Reply to Referee #1

by Marcel Lerch, Michael Zech & co-authors

Many thanks for considering me as reviewer of the manuscript entitled 'Human and livestock faecal biomarkers at the prehistorical encampment site of Ullafelsen in the Fotsch Valley, Stubai Alps, Austria – potential and limitations' by Lerch et al. The study is very interesting and addresses the analyses of faecal steroids in 37 soil samples and 2 faecal samples for the identification of the presence of cattle and sheep along humans at the Ullafelsen archaeological site. The Ullafelsen is an important (geo-)archaeological reference site and the analyses of faecal biomarkers gives further insights into human and/or animal occupation. The manuscript generally fits the scope of Biogeosciences, although I have major concerns about the methods, the results and the conclusions drawn from the analyses that need to be addressed by the authors before the manuscript could be considered for publication.

→ We are very grateful to anonymous Referee #1 for her/his encouraging words and the very constructive review helping us to further improve our manuscript. We will reply to each comments below.

General comments:

My major concern is that there are apparently no analyses presented of nearby reference soil samples to compare the steroid analyses of the archaeological soil samples to the natural background. Without comparison, there is no possibility to actually identify the presence of cattle & sheep/humans at the Ullafelsen. Bile acids (especially deoxycholic acid) as well as 5β-stigmastanol and coprostanol could be omnipresent in soil samples as wild animals contribute to low abundances of those faecal steroids in topsoils. Without reference samples you neither can characterise the changes in steroid patterns between archaeological samples and reference samples nor you can identify the enrichment of faecal steroids in the archaeological samples. Thus, how can you be sure, that the faecal steroids you find in your 37 soil samples are not just the natural background?

→ We agree with Referee #1 that additional faecal biomarker results for soil samples from reference soils will increase the impact of our manuscript. We therefore currently expand our data set with samples of non-archaeological soil profiles from the Fotsch Valley and will readily include those data during revision.

Furthermore, I don't understand the logic behind sampling two modern faecal samples of sheep and cattle. The data is compared to other studies, but the connection between the data of the faecal samples and the soil samples is not really discussed by the authors. The data from the analyses does neither give new insights into steroid patterns of herbivore faeces nor does it give any novel support of the steroid analyses of the soil samples. This issue must be either explained by the authors, why it was necessary to conduct analyses on modern faecal samples (How where the animals fed?), or it must be removed.

→ We prefer not to remove the modern faeces part from our manuscript. First, it helps in our opinion introducing our readership into steroid analyses and results. Second, our results show the presence of low amounts of chenodeoxycholic acid in modern cattle and sheep faeces. Prost et al. (2017) did not report on this previously. We will readily emphasize this more explicitly in our manuscript during revision.

Furthermore, I don't think it is necessary to present the data of laboratory replicate analyses of the faecal samples. The results are just a quality check that the samples are homogenous and that the laboratory method is working correctly. It is much more advisable to present data of field replicates (several sheep/cattle individuals) to present the natural variability of steroid patterns between animals.

→ We agree with Referee #1 that field replicates are desirable. Nevertheless, laboratory replicate analyses show that the method of the faecal biomarker analyses works correctly and our results are robust. We therefore prefer not to remove these results from the supplementary material.

The other major concern is, that you proposed (when I understood correctly), that there were humans, cattle and sheep present at the Ullafelsen. Consequently, I would expect that there is a mixed signal of herbivore steroids (5β-stigmastanol, deoxycholic acid) and human steroids (coprostanol, lithocholic acid), but your analyses show the presence of herbivore steroids. Is this a sign, that your steroid analyses presented in your study is the natural background of the region? I think, that this is very important for your study and it requires a discussion (along with the absence of reference samples) instead of one sentence in the conclusion ('Based on our faecal biomarker results, we cannot confirm the Mesolithic settlement of hunter-gatherers at the Ullafelsen').

→ Please allow us to raise the counter question "What is a natural background?" Undoubtedly, humans strongly influenced the Ullafelsen and its surroundings since the Mesolithic period. We therefore discuss/conclude from the absence of steroid contents and patterns indicating human faeces input that the Ullafelsen was at least not used as "toilet" (l. 382ff). With regard to the herbivore steroid contents and patterns, we will readily include in the discussion part of our revised manuscript a comparison with the reference site results. Our hypothesis is that the patterns are similar, but the contents are higher on the Ullafelsen, because of the more intensive grazing on the also for cattle and sheep very favorable Ullafelsen plateau (cf. increased δ15N values illustrated in Fig. 6 of Zech, Lerch et al. 2021. https://doi.org/10.5194/egqsj-70-171-2021). Still, please be aware that grazing and faeces input occurs on the reference sites, too.

I also wondered, if you could provide the limit of detection/quantification of your steroids, because your concentrations are quite low.

→ According to Prost et al. (2017), we consider the limit of detection/quantification to be 2 ng g^{-1} soil for all Δ^5-sterols, stanols and stanones and 5 ng g^{-1} soil for all bile acids. We will include this information in the revised manuscript.

Could you also please provide an explanation, why you detect chenodeoxycholic acid in your 37 soil samples? According to Prost et al. 2017, there is no chenodeoxycholic acid in cattle and sheep faeces, but it is present in goat (also wild?) and human faeces. Furthermore, the presence of chenodeoxycholic acid contradicts your statement, that there is a strong indication that there were cattle and sheep faeces present? This should be discussed in your manuscript.

→ A very good point. During revision, we will readily emphasize more explicitly that in contrast to Prost et al. (2017) our two modern faeces samples of cattle and sheep contain also low amounts of chenodeoxycholic acid. Furthermore, the results of our modern cattle and sheep faeces show also low contents of coprostanol and lithocholic acid, which are human-derived faecal biomarkers. However, given the low contents of these biomarkers combined with our evaluation of biomarker ratios (Fig. 7) we see no evidence for the input of human faeces in our soil samples.

Specific comments:

Abstract

Line 18-19: Instead of 'for contributing to a better understanding of pedogenesis and landscape evolution' highlight the main outcomes of your recent study.

→ Given the word limitations in the abstract, we prefer to maintain this sentence as it is. Further details are provided in the Introduction (ll. 58ff) and in the Material and Methods section (ll. 155ff).

Line 20: Why is it so important to look at faecal biomarkers at this specific site? Is it because it was unknown, if hunter-gathered also held animals during the Mesolithic period? Why is it important to identify animal biomarkers at this specific archaeological site? Please highlight this in your abstract.

→ "Importance" is not the right terminology. We will rewrite to "In order to study the human and/or animal faeces input at this relevant geoarchaeological site ... ".

Line 22: 'dominance of 5β-stigmastanol and deoxycholic acid for ruminants' is quite a clear statement. I would suggest to write something comparable like 'dominance of 5β-stigmastanol and deoxycholic acid point to/suggest the input of ruminant faeces.

→ Will be changed.

Introduction

Line 44: It is unclear to me, what 'human environment interaction' exactly means in this context?

→ Well, this is explained here in this introduction: Mesolithic hunter-gatherers used the Ullafelsen and its surrounding as summerly camp for hunting. The soils on the Ullafelsen are strongly impacted by this Mesolithic impact and latest since the Bronze Age alpine pastoralism changed the vegetation of the study area dramatically.

Line 61-65: this is an important information for the relevance of your study and should be highlighted in your abstract because it summarizes the reason, why you applied faecal biomarker analyses in your study.

→ Many thanks, we will readily follow your suggestion.

Line 68: This is not entirely correct, because bile acids are steroids. Please change this information to steroids (e.g. sterols, stanols, stanones and bile acids) or something similar.

→ Thank you very much, we will readily follow your suggestion to change the terminology of steroids.

Line 71: because you defined steroids previously, just use the term 'steroids' instead of 'steroid and bile acid.

→ Will be changed.

Line 71: I would not write 'organisms' but would specify this information to 'animals.

→ Will be changed.

Line 72-73: 'as well as their residues in soils and sediments' this part of the sentence does not make sense to me. Please rephrase.

→ Will be rephrased.

Line 73: the correct surname is 'von der Lühe'. Please change all the citations accordingly and the correct order in the reference list.

→ Will be changed.

Line 74: it is not only important to look at the plant Δ5-sterols but it is much more important to look at reference samples that were not altered by human perturbations.

→ We will carry out further analyses of reference soils in the Fotsch Valley.

Line 75: as already mentioned, please change this, because bile acids are also steroids.

→ Will be changed.

Line 76: You forgot to explain the importance/formation of stanones.

→ Will be added.

Line 81: I am not sure, if Prost et al. 2017 is the correct citation here. A much more important reference here is 'Lloyd et al. (2012) Tracing the flow-driven vertical transport of livestock-derived organic matter through soil using biomarkers'. Please add this reference and remove Prost et al. 2017. It might be also useful for the interpretation of your data.

→ Many thanks, we will readily follow your suggestion.

Line 76-87: this part is not well written and requires restructuring. In line 76 you introduce the 5β-stanol coprostanol and 5β-stigmastanol. In line 83 you explain how 5β-stanols are produced. Please restructure to the definition of your specific compounds and explain directly how they are formed and which Δ5-sterol is the precursor molecule. In the end you can explain, that once 5β-stanols are introduced into the soil, they could be further transformed to epi-5β-stanols.

→ Will be rephrased.

Line 81: this sentence does not allow 'potential leaching of steroids', because steroids are known to be associated to organic matter. So I would recommend to add an uncertainty to this sentence such as 'it has been shown that they were not leached into deeper soil horizons (Lloyd et al. 2012).

→ Many thanks, we will readily follow your suggestion.

Line 85: please change to 'microbially transformed'. It is much more logic to start with 'bile acids are formed from cholesterol in the liver -> via the bile into the gut of mammals as primary bile acids -> could be further mediated to secondary bile acids by microorganisms. Please add some examples of secondary bile acids in brackets.

→ Will be changed.

Line 101-107: The relevance is not clearly stated in the study. It is interesting to look at the faecal biomarkers at your archaeological site. What I still miss is the clear message that you assumed that hunter-gatherers at the Ullafelsen had animals with them.

→ Actually, it was never our assumption that hunter-gatherers at the Ullafelsen had animals with them. To make our motivation and hypotheses more clear, we will add to our research question (iii) the sentence: *"We hypothesize*

that human faeces input is detectable in the E horizon representing the Mesolithic living floor (LL), whereas livestock faces input dominates in the overlaying OAh horizon."

Material and Methods

Line 113: Is it common to write plant names with capital letter? Otherwise, please change.

→ Will be changed.

Line 132: 'faeces samples from cattle and sheep': this is not enough information. Are those samples from close-by herds? Natural samples? How were they collected and stored? How were the animals fed, because food does have an impact on the steroid pattern of faeces? This is important information for your interpretation. It is also known that steroid patterns could differ between individuals, so why did you sampled just one sample from one individual?

→ We will readily add additional information about the two faeces samples during revision. We also agree that further field replicates would be desirable. However, given that the focus of our study is on the (archaeological) soils, we currently do not plan field replicates for modern faeces samples.

Line 133: Do the soil samples from the trench serve as reference samples because they were not influenced by humans? Since the abstract, I was wondering how you are planning to compare your samples from the archaeological site and a reference sample would be very helpful for the interpretation of the data.

→ The soil samples from the close by trench profiles are also archaeological soil profiles on the Ullafelsen and cannot be used as reference samples. We will add data of reference soil samples from the Fotsch Valley during revision.

Line 167: please change <2 mm to ≤ 2 mm because you also include particles with a size of 2 mm.

→ Will be changed.

Line 167-168: I am confused, because in the section before, you mentioned that you already conducted TC and TN analyses which was published in Zech et al. (2021). How do these analyses differ from the analyses you mention here?

→ TC and TN values for the investigated soil samples were already published in Zech et al. (2021). We used these data also for our faecal biomarker analyses.

Line 172-173: Please remove this sentence 'Analyses took place…'

→ Will be removed.

Line 174-179: You weighed the samples before you applied the Soxhlet extraction. Please change the order of the sentences here.

→ Will be changed.

Line 179: what do you mean with 'neutral and acid lipids'. It was not explained before and the information must be included here. You can also say 'α-pregnanol' served as IS for sterols and stanols, isodeoxycholic acid served as IS for bile acids. What is the IS for the stanones?

→ Neutral lipids mean the fraction of sterols, stanols and stanones, while acid lipids mean the fraction of bile acids. We used pregnanon as IS for the stanones. 5α-cholestan was used as IS 2. We will add this information in the manuscript.

Line 180: Please include the information how you removed the solvent. I guess it was rotary evaporation?

→ Yes, we removed the solvent using rotary evaporator. Will be rephrased.

Line 181: Here is the definition of neutral lipids. This is too late, when you already mention them in line 179.

→ Will be changed.

Line 181: acidic lipids do also include fatty acids.

→ Will be added.

Line 186: I would say 'acidic fraction was methylated' instead of 'bile acids', because the fatty acids are included.

→ Will be rephrased.

Line 189: I think, 'Merck' is enough information and you can remove 'Sigma-Aldrich and 10187' (Is this the ordering number?)

→ Will be removed.

Line 190: you eluted the fatty acid methyl esters to waste, right?

→ Yes, this is correct.

Line 194: It is '5α-cholestane'. Please change.

→ Will be changed.

Line 194-195: You added the second internal standard to the reactivials? Or did you remove the derivatization agent with nitrogen and transferred the sample to GC-MS vials before you add ISII?

→ For the sterol, stanol and stanone fraction, we added IS2 after derivatization and drying under nitrogen. We transferred the sample solved in toluene together with IS2 into GC-vials.

→ For the bile acid fraction, we added IS2 into reactivials after derivatization and transferred it together with the derivatization agent into GC-vials.

Line 201: Merck' is enough information and you can remove 'Sigma-Aldrich and 10187'

→ Will be removed.

Line 203: It is '5α-cholestane'. Please change.

→ Will be changed.

Line 206: what is the concentration of the stock solution of the external standard?

→ The stock solution concentration of the external standards is ~10 µg ml^{-1}.

Line 207-208: This step does not make sense to me. Why did you add the first internal standard to the external standards in the same concentration? To be able to calculate the recovery of the first internal standard in your

samples you need the calibration line of the first internal standard. It is also not clear for what you used the second internal standard?

→ We added the internal standard 1 in the same concentration as the external standards for calculation of the external standard calibration line and to determine the steroid recoveries.

→ Internal standard 2 was added to remove measurement fluctuations. The peak area of the internal standard 2 was used as reference for the peak area calculation of each steroid compound we detected.

Line 214: Please change to 'injection in splitless mode'

→ Will be changed.

Line 218-257: this section is part of the Introduction and a little bit misplaced here. There are also no references to the equations (Eqn. 1-5) in the text, instead you use 'ratio 1-5'.

→ Many thanks, we will readily follow your suggestion and will add references to the equations.

Line 246: 'This transformation is induced by soil composting' is not a general way how epi-5β-stanols are formed. Composting requires specific aerobic conditions, while epi-5β-stanols are preferably produced under anaerobic conditions. Please remove this statement.

→ Will be removed.

Results and Discussion

Biomarker patterns of faeces from predominating animals

This whole paragraph was not really a discussion of the results and what kind of message you get from your calculated ratios. It was not clear to me, how the analyses of the faeces samples can be linked to the analyses of the soil samples and if there is any newly developed message you can draw from the ratios.

→ We compare our modern faeces results with data of Prost et al. (2017). Our results show similarities as well as differences in steroid patterns and ratios to Prost et al. (2017). We detected in our modern faeces samples a low content of chenodeoxycholic acid, while Prost et al. (2017) did not detect this bile acid compound in their samples of cattle and sheep faeces. Based on the steroid patterns of modern ruminant faeces from the Ullafelsen, we can evaluate the faecal biomarker results of our soil samples. Steroid ratios of modern faeces help us to identify the sources of dominant faeces input in soils.

Line 260-261: Again, bile acids are also steroids. You must either find a short term for sterols, stanols and stanones or you always name those three compounds.

→ Many thanks, we will readily follow your suggestion.

Line 263: I am not sure what you mean with 'robustness of our results' by repeating the analyses. It is more a quality check, if your method is working and if your faecal samples are homogenous.

→ "Robustness of our results" describes a quality check of the faecal biomarker analyses. We demonstrate our readership that the applied method works correctly and our results are reproducibility. We will replace "robustness" by "reproducibility".

Line 266: It depends also on how you treated your faeces samples after sampling.

→ Information will be added in the material and methods section.

Line 274: How do you interpret the results of your samples of ratio 1?

→ Results of ratio 1 of our modern faeces show typical ratios for cattle and sheep faeces, which are also reported by Prost et al. (2017). We will add this interpretation in the manuscript.

Line 273-278: This whole paragraph is difficult to read. Please rephrase. Also, it is not clear to me what is your interpretation of your calculated ratios? What kind of conclusion do you draw from your results of the calculated ratios?

→ We compare the ratios of our modern faeces results with data of Prost et al. (2017). In order to determine the origin of faeces input in soils, we calculated different ratios of modern ruminant faeces to get a reference ratio for our analysed soil samples. We will readily follow to your suggestion to rephrase this paragraph.

Line 288: In my opinion, three measurements of the same sample is not a quality control of the natural fluctuations of steroids in faeces, it is rather a control if the method and analyses worked well. In this case, I think it is not necessary do discuss it. You can discuss natural variations when you took field replicates of several sheep/cattle individuals.

→ Thanks a lot, we will consider your suggestion during revision.

Faecal biomarker contents and patterns in soils

Line 305: You 'assume' that the 'small contents of steroids in the subsoil horizons at the Ullafelsen are mainly caused by the influence of β-sitosterol due to the strong rooting in the soil matrix'. Please change.

→ Will be changed.

Line 338: You can only indicate the strong input of faeces from cattle and sheep when you compare your results to reference samples. Furthermore, you also found chenodeoxycholic acid in your samples, which is also an indication of the input of other herbivore faeces than cattle and sheep (see Prost et al. 2017). How do you explain the presence of chenodeoxycholic acid?

→ Data of reference soil profiles will be added during revision. In contrast to Prost et al. (2017), our faecal biomarker results of modern cattle and sheep faeces show also a low content of chenodeoxycholic acid. That´s how we explain the presence of this bile acid compound in our soils. We assume that the input of chenodeoxycholic acid by other wild animals or herbivore species at the Ullafelsen is negligible. We will emphasize this better during revision.

Conclusion

Please remove citations in the conclusion section, because it is not common. Furthermore, the conclusion contains parts of the discussion which should be moved to the discussion section.

→ Citations will be removed. Parts of the conclusion will be moved in the discussion section.

Technical corrections

Please check if your references are in a correct order e.g. in line 53 it is 'Geitner et al., 2011; Schäfer, 2011a' and in line 56 it is the other way round.

→ Will be changed.

Line 67: How do you order your citations here? After years? Please check your entire manuscript for the order of references and change accordingly (e.g. after years,…).

→ We will change the order of the citations after years.

Text is much easier to read when you include the common abbreviations of bile acids: deoxycholic acid = DCA, lithocholic acid = LCA,…

→ Will be changed.

All concentrations should be given in 'µg g-1' instead of 'µg/g'.

→ Will be changed.

Figures

Could you please provide graphs in higher quality? The axis are not readable and the lines of the bars are not consistent in thickness and color

→ Figures and graphs will be changed.