

Trust is good, control is better: technical considerations in blood microbiome analysis

We agree with Hornung *et al*¹ that studying blood microbiome is a major technical challenge with potential artefacts. At least three important challenges must be tackled:

1. Low amount of bacterial DNA in blood.²
2. High amounts of PCR inhibitors.
3. Bacterial DNA contaminants from environment, reagents and consumables.

Measuring, reducing and controlling bacterial contaminants are key elements of optimisations made on the molecular pipeline used in our study³ as well as eight published studies on blood microbiome.^{2,4-7} The studies from Salter *et al*⁸ and Laurence *et al*⁹ are useful to understand the burden of bacterial contaminants when working with low bacterial abundance samples. In former publications,^{2,10} we have described our procedure and the controls performed to address such contamination. One must be careful when using a fixed list of bacterial contaminants, as each experiment has its own contamination burden. Therefore, two different experiments done under different conditions, will not have the same contaminants. What is essential, as pointed out by Hornung *et al*, is to include and analyse negative controls in each experiment. Although not explicitly mentioned before, our study³ included the following negative controls:

- i. Extraction negative controls (water at DNA extraction step).
- ii. PCR negative controls (water at first PCR step).

We now present data from these control experiments. Abundance of 16S ribosomal RNA genes measured by quantitative PCR (qPCR) shows over 1000-fold difference between blood samples and extraction negative controls (figure 1A). Blood samples also exhibit significantly higher genus richness (figure 1B) and distinct microbiome compositions (figure 1C) compared with negative controls. Therefore, the technical contamination would have only a marginal impact in this study. Though we cannot exclude that a small fraction of the measured bacterial DNA corresponds to contamination, the contaminants are low and relatively homogenous between samples and should not influence the statistical tests performed.

Among the nine bacterial genera listed by Hornung *et al* as potential contaminants based on the literature, the negative control

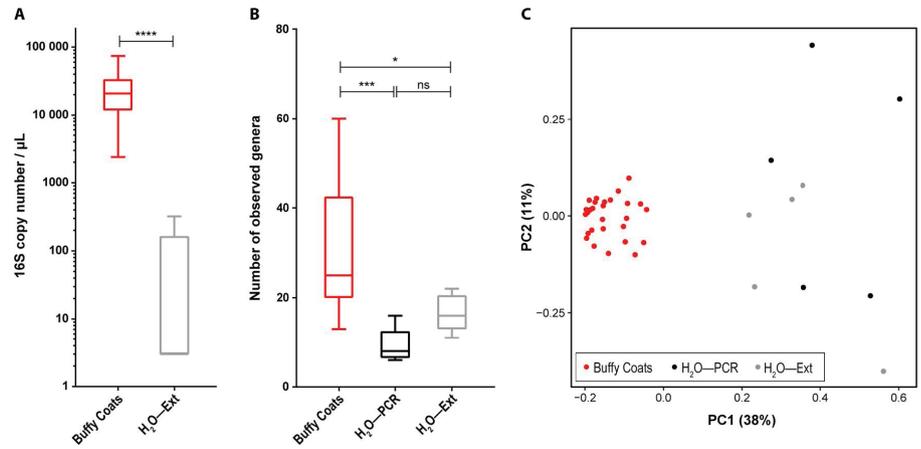


Figure 1 (A) qPCR-based 16S rRNA gene abundances are significantly higher in buffy coat samples than negative controls (H₂O—Ext) based on Mann-Whitney U test. Median 20800 versus 3 copies/μL; mean 24160 versus 67.2 copies/μL. (B) Buffy coat samples exhibit significantly higher genus richness than negative controls (H₂O—PCR and H₂O—Ext) based on Kruskal-Wallis test followed by Dunn's post hoc tests. (C) Principal coordinate analysis of the 16S rRNA gene sequencing data using Bray-Curtis dissimilarity measure shows clear separation of buffy coat samples from negatives controls (H₂O—PCR and H₂O—Ext). H₂O—Ext: molecular grade water added in an empty tube, extracted and analysed (qPCR and/or sequencing) at the same time as the samples. H₂O—PCR: molecular grade water added in an empty tube and amplified and sequenced at the same time as the extracted DNA of the samples. Statistical significance—*p<0.05; ***p<0.001; ****p<0.0001. qPCR, quantitative PCR; rRNA, ribosomal RNA.

sequencing data clearly show that eight of them were not contaminants in our study (figure 2A). These were either absent from negative controls or present in significantly lower relative proportions than in blood samples. The remaining genus, *Arthrobacter*, with similar relative abundance in samples/controls (figure 2A), could be considered a contaminant. When working with compositional data, it is important to note that relative abundance of contaminants in negative controls will be exaggerated. It should always be interpreted together with

quantitative data, such as qPCR abundances (figure 1A). Therefore, it is disputable whether *Arthrobacter* is a real contaminant given our data, but still possible. Additionally, we also found that *Escherichia/Shigella* relative abundance could suggest that it is a contaminant, but it is not uncommon to find it in blood. Consequently, we did not exclude *Arthrobacter* and *Escherichia/Shigella*, but they did not show clinically meaningful correlations and therefore were not discussed in our report.³ Two other taxa (*Bradyrhizobium* and *Ralstonia*) were

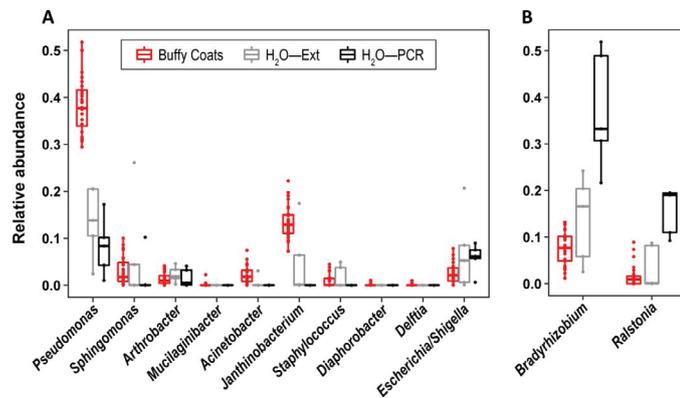


Figure 2 Comparison of bacterial genus relative abundances in buffy coat samples and negative controls (H₂O—PCR and H₂O—Ext). (A) Bacterial genera listed in the letter of Hornung *et al* as potential contaminants, and *Escherichia/Shigella*. (B) Two bacterial genera that were considered as likely contaminants and discarded from our previous letter. H₂O—Ext: molecular grade water added in an empty tube, extracted and analysed (qPCR and/or sequencing) at the same time as the samples. H₂O—PCR: molecular grade water added in an empty tube and amplified and sequenced at the same time as the extracted DNA of the samples. qPCR, quantitative PCR.

present in higher proportions in negative controls compared with samples (figure 2B), and thus were considered as likely contaminants and not considered further.³

Finally, contamination by skin bacteria is indeed a major challenge when using small volume of blood (20 µL) taken by skin puncture. However, in this study, 40 mL of blood was withdrawn. Moreover, portal, hepatic and atrial blood were collected using catheters not in contact with skin. Therefore, contamination from the skin is negligible in our study.

Overall, we second the concerns raised by Hornung *et al*, and through this letter highlight the important controls required in blood microbiome research.

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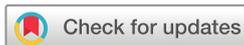
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