Author's response to reviews

Title: Polyphenolic compounds appear to limit the nutritional benefit of biofortified higher iron black bean (Phaseolus vulgaris L.)

Authors:

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Author's response to reviews: see over
Dear editor,

Please find attached the revised manuscript entitled “Polyphenolic compounds appear to limit the nutritional benefit of biofortified higher iron black bean (Phaseolus vulgaris L.)” authored by myself, Steve E Beebe, Spenser Reed, Jonathan J Hart and Raymond P Glahn. We very much appreciated the thorough comments received on this manuscript by the reviewers. We have considered all of the comments and revised the manuscript accordingly. As requested, we respond to the reviewers individual comments below. We believe that we have adequately addressed all of the reviewers comments and the manuscript is much improved as a result.

Please contact me should you have any questions or need additional information.

Thank you.

Sincerely,

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Polyphenolic compounds appear to limit the nutritional benefit of biofortified high iron black bean (Phaseolus vulgaris L.)

MS: 1994561875113087

Responses to Reviewer #1's comments (Visith Chawasit)

- **Comment:** P1/Front Appropriate title.
  **Response:** Thank you for your comment, as suggested the title was revised.

- **Comment:** P2/abstract/para 1 - It should be mentioned that bean is an affordable source of protein for developing countries.
  **Response:** Thank you for your comment. This point is mentioned in the introduction section (page 4, paragraph 3).

- **Comment:** I don’t agree to use the term “high”. Normally, the term “high” is classified as nutrient claim which should be based on a standard level. The term “higher” may be more appropriate.
  **Response:** Thank you for your comment. As suggested the term “higher” is now used in the text.

- **Comment:** What is the difference between “absorbable” and “bioavailable”? In this study, both terminologies were used.
  **Response:** Thank you for your comment and I am happy to clarify: “absorbable” refers to what is absorbed by the subject, and, “bioavailable” refers to the Fe available for absorption in the diet (Tako et al., 2011, 2012, 2013).

- **Comment:** P2/abstract/para 2 - Iron in mg/kg # kg of what?
  **Response:** Thank you for your comment. It is iron in mg/kg diet. The text was revised.

- **Comment:** Nothing mentioned on the other methods used.
  **Response:** Thank you for your comment and we apologize for not including additional information. The text was revised and information related to the methods used was added.

- **Comment:** Check with the journal on the use of abbreviation for the 1st time in the article (should BW mentioned as body weight in the first time).
  **Response:** Thank you for your comment. The text was revised.

- **Comment:** P2/abstract/para 3 - From the result, ratio of phytate/Fe were not significant between 2 meals and total polyphenols of each line was not indicated. However, the discussion mentioned on significant effect of phenolic types. Therefore, not only level but type should be mentioned as well.
Response: Thank you for your comment. As requested, the specific information was added in the text (abstract - third paragraph and starting at page 15 - first paragraph).

- **Comment:** The last sentence should not be mentioned.

Response: Thank you, the text was revised.

- **Comment:** P2/abstract/para 4 - absorbable Fe?

Response: Thank you for your comment. As was previously suggested (Tako et al., 2011, 2012, 2013), this term refers to the amount of Fe that is absorbed by the subject.

- **Comment:** higher-Fe beans, polyphenolics # polyphenolic compounds, in vitro should be in italic.

Response: Thank you for your comment. The text was revised as suggested.

- **Comment:** P4/introduction/para 1 - dietary iron additives? Does it mean “supplementation”??

Response: Thank you for your comment. By iron additives we meant iron supplementation. To avoid confusion the text was revised.

- **Comment:** logistics and affordability problems should be mentioned since they are the reasons for biofortification.

Response: Thank you for your comment. As suggested, this point was added in the text.

- **Comment:** P4/introduction/para 2 - absorbable Fe?

Response: Thank you for your comment. As was previously suggested (Tako et al., 2011, 2012, 2013), this term refers to the amount of Fe that is absorbed by the subject.

- **Comment:** The term “high” should be used carefully as mentioned above.

Response: Thank you for your comment. The text was revised.

- **Comment:** P5/introduction/para 2 - dry weight # dry basis

Response: Thank you for your comment. The text was revised.

- **Comment:** The study with piglets is the best animal model which is comparable to human. Why did they have to do this study again?

Response: Thank you for your comment. The objective of the current study was to compare the capacities of biofortified and standard, black bean lines to deliver Fe for Hb synthesis using a Fe deficient broiler chickens as the *in vivo* model. If these screening tools indicate that nutritional benefits exist, then human efficacy studies using these lines would be warranted and can proceed with greater confidence of success. This study was conducted to confirm previous results and by using a consecutive growth cycle of the higher-Fe black bean.
Also, in the current study we are able to demonstrate a specific polyphenolic compounds effect on Fe bioavailability (catechin, myricetin, kaempferol 3-glucoside and quercetin 3-glucoside, Table 4). As suggested, this is now indicated in the abstract (page 2, third paragraph) and discussion (Page 15, first paragraph) sections. Also, and as we previously demonstrated (Tako et al., 2010, 2011, 2012, 2013; Mahler et al., 2012), the poultry model have been used for nutritional research and was shown to be an excellent animal to model Fe bioavailability, as chicks respond quickly to malnutrition, and their micronutrient deficient phenotypes include poor Fe status, growth stunting, and organ hypertrophy. Also, this model agrees well with human cell line in vitro results (Tako et al., 2011, 2012, 2013).

- **Comment:** P6/introduction/para 1 - *in vivo*
  
  **Response:** Thank you, the text was revised.

- **Comment:** P6/mat. meth/para 3,4 - why don’t iron-free water such as reverse osmosis water is used? What is 18 omega water?
  
  **Response:** Thank you for your comment. We are using ultra-pure water (achieved by 18 Ω water system) to avoid any possible contaminations (Tako et al., 2011, 2013). In order to clarify this, the text was updated.

- **Comment:** P7/mat.meth/para 3 - day 42nd, 2-3 gr # 2-3 g, - 800C #80oC
  
  **Response:** Thank you for your comment. The text was revised.

- **Comment:** P8/ mat.meth/para 2,3 - absorbance # absorbency, *Gallus gallus*
  
  **Response:** Thank you. The text was revised accordingly.

- **Comment:** P9/ mat.meth/para 1 - In vitro in italics. Please check if the term bioavailability” is appropriate. Should it be “bioaccessibility”?*
  
  **Response:** Thank you. The text was revised accordingly.

- **Comment:** P9/ mat.meth/para 4 - m/z =?, Full terminology for UPLC/MS for the first mentioning.
  
  **Response:** Thank you. The text was revised accordingly.

- **Comment:** P10/ mat.meth/para 1,3 - TM should be added as a superscript in many places such as Waters Metabolynx, Waters, MassLynx, - use # uses, - Full terminology for ESI, - 5/s # is there a unit?
  
  **Response:** Thank you. The text was revised as suggested and the missing information was added (Page 10).

- **Comment:** Materials and Methods overall, need references in certain parts.
Response: Thank you. Additional references were added.

- **Comment:** Results May I suggest to combine results with discussion, which will be easier to understand and did not need to repeat the numbers in the Tables.

Response: Thank you for your comment and suggestion, the revised manuscript is more concise and therefore we would like to keep the results and discussion sections separated.

- **Comment:** Discussion - More discussion should be done on gene expression.

Response: Thank you for your comment. As requested, additional discussion of the gene expression results was added in the text (page 14, second paragraph).

- **Comment:** Number for day should be mentioned as 14th, 21st etc.

Response: Thank you. The text was revised.

- **Comment:** Discussion on polyphenolic profile is good.

Response: Thank you.

- **Comment:** Can the total phytate represent total phenolic compounds? Can we assume from the phenolic profile that the total values represent the total phenolic compounds? This is very important since the phytate/Fe ratios are not different in both meals. If the profile analysis can cover most of phenolic compounds, the total phenolic content should be reported in Table 4. Otherwise, the discussion should emphasize only on the profile but not content.

- Which kinds of phenolic compounds are the promoters of Fe uptake?
- Italics for in vitro and in vivo should be adjusted in many places.

Response: Thank you for your comment. Our results suggested that catechin, myricetin, kaempferol 3-glucoside and quercetin 3-glucoside (Table 4), may decrease/inhibit the dietary Fe bioavailability in the black beans that were tested. We adjusted the text and it is more concise to emphasize this point (abstract, page 2; and discussion, page 15). As for possible Fe promoters (in beans and other staple crops), the manuscript is under preparation. In vitro and in vivo are adjusted in the entire text.

- **Comment:** Conclusion – CONCLUSION

Response: Thank you, the text was revised.

- **Comment:** I don’t agree with the sentence “We conclude that …………..dietary staple.” It does not relate to the results.

Response: Thank you for your comment. We suggest that biofortified black beans show promise as a vehicle for increasing intakes of bioavailable Fe, as long as the bean polyphenols profile are
evaluated (as part of the screening process) and in order to improve the nutritional benefit of beans. This point is now part of the text.

- **Comment**: Abbreviations - BE # BW
  
  **Response**: Thank you. The text was revised.

- **Comment**: Acknowledgements Misspelling# ACKNOWLEDGEMENTS
  
  **Response**: Thank you. The text was revised.

- **Comment**: References Check for italics of the scientific names.
  
  **Response**: Thank you. We revised the references list.

- **Comment**: Table 4 The numbers for Caffeic acid should be incorrect.
  
  **Response**: Thank you. Table 4 was revised.

- **Comment**: Overall, well written and easy reading.
  
  **Response**: Thank you, we appreciate your review and very helpful comments.
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Responses to Reviewer #2’s comments (Maria Kapsokefalou)

- **Comment:** This is a well-designed study, conducted by a research group that has shown leadership in the field.
  
  **Response:** We very much appreciate the reviewer's approval of the significance of the scientific work that was conducted, thank you.

- **Comment:** Discussion and Conclusion: The authors measured selected phenolic compounds to further explain their results and showed that the high iron variety was high in some of the measured phenolics. Total phenolic content was not measured. Thus statements such as:
  
  “the high Fe beans were also higher in polyphenolic content than the low Fe beans. Thus it appears that the potential nutritional benefit from the higher Fe content was offset by the increased levels of polyphenolics.” (discussion pg 14), or, “Evidence suggests that the nutritional benefit of the biofortified beans is reduced by the presence of polyphenols”, are not fully supported by the experimental design and data. The reviewer suggests that the statements on phenolics should be carefully revised or omitted.

  **Response:** Thank you for your comment. As suggested by the reviewer the text was revised (abstract, page 2; discussion, page 15).

- **Comment:** The following comment is considered “Discretionary Revision”
  
  As the question on phenolics was not part of the objectives of the study and therefore not supported by the experimental design, the discussion section should not treat the subject in such detail. For example, authors may omit first paragraph, pg 15, and most of pg 15 and 16.

  **Response:** Thank you for your comment. We feel that the discussion related to bean polyphenols is important to the better understanding of the data reported and the need to profile bean polyphenolics as part of the screening process, however, and as suggested by the reviewer the text was revised and is now more concise.