

## **Review 'Human and livestock faecal biomarkers at the prehistorical encampment site of Ullafelsen in the Fotsch Valley, Stubai Alps, Austria – potential and limitations'**

### **General comments**

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.

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 5 $\beta$ -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?

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. 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.

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 (5 $\beta$ -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').

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

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.

### **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.

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.

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

## **Introduction**

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

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.

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.

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

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

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.

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

Line 74: it is not only important to look at the plant  $\Delta^5$ -sterols but it is much more important to look at reference samples that were not altered by human perturbations

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

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

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.

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

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).

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.

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.

## **Material and Methods**

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

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 sample just one sample from one individual?

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.

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

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?

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

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

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 ' $\alpha$ -pregnanol' served as IS for sterols and stanols, isodeoxycholic acid served as IS for bile acids. What is the IS for the stanones?

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

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

Line 181: acidic lipids do also include fatty acids.

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

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

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

Line 194: It is ' $5\alpha$ -cholestane'. Please change.

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

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

Line 203: It is ' $5\alpha$ -cholestane'. Please change.

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

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?

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

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'.

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

## **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.

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.

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.

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

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

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?

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.

### *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  $\beta$ -sitosterol due to the strong rooting in the soil matrix'. Please change.

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?

## **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.

## **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.

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,...).

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

All concentrations should be given in ' $\mu\text{g g}^{-1}$ ' instead of ' $\mu\text{g/g}$ '.

## **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 colour.