Journal of Genetics (2021)100:84
https://doi.org/10.1007/s12041-021-01326-x

RESEARCH NOTE

Gut microbiome architecture of wild greater one-horned rhinoceros: a vulnerable species from Kaziranga National Park, India

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Received 28 October 2020; revised 7 July 2021; accepted 11 July 2021

Abstract. *Rhinoceros unicornis*, also known as the greater one-horned rhinoceros (GoHR), is a vulnerable wildlife species found in the Indian subcontinent with an estimated global population of 3582, of which an estimated 2995 resides in India. The Kaziranga National Park of Assam is the home to ∼80.56% of the GoH population in India. Recent advances in genetics and microbial studies underscored the importance of gut microbial symbiosis as a crucial factor for host metabolic health and environmental interaction, particularly for higher mammals. Alteration of the normal microbiome can also be an indicator of chronic disease and infection. Freshly voided dung samples from nine dung heaps of free ranging or wild *GoH* rhinoceros were collected from Kaziranga National Park for mapping the gut microbial architecture through 16S-metagenomic approach. In our sample, the *GoH* gut harbours 168.8±12.55 (SE) bacteria-specific OTUs belonging to 21 phyla of which the gram-negative Proteobacteria is the most abundant phyla. Other abundant phylas found in the GoH gut are Firmicutes and Bacteroidetes. Although the GoH rhinoceros gut can utilize fibrous plant by microbial fermentation, the aerobic, nonfermenting *Acinetobacter* (20.7%), *Stenotrophomonas* (17.8%) and *Brevundimonas* (9.1%) constitute about 50% of all identified genus. Functional prediction of the GoH microbiome reveals that >50% of the bacteria present are involved in metabolism followed by cellular processes and information processing. A significant proportion (>1%) are associated with different diseases. In summary, our study characterized bacterial communities of nine wild GoH to identify some unique features and its implication in disease and survival of GoH.

Keywords. microbiome; Kaziranga; vulnerable species and herbivore; *Rhinoceros unicornis*.

Introduction

The greater one-horned rhinoceros (GoHR), *Rhinoceros unicornis*, is a vulnerable wildlife species found in the Indian subcontinent (Talukdar *et al*. 2008, IUCN/SSC Guidelines v. 1.0 2013). The GoHR have an estimated population of around 3747 individuals, spread across the foothills and grasslands of eastern Himalayas and Brahmaputra valley (The Rhino Research Centre, Cambridge, United Kingdom 2019, http://www.rhinoresourcecenter.com; WWF Report 2017, https://www.worldwildlife.org/species/greater-one-horned-rhino). Among the Indian population of about 2995 GoHR (80.00% of the global population), around 2664 of them are found in Assam and of these around 80.56% (2413 as per 2018 estimation) are found in Kaziranga National Park alone (The Hindu, 30 March 2018, https://www.thehindu.com/news/national/other-states/rhino-census-2018-

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Supplementary Information: The online version contains supplementary material available at https://doi.org/10.1007/s12041-021-01326-x.

Published online: 12 November 2021

kaziranga-now-has-2413-rhinos/article23393316.ece). The Kaziranga National Park of Assam occupies the centre stage to all conservation efforts for GoH, as it is the home to ~91% of the GoH population in India. Because of the vulnerable status of the species, an understanding of the pathophysiology and identification of a congenial environment for the survival of the species is of strategic importance. Recent advances in genetics and microbial studies underscored the importance of gut microbial symbiosis as a crucial factor for host metabolic health and environmental interaction, particularly for higher mammals.

The GoHR is a 'simple stomach mega herbivore' weighing up to 2.5 tones and after the elephant, it is the largest extant mammalian herbivore. It has the ability to utilize fibrous plant matter through microbial fermentation in the hindgut (Clauss *et al*. 2005). To digest the huge quantity of grass under an anaerobic environment, a specialized microbial community is essential in these herbivores (Flint 1997). The establishment of a specialized microbiome corresponding to a hindgut fermenter species in the wild is a coevolutionary process in herbivores and is dependent on the diet and habitat (Ley *et al*. 2008; Gibson *et al*. 2019). To understand the nutritional and digestive physiology as well as the functional and biological significance of the gut microbiome of GOH, we have predicted the functional profile of the microbial communities using 16s rRNA marker gene sequences. Our analysis reveals that the majority (>50%) of the bacteria in the microbiome are related to metabolism. Apart from that, we found around 18% of the gut bacteria are functionally predicted as involved in genetic information processing, followed by cellular processes (~10%) and environmental information processing (~10%). A significant percentage (>1%) of the bacteria are predicted to be harmful and are associated with different diseases. The gut microbiome needs to be studied for the vulnerable wildlife species like GoHR. Hence, a better understanding is necessary to facilitate the planning and implementation measures to address rehabilitation, disease outbreak and the survival of the vulnerable species like the GoHR.

To characterize the complex microbial populations in the GoHR gut, we harnessed the power of high throughput massively parallel sequencing. Our present study comprehensively mapped the gut microbial architecture among wild population of GoHR from India using the 16S metagenomic approach. To the best of our knowledge, the present study is the first of its kind, which uses high throughput sequencing to characterize the complex gut-microbial populations of the GoHR from the wild natural habitat.

Material and methods

Freshly voided dung samples (minimum 50 gm) from nine dung heaps (specifically inner masses of the dung heap to avoid possible environmental contaminants) of free ranging or wild GoHR were collected during the month of July 2018 from Kaziranga National Park. The study was approved by the Forest Department, Govt. of Assam for collecting the wild free ranging GoHRs' dung in noninvasive procedure. Dung samples (minimum 50 gm) were collected from the middle of the bolus and kept in sterile plastic containers without any preservatives, and was immediately despatched to the laboratory in an icebox. All possible efforts were made to collect freshly voided dung samples not older than the previous night. The selection was purely based on expert's inspection on the physical and visual parameters of the dung (moisture content, shine and presence of mucous layer, presence of maggots, dung beetles and external/surface fungal growth like mushrooms and toadstool). The dried samples with external fungal growth over the surface, dung heaps scratched and dispersed by wild fowls, birds and wild boars were not picked up for the study. DNA was extracted using the Qiagen Stool DNA kit. Amplicons were generated through 16S universal primer for variable regions between 3 and 4, and sequenced on Illumina-HiSeq and analysed through QIIME (v. 1.9.0) (Caporaso *et al*. 2010). Details are presented in a–c in electronic supplementary at http://www.ias.ac.in/jgenet/. Ethical approval for this study was obtained from Forest Department, Govt. of Assam and Institutional

Table 1. Represents the read count and QC for each sample (nine *R. unicornis*) of pair end 16S amplicon sequencing on Illumina HiSeq2500.

Sample ID	Sequence reads of each for R1 and R2 reads	Combined pairs	Dereplicated pairs	Nonchimeric pairs	R1 (% Q > 30)	R2 (% Q > 30)	Mean read length (bp)
10R	1117782	771661	749837	539411	85.53	76.26	251
1R	1330294	785547	651307	521009	85.82	74.64	251
2R	1081725	754076	717876	557801	86.21	78.01	251
3R	1165296	767913	742165	579629	86.45	77.8	251
4R	918327	613987	595758	419410	85.18	75.11	251
6R	1131494	801627	754089	525982	85.80	77.02	251
7R	986196	703975	669620	473580	86.20	76.78	251
8R	849181	525053	503198	395778	85.60	75.92	251
9R	894630	569181	551190	441477	86.43	76.66	251

ethic committee of College of Veterinary Science, AAU, Khanapara.

Results

In the present study, involving data from nine GoHR, we estimated that on an average, GoHR gut harbours 168.8±12.55 (SE) (range: 108–236) bacteria-specific OTUs. Sequence reads generated for the samples ranged from 1,330,294 to 894,630 (table 1) and across all samples, a total of 1,226,013 sequence reads were identified as bacterial, covering 21 phyla (table 1 in electronic supplementary material). The data reveals that overall the Proteobacteria is the most abundant phyla (minimum 19.09% and maximum 94.09%) of the individual GoHR guts, followed by Firmicutes (minimum 1.3% and maximum 60.8%) and Bacteriodets (minimum 1.2% and maximum 19.6%) among the samples (figure 1).

The bacteria-specific OTUs and alpha diversity indices (Shannon–Weaver, Simpson, Inverse Simson, Pielou's evenness (J) and Fisher alpha) for each individual GoHR is presented in table 2. The alpha diversity of a calf (7R) is different from the adult GoHR and it exhibits the lowest alpha diversity index. We computed the Bray–Curtis β-diversity index (BCDI) among the nine GoHR that are presented in figure 2. The BCDI among the nine wild GoHR ranged from 0.38 to 0.96.

Table 2. Alpha diversity index of *R. unicornis.*

	OTUs	Shannon	Simpson	Fisher alpha	Inverse Simpson
1R	197	1.30	0.54	14.44	2.10
2R	108	1.40	0.75	13.90	4.13
3R	161	2.30	0.79	14.05	4.86
4R	149	2.50	0.87	9.89	7.85
5R	182	2.60	0.89	11.32	9.13
6R	135	2.70	0.85	12.01	6.70
7R	236	0.90	0.41	7.72	1.69
8R	191	1.60	0.67	11.23	3.09
9R	160	3.09	0.90	17.60	10.05

A total of 39 OTUs, homogeneous across all nine GoHR, covering 90.4% (1,108,929 OTU reads) of total 1,226,013 bacterial sequence reads among all nine samples have been treated as the core OTUs (figure 3). Among the 39 core OTUs for nine GoHR, 18 OTUs identified the genus that covers 925,621 sequence reads (83.4%). Within the core OTUs, the genus *Acinetobacter* (20.7%), *Stenotrophomonas*

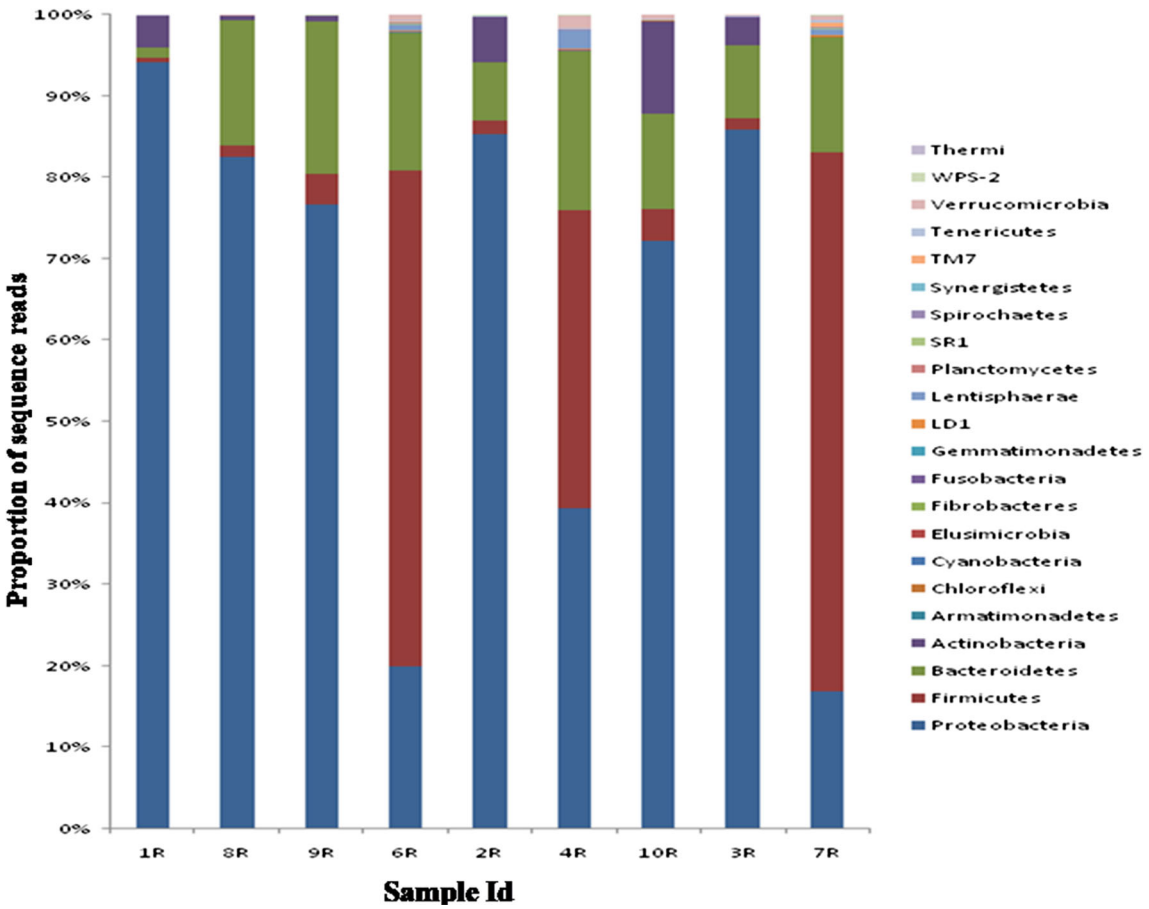

Figure 1. Represents the abundance of 21 annotated phyla for each nine wild *R. unicornis.*

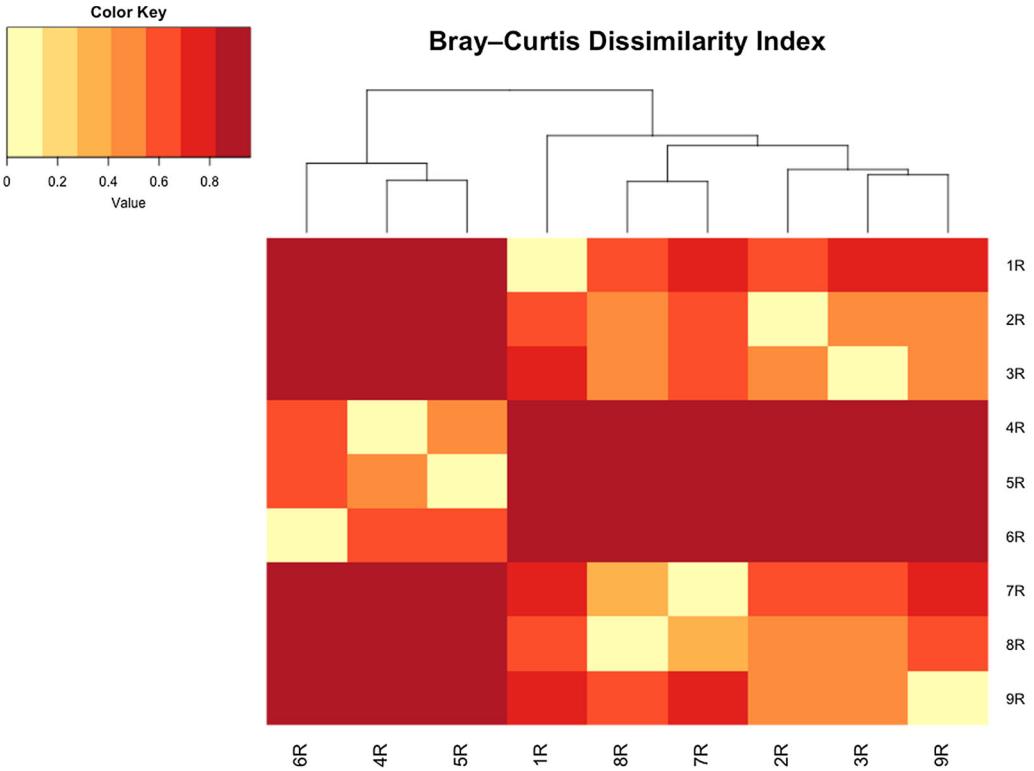

Figure 2. BCDI among the nine GoHR.

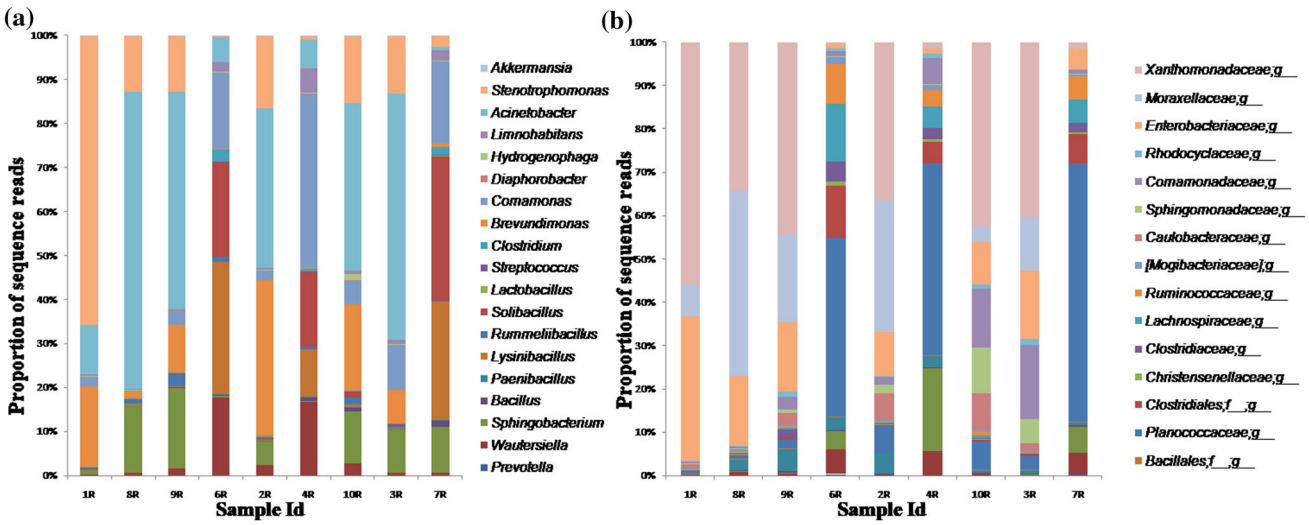

Figure 3. Thirty-nine OTUs are homogenus across the all nine *R. unicornis* that covers 90.4% of total 1,226,013 sequence reads that assigned for bacterial sequence among all nine samples, may exhibit as core. (a) OTUs identified as bacterial genus that are present in all nine *R. unicornis*. Among the 39 core OTUs, 18 OTUs identified the genus that covers 83.4% sequence reads. (b) OTUs identified as bacterial sequence as unidentified genus that are present in all nine *R. unicornis.* Among the 39 core OTUs, 16.5% bacterial sequence specific OTUs were unable to identify the genus.

(17.8%), *Brevundimonas* (9.1%) *Comamonas* (6.3%), *Solibacillus* (5.3%), *Lysinibacillus* (5.3%), *Sphingobacterium* (5.1%), *Wautersiella* (3.08%) are present in abundant manner, whereas *Limnohabitans*, *Rummeliibacillus*, *Clostridium*, *Bacillus*, *Hydrogenophaga*, *Lactobacillus*, *Streptococcus*, *Paenibacillus*, *Diaphorobacter*, *Prevotella*, *Akkermansia* also coexist as core with <1% among the all nine *R. unicornis* (figure 3a). Apart from the 18 genus-specific OTUs, we found another set of 21 bacteria-specific OTUs constituting 183,308 sequence reads (16.5% of total read). These OTUs lacked the resolution to identify the genus of the bacteria, but belonged to different taxonomic

groups like Coriobacteriaceae, Flavobacteriaceae, Planococcaceae, Xanthomonadaceae, Comamonadaceae, Enterobacteriaceae, Rhodocyclaceae, Moraxellaceae, Mogibacteriaceae, Christensenellaceae and Bacteroidales (figure 3b). For the nine GoHR, the number of OTUs that were identified as core, but lacked the resolution to identify the genus ranged from minimum 124 (0.0001%) sequence reads to a maximum of 53,088 (4.3% of total read) sequence reads. The OTUs belonging to the families Planococcaceae (4.3% of total read), Xanthomonadaceae (3.2% of total read) and Moraxellaceae (1.7% of total read) are the major ones for which the genus was not identifiable. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences through phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) reveals that the majority (50.2 ± 1.56%) of the bacteria in the microbiome are related to metabolism. Apart from that, we found around 18 per cent (17.8± 0.06) of the gut bacteria are functionally predicted as involved in genetic information processing. Other important functions predicted are cellular processes (9.7 ± 0.07%) and environmental information processing (9.9 ± 0.01%). A significant percentage (>1%) of the bacteria are predicted to be harmful and are associated with different diseases (figure 4).

Discussion

It is essential to initiate the studies for understanding the mutual symbiotic gut bacterial association and its dysbiosis through diseases and pathogens affecting the GoHR as a considerable number of cases of GoHR deaths due to pathogenic infection go unaddressed because of our ignorance. As per the IUCN's Disease and Parasite consideration (5.1.6), surveillance of source populations can establish the potential pathogen community present among the individuals of the population, thus enhancing our understanding of host–pathogen interaction for the species. Both of the above warrants a comprehensive characterization of wild GoHR gut microbiome. This study documents that the *R. unicornis* gut is dominated by gram-negative Proteobacteria, Firmicutes and Bacteriodetes. In our study, 0.14% of the GoH gut microbiota remains unclassified. The GoH rhino gut microbiome potentially differs from the white and black rhino, whose gut microbiome is dominated by Firmicutes and Cyanobacteria (Bian *et al*. 2013; Gibson *et al*. 2019). However, Proteobacteria dominated herbivore gut has been documented in extinct mega herbivores like mammoth (76.2%) and Wooly rhinoceros (19.8%) (Gibson *et al*. 2019). In these extinct species, Proteobacteria was followed by Firmicutes and Actinobacteri (Talukdar *et al*. 2008). In domesticated herbivores, a distinct microbial architecture has been observed. For instance, the gut of dairy cattle is dominated by Firmicutes (70.1%) followed by Bacteroidetes (8.1%), Actinobacteria (7.3%) and Proteobacteria (2.5%). Surprisingly though, dietary alteration including antibiotics did not reveal significant alteration of GI tract microbial architecture. 'Firmicutes dominated gut microbial architecture' was also documented in several carnivorous animals like leopard cats (63%), otters (88%) and raccoon dogs (90%), while Proteobacteria is present in high abundance among

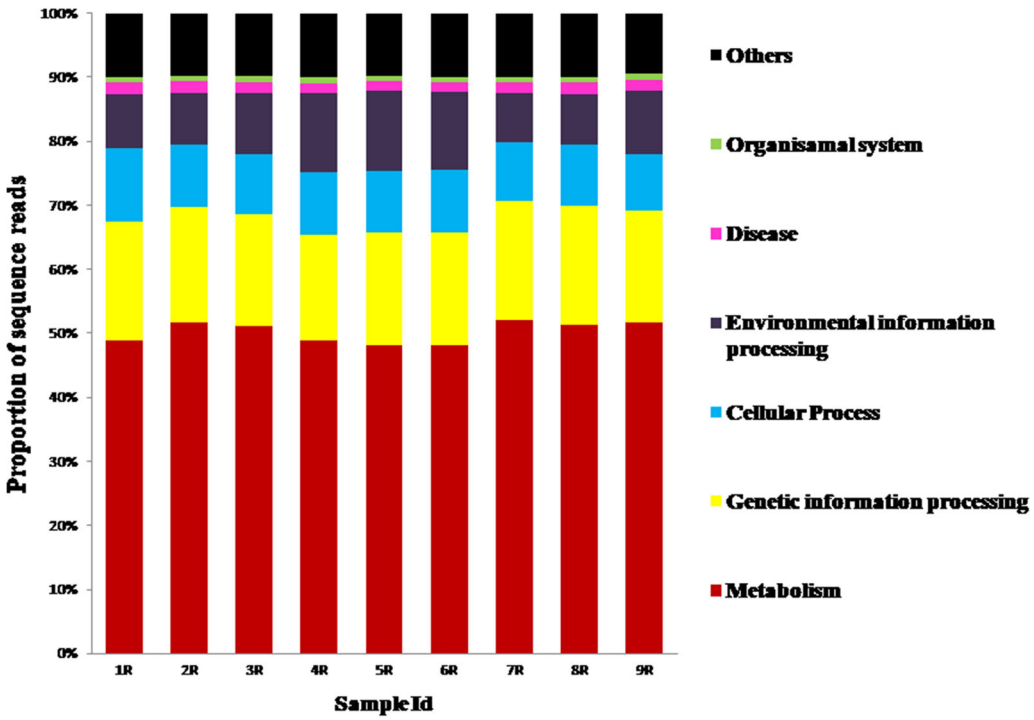

Figure 4. Represents the predictive functional profiling of microbial communities through PICRUSt.

leopard cats (range 10–80%). Habitat specific difference in bacterial community has also been documented for animals; e.g., the wild red panda gut is Proteobacteria dominated (40–80%) while in captivity it is dominated by Firmicutes (80–90%). The GoHR gut-microbiome also harbours soil bacteria and bacteria that are present in plants like *Solibacillus* (5.3%), *Lysinibacillus* (5.3%), *Sphingobacterium* (5.1%) *Limnohabitans*, *Rummeliibacillus* and *Hydrogenophaga* that are associated with plant are also present in <1% abundance. The presence of soil and plant associated bacteria in GoHR stool possibly exhibits its poor digestive herbivore nature. These bacteria are hence likely to be habitat specific, like in this case all the samples were collected from grasslands of Brahmaputra valley. *Brevundimonas*, *Prevotella* and *Clostridia* that are present in GoHR gut are associated with plant-derived fibre degradation among the herbivores (Mardanov *et al*. 2012). *Lactobacillus* and *Streptococcus* that are abundant inhuman gut was also present in limited abundance in all nine GoHR samples. These bacteria are likely to be the core and are crucial for energy metabolism and growth.

In summary, the study documents the composition of bacterial communities in the faeces of nine wild *R. unicornis* from the grasslands of Brahmaputra valley. Present data reveals the presence of a unique bacterial population that comprises admixture of plant, soil and animal associated bacteria. The core gut bacteria of *R. unicornis* exhibit its own distinctive microbial architecture that comprehensively differ from other rhinos (black rhino and white rhino) as well as other herbivores like horses, swine and dairy cattle (Flint *et al*. 2008; Daly *et al*. 2012; Mardanov *et al*. 2012). Although there are differences in the proportion of different bacteria in the GoHR, it is to be noted that after the functional prediction of the gut microbiome, the individual variation gets considerably reduced (figure 4). The data reveals that about 50% of sequence read corresponds to metabolism, 20% reads are involved to explain genetic information processing and 10% of reads are carrying the information on cellular process, environmental information processing.

This study initiates investigation of *R. unicornis* gut microbiome using a high throughput 16S metagenomic sequencing to map its gut. The observations may be crucial to understand the bacterial ecosystem of this vulnerable wildlife species and would enable us to better understand the involvement of those bacteria in varied range of functional prediction that includes host metabolism, genetic information processing, cellular network and diseases (figure 4). A comprehensive catalogue of the gut microbiome of GoHR provides an opportunity for disease surveillance using the easy and noninvasive route of gut microbiome analysis. It also initiates the required groundwork for possible habitat identification and rehabilitation of the species. The datasets generated for the current study are available with the corresponding author.

Acknowledgements

We would like to acknowledge the Chief Wildlife Warden of Assam for allowing us to initiate the study. We are thankful for the help and needful support provided by the Field Director, Kaziranga National Park, Divisional Forest Officer, East Assam Wildlife Division, Range Forest Officers and the forest guards for providing necessary assistance for carrying out this study and collect necessary samples. The authors sincerely acknowledge Mrs Soumita Dutta for laboratory assistance. We would like to thank Sri Ravi Singh, SG & CEO WWF India for his constant motivation and support to undertake challenging works. We thank the team members of WWF India especially Dr Dipankar Ghose and Dr Anupam Sarmah for their help and support during the study. This study was possible because of the support of our donors, especially WWF-US. The study was funded by World Wildlife Fund (WWF) US grant number PRO-927.

Author contributions

PK, SKP and AB were involved in the study design and data generation, and analysis. DP, AS and PK conducted the field work and sampling. NNB facilitated sample processing and coordinating between the two laboratories of College of Veterinary Science, AAU, Khanapara and Regional Medical Research Centre. CKB and CB were involved in data generation and analysis. All authors were actively involved in manuscript preparation.

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Corresponding editor: T. N. C. VIDYA