



Experimental validation of small mammal gut microbiota sampling from faeces and from the caecum after death

Dagmar Čížková¹ · Ľudovít Ďureje¹ · Jaroslav Piálek¹ · Jakub Kreisinger²

Received: 27 May 2020 / Revised: 7 May 2021 / Accepted: 7 May 2021 / Published online: 27 May 2021
© The Author(s), under exclusive licence to The Genetics Society 2021

Abstract

Data on the gut microbiota (GM) of wild animals are key to studies on evolutionary biology (host–GM interactions under natural selection), ecology and conservation biology (GM as a fitness component closely connected to the environment). Wildlife GM sampling often requires non-invasive techniques or sampling from dead animals. In a controlled experiment profiling microbial 16S rRNA in 52 house mice (*Mus musculus*) from eight families and four genetic backgrounds, we studied the effects of live- and snap-trapping on small mammal GM and evaluated the suitability of microbiota from non-fresh faeces as a proxy for caecal GM. We compared CM from individuals sampled 16–18 h after death with those in live traps and caged controls, and caecal and faecal GM collected from mice in live-traps. Sampling delay did not affect GM composition, validating data from fresh cadavers or snap-trapped animals. Animals trapped overnight displayed a slight but significant difference in GM composition to the caged controls, though the change only had negligible effect on GM diversity, composition and inter-individual divergence. Hence, the trapping process appears not to bias GM profiling. Despite their significant difference, caecal and faecal microbiota were correlated in composition and, to a lesser extent, diversity. Both showed congruent patterns of inter-individual divergence following the natural structure of the dataset. Thus, the faecal microbiome represents a good non-invasive proxy of the caecal microbiome, making it suitable for detecting biologically relevant patterns. However, care should be taken when analysing mixed datasets containing both faecal and caecal samples.

Introduction

Gut microbiota (GM) have an indisputable effect on different host phenotypes, including immunity, digestion efficiency and cognitive functioning (Turnbaugh et al. 2006; Round and Mazmanian 2009; Cryan and Dinan 2012). As such, host–GM symbiosis can represent an important extension of current evolutionary theory (Zilber-Rosenberg

and Rosenberg 2008). Most host vs. microbiota interaction studies over the past two decades have focused on the microbiota of animals held in captivity or humans. Such a narrow focus limits possibilities for gaining insights into the nature of such interactions from an evolutionary perspective (Hird 2017). For example, both captive-bred animals and westernised human populations harbour highly derived microbial communities (Clayton et al. 2016; Gomez et al. 2016; McKenzie et al. 2017; Rosshart et al. 2017), not necessarily reflecting mutual adaptations that have taken place during the co-evolutionary history of the GM and its host. Relaxed natural selection in captivity also restricts measurements of GM fitness effects.

To overcome these limitations, a growing number of studies have been describing the composition and function of symbiotic microbiota in wild animal populations. It has now been shown that GM are involved in modulation of a number of key ecophysiological and life-history traits in wild animal hosts and have the potential to increase their adaptation capacity (Alberdi et al. 2016). For example, GM allows for the utilisation and detoxification of a range of

Associate editor Xiangjiang Zhan.

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41437-021-00445-6>.

✉ Dagmar Čížková
dejsha@seznam.cz

¹ Institute of Vertebrate Biology, Czech Academy of Sciences, Brno, Czech Republic

² Faculty of Science, Department of Zoology, Charles University, Prague, Czech Republic

dietary components (García-Amado et al. 2007; Wang et al. 2014; Gomez et al. 2015; Kohl and Dearing 2016; Kohl et al. 2018; Kartzinell et al. 2019), and thus affects dietary niche width. Correlated changes between GM and other variables, such as host physiology (Dill-McFarland et al. 2014; Weldon et al. 2015; Amato et al. 2019), stress level (Vlčková et al. 2018; Stothart et al. 2019) and pathogenic infection (Kreisinger et al. 2015; Vasemägi et al. 2017; Aivelo and Norberg 2018) have brought valuable insights into interactions between the host, its GM and the environment under natural conditions. Some of these patterns have been further validated by transplantation of wild GM into experimental animal models (Sommer et al. 2016; Miller et al. 2016; Warne et al. 2017; Rosshart et al. 2017). As GM dysbiosis can have serious negative consequences on the viability of wild populations, there is an increasing need to integrate monitoring and maintenance of wildlife-associated microbial diversity into management and conservation practices (Trevelline et al. 2019).

Microbiota composition varies along the length of vertebrate gastrointestinal tracts (GIT; Zhao et al. 2015; Suzuki and Nachman 2016; Ericsson et al. 2016; Li et al. 2017; Yan et al. 2019; Tang et al. 2019) in connection with the different functions of each gut compartment (Donaldson et al. 2016). Intestinal compartments rich in diet-digesting bacteria are usually chosen for GM analysis, with the luminal contents or the caecum wall preferred for rodents, lagomorphs, most birds and herbivorous reptiles. However, GM sampling from live animals is invasive and, apart from cloacal/rectal swabs, difficult to manage in the field (Tang et al. 2020), while the killing of animals to sample their gut content is often illegal, e.g. in endangered species, and generally questionable from an ethical point of view (Zemanova 2020). Further, post-mortem sampling does not allow for repeated sampling of the same individual, which is advantageous when studying GM changes during ontogeny or when comparing with temporarily changing environmental variables (Kreisinger et al. 2017; Björk et al. 2019). Sampling of faecal microbiota (FM), on the other hand, has potential as a widely applicable non-invasive substitute of wildlife GM sampling (Zemanova 2019, 2020), assuming that it is feasible in the field and that FM constitutes a good proxy of GM.

While truly non-invasive collection of abandoned faeces is feasible for some large vertebrates (Menke et al. 2015), the majority of small animals need to be trapped to obtain faecal material. In such cases, the animals can be left to defecate after capture, or rectal/cloacal swabs can be taken, both of which, however, prolong handling and cause additional stress to the animal. Collecting faecal material directly from traps clearly represents a less invasive and time-consuming option; however, the main concerns with this approach are contamination of the faeces by microbes

from non-sterile traps (e.g. from previously trapped animals) and progressive changes in microbiota after defecation, as addressed by Kohl et al. (2015).

A number of recent studies have focused on the comparison of faecal and GM in various mammals (Stearns et al. 2011; Zhao et al. 2015; Yasuda et al. 2015; Li et al. 2017; Ingala et al. 2018; Stalder et al. 2019), including house mouse *Mus musculus* (Gu et al. 2013; Weldon et al. 2015; Suzuki and Nachman 2016; Tanca et al. 2017), as well as in birds (Stanley et al. 2015; Videvall et al. 2017; Yan et al. 2019). In those studies that included multiple intestinal compartments, the general trend appears to be that both alpha diversity and composition of microbiota from faeces tends to be more similar to those from the lower GIT (colons and caeca) than those in the small intestine (Stearns et al. 2011; Gu et al. 2013; Zhao et al. 2015; Yasuda et al. 2015; Suzuki and Nachman 2016; Li et al. 2017; Yan et al. 2019). This can be explained by the closer physical proximity of faeces and lower GIT compartments. Moreover, faecal and lower GIT microbiota tend to share the majority of their bacteria; hence, FM would appear to be suitable for detecting bacterial taxa present in the intestines. On the other hand, FM and lower GM appear to differ in taxonomic composition as they show substantial abundance shifts in bacteria at various taxonomic levels. In line with this, house mouse FM and GM are characterised by distinct functional profiles (Tanca et al. 2017).

In-depth systematic analysis of GM–FM abundance shifts, using high numbers of individuals, have been performed on chickens (Stanley et al. 2015; Yan et al. 2019) and (with fewer samples) ostriches and rhesus macaques (Yasuda et al. 2015; Videvall et al. 2017). Overall, there was a moderate bacterial abundance correlation between FM and GM, with the strength of correlation varying significantly between individually analysed bacterial taxa and the taxonomic level examined.

A profoundly important aspect of FM–GM resemblance for wildlife studies is that differences in microbiota composition and diversity between individuals must follow the same pattern when analysing faecal and intestinal microbiota. Wildlife microbiome research tends to be explorative in nature and is usually based on observing correlations between inter-individual differences in microbiota composition or diversity and variation in the traits of interest. Some studies across or within wild species have evaluated consistency in FM vs. GM changes in response to several variables (Weldon et al. 2015; Ingala et al. 2018; Stalder et al. 2019); however, their results do not address the general consistency of inter-individual GM and FM patterns. Indeed, there have been no straightforward analyses to date evaluating correlation strength between inter-individual differences in FM vs. GM (including composition and alpha-diversity measures), possibly due to the relatively high sample sizes needed for such comparisons.

Under some circumstances, such as when sampling road kills or during targeted decreases in population density (e.g. invasive species or animal pests), direct sampling of wildlife intestinal microbiota is appropriate, or at least justifiable (Dubois et al. 2017). GM may often be sampled as part of larger research projects with specific aims requiring an invasive approach, e.g. when sampling the inner organs or during parasitological examination. In live-trapped animals, GM samples can be taken immediately after scarification; however, it remains unknown whether changes occur in GM due to stress induced by the trapping process. If the animal is killed during the trapping process, as with snap-traps commonly used on rodents, the time delay between the animal's death and sampling can be as long as that between setting the trap and dissection of the animal. The few studies investigating links between ante-mortem and early post-mortem GM in humans indicate stability in GM composition for at least 2 days after death, with the caecal microbiome changing more quickly than rectal (Tuomisto et al. 2013; Pechal et al. 2018). However, animals with smaller body sizes are likely to show different timing of post-mortem changes compared to humans, e.g. a more rapid decline in corpse body temperature, which may substantially influence microbial dynamics (Brooks 2016). In line with this, Heimesaat et al. (2012) documented an early onset of post-mortem ileal microbiota changes in mice, with some bacterial taxa starting to overgrow as soon as 3 h after death. On the other hand, Lawrence et al. (2019) showed that rabbit caecal microbiota (CM) remained stable for 48 h after death at two different temperatures.

In this study, we conduct a controlled experiment to evaluate potential bias in GM due to (i) the trapping process, and (ii) to delayed post-mortem sampling, and examine the suitability of FM as a non-invasive proxy for GM. In particular, we assess differences in CM profiles in house mice from a conventional breeding facility that were (i) unexposed to the environment in live-traps, (ii) were kept alive in the trap overnight and sampled next morning and (iii) those that were euthanised, left lying on the trap and sampled with a one-night delay. Moreover, faeces from individuals included in the second treatment group were collected from the traps and used for subsequent comparison of FM vs. CM.

Material and methods

Experimental animals

This experiment used 52 house mice (*Mus musculus*) from eight families and four different genetic backgrounds (strains C3Ha, C3Hb, PWD and PWK [Laukaitis et al. 1997; Gregorová and Forejt 2000]) kept at the breeding

facility of the Institute of Vertebrate Biology in Studenec (licences for keeping and experimental work 61,974/2017-MZE-17214 and 62,065/2017-MZE-17214, respectively). For three families, we included breeding pairs (parents) and their offspring (brothers and sisters) into the experiment, while the other families were represented by offspring only. The breeding pairs were co-housed in a single cage before the experiment, while the offspring were caged separately after weaning at 21–24 days. Parental age ranged between 272 and 274 days, while offspring ranged between 34 and 45 days.

In a previous study, we showed that GM alpha-diversity and GM inter-individual variability were similar in wild mice and mice from our breeding facility, despite the two groups clustering separately based on GM composition (Kreisinger et al. 2014). Note, however, that this compositional difference was not due to differences in the presence/absence of bacterial taxa, but rather to moderate shifts in abundance. Importantly, the experimental setup allowed for us to control other variables, which would be hard to achieve in the wild. In particular, we introduced defined variability for biologically relevant traits, i.e. genetic background, family relationships and age, and the experiment was performed over a single night under stable conditions to minimise environmental variability. Despite this, we could not exclude the effect of some other factors, such as decreased stress susceptibility in captive animals, which could have resulted in an underestimation of GM changes due to stress induced by the trapping process. It is also possible that under environmental conditions different from those set in the experiment (e.g. temperature), there could be differences in the magnitude of microbiota changes.

Experimental procedures

The mice were divided into three treatment groups, (1) alive-in-trap (21 ind.), (2) dead-on-trap (22 ind.) and (3) control-in-cage (9 ind.). Each family comprised one to several alive-in-trap/dead-in-trap pairs and 1–2 control-in-cage individuals (detailed in Table S1). Each of the alive-in-trap mice was kept at room temperature overnight (16–18 h) in a wooden live trap previously used for trapping rodents. The mice were provided with 'breeding facility chew' but no water in order to mimic live-trapping conditions. After an alive-in-trap individual was put into the trap, its dead-on-trap counterpart was euthanised by cervical dislocation and left lying on the trap to simulate snap-trapping conditions. The traps were then placed on shelves in a single breeding facility box in a random order and ca. 40 cm apart. The control-in-cage individuals were left in their breeding cages. After 16–18 h, the alive-in-trap and control-in-cage mice were euthanised. Mice of all three groups were then

dissected and the caecum tips flash-frozen in liquid nitrogen and stored at -80°C . Faeces were collected from each trap immediately after dissection of the trapped individual was completed. This resulted in a mixture of faeces ranging from 0 to 18-h old, as would be expected for over-night trapping in the wild. The faecal samples were then stored in the same manner as the caecum tips. The experiment was performed over a single night in order to avoid any effect of temporal changes in the microbiota.

DNA was extracted from the caecum tips and from five randomly chosen faecal droppings per individual using the DNeasy PowerSoil HTP 96 Kit (Qiagen, Netherlands), following the manufacturer's instructions. Bacterial 16S rRNA amplicon sequencing libraries were constructed using two-step PCR, with technical duplicates prepared to account for noise due to PCR and sequencing stochasticity. A barcoding PCR was performed with the 16S rRNA primers S-D-Bact-0341-b-S-17 (CCTACGGGNGGCWGCAG) and S-D-Bact-0785-a-A-21 (GACTACHVGGGTATCTAATCC) amplifying the V3–V4 variable region. The primers were extended at the 5' ends with inline barcode sequences to increase multiplexing capacity and with 'tail' sequences serving as priming sites for the second PCR. The 10- μl PCR reactions consisted of 1x KAPA HIFI Hot Start Ready Mix (Kapa Biosystems, USA) and each primer at 0.2 μM and 4.6 μl of DNA template. These were incubated at 95°C for 3 min, followed by 28 cycles of $95^{\circ}\text{C}/30\text{ s}$, $55^{\circ}\text{C}/30\text{ s}$, $72^{\circ}\text{C}/30\text{ s}$ and a final extension at $72^{\circ}\text{C}/5\text{ min}$. Dual-indexed Nextera sequencing adapters were reconstructed during the second PCR, which followed the first PCR conditions except that it was performed in 20- μl volume, the concentration of each primer was 1 μM , 1.5 μl of the first PCR product diluted $\times 12.5$ was used as a template and the number of PCR cycles was 12. The products of the second PCR were quantified by 1.5% agarose gel electrophoresis, pooled equimolarly, purified with SpriSelect beads (Beckman Coulter, USA) and size-selected with Pippin Prep (Sage Science, USA) at 520–750 bp. The pool of libraries was sequenced using MiSeq (Illumina, USA) and v3 chemistry (i.e. $2 \times 300\text{-bp}$ paired-end reads) at the CEITEC Genomics Core Facility (Brno, Czech Republic).

Bioinformatic and statistical analysis

We used dada2 (Callahan et al. 2016) for quality filtering (reads with >1 expected error eliminated) and denoising of fastq files. The resulting Amplicon Sequence Variants (hereafter ASV) were checked for presence of chimeric variants using uchime (Edgar et al. 2011) and only those that were detected in both technical duplicates were retained (e.g. Pafčo et al. 2018). Taxonomic assignment was based on dada2 implementation of the RDP classifier (Wang et al. 2007;

posterior confidence threshold set to 80%) and the Silva reference database version 132 (Quast et al. 2013). The final dataset included 1468 ASVs and 788,238 reads (average coverage per sample: 10,798; range: 7269–18,533).

We rarefied the resulting abundance matrix to account for uneven sequencing depth (rarefaction threshold: 7269 reads per sample) and calculated Shannon diversities and number of observed ASVs in each sample for further alpha diversity comparisons. Differences in microbiota composition were analysed based on pairwise Bray–Curtis (accounting for ASV abundance) and binary Jaccard (accounting for ASV prevalence) dissimilarities, both being calculated for the rarefied dataset. We decided to use rarefaction for these analyses as (i) it is the most simple and one of the most transparent means of data normalisation, (ii) it is probably the only valid standardisation method for prevalence-based betadiversities and (iii) it is unlikely to be associated with any adverse effect on both Type I and II statistical errors (at least in the case of our data). The latter claim was supported by a nearly perfect correlation between Bray–Curtis dissimilarities for rarefied data vs. unrarefied data with read numbers converted to ASV proportions (Mantel test: $r = 0.9988$, $p = 0.0001$). The same was true for alpha diversity estimates, where diversity of rarefied dataset correlated perfectly with the unrarefied data if log-transformed sequencing coverage was included as a covariate (linear regression: estimate $[\pm\text{S.E.}] = 1.0104 [\pm 0.0064]$, $F_{2,70} = 13010$, $R^2 = 0.9973$, $p < 0.0001$ for ASV richness, and estimate $[\pm\text{S.E.}] = 1.0.9919 [\pm 0.0073]$, $F_{2,70} = 10880$, $R^2 = 0.9968$, $p < 0.0001$ for Shannon diversity).

Differences in GM alpha diversity between treatment groups were analysed using linear mixed-effect models (LMM), where the treatment group or sample type (i.e. CM vs. FM) were considered as categorical explanatory variables and family membership or individual identity as random effects. Observed ASV numbers were log-scaled to achieve normal distribution of residuals. Variation in GM composition between the treatment groups, genetic background and families was visualised using unconstrained Nonmetric Multidimensional Scaling (NMDS). Next, we applied constrained distance-based redundancy analysis (db-RDA; Legendre and Anderson 1999), where treatment group identity was included as a predictor and family membership and genetic background were considered as conditional variables. This means that GM differences due to families and genetic background were removed prior to statistical testing of the predictor variable (i.e. the treatment group) and their effect was also eliminated from the resulting ordination plots. In this way, even subtle GM differences caused by the treatment could be revealed. It is worth noting that this kind of model cannot be fitted by more commonly used community microbiology methods, e.g. PERMANOVA.

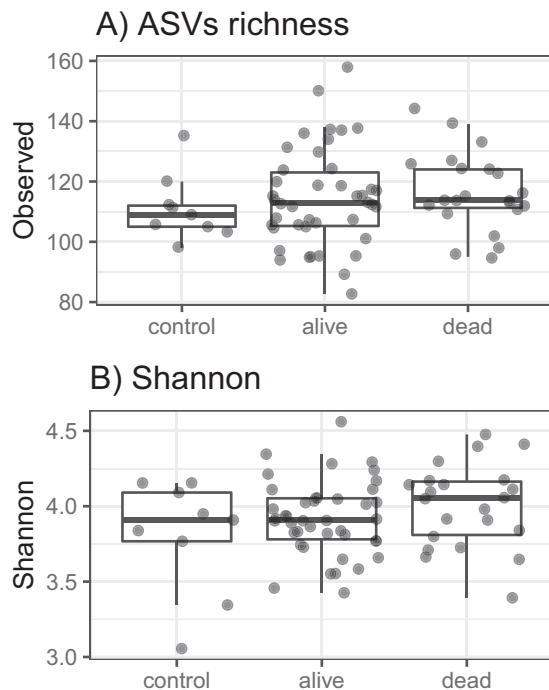


Fig. 1 Alpha diversity variation of caecal microbiota between control-in-cage (control), alive-in-trap (alive) and dead-on-trap (dead) mice. Alpha diversity comparisons were based on **A** number of observed ASVs and **B** Shannon diversity index. All differences between treatment groups were non-significant ($p > 0.05$) according to linear mixed-effect models.

Candidate ASVs, whose abundances differed among treatment groups, were identified based on generalised LMM (hereafter GLMM) with negative binomial distribution. GLMMs included per-sample read count for each ASV as a response and the same fixed and random effect structure as described above. GLMMs were fitted for unrarefied ASV read counts to avoid inflation of Type II statistical errors (McMurdie and Holmes 2014). To account for variation in sequencing depth between samples, log-transformed sequencing depth was specified as an offset. In the case of GLMM, the method of Benjamini and Hochberg (1995) was used for multiple testing corrections.

Correlations between FM and CM alpha diversity sampled in the same individual were tested using Pearson correlations (i.e. the number of observations equalled the number of individuals sampled). Correlations between microbiota compositions of the two sample types were tested by Mantel's tests, which directly compared composition-based distance matrices (either Jaccard or Bray–Curtis) and therefore the number of observations equalled to $(n^2 - n)/2$, where n = number of individuals sampled. All statistical analyses were conducted in R version 3.4.4. (R Core Team 2018). Annotated scripts used for data analysis are available in Supplementary materials S3 and S4.

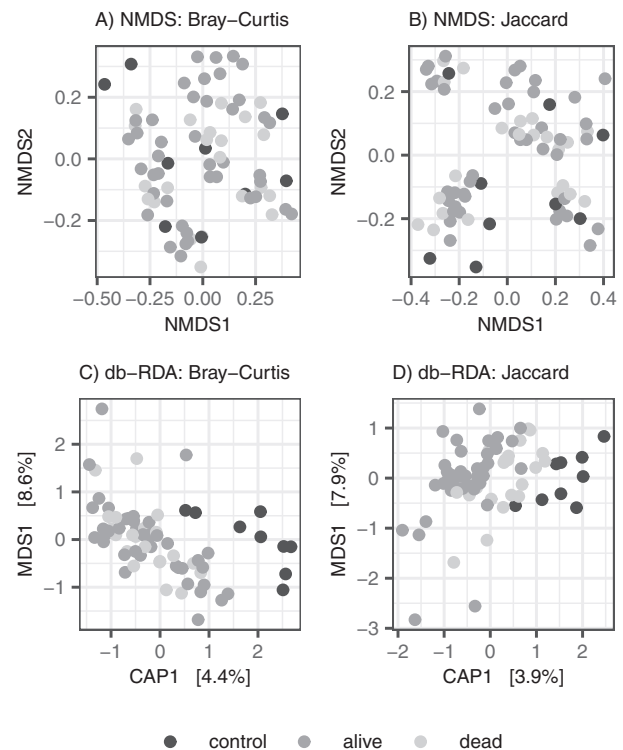


Fig. 2 Compositional variation of caecal microbiota between control-in-cage (control), alive-in-trap (alive) and dead-on-trap (dead) mice. Analysis was based on unconstrained ordinations (NMDS) and constrained ordinations (db-RDA) that maximise gradients between treatment groups and eliminate variation due to family/strain membership. Calculations were based on community divergence accounting for ASV prevalence (Jaccard) or abundance (Bray–Curtis).

Results

Effect of sampling delay and trapping on CM

Consistent with most previous studies on house mouse GM, the CM of all experimental individuals was dominated by bacteria from Clostridia, Bacteroides and Campylobacteria (Fig. S1).

There were no significant differences in caecal alpha diversity between control-in-cage individuals vs. alive-in-trap or dead-on-trap individuals (LMM: $\Delta D.f = 2$, $\chi^2 = 0.9271$, $p = 0.629$ for observed number of ASVs, and $\Delta D.f = 2$, $\chi^2 = 4.2078$, $p = 0.122$ for Shannon diversity). However, both Shannon diversity and number of ASVs exhibited a slight, non-significant increase in dead-on-trap mice (Fig. 1).

According to unconstrained NMDS, variation in GM composition between mice families and genetic backgrounds prevailed over systematic differences between treatment levels (Figs. 2 and S2). However, if we applied db-RDA to eliminate GM variation given by families/genetic backgrounds, we detected significant differences between the three groups (db-RDA: pseudo- $F_{(2,64)} =$

1.7499, $p = 0.005$ for prevalence-based dissimilarity, and pseudo- $F_{(2,64)} = 1.8875$, $p = 0.005$ for abundance-based dissimilarity). The first db-RDA axis tended to separate control samples from both live and dead groups ($F_{(1,64)} = 2.2338$, $p = 0.012$ for prevalence-based dissimilarity, and $F_{(1,64)} = 2.4706$, $p = 0.007$ for abundance-based dissimilarity), while the second db-RDA axis was non-significant ($F_{(1,64)} = 1.2865$, $p = 0.113$ for prevalence-based dissimilarity, and $F_{(1,64)} = 1.3145$, $p = 0.122$ for abundance-based dissimilarity). GLMMs identified two ASVs from the genus *Helicobacter* and an unassigned Prevotellaceae, whose abundance increased in alive-in-trap mice compared to controls (Fig. S3). However, there was no ASV-level difference between dead-on-trap vs. alive-in-trap mice.

Comparison of faecal and CM

Alpha diversity of caecal samples did not differ significantly from that of faecal samples (LMM: $\Delta D.f = 1$, $\chi^2 = 2.7641$, $p = 0.0964$ for ASV richness, and $\Delta D.f = 1$, $\chi^2 = 3.1185$, $p = 0.07741$ for Shannon diversity). But, the observed number of ASVs did not correlate between the faeces and caecum of the same individual (Pearson $r = 0.2909$, $p = 0.2007$), while the Shannon index did show a significant, though weak, correlation (Pearson $r = 0.4821$, $p = 0.0269$; Fig. 3).

Composition of faecal samples was tightly correlated with composition of CM (Mantel's test: $r = 0.8473$, $p =$

0.001 for abundance-based dissimilarity, and $r = 0.8933$, $p = 0.001$ for prevalence-based dissimilarity; Fig. 3). Importantly, both faecal and caecal samples showed divergence in microbiota composition between mice families and strains, with the effect size consistent between the two sample types (Fig. S5). Similarly, divergence calculated with a mixed dataset (i.e. pairwise dissimilarities between caecal vs. faecal samples, excluding caecum-faeces pairs from the same individuals) was consistent, with divergence in caecal or faecal samples only (Fig. 4).

Despite FM and CM composition being tightly correlated, and unconstrained NMDS ordination not suggesting any pronounced compositional divergence (Fig. S5), db-RDA accounting for within-individual covariance revealed systematic differences in microbial content between the two sample types (db-RDA: pseudo- $F_{(1,20)} = 7.9361$, $p = 0.001$ for abundance-based dissimilarity and pseudo- $F_{(1,20)} = 4.6582$, $p = 0.001$ for prevalence-based dissimilarity; Fig. S5). Based on GLMM, we identified five ASVs from the genera *Lactobacillus*, *Bacteroides* and *Anaeroplasm* that were over-represented in faecal samples (Fig. S4).

Discussion

To achieve reliable high-throughput profiling of host-associated microbial communities, considerable research

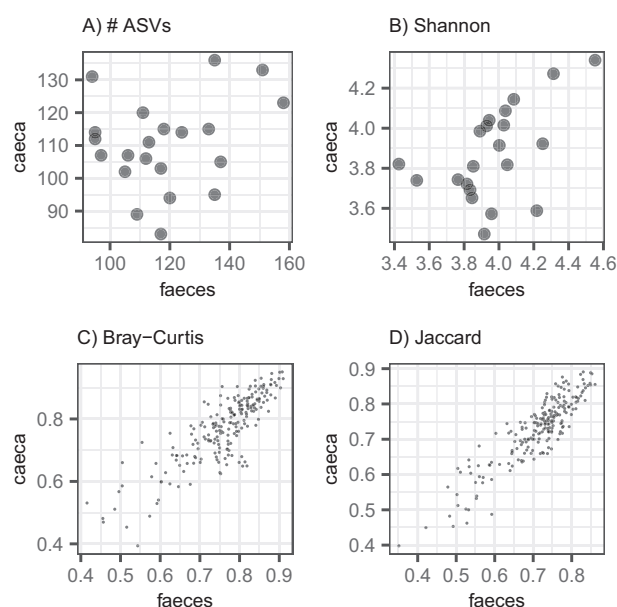


Fig. 3 Microbiota diversity and composition in caeca vs. faeces. Correlation in alpha diversity (A, B) and composition (C, D) between faecal and caecal samples obtained from the same individual (A, B) and from the same pair of individuals (C, D). Observed number of ASVs and Shannon diversity were used for alpha diversity analysis, while prevalence- (Jaccard) and abundance-based (Bray-Curtis) dissimilarities were used for compositional analysis.

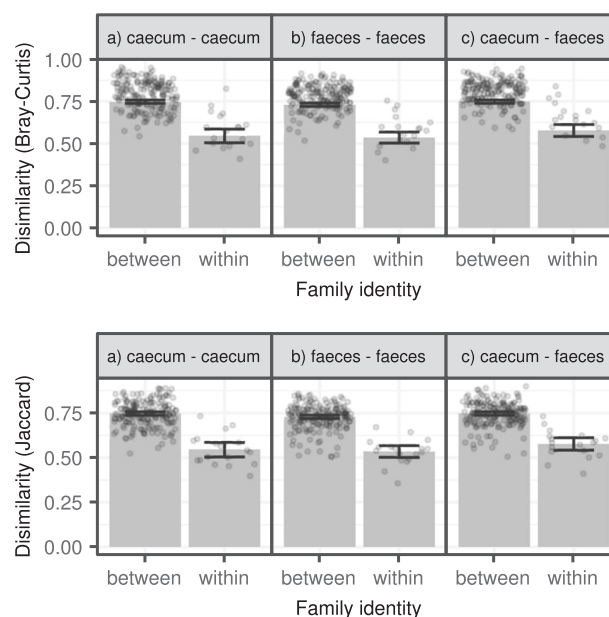


Fig. 4 Dissimilarity in microbiota composition between and within mouse families. Analysis was based on Bray-Curtis and Jaccard dissimilarities between individuals in A caecal microbiota, B faecal microbiota and C a combination of both sample types (excluding dissimilarities between the same sample types and the same individual). Also shown are actual observations, means and 95% bootstrap-based confidence intervals.

effort has been dedicated to the development and optimisation of suitable wet-laboratory, bioinformatic and statistical tools (reviewed in Pollock et al. 2018). Our study extends this endeavour by searching for optimal strategies for host-associated microbiota sampling in wild host populations.

Our data suggest that short-term exposure of live animals to the trap environment has a limited, but still measurable, effect on microbiota structure. In particular, two ASVs belonging to the genus *Helicobacter* and the Prevotellaceae family (Fig. S3) increased in animals that stayed in the traps overnight compared to those left in their cages. The fact that the difference was due to an abundance shift in bacteria already present in the mouse gut indicates that it was caused by altered physiology, possibly due to stress induced by enclosure in the trap, rather than exposure to a 'dirty' trap environment. In line with this reasoning, it has been shown that members of the Prevotellaceae family are involved in many aspects of host physiology, including homeostasis of energy metabolism, development of autoimmune diseases, response to hypoxia and acute and chronic stressors (De Filippo et al. 2010; Wu et al. 2011; Maslanik et al. 2012; Scher et al. 2013; Palm et al. 2014; Gorvitovskaia et al. 2016; Amaral et al. 2017; Suzuki et al. 2019; Stothart et al. 2019; Iljazovic et al. 2020). Nevertheless, to exclude the possibility of environmental transfer (though this is rather improbable for obligate anaerobes such as *Prevotella*), it would be necessary to include samples taken directly from the traps. To better distinguish between contaminating and resident gut bacteria, it would also be of great benefit to employ recent metabarcoding protocols based on the whole 16S rRNA region and long-read sequencing technologies (Earl et al. 2018; Callahan et al. 2019; Matsuo et al. 2020). Overall, it appears that overnight trapping with non-sterile live-traps has a negligible influence on the reliability of GM profiling.

Similarly, delayed microbiota sampling after the animal's death (16–18 h at room temperature) did not affect GM profiles. Clustering of GM samples followed the natural structure of the dataset (family membership, genetic background), with no visible effect of sampling delay. At the same time, there was no ASV over- or under-representation in the overnight-dead animals compared to those kept alive in traps. It follows, therefore, that sampling from fresh cadavers or snap-trapped individuals should provide reliable information on their GM content. When choosing between kill traps or live traps for the purposes of invasive GM sampling, animal welfare should be the main point considered as GM sample quality appears to be comparable under both settings. In this sense, use of snap traps prevents undue suffering from long-term enclosure in the trap, the resulting stress of which can sometimes prove fatal. Furthermore, snap trapping can be more efficient in terms of trapping success for many animal species. On the other hand, live traps allow for selection of the animals to be invasively sampled, i.e. non-target species, juveniles or

redundant individuals can all be released unharmed. Consequently, each study should carefully consider which traps to use based on their particular circumstances.

As in previous studies (Zhao et al. 2015; Yasuda et al. 2015; Weldon et al. 2015; Li et al. 2017; Ingala et al. 2018; Yan et al. 2019), FM composition differed from microbiota found in the caecum, which plays a crucial role in microbial fermentation processes in many mammalian species (Karasov and Douglas 2013), with faeces displaying enriched levels of the genera *Lactobacillus*, *Bacteroides* and *Anaeroplasm* (Fig. S4). Importantly, despite this difference, FM and CM were tightly correlated, both for ASV abundance and prevalence (Fig. 3). The proportionality of inter-individual composition differences between faeces and the caecum was further documented by the similarity in their association patterns. As with caecal samples, faeces followed the same biologically relevant variation patterns of the host, especially as regards divergence within and between social groups and genetic backgrounds, with divergence being similar for both sample types (Fig. S5). Somewhat surprisingly, similarity was even retained for mixed samples (i.e. divergence using faecal–caecal dissimilarity), suggesting that, for this type of analysis, the two sample types may be interchangeable (Fig. 4).

The average alpha-diversity of caecal samples, measured as number of observed ASVs and as Shannon index, did not differ significantly from that of faecal samples. However, at the individual level, alpha diversity in the caecum showed only weak or no correlations with alpha diversity in faeces. Taken together, these results suggest that faecal alpha diversity says little about caecal alpha diversity in the same individual, despite the population means of both communities being comparable.

Overall, we were able to show that faecal samples collected several hours after defecation were a suitable proxy for inter-individual differences in CM composition, but were a much worse proxy for differences in caecal alpha-diversity. Also, FM showed significant shifts in bacterial abundance compared to CM. Researchers should be aware of these two caveats if using the FM as a proxy of GM, and should consider their possible influence, depending on the type of analysis performed. Considering that FM sampling does not require the death of the animals studied, however, we encourage researchers to use FM, at least in correlative, divergence-based GM studies in wild animals similar to the house mouse. We further suggest that future studies should pay more attention to comparisons of FM–GM alpha-diversities in other wild-living species.

Data availability

Sequencing data associated with this study were archived in the European Nucleotide Archive, under the study accession

number PRJEB41530. Accession numbers of each sample are available in Table S1.

Acknowledgements This study was supported through the Czech Science Foundation, Project no. 19-19307S. Access to computing and storage facilities owned by parties and projects contributing to the National Grid Infrastructure MetaCentrum, provided under the programme ‘Projects of Large Infrastructure for Research, Development, and Innovations’ (LM2010005), is greatly appreciated. We also acknowledge the Genomics Core Facility, Central European Institute of Technology, Masaryk University, supported by the National Centre for Medical Genomics research infrastructure (LM2015091 funded by the Ministry of Education, Youth and Sports, Czech Republic). Especial thanks go to Filip Pardy and Boris Tichý for their support while obtaining the scientific data presented in this paper. The upkeep and management of the mouse colony were supported by the Czech Academy of Sciences under the Strategy AV 21 program.

Author contributions JK and DČ conceived the idea and designed the methodology; JP provided the experimental animals; DČ and LD collected the data; JK analysed the data; JK and DČ wrote the manuscript. All authors contributed to the drafts and gave final approval for publication.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

- Aivelo T, Norberg A (2018) Parasite-microbiota interactions potentially affect intestinal communities in wild mammals. *J Anim Ecol* 87:438–447
- Alberdi A, Aizpurua O, Bohmann K, Zepeda-Mendoza ML, Gilbert MTP (2016) Do vertebrate gut metagenomes confer rapid ecological adaptation? *Trends Ecol Evol* 31:689–699
- Amaral WZ, Lubach GR, Proctor A, Lyte M, Phillips GJ, Coe CL (2017) Social influences on *Prevotella* and the gut microbiome of young monkeys. *Psychosom Med* 79:888–897
- Amato KR, Sanders GJ, Song SJ, Nute M, Metcalf JL, Thompson LR et al. (2019) Evolutionary trends in host physiology outweigh dietary niche in structuring primate gut microbiomes. *ISME J* 13:576–587
- Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Ser B Methodol* 57:289–300
- Björk JR, Dasari M, Grieneisen L, Archie EA (2019) Primate microbiomes over time: longitudinal answers to standing questions in microbiome research. *Am J Primatol* 81:e22970
- Brooks JW (2016) Postmortem changes in animal carcasses and estimation of the postmortem interval. *Vet Pathol* 53:929–940
- Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP (2016) DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods* 13:581–583
- Callahan BJ, Wong J, Heiner C, Oh S, Theriot CM, Gulati AS et al. (2019) High-throughput amplicon sequencing of the full-length 16S rRNA gene with single-nucleotide resolution. *Nucleic Acids Res* 47:e103
- Clayton JB, Vangay P, Huang H, Ward T, Hillmann BM, Al-Ghalith GA et al. (2016) Captivity humanizes the primate microbiome. *Proc Natl Acad Sci U S A* 113:10376–10381
- Cryan JF, Dinan TG (2012) Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nat Rev Neurosci* 13:701–712
- De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S et al. (2010) Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc Natl Acad Sci U S A* 107:14691–14696
- Dill-McFarland KA, Neil KL, Zeng A, Sprenger RJ, Kurtz CC, Suen G et al. (2014) Hibernation alters the diversity and composition of mucosa-associated bacteria while enhancing antimicrobial defence in the gut of 13-lined ground squirrels. *Mol Ecol* 23:4658–4669
- Donaldson GP, Lee SM, Mazmanian SK (2016) Gut biogeography of the bacterial microbiota. *Nat Rev Microbiol* 14:20–32
- Dubois S, Fenwick N, Ryan EA, Baker L, Baker SE, Beausoleil NJ et al. (2017) International consensus principles for ethical wildlife control. *Conserv Biol J Soc Conserv Biol* 31:753–760
- Earl JP, Adappa ND, Krol J, Bhat AS, Balashov S, Ehrlich RL et al. (2018) Species-level bacterial community profiling of the healthy sinonasal microbiome using Pacific Biosciences sequencing of full-length 16S rRNA genes. *Microbiome* 6:190
- Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27:2194–2200
- Ericsson AC, Johnson PJ, Lopes MA, Perry SC, Lanter HR (2016) A microbiological map of the healthy equine gastrointestinal tract. *PLoS ONE* 11:e0166523
- García-Amado MA, Michelangeli F, Gueneau P, Perez ME, Domínguez-Bello MG (2007) Bacterial detoxification of saponins in the crop of the avian foregut fermenter *Opisthocomus hoazin*. *J Anim Feed Sci* 16:82–85
- Gomez A, Petrzalkova KJ, Burns MB, Yeoman CJ, Amato KR, Vlckova K et al. (2016) Gut microbiome of coexisting BaAka pygmies and Bantu reflects gradients of traditional subsistence patterns. *Cell Rep* 14:2142–2153
- Gomez A, Petrzalkova K, Yeoman CJ, Vlckova K, Mrázek J, Koppova I et al. (2015) Gut microbiome composition and metabolomic profiles of wild western lowland gorillas (*Gorilla gorilla gorilla*) reflect host ecology. *Mol Ecol* 24:2551–2565
- Gorvitovskaia A, Holmes SP, Huse SM (2016) Interpreting *Prevotella* and *bacteroides* as biomarkers of diet and lifestyle. *Microbiome* 4:15
- Gregorová S, Forejt J (2000) PWD/Ph and PWK/Ph inbred mouse strains of *Mus m. musculus* subspecies—a valuable resource of phenotypic variations and genomic polymorphisms. *Folia Biol* 46:31–41
- Gu S, Chen D, Zhang J-N, Lv X, Wang K, Duan L-P et al. (2013) Bacterial community mapping of the mouse gastrointestinal tract. *PLoS ONE* 8:e74957
- Heimesaat MM, Boelke S, Fischer A, Haag L-M, Loddenkemper C, Kühl AA et al. (2012) Comprehensive postmortem analyses of intestinal microbiota changes and bacterial translocation in human flora associated mice. *PLoS ONE* 7:e40758
- Hird SM (2017) Evolutionary biology needs wild microbiomes. *Front Microbiol* 8:725
- Ilijazovic A, Roy U, Gálvez EJC, Lesker TR, Zhao B, Gronow A et al. (2020) Perturbation of the gut microbiome by *Prevotella* spp. enhances host susceptibility to mucosal inflammation. *Mucosal Immunol* 14:113–124
- Ingala MR, Simmons NB, Wultsch C, Krampis K, Speer KA, Perkins SL (2018) Comparing microbiome sampling methods in a wild mammal: fecal and intestinal samples record different signals of host ecology, evolution. *Front Microbiol* 9:803
- Karasov WH, Douglas AE (2013) Comparative digestive physiology. *Compr Physiol* 3:741–783

- Kartzinel TR, Hsing JC, Musili PM, Brown BRP, Pringle RM (2019) Covariation of diet and gut microbiome in African megafauna. *Proc Natl Acad Sci U S A* 116:23588–23593
- Kohl KD, Dearing MD (2016) The woodrat gut microbiota as an experimental system for understanding microbial metabolism of dietary toxins. *Front Microbiol* 7:1165
- Kohl KD, Luong K, Dearing MD (2015) Validating the use of trap-collected feces for studying the gut microbiota of a small mammal (*Neotoma lepida*). *J Mammal* 96:90–93
- Kohl KD, Varner J, Wilkening JL, Dearing MD (2018) Gut microbial communities of American pikas (*Ochotona princeps*): Evidence for phyllosymbiosis and adaptations to novel diets. *J Anim Ecol* 87:323–330
- Kreisinger J, Bastien G, Hauffe HC, Marchesi J, Perkins SE (2015) Interactions between multiple helminths and the gut microbiota in wild rodents. *Philos Trans R Soc B Biol Sci* 370:20140295
- Kreisinger J, Čížková D, Vohánka J, Piálek J (2014) Gastrointestinal microbiota of wild and inbred individuals of two house mouse subspecies assessed using high-throughput parallel pyrosequencing. *Mol Ecol* 23:5048–5060
- Kreisinger J, Kropáčková L, Petrželková A, Adámková M, Tomášek O, Martin J-F et al. (2017) Temporal stability and the effect of transgenerational transfer on fecal microbiota structure in a long distance migratory bird. *Front Microbiol* 8:50
- Laukaitis CM, Critser ES, Karn RC (1997) Salivary androgen-binding protein (ABP) mediates sexual isolation in *Mus musculus*. *Evol Int J Org Evol* 51:2000–2005
- Lawrence K, Lam K, Morgun A, Shulzhenko NLöhr C (2019) Effect of temperature and time on the thanatomicrobiome of the cecum, ileum, kidney, and lung of domestic rabbits. *J Vet Diagn Invest* 31. <https://doi.org/10.1177/1040638719828412>
- Legendre P, Anderson MJ (1999) Distance-based redundancy analysis: testing multispecies responses in multifactorial ecological experiments. *Ecol Monogr* 69:1–24
- Li D, Chen H, Mao B, Yang Q, Zhao J, Gu Z et al. (2017) Microbial biogeography and core microbiota of the rat digestive tract. *Sci Rep* 7:45840
- Maslanik T, Tannura K, Mahaffey L, Loughridge AB, Bennison L, Ursell L et al. (2012) Commensal bacteria and MAMPs are necessary for stress-induced increases in IL-1 β and IL-18 but not IL-6, IL-10 or MCP-1. *PLoS ONE* 7:e50636
- Matsuo Y, Komiya S, Yasumizu Y, Yasuoka Y, Mizushima K, Takagi T et al. (2020) Full-length 16S rRNA gene amplicon analysis of human gut microbiota using MinIONTM nanopore sequencing confers species-level resolution. *bioRxiv*. <https://doi.org/10.1101/2020.05.06.078147>
- McKenzie VJ, Song SJ, Delsuc F, Prest TL, Oliverio AM, Korpita TM et al. (2017) The effects of captivity on the mammalian gut microbiome. *Integr Comp Biol* 57:690–704
- McMurdie PJ, Holmes S (2014) Waste not, want not: why rarefying microbiome data is inadmissible. *PLOS Comput Biol* 10: e1003531
- Menke S, Meier M, Sommer S (2015) Shifts in the gut microbiome observed in wildlife faecal samples exposed to natural weather conditions: lessons from time-series analyses using next-generation sequencing for application in field studies. *Methods Ecol Evol* 6:1080–1087
- Miller AW, Oakeson KF, Dale C, Dearing MD (2016) Microbial community transplant results in increased and long-term oxalate degradation. *Micro Ecol* 72:470–478
- Pafčo B, Čížková D, Kreisinger J, Hasegawa H, Vallo P, Shutt K et al. (2018) Metabarcoding analysis of strongylid nematode diversity in two sympatric primate species. *Sci Rep* 8:5933
- Palm NW, de Zoete MR, Cullen TW, Barry NA, Stefanowski J, Hao L et al. (2014) Immunoglobulin A coating identifies colitogenic bacteria in inflammatory bowel disease. *Cell* 158:1000–1010
- Pechal JL, Schmidt CJ, Jordan HR, Benbow ME (2018) A large-scale survey of the postmortem human microbiome, and its potential to provide insight into the living health condition. *Sci Rep* 8:5724
- Pollock J, Glendinning L, Wisedchanwet T, Watson M (2018) The madness of microbiome: attempting to find consensus “best practice” for 16S microbiome studies. *Appl Environ Microbiol* 84:e02627–17
- Quast C, Priesse E, Yilmaz P, Gerken J, Schweer T, Yarza P et al. (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* 41:D590–D596
- R Core Team (2018) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>
- Rosshart SP, Vassallo BG, Angeletti D, Hutchinson DS, Morgan AP, Takeda K et al. (2017) Wild mouse gut microbiota promotes host fitness and improves disease resistance. *Cell* 171:1015–1028.e13
- Round JL, Mazmanian SK (2009) The gut microbiome shapes intestinal immune responses during health and disease. *Nat Rev Immunol* 9:313–323
- Scher JU, Szczesnak A, Longman RS, Segata N, Ubeda C, Bielski C et al. (2013) Expansion of intestinal *Prevotella copri* correlates with enhanced susceptibility to arthritis. *eLife* 2:e01202
- Sommer F, Ståhlman M, Ilkayeva O, Arnemo JM, Kindberg J, Josefsson J et al. (2016) The gut microbiota modulates energy metabolism in the hibernating brown bear *Ursus arctos*. *Cell Rep* 14:1655–1661
- Stalder GL, Pinior B, Zwirzitz B, Loncaric I, Jakupović D, Vetter SG et al. (2019) Gut microbiota of the European Brown Hare (*Lepus europaeus*). *Sci Rep* 9:2738
- Stanley D, Geier MS, Chen H, Hughes RJ, Moore RJ (2015) Comparison of fecal and cecal microbiotas reveals qualitative similarities but quantitative differences. *BMC Microbiol* 15:51
- Stearns JC, Lynch MDJ, Senadheera DB, Tenenbaum HC, Goldberg MB, Cvitkovitch DG et al. (2011) Bacterial biogeography of the human digestive tract. *Sci Rep* 1:170
- Stothart MR, Palme R, Newman AEM (2019) It's what's on the inside that counts: stress physiology and the bacterial microbiome of a wild urban mammal. *Proc R Soc B Biol Sci* 286:20192111
- Suzuki TA, Martins FM, Nachman MW (2019) Altitudinal variation of the gut microbiota in wild house mice. *Mol Ecol* 28:2378–2390
- Suzuki TA, Nachman MW (2016) Spatial heterogeneity of gut microbial composition along the gastrointestinal tract in natural populations of house mice (EG Zoetendal, Ed.). *PLoS ONE* 11: e0163720
- Tanca A, Manghina V, Fraumene C, Palomba A, Abbondio M, Deligios M et al. (2017) Metaproteogenomics reveals taxonomic and functional changes between cecal and fecal microbiota in mouse. *Front Microbiol* 8:391
- Tang Q, Jin G, Wang G, Liu T, Liu X, Wang B et al. (2020) Current sampling methods for gut microbiota: a call for more precise devices. *Front Cell Infect Microbiol* 10:151
- Tang W, Zhu G, Shi Q, Yang S, Ma T, Mishra SK et al. (2019) Characterizing the microbiota in gastrointestinal tract segments of *Rhabdophis subminiatus*: dynamic changes and functional predictions. *MicrobiologyOpen* 8:e789
- Trevelline BK, Fontaine SS, Hartup BK, Kohl KD (2019) Conservation biology needs a microbial renaissance: a call for the consideration of host-associated microbiota in wildlife management practices. *Proc R Soc B Biol Sci* 286:20182448
- Tuomisto S, Karhunen PJ, Pessi T (2013) Time-dependent post mortem changes in the composition of intestinal bacteria using real-time quantitative PCR. *Gut Pathog* 5:35
- Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444:1027–1031

- Vasemägi A, Visse M, Kisand V (2017) Effect of Environmental Factors and an Emerging Parasitic Disease on Gut Microbiome of Wild Salmonid Fish. *mSphere* 2:e00418–17
- Videvall E, Strandh M, Engelbrecht A, Cloete S, Cornwallis C (2017) Measuring the gut microbiome in birds: Comparison of faecal and cloacal sampling. *Mol Ecol Resour* 18:424–434
- Vlčková K, Shutt-Phillips K, Heistermann M, Pafčo B, Petrželková KJ, Todd A et al. (2018) Impact of stress on the gut microbiome of free-ranging western lowland gorillas. *Microbiol Read Engl* 164:40–44
- Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 73:5261–5267
- Wang J, Linnenbrink M, Künzel S, Fernandes R, Nadeau M-J, Rosenstiel P et al. (2014) Dietary history contributes to enterotype-like clustering and functional metagenomic content in the intestinal microbiome of wild mice. *Proc Natl Acad Sci U S A* 111:E2703–2710
- Warne RW, Kirschman L, Zeglin L (2017) Manipulation of gut microbiota reveals shifting community structure shaped by host developmental windows in amphibian larvae. *Integr Comp Biol* 57:786–794
- Weldon L, Abolins S, Lenzi L, Bourne C, Riley EM, Viney M (2015) The gut microbiota of wild mice. *PLoS ONE* 10:e0134643
- Wu GD, Chen J, Hoffmann C, Bittinger K, Chen Y-Y, Keilbaugh SA et al. (2011) Linking long-term dietary patterns with gut microbial enterotypes. *Science* 334:105–108
- Yan W, Sun C, Zheng J, Wen C, Ji C, Zhang D et al. (2019) Efficacy of fecal sampling as a gut proxy in the study of chicken gut microbiota. *Front Microbiol* 10:2126
- Yasuda K, Oh K, Ren B, Tickle TL, Franzosa EA, Wachtman LM et al. (2015) Biogeography of the intestinal mucosal and luminal microbiome in the rhesus macaque. *Cell Host Microbe* 17:385–391
- Zemanova MA (2019) Poor implementation of non-invasive sampling in wildlife genetics studies. *Rethink Ecol* 4:119–132
- Zemanova MA (2020) Towards more compassionate wildlife research through the 3Rs principles: moving from invasive to non-invasive methods. *Wildl Biol* 2020. <https://doi.org/10.2981/wlb.00607>
- Zhao W, Wang Y, Liu S, Huang J, Zhai Z, He C et al. (2015) The dynamic distribution of porcine microbiota across different ages and gastrointestinal tract segments. *PLoS ONE* 10:e0117441
- Zilber-Rosenberg I, Rosenberg E (2008) Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. *FEMS Microbiol Rev* 32:723–735