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Gut microbiota diversity among humans, elephants, livestock and wild herbivores in Chitwan National Park bears implications for conservation medicine

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Gut microbiome influences host health and well-being. Co-occurring hosts may exchange disease-causing bacteria belonging to these microbial communities. Therefore, monitoring gut microbiota composition in wildlife and humans is paramount to prevent zoonotic diseases, thus protecting and strengthening public health. We characterized diversity and abundance of the gut microbiome bacterial component across *mahouts* (captive elephant trainers and handlers), their pachyderms, livestock and wild herbivores in and around Chitwan National Park (Nepal). Firmicutes and Bacteroidota were invariably the dominant phyla. In humans, the relative abundance of Firmicutes was higher, the alpha diversity lower and beta diversity different compared to other host categories. Livestock and wild herbivores displayed similar alpha and beta diversity due to the presence of Proteobacteria, Actinobacteriota and Verrucomicrobiota. Elephants had a higher alpha diversity, and a significant beta diversity compared to other mammals. Our results suggest that taxonomic affiliation and diet niche are the main drivers of gut microbiota composition. Nevertheless, *Mycobacterium* and other potentially pathogenic bacteria genera were detected in elephants and livestock other than wild herbivores. These findings shed light on microbiota sharing and interlinking in each environment, thereby highlighting the importance of conservation medicine to better our understanding of health in co-occurring host species.

Keywords Bacterial function, Human–wildlife–livestock–environment interface, Microbiome, Nepal, One Health, Zoonosis

The community of microorganisms (bacteria, viruses, archaea and protists) living in a shared environment is known as microbiota. This concept is tightly associated with the entire collection of genomic elements yielded by its members and known as microbiome[1]. These microbial communities may modulate host physiology[2] as

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well as the development of its immune system and functions such as digestion, playing also an important role in detoxification reactions[3]. In adult humans, the most abundant and diverse microbiota, accounting for more than five million different genes, are found in the digestive tract[4]. Alterations in gut microbiome composition were found to be associated with health conditions like obesity[5,6], diabetes[7,8], kidney diseases[9], skin immune-mediated disorders[10,11], and allergies[12]. On the other hand, a healthy gut microbiome is deemed to exert a strong influence on the overall host well-being[13] and energy balance[14], even though significant uncertainty remains in distinguishing cause from correlation in this research branch[15].

Gut microbiome studies have been traditionally based on stool samples and conventional culture-based techniques. However, a major drawback of this approach is that pathogens—often representing the main target of such studies—remained undetected, which happened frequently in the case of taxa new to science or at least to a certain geographic area[16]. Indeed, pathogen detectability with conventional techniques was highly reliant on culture conditions and test sensitivity, while methods based on PCR and Sanger sequencing worked satisfactorily only if the primers used amplified pathogen template DNA[17]. The advent of Next Generation Sequencing (NGS) techniques in the early 2000s revolutionized microbiome studies allowing to overcome these shortcomings, which not only translated into identifying unknown etiological agents but also into developing new tests for pathogen detection[18]. Over the last 2 decades, NGS has become a routine in microbial community analyses, with the vast majority of NGS studies on gut microbiome based on 16S rRNA metabarcoding (i.e., amplicon sequencing), which is used to identify bacterial taxa and estimate their abundance[19]. This approach, which relies on the use of universal primers to amplify relatively short DNA sequences, suffers, however, from some methodological drawbacks such as the limited taxonomic resolution and the possible incorporation of PCR-linked errors[20]. Today, shotgun metagenomics (a technique allowing to sequence the entire genomes of microbial communities as opposed to amplifying a single region used as barcode) is increasingly being used as a powerful tool to disentangle complex patterns of pathogen transmission among ectoparasites, animal reservoirs, and humans[21]. This approach, which stands in striking contrast with culture-based techniques that were able to isolate only 10–25% of the overall microbiota[22].

Moreover, a considerable portion of the investigations addressing the role of microbiome on animal health are focused on the implications on humans[23]. Most of these studies, based on NGS approaches, showed that habitat degradation poses animal species at risk not only because of reduced resources but also through microbiota depletion[24–26]. Such changes in the microbial community are known to affect host fitness, which may trigger a cascade effect through species interaction with important implications on their conservation and management[23]. On the other hand, social interactions are also an important driver of gut microbiota composition, as evidenced in wild (e.g., yellow baboon, *Papio cynocephalus*[27]) and feral (e.g., Welsh mountain ponies, *Equus ferus caballus*[28] animal populations as well as humans[29]). Likewise, host aggregation may increase the relative abundance of some microbial taxa—including pathogenic bacteria—while reducing the overall microbiota diversity[30,31].

Untangling multiple factors that influence gut microbiota composition is crucial to understanding the ultimate effect on animal and human health. In particular, the gut microbiome is one of the key drivers behind the biochemical pathways associated with diet and, as such, it is tightly associated with metabolic diseases[32]. Therefore, the characterization of the bacterial component—the largest one in the gut microbiome—and that of the interplay among multiple bacterial taxa is a fundamental prerequisite to better understand the role played by other microbial components (that also affect host well-being[33]), thus disentangling the complex mechanisms underlying a healthy and well-functioning gut microbiome. Likewise, understanding the reciprocal interactions between resident microbiotas in animal and human hosts sharing the same environment is crucial to predict (and, hence, prevent) the emergence and spread of zoonotic diseases[34] that has threatened public health and well-being at global level over the last few years like those caused by *Salmonella*[35].

In this study, we used a 16S rRNA-based metabarcoding approach to characterize the gut microbiome bacterial component across different host categories—namely humans, livestock, captive elephants and wild herbivores—in and around Chitwan National Park (southern Nepal) to evaluate its implications in a conservation medicine perspective[36]. We also evaluated the possible occurrence of potentially pathogenic *ESKAPE-E* bacteria, namely *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanni*, *Pseudomonas aeruginosa* and *Enterobacter* species[37], and *Mycobacterium*, a genus including species responsible for serious diseases in mammalian hosts—humans included[38]—as a constitutive member of the gut microbiota in all host species. Our hypothesis is that the exceptionally close contact between animals and people in Chitwan National Park facilitates the exchange of bacterial taxa, including those uncommon in human gut microbiota, raising potential sanitary concerns. We predict that the number of taxa shared between humans and animals is proportional to the intensity of contact, being highest with captive elephants, intermediate with livestock, and lowest with wild herbivores. If confirmed, this scenario underscores the need for heightened sanitary measures and monitoring in settings where humans, livestock, and wildlife co-occur—characteristic of many protected areas globally.

Materials and methods

Study area

Established in 1973 as the first national park in Nepal, Chitwan National Park (CNP) is a World Heritage hosting an astonishingly diverse flora and fauna[39]. CNP stretches over an area of 932 km^2 situated in the Terai lowlands across the flood plains of Rapti, Reu and Narayani rivers (Fig. 1). The climate of this area is primarily tropical and subtropical, dominated by the summer monsoon. Sal (*Shorea robusta*) forests cover most (70%) of CNP, while patches of grasslands (20%) hosting more than 50 different herbaceous species (including the elephant grass *Saccharum* spp.), lie scattered across it[40]. CNP is home to around 68 species of mammals, more than 576 species of birds, 49 species of reptiles and amphibians, 120 species of fishes and numerous invertebrates[41]. The greater one-horned rhino (*Rhinoceros unicornis*), the Bengal tiger (*Panthera tigris*), the Asian elephant (*Elephas*

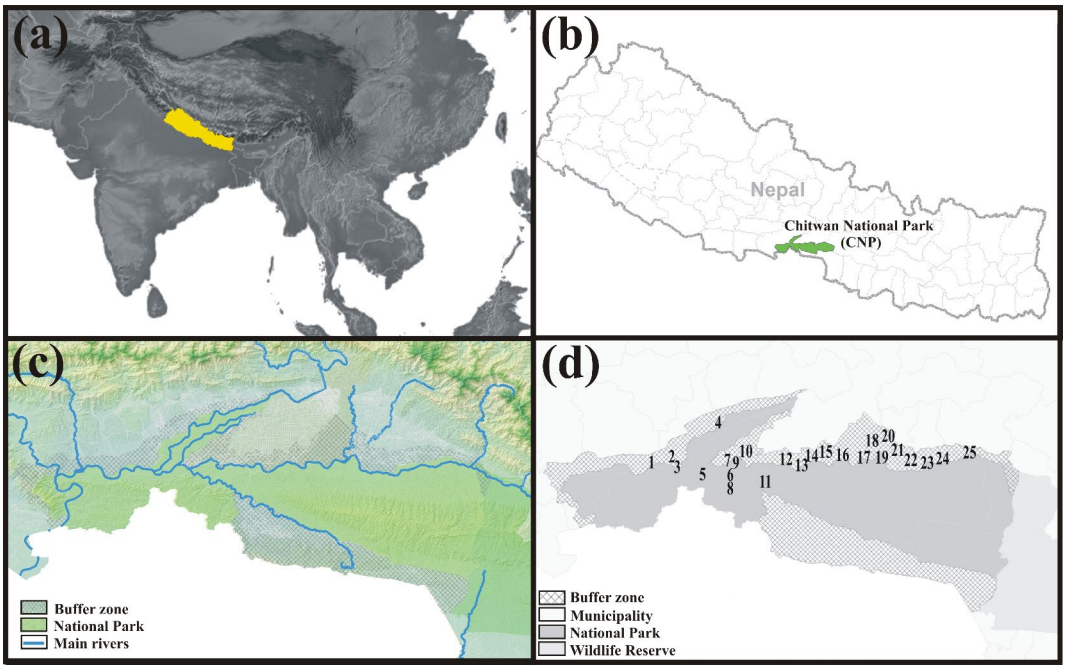

Fig. 1. Study area and sampling locations. (**a**) Map of southern Asia evidencing the location of Nepal (yellow shape). (**b**) Location of Chitwan National Park (green shape) in Nepal. (**c**) Physical map of Chitwan National Park and its buffer zone evidencing the main rivers. (**d**) Sampling locations within the park and the buffer zone (see Table S1 for further details). Maps were created in QGIS v3.30.0 (https://www.qgis.org/).

maximus) and the Gharial crocodile (*Gavialis gangeticus*) are some of the iconic flagship species found in CNP. Other charismatic species inhabiting the protected area are carnivores like the leopard (*Panthera pardus*), the dhole (*Cuon alpinus*), the sloth bear (*Melursus ursinus*), the Bengal fox (*Vulpes bengalensis*) and the striped hyena (*Hyaena hyaena*), as well as medium to large-sized herbivores such as the barking deer (*Muntiacus vaginalis*), the hog deer (*Axis porcinus*), the spotted deer (*Axis axis*), the sambar deer (*Rusa unicolor*) and the gaur (*Bos gaurus*) along with the wild boar (*Sus scrofa*). Elephant safari in the periphery of the park, designated as "buffer zone", was first allowed in 1996 to promote tourism and support the livelihoods of more than 45,500 people living around CNP[42]. Additionally, the buffer area also provides forest resources such as fuel wood, fodder and grass to the surrounding human communities, thus promoting human-wildlife co-existence[43–45]. This, however, entails a thinner human-wildlife interface and increased possibility of contact.

Sample collection, DNA extraction and species identification

A total of 176 fecal samples (Supplementary Table S1) from (i) humans (n = 16); (ii) livestock (n = 15, including three buffaloes *Bubalus bubalis*, one cow *Bos indicus*, six goats *Capra hircus* and five sheep *Ovis aries*); (iii) Asian elephants (n = 40, including one wild and 39 captive individuals); and (iv) wild herbivores (n = 105) were collected during the dry season (which also correspond to the coldest period of the year in Chitwan: https://www.raonline.ch/pages/np/visin2/np_climate01c.html), from December 2018 to February 2019 (all non-human samples) and in April 2021 (human samples). It could be argued that the best approach would have been collecting fecal samples directly from the rectum of wild herbivores to prevent any possible environmental or cross-contamination, but this was not feasible due to the strong restrictions applying to wildlife research in CNP. However, previous research on tiger gut microbiome based on non-invasive samples had evidenced a markedly distinct composition when compared with local soil microbiome profile[46] and, therefore, we have reasons to believe that gut microbiome profiling based on non-invasive samples is reasonably accurate. In the interest of clarity, we are aware that sample sizes are suboptimal for some taxa; nevertheless, while conclusive results cannot be drawn in such cases because of the limited statistical power, it should be noted that downstream analyses were mostly performed within the four above-mentioned category groups (i–iv). Moreover, we acknowledge that for an optimal sampling design it would have been best to collect human samples in parallel with animal samples, but the delay for the issuance of related permits first and the advent of COVID-19 pandemic later prevented us from doing so, which calls for some caution when performing comparative analyses. We selected *mahouts*, an ethnic group of people who are captive elephant riders, trainers or keepers, as human subjects in this study in view of their close contact (i.e., physical) primarily with the pachyderms (in the past, each *mahout* was assigned a single elephant and their working relationship endured for a lifetime, with the amount of time the keeper and the animal spent together being crucial to maximize its tameness and performance[47] but also with their own livestock. CNP *mahouts* mostly live indeed in the buffer zone with their families and are therefore in close contacts with the domestic animals their use for their own subsistence and wild animals living in the park, thus representing ideal candidates for zoonotic trasmission from all these animal groups. Fresh (i.e., dark, hydrated

and soft) fecal samples (or pellets) were selected based on specific guidelines[48] to maximize sample integrity and collected non-invasively for livestock and wild herbivores (for reasons of practicality we included the wild boar – that along with deer is typically known for being a reservoir of pathogens such as *M. tuberculosis*[49]—among herbivores even though it is most often referred to as an opportunistic omnivore[50]) from CNP to maximize sample integrity, while stool samples were collected for humans and captive elephants from its buffer zone, precisely Sauraha village (Fig. 1), immediately after defecation. All the fecal samples were collected in a 4 ml tube containing 2 ml DET buffer and transferred in cold chain to the National Trust for Nature Conservation (NTNC) laboratory in Sauraha before delivery to the Intrepid Nepal Pvt. Ltd. laboratory in Kathmandu. Each sample suspension (600 µl) was mixed to an equal volume of 95% ethanol and centrifuged at maximum speed for 1 min. The supernatant was discarded, and the precipitate was suspended in PBS (600 µl) prior to DNA extraction with the QIAmp fast DNA stool minikit (Qiagen, Hilden, Germany) following manufacturer's manual. DNA was finally eluted in ATE buffer (60 µl). Lack of bead-beating and extra heating steps might have limited the detection of thick-walled microorganisms such as gram-positive bacteria (including *Mycobacterium*), as mechanical disruption is known to enhance the release of nucleic acids[51]. That said, previous studies indicate that suboptimal cell lysis hardly affects the overall microbiome composition as opposed to the overall DNA yield[52]. Species identity of wild herbivore fecal samples was assessed by Sanger sequencing of a ~ 412 bp barcoding region of *cytochrome-b* (*cyt-b*) gene using universal primers mcb398-f (TACCATGAGGACAAATATCATTCT G) and mcb869-r (CCTCCTAGTTTGTTAGGGATTGATCG) as described in[53], and, hence, by BLAST search against the NCBI GenBank database with 97% sequence identity threshold.

Gut microbiota metabarcoding

The amplification of bacterial 16S rRNA was performed with universal primers 341F and 805R[54] targeting the V3–V4 hypervariable regions. We performed the PCR in a volume of 2.5 µl[55] using 1.25 µl of 2X Kapa HiFi Hotstart Ready Mix (Roche, Wilmington, MA, USA), 0.5 µl of each 1 pmol illumina forward (341F) and reverse (805R) primers, and 0.25 µl of template DNA. The thermal conditions were initial denaturation at 95 °C for 3 min, 35 cycles at 98 °C for 30 s, 65 °C for 30 s and 72 °C for 30 s, and final extension at 72 °C for 10 min. The reaction was performed in triplicate for each sample and the three PCR products pooled in a single volume of 7.5 µl for each sample. Aliquots of 1.5 µl each were run on a 1.5% (w/v) agarose gel stained with Gel Red Nucleic Acid Gel Stain (Biotium, Fremont, CA) in TAE buffer and visualized through a digital imaging system expecting an approximately 600 bp-long band. The remaining 5 µl of PCR products yielding successful amplification were cleaned-up by 9 µl of Ampure XP beads (Agencourt, Beckman Coulter Inc., La Brea, CA, United States). The required product was separated from the beads using a DynaMag 96 Side Magnet (Thermofisher Scientific, Waltham, MA, USA), washed with 70% ethanol (Emsure, Merck, Germany) and eluted in 15 µl of nuclease-free water. Subsequently, 3 µl of purified product were used for the indexing PCR along with 1.5 µl of Nextera XT Index 1 Primer (N7XX), 1.5 µl of Nextera XT Index 2 Primer (S5XX) for the Nextera XT index (FC-131–1001 or FC-131–1002), 7.5 µl of 2X KAPA HiFi Hotstart Ready Mix and 1.5 µl of nuclease-free water in a final reaction volume of 15 µl. PCR conditions were: 95 °C for 3 min, 8 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 5 min, and final extension at 72 °C for 5 min.

Individual libraries were quantified using the KAPA Library Quantification kit (Roche) by adding 6 µl of the KAPA SYBR FAST qPCR Mastermix with Primer Premix to 4 µl of diluted library DNA (100,000-fold). This 10 µl reaction volume was processed with the following PCR conditions: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C and annealing/extension/data acquisition at 60 °C for 45 s. Extraction and PCR blanks produced no bands in the gel electrophoresis and were therefore not included in downstream analyses. The quantified libraries were normalized to 4 nM using nuclease-free water and pooled in equal volumes to obtain a 4 nM pooled library that was later spiked with 4 nM PhiX and denatured using 0.2 N NaOH. The denatured library was neutralized using Hyb buffer to result into a 10 pM final library which was loaded using MiSeq Reagent kit v2 (600 cycle) (Illumina, USA) with a 2 × 250 bp paired-end configuration for sequencing. Raw data are available from the SRA database with accession number PRJNA880466. In the interest of clarity, individual samples from the same species were not pooled but sequenced separately; concerning downstream analyses, data were mostly compared across the above mentioned four category groups.

Sequence data processing and taxonomic analysis

We first checked the quality of the raw paired-end reads using FastQC v0.11.9[56], then filtered and trimmed short-length (< 235 bp) and low-quality (q-score < 30) bases with TRIMMOMATIC[57]. We processed the filtered reads using the QIIME2 v2021.11.0[58] pipeline. More specifically, we performed denoising of paired-end reads by trimming, merging and removing chimeric sequences using the DADA2 plugin[59] in QIIME2. We carried out a blast of the obtained Amplicon Sequence Variants (ASVs) against the SILVA 16S rRNA database v138[60] and classified their taxonomy for the phylum, class, genus, and species level using the QIIME2 pre-trained naïve Bayes classifier trained on the SILVA database. We aligned ASV representative sequences using MAFFT v7.490[61] and reconstructed a maximum-likelihood phylogenetic tree using FastTree v2.1.10[62].

Alpha and Beta diversity analysis

We calculated microbial diversity within (alpha diversity) and between (beta diversity) different host categories, based on the number of distinguishable taxa, using QIIME2 core diversity plugins after applying rarefaction with even sub-sampling of 10,000 sequences per sample (this value being deemed sufficient to capture most diversity data[63]). We grouped the samples based on their host category, namely humans, captive elephants, livestock, and wild herbivores. Rarefied abundance data were analyzed with Kruskal–Wallis pairwise test to evaluate the alpha-significance of the diversity across groups. For this purpose, we assessed the alpha diversity using two metrics, namely Faith's phylogenetic diversity (PD)[64], accounting for the phylogenetic relatedness

of the community members, and Shannon Diversity Index[65], based on the relative abundance of different taxa (α = 0.05). We visualized the alpha diversity matrices using R package ggplot2 v3.34[66]. The beta diversity was evaluated by both Bray–Curtis dissimilarity[67,68], based on abundance data, and Weighted as well as Unweighted UniFrac dissimilarities[69], a distance metric incorporating also phylogenetic information to compare samples in QIIME2. The Bray–Curtis matrix was used to perform a Permutational Multivariate Analysis of Variance (PERMANOVA)[70] with 999 permutations and test the significance of differences in microbial composition among host categories. Finally, principal coordinate analysis (PCoA) plots were generated using EMPeror visualization tool[71] to visualize grouping patterns based on different microbiome composition profiles.

Results

Host species

Cyt-b barcoding allowed to identify the species in all non-invasive samples (n = 105), which were assigned to spotted deer (n = 58), sambar deer (n = 1), hog deer (n = 2), greater one-horned rhino (n = 28) and wild boar (n = 16).

Gut microbiota composition

A total of 9,475,915 raw reads were obtained with a mean of 53,840.4 reads per sample (range: 63–723,447). After quality filtering, 8,954,989 reads were retained. High-quality reads (Q ≥ 30) were assigned to 48,570 ASVs with a total absolute frequency of 5,682,895 reads. The median ASV frequency per sample was 15,924 (range: 12–548,727). We retained 176 samples (100%) and 10,000 reads per sample (18.57%) after sample rarefaction (Fig. S1). Overall, Firmicutes (44.95%) and Bacteroidota (36.73%) were the most abundant phyla across all host categories. We also found Proteobacteria (5.56%), Actinobacteriota (3.26%), Verrucomicrobiota (3.13%), Planctomycetota (1.42%) and Patescibacteria (1.20%) (Fig. 2). When the different hosts were considered separately, we found that Firmicutes and Bacteroidota were invariably the most represented phyla, especially in humans (73.34% and 19.06%, respectively). Overall, we observed substantial taxonomic differences across hosts starting from the third most abundant phylum onwards. While Fusobacteriota (3.66%) and Proteobacteria (1.86%) were the other major phyla in humans, Verrucomicrobiota (12.28%) and Planctomycetota (2.31%) ranked in the same position in livestock and Fibrobacterota (3.17%) in captive elephants. When examining the wild herbivores, we found that the other major phyla were Actinobacteriota (10.28%) and Proteobacteria (7.06%) in the hog deer; Fusobacteriota (3.15%) and Patescibacteria (2.59%) in the sambar deer; Proteobacteria (7.91%), Actinobacteriota (7.43%), Verrucomicrobiota (2.63%) and Patescibacteria (2.49%) in the spotted deer; Actinobacteriota (6.46%) and Proteobacteria (5.08%) in the wild boar; Proteobacteria (5.66%) and Planctomycetota (2.94%) in the greater one-horned rhino (Fig. 2). Information on less abundant phyla as well as lower taxonomic categories up to the genus level are provided in Supplementary Table S2, along with the number of reads per taxa and host categories, as well as in Figs. S2–S4; the number of shared genera is shown in Fig. 3.

Concerning the possible presence of *ESKAPE-E* bacteria, we detected the occurrence of *Enterococcus* in greater one-horned rhino (n = 1; 3.6%) and wild boar (n = 3; 18.8%); *Staphylococcus* in captive elephant (n = 2; 5.1%), livestock (n = 1; 6.7%) and spotted deer (n = 1; 1.7%); *Pseudomonas* in captive elephant (n = 4; 10.3%), spotted deer (n = 6; 10.3%), wild boar (n = 2; 12.5%) and greater one-horned rhino (n = 4; 14.3%); *Enterobacteriaceae* in captive elephant (n = 11; 28.2%), spotted deer (n = 9; 56.3%) and wild boar (n = 9; 56.3%); *Acinetobacter* in all host species except for humans, livestock and sambar deer (Supplementary Table S2). We also detected the presence of *Mycobacterium* in the fecal microbiota of captive elephant (n = 2; 5.1%), sheep (n = 1; 20%), hog deer (n = 1; 50%), spotted deer (n = 17; 29.3%), greater one-horned rhino (n = 1; 3.6%) and wild boar (n = 2; 12.5%). In one spotted deer individual (1.9%), we detected *M. goodii*.

Alpha diversity of gut microbial communities across host categories

PD average values ranged from 23 in humans to 69 in captive elephants, with intermediate but still comparatively high values in livestock (60) and wild herbivores (58) (Table 1; Fig. 4). The PD did not differ significantly between these two host categories (Kruskal–Wallis: H = 0.019, $p = 0.890$) but did across all other pairs: livestock versus captive elephant (Kruskal–Wallis: H = 7.762, $p = 0.005$); captive elephant versus human (Kruskal–Wallis: H = 31.189, $p = 2.34E{-}08$); captive elephant versus wild herbivores (Kruskal–Wallis: H = 21.749, $p = 4.59E{-}06$); livestock versus human (Kruskal–Wallis: H = 21, $p = 4.59E{-}06$); and human versus wild herbivores (Kruskal–Wallis: H = 36.350, $p = 1.65E{-}09$).

The average value of Shannon diversity ranged from 4.47 in humans to 7.99 in captive elephants, while it was 7.83 in livestock and 7.74 in wild herbivores (Table 1; Fig. 4). No significant differences in Shannon diversity were observed between captive elephants and livestock (H = 2.506; $p = 0.113$) as well as captive elephants versus wild herbivores (H = 0.912; $p = 0.339$) nor livestock versus wild herbivores (H = 0.171; $p = 0.679$). Conversely, significant values were obtained when comparing human versus captive elephants (H = 29.85; $p = 4.67E{-}08$), human versus livestock (H = 21; $p = 4.59E{-}06$) and human versus wild herbivores (H = 37.56; $p = 8.86E{-}10$).

Beta diversity of gut microbial communities across different host species

We found significant differences in Bray–Curtis beta diversity (PERMANOVA; Pseudo-F = 10.47; $p = 5E{-}04$) as well as Weighted (PERMANOVA; Pseudo-F = 16.18; $p = 5E{-}04$) and Unweighted (PERMANOVA; Pseudo-F = 13.61; $p = 5E{-}04$) Unifrac beta diversity between all host categories (Fig. 5).

Discussion

Gut microbiome makeup is widely recognized as a key driver of human and animal health[72]. The recent advent of NGS has offered a suite of increasingly powerful and affordable tools to get insights into the abundance

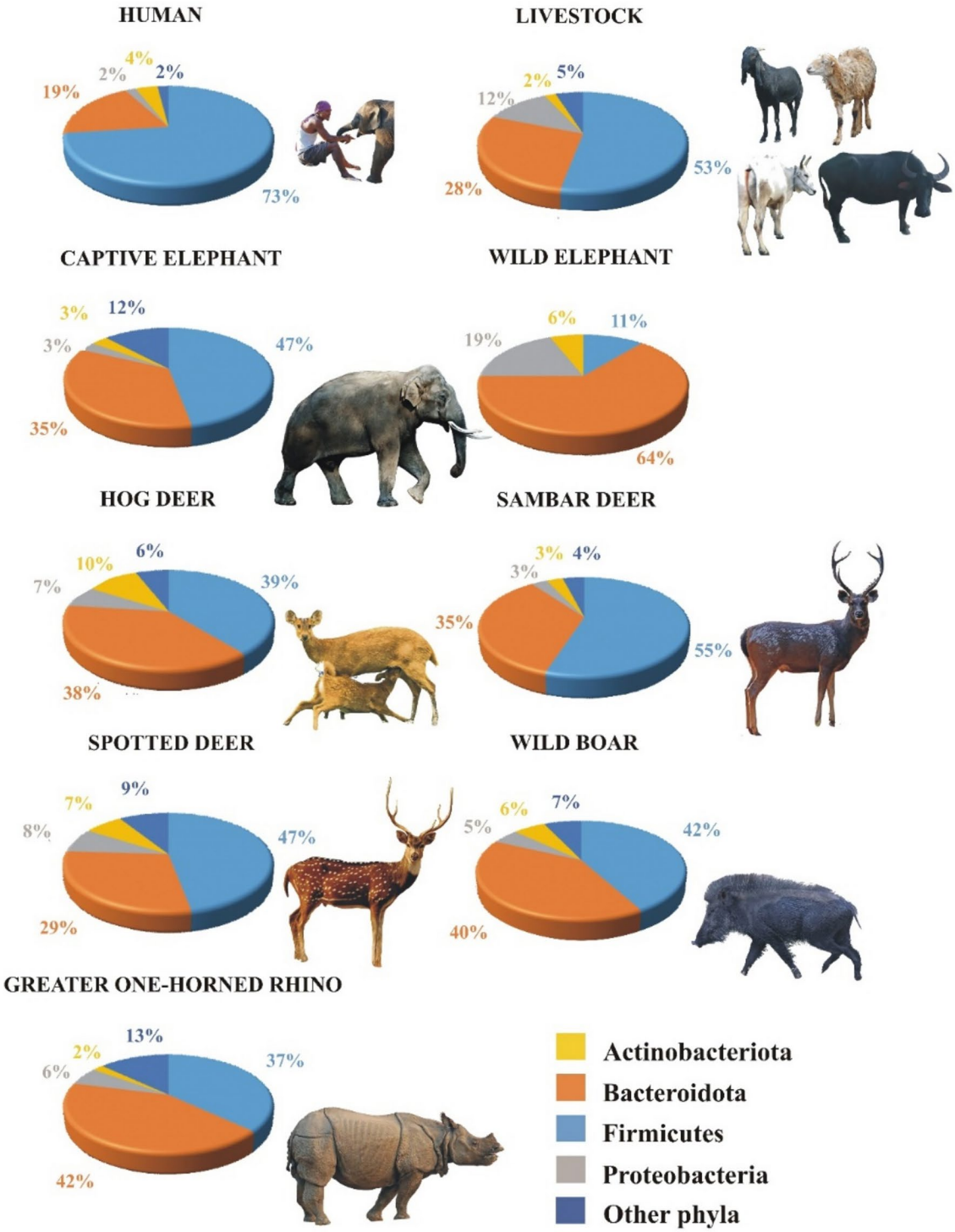

Fig. 2. Phyla composition of gut microbiota across humans (n = 16), livestock (n = 15), captive elephants (n = 39) and wild herbivores (hog deer: n = 2; sambar deer: n = 1; spotted deer: n = 58; wild boar: n = 16; greater one-horned rhino: n = 28). Animal photos (modified) are not to scale; for credits see the Acknowledgements and Appendix S1.

and structure of such microbial communities, delivering massive amount of information of evolutionary, conservation and management interest. Importantly, these data are key for protecting and strengthening public health. In our study, we characterized the gut microbiome in humans and herbivores (both captive/domesticated and wild) in a human-wildlife interface ecosystem to investigate their interactions at the microbiome level.

We found that humans had a significantly lower alpha diversity and different beta diversity than the other host groups surveyed in this study despite their physical proximity and frequent interactions. This result is not

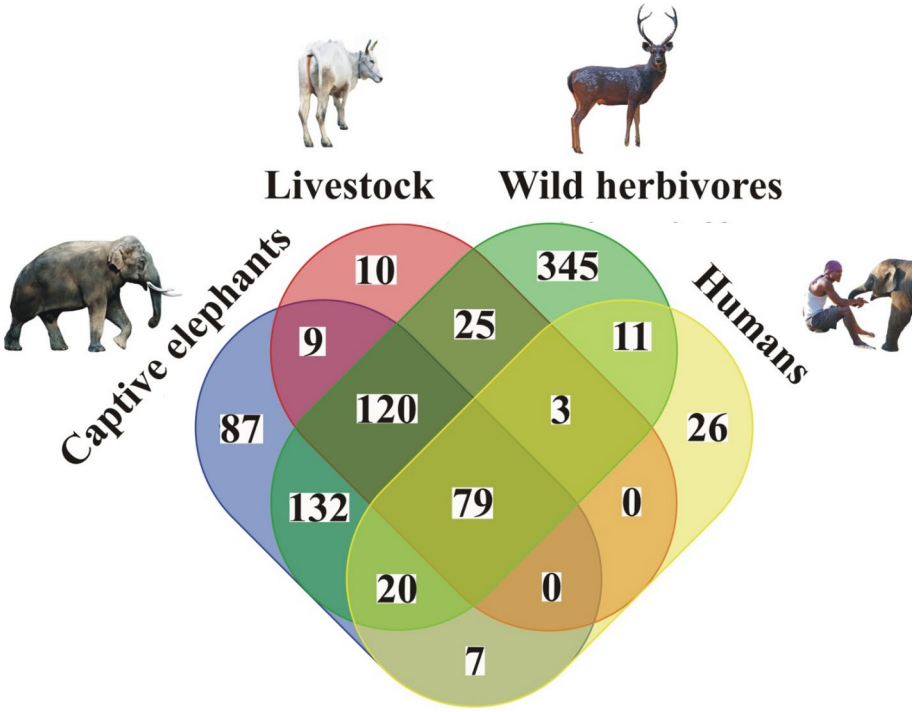

Fig. 3. Sharing of bacterial genera across the gut microbiota of humans (*mahouts*), livestock, captive elephants and wild herbivores. The 79 genera common to all four host categories represent the following percentages: 54.11% in humans; 32.11% in livestock; 17.4 in captive elephants and 10.75% in wild herbivores. Animal photos (modified) are not to scale; for credits see the Acknowledgements and Appendix S1.

	PD	St. dev	SD entropy	St. dev
Captive elephants	69.077	10.865	7.993	0.745
Livestock	60.344	8.418	7.834	0.415
Wild herbivores	58.105	12.279	7.744	1.123
Humans	23.461	8.134	4.477	0.645

Table 1. Faith's phylogenetic diversity (PD) and Shannon diversity (SD) values with standard deviation (St. Dev).

surprising considering that several factors—primarily dietary differences—are known to affect gut microbiome composition and diversity even among phylogenetically close host groups[73,74]. Firmicutes, which are known to play a major role behind systemic immunity[75] and human health as a whole[76,77], stood out among the gut microbiota of *mahouts*. Bacteria from this phylum aid in breaking down carbohydrates, including those found in dietary fiber and resistant starch, which cannot be digested by the host enzymes alone[78]. In contrast, all the other herbivorous mammals shared similar proportions of Firmicutes and Bacteroidota (which are key to ferment polysaccharides and otherwise indigestible carbohydrates[79]), that were invariably the most represented phyla in line with what was observed across a number of other mammalian species[80], humans included[81], and whose balanced ratio is known to be tightly associated with homeostasis maintenance[82]. Likewise, both alpha diversity metrics showed that the gut microbiota community of livestock and wild herbivores was comparable both in terms of composition and relative abundance. This outcome is plausible in that all the members of these two host categories belong to former order Ungulata and to the extant order Artiodactyla, with the only exception of the greater one-horned rhino (which instead belongs to order Perissodactyla) and have largely overlapping dietary niches. Moreover, livestock and wild herbivores often co-occur and compete for the same trophic resources in CNP buffer zone. On the other hand, the beta diversity differed significantly between livestock and wild herbivores. These results could be explained in view of the overrepresentation of some host species, namely wild boar, greater one-horned rhino and especially spotted deer. The latter, other than being by far the most abundant wild herbivore of the Indian subcontinent and representing the major prey species for the resident large carnivore guild[83], has a broad ecological niche, which could reflect its high microbiome beta diversity.

The taxonomic resolution of the 16S region used in this study did not allow us to get to the species level in most cases. Nevertheless, when looking at the presence of *ESKAPE-E* bacteria (which are mostly environmental or commensal taxa occasionally infecting immunodepressed subjects[84]), none of the six bacterial genera at issue

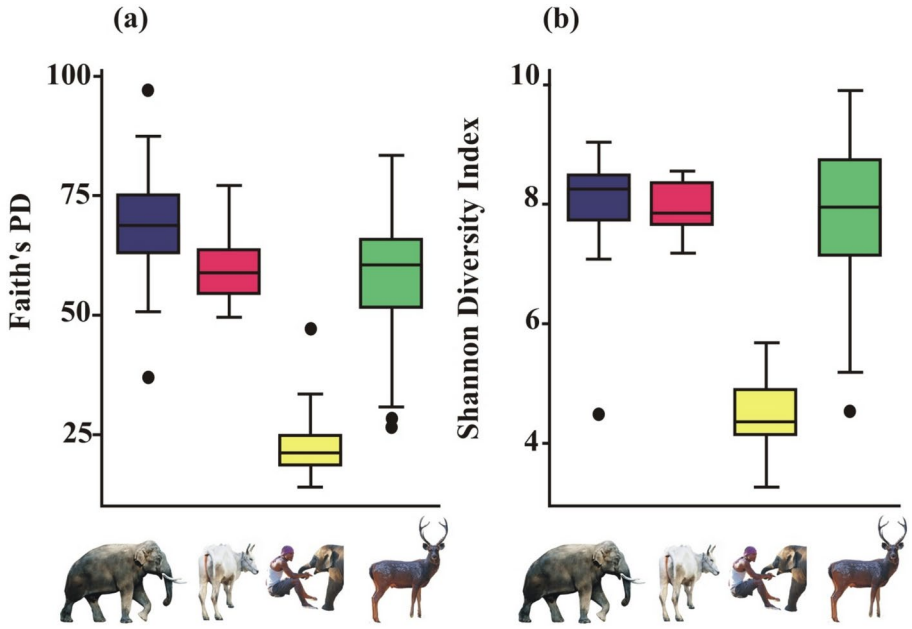

Fig. 4. Alpha diversity based on Faith's phylogenetic diversity (**a**) and Shannon diversity metrics (**b**) (α = 0.05) across different host categories: captive elephants, livestock, humans and wild herbivores (left to right). Animal photos (modified) are not to scale; for credits see the Acknowledgements and Appendix S1.

was found in humans nor in sambar deer, but four were found in captive elephants and one in livestock, which calls for caution in a conservation medicine perspective. Moreover, four of these genera were identified in spotted deer and wild boar, and three in the greater one-horned rhino. These findings highlight key wild herbivores as potential sources of zoonoses and emphasize the importance of examining host eco-behavioral characteristics that may explain this pattern. Interestingly, we also detected *Mycobacterium* in 20% of the spotted deer surveyed in this study (including *M. goodii*, recently defined as an emerging nosocomial pathogen[85]) along with a few other herbivorous mammals including captive elephants[86]. Since mycobacteria are commonly found in soils as well as ground and surface water bodies[87], this finding may be indicative of some forms of environmental contamination of collected fecal samples. Considering that some pathogenic bacteria causing tuberculosis in both humans and animals belong to this bacterial genus, this finding should spur further investigations for public health reasons as well as to understand the role of these bacteria in the normal microbial flora of these mammalian hosts. For this purpose, shotgun metagenomics will provide the taxonomic resolution needed to identify not only *Mycobacterium* and *ESKAPE-E* bacteria to the species level species, but also to collect valuable information on non-bacterial microbiota components—such as protists, fungi and viruses—that might also be relevant to public health.

We observed that captive elephants displayed a significantly higher phylogenetic diversity of bacterial taxa in their gut microbiome, while species richness and evenness quantified as alpha diversity was comparable to that of livestock and wild herbivores (Fig. 4). On the contrary, the beta diversity of captive elephants was significantly different from that of all the other host categories, their *mahouts* included. Elephants, though sharing their diet with other herbivores, are phylogenetically distinct (Proboscidea). Other resident megafauna, the greater one-horned rhino, shared several similarities with the elephants (Fig. 5).

When the taxonomic levels below the phylum were considered, a marked difference in microbiome composition emerged between captive and wild elephants, with the former showing, along with humans, the highest proportion of Lachnospirales and the latter yielding much higher proportion of Caulobacterales and Sphingobacteriales than any other host category (Fig. S3). Captive elephants are given a variety of food throughout their lifetime, which makes it difficult to assess which diet regime corresponds to their healthier gut microbiota community. Addressing this specific question in the near future is certainly among the research priorities of the studies that can be carried out in Chitwan[88,89].

Despite a constitutive proportion of common genera (the highest share of *mahouts* being with their pachyderms and not with their livestock: Fig. 3)—the assemblages of humans, livestock, captive elephants, and wild herbivores inhabiting CNP and its buffer zones still retain well-differentiated gut microbiota communities despite their close interactions. Livestock and captive elephants spend their entire lifetime in and around human settlements and are mostly dependent on humans for food and shelter. It is worth mentioning that contrasting evidence do occur, as evidenced by the substantial share of bacteria (including potentially pathogenic taxa) and their resistance genes between veterinary students and the livestock they had been in close contact with over just 3 months[90]. Conversely, our results aligned with conventional wisdom postulating that microbiome is primarily shaped by the host taxonomic position and only secondarily by their diet niches and environment[91–93]. Our hypothesis regarding the sharing of bacterial components uncommon in human gut microbiota, as well

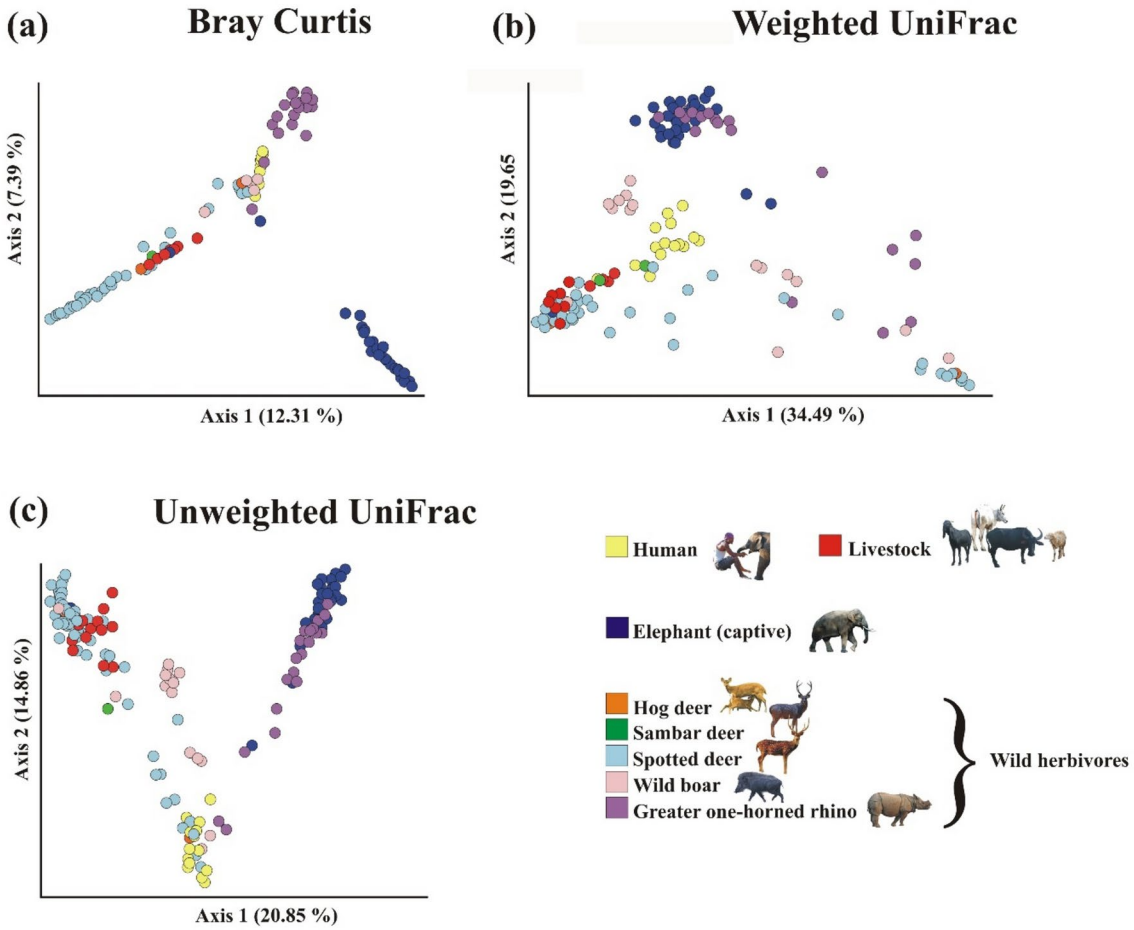

Fig. 5. Beta diversity based on: (**a**) Bray–Curtis, (**b**) Weighted and (**c**) Unweighted Unifrac distance metrics. Animal photos (modified) are not to scale; for credits see the Acknowledgements and Appendix S1.

as our prediction that taxa (i.e., genera) would correlate with the intensity of host contact, was not supported with the limited dataset analyzed. Increasing the sample size in future studies may provide a more definitive answer to this hypothesis. Nonetheless, we obtained valuable insights with significant implications for zoonotic transmission and disease management. Specifically, the comparatively high prevalence of potentially pathogenic taxa in spotted deer and wild boar underscores the need for caution, as these species, often consumed illegally as preferred wildlife meat, are confirmed to be primary sources of zoonotic infections worldwide[94,95].

Conclusions

Our research revealed the bacterial composition and diversity of the gut microbiome of various host species in a popular national park in Asia with high human and animal densities, thus providing information on microbiome sharing and interactions within an ecosystem with increasing contacts and fast thinning interface between people and wildlife. This is particularly relevant in a conservation medicine framework from One Health approach[96,97]—highly reliant on the pursuit of holistic knowledge dealing with all the possible relationships connecting three major One Health components, gut microbiome included. The findings of this study underscore the need for actions to limit human interactions with both wild boars—whose steadily increasing population in Nepal has led to crop raiding and significant human-wildlife conflict[98]—and spotted deer. Additionally, we also advocate raising awareness about the risks associated with wildlife meat consumption and the potential transmission of zoonotic diseases. We recommend conducting similar investigations in other study systems, protected areas and microbiota associated with different body micro-environments, such as the skin surface and the respiratory tract.

Data availability

The 16S sequence fastq data generated by Illumina MiSeq in the present study were deposited in the NCBI SRA database under BioProject accession number PRJNA880466, which can be accessed via https://www.ncbi.nlm.nih.gov/bioproject/PRJNA880466/. Similarly, the cyt-b partial sequence data were deposited in NCBI GenBank database under accession numbers OQ127588-OQ127636 and OP314810-OP314869, these data can be accessed using the accession numbers via https://www.ncbi.nlm.nih.gov/nuccore.

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Author contributions

R.M.R.: writing—original draft (lead); investigation (lead); formal analysis (lead); writing—review and editing (equal); G.F.: Methodology (supporting); validation (supporting); Visualization (lead); Writing—review and editing (lead); P.M.: Data curation (lead); Formal analysis (equal); Software (equal); P.G.R.: Data curation (supporting); R.N.: Data curation (supporting); Investigation (equal); Formal analysis (equal); R.R.: Investigation (equal); Formal analysis (equal); S.S.: Formal analysis (equal); Software (equal); A.S.: Resources (equal); Project administration (equal); C.G.: Conceptualization (equal); Funding acquisition (equal); Project administration (equal); Resources (equal); supervision (lead); validation (equal); Writing—review and editing (equal); P.C.A.: Conceptualization (equal); Project administration (supporting); supervision (supporting); J.D.L.F.G.: Conceptualization (supporting); Project administration (equal); Writing – review and editing (equal); supervision (supporting); validation (equal); J.Q.: Methodology (equal); validation (supporting); D.K.: Conceptualization (lead); Project administration (lead); supervision (supporting).

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Declarations

Competing interests

The authors declare no competing interests.

Ethics statement

The ethical approval of the study was provided by the Nepal Ethical Review Board of Nepal Health Research Council (IRC number 312/2018). All individuals gave written informed consent before participation during the study. The experimental protocol used in the present study was approved by the Bioethics Committee of BIOPOLIS/CIBIO-InBIO (University of Porto). Studies were conducted in accordance with the principles of the Declaration of Helsinki.

Additional information

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