Journal of
Zoological and Botanical Gardens

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Review

Conservation Innovations and Future Directions for the Study of Rhinoceros Gut Microbiome

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Abstract: Rhinoceros are among the largest and most endangered herbivores in the world. Three of the five extant species are critically endangered, with poaching and habitat loss contributing heavily to declines. The gut microbiome is an essential facet of host health and digestion, mediating a variety of immune and physiological functions. Certain microbes have the potential to serve as biomarkers for reproductive outcomes and predictors of disease susceptibility. Therefore, assessing gut microbial dynamics in relation to wild and managed rhinoceros populations has particular relevance for zoos and other conservation organizations that maintain assurance populations of these charismatic megafauna. The functional gut microbiomes associated with all rhinoceros species remain poorly studied, and many published reports are limited by small sample sizes and sex biases. In this review, we synthesize current research to examine the rhinoceros gut microbiome under human management and resulting conservation implications, address common pitfalls of wildlife gut microbiome studies, and propose future avenues of research in this field.

Keywords: black rhinoceros; endangered species; gut microbiome; greater one-horned rhinoceros; southern white rhinoceros; Sumatran rhinoceros; conservation

Citation: Burnham, C.M.; Ange-van Heugten, K.; McKenney, E.A.; Minter, L.J.; Trivedi, S. Conservation Innovations and Future Directions for the Study of Rhinoceros Gut Microbiome. *J. Zool. Bot. Gard.* **2023**, *4*, 396–412. https://doi.org/10.3390/jzbg4020030

Academic Editor: Steven Monfort

Received: 19 December 2022
Revised: 10 April 2023
Accepted: 18 April 2023
Published: 2 May 2023

1. Introduction

The mammalian gut microbiome is inextricably connected to host health and function; it influences disease susceptibility and metabolic dysregulation in managed wildlife, including the prevalence of gastrointestinal disorders [1–3]. Beyond disease, microbes within the gut affect host nutrition and may serve as biomarkers for reproductive dysfunction [4,5]. From both a conservation and animal welfare standpoint, understanding the gut microbiome is of critical importance for managing imperiled species [3,6].

Rhinoceros are some of the largest and most endangered terrestrial megafauna on the planet. The *Rhinocerotidae* family is divided into five extant species: the white rhinoceros (*Ceratotherium simum*), the black rhinoceros (*Diceros bicornis*), the greater one-horned (or "Indian") rhinoceros (*Rhinoceros unicornis*), the Sumatran rhinoceros (*Dicerorhinus sumatrensis*), and the Javan rhinoceros (*Rhinoceros sondaicus*). Of those species, three (black, Sumatran, and Javan rhinoceros) are classified as critically endangered by the International Union for Conservation of Nature, while the greater one-horned is classified as vulnerable. White rhinoceros are divided into two subspecies, the northern white rhinoceros (*C. s. cottoni*) and the southern white rhinoceros (*C. s. simum*); the northern white rhinoceros is functionally extinct, while the southern white rhinoceros is considered near threatened [7]. Alarmingly, there are fewer than 100 individuals left within either the Javan or the Sumatran rhinoceros populations [8,9]. The greatest threat to most rhinoceros species is poaching: their keratinous horns are highly desired for traditional medicine in China and Vietnam and fetch a lucrative price in illicit markets [10,11]. Habitat fragmentation and loss are secondary

drivers of population declines, isolating reproductive-aged individuals and limiting mating opportunities [8,9].

Given the precipitous decline of rhinoceros numbers in the wild, healthy assurance populations should be maintained under human management. Past conservation efforts focused on targeted translocations and consolidation of populations successfully revived both the southern white rhinoceros and greater one-horned rhinoceros populations from fewer than 100 individuals at the end of the 19th century to over 18,000 and 3500 estimated individuals, respectively [10,11]. However, subsequent generations of ex situ rhinoceros populations bred under human management face problems that limit their progenitive success including infertility, early embryonic death, irregular/lack of cycling, and several disease states uncommon in wild populations including hemolytic anemia and renal failure [12–16]. Fewer than 50% of managed southern white rhinoceros are reproductively successful, and nearly 50% of females over the age of 15 develop a reproductive tract pathology [15]. Almost half of greater one-horned rhinoceros calves are stillborn, regardless of whether the female is primiparous or proven [15,17,18]. Gut microbial studies may elucidate the etiology of these deleterious conditions and therefore better inform future management of the diet, health, and pairing of animals under human care. This could be accomplished through the identification of gut microbial taxa associated with specific deleterious conditions, the presence or absence of which may be used as a prognostic indicator of infection. These taxa are potential therapeutic targets and resulting diseased states could possibly be ameliorated with microbial interventions (i.e., probiotic supplementation or fecal microbiota transplant [19]). Studbook management could similarly benefit; for example, a young female rhinoceros entering the breeding population and paired to a genetically valuable mate could be evaluated for microbial biomarkers that predict reproductive failure and may be quickly exchanged for a more viable candidate and/or treated with hormone therapies or microbial interventions. Monitoring changes in microbial communities due to environmental factors may also spur husbandry changes (e.g., by tracking the microbiome while implementing less rigorous cleaning procedures, it may be possible to achieve a more diverse gut microbiome without any adverse effects on animal health while also providing the added benefit of reducing staff workload). These strategies may become increasingly feasible and effective with further research.

Here, we review the literature on rhinoceros gut microbiomes with emphasis on the conservation of four rhinoceros species (black, white, greater one-horned, and Sumatran rhinoceros). Our aims were to collect, summarize, and evaluate relevant research on this topic, illuminate contradictory findings within rhinoceros gut microbiome studies, identify common pitfalls in those studies, and propose avenues for innovative future research. The literature review was performed by searching PubMed, Google Scholar, and North Carolina State University's Summon database for pertinent articles. Keywords included "rhinoceros" and "gut microbiome", as well as the adjacent term "fecal microbiome." Common synonyms and abbreviations for "gut" were also searched, including "gastrointestinal" and "GI". Literature was excluded for the following criteria: being too broad in focus; pertaining to gut microbiomes within non-rhinoceros species specifically, including rhinoceros beetle species; being outside the scope of the review (e.g., metagenomic analyses or evolutionary microbial convergence). Non-peer reviewed literature was also excluded. PubMed returned nine results for "rhinoceros gut microbiome", of which five were relevant to this review. North Carolina State University's Summon directory returned 231 results, of which six were relevant. Google Scholar was the least discriminant literature database, returning 422 results containing the keywords "rhinoceros" and "gut microbiome." After exclusion criteria, 12 total relevant rhinoceros gut microbiome-specific papers were discovered (including all previously discovered papers from other databases), which is the sum-total of published literature on this topic to-date to the best of our knowledge; this is unsurprising given the relative novelty of the gut microbiome field (e.g., the two earliest publications were published in 2013 and 2017, with the brunt published in 2019 and beyond). One paper relating to the microbiome composition of the extinct woolly rhinoceros (*Coelodonta*

antiquitatis) published by Mardanov et al. [20] was excluded after further review, as it was of dubious significance to current conservation efforts of rhinoceros species and involved microbial DNA collected from intestinal contents of a single animal that was dead for an indeterminant amount of time before being preserved in permafrost ~40,000 years ago. Based on these factors, it is unlikely that the results of the microbial community analysis accurately reflect the microbial community present in the living animal. Thus, 11 papers pertaining to gut microbiome in rhinoceros species were evaluated in this review. There are currently no publications that characterize the microbiome of Javan rhinoceros, given the elusive nature of wild populations and the absence of any individuals under ex situ human management; therefore, we are unable to include this species.

2. The Animal Gut Microbiome under Human Management and Implications for In-Situ/Ex-Situ Conservation

A microbiome can be defined as a microbial community occupying a given host or habitat, forming a dynamic micro-ecosystem with commensal, symbiotic, and pathogenic interactions [21,22]. The gut microbiome specifically comprises microbes in the gastrointestinal tract, and mediates host physiology including immune function, metabolism, and digestion [23,24]. Microbial interactions with the host are varied and may be diffuse/indirect, though bidirectional interactions via the gut-lung, gut-brain, and gut-liver axes have recently been described in human and mouse models [25–27]. Atypical shifts in the gut microbiome may lead to a persistent imbalance in diversity and composition of gut communities with downstream consequences for host health. This imbalance, termed "dysbiosis", is usually associated with decreased microbial taxonomic richness and potential increase of opportunistic pathogenic species known as "pathobionts" [28–30]. Overgrowth of pathobionts can result in a diseased state in the host, which may be associated with compromised immune systems due to stress or infection in managed animals [2,31].

Gut microbial taxa are often associated with specific host species, disseminating vertically between parents and progeny [32–35]. However, microbiomes vary widely among individuals, even within host species; this individual variance makes up a host's microbial "cloud" [36]. Individual variation may be attributed to diet [37–39], gut morphology [40], feeding strategy [41,42], geographic location [43,44], housing facility [16], season of sampling [44–47], health status [48], sex [49,50], age [51,52], and a variety of other factors. As an individual matures, their microbiome continues to develop toward a stable climax community via both diet and horizontal transfer from the environment and conspecifics [53–55]. Regardless of this horizontal uptake, much of the host microbiome cannot exist independently from that host [56]. If these host-specific microbial species are lost due to some disturbance such as antibiotic usage, they cannot be recovered via the environment and will not be passed to offspring. It is hypothesized that this loss would be compounded in zoos and other conservation facilities, where limited population sizes and reproductive partners may lead to a generational loss of microbial diversity that is nearly impossible to reestablish [57].

Previous comparisons of managed and wild gut microbiomes revealed that some managed animals have reduced microbial diversity in comparison to their wild conspecifics [3,58–62]. There are many proposed explanations for this potential trend, including the lack of "natural" dietary diversity, relative sterility of the managed environment, lack of interaction with allospecifics, and the necessary usage of antibiotics to treat sick animals. Loss of wild-type microbial diversity can lead to the loss of specific microbial species crucial in degrading certain plant toxins and fibers. While this may be of minimal consequence to animals under human management who are often fed easily digestible diets, it jeopardizes wild translocation or reintroduction attempts, especially for herbivores [3,63]. In addition, managed animals with dysbiotic microbiomes may be more susceptible to pathogens and disease in the wild, further impeding reintroduction efforts and potentially spreading deleterious effects to wild conspecifics exposed to released individuals [3,64]. Despite this,

managed animals translocated in situ may acquire a wild-type microbiome and experience increased microbial diversity, given enough time [65,66].

There is, however, ample room for skepticism regarding the validity of many of these managed versus wild microbiome inferences. Population sizes across many of the comparative studies were low ($n < 15$), as is the case with most studies involving rare and endangered animals. As mentioned previously, microbial diversity and community composition can also vary due to age of the animal, season of sampling, geographic location, and zoological facility. These confounding factors prevent the extrapolation of the results of studies based on a single managed facility or single wild population location to the species as a whole.

3. The Rhinoceros Gut Microbiome

Several studies have characterized the gut microbiomes of managed and wild members of the *Rhinocerotidae* family including the southern white rhinoceros, black rhinoceros, Sumatran rhinoceros, and greater one-horned rhinoceros [16,67–69]. Roth et al. [16] compared the major microbial taxa associated with four rhinoceros species (white rhinoceros, $n = 13$; black rhinoceros, $n = 6$; Sumatran rhinoceros, $n = 3$; greater one-horned rhinoceros, $n = 9$) and found similar patterns of gut community composition across hosts, with Firmicutes (range; 51–66.3%) and Bacteroidetes (23.4–39.8%) dominating, followed by Verrucomicrobia (1.9–7.6%), Spirochetes (1.1–3.1%), Actinobacteria (0.03–1.04%), and Fibrobacteres (0.19–2.14%). Different rhinoceros species have different herbivorous feeding strategies: white rhinoceros are obligate grazers, while the black, Sumatran, and Javan rhinoceros are all browsers [70]. The greater one-horned rhinoceros is a primarily grazing species but will also browse depending on food availability, making it difficult to categorize [71,72]. Due to the significant influence of diet on gut microbiome, one would expect the different species to cluster together in microbial composition by feeding strategy. There is some validity to this, as Sumatran and black rhinoceros host greater proportions of Bacteroidetes (~34%) compared to southern white (~23%) and greater one-horned rhinoceros (~18%) [16]. The black and Sumatran rhinoceros also had less microbial diversity (Shannon diversity; $p < 0.001$) than the other two species [16]. These results diverge from expected outcomes based on phylogeny, managed diet, and historic geographical range. Incidentally, both black and Sumatran rhinoceros are prone to iron overload disorder (IOD) under human management, though further research is necessary to assess if there is a microbial mechanism behind this trend [16]. McKenzie et al. [3] identified *Rhinocerotidae* to be unique as one of the few taxonomic families to actually experience an increase in microbial diversity under human management. Nevertheless, the low sample size of 13 individuals from only two species ($n = 6$ white rhinoceros; $n = 7$ black rhinoceros) limit the broader applicability of this finding. It is also unclear whether increased microbial diversity in a managed population is beneficial or indicative of dysbiosis, especially when "core" microbiomes (i.e., which taxa are shared across all individuals) are difficult to establish. It should be noted that all current studies into rhinoceros gut microbiome have been conducted using fecal microbiome as a proxy, as sampling of the gastrointestinal tract is invasive and generally not practicable. General characteristics of four rhinoceros species and their associated microbiomes are listed in Table 1.

Table 1. Rhinoceros species/subspecies demographics and characteristics of their associated gut microbiomes. Bacterial phyla percentage ranges represent the lowest and highest average relative abundance values presented across the published literature.

	Southern White Rhinoceros (*Ceratotherium simum simum*)	**Black Rhinoceros (*Diceros bicornis*)**	**Greater One-Horned Rhinoceros (*Rhinoceros unicornis*)**	**Sumatran Rhinoceros (*Dicerorhinus sumatrensis*)**
IUCN status	Near threatened [10] [1]	Critically endangered [72]	Vulnerable [11]	Critically endangered [9]
Number in wild	~18,000 [10]	~5630 [72]	~3588 [11]	<80 [9]
Social group size [15]	Bulls solitary; cow-calf pairs; female and adolescent groups of <16 individuals	Solitary; cow-calf pairs	Solitary; cow-calf pairs	Solitary; cow-calf pairs
Feeding strategy [15]	Grazer	Browser	Primarily grazer, some browsing	Browser
Bacteroidetes (%)	21 [73]–55 [68]	18 [w] [62]–49 [69]	2 [w] [74]–30 [68]	39 [16]
Firmicutes (%)	23 [67]–72 [16]	26 [69]–64 [16]	20 [w] [75]–78 [16]	56 [16]
Proteobacteria (%)	<1 [67,73]	<1 [16]–24 [w] [62]	1 [16]–63 [w] [75]	<1 [16]
Verrucomicrobia (%)	<1 [69,73]	<1 [16]–3 [69]	<1 [16]–15 [w] [74]	<1 [16]

[1] References for published data presented in []. [w] Wild rhinoceros data.

3.1. White Rhinoceros

White rhinoceros are divided into two subspecies: the northern white rhinoceros and southern white rhinoceros. The Northern white rhinoceros is functionally extinct, with only two nonreproductive female individuals left [10]. Given the rarity of the species, there are no associated gut microbiome publications. All published literature reviewed hereafter concerns the gut microbiome of southern white rhinoceros. Southern white rhinoceros are obligate grazers native to the grasslands of South Africa, Kenya, Namibia, and Zimbabwe [10]. They are the most numerous of the five rhinoceros species with an estimated 18,000+ individuals, the majority of which (>90%) reside within South Africa [10]. Regardless, they are considered Near Threatened by the IUCN due to rampant poaching within their range, which has caused the population to decline 15% between the latest surveys in 2012 and 2017 [10]. Despite previous success with translocated managed populations, zoo-born generations following the founder females face a variety of reproductive issues [15,68]. Some researchers hypothesize that human-formulated diets and hormonal imbalances (e.g., dietary estrogen levels) are partially responsible, as white rhinoceros fertility levels have been shown to correlate to fecal phytoestrogen profiles [68,76]. Phytoestrogens are derived from plants and compete for the same receptors as native mammalian estrogen; they are especially prevalent in the nutrient-rich legume hay and soy/alfalfa-based concentrates once typical in formulated diets [76]. Williams et al. [68] suggested that reproductive outcomes in white rhinoceros may be linked to the presence or absence of gut microbiota that convert dietary phytoestrogens into usable metabolites. The authors identified 77 operational taxonomic units (OTUs) significantly associated with phytoestrogen concentrations, noting that the study population of white rhinoceros (n = 6) exhibited significantly higher abundances of phytoestrogens than greater one-horned rhinoceros (n = 2) at the same facility. In addition, two OTUs (unclassified members of *Lachnospiraceae* and the RC9 gut group OTU 46) were significantly associated with increased fertility (measured by pregnancies achieved/calves born) in rhinoceros, while four OTUs (unclassified members of *Bacteroidales*, YRC22 group, RC9 gut group OTU 92, and *Prevotella* spp.) correlated to decreased fertility [68].

While certain major taxa (e.g., Bacteroidetes and Firmicutes) were consistently detected in managed southern white rhinoceros microbiomes across studies, the abundances and rankings of those taxa varied substantially despite all studies utilizing similar methods (i.e., 16S rRNA sequencing of microbial DNA extracted from frozen samples). Cersosimo et al. [69] and Williams et al. [68] identified Bacteroidetes as the most common phyla (41.6–55%) followed by Firmicutes (29–33%), while Burnham et al. [73], Bian et al. [67] and Roth et al. [16] identified Firmicutes as the most common phyla (44–55%), followed by Bacteroidetes (21–35%). Kothmann et al. [77] analyzed the microbiome of n = 8 rhinoceros

but did not assess phyla abundance, instead opting for phylogenetic class comparisons. Kothmann et al. [77] identified the Bacteroidetes class Bacteroidia as most common (~60%), followed by the Firmicutes class Clostridia (~23%). Lentisphaerae was also identified as a major phylum (>1% relative abundance) by both Bian et al. [67] and Cersosimo et al. [69], but only Cersosimo et al. [69] detected Tenericutes at major abundance (1.43%) among n = 3 female southern white rhinoceros. Cersosimo et al. [69] also found that all three white rhinoceros hosted similar microbial communities among individuals, likely due to both social behavior and shared diet [69]. White rhinoceros are the most social of the five extant species, enabling microbial transfer via physical contact among herd members [15,70]. Southern white rhinoceros in the study boasted greater Shannon diversity and richness, as well as higher relative abundances of Firmicutes, Fibrobacteres, and Spirochaetes, but fewer Verrucomicrobia compared to black rhinoceros at the same facility [69]. Kothmann et al. [77] did not find significant interactions between sexes, though comparison of the core microbiome by age classes revealed higher alpha diversity values in young ($\leq$6 years old) versus old rhinoceros ($\geq$6 years old). Burnham et al. [73] also found significant differences in gut microbial diversity among four age classes (juvenile, subadult, adult, geriatric) of n = 10 zoo-managed southern white rhinoceros. In addition, Burnham et al. [73] found significant differences in alpha and beta diversity depending on seasonality of sampling.

3.2. Black Rhinoceros

The black rhinoceros is a browsing herbivore endemic mostly to the bushveld and savannahs of South Africa, Namibia, Zimbabwe, and Kenya [78]. Black rhinoceros population size was estimated to total 5630 in 2018. They are considered critically endangered due to poaching, which peaked between 1960 and 1995 and reduced populations by 98% [78,79]. Black rhinoceros breed readily in zoos, but managed animals can be afflicted by diseased states that are uncommon in wild populations, such as iron-overload disorder, hepatopathy, hemolytic anemia, and ulcerative dermopathy [12,15]. The few comparisons between the gut microbiomes of managed and wild black rhinoceros have yielded incongruent findings: McKenzie et al. [3] detected higher alpha diversity in managed populations (n = 7; 6 managed vs. 1 wild), while Gibson et al. [62] did not detect significant differences in alpha diversity between wild and managed populations (n = 25; 17 wild vs. 8 managed). However, Gibson et al. [62] did find that microbial community composition, as measured by beta diversity, significantly differed between managed black rhinoceros and their wild counterparts. The top phyla (Table 1) present in wild black rhinoceros microbiomes were Firmicutes (51%), Proteobacteria (23.6%) and finally Bacteroidetes (17.6%), while the top phyla in managed rhinoceros were Firmicutes (48%) and Bacteroidetes (42.4%) [62]. Managed black rhinoceros host greater proportions of microbes typically found in domestic ruminants, suggesting that wild-type functional bacterial communities are replaced with those of human-managed livestock [62]. Gibson et al. [62] also revealed that managed black rhinoceros microbiomes code more glycolysis and amino acid synthesis pathways compared to free-ranging black rhinoceros, indicating microbial adaptation to diets containing less fiber and more glucose than items foraged in situ. Optimization of managed black rhinoceros nutrition, administration of probiotics, and fecal microbial transplantation were recommended to restore wild-type gut microbes [62].

Diet-induced shifts in gut microbial membership and functionality may also contribute to reproductive challenges in managed black rhinoceros populations. For example, irregular ovarian activity and obesity are known drivers of limited progenitive output [80–82]. Antwis et al. [82] found significant differences in black rhinoceros gut microbial composition associated with individual, facility, and phase of ovarian cycle. Specifically, *Aerococcaceae*, *Atopostipes*, *Carnobacteriaceae*, and *Solobacterium* all correlate significantly with reproductive success, pregnancy, and high fecal progestogen concentrations [82]. Human-formulated diets may therefore impact reproduction by selecting against bacteria associated with the production of hormones necessary for breeding success in black rhinoceros [82].

Iron overload disorder is an affliction affecting browsing rhinoceros under ex situ human management [83]. This disorder describes hemosiderosis, or excessive accumulation of iron within organ tissue, which has the potential to cause deleterious downstream effects. While reviews of black rhinoceros mortality records do not generally list hemosiderosis or hemochromatosis as cause of death, high iron stores correlate with increased susceptibility to disease and infection in this species [16,83], and death due to infectious disease accounted for 22% of managed black rhinoceros deaths over a 16-year period [12,14,84,85]. As gut microbial composition is directly affected by iron stores in the body and certain microbiota can affect iron absorption, the relationship between the rhinoceros microbiome and IOD is relevant to IOD management [16,86,87]. Roth et al. [16] compared the microbiomes of black and Sumatran rhinoceros (IOD-susceptible) species to that of southern white and greater one-horned rhinoceros (IOD-resistant) in an attempt to discern which, if any, microbial taxa are associated with compromised health/IOD. Black and Sumatran rhinoceros hosted less microbial diversity than the IOD-resistant species, and lacked Coriobacteriaceae, Prevotellaceae, and Clostridium XlVa that were detected in greater one-horned rhinoceros. Black rhinoceros also had the most distinct fecal metabolome (the total collection of metabolites within an organism) of the species studied. While age, sex, and fecal metabolomic profile did not significantly drive compositional differences among individuals, IOD-susceptible species hosted more consistent but less diverse microbial communities than IOD-resistant species [16].

Cersosimo et al. [69] detected significant dissimilarities among three black rhinoceros studied at a single facility, in contrast to their white rhinoceros counterparts. This variation likely results from black rhinoceros' variable browse-based diet and less social behavior. While the black rhinoceros is likely more social than previously proposed, they do not often interact closely with conspecifics and thus limit microbial transmission and community homogenization within the population. High inter-individual variation within black rhinoceros appears to be typical across both managed and wild populations, as noted in other studies [62,69,82].

3.3. Greater One-Horned Rhinoceros

Greater one-horned rhinoceros are classified as vulnerable by the IUCN, with an estimated 3588 individuals occupying parts of Nepal and northeastern India [11]. The majority of the population (>70%) resides within the Assam state of India, mostly in the floodplains and grasslands of Kaziranga National Park [11]. While poaching remains a threat to this species, recent declines are attributed more to habitat loss stemming from agricultural encroachment and decreases in habitat quality due to invasive species (i.e., the vigorous climbing vine *Mikania micrantha* decimating native grasses and browse [88]). Managed populations reproduce successfully in large enclosures ex situ, but females are still prone to miscarriage and nearly 50% of offspring are stillborn [15].

Four analyses of the greater one-horned rhinoceros gut microbiome have been published, beginning in 2019 [16,68,74,75]. Borah et al. [74] evaluated the gut microbial community composition of n = 10 animals sampled within Kaziranga National Park in the Indian state of Assam and found them to be dominated by Firmicutes bacteria (74.9%), as is prevalent in other rhinoceros species. The next most common phylum was Verrucomicrobia (14.8%), followed by Proteobacteria (6.9%) and Bacteroidetes (2.29%). Interestingly, a more recent study published in 2021 by Kakati et al. [75] examined feces from n = 9 greater one-horned rhinoceros within the same Kaziranga National Park as Borah et al. [74] and found Proteobacteria (range; 19.9–94.09%) was the most abundant bacterial phyla within these animals, followed by Firmicutes (1.3–60.8%) and Bacteroidetes (1.2–19.6%). This was the highest relative abundance of Proteobacteria noted in any rhinoceros species, with the next highest being 24% abundance found in wild black rhinoceros (Table 1). Both Kakati et al. [75] and Borah et al. [74] utilized similar methodologies with 16s rRNA sequencing and OTU analysis, though neither were able to directly state how long the rhinoceros fecal samples sat unpreserved in ambient temperature before collection. Borah et al. [74]

collected samples "during the morning hours" while Kakati et al. [75] sampled from dung heaps "not older than the previous night"; lack of stabilization may have contributed to changes in the microbial community profile present within the feces. The results of both studies contrast with those from other rhinoceros species and Williams et al. [68], who found Bacteroidetes at the second highest abundance in greater one-horned rhinoceros. Indeed, the occurrence of Verrucomicrobia at >10% relative abundance is more typical in horses, the domestic animal model for rhinoceros [74,89], and in managed animals compared to wild conspecifics [3]. Still, the greater one-horned rhinoceros is the only member of the *Rhinoceros* genus for which we have microbial data. The differences in community structure could thus stem from differences in phylogeny, the evolutionary history of the host, which is one of most dominant drivers of animal gut microbiome diversity and composition [35,90].

Roth et al. [16] compared the gut microbiomes of $n = 9$ greater one-horned rhinoceros (four male, five female) to the other three species across four facilities and found that while greater one-horned rhinoceros and southern white rhinoceros hosted the most similar community composition, both species still hosted distinct microbiomes. Greater one-horned rhinoceros microbiomes in this study were also not affected by facility-, age-, or sex-based based variation [16]. Unlike white rhinoceros, greater one-horned rhinoceros do not experience declines in fertility driven by high dietary phytoestrogen, though their feeding strategy is >85% grazing and they consume similar managed diets of soy/alfalfa-based pellets and hays [68,72]. Williams et al. [68] sampled $n = 2$ female greater one-horned rhinoceros and found different microbial communities and significantly higher intersample diversity compared to white rhinoceros; this trend is likely driven by the addition of browse and produce to the greater one-horned rhinoceros diet, though the extremely small sample size makes inferences challenging.

3.4. Sumatran Rhinoceros

The Sumatran rhinoceros is native to three protected ranges within the tropical rainforests and montane moss forests of Sumatra, where its current biggest threat is negative Allee effects stemming from inbreeding of isolated populations [9]. There are fewer than 80 individuals remaining in the wild, and of the estimated eight individuals housed in zoos and sanctuaries, only two are currently reproductive; with a declining population, it is one of the most endangered rhinoceros species [9]. Consequently, there is minimal information regarding the gut microbiome in Sumatran rhinoceros.

Roth et al. [16] published the only study concerning Sumatran rhinoceros microbiome in an attempt to ascertain possible links between the microbiome and disease in IOD-susceptible rhinoceros. Characterization of the microbiome communities within $n = 3$ managed Sumatran rhinoceros (2 male, 1 female) revealed relative abundances of major microbial phyla similar to black rhinoceros, despite differences in evolutionary histories and managed diets provided by the facilities. Bacteroidetes were more prevalent in Sumatran rhinoceros than in white or greater one-horned rhinoceros, though the phylum was still second in abundance to Firmicutes. In the Roth et al. [16] study, only 88% of the microbial DNA reads from Sumatran rhinoceros were able to be mapped to the phylum level, unlike the other three species which had a mapping success rate of 97–98%. Institutional impacts on microbial diversity could not be inferred because all three rhinoceros were housed at the same facility. These animals' ages spanned six years to 33 years old and were sampled in three different months corresponding to two different seasons. The authors were unable to detect significant differences in microbial composition and diversity due to seasonality, though more unmapped reads were detected during the winter sampling period for Sumatran rhinoceros, accompanied by a decline in Firmicutes and Verrucomicrobia abundance. Larger sample sizes would be necessary to statistically confirm this trend.

4. Common Pitfalls within Rhinoceros Gut Microbiome Studies

The study of the gut microbiome in wildlife elicits particular challenges. Inter-individual variation may mask the effects of geographic location, diet, reproductive groups [91], or body condition [92], making it difficult to identify what constitutes a "typical" or core microbiome for a given host species, much less to detect statistically significant differences among populations [93]. Limited populations and low sample sizes inherent to endangered and elusive species further compound these issues. Studies performed within a single population in a single geographic region cannot be extrapolated to the species as a whole. Likewise, deviations from a species' proposed core microbiome do not always correlate to a diseased state, and thus have questionable clinical relevance. In addition, the abundance of certain taxa does not correlate to their functional impact on the host [94]. Microbial species present at <1% abundance are widely considered non-major taxa and yet may contribute disproportionately to metabolite production and other physiological factors [68]. The fecal microbiome is commonly used as a proxy for host gut microbiome, as collection of fecal material is relatively non-invasive. However, recent research has proposed that feces is inadequate at representing all microbial taxa present in the contents and mucosa across the entirety of the gastrointestinal tract [95–98]. Intestinal biopsy is necessary to access those microbes; but that level of invasive sampling is impracticable for all but the most extreme studies utilizing living hosts. Thus, studies of sensitive species must continue to use fecal proxies until novel sampling approaches or more sensitive methods are devised.

The comparison of wild and managed wildlife populations necessitates fieldwork, but the proper preservation of fecal material and stabilization of the microbiota within the sample (e.g., through the gold standard method of freezing at −80 °C) becomes challenging under field conditions. As microbial growth on fresh fecal samples is likely accelerated in humid tropical climates (where one might sample Javan or Sumatran rhinoceros), sample integrity (e.g., the innate microbial community composition) is compromised faster in unpreserved feces. In addition, behavior of the target species may create difficulties for experimental design. For example, white rhinoceros often defecate in middens, making it challenging for researchers to confidently assign fecal samples to individuals unless they witness defecation or invasively sample while the animal is anesthetized [99].

When analyzing previously published gut microbiome data, differences in sequencing and bioinformatic methodology introduce confounding effects that render studies incomparable. For example, the classification of microbial DNA reads into OTUs was standard practice in microbiome studies until amplicon sequence variants (ASVs) were adopted as the preferred method for classification. ASVs are single DNA sequences identified prior to amplification, removing possible sequence errors introduced during amplification [98]. ASVs can also be distinguished by a single nucleotide and do not need similarity thresholds, making them more accurate to true taxonomy than OTUs [100]. ASVs and OTUs cannot be compared directly due to these differences, leading to questions of validity in relating or combining results from previous studies that utilized OTU-based microbiome analysis [100].

Similarly, innovations in sequencing technology have made shotgun sequencing more affordable, rendering 16S rRNA amplicon sequencing less desirable. Shotgun sequencing characterizes the entire microbial community and is better at detecting rare taxa in comparison to 16s rRNA sequencing. While broad biological patterns in community characterization are consistent across different sequencing methods, shotgun sequencing also allows the analysis of functional relationships between host and microbiota [101,102].

With these challenges in mind, we reviewed 11 relevant publications on the rhinoceros gut microbiome and identified five frequent pitfalls. Common pitfalls in rhinoceros studies were compiled from the limitations identified within the literature, whether stated explicitly by the authors or gleaned implicitly from the experimental methodology. These pitfalls, ranked by incidence in the literature, are: Low population sizes in studies (11); Non-standardized sequencing and bioinformatic techniques (which affects downstream results

and prevents robust comparisons between studies) (11); Unequal distributions of rhinoceros populations compared across facilities/wild populations (4); Non-standardized sample collection (i.e., an unclear amount of time between defecation and collection (2), or sample collection occurring during non-standardized time of year(s) (2)); Inter-individual variation confounding results (1). Pitfalls can be visualized in Figure 1 and Table 2.

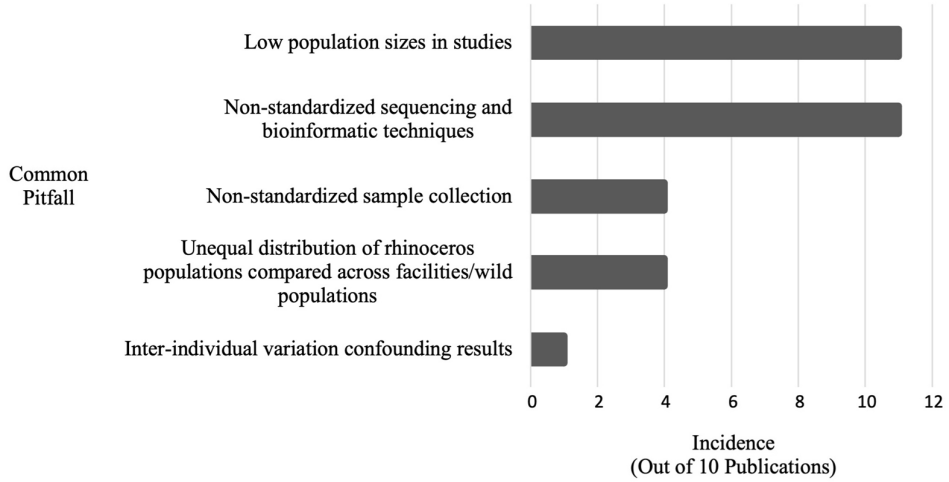

Figure 1. Incidence of common pitfalls within n = 11 rhinoceros gut microbiome publications published between 2013–2022.

Table 2. Incidences of common pitfalls in n = 11 rhinoceros gut microbiome research publications (2013–2022).

Common Pitfall	Incidence	Publication(s)
Low population sizes in studies	11	[3,16,62,67–69,73–75,77,82]
Non-standardized sequencing and bioinformatic techniques	11	[3,16,62,67–69,73–75,77,82]
Unequal distributions of rhinoceros populations compared across facilities/wild populations	4	[3,16,62,82]
Non-standardized sample collection (unclear amount of time between defecation and sample collection)	2	[74,75]
Non-standardized sample collection (time of year/season in which stool specimens were collected not standardized)	2	[16,62]
Inter-individual variation confounding results	1	[82]

5. Addressing Pitfalls and Future Directions

Rhinocerotidae gut microbiome research is still in its infancy. Studies are only now beginning to branch out from simple overviews of community composition and diversity indices to functional analyses of microbial interactions with their hosts. We have proposed several future directions of research below to address both common pitfalls and gaps in the current literature. Pitfalls stemming from flawed experimental design, such as uncontrolled or unknown time between defecation and sample collection, may be rectified by simple methods such as increased animal monitoring. Other pitfalls require more complex solutions, as suggested below.

5.1. Addressing Non-Standardized Sequencing and Bioinformatic Methodology Pitfalls through Collaboration

The use of different sequencing and bioinformatic methods makes valid comparisons between microbiome studies difficult, even when the host species is the same. We call for a standardization of methods used to evaluate the microbiomes of imperiled wildlife, such that results from multiple studies can be confidently compared and amassed to give broader context with fewer technical artifacts. Given the pace of technological advancement in the field, this consensus will need to be revised every few years. Standardization can be achieved in several ways, including: presentation and discussion of the issue (including research needs, feasibility, and current methodologic recommendations) at appropriate conferences and Association of Zoos and Aquariums meetings; publications of "Letter to the Editor" regarding the topic; development of a collaborative network. Of particular interest is the building of that collaborative network, which may include established authors and individuals interested in pursuing research on the gut microbiome of rhinoceroses. A centralized website could serve as a repository of resources including a catalog of current relevant publications and pre-prints, as well as tools for future researchers including permit requirements, fecal storage technology recommendations (with crucial information about methods that do not work), and a directory of peers.

5.2. Addressing Individual- and Population-Related Pitfalls through the Establishment of Reference Datasets across Managed Facilities and Wild Populations

Establishing core microbiomes for whole species is challenging given the considerable variation among individuals. However, a broad comparative database of species-specific microbiomes across populations in situ and ex situ would enable health assessment based on deviations from established "typical" communities for populations in a given facility or region. Ex situ sampling would include many (preferably all) individual rhinoceros in each species managed in facilities across the world, to quantify the impacts associated with different geographic locations and management strategies. Sample sizes of this scale would alleviate previous biases in data and inform apparent but statistically unsubstantiated trends. More complete diet records for studied animals are also necessary for robust inferences, as diet is one of the major drivers of variation in the gut microbiome [34]; even analyzing the microbiome of dietary items (e.g., browse or produce) may clarify observed differences in microbial community composition among populations which are currently attributed to broad factors such as seasonality or geographic range. General "reference" microbiomes could then be produced for facilities based on variables such as age and sex of the animal and could potentially be used prognostically to predict reproductive dysfunction, disease susceptibility, and nutritional problems.

Wild vs. managed comparisons would be another critical facet of this design, as wild populations of rhinoceros are generally more reproductively successful and less prone to certain health issues (e.g., obesity and enteritis) [12,15]. The few previous comparisons of wild vs. managed population microbial diversity levels have produced incongruent results, likely due to limited sample sizes [16,62]. Further research is thus necessary to both quantify whether rhinoceros species truly host higher microbial diversity under human management and to make more robust inferences as to why the trend is present and how differences might be functionally/clinically significant.

For researchers interested in investigating wild rhinoceros microbiome, it is important to note that transfer of fecal samples across country borders is typically less complicated than transfer of blood or other biologics. While experimental designs involving invasive sampling practices (i.e., per rectal sampling) may require Convention on International Trade in Endangered Species (CITES) import and export permits, for field research projects conducted by labs based in the United States, fecal samples freely collected off the ground and properly preserved are considered wildlife byproducts and are exempt from this requirement per the US Code of Federal Regulations (50 CFR 23.16). These samples may be imported into the US without a CITES permit, though we do recommend reaching out to

the US Fish and Wildlife Service agent at your port of entry for confirmation. Additionally, confirm with the Centers for Disease Control and Prevention as well as United States Department of Agriculture Animal and Plant Health Inspection Service that your study animals and experimental design are exempt from permit requirements before attempting to import any samples.

5.3. Future Avenue of Research: Address Reproductive Dysfunction

Human-managed populations of rhinoceros species tend to exhibit reproductive pathologies and disorders seldom seen in wild populations [15,16,68,82]. While manipulation of diet and social groupings and the administration of hormonal therapies have alleviated some reproductive issues, the underlying mechanisms are only beginning to be understood. Investigating the role of the gut microbiome is of interest, as studies in both black and white rhinoceros revealed associations between certain gut microbial taxa and reproductive success/fertility [68,81]. Similar associations may exist in managed greater one-horned rhinoceros, for whom nearly 50% of pregnancies end in stillbirths or miscarriages [15,17,18]. Tracking microbial diversity and community changes in concert with reproductive hormone cycles may also elucidate bidirectional interactions [82,103]. Identification of microbial biomarkers for reproductive success or failure would inform population management by studbook administrators and animal care teams.

5.4. Future Avenue of Research: Fill Species Gaps in the Literature

The gut microbiomes of Javan rhinoceros have never been studied, given the elusive nature of the critically low extant population. There are inherent challenges to sampling these individuals, as the entirety of the population (<100 individuals) exists within dense lowland tropical rainforests in the protected and patrolled Ujung Kulon national park on the island of Java [8]. Park managers and staff tasked with monitoring the population rarely make visual contact with the rhinoceros, instead observing presence using camera trap data and monitoring health noninvasively via fecal samples [104,105]. These banked samples plus more recently collected feces represent a reservoir of untapped microbiome data, assuming some identifying information about the corresponding individual is available [97]. The greatest challenge is to minimize the time between defecation and collection, as the abundances of certain microbial taxa will shift after environmental exposure, particularly in hot and moist tropical climates. Unpreserved samples stored indoors at room temperature (20–25 °C) under controlled settings exhibit significant changes in composition and diversity indices after 24 h [106,107]. Regardless, the Javan rhinoceros gut microbiome presents novel and useful information, especially for comparisons against the other member of the *Rhinoceros* genus, the greater one-horned rhinoceros.

The gut microbiome is inextricably tied to life processes including health and reproductive success. As such, noninvasive fecal samples provide an important tool for management of endangered populations in situ and under human management. Standardized methods and a global database could reveal microbial dysbiosis that detrimentally impact host health, ultimately informing management strategies.

Author Contributions: Conceptualization, C.M.B. and S.T.; writing—original draft preparation, C.M.B.; writing—review and editing, C.M.B., K.A.-v.H., L.J.M. and E.A.M.; supervision, S.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Data sharing not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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