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Conservation and captivity status influence the diversity of the mammalian gut microbiome

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SUMMARY Animal biodiversity is decreasing globally despite large-scale animal conservation efforts, such as providing care in captivity, captive breeding, and reintroduction programs. Just as in humans, the animal gut microbiome influences host health. Thus, there is a need to study the link between conservation status, captivity, and the gut microbiome to provide insight into improving current conservation strategies. In this study, we used a dataset of 16S rRNA gene sequences collected by McKenzie *et al*. to determine the effects of conservation status, diet type, diet breadth, and captivity on the mammalian gut microbiome. Our findings revealed significant differences in microbial communities between conservation status groups. Additionally, we found that diet type, diet breadth, and captivity significantly contributed to gut microbial diversity. Specifically, differences in gut microbial diversity between low- and high-risk conservation groups were statistically significant in both captive and wild mammals. Captive mammals shared more microbial genera between low- and high-risk conservation groups compared to wild mammals. Notably, high-risk mammals in captivity displayed fewer differentially abundant bacterial genera associated with pathogenicity than their wild counterparts. These results indicate that both conservation and captivity status affect mammalian gut microbial diversity, illuminating the significance of the gut microbiome in regulating species survival and the impact of human intervention on mammalian health via conservation activities.

INTRODUCTION

Despite extensive global animal conservation efforts, more and more species are threatened by human activities including climate change, habitat fragmentation, and the spread of infectious diseases (1, 2). Threatened species are added to the ecological watchlist at an alarming rate, which is 1,000 times higher than the natural rate of extinction (3, 4). Restoration and conservation projects can be costly and time-consuming, necessitating the prioritization of high-risk species, identification of factors which may influence an animal's extinction risk, and more effective restoration and reintroduction methods (5). The largest database of biological species conservation is the International Union for Conservation of Nature (IUCN) Red List, which classifies organisms by their likelihood of extinction (6).

A key element to current conservation efforts is captivity, which facilitates intensive care for endangered species to thrive and provides the opportunity to reverse the extinction process (7). The gut microbiome is interconnected with habitat loss, availability of food, individual health, and the probability of extinction (8, 9). Previous research has shown that captivity is associated with lower gut microbial diversity in captive animals than in wild animals (10), converging towards a humanized gut microbiome with the presence of taxa commonly found in the human gut (11, 12). These results are speculated to be linked to interactions with human captors and changes in diet, including substitutions with items not found in the animals' natural habitat and reduction in the range of food sources (11). Dysbiosis, or a perturbation in the gut microbiome, is correlated with an increased risk of disease in humans (13) as well as in animals (14), which poses an important consideration when monitoring endangered species in captivity.

Discerning the effects of external and internal factors contributing to gut microbiome turnover is crucial in aiding conservation efforts. However, previous studies have been limited by their scope and size of their datasets (11, 15). McKenzie *et al*. (15) acquired a larger dataset by collecting fecal samples from 41 different mammalian species across six

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orders to explore the effects of captivity, along with other factors, such as diet and fermentation types, on mammalian gut microbiota. The dataset contains 16S rRNA gene sequencing (V4 region) information from each sample, allowing for taxonomic identification of the gut microbiome and downstream analysis (15). Other research groups have used the same dataset to elucidate the impact of diet on the animal gut microbiota (16–18). Different diet types were found to correlate with different microbial diversity and composition at the class taxonomic rank (16–18). Despite extensive research into the effects of diet and captivity on the gut microbiota, few studies have determined the relationship between the gut microbiome and conservation status.

To fill this research gap, our study aims to determine the effects of conservation status, diet type, diet breath, and captivity on the mammalian gut microbiome using the dataset collected by McKenzie *et al*. (15), with a focus on conservation and captivity status. We hypothesize that: 1) animals of different conservation status exhibit different gut microbiomes, 2) differences in gut microbial diversity between low- and high-risk mammals varies with diet type and diet breadth, and 3) captivity is associated with a reduced contribution of conservation status on gut microbial diversity and composition because the animals' environment and diet change due to human influence. Since dysbiosis in the gut tends to manifest itself as a loss of microbial species diversity (8), we predict that there will be a trend of decreasing gut microbial species diversity in species experiencing the least to most extinction risk. Additionally, we predict that a greater difference in gut microbial diversity will be found in carnivores or mammals with lesser diet breadth. Lastly, we predict that the gut microbiota of captive animals will become more similar between different conservation statuses.

METHODS AND MATERIALS

Data information. The dataset was obtained from a previous study by McKenzie *et al*., which investigated the effects of captivity on the mammalian gut microbiome (15). For each of the 296 samples, there was associated metadata including mammal origin, captivity status, conservation status, diet type, and the number of diet categories. The conservation status of the mammals was classified based on the IUCN Red List with five possible groups (Table S1): least concern (e.g., aardvarks, impala, springboks), near threatened (e.g., onagers, zebras), vulnerable (e.g., giant anteaters, cheetahs), endangered (e.g., ring-tailed lemurs, wild dogs), and critically endangered (e.g., western gorillas, Eastern black rhinoceros). Diet types were classified as carnivorous, herbivorous, or omnivorous. The number of diet categories was calculated based on the Eltonian trait diet categories (19), which may be fruit, invertebrate, nectar, plant-other, seeds, scavengings, warm blooded vertebrates, fish, or unknown vertebrates.

Processing 16S rRNA sequences. The raw sequence reads were imported and demultiplexed in Quantitative Insights Into Microbial Ecology version 2 (QIIME2) (20). A trim length of 234 nucleotides was applied based on a minimum threshold of median Phred score of 30 for quality. The demultiplexed sequences were trimmed, denoised, and filtered using the QIIME2 plugin Divisive Amplicon Denoising Algorithm 2 (DADA2) (21). We processed high-quality reads into amplicon sequence variants (ASVs) and assigned taxonomy to ASVs using a 16S rRNA reference library based on the SILVA database (22–24). ASVs representing mitochondria or chloroplasts were removed. To analyze the effects of diet type (carnivores, herbivores, or omnivores), diet breadth (single diet category or multiple diet categories), and captivity status (captive or wild) on the relationship between conservation status and gut microbial diversity, the samples were then subsetted based on these factors. Since the number of omnivore samples were not sufficient for downstream analysis, the omnivore subset was removed. To maintain enough samples for analyses, we performed our analyses of the three factors (diet type, diet breadth, and captivity status) using two conservation status groups (6): 1) the "low-risk" category, composed of least-concern and near-threatened mammals, and 2) the "high-risk" category, composed of vulnerable, endangered, and critically-endangered mammals.

Beta diversity analyses. Each sample was rarefied to 83,570 reads for the overall dataset analysis of conservation status, 80,508 reads for every subset of diet type and captivity status, or 89,808 reads for every subset of diet breadth to maximize the number of retained samples and represented features. To determine differences in gut microbial communities between the two categories of grouped or ungrouped conservation statuses, we used QIIME2 to calculate pairwise permutational analysis of variance (PERMANOVA) for Jaccard distance, Bray-Curtis dissimilarity, unweighted UniFrac, and weighted UniFrac metrics. We used 999 permutations for each beta diversity metric with a *p*-value cutoff of 0.05 to determine statistical significance.

Alpha diversity analyses and data visualization. Processed taxonomic and phylogenetic data from QIIME2 were imported into R (version 2022.2.1) (25) using the file2meco package (version 0.2.2) (26). A new metadata file was generated with an additional row specifying data type as per microeco analysis requirements. ASVs were filtered by a minimum relative abundance threshold of 0.05% to remove low-abundant reads.

To visualize alpha and beta diversity between low- and high-risk mammals and their captivity status we produced boxplots and principal coordinates of analysis (PCoA) plots using the microeco (version 0.7.1) (26) and ggplot2 (version 3.3.5) (27) packages. Ellipses were added to PCoA plots to identify microbial community clustering. To determine whether differences in microbial diversity between low- and high-risk mammals were statistically significant, we used Kruskal-Wallis tests to calculate Shannon diversity indices. To investigate the similarities in microbial richness between conservation status categories, the number of unique and overlapping genera between low-risk and high-risk were plotted as Venn diagrams in R using the microeco package (26) for each subset of captivity status.

Differential abundance analysis. The overlapping taxa were explored by first importing QIIME2-generated sample subsets, rooted phylogenetic tree, and taxonomy information file into R using the phyloseq package (version 1.34.0) (28). ASVs were filtered by a minimum relative abundance threshold of 0.05%. Differential abundance analysis was conducted using the DESeq2 package (version 1.30.1) (29) and plotted with ggplot2 (27) to compare low- and high-risk mammals at the genus level.

Data availability. Mammalian 16S rRNA sequence data and metadata used for this project can be found on the NCBI BioSample database (30) with the accession code PRJEB29017. New metadata files, QIIME2 command line scripts for data processing, filtering, metadata grouping, and beta diversity analyses, as well as R scripts for beta diversity and differential abundance analyses, can be found within the supplementary materials.

RESULTS

Mammals of different conservation statuses displayed significant differences in gut microbial diversity. To test our first hypothesis that mammals of different conservation statuses have different gut microbiomes, we compared pairwise PERMANOVAs for four beta diversity metrics: Jaccard distance, Bray-Curtis dissimilarity, unweighted UniFrac, and weighted UniFrac. There were significantly different microbial communities between at least one pair of conservation statuses for each metric, but Jaccard distance, Bray-Curtis dissimilarity, and unweighted UniFrac showed greater statistical significance than weighted UniFrac (Table 1). Notably, the pairs with no statistical significance in Jaccard distance, Bray-Curtis dissimilarity, and unweighted Unifrac were primarily comparisons between vulnerable, endangered, or critically-endangered mammals (Table 1).

Mammals of different conservation statuses displayed significant differences in gut microbial diversity depending on diet type, diet breadth, and captivity status. To test our second hypothesis that differences in gut microbial diversity between low- and high-risk mammals varies with diet type, diet breadth, and captivity status, we ran a similar analysis as our first hypothesis but used samples subsetted based on each factor to compare low- and high-risk mammals. For diet type, carnivores or herbivores with different conservation statuses had significantly different microbial communities when we used Jaccard distance and unweighted UniFrac metrics, but when we used Bray-Curtis dissimilarity and weighted

TABLE. 1 Pairwise PERMANOVA results of mammals according to conservation status. Df indicates degrees of freedom for groups 1 and 2, respectively. Significant *q*-values ($q < 0.05$) are marked with an asterisk.

Group 1	Group 2	df	Jaccard		Bray-Curtis		Unweighted UniFrac		Weighted UniFrac	
			pseudo-f	*q-value*	*pseudo-f*	*q-value*	*pseudo-f*	*q-value*	*pseudo-f*	*q-value*
Critically endangered	Endangered	10, 19	1.391	0.033*	1.505	0.044*	1.697	0.066	2.051	0.078
	Vulnerable	10, 11	2.443	0.103	3.058	0.154	3.230	0.146	3.282	0.272
	Near threatened	10, 8	1.780	0.014*	1.8601	0.010*	2.322	0.018*	1.598	0.161
	Least concern	10, 34	1.229	0.003*	1.1845	0.003*	1.338	0.003*	1.201	0.040*
Endangered	Vulnerable	9, 11	2.410	0.199	2.7941	0.226	3.206	0.256	3.034	0.272
	Near threatened	9, 8	1.907	0.017*	1.7661	0.044*	3.434	0.023*	3.228	0.058
	Least concern	9, 34	1.160	0.003*	1.1381	0.003*	1.160	0.005*	1.201	0.040*
Near threatened	Vulnerable	8, 11	3.422	0.038*	3.9753	0.061	5.713	0.066	5.262	0.116
Least concern	Vulnerable	34, 11	2.667	0.003*	3.1710	0.003*	3.635	0.003*	2.101	0.078
	Near threatened	34, 8	1.563	0.003*	1.4529	0.003*	1.984	0.003*	1.992	0.020*

UniFrac, only the herbivore subset showed significantly different communities between low- and high-risk groups (Table 2). Herbivore samples also had microbial communities that were more distinct between low- and high-risk groups than carnivores (Table 2).

TABLE. 2 PERMANOVA results of mammals according to diet type, diet breadth, and captivity status. Groups are composed of least-concern and near-threatened (low-risk) and vulnerable, endangered, and critically-endangered (high-risk) mammals. Df indicates degrees of freedom for groups 1 and 2, respectively. Significant *q*-values ($q < 0.05$) are marked with an asterisk.

Diet type										
Group 1	**Group 2**	**df**	**Jaccard**		**Bray-Curtis**		**Unweighted UniFrac**		**Weighted UniFrac**	
Carnivorous			*pseudo-f*	*q-value*	*pseudo-f*	*q-value*	*pseudo-f*	*q-value*	*pseudo-f*	*q-value*
Low-risk	High-risk	7, 9	1.799	0.007*	1.557	0.065	1.829	0.02*	1.488	0.177
Herbivorous			*pseudo-f*	*q-value*	*pseudo-f*	*q-value*	*pseudo-f*	*q-value*	*pseudo-f*	*q-value*
Low-risk	High-risk	30, 23	3.463	0.001*	4.437	0.001*	5.500	0.001*	3.486	0.001*
Diet breadth										
Group 1	**Group 2**	**df**	**Jaccard**		**Bray-Curtis**		**Unweighted UniFrac**		**Weighted UniFrac**	
Single diet category			*pseudo-f*	*q-value*	*pseudo-f*	*q-value*	*pseudo-f*	*q-value*	*pseudo-f*	*q-value*
Low-risk	High-risk	32, 23	2.884	0.001*	3.518	0.001*	3.666	0.001*	2.310	0.048*
Multiple diet categories			*pseudo-f*	*q-value*	*pseudo-f*	*q-value*	*pseudo-f*	*q-value*	*pseudo-f*	*q-value*
Low-risk	High-risk	6, 9	1.461	0.019*	1.387	0.023*	1.535	0.055	1.344	0.215
Captivity status										
Group 1	**Group 2**	**df**	**Jaccard**		**Bray-Curtis**		**Unweighted UniFrac**		**Weighted UniFrac**	
Captive			*pseudo-f*	*q-value*	*pseudo-f*	*q-value*	*pseudo-f*	*q-value*	*pseudo-f*	*q-value*
Low-risk	High-risk	38, 29	2.678	0.001*	3.095	0.001*	3.137	0.001*	1.954	0.057
Wild			*pseudo-f*	*q-value*	*pseudo-f*	*q-value*	*pseudo-f*	*q-value*	*pseudo-f*	*q-value*
Low-risk	High-risk	6, 4	1.914	0.021*	1.890	0.041*	2.221	0.042*	2.014	0.096

For diet breadth, we observed significantly different gut microbial communities with Jaccard distance and Bray-Curtis dissimilarity for both mammals with single or multiple diet categories between different conservation statuses, while significantly different microbial communities between conservation statuses for mammals with a single diet category were found using the unweighted and weighted UniFrac metrics (Table 2). The disparity between conservation statuses was greater for mammals with a single diet category than those with multiple diet categories (Table 2).

For captivity status, we observed statistically significant differences in microbial community for both captive and wild mammals between different conservation statuses using Jaccard distance, Bray-Curtis dissimilarity, and unweighted UniFrac metrics (Table 2; Figure S1). We observed no statistical difference when using the weighted UniFrac metric (Table 2; Figure S2). The disparity between conservation statuses was greater for captive mammals than in wild mammals (Table 2). Since previous studies have extensively analyzed the effects of diet on the mammalian gut microbial diversity in this dataset, we decided to focus on only captivity status hereafter and its effects on the relationship between conservation status and the mammalian gut microbial diversity. We found that low- and high-risk groups had more distinct microbial communities for captive mammals (unweighted Unifrac PERMANOVA: $F_{28,39}$ = 3.137, q = 0.001) (Figure 1A) than wild mammals (unweighted UniFrac PERMANOVA: $F_{4,6}$ = 2.221, q = 0.042) (Figure 1B).

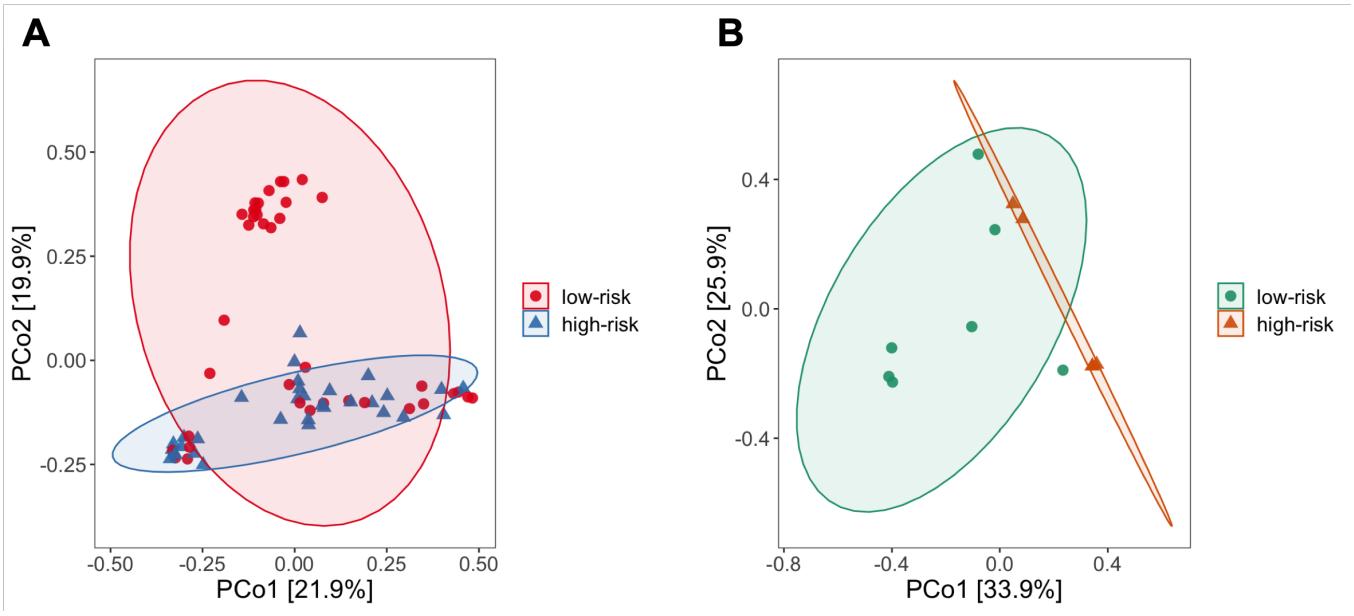

FIG. 1 Conservation status influences gut microbial diversity. Unweighted UniFrac principal coordinates analysis is plotted for captive (A) (q = 0.001) and wild (B) (q = 0.042) mammals. Groups are composed of least-concern and near-threatened (low-risk) and vulnerable, endangered, and critically-endangered (high-risk) animals.

Conservation status significantly increases alpha diversity in gut microbiomes of low-risk mammals than high-risk mammals in captivity but not in the wild. To test our third and final hypothesis that captivity is associated with a reduced contribution of conservation status on gut microbial diversity and composition as a result of anthropogenic changes in the animals' environment and diet, we calculated alpha diversity for each group using the Shannon diversity index. Statistical analysis using the Kruskal-Wallis test revealed there was a significant difference (p = 0.001) between low- and high-risk groups in captive mammals (Figure 2A). Low-risk captive mammals showed a higher gut microbial diversity, indicating more unique microbial communities in terms of richness and abundance, when compared to high-risk captive mammals (Figure 2A). However, there was no significant difference (p = 0.530) based on conservation status in wild mammals (Figure 2B).

Captive mammals have more shared microbial taxa between low- and high-risk groups than wild mammals. To investigate the number of shared and unique microbial taxa

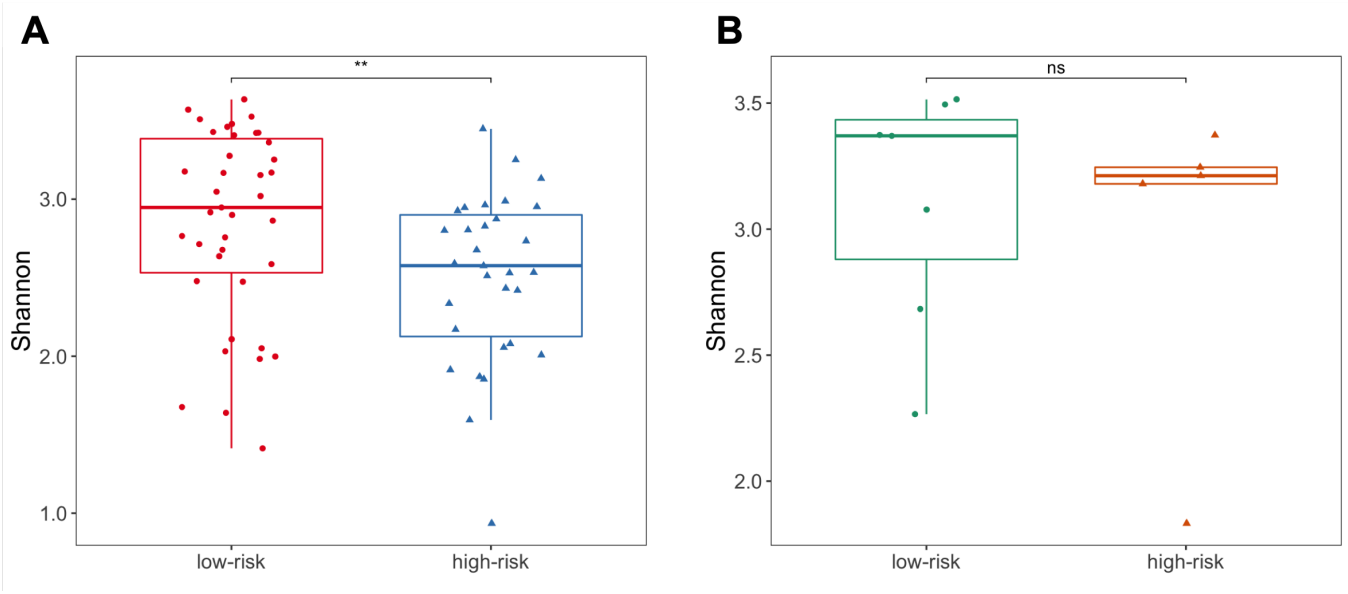

FIG. 2 Gut microbial diversity is greater in low-risk mammals than high-risk mammals in captivity. Boxplots are made using Kruskal-Wallis tests of Shannon diversity index for captive (A) (p = 0.001) and wild (B) (p = 0.530). Groups are composed of least-concern and near-threatened (low-risk) and vulnerable, endangered, and critically-endangered (high-risk) animals. Asterisks indicate significant p-values ($p < 0.05$), while ns denotes no significance.

between different conservation statuses for captive and wild mammals, we generated Venn diagrams of shared taxa at the genus level in R using the microeco package. We found that, of 106 genera present in the captive samples, 83 genera (78.3%) were shared between the low- and high-risk groups (Figure 3A). In wild mammals, the low- and high-risk groups shared 43 out of 100 genera (43%) (Figure 3B). In both wild and captive mammals, the low-risk group had more unique microbial genera (12.3% for captive, 44% for wild) than the high-risk group (9.4% for captive, 13% for wild). Moreover, the difference in the numbers of unique microbial taxa between conservation statuses was greater in wild mammals than in captive mammals.

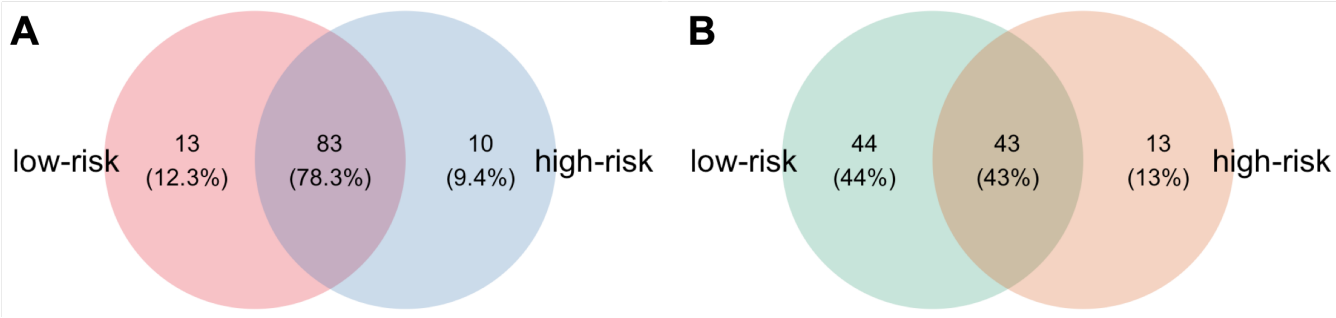

FIG. 3 Venn diagrams of shared microbial taxa between mammals of different conservation and captivity status. Presence of microbial taxa are resolved to the genus level for captive (A) and wild (B) mammals. Groups are composed of least-concern and near-threatened (low-risk) and vulnerable, endangered, and critically-endangered (high-risk) animals.

Mammals in captivity display more differentially abundant genera between low- and high-risk groups than mammals in the wild. Using the low-risk group as the reference and a minimum relative abundance threshold of 0.05% to filter for significantly abundant taxa, we determined that mammals in captivity have 20 differentially abundant genera (Figure 4A), while mammals in the wild have only 14 (Figure 4B). Of these differentially abundant taxa, *Alistipes* and *Bacteroidales RF16 group* were found to be the only genera that were differentially abundant in both captive and wild mammals. *Alistipes* was $2^{4.943}$ and $2^{27.731}$

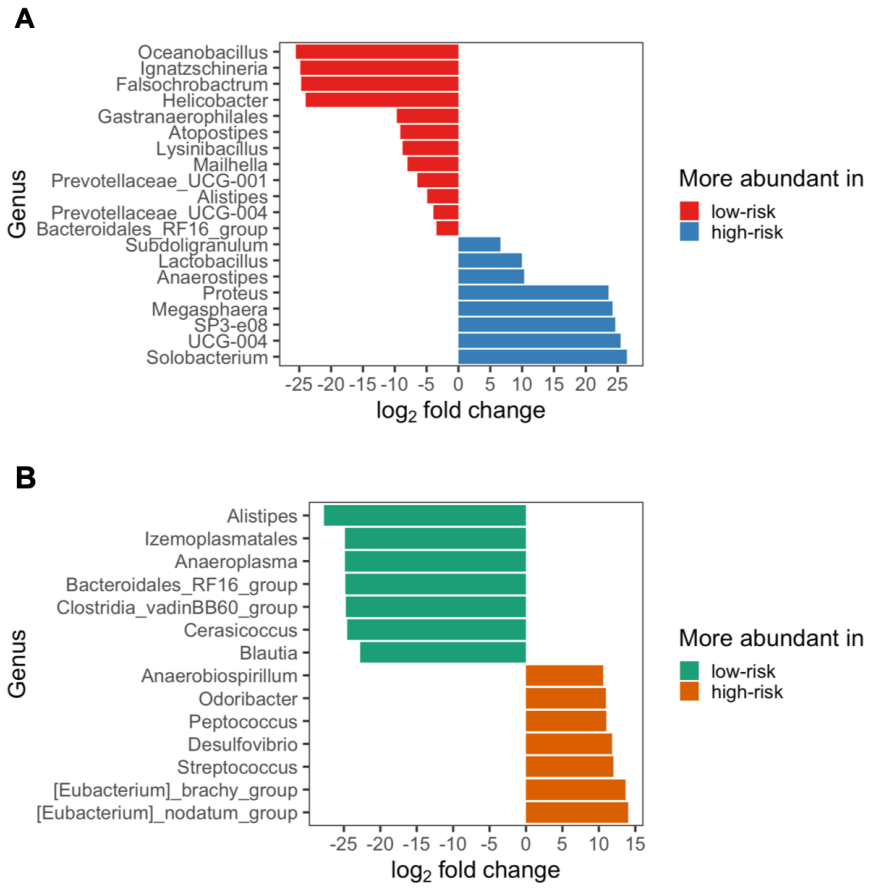

FIG. 4 Differential abundance analysis of mammals of different conservation and captivity status. Bar graphs represent the $\log_2$-fold change of the significantly differentially abundant genera ($p < 0.05$) in captive (A) and wild (B) mammals. Groups are composed of least-concern and near-threatened (low-risk) and vulnerable, endangered, and critically-endangered (high-risk) animals. Reference is to the mean abundance.

times more abundant in low- than high-risk mammals in captivity and the wild, respectively (Table S2). *Bacteroidales RF16 group* was $2^{3.44}$ and $2^{24.81}$ times more abundant in low- than high-risk mammals in captivity and the wild, respectively (Table S2). Notably, *Solobacterium* was the most differentially abundant genus ($2^{26.450}$) in captive mammals, while *Alistipes* was the most differentially abundant genus in wild mammals (Table S2).

DISCUSSION

Conservation status may affect mammalian gut microbial diversity. Our study explores the effects of conservation status on the gut microbial diversity of mammals. We found that gut microbial beta diversity is significantly different between different conservation statuses (Table 1), supporting our first hypothesis that conservation status has an effect on mammalian gut microbial diversity. Moreover, we generally did not observe significant differences in gut microbial diversity between the vulnerable, endangered, and critically-endangered groups, indicating their gut microbial communities are similar (Table 1). Collectively, these represent the high-risk group, which is consistent with the ranking of conservation statuses in the IUCN Red List (6). Since conservation status is not a true biological property but an artificial one, the differences in gut microbial diversity observed between each status suggests human perception and conservation efforts may be more directed towards the high-risk species, leading to anthropogenic changes in biotic or abiotic factors in their environment which in turn alter their gut microbial diversity (31, 32). Simultaneously, the gut microbiome may predispose a species to a greater extinction risk as it modulates host health (14, 15). Our research is an observational study that provides correlational evidence as opposed to a randomized or longitudinal study, preventing the conclusion of whether conservation status or the gut microbiome is the causal factor.

Interestingly, differences in gut microbial diversity between conservation statuses appear to be driven by richness, abundance, and phylogenetic distance when assessed individually by the Jaccard distance, Bray-Curtis dissimilarity, and unweighted UniFrac metrics, but not

when assessed jointly by the weighted UniFrac metric (Table 1). This discrepancy may be because unweighted and weighted UniFrac methods are more sensitive to changes in rare or abundant lineages and less sensitive to moderately abundant lineages (33). Regardless, we show that the richness, abundance, and phylogenetic distance components of beta microbial diversity are undoubtedly influenced by conservation status.

Diet type, diet breadth, and captivity status modulate the effect of conservation status on mammalian gut microbial diversity. To determine the possible mechanisms by which conservation status may influence gut microbial diversity, we focused on three possible modes of anthropogenic change: diet type, diet breadth, and captivity status. We found that an herbivorous diet is associated with a greater effect on gut microbial diversity between conservation statuses than a carnivorous diet (Table 2), which does not support our prediction for the second hypothesis. Such a trend may be explained by intrinsic differences in gut microbiome stability between mammals of different diet types. Previous studies have found that the fecal microbiota is relatively similar between herbivores, while carnivores exhibit greater variability both between and within species (34). Since the carnivorous diet inherently results in greater fluctuations, the effect of conservation status would only be a minor contributor to variability in their microbial diversity. Our results also suggest that conservation status affects the abundance component of gut microbial diversity in herbivores but not carnivores (Table 2). Studies that used this dataset and other sources of data observed that herbivores display greater gut microbial richness and abundance than omnivores and carnivores (16, 35). This has been attributed to herbivores requiring numerous microbes for cellulose and xenobiotic digestion (17, 34, 36). Carnivores have adapted to a relatively less consistent feeding strategy which may mean that fluctuations in their gut microbial abundance are more common (37), but abundance changes to the herbivore microbiota may disrupt their digestion (38), subjecting them to health and extinction risk.

For diet breadth, the number of diet categories impacted the richness and abundance of the gut microbial diversity (Table 2). This result supports our second hypothesis as conservation status has a more pronounced effect on mammals with a single food source than those with multiple. Consistent with a previous study on this dataset, a greater diet breadth was associated with a greater difference in bacterial community (15). Since mammals with less diet breadth may be more sensitive to changes in the availability of specific diet categories, a high-risk conservation status may signal environmental disruption and hence reduced food availability (39, 40). However, our results indicate phylogenetic distance was not affected by diet breadth (Table 2), potentially because our method did not consider the consumed proportions of each food source. A previous finding in this dataset indicates that the dominant diet category influenced phylogenetic distance in herbivores and carnivores but not the abundance in carnivores (17). Thus, a multiple-category diet with one dominant food source may not differ appreciably from a single-category diet.

For captivity status, contrary to our third hypothesis, our results showed a greater effect of conservation status on the gut microbial diversity of captive mammals than on wild mammals through richness, abundance, and phylogenetic distance (Table 2; Figures 1, 2). This is potentially due to conservation activities in captivity, including an increase in food availability and access to veterinary services (41, 42). These factors may lead to an artificial divergence in the gut microbial diversity between low- and high-risk mammals. Interestingly, richness, abundance, and phylogenetic distance individually responded to differences in conservation and captivity statuses, but jointly produced a statistically insignificant microbial diversity, a trend similar to the overall conservation status, which may indicate a limitation in the sensitivity of the UniFrac methods (33).

Captivity may alter mammalian gut microbial diversity differently depending on conservation status. McKenzie *et al*. found that for most mammalian hosts, the alpha gut microbial diversity remained unchanged or decreased in captivity, except for the rhinoceros (15). Consistent with their findings, we found decreased microbial diversity in terms of richness and abundance in high-risk mammals compared to low-risk mammals in captivity (Figure 2). Differences in gut microbial diversity were statistically significant when evaluated using beta diversity (Table 2; Figure 1) but not alpha diversity metrics (Figure 2). The alpha diversity metrics suggest that mammals with similar extinction risk harbor more similar gut microbiota in the wild than in captivity. The difference in alpha diversity in wild mammals

may have been insignificant because of the small sample size of high-risk wild mammals (Table S1). While previous studies have established that captive animals have lower gut microbial diversity compared to their wild counterparts, there are no studies to the best of our knowledge that explain why this decrease in microbial diversity is more pronounced in high-risk mammals.

The disproportionate effect of captivity on low- and high-risk mammals may be explained by a difference in human intervention in captivity. Studies on mammalian conservation efforts mention that more funds, and thus more veterinary care, are allocated to the care of high-risk captive animals than those of low-risk (43). One of the main objectives of captivity of a high-risk species is a high rate of captive breeding, which may allow for the species' reintroduction to the wild and a decrease of its extinction risk (44, 45). However, one of the main obstacles to successful captive breeding are persistent bacterial infections, which are subsequently treated with antibiotics and lead to a dysbiosis of the mammalian gut microbiome (32). Recently, Power *et al.* (46) detected antibiotic-resistant bacteria in approximately half of wallabies bred in captivity compared to wild populations. An increasing number of studies (5, 32, 41, 47) are calling for conservation efforts to include microbiome management, but much research still needs to be done as there still is no clear understanding of what constitutes an 'ideal' mammalian gut microbiome (15, 47).

Captivity leads to a convergence of mammalian gut microbial composition between conservation statuses. Our findings suggest that low- and high-risk mammals in captivity have significantly similar microbial composition than low- and high-risk mammals in the wild (Figure 3). This supports our hypothesis that conservation status has a reduced contribution to gut microbial composition in captivity due to human influence. This is consistent with previous studies which have found that the gut microbial diversity of captive mammals converged towards a human gut microbiome (11, 18). Furthermore, mammals in the wild may require a wider range of microbial taxa to digest different food sources. Previous research has found that endangered primates in the wild possess microbes responsible for degrading xenobiotics, including tannins and other toxins, but these taxa are lost in environments where the plant species have been eliminated by either captivity or human deforestation activities (9). Exposure to toxins is crucial to maintaining numerous gut microbes involved in detoxification as observed in woodrats (48, 49). Their findings may be extrapolated to an artificial environment, in which the diet of captive mammals may be altered, providing a different set of xenobiotics and thus inducing different metabolic pathways in their gut microbiome (50). Furthermore, gut microbial diversity of mammals with specialized diets is more affected by captivity (51). Since a narrower diet predisposes animals to greater risk of extinction, they are also more likely to be classified as a high-risk mammal.

Differential abundance of taxa that are shared between high- and low-risk mammals in captivity may be influenced by a third variable. Although captive mammals display a greater number of shared, unique taxa between low- and high-risk mammals than their wild counterparts, they also have more differentially abundant genera (Figure 4). This may suggest that, although mammals of different conservation statuses in captivity display more similar gut microbiota—which is consistent with our hypothesis—there is another factor that drives the differences in relative proportions of those shared taxa between high and low-risk groups. This third-variable may be diet, since diet is a dominant modulator of the gut microbiome (52). Thus, further research is required to determine if diet is a confounder in our observations.

Bacteria related to pathogenicity have significant differences in abundance between conservation statuses. *Alistipes* and *Bacteroidales RF16 group* are the only genera that are differentially abundant between high- and low-risk groups in both captive and wild mammals (Figure 4, Table S2). As these groups are conserved between different conservation and captivity statuses, they do not influence gut microbial diversity in terms of richness. Although little is known about the function of *Bacteroidales RF16 group*, its relative abundance has been negatively correlated with isobutyrate production and digestive health (53, 54). Contrasting evidence states that *Alistipes* may be pathogenic and related to diseases like anxiety, myalgic encephalomyelitis, and depression in some hosts, but associated with health-protective liver, colon, and autoimmune functions in others (55). *Solobacterium* and *Alistipes* are the most differentially abundant genera in captive and wild mammals, respectively, having the greatest $\log_2$-fold changes (Figure 4, Table S2). Whereas *Solobacterium* is more

abundant in high-risk captive mammals, *Alistipes* is more abundant in low-risk wild ones. *Solobacterium* is associated with the pathogenicity of diseases such as halitosis and other human infections (56). This suggests that high-risk mammals in captivity may still possess a gut microbiota that is more associated with disease than their low-risk counterparts. Further research on these new and unclassified microbes must be conducted to support more conclusive findings about their roles in modulating animal gut health.

Captivity may have a greater effect on the reduction of pathogenic bacteria in high-risk mammals. More bacterial genera commonly associated with pathogenicity are found to be differentially abundant in high-risk mammals in the wild than high-risk mammals in captivity (Table S2). Notably, six of the seven of the genera that were more abundant in high-risk wild mammals are associated with pathogenicity. Examples include *Streptococcus*, which has a pathogenic role in many swine and human infections (57), *Desulfovibrio*, of which many species are implicated in human opportunistic infections and disease (58), and *Anaerobiospirillum*, which has been associated with bacteremia and diarrhea in humans (59). In contrast, only one of eight differentially abundant genera, *Proteus*, is known to be associated with pathogenicity in high-risk captive mammals (Table S2) (60). This suggests that captivity may contribute to a reduction in the abundance of pathogenic bacteria in high-risk mammals.

On the contrary, this pattern is not observed in low-risk mammals. In fact, captive, low-risk mammals demonstrate a higher ratio of differentially abundant pathogenic bacterial genera than their wild, low-risk counterparts (Table S2). Differentially abundant genera in low-risk mammals include *Helicobacter*, which is more abundant in low-risk captive mammals, and *Alistipes*, which is more abundant in low-risk wild mammals. Many *Helicobacter* species have been characterized in the pathogenesis of gastric and enterohepatic diseases in humans and other animals (61), while *Alistipes* may have a role in cancer, inflammation, and mood disorders (55, 62).

Altogether, these findings suggest that captivity has a greater effect on the reduction of pathogenic bacteria in high-risk mammals than low-risk ones. This may have critical implications on conservation decisions to move high-risk animals from the wild into captivity. In captivity, the health and reproductivity of endangered animals are often improved by human intervention, such as antibiotic use, in preparation for species reintroduction to their natural habitat (44, 46). In the wild, however, pathogens in the digestive system may predispose a species to extinction in the form of population reduction from infectious diseases (4). For example, globalization and introduction of foreign flora have presented exotic pathogens in the Kenyan buffalo, reducing their population by over 90% in 10 years (4). Longitudinal studies of the mammalian gut microbiome should be conducted to conclude whether it is the presence of pathogenic bacteria that causes a species to be endangered, or environmental risk factors for extinction that ultimately are reflected as a change in the mammal's gut microbiome.

However, we cannot definitively conclude that captivity is beneficial for high-risk mammals, since these findings are limited in the context of pathogenic bacteria, and do not imply broader implications of why there may be a reduction in the abundance of these microbes. Animal health and reproduction in captivity is often treated through administration of medications and hormones (32, 44, 46), but these strategies may not prove to be beneficial for species' reintroduction into the wild. Thus, further research on the implications of current conservation strategies such as the use of probiotics (32) on the mammalian gut microbiome is necessary to construct appropriate strategies for microbiome modulation and long-term species conservation. It should be noted that, while our findings are limited to the taxa shared between high- and low-risk groups, it will be necessary to investigate the profile of taxa that are unique to each group to understand the full picture.

Limitations The primary limitation in our study is the number of samples available, especially for wild mammals. Due to low sequence quality, only five samples from three species of wild high-risk mammals were retained after filtering. The observed trends in high-risk mammals might potentially be species-specific, and generalization to a larger population requires future studies with a wider range of high-risk mammals. Furthermore, the retained wild samples originated from only one location. Geography was suggested to play a role in

the gut microbial diversity (18), hence future studies should collect more wild samples from various locations. By considering the excessive sample loss for wild samples compared to captive samples, future studies could evaluate whether geography is a confounding variable.

Other limitations include the inability to resolve microbes to the species level. While determining the differential abundance of microbes at the genus level is informative, it is the characteristics of certain species such as pathogenicity that impact the specific outcome of the mammals in captivity and in the wild. To understand how captivity impacts the population of microbial species and the health of mammals, future studies may improve the taxonomic resolution by whole genome sequencing of specific microbes of interest. Similarly, our study only investigated the overlapping taxa between the two grouped conservation statuses for captive and wild mammals, but the unique taxa were not identified because of time constraints. The unique taxa are equally important in evaluating the health of captive and wild mammals of different conservation statuses.

Conclusions Our study investigated the effects of conservation status on the gut microbial diversity of mammals, including potential trends specific to different diet types, diet breadths, and captivity statuses. The results support our hypotheses that conservation status influences mammalian gut microbial diversity, which might be additionally modulated by diet type, diet breadth, and captivity status through richness, abundance, or phylogenetic distance individually. Captivity is shown to result in a lower gut microbial diversity for the high-risk group compared to the low-risk group. Moreover, the taxonomic composition of the gut microbiome of mammals between different conservation statuses converges in captivity compared to in the wild. High-risk mammals in captivity also display fewer differentially abundant bacterial genera that are associated with pathogenicity than their wild, high-risk counterparts, suggesting that captivity may have a greater effect on the abundance of pathogenic bacteria in high-risk mammals than low-risk ones. While validation through comparisons with future studies using larger sample sizes is necessary, our findings provide implications for improving animal conservation efforts through the effects of captivity on endangered species and their gut microbiomes.

Future Directions Our study examined the effects of conservation status, diet type, diet breadth, and captivity status as independent variables influencing the gut microbial diversity of mammals. In nature, however, these factors do not act independently. To elucidate how these factors interact to affect gut microbial diversity and composition, future studies should combine these factors to discern whether the effects are causal and temporal (e.g., Do loss of food sources and subsequent changes in diet breadth lead to changes to an animal's microbiota, leading to an increase in extinction risk, and resulting in a change in captivity status? Or, does a species' extinction risk lead to changes in its captivity status, subsequently influencing its diet breadth and gut microbiota?), or synergistic (e.g., Is conservation status influenced by the effects of both diet type and diet breadth, simultaneously?).

Future studies could also expand on our study to classify the taxa that are significant indicators in each conservation status category and detect how these distributions of taxa shift across conservation statuses using Threshold Indicator Taxa ANalysis (TITAN) (63). As a sensitive and precise method of measuring community thresholds, TITAN outputs can also be used to model species sensitivity, thus presenting novel biodiversity conservation applications (63). Future studies could also conduct Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis on the dataset to examine the functional composition of the gut microbial community of mammals at various conservation statuses and changes in composition diet breadth or captivity (64). This analysis could inform how functions such as metabolism or digestion could be impacted by changes in gut microbiota composition due to these factors at various levels. Although the consideration of gut microbiome management is crucial in successful conservation strategies, the characterization of an 'ideal' mammalian gut microbiome is unclear (15, 47). Additional research into microbiome analysis will provide an invaluable understanding to potential drivers of extinction.

To provide a broader and more generalized perspective on the effects of species conservation on the gut microbiome, datasets with other classes of animals such as insects,

amphibians, and reptiles could be collected and studied. Specifically, the majority of animals threatened with extinction are amphibians (6), highlighting the need to collect samples from non-mammalian animals.

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CONTRIBUTIONS

For data analysis, Lai worked on the data processing and diversity metrics generation in QIIME2. Lee worked on microeco analysis in R and figure formatting. Quintana generated the preliminary visualization of beta diversity metrics significance using PCoA plots. Hui worked on the differential abundance plots in R and collaborated with Lee on figure formatting. For manuscript composition, Lee contributed to the abstract, introduction, methods, and results for the R workflow and generated the list of references. Quintana conducted the initial literature review and collaborated with Lee on the introduction, with Lai on the discussion, and with Hui on the annotation of differential bacterial genera. Hui wrote the future directions and the differential abundance analysis results and discussion. Lai wrote the materials and methods for the QIIME2 workflow, results, and discussion, and the limitations and conclusion. All authors collaboratively edited the manuscript.

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