Author's response to reviews

Title: Metabolomics reveals the metabolic shifts following an intervention with rye bread in postmenopausal women- A randomized control trial

Authors:

Ali A Moazzami (Ali.Moazzami@lmv.slu.se)
Isabel Bondia-Pons (ibondiapons@unav.es)
Kati Hanhineva (kati.hanhineva@uef.fi)
Katri Juntunen (Katri.Juntunen@uef.fi)
Nadja Antl (nadja.antl@hotmail.com)
Kaisa Poutanen (Kaisa.Poutanen@vtt.fi)
Hannu Mykkänen (hannu.mykkanen@uef.fi)

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

On behalf of all co-authors, I would like to thank you for considering our manuscript and sending us relevant reviews comments. I also would like to mention that all the reviewers’ comments have been pondered and we either made appropriate changes to the manuscript or replied to the comments. Two major comments of reviewer 2 regarding providing figure for multivariate statistic analysis and providing the absolute concentrations of the discriminative metabolites have been responded by adding the required information to the manuscript in Figure 1 and Table 2 respectively. The reviewers’ comments are in black and our replies are in blue and anything referring to the manuscript text is in bold. In addition, we have highlighted (in yellow) the parts of the manuscript, which were changed compared to the previous version. I hope that our revision will be found appropriate for publication and we look forward to hearing from you.

Sincerely yours

Ali Moazzami

Co-responding Author

Referee 1

Metabolomics reveals the metabolic shifts following an intervention with rye bread in postmenopausal women- A Randomized control trial

By
Ali A Moazzami, Isabel Bondia-Pons, Kati Hanhineva, Katri Juntunen, Nadja Antl, Kaisa Poutanen, Hannu Mykkänen

1. Is the question posed by the authors new and well defined?
The Authors in their research of nutritional and health benefits of high fibre diet (whole grain rye or wheat) extend present studies and describe them in well written article.

Comment: we would like to thank the reviewer for this comment.

2. Are the methods appropriate and well described, and are sufficient details provided to replicate the work? 
The scheme of the studies and analytical part are well described in details that allow replicate presented in the article work

Comment: we would like to thank the reviewer for this comment.

3. Are the data sound and well controlled? 
Presented data are well described and controlled

Comment: we would like to thank the reviewer for this comment.
4. Does the manuscript adhere to the relevant standards for reporting and data deposition?
The manuscript is written in the appropriate style.

Comment: we would like to thank the reviewer for this comment.

5. Are the discussion and conclusions well balanced and adequately supported by the data?
There is good balance between the data and their discussion. However, although the statistical analyses indicate the health benefits of WG diet, one would rather expect large differences. It is clearly seen by the Authors indicating this in the last sentence of the Discussion. Maybe longer duration of the WG treatment will be needed for obtaining more sound results.

Comment: We agree that in future studies longer intervention period might have produced more clearcut results on some of the parameters. However, the intervention was originally designed to investigate the effects of rye bread on lipid and glucose metabolism, and therefore 8-week periods were applied.

6. Do the title and abstract accurately convey what has been found?
The title and abstract are adequate to the results of described research.

7. Is the writing acceptable?
Writing is good and acceptable

Comment: we would like to thank the reviewer for this comment.

A. Kozubek
Referee 2

Reviewer: Christian C Yde

Reviewer's report:
Review: Metabolomics reveals the metabolic shifts following an intervention with rye bread in postmenopausal women- A Randomized control trial
The manuscript is very well written, and the study is interesting providing differences in two metabolic pathways. Not many details of the multivariate data analysis have been given (no figures), and it is difficult to follow this part of the data handling, and absolute concentrations of important metabolites should be included.

Response: We would like to thank the reviewer regarding his positive comments. All the steps taken in data analysis have been explained in material and method. We have also presented all the statistical data i.e. rank products and p-values in Table 2. However, in agreement with the reviewer’s comment, more information regarding the validation of the multivariate model was added to Material and Methods (Page 9 Line 9-11) and in addition, Figure 1 presenting the rank products was added to Results to make the data analysis more clear.
Moreover, in agreement with the reviews comment, the absolute concentrations of the metabolites, which their corresponding NMR signals were found discriminative in multivariate analysis, were calculated from the NMR spectra and were added to Table 2 (Material and Method Page 9 Line 11 and 20). We have applied a quantitative approach from the beginning by filtering the plasma to take away protein and adding internal standard
(see vide infra). The calculations of the absolute concentrations were performed using NMR Suite 7.1 profiler (ChenomX Inc, Edmonton, Canada) after correction for overlaps (e.g. betaine and glucose).

**Major Compulsory Revisions**

**Objective:** Why go for NMR-based metabolomics and not as in paper [15] LC-MS (or both techniques) if you want to “achieve a more comprehensive understanding of modulation in metabolic profile following an intervention”?

**Response:** We agree with the review that using LC-MS in addition to NMR could add to the comprehensive understanding regarding the metabolic effect of rye products. MS analysis have been already performed on these intervention [1] (Reference was mentioned in manuscript Page 5 Line 9) and the present NMR analysis is to achieve further insight. In addition, we used NMR metabolomics after it was proven successful in our previous metabolomics study on prostate cancer patients after intervention with rye products [2]. NMR analysis is a reproducible approach and can provide quantitative data (see vide infra), which are important factors in detecting the treatment effects in presences of large interindividual differences between subjects. Intriguingly, we have observed a consistent metabolic effect of rye intervention on betaine and dimethylglycine in two separate interventions with different subject populations (men with prostate cancer and healthy postmenopausal women) using the NMR-based metabolomics analysis.

Please show the rank products. Did you validate the MLPLS-DA model as in paper [22] (cross model validation, variable selection and permutation testing)? I don’t understand why you need to do a t-test on the biomarkers found by MLPLS-DA (t-test does not take the covariance into account)? But you are right that p-values are more “widely recognizable”. Your data handling approach is very similar to Yde et al (2011) Br J Nutr 107:1603-1615.

**Response:** The validation of multivariate model has been performed according to the previous publications [3] (Ref 22 on the manuscript). However, in agreement with the reviewer’s comment, we added more information regarding multivariate data analysis and validation to Material and Methods (Page 9 Line 9-11) and Results Figure 1.

The rank products have been presented in Table 2 and Figure 1. As reviewer has also pointed out, based on the experience from our previous publication, we have found reporting the p-values from t-test useful since MLPLS-DA and rank products are less recognized [2]. In previous version of the manuscript, we first assigned the discriminative metabolites using MLPLS-DA and then we applied t-test just on those NMR buckets we found already different using multivariate approach. In agreement with review’s comments, we took away the p-values of NMR bucket from Table 2. However, in present version, we have calculated the absolute concentrations of the metabolites with discriminative NMR signals in MLPLS-DA and then performed paired t-test on absolute concentrations, which are presented in the last column of Table 2.

To avoid misunderstanding we have added a comment to the material and method pointing at the fact the t-test does not take the covariance into account (Page 9 Line 16).
As the reviewer commented, our statistical analysis approach is very similar to their recent work Yde et al (2012) [4]. This reference was added to the Material and Method (Page 9 Line 16).

Please show a representative NMR spectrum. Why don’t you do a more targeted approach. I like that you have measured on ultra-filtrated samples. Normally, you can’t determine absolute concentrations in a blood sample because the internal standard interacts with lipoproteins (there are also many issues with signal overlap). But why haven’t you determined any absolute concentrations in the study? You could have performed MLPLS-DA on the absolute concentration determined from spectral integration instead of the buckets.

Response: We did not add a NMR spectrum since the signals, we referred to have been well annotated in present literature [5] and our previous publication [2]. The authors are grateful that the reviewer has pointed out the significance of their approach regarding using filtered serum samples for NMR metabolomics analysis. We filtered proteins, which otherwise could cause broad overlapping signals on the NMR spectra. In addition, as the reviewer pointed out, after filtering the serum, an internal standard could be added to the serum filtrate without any interactions with lipoproteins. These two steps in our study made the quantitative approach possible (Page 7 Line 14). We have used spectral analysis approach by applying MLPLS-DA on spectral buckets, since it is a standard approach in NMR metabolomics and in addition, we found this approach successful in our previous study after rye intervention on prostate cancer patients [2]. However, in agreement with review’s comment, we calculated the absolute concentration of the discriminative metabolites from our quantitative spectral data using NMR Suite 7.1 profiler (ChenomX Inc, Edmonton, Canada), which is presented in Table 2.

What about the betaine signal at 3.88 ppm. Is this in agreement with the 3.273 ppm signal (do both signals have important RPs)? Has betaine been assigned with 2D-NMR?

Response: In the present study betaine was also assigned with 2D NMR. Increase in the intensity of betaine NMR signal (3.273 ppm) has also been reported after consumption of whole grain rye by another other group, which we referenced to in our manuscript [6] (Page Line). The betaine signal at 3.91 ppm was not found discriminative in MLPLS-DA even though it was higher in rye group. However, we calculated the absolute concentration of betaine from signal at 3.273 after correction for overlap with glucose signals using NMR Suite 7.1 profiler (ChenomX Inc, Edmonton, Canada).

Minor Essential Revisions
Title: Please change “Randomized” to “randomized”
Response: The changes were made according to reviewer’s comment.

In the opinion of the reviewer the first paragraph has nicely been stated in the introduction. So no need to write these lines again.
Furthermore, Wang et al. (Nature 472: 57-82) have recently shown a dubious health-effect of betaine intake.
Response: We appreciate reviewer’s suggestion regarding new references. The references were added to the manuscript (Page 13 Line 16 and Page 14 Line 10 and Last line of Discussion).

Discretionary Revisions
Consider to cite more papers from other groups for metabolomics studies. Ex. Fardet et al. (2007) J Nutr 137:923-929.
Response: In agreement with review’s comment the reference was added to the manuscript (Page 4 Line last).
Quality of written English: Acceptable
Statistical review: Yes, and I have assessed the statistics in my report
References


