Author’s response to reviews

Title: High phosphorus intake and gut-related parameters - results of a randomized placebo-controlled human intervention study

Authors:

Ulrike Trautvetter (ulrike.trautvetter@uni-jena.de)
Amélia Camarinha-Silva (Amelia.Silva@uni-hohenheim.de)
Stefan Lorkowski (Stefan.Lorkowski@uni-jena.de)
Gerhard Jahreis (Gerhard.Jahreis@uni-jena.de)
Michael Glei (Michael.Glei@uni-jena.de)

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Author’s response to reviews:

Revision Letter

Dear Editor,

Thank you very much for reviewing the article “High phosphorus intake and gut-related parameters – results of a randomized placebo-controlled human intervention study” (NUTJ-D-17-00259). We also think the reviewers for the careful evaluation of our manuscript. The reviewers’ comments helped us to significantly improve the quality of our manuscript.

Please find in the following our point-by-point reply to the reviewers’ comments accompanied by explanations of the changes made in the revised manuscript.

Yours sincerely,

Prof. Dr. Michael Glei
Reviewer #1:

1. Were the SCFA measured as umol/dry weight or umol/wet weight? It would be useful to state briefly how the results were obtained so that the reader does not have to dig to other references to understand the units. Similarly for fat content. Why this is important of course is that water content of the stool could explain the results regarding SCFA and nothing to do with bacterial activity per se. Can the authors clarify please?

We acknowledge the kind evaluation of our study by the reviewer and are grateful for these helpful comments that helped us to improve the overall quality of our study. The SCFA and fat concentrations in faeces were analysed in fresh faeces. We corrected the unit in the figure to “µmol/g fresh faeces” (Figure 3, Additional Figure 1 and Additional Figure 2) and added a short description of the analysis to the Methods section. Furthermore, we added the aspect of the water content in the discussion section. The respective sections read now as follows:

Lines 144-163:

Faecal analysis

At the evening before or at the morning of the blood sampling, the subjects were encouraged to collect one whole defecation in provided boxes and to store the sample in a cool dark place. The faecal samples were transported to the study centre at the day of the blood sampling after placebo and eight weeks of intervention. Each specimen was weighed and homogenised. Faecal pH value was measured using a glass pH electrode (InLab 420 electrode, MP 225; Mettler Toledo GmbH, Giessen, Germany). Faeces samples were aliquoted for the respective analysis (faecal fat and FW preparation) and stored at -20 °C until use. For SCFA analysis, 1 g fresh faeces were diluted with 2 ml distilled water, mixed and stored at -20 °C until use.

Faecal fat

Faecal fat was measured as ether extract after acid hydrolysis by conventional Soxhlet Extraction on a SOXHERM 2000 automatic (C. Gerhardt, Königswinter, Germany) [13].

Short-chain fatty acids in faeces

SCFA analysis was performed as published by [17]. Briefly, faeces-water mixtures were thawed and thoroughly mixed. After centrifugation (6000 x g, 15 min), 500 µl of the supernatant were added with 50 µl i-caproic acid (internal standard), mixed and centrifuged (6000 x g, 15 min). For gas chromatographic measurements, 1 µl of the solution was used (Shimadzu model GC 17A, Shimadzu, Kyoto, Japan).

Lines 358-359
These effects are independent of the water content of the faeces, since faecal dry matter did not change due to the interventions (data not shown).

2. How does the subjective scoring of gut symptoms inform this - is there any relationship between reported diarrhoea or constipation and SCFA?

Thank you very much for this valuable comment. The subjects did not score the gut symptoms. Instead, they were encouraged to report whether they had problems with diarrhoea, obstipation, flatulence or undefined stomach ache (lines 131-138). The presented results are not suitable to establish a relationship between reported diarrhoea/constipation and SCFA and therefore we prefer not to discuss this issue. We hope that the reviewer understands our decision.

Lines 145-147

Supplement tolerance questionnaire

After placebo as well as after four and eight weeks of supplementation, subjects were encouraged to fill out a questionnaire about diverse health aspects for the last weeks. One question aimed to assess complaints regarding gut health. The subjects should report, whether they had problems with diarrhoea, obstipation, flatulence or undefined stomach ache. In the case of such problems, the subjects were asked to state, whether these problems could be due to the consumption of the test products (supplemented or non-supplemented powder).

3. Both in the SCFA results and the microbiome results, the opening sentence states that overall no significant differences were found. Then the next sentence (in each section) goes on to describe significant differences albeit at gender level. The authors should consider being clearer here as the statements seem diametrically opposed. In the opening sentence (in each section), I presume you mean "When men and women were considered together"... and in the subsequent sentence I presume you mean "In gender specific analysis of the data"....? Please clarify? What firm conclusions can be drawn from a sub-analysis on n=3 per group?

Thank you for this valuable suggestion. We revised the Results section accordingly (see below) and hope that it is better described now. The subgroup is small, therefore we considered this fact as a major limitation of the present study.

Lines 272-274

Considering men and women together, the interventions did not affect the concentrations of total SCFA or the concentrations of the main SCFA acetate, propionate and butyrate compared to placebo (Figure 3).
A gender-specific analysis revealed that acetate and total SCFA concentrations were significantly higher in the P1000/Ca1000 group compared to the P1000/Ca0 group after eight weeks, but only in male subjects (Additional Figure 1).

The interventions with phosphorus did not significantly affect the TI as a marker for genotoxic activity of the FW (Figure 4B), considering men and women together and separately.

Considering all subjects of the study subgroups (n = 5 for each intervention), no differences of the microbiome were observed between placebo and eight weeks of intervention as well as between the different intervention groups after eight weeks.

The most important limitations of our study are (i) the restricted one portion faecal collection and (ii) the determination of gut community in a limited subgroup with only a few subjects.

4. Minor Comments:

Line 47 should read "significantly"

Thank you for this comment. We changed this accordingly and the revised text reads now as follows (line 50).

Men of the P1000/Ca1000 intervention had a significantly different gut microbial community compared to the men of the P1000/Ca0 and P1000/Ca500 ones.

5. Line 107 - "wellness" would be better than "health" in this context which is rather meaningless in terms of an inclusion criterion.

We appreciate this helpful suggestion and replaced the word “health” with the word “well-being”. The respective sentence reads now as follows (lines 110-112)

Eligibility criteria for participants were an age between 18 and 60 years as well as mental and physical well-being.
6. Figure 1 implies that there was a control group (Placebo; n=66) which operated in parallel to the 3 intervention groups. The text implies that placebo was 2 weeks (n=66) followed by randomisation to one of three groups. Figure 1 should reflect this sequence of events more clearly.

Thank you very much for this valuable hint. We have chosen another image format and revised the figure accordingly.

Reviewer #2:

1. The authors conducted a double-blinded, placebo-controlled and parallel design trial to evaluate if high phosphorus supplementation affects gut-related parameters independent of calcium intake. This is an interesting article. The followings are my comments and suggestions:

We thank the reviewer 2 for the careful evaluation of our study and the valuable comments that helped us to improve the quality of our manuscript.

2. Abstract:

Line 27: Is this modulation beneficial or harmful?

We thank the reviewer for this valuable suggestion and added “beneficial” in the sentence. The respective sentence (lines 26-29) reads now as follows:

In the small intestine, a part of the ingested phosphate and calcium precipitates to amorphous calcium phosphate (ACP), which in turn can precipitate other intestinal substances, thus leading to a beneficial modulation of the intestinal environment.

3. Line 30: Please indicate mean age and BMI, and gender of the participants (number of men/women)

We thank the reviewer for this suggestion and added accordingly the information to the abstract. The sentence reads now as follows (lines 32-34):

Sixty-two healthy subjects (men, n=30; women, n=32) completed the double-blind, placebo-controlled and parallel designed study (mean age: 29±7 years; mean BMI: 24±3 kg/m2).
4. Lines 46, 51 and in many parts of the manuscript: The intervention was not only with phosphorus. So I suggest changing to "calcium and phosphorus"

We appreciate this helpful suggestion, but we cannot change to “calcium and phosphorus”, because one intervention group get only phosphorus (P1000/Ca0) and for this group the statement would be wrong. However, to address the reviewer’s request we tried to clarify the statements as follows:

Lines 49-51

None of the interventions markedly affected cyto- and genotoxic activity of FW. Men of the P1000/Ca1000 intervention had a significantly different gut microbial community compared to the men of the P1000/Ca0 and P1000/Ca500 ones.

Lines 53-54

Supplementations did not cause increased intestinal distress.

Lines 289-293

Based on our results that PBS (negative control) caused no genotoxic damages (TI 7 ± 2%) and H2O2 caused strong genotoxic effects (TI 59 ± 15%), the FW matrix showed only moderate genotoxicity independent of the phosphorus and calcium interventions (mean TI for all subjects 25 ± 13%).

5. Line 39 (abstract), and line 89 (background): I'm not familiar with the term "compatibility" in this context - is it the best term?

We are thankful for this suggestion and changed the word "compatibility” to “tolerability”.

The respective revised sentences read as follows:

Lined 42-43

By questionnaire evaluation we examined tolerability of the used phosphorus supplement.

Lines 93-95

In addition, we examined the tolerability of the used phosphorus supplement with respect to gut distress, such as diarrhoea or stomach ache, by questionnaire evaluation.
6. **Background**

Line 63: I would consider deleting this sentence and directly indicate what has been discussed (ex: mortality, bone health, cardiovascular disease, etc.)

We thank the reviewer for this hint and revised as well as clarified the sentence which reads now as follows

Lines 66-68

Dietary phosphate and serum phosphate concentrations of healthy people and patients with chronic kidney disease have been discussed critically in the last years, regarding bone, cardiovascular health and mortality [1-4].

7. **Line 68: recommended intake for adults and the elderly**

We thank the reviewer for this suggestion and added accordingly the information to lines 72-74 which read now as follows.

According to the National Health and Nutrition Examination Survey of the USA, dietary phosphorus intake of Americans exceeds the daily recommended intake of 700 mg phosphorus for adults and the elderly [4, 7].

8. **Lines 72-75: Rewrite or delete the sentence (unnecessary information)**

We appreciate this advice, but we think that this sentence contains a very important information, because the mentioned study is the one from which the data/samples of the present manuscript are derived from. To address the reviewer’s request we revised the sentence as follows (lines 76-79):

In 2014, our department determined the influence of a high phosphorus intake in combination with different calcium supplies on phosphorus, calcium, magnesium and iron metabolism as well as fibroblast growth factor 23 in a human intervention study [10].

9. **Line 87: Please give a reference for your statement "above-mentioned study"**

We thank the reviewer for this valuable suggestion. We accordingly added the following reference and revised the statement (lines 90-93).
In the study presented here, we analysed faecal samples from the subjects of the above-mentioned study [10] regarding cytotoxicity and genotoxicity of faecal water (FW), faecal concentrations of SCFA and fat as well as the composition of the gut microbiome (in a subgroup of study subjects).

10. Methods

How has free-living intake been monitored? Did the participants receive any guidance?

Lines 93-100: Did they monitor supplement intake?

We thank the reviewer for this question. The free-living intake has been monitored by dietary records three days before the blood sampling. The results and guidance have been already published by Trautvetter et al. and are now briefly addressed in lines 236-239 of the revised manuscript. Also, at each study visit, the subjects were encouraged to report in a questionnaire if they had taken the supplement every day and in case of “no” they could justify. Despite of this monitoring, we can see the compliance in the renal phosphorus excretion. In case the subjects did not take the supplement, the renal excretion wouldn’t be increasing due to phosphorus supplementation. We added some information to the Methods section (lines 99-101) as follows to clarify this issue:

Lines 241-246

Baseline characteristics of subjects who completed the study and nutrient intake (three-day dietary record) have been published elsewhere [10]. Briefly, age, BMI, serum 25-hydroxyvitamin D, kidney function as well as intake of fat, protein and carbohydrates were not significantly different between the three study groups. Phosphorus and calcium intake increased significantly after the respective supplementations.

Lines 103-105

Participants were encouraged to drink the sherbet powder twice a day diluted in 250 ml water and to document when the sherbet powder was not consumed.

11. Line 105: body weight and BMI should have been included as eligibility criteria

We appreciate this advice, but we are not able to include body weight and BMI as eligibility criteria, since these two aspect were not defined as such criteria (see study design published at ClinicalTrials.gov).
12. Line 132: Was the supplement tolerance questionnaire referred to at line 132 validated for assessment of health aspects in adult population? Please specify, with appropriate reference(s).

We are thankful for this comment. The tolerance questionnaire was only validated by using it in our group. We used this questionnaire for a lot of studies primarily to get information of the subjects’ well-being. In the present study, this questionnaire was analysed more in detail for the first time. In this course we noticed limitations of this questionnaire and state this now in the revised manuscript (lines 451-453) as follows:

Furthermore, the interpretation of the questionnaires is limited, since the subjects reported only whether they had problems but not the frequency and intensity of disturbances.

13. Line 139: Please provide information about faecal sample collection and quality assurance processes used.

Thank you very much for this valuable suggestion, we accordingly added some additional information about the faecal sample collection to the Methods section (lines 145-147), which reads as follows:

At the evening before or at the morning of the blood sampling, the subjects were encouraged to collect one whole defecation in provided boxes and to store the sample in a cool dark place.

14. Lines 145 (faecal fat analysis) and 147 (SCFA analysis): Please provide the technique used (ex: HPLC)

We appreciate this advice and added the information about faecal fat and SCFA analysis to the Methods section. The respective sections read as follows (lines 154-163):

Faecal fat

Faecal fat was measured as ether extract after acid hydrolysis by conventional Soxhlet Extraction on a SOXHERM 2000 automatic (C. Gerhardt, Königswinter, Germany) [13].

Short-chain fatty acids in faeces

SCFA analysis was performed as published by [17]. Briefly, faeces-water mixtures were thawed and thoroughly mixed. After centrifugation (6000 x g, 15 min), 500 µl of the supernatant were added with 50 µl i-caproic acid (internal standard), mixed and centrifuged (6000 x g, 15 min). For gas chromatographic measurements, 1 µl of the solution was used (Shimadzu model GC 17A, Shimadzu, Kyoto, Japan).
15. Line 171: as proposed by Oberreuther-Moschner et al.

Thank you for this suggestion; we revised the sentence (line 182), which reads as follows.

Genotoxicity of FW was tested in HT29 cells as proposed by Oberreuther-Moschner et al. [22].

16. Statistics: Please report sample size calculation

The reviewer highlighted an important issue. Sample size calculation was done for the primary outcomes and published by Trautvetter et al. (2016). We state this in the Statistics section of the revised manuscript (lines 220-222) as follows:

Power calculation for the primary outcome (plasma phosphate concentration) of the human intervention study was published elsewhere [10].

17. Line 220: P < 0.05 or P < 0.05?

The microbial composition was analysed with different statistical methods and therefore p < 0.05 was used (line 238-239).

OTU abundances were considered significantly different for p < 0.05.

18. Results:

It would be interesting to report and discuss the mean Ca and P intake, and the Ca/P ratio during the study periods

We thank the reviewer for this suggestion. The mean calcium and phosphorus intakes were already published by Trautvetter et al. (2016). We address this issue now in lines 241-246 of the revised manuscript which read as follows:

Baseline characteristics of subjects who completed the study and nutrient intake (three-day dietary record) have been published elsewhere [10]. Briefly, age, BMI, serum 25-hydroxyvitamin D, kidney function as well as intake of fat, protein and carbohydrates were not significantly different between the three study groups. Phosphorus and calcium intake increased significantly after the respective supplementations.
19. Figure 2B: Please report the sample size each group

Table 1: Please report the sample size in each group

We thank the reviewer for this suggestion and added the sample size in Figure 2B, Table 1. In addition, we added the sample size per group in the legends of Figure 3, Figure 4, Additional Figure 1 and Additional Figure 2 as shown below.

Line 489 legend of Figure 3
P1000/Ca0: n = 16; P1000/Ca500: n = 19; P1000/Ca1000: n = 19;

Lines 497-498 legend of Figure 4
A: P1000/Ca0: n = 15; P1000/Ca500: n = 19; P1000/Ca1000: n = 17
B: P1000/Ca0: n = 10; P1000/Ca500: n = 8; P1000/Ca1000: n = 15

Line 712 legend of Additional Figure 1
P1000/Ca0: n = 7; P1000/Ca500: n = 9; P1000/Ca1000: n = 8

Line 722 legend of Additional Figure 2
P1000/Ca0: n = 9; P1000/Ca500: n = 10; P1000/Ca1000: n = 11

20. Line 234: spelling "and" P1000/Ca500...

Thank you for this comment. We accordingly revised the sentence (line 256):
After P1000/Ca0 and P1000/Ca500 intervention, the faecal pH value did not change (Table 1).

21. Lines 237-238: It would be better to consider results without this outlier

We appreciate this suggestion. But in the interest of completeness, we decided to consider always all study results including outliers.

22. Line 252: It is needed to provide the figure reference "(Figure 3)" after "the P100/Ca0"

Thank you for the helpful hint. We added the information as requested (line 274-276):
Certainly, concentrations of total SCFA and acetate were significantly higher after eight weeks of P1000/Ca500 supplementation compared to the P1000/Ca0 (Figure 3).
23. Discussion

The reader may well wonder why some parameters (total SFCA, acetate, Clostridium, etc.) differed only for men - reasons for why this occurred need to be covered in detail in the discussion.

The reviewer highlighted an important issue and we extended our discussion to address this issue. The respective section reads now as follows (lines 387-390):

The partly gender-specific effects might be a result of sex-specific diet effects on the human microbiome as proposed by Bolnick et al. [38]. Animal and human studies showed, that gender influences microbial composition in the gut [38-41] and, thus, the formation of SCFA [42], too.

24. Line 355: I did not understand which groups differed ("two groups" or "only compared to P100/Ca500 group")

Thank you for your comment on this issue. We deleted this sentence, because we accidently mixed up results.

25. Lines 407-408: How was the intake of calcium in this study?

We appreciate this advice and added the requested information on calcium intake to the respective sentence (lines 432-435), which reads now as follows:

Possibly, this could be caused by the normal calcium intake that was used as placebo here (approx. 900 mg calcium/d) [10] and by Ditscheid et al. (approx. 1000 mg calcium/d) [13], whereas in the studies by Boon et al. (400 mg calcium/d) [51] and Jacobsen et al. (500 mg calcium/d) [53] low calcium diets were used as controls.

26. Line 410: Please give a reference number for Grimm et al. (reference 15); Line 415: Would it be reference 15 instead of 10?

We thank the reviewer for this comment and the careful reading. We added the reference and deleted the next sentence, because the statement was confusing in the context of the following sentences. The revised section (lines 430-433) reads now as follows:

In a phosphate supplementation study from Grimm et al., a phosphate-rich diet (additional 1436 mg phosphorus/d) was associated with intestinal distress, soft stool or mild diarrhoea. The
authors concluded that the symptoms are a consequence of an osmotic effect of the added polyphosphates in the intestinal lumen [15].

27. Line 419: Why were the problems attributed to the sherbet powder? Was the sensorial analysis of the product evaluated?

We thank the reviewer for this question. We sensorial tested the sherbet powder with and without phosphorus and calcium additives with experienced staff of our department. The problems which were reported from the subjects were independent of the phosphorus and calcium additives. This is stated in line lines 444-449:

The majority of the subjects mentioned a connection to the test product. Analysis of the questionnaires showed that the reported disturbances were independent of the phosphorus and calcium supplementation and time-point of intervention. Therefore, we consider the gut problems as a result of the sherbet powder consumption in general and not as a result of the phosphorus and calcium supplementation.