Unraveling Differences in Fecal Microbiota Stability in Mammals: From High Variable Carnivores and Consistently Stable Herbivores

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Abstract

Background

Through the rapid development in third generation sequencing methods and tools, microbiome studies on a various number of species were performed during the last decade. This advance makes it possible to analyze hundreds of samples from different species or animal groups at the same time in order to obtain a general overview of the microbiota. However, there is still uncertainty on the variability of the microbiota of different animal groups and on whether certain bacteria within a species are subject to greater fluctuations than others. This is largely due to the fact that the analysis in most extensive comparative studies is based on only a few samples per species or per study site. In our study, we aim to close this knowledge gap by analyzing multiple individual samples per species from four representative mammalian groups: Ruminantia, Perissodactyla, Canoidea and Feloidea held in different zoos. To assess microbial diversity, 621 fecal samples from 31 species were characterized by sequencing the V3-V4 region of the 16S rRNA gene using Illumina MiSeq.

Results

We found significant differences in the consistency of microbiota composition and in fecal microbial diversity between carnivore and herbivore species. Whereas the microbiota of carnivores is highly variable and inconsistent within and between species, herbivores show less differences across species boundaries. Furthermore, low-abundant bacterial families show higher fluctuations in the fecal microbiota than high-abundant ones.

Conclusions

Our data suggest that microbial diversity is significantly higher in herbivores than in carnivores, whereas the microbiota in carnivores, unlike in herbivores, varies widely even within species. This high variability has methodological implications and underlines the need to analyze a minimum amount of samples per species. In our study, we found considerable differences in the occurrence of different bacterial families when looking at just three and six samples. However, from a sample number of 10 onwards, these variations greatly decreased in most cases and led to constant and more reliable results.

Background

Due to intensive research in the field of microbiome science and further development of 3rd generation sequencing, the tasks and importance of gastrointestinal microorganisms, especially the production of short-chain fatty acids (SCFA) serving the host organism as energy supply are now well described [1, 2, 3]. In recent years, a lot of research has been conducted to analyze the composition and factors influencing the microbiome for various animal groups using two different approaches. The first often-used study

design focusses on a single species or on a specific animal group. Here, multiple samples per individual or species are analyzed representing one or several time points. Especially farm animals e.g. cattle [4, 5, 6], pigs [7, 8, 9] or sheep [10, 11], have been largely analyzed due to their importance in agriculture. The microbiota of some exotic species, especially highly endangered species such as black rhinos [12], koalas [13] or Tasmanian devils [14], has also been described in more detailed studies. The advantage of this study design is that the microbial composition and diversity of the species studied can be compiled in detail and comprehensively. Moreover, further factors influencing the microbial composition can also be determined in in-depth statistical analyses.

The second study design focusses on an overall comparison within or between groups of animals e.g. terrestrial [15, 16, 17, 18, 19] and marine mammals [20], marsupials [21] or birds [22]. In contrast to the former approach, studies involving a large number of species are usually based on a smaller number of samples per species or collection site. A possible disadvantage of this approach could be a bias in the results of these analyses due to the limited number of samples per species studied. Especially for carnivore studies, there are notable inconsistencies across different studies. For instance, two lion samples show a dominance of *Fusobacteria* and *Firmicutes* in one study [17], while three lion samples of another one lack of *Fusobacteria* and instead contain *Actinobacteria* [16]. A similar pattern occurs in studies on different tiger and fox subspecies. While about half of the samples in one study [17] consist of *Proteobacteria* and *Fusobacteria* respectively, another study found large differences for those microbial families [23]. The abovementioned examples raise the question of whether a minimum number of samples is needed to describe the microbiota of a carnivore species. In addition, the issue remains whether there are systematic groups of animals that are more susceptible to microbial fluctuations, or whether this is due to specific bacterial species.

We aim to integrate the abovementioned approaches by analyzing a comprehensive dataset of four major mammalian groups (Canoidea, Feloidea, Perissodactyla and Ruminantia) to identify differences within or between those. As representatives of these groups each have a characteristic digestive system and rely on a different diet, these groups are well suited to test for variation in their microbial composition. The digestive tract of the Carnivora is short and - beside that of the Insectivora - also one of the least complex among mammals. It is characterized by a short intestine and colon as well as a small cecum. Carnivora are among the hindgut fermenter, which have the highest microbial activity in the appendix, colon and rectum [24, 25]. In general, individuals of this order show only slight adaptations to microbial fermentation, since they rely on easily digestible protein-rich nutrition and have lower glucose needs [26, 27]. Analyses of 16S rRNA gene have shown a low bacterial diversity in the stomach of carnivores but that diversity increases steadily within the subsequent intestinal sections [28]. In contrast to carnivores, herbivores such as Perissodactyla and Ruminantia depend on microbial fermentation for cellulose degradation. To enable fermentation, many herbivores have an enlarged, compartmented stomach or caecum as the place for microbial fermentation as well as a longer retention time in the digestive tract [29, 30].

In order to create such a widespread dataset, microbiome analyses of zoo-housed animals are suitable in different ways. First, it is necessary to know as many boundary conditions as possible to create a representative dataset using multiple samples per species, individuals and collection sites. In this regard, zoos offer a nearly perfect environment because the general conditions such as nutrition, age and pedigree of the animals are well-known. Second, microbiome research is of great interest for the zoos to improve animal welfare. Finally, the microbiota influences a variety of physiological and behavioral processes and, accordingly, a healthy microbiota is correlated with an animal's fitness. Other aspects that are largely unclear so far include possible changes in the microbiome in specific situations such as animal transport, animal socialization or feed conversion. With a meaningful dataset, deviations from the species-specific references can be identified and potential treatments initiated.

Results

In total we sequenced 621 fecal samples of 31 zoo-housed carnivore and herbivore species. After quality filtering and read merging, the dataset consists of 29,777,361 sequences with an average of 47,796 sequences per sample. Following the DADA2 pipeline in QIIME 2, we identified 21,058 different features across all samples (2,315 to 134,414 features per sample). The most common feature is represented 686,398 times in 292 samples.

Composition of fecal microbiota of major mammalian groups

We found significant differences between herbivores and carnivores in the microbial composition (ANOSIM statistic: $R = 0.50$, $p < 0.001$, number of permutations: 999, distance="bray") as shown in Fig. 1B. The four major bacterial families across all herbivore species are *Spirochaetaceae*, *Lachnospiraceae*, *Rikenellaceae* and *Oscillospiraceae*. *Spirochaetaceae* represent on average 15.9% of the microbiota of herbivore species and within these, they are twice as common in Perissodactyla than in ruminants. While this family is equally distributed across perissodactylan species, within the ruminants it only occurs in larger proportions in giraffes and okapis. In contrast, we found on average 20.2% of *Lachnospiraceae* in Perissodactyla and only 11.2% in ruminants, where larger proportions were observed in reindeer. *Rikenellaceae*, the third most-common family in herbivorous species, constitutes on average to 16.1% of the fecal microbiota of ruminants and to 12.6% that of Perissodactyla. With respect to the *Oscillospiraceae*, we found notable differences between both herbivore groups. While this family is equally spread across nearly all ruminants, it only appears in tapirs and black rhinoceros in greater proportions of all Perissodactyla. Besides those four major families, we identified *Bacteroidaceae* in many ruminants and an uncultured bacterium *p-251-o5* of the Bacteroidales order in Perissodactyla, especially in the grevy's zebras. Other bacterial families such as *Tanerellaceae*, *Erysipelotrichaceae*, *Clostridiaceae*, *Fusobacteriaceae* and *Enterobacteriaceae* constitute on average less than 5% to the microbiota across all herbivore species.

The most dominant bacterial family in carnivore species is *Fusobacteriaceae*, occurring on average in 23.2% of all Felidae and in 23.3% of all Canidae. However, within the Canidae, this family is low-abundant

in red pandas and brown bears as it constitutes to less than 5% of both fecal microbiotas. The distribution of *Clostridiaceae*, the second dominant family in carnivores, is on average similar for Felidae and Canidae. *Clostridiaceae* form a large proportion of the microbiota, accounting for more than 30%, in both bears and red pandas. Those species also differ from other Canidae with regard to *Bacteroidaceae*. Whereas this family is frequently found in most carnivores, it is low-abundant (< 2%) in the red pandas, brown bears, polar bears and fossas. Additionally, we found on average 16.0% *Peptostreptococcaceae* in Felidae and only 9.9% of this family in Canidae, but the value calculated for Felidae is mostly influenced by its high abundance of 33.0% in fossas. Beside these major bacterial families in carnivores, some others are largely represented in both bear species and red pandas. For example, we found that *Enterobacteriaceae* contribute on average 25.3% to the fecal microbial composition in red pandas, to 22.7% in polar bears and to 20.4% in brown bears. Furthermore, *Erysipelotrichaceae* are more dominant in brown bears and red pandas than in other Canidae. With regard to the Felidae, *Lachnospiraceae* are another dominant family being equally distributed across all sampled felid species. Other bacterial families such as *Spirochaetaceae*, *Lachnospiraceae*, *Rikenellaceae* and *Oscillospiraceae*, which were dominant in herbivorous species, accounted for less than 5% of the carnivore microbiota.

Microbial diversity within and between herbivores and carnivores

The microbial diversity measured by effective number of species differs highly significantly between carnivores and herbivores (Fig. 1C) (Kruskal-Wallis: $p < 0.001$, $df = 3$, Dunn Test with Bonferroni correction $p < 0.001$). Also, these differences are highly significant regarding the Shannon index and species richness (Fig. 2). Consequently, carnivorous species show a strongly reduced microbial diversity compared to herbivorous species. In contrast, alpha diversity within the two groups is quite similar across all measurements. For example, within the Caniformia the mean ENS per species varies between 51 in polar bears and 229.2 in arctic foxes, which is comparable to the variation within the Feliformia (94.5–217.6). In contrast ENS values in herbivores are much higher, ranging between 679 in black rhinoceros and 2,869.9 in the plains zebras and between 1,036.2 in common elands and 2107.6 in bongos for ruminants respectively.

Regarding the beta diversity, the principal coordinate analysis (PCoA) of the weighted UniFrac distance matrix explains a total of 63.3% of data variability within the first three main axes. The permutation test for homogeneity of multivariate dispersions shows homogeneity within the four animal groups ($F = 0.670$, $p = 0.570$, permutations = 999) as well as within carnivores and herbivores respectively ($F = 1.345$, $p = 0.250$, permutations = 999). The following ADONIS test for differences between each group was significant for the four species groups ($R^2=0.020$, $p < 0.001$, permutations = 999) but not for the diet type ($R^2=0.004$, $p = 0.065$, permutations = 999), which illustrates the differences between carnivore and herbivore species. This is also confirmed by the PCoA of the unweighted UniFrac measurement (Fig. 3). Similar to the weighted UniFrac, the homogeneity of dispersion is given for both, the animal groups ($F = 0.670$, $p = 0.570$, permutations = 999) and diet types ($F = 1.345$, $p = 0.260$, permutations = 999). For this metric, we found significant differences between diet types (ADONIS: $R^2=0.004$, $p < 0.05$, permutations = 999) and animal groups ($R^2=0.020$, $p < 0.001$, permutations = 999). Even if slightly less data variability

(46.3%) is explained by this distance matrix, it shows a recognizable pattern within the Carnivora. At a group-specific level, the Carnivora are divided into three clusters (Fig. 3B). The first cluster, closest to the Perissodactyla, consists of the polar and brown bear as well as the red panda samples. A little distant from these lies the center of the second cluster, made of the big and small cats as well as the South American Cerdocyonina represented by the bush dog and maned wolf samples. Finally, the third cluster, which is most distant from the herbivorous species is composed of the Vulpini group (fennec fox, arctic fox, bat-eared fox) and the African wild dog samples. Overall, it can be observed that the samples of the Perissodactyla and Ruminantia are more similar to each other than those of the carnivorous species.

For a more detailed analysis of this high variation within the Carnivora, which is in contrast to the herbivore species, we compared one representative species for each of the four groups with respect to the taxonomic assignment. Figure 4 and Fig. 5 show all samples of lions, brown bears, wildebeests and plains zebras. The results clearly show that the variability within the zebra and wildebeest samples is lower compared to the lion and brown bear samples. More detailed, we identified 19 microbial families that consist to more than 5% of the microbiota within the brown bear samples across four zoos. Those families vary largely in their occurrence, which is measured by the coefficient of variation (CV). Thus, families occurring in at least five samples which show the greatest fluctuations are *Staphylococcaceae* (2.0% ± 6.6%, CV = 3.2) which are represented in seven brown bear samples across half of the sampled zoos. In addition to this family, *Moraxellaceae* (3.1% ± 7.9%, CV = 2.5), *Streptococcaceae* (3.0% ± 6.7%, CV = 2.2) and *Lachnospiraceae* (2.7% ± 5.8%, CV = 2.1) also show large deviations within the bear samples. Bacterial families which make up greater proportion of the microbiota and are more common across all samples appear to be more stable. Some of those are *Erysipelotrichaceae* (10.2% ± 9.8%, CV = 1.0), *Peptostreptococcaceae* (15.9% ± 11.2%, CV = 0.7) and *Clostridiaceae* (25.0% ± 14.3%, CV = 0.6). The lion samples show a similar pattern, for which we identified 21 bacterial families (> 5%). Again, *Peptostreptococcaceae* (11.0% ± 9.6%, CV = 0.9) as a common family in all samples, as well as *Lachnospiraceae* (7.5% ± 6.2%, CV = 0.8) and *Fusobacteriaceae* (17.8% ± 12.1%, CV = 0.7) are among the most stable bacterial families in the lion microbiota. In contrast *Coriobacteriaceae* (1.8% ± 3.5%, CV = 1.9), *Erysipelotrichaceae* (5.0% ± 7.8%, CV = 1.6) and *Enterobacteriaceae* (5.7% ± 10.7%, CV = 1.9) are largely responsible for the high variability within the samples.

In contrast, the zebra samples show less variance in their taxonomic composition. Here, the greatest variability occurs among the *Bacillaceae* (2.0% ± 4.9%, CV = 2.4), *Oscillospiraceae* (1.8% ± 3.0%, CV = 1.7) and *Fibrobacteraceae* (1.6% ± 3.1%, CV = 1.9). In contrast, the most equally spread bacterial families are *Rikenellaceae* (7.2% ± 3.7%, CV = 0.5), *Spirochaetaceae* (15.6% ± 6.8%, CV = 0.4) and *Lachnospiraceae* (11.3% ± 3.6%, CV = 0.3). Similar to the plains zebra, the wildebeest samples also reveal less variability compared to carnivores. Again, the greatest variation is found in least abundant bacterial families. Within those are e.g. *Micrococcaceae* (3.0% ± 5.4%, CV = 1.8), *Clostridiaceae* (1.2% ± 2.3%, CV = 1.9) or *Moraxellaceae* (1.8% ± 3.7%, CV = 2.0). Compared to that, lesser variation between samples is found in families that are high-abundant as *Oscillospiraceae* (8.1% ± 2.9%, CV = 0.4), *Lachnospiraceae* (5.6% ± 2.3%, CV = 0.4) or *Prevotellaceae* (10.8% ± 5.5%, CV = 0.5). Further results are shown in detail in an additional file (see Additional file 2).

To further investigate the variability of the most abundant bacterial families in herbivores and carnivores, Fig. 6 shows the CV plotted against the number of samples and against the total percentage of occurrence. On the one hand, this underlines the fact that the CV of the most abundant bacterial families in herbivorous animals is in general lower than that of carnivorous animals. On the other hand, low-abundant bacterial families that are present in almost all fecal samples of an animal group, show greater deviations between samples (e.g. *Enterobacteriaceae*). In contrast, the CV for high-abundant families (e.g. *Clostridiaceae* and *Fusobacteriaceae*) is much lower. Considering the number of samples analyzed, it is noticeable that the CV does not necessarily decrease with regard to a larger number of samples being analyzed. To examine whether this effect is possibly due to species-specific differences, we created randomized subsets of bacterial families that occur in more than 7% of all herbivore or carnivore species, because low-abundant families seem to seem to have a higher variability per se as shown before. Within all species, this results in a decreased coefficient of variation as the number of samples increases (Fig. 7). In addition, species-specific differences become visible. For example, giraffes show a constantly low variability in both bacterial families, even when only a few samples are considered. In contrast, wildebeests and plains zebras are more variable when only a small number of samples are taken into account and first stabilize at a sample number of 15 in both analyzed bacterial families. Within carnivores, the tiger samples show a constant CV for all bacterial families from a sample number of n = 10. Even if the variability within the lion samples is higher compared to the tiger ones, they also become stable from a sample number of 10 onwards. Besides species-specific differences we also found differences in the variability between bacterial families in the brown bear. While the pattern for *Peptostreptococcaceae* and *Clostridiaceae* is the same as in tigers and lions, the high CV values of the *Fusobacteriaceae* is not noticeably declining with an increased sample size. Detailed results are shown in the additional file 3 (see Additional file 3).

Microbial indicators for herbivore and carnivore animals

Indicator families were analyzed for each of the four groups and each group combination using the IndVal.g function. We identified a total of 276 indicator families, most of them for herbivores, especially for Perissodactyla (Table 1). With 18 indicator families, Canoidea and Feloidea share less indicators than Perissodactyla and Ruminantia and only minor proportions of indicator families were found in combinations of herbivore and carnivore species. The complete results are presented in the additional file (see Additional file 5).

Table 1
Microbial indicators for different animal groups and their combination. Indicators were assigned at microbial family level.

Group	Number of indicator species
Canoidea	10
Feloidea	6
Perissodactyla	43
Ruminantia	16
Canoidea + Feloidea	18
Perissodactyla + Ruminantia	42
Canoidea + Perissodactyla	1
Canoidea + Ruminantia	3
Feloidea + Perissodactyla	2
Canoidea + Feloidea + Perissodactyla	3
Canoidea + Feloidea + Ruminantia	6
Canoidea + Perissodactyla + Ruminantia	4
Feloidea + Perissodactyla + Ruminantia	2

Almost all predicted indicator families show high A values, meaning that this indicator only occurs in the tested group, but is not necessarily spread across all of its members. In contrast, the B values, showing the distribution of an indicator across all group members are much more variable. Indicator families restricted to Canoidea are *Gemellaceae* (A = 1.00, B = 0.03) and *Xiphinematobacteraceae* (A = 1.00, B = 0.02), but they do not occur in all of the samples. Regarding the Feloidea, no exclusive indicators were found. However, *Coriobacteriaceae* (A = 0.88, B = 0.88) are strongly related to this group and distributed among nearly all members. In general, all indicator families associated to the carnivore groups show low B values which might be a further indication of greater diversity within the two groups as seen in the PCoA analysis. However, this view changes when one considers the indicator families that occur in both the Feloidea and the Canoidea. In particular, *Enterobacteriaceae* (A = 0.98, B = 0.94), *Clostridiaceae* (A = 0.96, B = 0.95) and *Fusobacteriaceae* (A = 0.99, B = 0.83) occur in almost all carnivore species and appear to be clear indicator families for those in general. Additionally, these families are also the most dominant ones in the fecal microbiota composition of carnivores (Fig. 1b).

In contrast, more indicator families were found in herbivores. *Fibrobacteraceae* (A = 0.81, B = 0.97), *Synergistaceae* (A = 1.00, B = 0.75), *Defluviitaleaceae* (A = 0.88, B = 0.80) and *Methanocorpusculaceae* (A = 0.79, B = 0.88) occur almost exclusively in Perissodactyla and are present in almost all species. For ruminant species, one of the most prominent indicators are *Barnesiellaceae* (A = 0.89, B = 0.72) and *Atopobiaceae* (A = 0.73, B = 0.46), which occur in many members of this group. Looking at the combined

indicators of Perissodactyla and ruminants, many microbial families are found almost exclusively in those two groups and are present in all of their members. Again, those indicator families are among the most dominant ones in the taxonomy plot (Fig. 1b) i.e. *Spirochaetaceae* (A = 0.99, B = 1.00), *Rikenellaceae* (A = 0.96, B = 0.99) and *Oscillospiraceae* (A = 0.87, B = 0.90).

Discussion

We found significant differences in the fecal microbial composition between herbivore and carnivore species. The most dominant bacterial families found in herbivore species are *Spirochaetaceae*, *Lachnospiraceae*, *Rikenellaceae* and *Oscillospiraceae*. The first two mentioned occur more frequently in Perissodactyla, whereas the latter two appear on average more often in ruminants. Those results are in line with the in-depth study on African herbivores [19], who also found *Oscillospiraceae* as the most dominant family in ruminants such as giraffes, cattle or hartebeests. Nevertheless, our study showed greater proportions of *Rikenellaceae* in ruminants. Both, *Oscillospiraceae* and *Rikenellaceae*, have recently been characterized as herbivore specific bacteria in a covariance network analysis [18], with *Oscillospiraceae* being a major player in cellulose degradation and therefore being related to a herbivore and fiber-rich diet [31]. Another link to the study on African herbivores [19] is the appearance of *Spirochaetaceae*, especially in zebras as representatives of Perissodactyla. Similar to *Oscillospiraceae*, this family is responsible for fiber digestion and therefore essential for the herbivore digestive system [32, 33]. Besides *Spirochaetaceae*, we found *Lachnospiraceae* as another main family in Perissodactyla. This family has been detected in the human intestine as well as in the rumen and digestive system of different mammals [34, 35]. Bacteria belonging to this family such as *Roseburia* or *Lachnospira* are involved in the production of SCFAs by hydrolyzing sugars (e.g. starch) and were found to be associated with the consumption of plant protein and fiber [36, 37]. Additionally, the abundance of *Lachnospiraceae* can decrease with regard to a high-protein diet indicating a minor role in protein metabolism [38]. Those major bacterial families found in herbivorous animals are mainly capable of carbohydrate digestion like starch or maltose allowing the host to gain enough energy from the plant-based diet.

In contrast, the main bacterial families found in carnivore species are *Fusobacteriaceae*, *Clostridiaceae*, *Bacteroidaceae* and *Peptostreptococcaceae*. *Fusobacteriaceae* are often linked to a high-fat and protein-based diet and were observed in different carnivores with *Fusobacterium* previously being classified as a carnivore specific bacterium [18, 39]. This bacterial family is able to produce SCFAs using carbohydrates or amino acids [41] and it has been shown that *Fusobacteriaceae* are more common in carnivorous Carnivora than in omnivorous or herbivorous Carnivora [40], which is consistent with our study. Both, *Clostridiaceae* and *Bacteroidaceae*, being dominant in carnivore families in our study have already been detected in the gastrointestinal microbiota of different predators [18, 39, 42, 43]. While *Clostridiaceae* appear to be important for protein metabolism, *Bacteroidaceae* occur in combination with a fiber-rich diet and are not affected by protein intake [44, 45, 46]. In summary, our results show the highest proportion of *Bacteroidaceae* in bat-eared foxes as well as the highest proportion of *Clostridiaceae* in polar bears, which partly matches this theory. However, we could not find major differences for these two bacterial families.

Beside significant differences in the taxonomic assignment between carnivore and herbivore mammals, we also found a significantly higher microbial alpha diversity in herbivore species compared to carnivores. This might be due to the more complex digestive system of herbivorous species and their dependence on microbes to break down cellulose. This relationship has been shown previously for several species [23, 35, 39, 40, 47]. Furthermore, herbivorous mammals are known to rely on microbial metabolic pathways to a greater extent than carnivores [18].

In addition to confirming previous studies on the carnivore microbiota, we have also found some species that deviate from previous assumptions, namely both bear species, the red panda as well as the fossa. Contrary to the other carnivores, *Fusobacteriaceae* only occur in minor proportions within red pandas and brown bears, but *Erysipelochtrichaceae* are enriched in this animal. Furthermore, both bear species as well as the red pandas consist of major proportions of *Enterobacteriaceae* but only of minor proportions of *Bacteroidaceae* - similar to the fossa. Within the PCoA plot of beta diversity (Fig. 3B), the fossa samples lie within those of other felids, whereas the two bear species as well as the red pandas form a separate cluster apart from the Feloidea and the Canidae. The most influencing factors for fecal microbiota composition are described to be diet and phylogeny [17, 32, 48]. Because the omnivorous diet of the analyzed bears was similar to that of the other Canidae, and even the red pandas were fed an omnivore diet in half of the analyzed zoos, it is unlikely, that this separation is mainly influenced by diet. Another factor influencing the microbial composition is the host phylogeny. Bears, red pandas and fossa all evolved separated from other members of the respective group. The fossa as a Malagasy carnivore evolved distinct from other Felidae as a sister group to the Herpestidae about 18–24 Mya ago [49, 50]. Regarding the Caniformia, the Arctoidea clade split in a rapid radiation about 43 Mya in three superfamilies Ursoidea, Pinnipedia and Musteloidea. Within these, the Ursidae evolved about 18 Mya ago, whereas the Ailuridae evolved about 33 MYA ago as a sister clade to Mephitidae, Procyonidae and Mustelidae [51, 52, 53, 54]. In recent years, the theory of co-evolution between host and microbes arose and continues to be proven. It states that bacterial symbionts adapt to e.g. dietary changes of the host and the host in turn adapts to the changed microbiota [16, 17, 55, 56]. Although this was not analyzed in this study, our results may suggest a co-evolution between gut microbes and host phylogeny in different mammalian groups. Furthermore, the results indicate that there are clear differences between herbivore and carnivore species but that there are several deviations from previously published gut microbiotas.

Close similarity in the fecal microbiota of herbivores and great diversity within the carnivores

Beside significant differences between herbivore and carnivore species, our results reveal a closer similarity in the fecal microbiota of herbivores compared to higher deviations in carnivores. Although there are several studies that describe either a distinct clustering of herbivores and carnivores due to differences in diet or phylogeny or a clustering of herbivorous carnivores to other Carnivora [16, 23, 39, 40, 48], none of them has yet referred to the expansion of these clusters. On the other hand, we found much more indicator species for herbivores than for carnivores and the carnivore indicators are not distributed across all species. This finding confirms the deviations within the carnivores. Furthermore, these differences can be seen using example species from each group (Figs. 4 and 5). Here, the general

variability of microbial families occurring in at least 5% per sample is lower in wildebeests and zebras than in brown bears and lions. In addition, the coefficient of variation is much higher in low-abundant microbial families compared to high-abundant families. One explanatory approach for the higher deviation in carnivores is the diet. While herbivores are mostly fed on hay, alfalfa or grass throughout the year, the diet and its composition is more variable in carnivores. Especially omnivorous carnivores such as most Canoidea are fed on a variety of food sources as fresh and kibble meat, fruits, vegetables or insects. But even hypercarnivore species undergo daily changes in meat origin or preparation (e.g. whole-body or sheer meat). For canids and felids, it is proven that the fecal microbiota is greatly altered by diet and dietary changes. Especially changes in the proportion of carbohydrates and protein influence the necessary gut bacteria, i.e. *Prevotella* or *Fusobacteria* respectively [42, 43, 57, 58, 59].

These differences in the microbial variability of carnivorous fecal samples also have important methodological implications. It is therefore necessary to adapt the number of samples being analyzed to the species to be studied in order to obtain meaningful results. Herbivores are very similar in terms of their microbial composition. In ruminants, *Oscillospiraceae*, *Lachnospiraceae*, and *Rikenellaceae* appear to dominate as the major bacterial families [16, 19] and this is evident in studies using different sample sizes. For example, the core results of a study on five giraffe samples are consistent to a similar study on more than 50 giraffe samples and the same pattern can be seen in regard to studies on elands [19, 23, 60] or zebras representing Perissodactyla [16, 19]. These results are in line with the low CV that we found in herbivores. Nevertheless, we found species-specific differences for the major bacterial families within herbivores as well. In particular, giraffes show very low variability in *Rikenellaceae* and *Prevotellaceae*, so these differences should be visible even in very few samples analyzed. In contrast, wildebeest samples are highly variable for those two families, resulting in the need to analyze at least 15 samples to control for this variations.

In contrast, the carnivore microbiota in general is much more variable which is expressed in a higher CV compared to that of herbivores. Especially within this group of animals, it is therefore important to analyze a reliable number of samples in order to characterize the microbiota. This is also illustrated by the fact that previous studies on carnivores yield significantly different results on the composition of the fecal microbiota. For example, studies using just two or three fecal fox, polar bear or bush dog samples [16, 23] found great differences in the proportion of *Prevotellaceae* and *Fusobacteriaceae*. The same pattern was observed for Feloidea, in studies on just a few cheetah and lion samples which could only detect minor proportions of *Fusobacteria*, whereas a study using more than 60 animals reported about 20% *Fusobacteria* in cheetahs [16, 61, 62, 63]. In this study, we found *Fusobacteriaceae* across all carnivore species in highly different proportions. Within the brown bear samples this family is present on average in 4.3%, which explains the high coefficient of variation even when using a high amount of samples. But also within the lion and tiger samples, in which the proportion of *Fusobacteriaceae* with an average of 18.3% and 23.5% is considerably higher, the CV for this family only becomes constant with 10 samples being analyzed (Fig. 7). This strengthens, on the one hand, our finding that low-abundant bacterial families are more variable in the fecal microbiota of mammals and, on the other hand, that it is

necessary to include multiple samples in the analysis as this reduces uncertainties that can occur with small numbers of samples (n = 3 or 6).

Considering the highly variable microbiota of Canoidea and Feloidea and the more constant microbiota of Ruminantia and Perissodactyla, it is important to select an appropriate number of samples for further analysis. Depending on the methodological approach, it should be noted that low-abundant bacterial families are often subject to greater fluctuations than high-abundant ones, and that there seem to be species-specific differences in microbiota variability within these animal groups.

Conclusions

To the best of our knowledge, this is the first study focusing on the microbiota variability of a wide range of carnivore and herbivore mammals by analyzing multiple samples per species in different locations. Our results support already existing theories such as a greater alpha diversity in herbivores or the general description of major bacterial families in both groups. Additionally, we found some species as the brown and polar bear, red panda or fossa that deviate from other members of their diet group. Phylogeny and host-microbe co-evolution may have a greater effect on fecal microbial composition here. In contrast, we show that the microbiota of ruminants and Perissodactyla are more similar within the respective group than carnivores. This results in a lower minimum number of samples that need to be analyzed to decipher the total fecal microbial diversity. For most of the bacterial families and animal species studied, our results show larger deviations when only a few samples (n = 3 or 6) are considered. In general, these deviations become smaller when 10 samples or more are considered and should thus be sufficient to provide a good insight into the fecal microbiota.

For further research, it will be interesting to investigate whether the greater variability of the Carnivora microbiota also applies in short-term time series analyses of a few days and which bacterial families remain constant or contribute to daily fluctuations in the fecal microbial composition.

Methods

Sample collection

Between April 2018 and August 2020, 621 samples were taken from 31 carnivore and herbivore species in a total of 20 German zoos (see Additional file 1). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the zoos. For each species, at least five samples across three different zoos were collected (except for *Vulpes lagopus*, *Equus zebra* and *Panthera onca*). Non-invasive sampling was mostly performed during the daily cleaning routines of the enclosures in cooperation with the keepers. Only fresh fecal samples of different individuals were collected in previously disinfected 50mL centrifuge tubes using sterile inoculation loops. When individual differentiation was not possible, fresh samples were collected from different locations in the enclosure to increase the likelihood that the samples are derived from different individuals in the mixed-species

enclosure. In the next step a subsample was taken from the center of the feces and transferred to a sterile 2mL cryotube, which was then immediately stored in liquid nitrogen. For further processing the samples were delivered to StarSEQ GmbH in Mainz, Germany. Here, the samples were preprocessed with the Precellys® Evolution Homogenizer (Bertin Instruments, Rockville, USA) and DNA extraction was performed using the QIAamp® PowerFecal DNA Kit (Qiagen, Hilden, Germany). The DNA concentration in all extracts was measured using a NanoDrop spectrophotometer (Thermofisher, Massachusetts, USA).

16S rRNA gene sequencing and data processing

PCR amplicons for the V3 - V4 region of the 16S rRNA gene were generated with primer pair 341F and 806R. Pooled amplicons were sequenced with the Illumina MiSeq 2×250 v3 kit for 600 cycles at StarSEQ GmbH. To control for sequencing quality, a 25% PhiX control library was added to the run. Samples were processed following the QIIME 2 [65] pipeline. After demultiplexing, DADA2 [66] was used to call exact sequence variants (ESVs) and a phylogenetic tree was inferred for all sequences based on a sequence alignment generated by MAFFT. Low-abundant features as well as chloroplast and mitochondrial sequences were removed from the dataset. The taxonomic assignment of ESVs was performed using a pre-trained naive Bayes classifier [67] based on SILVA 138 full-length sequences [68]. Features occurring in less than 10 sequences were removed from the dataset. The following statistics were performed in R version 3.6.3 [69] using the packages vegan [70] and FSA [71]. To test for differences in the taxonomic composition between the four mammalian groups, ANOSIM test was performed on dissimilarity matrices with Bray-Curtis distances. Alpha diversity was determined by Shannon index, the effective number of species (ENS) and richness which were calculated using QIIME2. Afterwards, differences between groups were tested using the Kruskal-Wallis test, followed by a post-hoc Dunn Test with Bonferroni correction in R. Beta diversity was also calculated in Qiime2 core-metrics on the ESV table using unweighted and weighted UniFrac distances. Subsequently, a test for homogeneity of dispersion and the Adonis test for differences between groups were performed on the four species groups as well as on diet type (herbivore, carnivore). To calculate differences in the occurrence of bacterial families within carnivores and herbivores, CV was applied to the respective major bacterial families. Furthermore, randomized subsets of the taxonomic assignment of the wildebeest, giraffe, plains zebra, tiger, brown bear and lion were created with sample numbers of n = 3, 6, 10, 15, 20 and 25 with three replicates each. For the most-abundant bacterial families the CV was calculated on those replicates. Furthermore, indicator species for each group were identified using the indicspecies R package [72]. The IndVal value calculates the associations between species and sites, followed by a permutation significance test (n = 999, $\alpha = 0.05$). Indicators were assigned at microbial family level. To create an approximate host phylogeny, the TimeTree database was used on the involved species names.

List Of Abbreviations

SCFA: Short chain fatty acids

ESV = exact sequence variant

ENS = effective number of species

CV = coefficient of variation

Declarations

Ethical Approval and Consent to participate

All procedures were performed in accordance with international guidelines and regulations for the use of animals in research.

Consent for publication

Not applicable.

Availability of supporting data

Raw amplicon sequencing data have been deposited on NCBI's SRA (sequence read archive; accession PRJNA716130). All other data generated or analyzed during the current study are included in the manuscript and its additional files.

Reviewer Link:

https://dataview.ncbi.nlm.nih.gov/object/PRJNA716130?reviewer=nddpiikt6t8ivk912j6kmi1nib

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceptualization and Study design: FZ, ALB and PWD; Writing original draft: FZ; Data collection: FZ; Bioinformatic and statistical analysis: FZ; Data interpretation and Visualization: FZ, ALB and PWD; Project administration and Funding acquisition: PWD; Review and editing: FZ, ALB and PWD.

All authors read and approved the final manuscript.

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Authors' information

Not applicable

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Figures

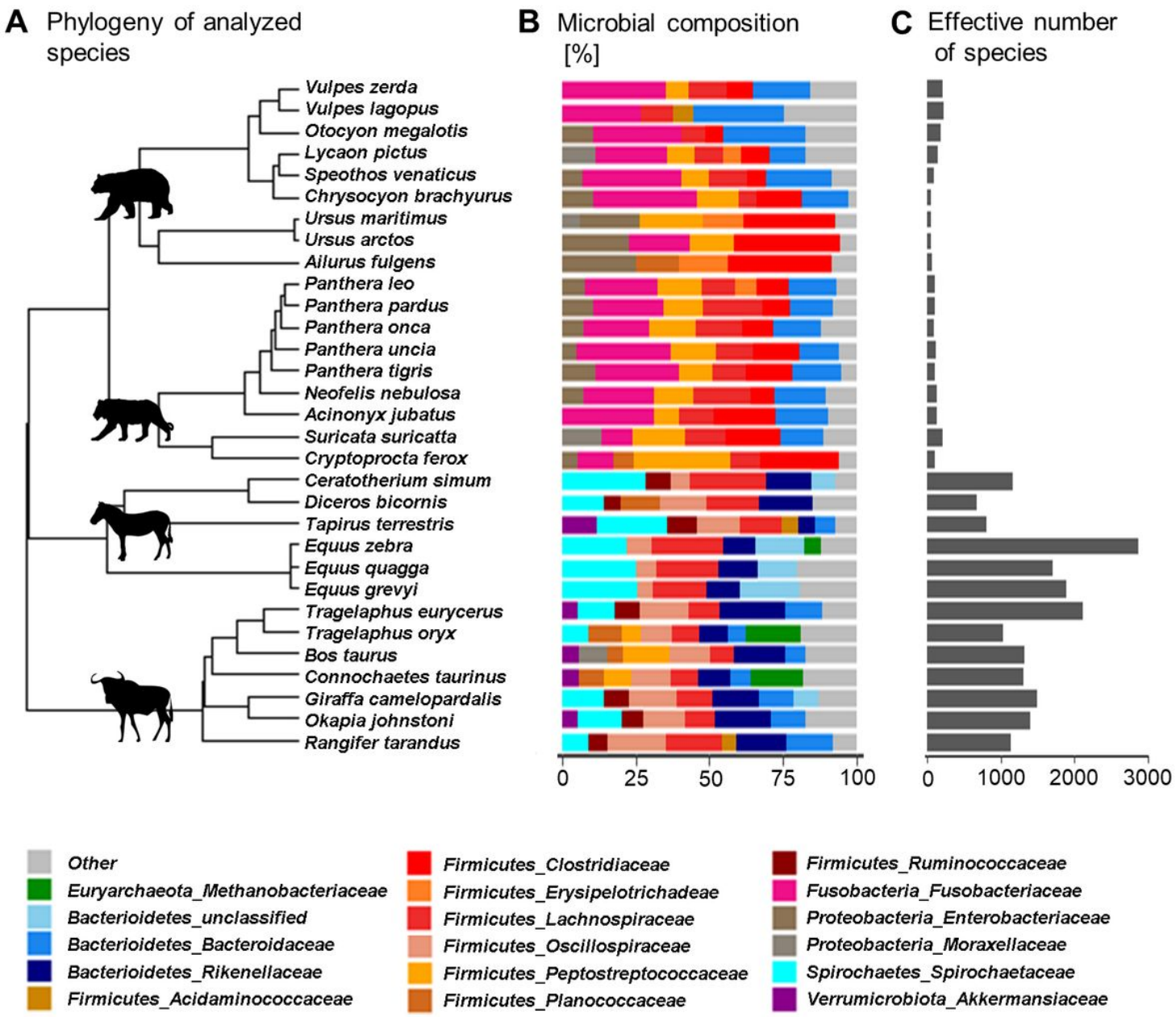

Figure 1

Variation in the fecal microbiota of mammals. A) Phylogeny of the analyzed 31 species based on TimeTree database [73]. B) Average composition of the fecal microbiota per species. Microbes that occur in less than 5% are summarized under “Other“. ANOSIM on the four groups: permutations = 999, distance=bray, R=0.496, p<0.001. C) Average fecal diversity per species presented as number of effective species. Kruskal-Wallis on the four groups: p<0.01, df=3, Dunn Test with Bonferroni correction p<0.001.

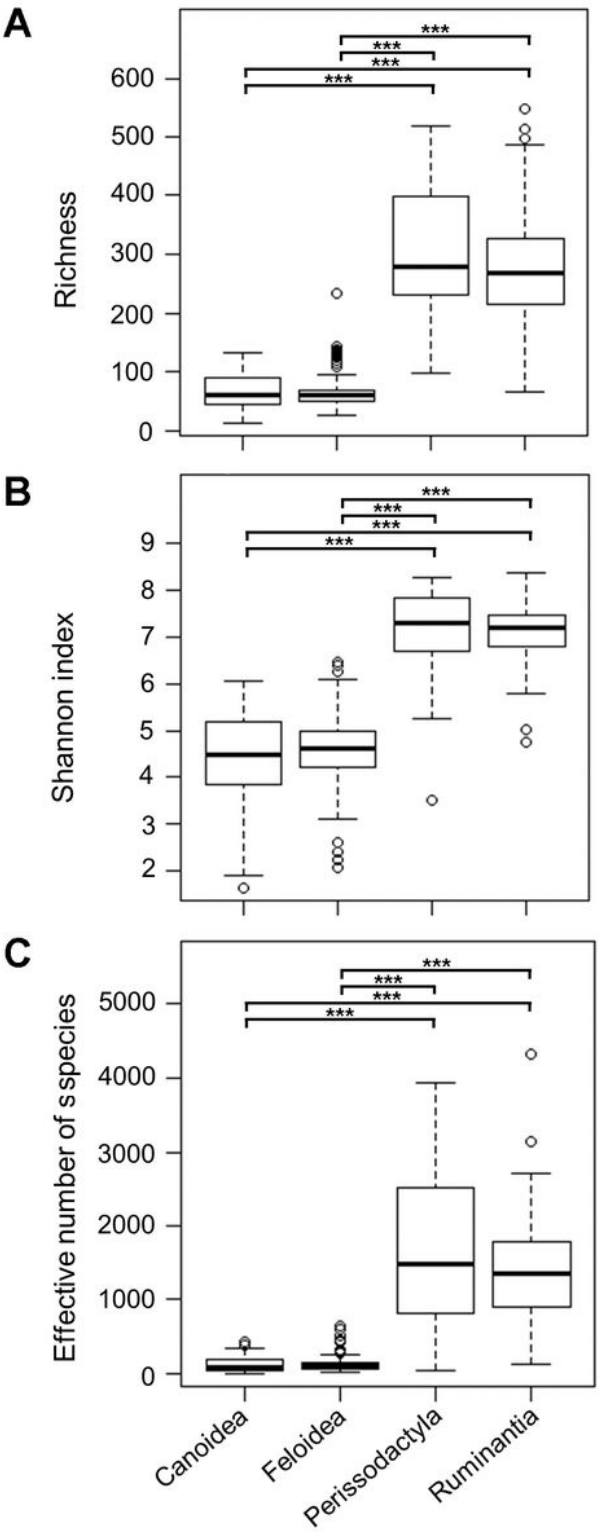

Figure 2

Alpha diversity of carnivores (Canoidea and Feloidea) and herbivores (Ruminantia and Perissodactyla) measured as species richness (A), Shannon index (B) and effective number of species (C). Kruskal-Wallis $p<0.01$, df=3, Dunn Test with Bonferroni correction $p<0.001$ for all diversity indices. Statistical results for pairwise comparisons are presented in an additional file (see Additional file 4).

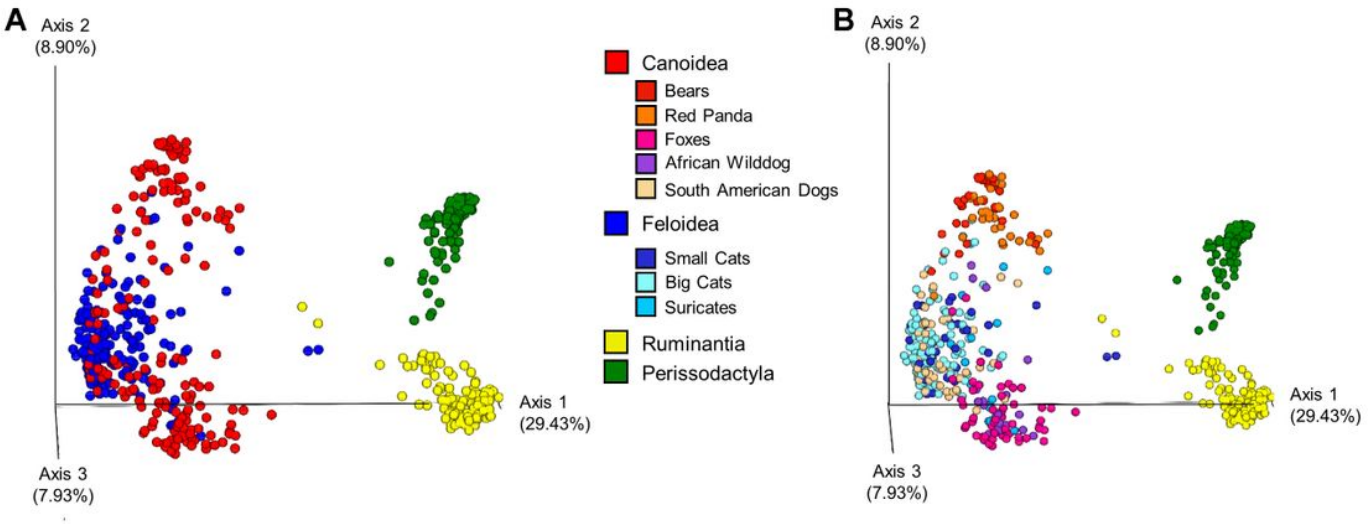

Figure 3

Beta diversity of carnivores and herbivores calculated on an unweighted UniFrac distance matrix. Differences in samples are shown based on the four analyzed groups (A) and on a more detailed division of carnivore groups (B) as shown in the figure legend. The proportion of data explained by this measurement is shown in brackets for each axis.

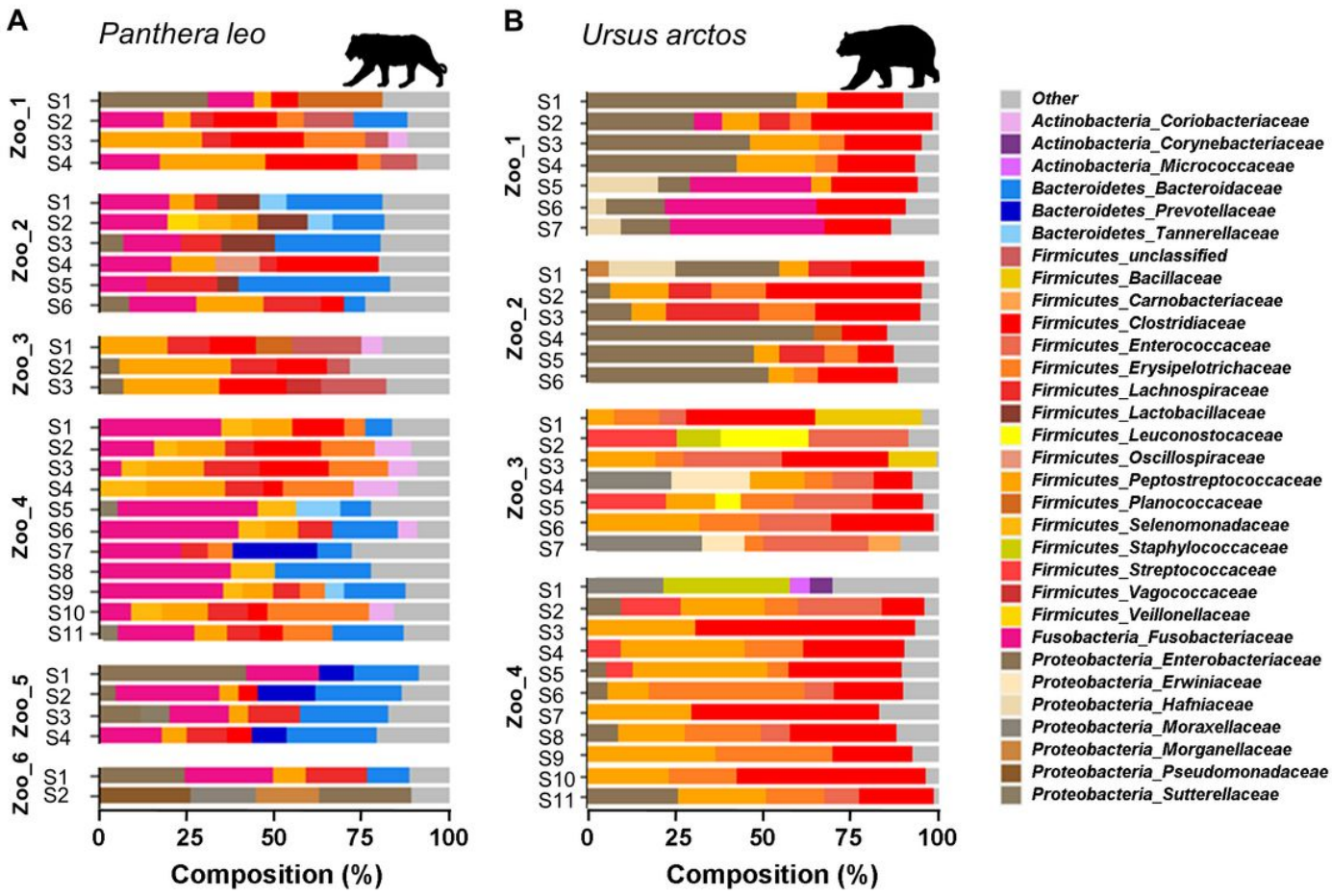

Figure 4

Variability in the fecal microbiota of carnivore species. The composition is shown for the lion as a representative of the Felidae (A) and for the brown bear as a representative of the Canidae (B), including all samples that have passed the analysis. Microbes that occur in less than 5% are summarized under “Other“.

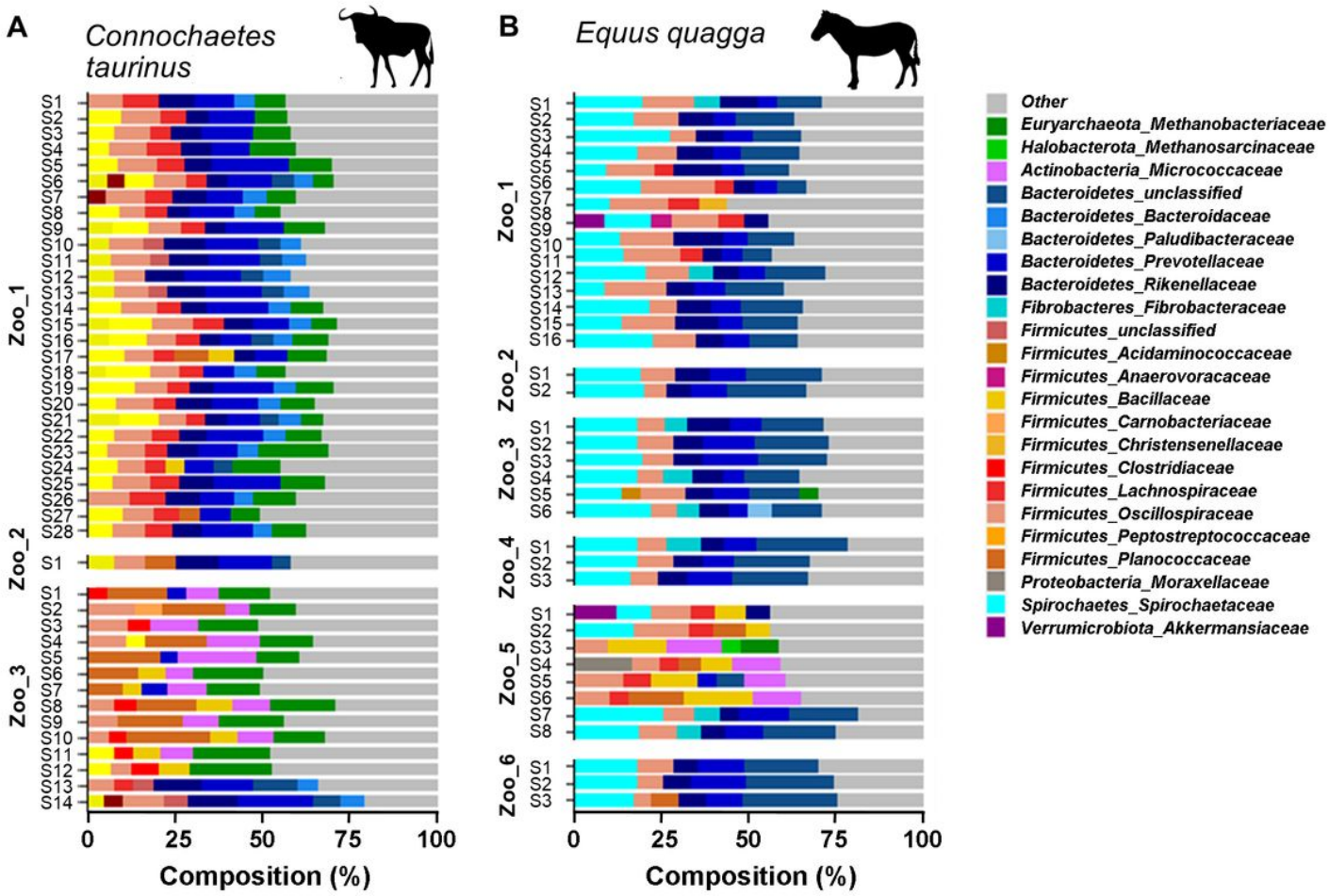

Figure 5

Variability in the fecal microbiota of herbivore species. The composition is shown for the wildebeest as a representative of the Ruminantia (A) and for the plains zebra as a representative of the Perissodactyla (B), including all samples that have passed the analysis. Microbes that occur in less than 5% are summarized under “Other“.

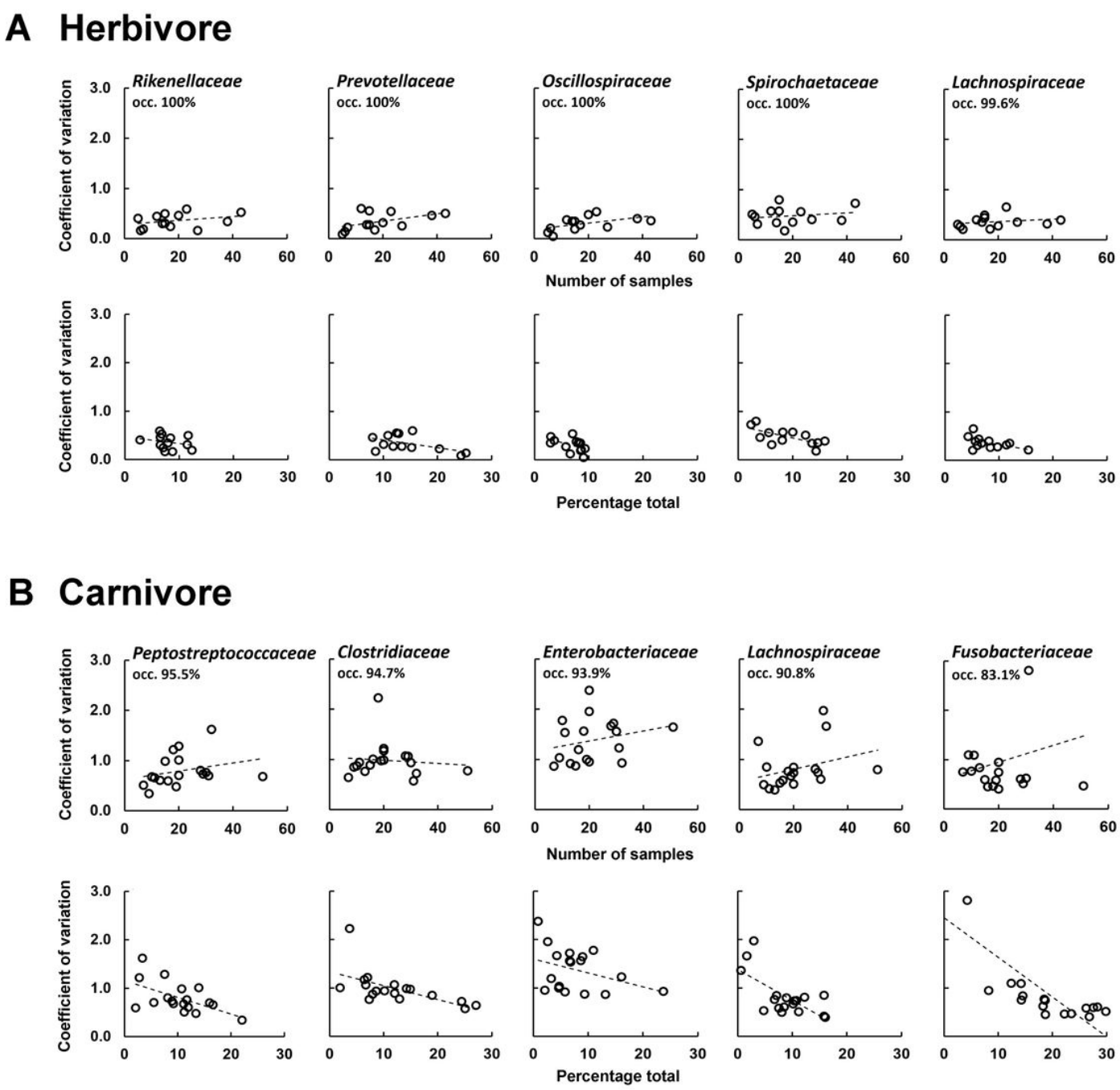

Figure 6

Coefficient of variation of different bacterial families for all herbivores (A) and carnivores (B) plotted against the number of samples (top row in each case) and against the total percentage of occurrence (bottom row in each case). The tendency is indicated by a linear regression line. The occurrence in the total sample is given for each bacterial family.

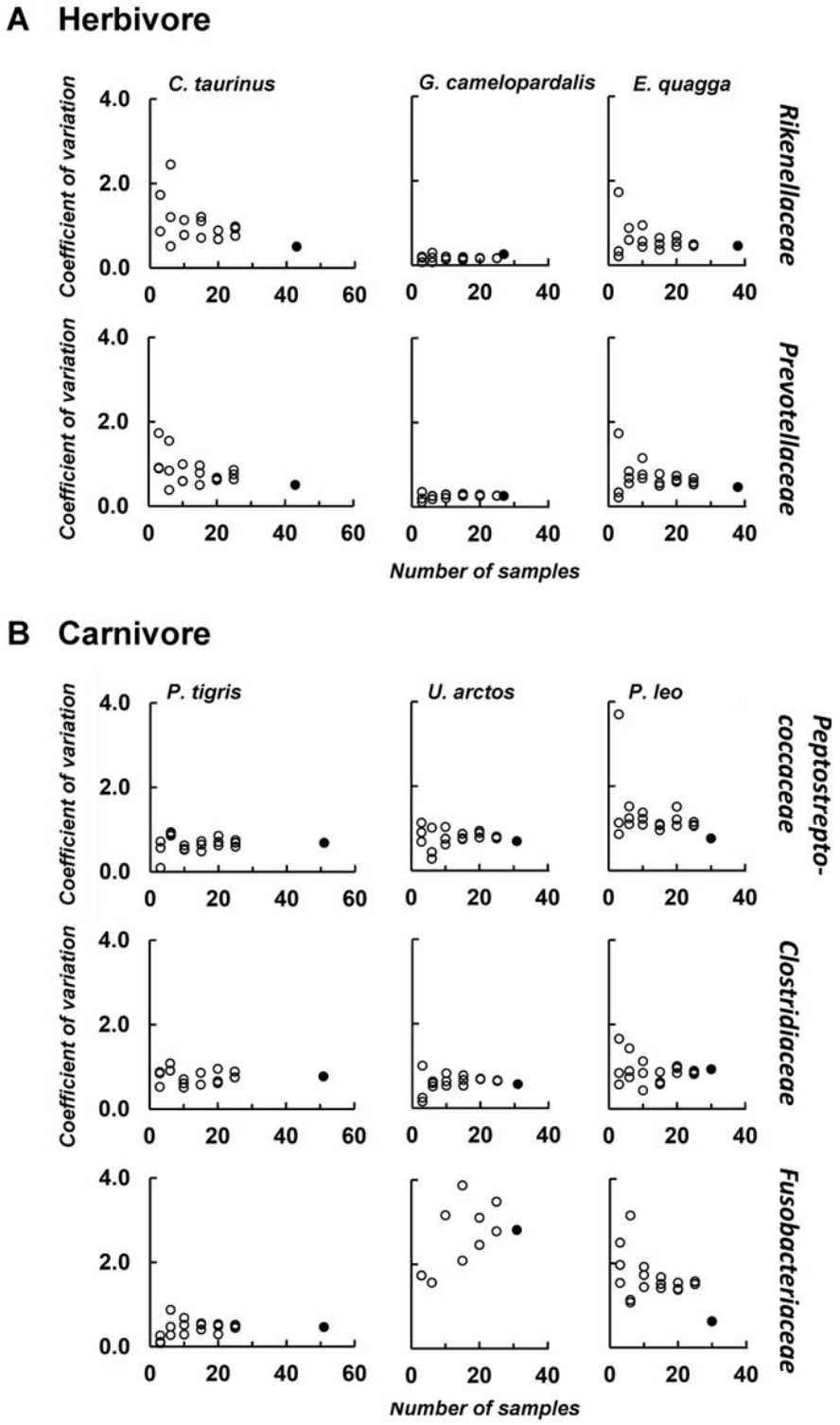

Figure 7

Coefficient of variation of different bacterial families for selected herbivorous (A) and carnivorous (B) species. Shown are randomized subsets (unfilled circles) for a different number of samples as well as the entire data set (filled circles).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.xlsx
- Additionalfile2.xlsx
- Additionalfile3.xlsx
- Additionalfile4.xlsx
- Additionalfile5.txt