP Nick-End Labeling) is a commonly used method for detecting cell apoptosis. I am very sorry that there are still many issues that need further correction in this revised draft. The remaining part of this paragraph mainly explains the specific TUNEL experimental operation process.

• Line 345: fix "dominated by 3-5 peptides" to tri and pentapeptides to match the remainder of the document.

A: Line 348. Thanks, it has been corrected.

• Line 364: "As shown in Figure 2A, on 1-12thof the experiment,…" do you mean days 1 to 12 of the experiment?

A: Line 367. Thanks, it should be day 1-12.

• Line 365: "on day 13th" should be day 13 or the 13th day of the experiment

A: Line 368. Thanks, It has been corrected.

• Lines 365- 367: "On day 13th, oral administration of C. difficile to mice. C. difficile was quantified by selective culture counting of faeces collected from mice on the first day of infection, …" There are many issues here! What amount of C. difficile was administered? Was C. difficile truly quantified by counting? The remaining review comments and methods suggests qPCR was used?

A: Line 370. Thanks, mice were orally administered 0.2 ml of *C. difficile* at a concentration of 1×10^6 CFU/mL.

In this study it was qPCR to identify whether *C. difficile* was successfully infected or not. Plate counts were mainly used for the standard curves of the pre-existing C.

difficile standards. The text has been amended to read "qPCR fluorescence quantification of *C. difficile* in the faeces of mice collected on the first day of infection gave a logarithmic value of *C. difficile* colonisation of 9.12 ± 0.47 (log10 CFU/g), indicating that *C. difficile* had successfully colonised the faeces."

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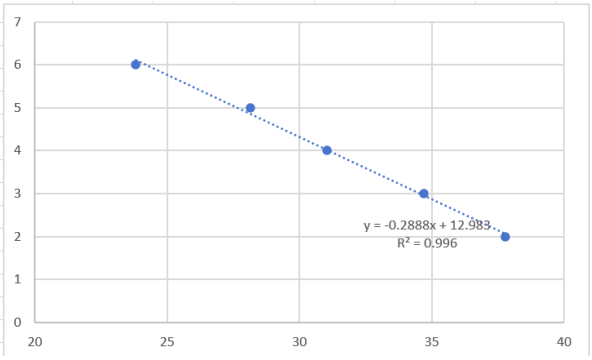

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• Lines 369-373: Again many issues here. Frequency and delivery mode are not clear, the other controls have not been described etc. This needs significant work.

A: Line 366. We are very sorry, we have rewritten it and have revised it to " The Ti and Da groups were orally administered 400 mg/kg bw per day for two consecutive weeks of *E. japonicus* peptide and *G. max* peptide solutions, respectively. At the end of the trial with 2 weeks, diarrhea gradually stopped, stools became soft, and weight gradually recovered in the treated group compared with the model group. ", and the mode and frequency of giving has been clarified.

• Lines 529-540: There are so many different errors in this section that need to be addressed.

A: Line 533. Thank you very much, the paragraph has been reorganised with the following changes:

(1) Changed"*Clostridioides dificileinfection*" to “*Clostridioides difficile infection*”.

(2) The causative mechanism of CDI has been modified to "a dysbiosis of the intestinal flora caused by the heavy use of antibiotics, which results in the proliferation of C. difficile, leading to CDI"

(3) Duplicate statements on lines 532-534 have been deleted.

Grammatical errors

A: Thank you, the statement has been checked for grammatical errors.

Errors in how CDI is established (while surface proteins may contribute to dysbiosis this is not how CDI is established, and many years of literature supports the idea that antibiotics facilitate the majority of dysbiotic events that induce CDI)

A: Line 535. Thanks, it has been changed to " The overuse of antibiotics can lead to disturbances in the gut flora, which can lead to an increased risk of CDI."

Clostridium is referenced, which suggests this may be copied from elsewhere.

A: Line 533. I'm sorry, we've changed "*Clostridium*" to "*Clostridioides*".

There are repeated sentences on lines 532-534.

A: Line 536. Thanks, have changed it to "The two toxins TcdA and TcdB, can destroy intestinal cells, change the cytoskeleton and release inflammatory factors , followed by the clinical symptoms associated with *C. difficile infection*."

Reviewer #2 (Remarks to the Author):

The manuscript looks significantly better now, after the revisions. From my understanding, the manuscript is ready to be accepted after these issues are addressed.

Just some minor corrections needed

Line 40 and elsewhere: The correct term is UPLC-MS/MS

A: Line 133, etc. Thank you for your suggestions, changes have been made.

Line 167 and elsewhere: The term 35 °C is with spaces in some part, and without spaces in others. The correct way is to put it as 35°C (no spaces)

A: Line 173. Thank you very much. Have removed all the spaces before the °C.

Line 259: Do the authors mean cm^2 here?

A: Line 266. Thanks, it has been corrected.

Reviewer #3 (Remarks to the Author):

Thank you to the authors for carrefully revising their manuscript, which as been strongly improved. Authors have replied to my major concerns. I still have some minor comments:

Figure 2F caption: Please replace pro-inflammatory factors by anti-inflammatory factors regarding IL-4 and IL-10.

A: Line 731. Thanks, it has been corrected.

Line 529: Please correct "*Clostridioides dificileinfection*"

A: Line 533. Thanks, it has been corrected.