Author's response to reviews

Title: A pilot placebo-controlled, double-blind, and randomized study on the cognition-enhancing and anti-stress benefits of a proprietary chicken meat ingredient in healthy subjects

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Author's response to reviews: see over
Dear Dr. Kumagai,

Re: MS: 1825039980901549

Title of Manuscript: A pilot placebo-controlled, double-blind, and randomized study on the cognition-enhancing and anti-stress benefits of a proprietary chicken meat ingredient in healthy subjects Zain M Azhar, Jamil O Zubaidah, Khin ON Norjan, Candy YJ Zhuang and Fai Tsang

Thank you very much for considering our manuscript for publication in the Nutrition Journal. The combined constructive advice from you and the referees has been very helpful for our revision of the manuscript, and we now submit our appropriately revised manuscript and seek your acceptance. Together with the revised manuscript, we have also prepared the point-to-point responses to the editorial and each of the referees.

Overall, we have made the following major revisions:

1. We have amended the title of our manuscript to “A pilot placebo-controlled, double-blind, and randomized study on the cognition-enhancing benefits of a proprietary chicken meat ingredient in healthy subjects”.
2. We have taken the referees’ advice to focus our discussion on the cognition-enhancing benefits of CMI-168 and removed the sections discussing its anti-stress effects. As such we have removed related discussions on the role of stress in cognition in the Abstract, Background, Results, Discussion and Conclusion.
3. We have clarified in the relevant sections (Abstract, Background, Methods, Discussion) of the revised manuscript the properties of CMI-168 in relation to essence of chicken (EOC) and the scientific objective of the current study on CMI-168.

4. We have confirmed that the Digit Span test we conducted in this study was Digit Span Backwards as it provided more selective information on the effects of CMI-168 on the promotion of working memory. This description and explanation have been inserted in the Methods and Results sections.

5. We have also changed the naming of the baseline measurement to time 0 instead of week 1 to avoid confusion to readers. We have confirmed that the baseline measurement was indeed the time 0 measurement before the commencement of supplementation of either placebo or CMI-168.

6. We have confirmed that the sample / power calculation was performed using Centre for Disease Control software, Epi Info™ 7 to ensure that the sample size of a total of 20 subjects was sufficient to detect differences.

Together with this cover letter, we have also appended the point-by-point responses to the editors and referees respectively. We sincerely thank the editors and referees for the highly constructive advice on our manuscript and hope we have complied with the requirements sufficiently for acceptance of the revised manuscript.

Sincerely yours,
Fai TSANG
RESPONSES TO THE ADVICE ON FORMATTING CHANGES

Competing interest:
We have inserted the part mentioning competing interest before the reference list.

Acknowledgement:
We have inserted the Acknowledgement before the reference list.

Authors’ contributions
We have inserted the Authors’ contributions section before the reference list.
RESPONSES TO THE CONCERNS RAISED BY REFEREES

Referee 1: Chaur-Jong Hu

Major Compulsory Revisions

1. In the abstract and discussion, the authors claimed CMI-168 contains anti-stress properties, but there are no data supported this. The data show cognitive improvement in individuals under mild stress and the authors emphasized the impacts of stress on cognition. However, there was no evidence showing the effects of CMI-168 on stress status reduction. Therefore, the authors are suggested to focus on the cognitive enhancement instead of anti-stress properties of CMI-168.

Our response:
We greatly appreciate the constructive and valuable advice from the referee. We have taken the referee’s advice and have changed our focus to cognitive enhancement instead of anti-stress effects of CMI-168.

2. The interval for testing is short. Therefore, it could raise a concern of learning effects of these tests. There is improvement even in control group with comparison between those tests at week 6 and week 8. The investigators are suggested to discuss this point.

Our response:
We have analyzed the time-dependent changes in the measures of cognitive performance for both the placebo and CMI-168-supplemented group and confirmed that the changes in cognitive performance over time for placebo group were not statistically significant ($p > 0.05$), whereas there was a time-dependent improvement in the cognitive performance of those subjects supplemented with CMI-168 ($p < 0.01$).

3. The authors are suggested to discuss the clinical significances of findings showed in figure 3 and figure 4.

Our response:
As advised by the referee, we have added more discussion on the clinical significance of findings shown in Figures 3 and 4 in the Discussion section. Briefly, RAVLT is a cognitive assessment of verbal learning capabilities. One of the interesting components is the employment of interference (both proactive and retroactive) to examine the learning efficiency under the influence of interference or confusion (Figure 3). Interference susceptibility of executive functioning is often associated with the integrity of the frontal lobes, and/or to memory functions (consolidation and storage) associated with temporal lobe structures. The argument for the involvement of executive/frontal functions is supported by observations of increased susceptibility to proactive interference (PI) [References 17-19, 21 in the revised manuscript] and retroactive interference (RI) [Reference 16 in the revised manuscript]. The notion of anterior cerebral dependence in interference susceptibility has also been supported by functional neuroimaging [Reference 20 in
the revised manuscript]. Additionally, relative to PI, RI trial is more selectively associated with various components of executive functioning. However, apart from the proposed association between interference and executive functions, it is also possible that interference susceptibility may relate to memory functioning (the ability to retain or consolidate information over time). Within this framework, the recall performance on interference trials may reflect the workings of the memory system, and would thus be expected to correlate with other measures of memory. From our results shown in Figure 3, subjects supplemented with CMI-168 showed significantly ($p<0.01$) lower susceptibility to both proactive and retroactive interferences. This could be related to the cognitive benefits of CMI-168 to both the functions of prefrontal cortex and maintenance of efficient memory functions under the influence of both PI and RI. These findings corroborate well and substantiate the findings in Figure 1, which showed CMI-168-mediated benefit in working memory. In addition, as shown in Figure 4, CMI-supplemented subjects demonstrated enhanced performance in the delayed recall and memory recognition tasks. This shows that CMI-168 facilitated the memory related processes, particularly those in association with memory consolidation. This is important to the learning process as it would facilitate successful retention and processing of newly learned information for subsequent retrieval and application in future.
Referee 2: Justin Karr

Major Compulsory Revisions

1. The introduction reads well, leading the reader into a discussion on stress in relation to cognition. The background evidence on EOC appears relatively limited, emphasizing the importance of the current study; however, the authors should explain the active ingredient of EOC that may lead to these hypothesized cognitive gains or anti-stress mechanisms. Just as fish oil contains omega-2 for heart health, what does EOC contain to benefit cognition and stress? For example, if EOC modulates GC levels, what component of EOC (e.g., specific biomolecule) does this modulation?

Our response:

CMI-168 was developed as a result of engineering a more efficacious ingredient through optimization of the proprietary process that produces EOC. Currently, investigations on the bioactive responsible for the cognition-enhancing efficacy of CMI-168 are underway, the isolation, identification and verification of which will take another 2 to 3 years.

2. The authors should give a rationale as to why predominantly memory-related tasks were included in the cognitive battery. Is there a reason to think EOC will specifically benefit memory? Same for BAI, BDI and overall functioning (this is explained in the psychological profiles, but should be described earlier in the text for the reader to understand the rationale). The selection of these dependent variables is unclear.

Our response:

Our previous human intervention studies [References 4-6 in the manuscript] showed that EOC is beneficial to learning and memory, particularly under stressful conditions. CMI-168 was developed through modification of the proprietary process that produces EOC. Preliminary in vivo characterization of the cognition-enhancing effects of CMI-168 has shown that it is particularly beneficial to the efficiency of working memory and hippocampal-related memory processes/functions (data not shown). Hence, in our current study, we aimed to confirm its cognition-enhancing efficacies, focusing on understanding how it might be beneficial to the processes of learning and memory.

BAI and BDI are standard battery of clinical psychological assessments of potential and severity of depression and anxiety symptoms. The purpose of BAI and BDI assessments, together with General Health Questionnaire and Sheehan Disability Scale, is to ensure that all participants were normal and free of any psychiatric disorders.

3. The Digit Span Forwards does not effectively involve a working memory component. It is sometime considered a task of rote recall or could be termed a measure of “short-term memory storage capacity.” The Digit Span Backwards however involves the manipulation of information
within the phonological buffer, and the authors can call it a measure of working memory. The RAVLT is not a measure of episodic declarative memory, but instead a measure of verbal memory and learning. This should be re-worded in the text. The results section contains a better explanation of each task, and the authors should move these descriptions into their measurement section.

Our response:
We have further clarified with the first author, ZMA who was in charge of the study design and supervised the study, that the Digit Span we reported here was Digit Span Backwards. As one of our key objectives of the current study was to understand how CMI-168 could be beneficial to the cognitive resource optimization for effective information processing and manipulation, Digit Span Backwards task was employed in this study. As the referee has rightly advised, Digit Span Backwards is a measure of working memory involving the manipulation of information within the phonological buffer. We have amended the descriptions of Digit Span Backwards, and RAVLT in the manuscript.

4. For statistics, explain the factorial composition of the two-way ANOVA design. Also, the data is repeated, nested within individuals. Was a repeated measures ANOVA used? If so, was sphericity met? Not using RMANOVA would make the results unreliable, which would be a fundamental flaw in the analysis.

Our response:
Yes, we used repeated measure ANOVA and sphericity was met.

5. Also, in the results section, the authors should clarify where all the reported p-values are coming from. Are they based on pairwise comparisons? Were the F-tests significant for the interactions? Through its current presentation, the reader does not know the results of all important analyses. Although the figures help, consider a table with means and SDs along with F-values, t-values, and p-values for each respective test and measure. This type of presentation makes reading far clearer and assists meta-analysts if they review your study later on.

Our response:
Yes, the reported p-values are based on pairwise comparison and the F-tests were significant for the interactions. We greatly appreciate the referee’s recommendation of the providing a table of statistical analyses to assist future meta-analysis. The current study is a preliminary study aiming to provide initial indication of the cognition-enhancing effects of CMI-168 and it might not be useful / appropriate for future meta-analysis study. We are now planning a much larger multi-centered (3 countries) human study to further investigate the benefits of CMI-168 in cognitive function, which will be more suited for such meta-analysis.

6. Explain why baseline (day 0) testing scores were not included in the design?

Our response:
We have clarified with the authors who directly conducted the measurements and understood that in our Figures, “Week 1” was in fact Day 0 and that is the baseline measurement. Week 1 meant the 1st week of measurement where all participants were assessed of their baseline cognitive performance and then issued with the supplements (either placebo or CMI-168). To avoid confusion, we have changed the labeling of the time axis and time points of measurements are (time) 0, 6 weeks, and 8 weeks.

7. The beginning of the discussion focuses in extensive detail on mechanisms of stress and cognition; however, the results say very little about stress in relation to cognition. This focus on stress in the conclusions should be reduced, remaining within the scope of the observed results. Although the participants had stress-related concerns, the authors do not know if their baseline performances were impaired due to stress. In turn, cognitive improvement may be a function of the EOC improving normal cognitive functioning rather than stress-impaired functioning.
Our response:
As recommended, in the revised manuscript, we have focused our emphasis on discussing the cognitive-enhancing benefits of CMI-168 rather than its anti-stress effects.

8. On Page 14 of the manuscript, the authors essentially repeat their design in full. This is unneeded and should be removed, as the reader would have already read through the full design.
Our response:
We have removed the section as advised.

9. In the conclusion, the authors claim that EOC bears anti-stress effects. There is no evidence of this, as no DV measured stress and saw any improvement.
Our response:
We have removed the anti-stress conclusion as advised.

Minor Essential Revisions:

1. For the study design, did the participants self-administer the supplement? Please clarify.
Our response:
The participants self-administered the supplement and this has been added into the manuscript as advised to clarify.

2. Also, why six weeks and a two week follow-up? Explain the rationale for this time sampling.
Our response:
To rule out the possibility of a temporary equilibrating adjustment of function due to supplementation of CMI-168, rather than a sustainable improvement of cognitive function, we conducted another cognitive assessment two weeks after termination of supplementation. This
helps to address the doubt that the observed enhancement throughout the supplementation period of CMI-168 was merely due to a temporary adjustment of brain environment (e.g. synaptic activity). As shown in all the cognitive tests, the consistency of improvement in cognition was maintained even 2 weeks after termination of supplementation. This observation lends support to a sustained physiological change that resulted in consistent enhancement of function due to supplementation of CMI-168. This sustained modification could be possible through CMI-168-mediated optimization of cognitive resources in the brain that results in efficient information processing and retention. It is also possible that there could be certain level of persistent changes in the brain circuitry or synaptic properties that is correlated with the cognitive function. However, this could only be verified in our future study to further explore the underlying mechanisms responsible for the observed cognitive enhancement.

3. For the sample characteristics, explain whether concealed allocation occurred (i.e., the person ruling participants eligible for the trial did not know their group allocation when determining eligibility).
   Our response:
   We have confirmed that concealed allocation occurred. This has been added into the Sample Characteristics section as advised.
Referee 3: Jonathan Ling

Major Compulsory Revisions

1. Ten participants in each group was large enough to detect differences, however because we know so little about the participants there remains the possibility that the results could have been due to chance. It is also possible that participants in one group could have had a protein-deficiency that was simply addressed by EoC. A sample/power calculation should be included to demonstrate to readers that 20 participants were indeed sufficient.

Our response:
We did a sample/power calculation using Epi Info™ 7 software. We have added the description into our revised manuscript. Based on the calculation of a power of 80% and confidence level of 95%, 20 participants were indeed sufficient.

2. In order to enable comparisons with other studies that have examined EoC, the authors should state more clearly (if this is the case) that the substance being tested is EoC, and that it is only the means of extraction that is proprietary. As written, it is possible that readers could misinterpret the information (eg in section headed CMI-168) presented and believe that bioengineering of the EoC itself has taken place (which is implied by the first line of the conclusion).

Our response:
Thank you very much for pointing out the potential misinterpretation. In an attempt to engineer a more efficacious ingredient through modification of the proprietary process that produces EOC, CMI-168 was developed. In the current study, CMI-168, not EOC, was examined of its cognition-enhancing efficacies. We have taken the referee’s advice and changed the wordings to clarify the test material used for this study.

3. In the discussion, prior work is referred to that has examined the effect of acute stress on cognitive processing. This is different to the mild stress experienced by the participants in the current study. This needs to be clarified. Related to this, is it being argued that the mechanisms for acute stress and mild stress are the same? I think this is unlikely; more probably is that there are qualitative differences that are at least present in the subjective experience of stress. The relevance of the research on acute/chronic stress needs to be made clearer.

Our response:
We have re-aligned our focus of the current study to be on the cognition-enhancing effects of CMI-168 rather than its anti-stress effects. As such we have removed the discussion on stress.
4. Is there any evidence to suggest that ‘the constantly stressful environment might pose a continuous homeostatic imbalance…’? Is there anything uniquely stressful about modern environments? It is argued elsewhere in the manuscript that some stress (ie mild stress) can be helpful. This calls into question the whole reference to stress throughout the document. The focus would be better on the cognitive issues throughout the paper. Reference to stress could be removed from the manuscript without affecting the conclusions drawn.

Our response:
As advised, we have shifted out focus to discuss on the cognition-enhancing effects of CMI-168 to avoid confusion to readers on the stress-related discussion.

5. Reference to the anti-stress properties are inappropriate as there is no evidence to support this (the results show no differences between the groups) and should be removed from all sections of the manuscript.

Our response:
We have removed the sections as advised.

Minor Essential Revisions
1. The research question posed – does supplementation with a proprietary chicken product improve cognitive performance is clearly stated. Evidence is provided for the impact of similar supplements on cognition, therefore the current study represents an incremental advance on earlier work. The current study differs in that proprietary technology has been used to extract the chicken essence; a case needs to be more strongly made for the advances made by the current study.

Our response:
As advised, we have rephrased the research question to clarify the objective of the current study.

2. Discussion of the HPA axis, etc. (the first part of the introduction) is not clearly linked to the rest of the introduction, and can probably be deleted.

Our response:
We have deleted the discussion of HPA axis and stress-related discussion in our revised manuscript.

3. More information is needed about the participants in this study. This information should be placed in the subject section, and not in a separate section on sample characteristics in the results. Where were they recruited from? How were they recruited? Did any drop out (we only have the final numbers in the study presented in the results)? Was compliance measured (e.g. were
participants encouraged to return any unused capsules to the research team)? Were participants asked if they had complied?
Our response:
We have amended as suggested. All were walk-in patients or referred by their general practitioners for counseling/psychotherapy for stress-related issues. Compliance measure: subjects were to bring the remainder tablets before they were given new ones in the visits.

4. What exactly is meant by ‘An independent investigator… maintained a record of all the samples’?
Our response:
The investigator who assessed the participants at the clinic did not have any information with regards to the group allocation and supplements that the subjects got as all this information was kept by another independent investigator who did not assess their cognitive function and psychological well-being.

5. Is data available in relation to whether participants were (for example) vegetarian/vegan?
Our response:
All participants were on mixed diets.

6. In some instances the placebo group is referred to as the control, in others the supplemented group is referred to as the test group. Reference to each group should be made consistent throughout.
Our response:
We have amended as suggested.

7. What does GCP stand for? CMI-168 is referred to as CMI-528 in the Placebo section of the Method; missing ‘the’ before Beck Inventory’ in Measurement section of Method. I don’t think we need to know the concurrent validity of the BAI with the Hamilton rating. ‘Efficacious’ should be efficacious in the discussion.
Our response:
GCP stands for Good Clinical Practice. We have amended the typing errors.

8. The part of the paragraph in the discussion starting ‘In the current study…’ and going down to ‘…and episodic memory’ is a repeat of the abstract and should be deleted.
Our response:
We have deleted as suggested

9. Much is made of the difference between digits backward and digits forward measures in the results, but separate data are not provided to compare them.
Our response:
We have further clarified with the first author, ZMA who was in charge of the study design and supervised the study, that the Digit Span we reported here was Digit Span Backwards. As the referee has rightly advised, Digit Span Backwards is a measure of working memory. We have amended the descriptions of Digit Span Backwards, and RAVLT in the manuscript and have clarified the exclusion of Digit Span Forwards in this study. The main reason that we did not employ Digit Span Forwards measurement in this study is because we wanted to examine more specifically working memory using Digit Span Backwards and Letter-Number-Sequencing, whereas the components in RAVLT provided a more comprehensive assessment of memory processes that are relevant to learning.

10. The standard of the English is good. The figures are generally fine, although the error bars are difficult to see in some instances.