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GUT MICROBIOME DIVERSITY OF THREE RHINOCEROS SPECIES IN EUROPEAN ZOOS

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Abstract: The wild rhinoceros populations have declined drastically in the past decades because the rhinoceros are heavily hunted for their horns. Zoological institutions aim to conserve rhinoceros populations in captivity, but one of the challenges of *ex situ* conservation is to provide food sources that resemble those available in the wild. Considering that the mammalian gut microbiota is a pivotal player in their host's health, the gut microbiota of rhinoceros may also play a role in the bioavailability of nutrients. Therefore, this study aims to characterize the fecal microbiome composition of grazing white rhinoceros (WR; *Ceratotherium simum*) and greater one-horned rhinoceros (GOHR; *Rhinoceros unicornis*) as well as the browsing black rhinoceros (BR; *Diceros bicornis*) kept in European zoos. Over the course of 1 yr, 166 fecal samples in total were collected from 9 BR ($n = 39$), 10 GOHR ($n = 56$), and 14 WR ($n = 71$) from 23 zoological institutions. The bacterial composition in the samples was determined using 16S rRNA gene Illumina sequencing. The fecal microbiomes of rhinoceros clustered by species, with BR clustering more distantly from GOHR and WR. Furthermore, the data report clustering of rhinoceros microbiota according to individual rhinoceros and institutional origin, showing that zoological institutions play a significant role in shaping the gut microbiome of rhinoceros species. In addition, BR exhibit a relatively higher microbial diversity than GOHR and WR. BR seem more susceptible to microbial gut changes and appear to have a more diverse microbiome composition among individuals than GOHR and WR. These data expand on the role of gut microbes and can provide baseline data for continued efforts in rhinoceros conservation and health status.

INTRODUCTION

Rhinoceros species are among the most threatened species in the world because they are heavily hunted for their horns and a loss of habitat greatly reduces reproductive opportunities. The family Rhinocerotidae consists of five extant species: the black rhinoceros (BR; *Diceros bicornis*) and the white rhinoceros (WR; *Ceratotherium simum*) from Africa and the greater one-horned or Indian (GOHR; *Rhinoceros unicornis*), the Javan (JR; *Rhinoceros sondaicus*), and the Sumatran (SR; *Dicerorhinus sumatrensis*) rhinoceros from Asia. The number of wild rhinoceros has declined drastically in the past decades, with the International Union for Conservation of Nature currently listing the BR, JR, and SR as critically endangered, the GOHR as vulnerable, and the WR as near threatened.[18] BR, WR, and GOHR are kept and bred in zoological institutions worldwide, whereas the remaining populations of JR and SR reside only in protected areas in Indonesia.

Captivity is associated with several health problems in mammals and birds, such as issues with reproduction, obesity, and metabolism.[4,7,11,17] Changes in living environment and lifestyle as a consequence of captivity, including changes in temperature, diet, healthcare, surroundings, and increased exposure to humans, are known to shape the composition and function of the mammalian gut microbiome.[26] The gut microbiome provides a range of essential functions, such as digestion of complex food sources and signaling to the host immune system, and under captivity is proposed as an indicator of host condition.[10,25] Captive BR are known to suffer from iron accumulation (iron overload disorder), reduced insulin sensitivity, and increased inflammatory and oxidative stress.[29–31,36]

One of the challenges of *ex situ* conservation of rhinoceros is that the food sources in the wild may not match the food sources available in zoos.[6] Finding a suited food source for BR is considerably more difficult than for WR or GOHR.

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BR are browsers and primarily feed on shrubs, leaves, twigs, branches, and bark.[15] WR are grazers, with a diet consisting solely of grasses.[38] GOHR are also mostly grazers, but have a slightly mixed diet with some aquatic plants and browse.[16,20] A complex collaboration takes place between the host and the microbiome in the conversion and sharing of nutrients and trace elements, closely linking the bioavailability of these constituents to the host.[25,35,42] It has recently been suggested that a reduced gut microbiome diversity and metabolome differences are associated with an increased risk for iron overload disorder in BR and SR versus GOHR and WR.[34]

This study aims to complement existing knowledge on the rhinoceros gut microbiota through characterization of the fecal microbiome composition of WR, GOHR, and BR from 23 zoological institutions in Europe and investigates whether a clustering of gut microbiome composition by institutional origin could be observed for the different rhinoceros species.

MATERIALS AND METHODS

Sampling

Over the course of 1 yr (November 2017–December 2018), fecal samples were collected from BR ($n = 9$), GOHR ($n = 10$), and WR ($n = 14$). The original sampling schedule intended to collect fecal samples with interval periods of 2 mon. However, samples could not always be collected within the set sampling constraints; therefore, for the 33 individuals followed, only 17 complete sets of samples were obtained. Fecal samples from rhinoceros were collected using the following instructions (a sampling protocol was provided to each institution with sampling instructions and pictures): one fecal ball was collected within a time interval of maximum 2 h postdeposit. The fecal ball was picked up by the keepers from the enclosure with sterile gloves and placed on a sterile surface (the inside of the package of the sterile gloves). The fecal ball was divided into two halves by the keeper by using the sterile gloves. Samples were collected from the upper, middle, and lower inside of the ball and placed directly in the test tube by the gloved hand (a second person opened the test tube). Fecal samples were put inside a 9-ml collection tube containing DNA/RNA Shield (Zymo Research, Irvine, CA 92614, USA) not exceeding one third of the test tube volume. Samples were homogenized by inverting the tubes a few times, after which samples were sent to the central lab (BaseClear B.V., 2333 BE Leiden, The Netherlands) at room temperature and by regular mail. Previous internal stability studies at the central lab showed that the fecal microbiome composition did not change significantly under these ambient conditions for periods of several weeks (unpubl. data). Samples were stored upon arrival at the central lab at −80°C until further processing. Participating zoological institutions are represented by their geographical location in Supplemental Figure 1, and sample details are summarized in Table 1.

Microbial DNA extraction from fecal samples

The Quick-DNA Fecal/Soil Microbe Miniprep kit (Zymo Research) was used to extract bacterial DNA in an unbiased manner. In brief, samples were taken from the −80°C freezer and thawed on ice. Using disposable plastic spoons, two scoops of stool sample (±150 mg) were added in ZR BashingBead lysis tubes (Zymo Research) containing 0.1- and 0.5-mm bashing beads. The tubes were transferred to a Precellys 24 bead beater (Bertin Instruments, 78180 Montigny-le-Bretonneux, France) and homogenized three times at 5,500 rpm for 45 s, with 30-s pauses. The homogenate (400 µl) was subsequently used for DNA extraction according to the manufacturer's instructions, including an additional cleanup step using Zymo-Spin III-HRC filters (Zymo Research) for the removal of PCR inhibitors. DNA concentrations were quantified by fluorometric analysis.

16S rRNA gene PCR amplification and sequencing

PCR amplicon library preparation was performed based on the 16S Metagenomic Sequencing Library Preparation protocol (https://support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html). In short, barcoded amplicons from the V3-V4 region of 16S rRNA genes were generated using a two-step PCR. Ten nanograms of genomic DNA was used as template for the first PCR, with a total volume of 50 µl, by using 341F (5′-CCTACGGGNGGCWGCAG-3′) and 785R (5′-GACTACHVGGGTATCTAATCC-3′) primers appended with Illumina adaptor sequences. The following PCR conditions were used: 98°C for 30 s, followed by 25 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 30 s, with a last extension at 72°C for 10 min. The PCR products were purified, and the sizes of the PCR products were checked on a Fragment Analyzer (Agilent Technologies, Santa Clara, CA 95051,

Table 1. Rhinoceros fecal samples received from 23 different zoological institutions at six visits (I–VI) between November 2017 and September 2018. Three institutions could not sample in July 2018, but collected additional samples in November 2018, mentioned herein as visit VII. Two samples, indicated as "xx," were received double and treated as a second sample from the same visit. Lacking samples are indicated with a dash (—).

Rhinoceros[a]	Zoo	Visit I, Nov	Visit II, Jan	Visit III, Mar	Visit IV, May	Visit V, Jul	Visit VI, Sep	Visit VII, Nov
Black 01	Zoo 01	x	x	x	x	x	x	
Black 02	Zoo 01	x	x	x	x	x	x	
Black 03	Zoo 01	x	x	x	x	x	x	
Black 04	Zoo 02	x	x	x	x	x	x	
Black 05	Zoo 02	x	x	x	x	x	x	
Black 06	Zoo 03	x	x	x	—	—	—	
Black 07	Zoo 03	x	x	x	—	—	—	
Black 08	Zoo 04	—	—	—	x	—	—	
Black 09	Zoo 04	—	—	—	xx	—	—	
GOH 01	Zoo 01	x	—	x	x	x	x	
GOH 02	Zoo 02	x	x	x	x	x	x	
GOH 03	Zoo 05	x	x	x	x	x	x	
GOH 04	Zoo 06	x	x	x	x	—	x	
GOH 05	Zoo 07	x	x	x	x	x	x	
GOH 09	Zoo 08	x	x	x	x	—	x	
GOH 10	Zoo 09	x	x	x	x	x	x	
GOH 11	Zoo 10	x	x	x	—	x	x	
GOH 12	Zoo 11	x	x	x	x	x	x	
GOH 13	Zoo 12	x	x	x	x	—	x	x
White 01	Zoo 05	x	x	x	x	x	x	
White 02	Zoo 06	x	x	x	x	x	x	
White 03	Zoo 07	x	x	x	x	x	x	
White 04	Zoo 13	x	xx	x	x	—	x	
White 05	Zoo 14	x	x	—	—	—	—	
White 06	Zoo 15	x	x	x	x	x	x	
White 07	Zoo 16	x	x	x	x	x	x	
White 08	Zoo 17	x	x	—	x	—	—	
White 10	Zoo 18	x	x	—	—	—	—	
White 11	Zoo 19	x	x	x	x	—	x	x
White 12	Zoo 20	—	x	x	x	x	—	
White 13	Zoo 21	x	x	x	x	—	x	x
White 14	Zoo 22	x	x	x	x	x	x	
White 16	Zoo 23	x	x	x	x	x	x	

[a] Black, black rhinoceros; GOH, greater one-horned rhinoceros; White, white rhinoceros.

USA) before quantification by fluorometric analysis. Purified PCR products were used for the second PCR in combination with sample-specific barcoded primers (Nextera XT index kit, Illumina, San Diego, CA 92122, USA). The following PCR conditions were used: 98°C for 30 s, 6 cycles of 98°C for 10 s, 55°C for 30 s, 72°C for 30 s, and a last extension at 72°C for 5 min. Subsequently, PCR products were purified, checked on the Fragment Analyzer (Agilent Technologies), and quantified by fluorometric analysis. The yielded libraries were multiplexed, clustered, and sequenced on two separate PE300 Illumina MiSeq runs with the paired-end (2x) 300-bp protocol and indexing.

16S rRNA gene sequence analysis

The sequencing runs were analyzed by the BaseClear bioinformatics department by using the Illumina CASAVA pipeline (v1.8.3) with demultiplexing based on the sample-specific barcodes. The raw sequencing data were processed by removal of sequence reads of too low quality (only "passing filter" reads were selected) and discarding reads containing adaptor sequences or PhiX control with an in-house filtering protocol. A quality assessment on remaining reads was performed using the FASTQC quality control tool (v0.10.0; https://www.bioinformatics.babraham.ac.uk/projects/fastqc). For data processing, Illumina-paired reads were merged into single reads (pseudoreads) through sequence overlap, after removal of the forward and reverse primers. Chimeric pseudoreads were removed, and the remaining reads were aligned to the RDP 16S rRNA gene databases. Based on the alignment scores of the pseudoreads, the taxonomic depth of the lineage is

based on the identity threshold of the rank: species 99%, genus 97%, family 95%, order 90%, class 85%, and phylum 80%. Abundance of genera was calculated as a percentage of the total number of sequences identified in each sample.

Statistical analysis

To assess variation of microbial phyla and genera, relative abundances were computed for each sample. Alpha diversity metrics were calculated with the phyloseq package (v.1.30.0).[27] Wilcoxon rank-sum tests (Benjamini–Hochberg false discovery rate corrected) were used to test for significant differences in relative abundance and alpha diversity metrics according to rhinoceros species. A redundancy analysis (RDA), with ANOVA (unrestricted permutation test), was calculated using the vegan package (v.2.5-6) to assess correlations between 16S rRNA gene composition data (at the genus level) and sample characteristics (e.g., rhinoceros species).[28] This approach was used to reduce the dimensionality of the datasets by transforming a large set of variables into a smaller set that still contained most of the information.[19] A principal component analysis (PCA) reduces data by geometrically projecting them onto lower dimensions called principal components (PC), with the aim of showing the best summary using a small number of PC.[21] An RDA can be considered extension of a PCA in which the axes (principal components) are constrained to be linear combinations of the environmental variables.[33] Two datasets were necessary: one dataset for the species data (dependent variables), containing the sequenced microbiota, and one dataset for the environmental variables (independent variables) containing names, origins, and sampling dates. These calculations were performed to find the best ordination for the represented data. The interest of an RDA is to represent not only the main patterns of species variation as much as they can be explained by the measured environmental variables but also to display correlation coefficients between each species and each environmental variable in the dataset.[33] Considering the microbiome abundance data are not normally distributed and that the determination of the different groups through multivariate analysis, permutation tests were used. *P*-values were calculated as the proportion of the values of the statistic for all possible reorderings (permutations) of the observations when the groups were in fact not different.[1] The RDA shows the 10 taxa (bacterial groups) that are most discriminating for the diagram. In practice, this means that five taxa show the strongest positive correlation and five taxa the strongest negative correlation with the variable being included in the RDA. Figures were generated in RStudio using R statistical software (v.3.6.1) with the vegan (v.2.5-6), tidyverse (v.1.3.0), phyloseq (v.1.30.0), cowplot (v.1.1.1), and ggplot2 (v.3.3.3) packages.[27,28,32,44–46] Significance brackets for ggplot2 were generated using the ggsignif package (v.0.6.1; https://CRAN.R-project.org/package=ggsignif).

RESULTS

In total, 166 fecal samples were collected from 9 BR ($n = 39$), 10 GOHR ($n = 56$), and 14 WR ($n = 71$) (Table 1). In general, six fecal samples were collected per rhinoceros. However, for one BR only one sample was collected, for one BR two samples were collected, and for two BR three samples were collected. For two WR two samples were collected, for one WR three samples were collected, for one WR four samples were collected, and for four GOHR five samples were collected. Furthermore, all fecal samples were collected in a total of seven visits over a period of 12 mon, where samples from the different rhinoceros per visit were collected predominantly in 1 and 2 mon apart between visits. The BR were housed in four different zoos, whereas each of the GOHR and WR were located in different zoos, 10 and 14, respectively (Table 2). For the rhinoceros from which samples were collected, none of the zoos had at least one of each of the different rhinoceros species. Two zoos had a least one BR and a GOHR, and three zoos had one GOHR and one WR from which samples were collected (Table 2). DNA extraction resulted in sufficient DNA yield for all the samples, with an average concentration of 123 ± 40.3 ng/μl. PCR amplification for the 16S rRNA gene V3-V4 region was successful for all samples and subsequent sequencing generated a total of 567,343 and 2,526,970 quality-filtered sequence reads. Although the average number of reads per sample (7,466 ± 887 and 28,716 ± 5,095, respectively) for the two separate PE300 runs differed substantially, the number of reads per sample was sufficient for downstream analysis. Additional quality-control checks also showed that there was no statistically significant difference between the microbiome profiles found in both runs ($p = 0.540$, RDA not shown). The Good's coverage index for the respective runs estimated sufficient completeness of sampling, with a calculated mean coverage of 98.1 ± 0.32 and 99.4 ± 0.18%. Despite these indications that sample preparation and sequencing did not lead

Table 2. Number of sampled rhinoceros per zoological institution.

Zoo	Rhinoceros[a]	Black	GOH	White
Zoo 01	Black 01, 02, 03; GOH 01	3	1	
Zoo 02	Black 04, 05; GOH 02	2	1	
Zoo 03	Black 06, 07	2		
Zoo 04	Black 08, 09	2		
Zoo 05	GOH 03; White 01		1	1
Zoo 06	GOH 04; White 02		1	1
Zoo 07	GOH 05; White 03		1	1
Zoo 08	GOH 09		1	
Zoo 09	GOH 10		1	
Zoo 10	GOH 11		1	
Zoo 11	GOH 12		1	
Zoo 12	GOH 13		1	
Zoo 13	White 04			1
Zoo 14	White 05			1
Zoo 15	White 06			1
Zoo 16	White 07			1
Zoo 17	White 08			1
Zoo 18	White 10			1
Zoo 19	White 11			1
Zoo 20	White 12			1
Zoo 21	White 13			1
Zoo 22	White 14			1
Zoo 23	White 16			1

[a] Black, black rhinoceros; GOH, greater one-horned rhinoceros; White, white rhinoceros.

to strong technical biases, one sample was excluded from analysis due to a low read count and one sample displaying a deviating profile was excluded from further RDA analysis.

The microbiota profiles of all rhinoceros were represented by 1,415 genera. After initial data analysis, relative microbial profiles were generated for all samples (Fig. 1). Taxonomic classification revealed that the mean relative profiles of all rhinoceros species were dominated by Firmicutes (46.8 ± 7.37%) and Bacteroidetes (26.9 ± 5.14%), with lower abundances of Spirochaetes (4.2 ± 2.15%), Fibrobacteres (3.5 ± 3.03%), Planctomycetes (2.3 ± 1.17%), Proteobacteria (1.3 ± 1.42%), and Euryarchaeota (1.1 ± 0.63%). The remaining rare microbial phyla consisted of Chlamydiae, Lentisphaerae, Actinobacteria, Tenericutes, Cyanobacteria, Verrucomicrobia, Chloroflexi, Synergistetes, and Elusimicrobia. The 16 most abundant phyla and unclassified taxa comprise up to 99.9% of the data. For BR, GOHR, and WR, respectively 7.5 ± 2.02, 10.9 ± 1.64, and 10.0 ± 1.29% of the reads were unclassified. The mean Firmicutes-to-Bacteroidetes ratio was 2.93 ± 2.7 for BR, 1.57 ± 0.29 for GOHR, and 1.68 ± 0.36 for WR.

For a more in-depth analysis, the microbiota profiles of the BR, GOHR, and WR were analyzed by RDA (Fig. 2A). As anticipated from the qualitative inspection of the microbial profiles, the bacterial microbiomes of the different rhinoceros species grouped separately ($P = 0.001$; Fig. 2A). BR had a more distinct bacterial profile than the microbiota of GOHR and WR, which grouped more closely together. RDAs comparing between two rhinoceros species were computed to indicate the most discriminative taxa. *Anaerotruncus*, *Oscillospira*, *Oscillibacter unclassified Cyanobacteria*, and *Butyricicoccus* were most discriminative for the microbial profiles of BR compared with the bacterial microbiota of GOHR, for which *Bittarella*, *Acholeplasma*, *Anaerosporobacter*, *Anaeroplasma*, and *Acidaminococcus* were most discriminative (Fig. 2B). Comparing the microbiota of BR and WR indicates an enrichment of *unclassified Chlamydia*, *unclassified Cyanobacteria*, *Oscillospira*, *Oscillibacter*, and *Butyricicoccus* in BR and *Bittarella*, *Acholeplasma*, *Anaerosporobacter*, *Anaeroplasma*, and *Erysipelothrix* in WR (Fig. 2C). Distinctions between the microbiota of GOHR and WR were most apparent when looking at *Mariniphaga*, *Methanomicrobium*, *unclassified Euryarchaeota*, *Prolixibacter*, and *unclassified Thermoplasmata* in GOHR and *Schwartzia*, *unclassified Marinilabiliaceae*, *Prevotella*, *Mucinivorans*, and *Lentimicrobium* in WR (Fig. 2D).

RDA analysis also revealed that the compositional profiles of individual BR grouped independently from that of other BR, indicating that the BR appear to have individual specific microbial compositions ($P = 0.001$; Fig. 3A). To determine the impact of the visits over time on the microbiota composition, additional PCA analyses were performed. This showed that there was no clear clustering of the compositional profiles per visit but that there was a stronger clustering of the samples per rhinoceros. This indicates that the impact of the season was smaller than the subject specific impact (Supplemental Fig. 2). Moreover, it became clear that BR from the same zoological institution shared a similar bacterial microbiome, because their fecal profiles grouped together. This difference in location dependent profiles was found to be significant ($P = 0.001$; Fig. 3B). Different GOHR and especially WR were harder to distinguish from each other in the RDA, but were still found to be significantly different ($P = 0.001$ [Fig. 3B] and $P = 0.001$ [Fig. 3C], respectively). GOHR and WR appear to have more similar bacterial microbiomes than BR.

Shannon and Simpson indices were computed at the species level for each rhinoceros species to identify differences in alpha diversity. The alpha diversity for the different rhinoceros species were very similar, albeit that the alpha diversity

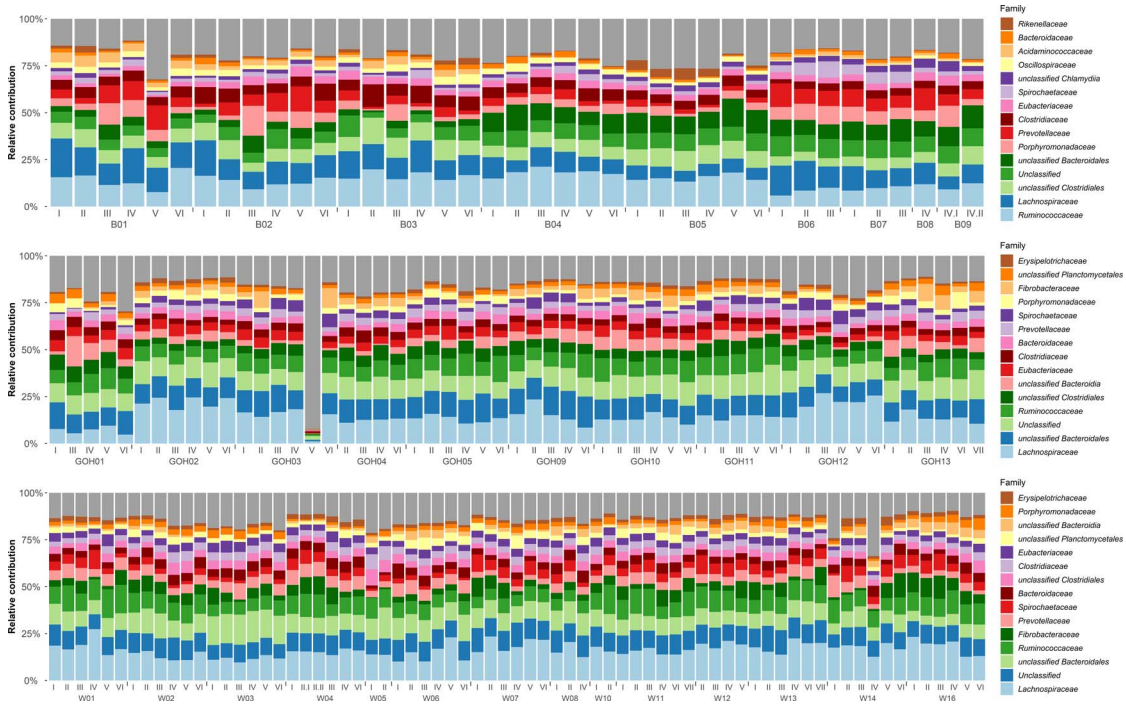

Figure 1. Relative composition of the fecal microbiome community from black rhinoceros (B), greater one-horned rhinoceros (GOH), and white rhinoceros (W) at the family level, with the 15 most abundant microbial families represented for each rhinoceros species. All remaining families are presented in gray. Different sampling time points of each rhinoceros are depicted with Roman numerals as presented in Table 1.

of BR microbiomes was significantly higher, followed by WR and GOHR, respectively (Fig. 4A).

Differences between relative community composition of the most abundant phyla were observed between rhinoceros species (Fig. 4B). Firmicutes and Proteobacteria were significantly more abundant in BR microbiomes than in microbiomes of GOHR and WR, whereas in the GOHR and WR microbial communities significantly higher abundances of Spirochaetes, Fibrobacteres, and Planctomycetes were found. Spirochaetes and Fibrobacteres were furthermore elevated in the profiles of WR compared with the profiles of GOHR. Euryarchaeota were found to be relatively more abundant in the microbiomes of BR and GOHR.

DISCUSSION

In this study, the gut microbiome diversity of the BR, GOHR, and WR from European zoos was investigated through 16S rRNA gene–based microbial profiling of fecal samples. Whereas BR and WR both reside in Africa and are more closely related to each other than to Asian GOHR, the microbial profiles of WR presented herein clustered more closely with GOHR profiles than with BR.[23] These findings are in agreement with previously published data and complement these other studies with the analysis of a considerable cohort of rhinoceros housed in different European zoos.[23,34] Moreover, Roth et al.[23] also included SR in their study and found that the fecal of the microbial profiles of BR and SR clustered more closely than to those of the GOHR and WR. Based on host phylogenetic analysis, SR are more closely related to GOHR than to BR or WR.[23] Although BR consume more grasses in their zoo diet than SR, it is considered that WR are grazers, GOHR are intermediate grazers-browsers, and SR and BR are browsing species. These findings suggest that feeding habit may be one driver of the microbiome of captive rhinoceros in relation to host phylogeny. Moreover, considering that microbial gut communities of captive and wild animals vary greatly, diet, gut physiology, and host phylogeny appear to be important factors shaping the gut microbial composition.[10,22]

Many studies have reported a lower gut richness or diversity in captive animals, although opposing results have also been reported (for review, see[10]). A study comparing the microbiomes

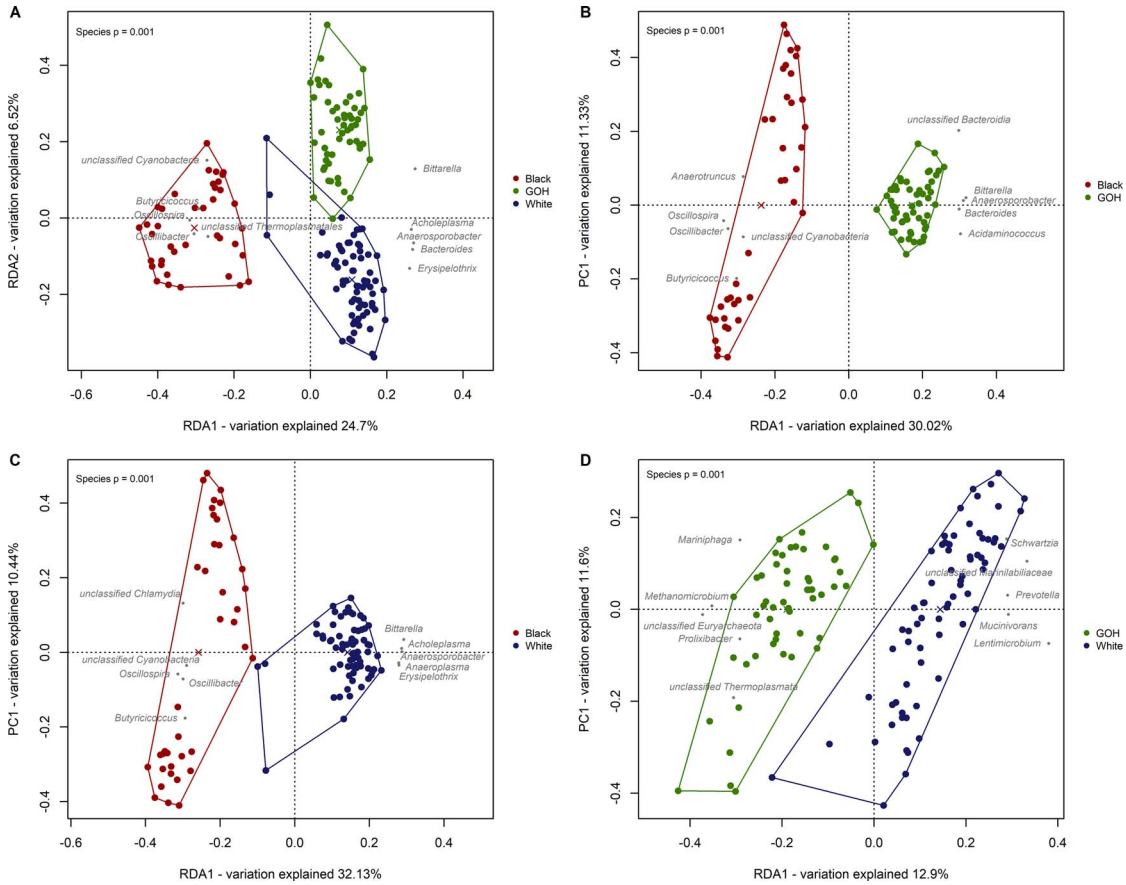

Figure 2. Redundancy analysis (RDA) of microbial composition at the genus level for (A) black rhinoceros (Black), greater one-horned rhinoceros (GOH), and white rhinoceros (White); (B) Black and GOH; (C) Black and White; and (D) GOH and White. The genera in gray represent the 10 taxa with the strongest drivers of the microbial composition where the subject or zoological institution was used as explanatory variable. PC, principle component.

of different captive rhinoceros species reported a lower microbial diversity in SR and BR than GOHR and WR.[34] Browsing BR receive a more diverse fiber-rich diet, and unlike their grazing cousins, they were expected to have a higher gut diversity. Alpha diversity analysis of these data revealed a comparable bacterial fecal diversity for the different rhinoceros species, although a significant difference was observed between BR, GOHR, and WR. These differences in studies are likely due to the inclusion of a different rhinoceros cohort from different zoos. The gut microbiomes also showed some similarities, with Firmicutes and Bacteroidetes being the two most abundant phyla across all three rhinoceros species, together representing on average 74% of the relative abundance. As in most mammals, these phyla are in majority and were also found to be the two most abundant phyla in previous rhinoceros microbiome studies.[2,5,13,26,34,47] Elevated ratios of Firmicutes to Bacteroidetes have been associated with obesity in humans and some animals, suggesting a value of this ratio as an indicator for health, favoring a shift toward Bacteroidetes.[24] Although the Firmicutes-to-Bacteroidetes ratio differed between individual rhinoceros, herein, on average, higher abundances of Firmicutes were found in the microbiome profiles of BR than in GOHR and WR. Considering the limited data available on the physiological status of the rhinoceros included in this study, the link between the Firmicutes-to-Bacteroidetes ratio and health could not be further explored herein. Moreover, ratios do not seem to be consistent between studies, either due to technical differences, such as PCR primer choice, or no strong correlation exists.[12,37,40]

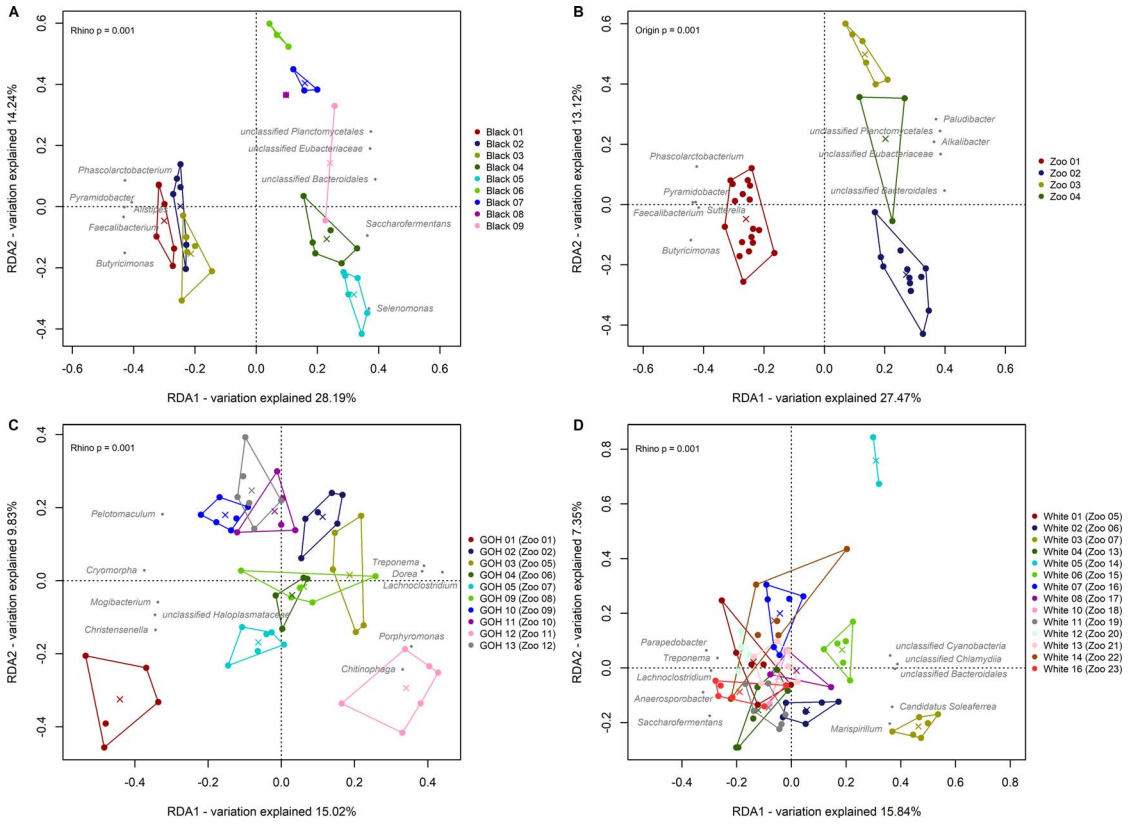

Figure 3. Redundancy analysis (RDA) of microbial composition at the genus level for (A) individual black rhinoceros (Black), (B) Black from different zoological institutions, (C) individual greater one-horned rhinoceros (GOH) that were each from a different zoo, and (D) individual white rhinoceros (White) that were each from a different zoo. The genera in gray represent the 10 taxa with the strongest drivers of the microbial composition where the subject or zoological institution was used as explanatory variable.

These data demonstrate that the zoological institution plays a role in the microbial buildup of rhinoceros gut microbiomes. In contrast to other studies where different rhinoceros from U.S.-based zoos were sampled, herein the fecal microbial composition of a relatively large cohort of rhinoceros from different European zoos was explored. The data showed clustering of the rhinoceros subject and species. Moreover, the BR microbiota showed a less strong clustering by zoological institution. A similar clustering of samples from BR subjects and clustering of the rhinoceros species was obtained in Cersosimo et al.[5] Although only single samples were obtained from the rhinoceros subjects in Roth et al.,[34] their data showed a clustering of the microbiota per species. The BR data consisted of full sampling sets from two zoological institutions (Zoo 01, $n = 3$ BR and Zoo 02, $n = 2$ BR) and partial sampling sets from two more zoological institutions (Zoo 03, $n = 2$ BR and Zoo 04, $n = 2$ BR). The incomplete sampling sets also indicated grouping based on zoological institution, further suggesting an impact of institutional origin on the BR microbiome. Differences between the microbiomes of individual GOHR and WR from different zoological institutions were found, but they could not conclusively be attributed to institutional origin because the dataset did not contain multiple GOHR or WR from one single zoological institution.

Through RDA analysis, the determination was made as to which bacterial genera were most discriminative for different rhinoceros species. The RDA analysis indicated that *Anaerotruncus*, *Oscillospira*, *Oscillibacter*, *Butyricicoccus* were most abundant in the profiles of BR. These genera are all relatively closely related, belonging to the Clostridial cluster IV in the Firmicutes phylum.[9,14] *Butyricicoccus* and *Oscillospira* are butyrate-producing genera and have recently been

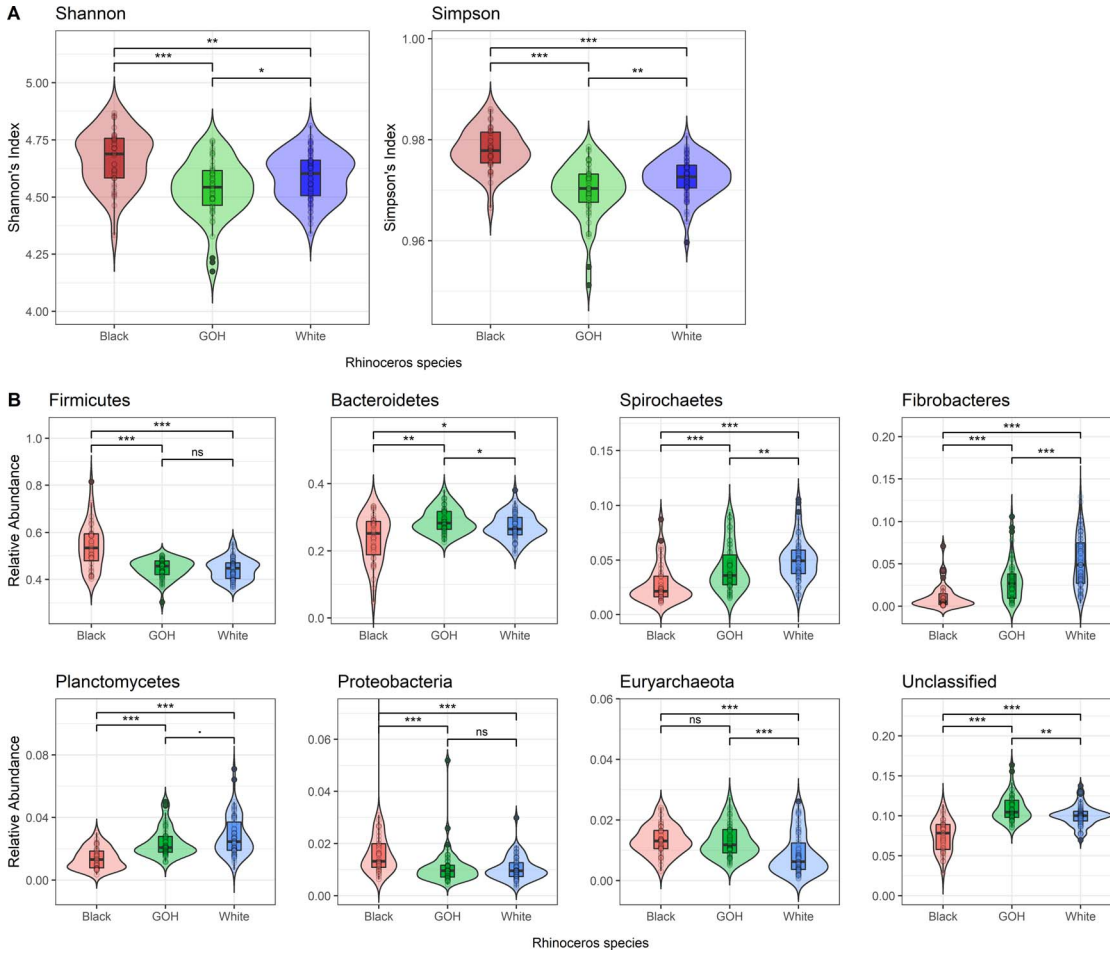

Figure 4. Alpha diversity and relative differential abundance. (A) Shannon and Simpsons diversity indices of the gut microbiota from black rhinoceros (Black), greater one-horned rhinoceros (GOH), and white rhinoceros (White) at the species level. (B) Relative abundances of different microbial phyla with a mean relative abundance above 1%, according to rhinoceros species. Significant differences were calculated by Wilcoxon rank-sum tests; Benjamini–Hochberg false discovery rate corrected (***$P < 0$, **$P < 0.001$, *$P < 0.01$, ·$P < 0.05$, $^{ns}P < 0.1$).

evaluated as potential probiotic candidates.[3,14,48] Considering that the findings herein, obtained from European-housed BR, are in agreement with those of two recent studies that reported a higher abundance of short-chain fatty acids, such as butyrate, in the fecal metabolome of U.S.-housed BR, the association of butyrate production and BR appears to be justifiable.[5,34] *Bittarella*, *Acholeplasma*, *Anaerosporobacter*, and *Anaeroplasma* were most abundant in both GOHR and WR profiles, compared with that of BR. *Acholeplasma* and *Anaeroplasma* belong to the class Mollicutes, whereas *Bittarella* and *Anaerosporobacter* belong to the class Clostridia. RDA analysis comparing the communities of GOHR and WR indicated that *Mariniphaga*, *Methanomicrobium*, *unclassified Euryarchaeota*, *Prolixibacter*, and *unclassified Thermoplasmata* were most abundant for GOHR microbiomes and that *Schwartzia*, *unclassified Marinilabiliaceae*, *Prevotella*, *Mucinivorans*, and *Lentimicrobium* were most discriminative for WR microbiomes. *Mariniphaga* and *Prolixibacter* both belong to the family Prolixibacteraceae within the phylum Bacteroidetes, whereas *Methanomicrobium*, *unclassified Euryarchaeota*, and *unclassified Thermoplasmatales* all belong to the phylum Euryarchaeota in the Archaea domain. *Methanomicrobium* is a methanogen, part of a group of Archaea that are known to harbor the digestive tract of herbivorous mammals and produce enteric methane as a by-product of feed

fermentation in the gut.[39,43] A link has been suggested between methanogen abundance and diet type, positively correlating methanogen abundance with fiber content in the diet. Considering that the diet of the rhinoceros was not recorded during our study, a link between the observed methanogens and fiber content could not be made. In addition, the coverage of specific archaeal groups may be selective or insufficient due to primer-related biases of the 16S rRNA primers used in this study. Therefore, the link between dietary fiber content and rhinoceros gut methanogens remains to be explored. *Schwartzia* are known to use succinate as the sole energy source, which is produced in large amounts during bacterial fermentation of dietary fiber.[41] *Unclassified Marinilabiliaceae*, *Prevotella*, *Mucinivorans*, and *Lentimicrobium* all belong to the phylum Bacteroidetes. *Prevotella copri* diversity in the human gut is reportedly affected by diet and linked to fiber-rich diets with enhanced carbohydrate catabolism.[8]

In conclusion, we identified significant differences in BR, GOHR, and WR gut microbiome composition. Our data report clustering of rhinoceros microbiomes according to species, individual rhinoceros, and institutional origin. These findings complement and are in agreement with previously published data and provide more insight into the significant role that zoological institutions play in shaping the gut microbiome of different rhinoceros species.[5,34] This study shows that BR appear to have a more diverse microbiome composition among individuals than GOHR and WR. Our data also show that the microbiome does not vary over time-season substantially. One could perhaps have expected that the diet would be the greatest contributor to the microbiome composition, considering that the diet at the institutions varies a lot during winter and summer, at least for the browsing species. This would suggest that the microbiome might be mainly based on feeding habit instead of the food that they are offered. Our data expand on the understanding of microbial communities in captive rhinoceros populations and can provide baseline data for continued efforts in conservation and health of rhinoceros species.

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Supplementary Figure 1. Geographical location of participating European zoological institutions. A complete overview of rhinoceros subject per zoological institution is summarized in Table 1.

Supplementary Figure 2. Principal Component Analysis of microbial composition at the genus level for (A) BRs grouped per subject; (B) BRs grouped per visit; (C) GOHRs grouped per subject; (D) GOHRs grouped per visit; (E) WRs grouped per subject; (F) WRs grouped per visit.