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RESEARCH ARTICLE

Effect of fecal preservation method on captive southern white rhinoceros gut microbiome

Christina M. Burnham[1] | Erin A. McKenney[2] | Kimberly Ange-van Heugten[1] | Larry J. Minter[3] | Shweta Trivedi[4]

[1]Department of Animal Science, 120 W Broughton Dr., North Carolina State University, Raleigh, NC 27607, USA

[2]Department of Applied Ecology, 100 Brooks Ave, North Carolina State University, Raleigh, NC 27607, USA

[3]Hanes Veterinary Medical Center, North Carolina Zoo, 4401 Zoo Parkway, Asheboro, NC 27205, USA

[4]Department of Animal Science, 123 Polk Hall, 120 W Broughton Dr., North Carolina State University, Raleigh, NC 27607, USA

Correspondence

Shweta Trivedi, Department of Animal Science, 123 Polk Hall, 120 W Broughton Dr, North Carolina State University, Raleigh, NC 27607, USA.
Email: strived@ncsu.edu

Abstract

The southern white rhinoceros (*Ceratotherium simum simum*) faces an uncertain future in the wild due to increased poaching pressure and habitat fragmentation, thus the management of reproductively successful populations is of critical importance. Successful reproductive outcomes in rhinoceros may be mediated by diet and gut microbial diversity; therefore, understanding gut microbial dynamics within and between captive and wild populations may help improve conservation efforts. Accordingly, gut microbiome preservation methods are needed that are practical for *in situ* field sampling of wild populations. We evaluated the efficacy of 3 different preservation methods over 2 timepoints for stabilizing microbial communities in feces from southern white rhinoceros (n = 10) at the North Carolina Zoo in Asheboro, North Carolina, USA, during July–September 2020 and January–March 2021. Samples were immediately frozen at −80°C, stored in PERFORMAbiome™·GUT (PB) tubes or stored in 95% ethanol at ambient temperatures (to simulate field conditions), and processed after 14 or 230 days post-collection. We quantitatively compared alpha and beta diversity across microbial communities and identified taxa that were enriched in each treatment group. Samples preserved in 95% ethanol consistently harbored lower Shannon diversity index (SHDI) and Simpson's diversity (SDI) values compared to Frozen and PB

samples. This trend was apparent in both Ethanol day-14 samples (SHDI 4.94; SDI 0.98) versus Frozen day-14 (SHDI 5.19; $W = 518$, $P < 0.001$; SDI 0.99; $W = 476$, $P < 0.001$) and PB day-14 (SHDI 5.15; $W = 430$, $P < 0.01$; SDI 0.99; $W = 1075$, $P = 1$) samples, and in Ethanol day-230 samples (SHDI 4.48; SDI 0.97) versus Frozen day-230 (SHDI 5.18; $W = 0$, $P < 0.05$; SDI 0.99; $W = 0$, $P = 0.032$) and PB day-230 (SHDI 5.23; $W = 0$, $P < 0.05$; SDI 0.99; $W = 0$, $P = 0.032$) samples. Ethanol day 230 samples differed ($P < 0.05$) from all other treatments in both alpha and beta diversity indices. Notably, frozen and PB preservation methods maintained compositionally similar microbial communities across both time points. Our results indicate that PB tubes stored at ambient temperatures perform similarly to freezing at −80°C, highlighting their utility for microbiome fieldwork applications. Identifying optimal and versatile microbiome preservation techniques will enable future studies of the gut microbiome in reproductively-successful wild populations, an effort central to conservation efforts in the southern white rhinoceros and other threatened species.

KEYWORDS

Ceratotherium simum simum, fecal preservation method, microbiome, North Carolina, PERFORMAbiome, southern white rhinoceros, 95% ethanol

Managing reproductively-successful assurance populations is imperative for the conservation of southern white rhinoceros (*Ceratotherium simum simum)* and other species facing an uncertain future due to anthropogenic pressures. However, captive populations of southern white rhinoceros in zoological facilities have historically experienced poor fertility and post-copulatory reproductive failure (Swaisgood et al. 2006, Metrione and Eyres 2014). By contrast, wild populations of rhinoceros and those managed in reserves *in situ* are reproductively successful with the total species estimate having risen from 50 individuals at the end of the 19th century to >18,000 wild individuals in December 2017 (Emslie 2020). Female fertility levels appear to vary with both their fecal phytoestrogen profiles and the presence of specific gut bacteria that metabolize those phytoestrogens (Tubbs et al. 2016, Williams et al. 2019), suggesting that reproductive outcomes in captive southern white rhinoceros may be facilitated in part via the gut microbiome. Fieldwork is needed to understand how captive populations differ from their wild conspecifics; however, the preservation and stabilization of gut microbial communities can be logistically challenging to achieve in the field.

The stability of fecal microbial communities begins to decline after 24 hours of storage at ambient temperature (Cardona et al. 2012, Carroll et al. 2012, Tedjo et al. 2015). Refrigerating samples at 4°C halts community degradation only up to 72 hours, after which the microbial community structure shifts to favor the growth of psychotropic and psychrophilic bacteria able to reproduce at low temperatures (Choo et al. 2015, Tedjo et al. 2015, Wu et al. 2019). As such, samples refrigerated for >72 hours must be frozen. Though immediate freezing at −80°C is

the gold standard for preserving fecal samples for microbiome research, many field sites are remote and may lack electricity, ultra-low freezers, or both (Choo et al. 2015, Song et al. 2016). In addition, any freeze-thaw cycles that occur during the transport of fecal samples to and from storage sites further jeopardize DNA quality (Song et al. 2016). To maximize the quality (e.g., molecular weight) of DNA extractions, samples must therefore be either 1) processed (i.e., DNA extraction and possibly sequencing) where they are frozen, 2) transported at ambient temperature or on ice to a lab for possessing, at the risk of sample integrity, or 3) transported at ultra-low temperatures, which is not possible at all sample sites (Song et al. 2016, Wu et al. 2019).

In an attempt to circumvent the pitfalls outlined above, many field researchers store fecal samples in 95% ethanol in an effort to halt microbial reproduction until the samples can be transported to a lab for DNA extraction (Wu et al. 2019). However, samples preserved in ethanol *in situ* can also be difficult to travel with as large quantities of ethanol (>1 L) must be shipped via private carrier, which is much more expensive and logistically challenging compared to transport via passenger plane (International Air Transport Association 2021). Further, several studies have indicated that samples preserved in ethanol consistently return low DNA yields after extraction, potentially affecting downstream applications (Vlčková et al. 2012, Hale et al. 2015, Song et al. 2016).

PERFORMAbiome™·GUT (PB), a proprietary fecal preservation method and storage device produced by DNA Genotek (Ottawa, Ontario, Canada), offers potential benefits for microbiome fieldwork, including the ability to maintain DNA integrity at temperatures from −20°C to 50°C and to maintain a stable microbial profile at room temperature for 60 days (DNA Genotek 2019). PERFORMAbiome·GUT tubes have been internally validated by DNA Genotek for use in domestic dogs, cats, and horses. However, only 2 published studies have used PB tubes, one for dog samples (Lin et al. 2020) and the other for harbor (*Phoca vitulina*) and grey (*Halichoerus grypus*) seals (Steinmetz et al. 2021). All 3 species possess relatively short, simple digestive tracts lacking a functional cecum (Mead 2009, Smith et al. 2009). PERFORMAbiome·GUT tubes have not been externally validated for preservation of feces from other species with diverse feeding strategies and gut morphologies, or under varying time constraints. We compared the effects of preservation method and time on the gut microbial composition and structure of fresh fecal samples collected from captive southern white rhinoceros. Rhinoceros are large grazing herbivores that have evolved a complex gut morphology including a large cecum and sacculated colon to facilitate digestion of their high-fiber diet, and thus provide a novel and compelling species in which to test the efficacy of different methods for preserving the gut microbiome in herbivores.

The objectives of our research were twofold: 1) to compare the efficacy of different fecal preservation techniques (immediate freezing at −80°C, PB tubes, and 95% ethanol) for preserving fecal microbial community structure, and 2) to test the limitations of those preservation methods over 14 days and 230 days. We hypothesized that 95% ethanol-preserved and PB-preserved samples would perform similarly to −80°C controls in preserving microbial community composition in fecal samples over a 14-day period. We also hypothesized that microbial community composition in PB samples would shift from 14 to 230 days of storage at ambient temperature, due to degradation of both preservative solution and sample. We did not expect the microbial profile of samples preserved in 95% ethanol to change significantly over 230 days, given its past validation as a long-term storage preservative for fecal samples (Song et al. 2016).

STUDY AREA

Our study took place at the North Carolina Zoo, specifically a 16-ha grasslands habitat and accompanying holding facilities. The zoo itself is situated in Asheboro, North Carolina, USA, over 800 ha of Piedmont plateau within the Uwharrie Mountains. This area is part of the Carolina Slate Belt ecoregion, characterized by irregular plains, rounded hills, and low linear ridges (Griffith et al. 2002). Deposits of silty soil and clay-rich saprolite cover volcanic slate and granite bedrock; this bedrock is close to the surface, forming monadnocks in some areas (Griffith et al. 2002). Woody vegetation in this ecoregion was dominated by oak-hickory-pine forest and mixed-oak forest.

Within the zoo grasslands habitat, vegetation was previously described as primarily fescue (*Festuca arundinacea*), annual ryegrass (*Lolium multiflorum*), Bermuda grass (*Cynodon dactylon*), and white clover (*Trifolium repens*; Wood et al. 2020). Elevation ranged from 185 m on the eastern edge of the habitat to 205 m on the western edge.

Daily access to zoo habitat by the study population varied depending on temperature and incidence of cyanobacterial blooms in accessible water sources. During the summer sampling period (July–September 2020), the temperature ranged from 17–33°C, with a daily average temperature of 26°C. During the winter sampling period (January–March 2021), the daily temperature was more variable and ranged from −4–26°C, with a daily average temperature of 4.5°C during January and February and 12°C during March. Asheboro had a humid subtropical climate with rainfall dispersed roughly evenly throughout the year. The average annual precipitation was 1,184 mm, with annual snowfall constituting 80 mm and mostly taking place between January and February 1991–2020 (Asheboro 2 W; National Climatic Data Center 2022).

METHODS

Sample population

We collected fecal samples from a population of 10 southern white rhinoceros (9 females, 1 male) managed at the North Carolina Zoo. The animals ranged in age from juvenile (n = 2; <3.5 yr old), subadult (n = 2; 3.5–7 yr old), adult (n = 4 F and n = 1 M; 8–40 yr old), to geriatric (n = 1; >40 yr old). We assigned age classes to individuals (Table 1) based on a modified version of the white rhinoceros age-class system previously published by Emslie et al. (1995). All individuals were dewormed with 0.01 g/kg Rumatel® Cattle Dewormer (morantel tartrate 19.4% solid granules, Phibro Animal Health, Teaneck, NJ, USA) once a day for 2 days on 9–10 February 2021. No animals received antibiotics during the study period, though the geriatric female G1 received oral phenylbutazone to treat arthritis symptoms.

Adult, subadult, and juvenile females were housed in the rhinoceros barn, while M1 and G1 were housed separately in the rhinoceros annex building. Groups in the rhinoceros barn were rotated between available stalls and had access to outdoor sand, grass, and asphalt paddocks during temperate weather (>4.5°C). Access to the

TABLE 1 Summary of individual characteristics of N = 10 southern white rhinoceros (*Ceratotherium simum simum*) sampled at the North Carolina Zoo in the North Carolina Zoo, Asheboro, USA, between 2020–2021.

Individual	Age (yr)	Class	Sex
J1	1	Juvenile	Female
J2	1	Juvenile	Female
S1	3	Sub-adult	Female
S2	3	Sub-adult	Female
F1	15	Adult	Female
F2	24[a]	Adult	Female
F3	29	Adult	Female
F4	33[a]	Adult	Female
M1	30	Adult	Male
G1	52[a]	Geriatric	Female

[a]Age estimates for wild-caught individuals.

16-ha grasslands habitat was prohibited during the July–September 2020 sampling period due to a toxic cyanobacterial bloom in the freshwater pond, however the rhinoceros regained access to the habitat during the January–March 2021 sampling period when temperatures rose above 4.5°C. When outdoor access was restricted, the animals were rotated in pairs between combined stalls so that each individual was equally exposed to all group members and indoor environments. In the annex building, M1 and G1 were housed in separate stalls and were allowed access to grass paddocks when temperatures rose above 2°C.

Diets for adult and subadult rhinoceros consisted of 1.36 kg of Mazuri® Wild Herbivore Diet Hi-Fiber (St. Louis, MO, USA) pellet year-round, while juveniles were provided 0.68 kg of pellet. When outdoor grazing was available during the summer, adults and subadults were offered 4.5 kg of timothy hay (*Phleum pretense*) per animal daily. During the winter, adults and subadults were offered 18 kg of timothy hay. Supplementary feed in the form of timothy hay cubes, orchard grass (*Dactylis glomerate*), and alfalfa hay (*Medicago sativa*) were offered in rotation for training and enrichment, though these constituted less than 20% of the daily diet. The geriatric female rhinoceros G1 received a slightly different diet than the main population for welfare purposes, consuming 5.44 kgs of Wild Herbivore feed and 11 kg of timothy/orchard grass mixed hay each day. Rhinoceros G1 also had access to outdoor grazing areas and was provided with timothy hay cubes and alfalfa hay for training and enrichment.

Sample collection and storage

Zoo staff attempted to collect fecal samples from each individual once per month from July through September in 2020 and January through March in 2021. The sampling intervals remained consistent throughout, beginning on the 20th day of the month and extending until the end of that month. Staff collected a minimum of 2 grams of feces from each individual within 30 min of defecation and aliquoted each sample across 3 preservation methods: immediate freezing at −80°C in a Whirl-Pak® bag (Nasco, Fort Atkinson, WI, USA), 15 mL conical Falcon® tubes (Corning, New York, NY, USA) containing 95% ethanol, and PB tubes containing a proprietary preservative solution (Figure 1). We stored samples preserved in 95% ethanol and PB tubes at ambient temperature for a minimum of 14 days to simulate field conditions. Due to logistical constraints, we were unable to collect from every individual each month. The total number of samples per method were as follows: Frozen day-14 (n = 51), Ethanol day-14 (n = 43), PB day-14 (n = 39). We stored an additional subset of frozen (n = 6), PB (n = 6), and 95% ethanol (n = 6) samples from August 2020 for an additional 222–230 days before extraction to test the longitudinal efficacy of the 3 preservation methods (hereafter referred to as the day-230 time point). Samples from rhinoceros S1, S2, F3, F4, M1, and G1 were used for this day-230 subset. A total of 151 samples were collected and processed by the end of the study.

We were unable to utilize PB tubes per manufacturer recommendations due to the grated secondary tube top and the physical nature of the rhinoceros' fecal samples, which included long fibrous hay pieces. The fecal hay created a mat on the grate that could not be pushed into the preservative solution, limiting the amount of preserved fecal material and decreasing downstream DNA extraction yields. As such, we modified the procedure, whereby zoo staff removed the grated tube top during sampling and manually pushed the feces into solution using a sterile spatula, ensuring immersion of all fecal material.

DNA extraction

Immediately prior to DNA extraction, we transferred the fecal samples immersed in solution (i.e., preserved in 95% ethanol or PB tubes) to microcentrifuge tubes and centrifuged at 15,000 times gravity for 1 minute to concentrate the sample; the supernatant was discarded. While this was a successful technique for PB samples, 95% ethanol samples routinely returned insufficient concentrations of extracted DNA. Therefore, in March 2021 another

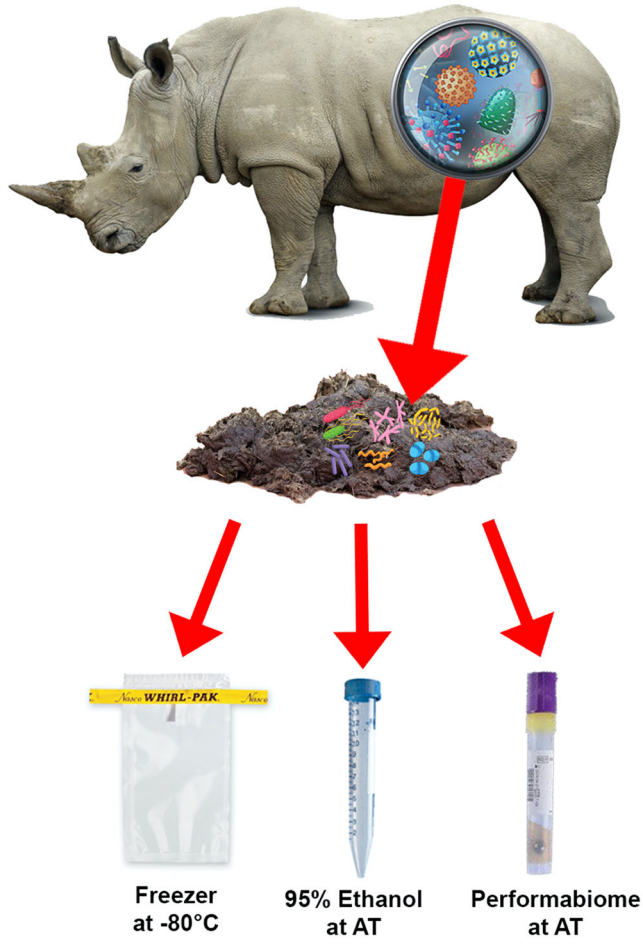

FIGURE 1 Overview of fecal storage and preservation methodology. Fecal samples from $N = 10$ captive southern white rhinoceros (*Ceratotherium simum simum*) at the North Carolina Zoo (USA) were stored at −80°C and at ambient temperature (AT) in 95% ethanol and in proprietary PERFORMAbiome™·GUT (PB) tubes. Samples were stored for either 14 or 230 days between 2020 and 2021.

technique was applied involving the evaporation of excess ethanol from the feces under a fume hood. Extractions from the evaporated samples yielded sufficient quantities of DNA for sequencing without additional processing.

We extracted DNA using the PowerFecal Pro DNA Kit (QIAGEN, Germantown, MD, USA) per manufacturer recommendations with the following modification: after we placed samples in the PowerBead Pro tubes and vortexed briefly to mix, they were subjected to bead beating at 4 m/s for 4 minutes using a FastPrep-24 bead beater (MP Biomedicals, Santa Ana, CA, USA) as opposed to being vortexed at maximum speed for 10 minutes; this bead beating speed was previously validated for use in DNA extraction for 16S rRNA V3–V4 region sequencing (Zhang et al. 2020). We eluted the extracted DNA in 15–100 µl of elution buffer (10 mM Tris); frozen and PB samples routinely returned high concentrations of DNA and were eluted in manufacturer recommended 100 µl of elution buffer, while samples that routinely returned minimal DNA concentrations were eluted with a minimum of 15 µl of elution buffer in order to increase concentrations for sequencing. We measured nucleic acid quantity and quality using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and stored the extracted DNA in elution tubes at −20°C until the end of the sampling season. Any samples that continuously

produced low DNA yields were extracted in duplicate following manufacturer recommendations up until the final eluting step, when an additional modification was added: one replicate was eluted, then that eluate was pipetted onto the filter membrane containing DNA from the second replicate and centrifuged again, effectively doubling the final DNA yields.

Sequencing

We sent DNA aliquots to the Genomic Sciences Laboratory at North Carolina State University for sequencing of the variable V3 and V4 regions of the 16S rRNA gene using established methods (Illumina 2013). Primers 341F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and 805R (GTCTCGTGGGCT CGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) were used to create amplicons ~460 base pairs in length (Klindworth et al. 2013).

We imported raw sequencing data to the CLC Genomics Workbench (v21.0.4) with Microbial Genomics Module plugin (QIAGEN, Germantown, MD, USA) for data quality control and operational taxonomic unit (OTU) clustering. We joined FastQ files via the CLC default Illumina platform parameters and paired the forward and reverse reads. Reads were trimmed with a 0.05 quality limit and an ambiguous limit of 2. Read length thresholds were set between 15 and 1,000 nucleotides. We used the SILVA 16S reference database (v132; https://www.arb-silva.de) to define OTUs based on a 97% taxonomic similarity cutoff and generated an OTU abundance table for all 151 samples; the abundance table was reformatted for downstream analysis in R (version 4.0.2, R Core Team 2022).

Bioinformatics and statistical analysis

We performed all statistical analyses using R (version 4.0.2, R Core Team 2022). We obtained a total of 59,680,646 16S rRNA sequence reads from 151 samples, with an average of 395,236 ± 12,585 (mean ± SEM) reads per sample (coverage min–max = 172,600–708,948 reads). After filtering 211,797 chimeric reads (i.e., reads corresponding to 2 or more reference sequences), we identified 14,247 unique OTUs. We retained 150 samples after rarefaction to 29,265 reads; rarefaction was performed using the mctoolsr package (Leff 2017) in R. Rarefaction of the dataset to a set read threshold introduces variable *P*-value results due to random subsampling. Thus, 50 seed values (reproducible rarefaction permutations) were set, and the resulting histogram of the 50 produced *P*-values was evaluated to validate significance for each statistical comparison. The updated OTU abundance table was used to calculate taxonomic relative abundance as well as alpha and beta diversity indices by utilizing the vegan (Oksanen et al. 2019) and mctoolsr

TABLE 2 Summary of alpha diversity estimates in fecal samples from *N* = 10 southern white rhinoceros (*Ceratotherium simum simum)* in the North Carolina Zoo, Asheboro, USA, stored via freezing at −80°C, 95% ethanol, and PERFORMAbiome™·GUT (PB) for 14 and 230 days in 2020–2021.

	Species richness	Shannon diversity index	Simpson diversity index
Ethanol Day 14	873.98	4.94	0.98
Ethanol Day 230	754.83	4.48	0.97
Frozen Day 14	888.74	5.19	0.99
Frozen Day 230	866.17	5.18	0.99
PB Day 14	880.62	5.15	0.99
PB Day 230	860.00	5.23	0.99

packages. Specifically, we measured alpha diversity using 3 distinct metrics (Table 2). Richness measures the number of species present, which correlates to the breadth of niche space available to microbial taxa in the gut. Shannon and Simpson's diversity indices both quantify the complexity of a community by incorporating both the total number of taxa and the relative abundance of taxa. However, the Shannon index weights rare species more heavily than common species and is thus sensitive to small changes in diversity, while Simpson's index gives greater weight to more dominant species. Together, the 3 indices provide a holistic overview of dominance dynamics within a sample. Kruskal-Wallis tests and pairwise Wilcoxon rank sum tests with Bonferroni corrections were used to assess and identify significant differences in microbial alpha diversity across preservation methods and were conducted with the stats R package (R Core Team 2022).

To analyze microbial beta diversity, we standardized the relative abundance of each OTU using the Hellinger transformation, then calculated Bray-Curtis dissimilarity with the vegan R package to create distance matrices. Eigenvectors and eigenvalues were calculated from the distance matrices to create multidimensional scaling (MDS) plots, and we utilized permutational multivariate analysis of variance (PERMANOVA) analyses from the vegan R package to assess differences in community composition. We adjusted PERMANOVA *P*-values using a False Discovery Rate correction (Landis et al. 2021). We also conducted a multivariate equivalent of Levene's test for homogeneity of variances using the betadisper function in the vegan R package to test whether the assumptions of PERMANOVA were violated due to differences in dispersion within groups. Additionally, we used Linear discrimination analysis Effect Size (LEfSe; https://huttenhower.sph.harvard.edu/galaxy/) to assess differential abundance; LEfSe is an algorithm that determines which microbial taxa most likely to explain differences between classes (i.e., preservation method) and estimates Linear Discriminant Analysis (LDA) scores to assess biological relevance through effect size of those differentially abundant taxa. The LEfSe program utilizes factorial, Kruskal-Wallis sum-rank tests to evaluate taxa with significant differential abundance with respect to classes, then uses an unpaired, Wilcoxon ranked-sum test to assess that significance (Segata et al. 2011). A class that is differentially abundant in a certain microbial taxon when compared to a different class is considered significantly enriched by that taxon. All Lefse comparisons below were made against frozen day-14 controls.

RESULTS

Taxonomic relative and differential abundance

Firmicutes (average relative abundance 56%) was the dominant phyla across all individuals, followed by Bacteroidetes (20%), Spirochetes (9%), Fibrobacter (8%), Kiritimatiellaeota (2%), Actinobacteria (1%), and Lentisphaerae (1%). Phylum and genus level bar charts indicated apparent differences in community composition across different preservation methods and time points (Figure 2), which were confirmed as significant differences between methods based on our LEfSe analysis. Here we report all differentially significant taxa with a linear discriminant analysis (log 10) score ≥3 (hypothesized to be the lower limit for biological relevance; de la Cuesta-Zuluaga et al. 2017, Saito et al. 2019; Figure 3). We also produced a full output of our linear discriminant analysis scores and *P*-values for each treatment comparison (available as a spreadsheet in Supporting Information).

Ethanol samples from both timepoints were enriched in Actinobacteria (for day-14, LDA 3.68, $P = 0.003$; for day-230, LDA 4.18, $P = 0.022$; Figure 3). The enrichment in Actinobacteria was mainly driven by members of the order Corynebacteriales (for day-14, LDA 3.35, $P = 0.014$; for day-230, LDA 3.95, $P = 0.024$) in both treatments, and ambiguous taxa belonging to the *Blastococcus (LDA 3.13)*, *Kineococcus (LDA 3.05)*, and *Plantactinospora* (LDA 3.10) genera in Ethanol day-230 samples ($P = 0.004$). In addition, Ethanol day-230 samples were enriched in the Actinobacteria class Coriobacteriia, driven by abundance of Coriobacteriales (LDA 3.67, $P = 0.04$). The bacterial phylum Firmicutes was also enriched in both ethanol treatments, driven by members of the classes Bacilli (for day-14, LDA 3.07, $P < 0.001$; for day-230, LDA 3.07, $P = 0.027$) and Clostridia (for day-14, LDA 4.39, $P = 0.001$;

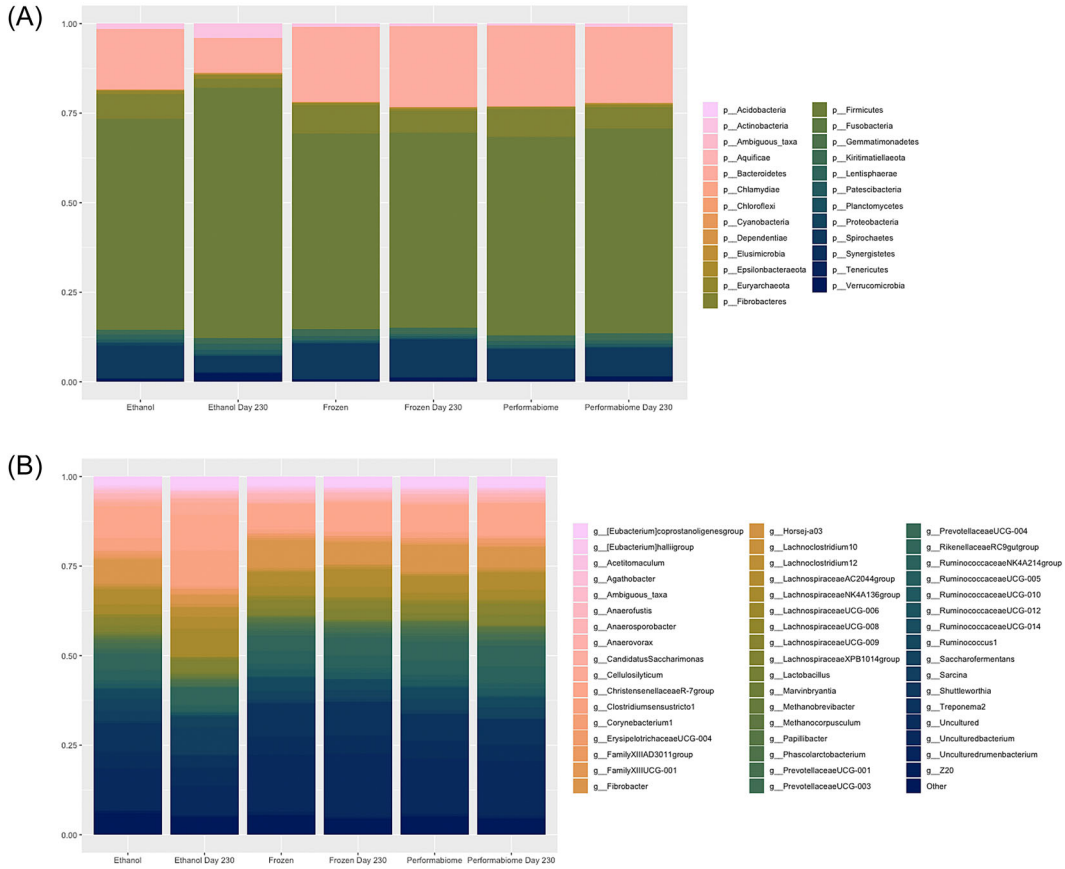

FIGURE 2 (A) Phylum- and (B) genus-level bar charts representing microbial community composition across fecal samples from $N = 10$ southern white rhinoceros (*Ceratotherium simum simum*) in the North Carolina Zoo, Asheboro, USA, preserved via freezing at −80°C, 95% ethanol, and PERFORMAbiome™·GUT (PB) at day 14 and day 230 in 2020–2021.

for day-230, LDA 5.07, $P = 0.015$). Within the Clostridia order, the Clostridiales family was most represented in both ethanol treatments, with enrichment driven by abundances of Lachnospiraceae (specifically, *Cellulosilyticum* (for day-14, LDA 4.18, $P < 0.001$; for day-230, LDA 4.74, $P = 0.003$); *Lachnoclostridium 12* (for day-14, LDA 3.99, $P < 0.001$; for day-230, LDA 4.78, $P < 0.001$); *Lachnospiraceae NK4A136 group* (for day-230, LDA 3.59, $P < 0.001$), *Lachnospiraceae UCG-010* (for day-230, LDA 3.07, $P = 0.035$)) and Clostridiaceae (specifically, *Sarcina* (for day-14, LDA 3.31, $P < 0.001$; for day-230, LDA 3.84, $P < 0.001$); *Clostridium sensu stricto 1* (for day-14, LDA 3.88, $P < 0.001$; for day-230, LDA 4.15, $P < 0.001$); *Clostridium sensu stricto 11* (for day-230, LDA 3.87, $P = 0.003$); *Clostridium sensu stricto 13* (for day-230, LDA 3.68, $P < 0.001$)). Members of the class Gammaproteobacteria (LDA 3.20, $P = 0.002$) and its family Enterobacteriaceae (LDA 3.14, $P = 0.008$) were enriched specifically in Ethanol day-14 samples, while day-230 samples were specifically enriched in the Enterobacteriaceae genera *Buttiauxella* (LDA 3.23, $P = 0.004$) and *Serratia* (LDA 3.29, $P = 0.004$). Day-14 samples were also enriched in members of the Bacteroidetes genus *Prevotellaceae UCG-004* (LDA 3.28, $P = 0.042$) while day-230 samples were enriched in an ambiguous Bacteroidetes taxon from the genus *Flavisolibacter* (LDA 3.31, $P = 0.004$). Lastly, Ethanol day-230 samples were enriched in Firmicutes family XVIII *Symbiobacterium* (LDA 3.10, $P = 0.004$), an uncultured bacterium from the Patescibacteria genus *Saccharimonadaceae* (LDA 3.23, $P = 0.004$), and the phyla Verrucomicrobia, driven by uncultured bacteria from the order LD1-PB3 (LDA 4.63, $P = 0.002$).

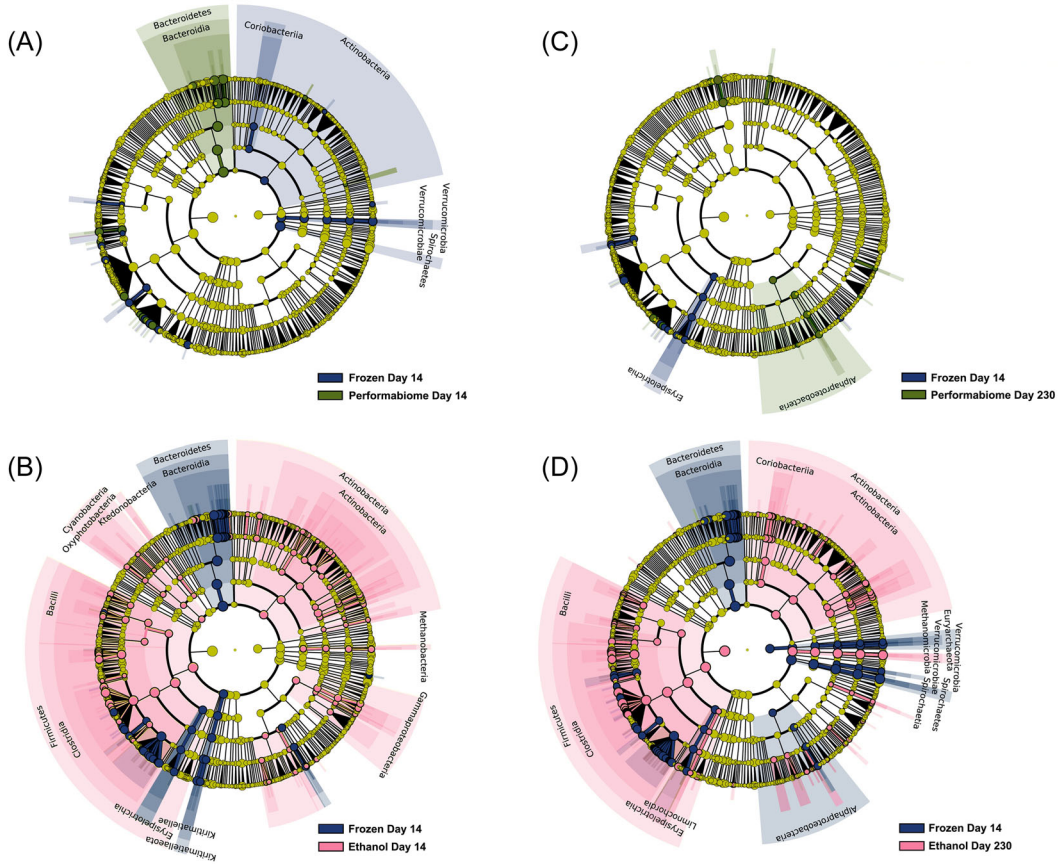

FIGURE 3 Linear discriminant analysis Effect Size cladograms comparing differentially abundant taxa in samples frozen at −80°C for 14 days to (A) PERFORMAbiome™·GUT (PB) and (B) ethanol preservation methods at day 14 and (C) PB and (D) ethanol preservation methods at day 230. Fecal samples were collected from $n = 10$ southern white rhinoceros (*Ceratotherium simum simum*) at the North Carolina Zoo, Asheboro, USA, in 2020–2021.

Comparisons of abundant taxa within Frozen day-14 samples versus both ethanol treatments revealed enrichment of frozen samples with Bacteroidetes (LDA 4.69, $P = 0.002$), specifically *Bacteroidales RF16 group* (LDA 3.00, $P < 0.001$), *Bacteroidales UCG-001* (LDA 4.21, $P < 0.001$), *Bacteroidetes BD2-2* (LDA 4.13, $P = 0.007$), *CAP-aah99b04* (LDA 3.42, $P < 0.001$) and *F082* (LDA 4.26, $P < 0.001$; Figure 3). Frozen samples were also enriched in members of the Firmicutes class Erysipelotrichia (LDA 3.34, $P = 0.019$), driven by enrichment of a bacterium within the *Anaerorhabdus furcosa group* (LDa 3.48, $P < 0.001$), as well as Lachnospiraceae members of the *Acetivibrio ethanolgignens group* (LDA 3.50, $P < 0.001$) and *Eubacterium oxidoreducens group* (LDA 3.49, $P = 0.004$). Several members of Ruminococcaceae were enriched in frozen samples, including *Eubacterium coprostanoligenes group* (LDA 3.16, $P = 0.001$), *Ruminiclostridium 1* (LDA 3.24, $P < 0.001$), and *UCG-010* (LDA 3.55, $P < 0.001$). Taxa enriched specifically in Frozen day-14 samples when compared to Ethanol day-14 samples included members of the phylum Kiritimatiellaeotaa (LDA 3.46, $P = 0.002$), driven by ambiguous taxa within the order WCHB1-41 (LDA 3.34, $P = 0.003$). A variety of taxa were enriched specifically in Frozen day-14 samples compared to Ethanol day-230 samples, including the Firmicutes families XIII (specifically *Anaerovorax* (LDA 3.05, $P = 0.001$)) and Peptococcaceae (LDA 3.03, $P < 0.001$) as well as the genera *Prevotellaceae UCG-001* (LDA 3.03, $P = 0.007$), *Fusicatenibacter* (LDA 3.26, $P = 0.002$), *Lachnospiraceae UCG-006* (LDA 3.07, $P < 0.001$), *UCG-008* (LDA 4.02, $P < 0.001$), and *UCG-009* (LDA 3.97, $P = 0.005$), *Ruminiclostridium 9* (LDA 3.85, $P = 0.014$), *Ruminococcaceae V9D2013 group* (LDA 3.03,

P < 0.001), as well as *Ruminococcaceae UCG-007* (LDA 3.41, *P* < 0.001) and *UCG-010* (LDA 3.99, *P* < 0.001; Figure 3). In addition, Frozen day-14 samples were also enriched in an uncultured taxa within the order Rhodospirillales (LDA 3.08, *P* = 0.005) and members of the phylum Spirochaetes (LDA 4.78, *P* = 0.01), driven by enrichment of *Treponema 2* species (LDA 3.85, *P* = 0.007). Lastly, the frozen samples were enriched in members of the domain Archaea (LDA 3.73, *P* = 0.033), specifically dominated by Methanomicrobia (LDA 3.74, *P* = 0.019), when compared to Ethanol day-230 samples.

As with ethanol treatments, both PB treatments were compared to Frozen day-14 samples (Figure 3). The PB day-14 samples were enriched in the phylum Bacteroidetes, driven by members of the order Bacteroidales (LDA 4.15, *P* = 0.005) and families Bacteroidales UCG-001 (LDA 3.75, *P* = 0.016) and F082 (LDA 4.15, *P* = 0.007; Figure 3). The PB day-14 samples were also enriched in members of the Actinobacteria family Ilumatobacteraceae (LDA 3.00, *P* = 0.045) and Firmicutes Family Eubacteriaceae (LDA 3.25, *P* < 0.001), specifically members of the genus *Anaerofustis* (LDA 3.26, *P* < 0.001), and genus *Ruminococcaceae UCG-014* (LDA 3.24, *P* = 0.046). The PB day-230 samples were enriched in members of the Bacteroidetes family Muribaculaceae (LDA 3.83, *P* = 0.048) and Firmicutes genera *Lachnospiraceae UCG-006* (LDA 3.23, *P* < 0.001), *Ruminiclostridium* (LDA 3.12, *P* = 0.025), *Ruminococcaceae UCG-014* (LDA 3.52, *P* = 0.011).

In contrast, frozen samples compared to PB day-14 samples were enriched in members of the Firmicutes genera *Ruminiclostridium 9* (LDA 3.68, *P* = 0.047) and *Ruminococcaceae UCG-010* (LDA 3.44, *P* < 0.001), as well as the phyla Spirochaetes (LDA 4.16, *P* = 0.022) and Verrucomicrobia, driven by uncultured bacteria in the order LD1-PB3 (LDA 3.56, *P* = 0.046). Comparisons against PB day-230 samples revealed enrichment of frozen samples with Firmicutes class Erysipelotrichia (LDA 3.38, *P* = 0.011), driven by uncultured members of the genus *Anaerorhabdus furcosa* group (LDA 3.26, *P* = 0.022; Figure 3). In addition, several uncultured members of the Firmicutes family XIII (LDA 3.50, *P* = 0.021) and members of the Firmicutes genera *Lachnospiraceae UCG-008* (LDA 3.60, *P* = 0.024), *Ruminiclostridim 9* (LDA 3.71, *P* = 0.045), and *Ruminococcaceae UCG-010* (LDA 3.56, *P* = 0.008) were enriched.

Frozen day-14 samples were also compared to Frozen day-230 samples. Day-14 samples were enriched in Firmicutes families XIII (LDA 3.54, *P* = 0.028) and Ruminococcaceae (LDA 4.12, *P* = 0.017), driven by uncultured members of the genera *Ruminiclostridium 9* (LDA 3.67, *P* = 0.029) and *UCG-010* (LDA 3.49, *P* = 0.022), as well as the phylum Lentisphaerae (LDA 4.02, *P* = 0.018), driven by enrichment of members of the class Oligosphaeria (LDA 3.93, *P* = 0.006) and genus *Horsej-a03* (LDA 3.64, *P* = 0.015). Frozen day-230 samples were enriched in uncultured clostridium species from the genera *Clostridium sensu stricto 1* (LDA 3.49, *P* = 0.022), *Lachnoclostridium 12* (LDA 3.96, *P* = 0.022), and *Ruminococcaceae UCG-014* (LDA 3.13, 0.028).

Alpha diversity

Ethanol day-14 samples harbored significantly different alpha diversity compared to both Frozen day-14 and PB day-14 samples, as measured by Shannon (Frozen: *P* < 0.001, *W* = 518; PB: *P* = 0.002, *W* = 430) and Simpson's diversity indices (Frozen: *P* < 0.001, *W* = 476; PB *P* < 0.001, *W* = 405; Table 3; Figure 4). Ethanol day-230 samples differed from all 5 other treatments and timepoints (*P* < 0.05). Median values for alpha diversity measures were lower in samples from ethanol treatments compared to all other preservatives (Figure 4).

Beta diversity

Multidimensional scaling (MDS) plots revealed distinct clusters driven by the 6 preservation treatments, though there was overlap among treatments (Figure 5). Frozen and PB samples had the most similar bacterial community structures, though frozen samples from both time points yielded the most consistent and least variable clusters compared to all other treatments. The PERFORMAbiome·GUT samples performed similarly to freezing, with the

TABLE 3 Wilcoxon statistical comparisons of alpha diversity measures across fecal samples from $N = 10$ southern white rhinoceros (*Ceratotherium simum simum)* in the North Carolina Zoo, Asheboro, USA, stored via freezing at −80°C, 95% ethanol, and PERFORMAbiome™·GUT (PB) for 14 and 230 days in 2020–2021.

				Species richness		Shannon diversity index		Simpson's diversity index	
Treatment 1	Treatment 2	n1	n2	Statistic (W)	Adj. *P*-Value	Statistic (W)	Adj. *P*-Value	Statistic (W)	Adj. *P*-Value
Ethanol Day 14	Ethanol Day 230	43	6	220	0.087	239	0.003**	220	0.055
Ethanol Day 14	Frozen Day 14	43	50	1006.5	1	518	<0.001***	476	<0.001***
Ethanol Day 14	Frozen Day 230	43	6	117	1	51	0.228	42	0.088
Ethanol Day 14	Performabiome Day 14	43	39	805	1	430	0.002**	405	<0.001***
Ethanol Day 14	Performabiome Day 230	43	6	139.5	1	41	0.078	30	0.018*
Ethanol Day 230	Frozen Day 14	6	50	21	0.01**	1	0.001**	8	0.003**
Ethanol Day 230	Frozen Day 230	6	6	3	0.228	0	0.032*	0	0.032
Ethanol Day 230	Performabiome Day 14	6	39	18	0.015*	0	<0.001***	10	<0.001***
Ethanol Day 230	Performabiome Day 230	6	6	3.5	0.37	0	0.032*	0	0.032*
Frozen Day 14	Frozen Day 230	50	6	143	1	150	1	125	1
Frozen Day 14	Performabiome Day 14	50	39	1008.5	1	1082	1	1075	1
Frozen Day 14	Performabiome Day 230	50	6	179.5	1	124	1	89	1
Frozen Day 230	Performabiome Day 14	6	39	127.5	1	134	1	148	1
Frozen Day 230	Performabiome Day 230	6	6	23	1	14	1	10	1
Performabiome Day 14	Performabiome Day 230	39	6	140	1	78	1	55	0.566

Adj.- Adjusted.

exception of one outlying value (a PB day-14 sample from July belonging to individual S1). Ethanol samples were highly variable, with large, indistinct community clusters. Ethanol day-230 samples shared overlap almost exclusively with Ethanol day-14 samples and differed the most from the other treatments; for example, Ethanol day-230 samples shared no overlap with frozen samples at either time point or with PB day-230 samples and showed minimal overlap with the PB day-14 samples (Figure 5).

Pairwise comparisons of beta diversity using PERMANOVAs on the Bray-Curtis distance matrix substantiated several significant differences between preservation methods that were visible via MDS plot (Table 4). Ethanol day-14 samples differed from all but Frozen day-230 samples ($P < 0.05$), and Ethanol day-230 samples differed significantly from all other preservation treatments. Frozen day-14 samples differed from all non-frozen preservation treatments. There were no differences in community composition between timepoints for either Frozen or PB samples. A post hoc analysis of homogeneity among methods revealed significant differences ($P < 0.001$) in dispersion within the

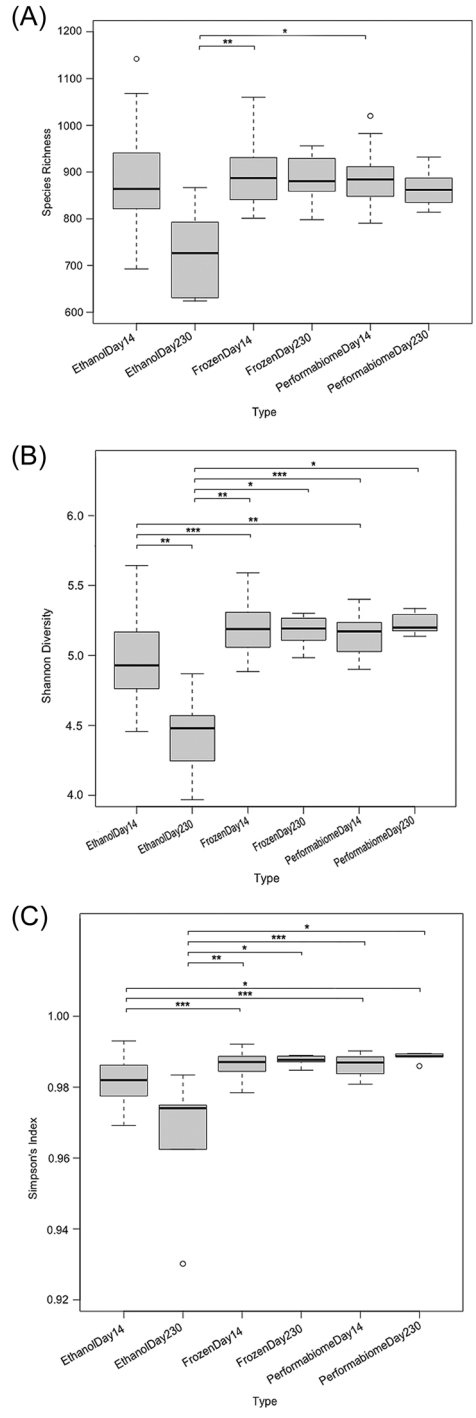

FIGURE 4 Boxplots comparing alpha diversity as measured by (A) species richness, (B) Shannon diversity, and (C) Simpson's diversity indices across freezing at −80°C, ethanol, and PERFORMAbiome™·GUT preservation methods at day 14 and day 230 for $N = 10$ southern white rhinoceros (*Ceratotherium simum simum*) in the North Carolina Zoo, Asheboro, USA, in 2020–2021. Error bars represent standard error of the mean. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

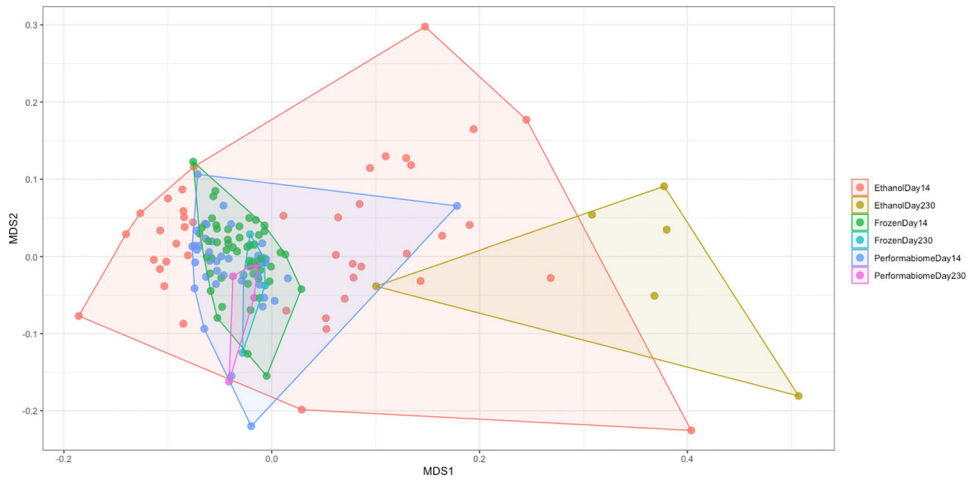

FIGURE 5 Multidimensional scaling plot comparing freezing at −80°C, ethanol, and PERFORMAbiome™·GUT (PB) preservation methods at day 14 and day 230 across all 3 preservation methods for $N = 10$ southern white rhinoceros (*Ceratotherium simum simum*) in the North Carolina Zoo, Asheboro, USA, in 2020–2021. Each point represents one fecal sample from one of $n = 10$ southern white rhinoceros, preserved using 1 of 6 preservation methods.

TABLE 4 Permutational multivariate analysis of variance (PERMANOVA) statistical comparisons of Bray-Curtis dissimilarity in microbial community composition across fecal samples from $N = 10$ southern white rhinoceros (*Ceratotherium simum simum)* in the North Carolina Zoo, Asheboro, USA, stored via freezing at −80°C, 95% ethanol, and PERFORMAbiome™·GUT (PB) for 14 and 230 days in 2020–2021.

	Frozen Day-14	Ethanol Day-14	PB Day-14	Frozen Day-230	Ethanol Day-230
Ethanol Day 14	0.003[a]	-	-	-	-
PB Day 14	0.003[a]	0.003[a]	-	-	-
Frozen Day 230	0.188	0.094	0.096	-	-
Ethanol Day 230	0.003[a]	0.003[a]	0.003[a]	0.009[a]	-
PB Day 230	0.017[b]	0.024[b]	0.096	0.579	0.006[a]

[a]$P < 0.01$.
[b]$P < 0.05$.

majority of pairwise comparisons. Of the methods, PB day-230 data were not significantly different (i.e., $P > 0.05$) in dispersion when compared to Frozen day-14, Frozen day-230, and PB day-14 data.

DISCUSSION

Proper sample preservation for microbiome studies is of the utmost importance for producing accurate and reproducible results. Freezing at −80°C has long been touted as the gold standard method for sample preservation due to its ability to halt biological function without damage to genomic material (Choo et al. 2015). However, freezing at −80°C has logistical limitations, especially for field research, whether samples are collected *in situ* abroad or locally (Song et al. 2016). We therefore aimed to determine whether 95% ethanol or the novel PB storage method is better suited for longitudinal storage of herbivore gut microbiome samples at ambient temperature, for periods of 14 days and ~230 days. Our results indicate that PB provides preservation similar to freezing at −80°C,

even when storage exceeds manufacturer recommendations by an order of magnitude (i.e., 230 days versus the 60-day recommended maximum).

The most abundant phyla detected across all preservation methods were Firmicutes, Bacteroidetes, Spirochetes, and Fibrobacteres, which is in line with several other studies of Rhinocerotidae microbiome (Bian et al. 2013, Roth et al. 2019, Williams et al. 2019). Actinobacteria have also been found in rhinoceros before, though neither Kiritimatiellaeota nor Lentisphaerae have previously been noted in this host family. However, both of the latter phyla have been previously identified in the *Equus* genus, and horses are the domestic animal model for rhinoceros digestion and nutrition (Arnold et al. 2021, Ericsson et al. 2021). Kiritimatiellaeota has also been identified in other hind-gut fermenters including Baird's tapir (*Tapirus bairdii*) and Asian elephants (*Elephas maximus*; Kandel et al. 2020, Yanez-Montalvo et al. 2021).

We detected differentially enriched taxa per method, including samples subjected to immediate freezing at −80°C. Frozen samples do not escape changes in composition levels over time, and generally contain higher ratios of Bacteroidetes compared to nonfrozen samples, and this discrepancy is thought to arise due to alterations in cellular structure that gram-positive bacteria experience when frozen (Bahl et al. 2012, Fouhy et al. 2015). Higher abundances of Bacteroidetes were apparent in Frozen day-14 samples when compared to ethanol samples from both time points, but not when compared to PB day-14 samples; the PB day-14 samples contained a higher abundance of Bacteroidetes than the frozen controls. Methanomicrobia (Archaea) were also enriched in frozen samples, but only in comparison to Ethanol day-230 samples. Archaea have been previously isolated in rhinoceros, and methanogens are thought to play a crucial role in increasing the fermentation efficiency in rhinoceros and other hind-gut fermenters (Luo et al. 2013, Moissl-Eichinger et al. 2018). Archaea have proven difficult to cultivate in laboratories, so the development of molecular sequencing methods such as 16S rRNA sequencing has led to increased understanding of their role in microbiome ecology (Moissl-Eichinger et al. 2018). As archaea are extremophiles, it is unsurprising that some psychrophilic members of that domain would experience increased abundance in samples frozen at −80°C.

The most promising outcome of our study was the performance of PB samples over time. PERFORMAbiome·GUT manufacturer instructions state that samples have a shelf-life limited to 60 days at ambient temperatures between −20°C and 50°C (DNA Genotek 2019), a length of time that already made PB a preferred preservation method for field studies. However, even after 230 days of storage, PB samples still yielded microbial profiles comparable to samples frozen at −80°C, as measured by both alpha and beta diversity indices. Notably, while there were significant differences in beta diversity between PB and frozen samples at day 14, the differences became insignificant by day 230 of storage. It is also important to note that while dispersion of the data was significantly different for the majority of pairwise comparisons of Bray-Curtis dissimilarity between methods (thus violating an assumption of PERMANOVA), PB day-230 data were not significantly different in dispersion when compared to Frozen day-14, Frozen day-230, and PB day-14 data. The storage solution within PB devices is proprietary and thus we cannot speculate what specific ingredients might affect compositional differences over time or between PB and other preservation methods. However, PB is a derivative of the OMNIgene®·GUT product (DNA Genotek 2019), which was produced and formulated to optimize home-based human fecal sample collection for gut microbial profile analysis. OMNIgene·GUT has been shown in previous studies to perform similarly to −80°C controls and technical replicates when compared against a variety of other preservation methods (Choo et al. 2015, Song et al. 2016).

The use of 95% ethanol as a preservative has been common for long-term storage of many biological materials from months to years, though a previous microbiome study has only validated its usage for preserving community composition in human and dog fecal samples up to 56 days (Song et al. 2016). This longitudinal storage capability was limited in our rhinoceros system, as the ethanol samples stored for 230 days underwent significant decreases in species richness, Shannon diversity, and Simpson's diversity. Interestingly, ethanol samples stored for only 14 days were also significantly different from controls frozen at −80°C, as measured by both alpha and beta diversity indices. Differences were driven by differentially

abundant taxa, specifically Actinobacteria, which was enriched at both time points. Actinobacteria have been previously shown to undergo ethanol-induced expansions in mice and are likely tolerant of high-alcohol environments (Bull-Otterson et al. 2013), thus, Actinobacteria may not be deleteriously affected by long term ethanol storage the way other microbial taxa would be. Actinobacteria are also resilient to another preservation method, fecal occult blood test (FOBT) cards (Moossavi et al. 2019). A separate issue with the use of 95% ethanol as a fecal preservation method was the consistently low DNA extraction yields, a challenge which has been documented widely in the literature (Vlčková et al. 2012, Hale et al. 2015, Song et al. 2016). This setback required additional time and funds to extract samples in duplicate, whereas frozen and PB samples consistently yielded ample, high-quality DNA.

Limitations of our study included a small sample size, especially for day-230 samples which represent a subset of the study population. In addition, we modified sample processing for ethanol samples toward the end of the DNA extraction period though statistical comparisons revealed consistent, significant differences between 95% ethanol and other day-14 preservation treatments across the full study period. The volume of fecal material preserved via different methods also varied due to sample collection constraints, so we cannot unequivocally state that DNA yields for one method differed significantly from another (although differences were apparent in practice). Lastly, we realize there may be variation stemming from different sequencing runs (i.e., batch effects) for samples collected in July–September of 2020 versus samples collected in January–March of 2021, however, by using the same laboratory, procedure, and technicians to process all samples, we minimized that variation to the greatest extent possible. Future studies should further evaluate the performance of PB preservation methods across additional parameters, including the upper limit of physical material that can be preserved using the proprietary solution without overwhelming it.

RESEARCH IMPLICATIONS

Of the preservation methods utilized in our study, PB tubes performed most similarly to freezing immediately at −80°C. Observed gut microbial community profiles remained consistent (i.e., statistically similar) between frozen and PB preservation treatments across both time points. The PB devices showed a remarkable consistency in stable preservation of microbial community composition over time at ambient temperatures, even beyond the manufacturer's storage period recommendations. The PB tubes also yielded microbial community profiles similar to profiles observed for the gold standard of freezing at −80°C, with only limited differences in beta diversity. The usage of 95% ethanol as a preservative was inadequate for both short (14 days) and long-term storage (230 days) of samples at ambient temperature. In contrast to studies evaluating the efficacy of ethanol as a microbiome preservation method used for either dog or human host subjects, our results indicated that ethanol may not provide optimal storage for fecal samples from herbivorous species with complex gastrointestinal tracts. We suggest that PB devices provide a viable solution to the challenges associated with microbiome fieldwork.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

ETHICS STATEMENT

This study involved noninvasive sampling of vertebrate animals and was approved by the North Carolina State Institutional Care and Use Committee (No. 20-260-O).

ORCID

Shweta Trivedi http://orcid.org/0000-0001-5101-7740

REFERENCES

Arnold, C. E., R. Pilla, M. K. Chaffin, J. L. Leatherwood, T. A. Wickersham, T. R. Callaway, S. D. Lawhon, J. A. Lidbury, J. M. Steiner, and J. S. Suchodolski. 2021. The effects of signalment, diet, geographic location, season, and colitis associated with antimicrobial use or *Salmonella* infection on the fecal microbiome of horses. Journal of Veterinary Internal Medicine 35:2437–2448.

Bahl, M. I., A. Bergström, and T. R. Licht. 2012. Freezing fecal samples prior to DNA extraction affects the Firmicutes to Bacteroidetes ratio determined by downstream quantitative PCR analysis. FEMS Microbiology Letters 329: 193–197.

Bian, G., L. Ma, Y. Su, and W. Zhu. 2013. The microbial community in the feces of the white rhinoceros (*Ceratotherium simum*) as determined by barcoded pyrosequencing analysis. PLoS ONE 8(7):e70103.

Bull-Otterson, L., W. Feng, I. Kirpich, Y. Wang, X. Qin, Y. Liu, L. Gobejishvili, S. Joshi-Barve, T. Ayvaz, J. Petrosino, et al. 2013. Metagenomic analyses of alcohol induced pathogenic alterations in the intestinal microbiome and the effect of *Lactobacillus rhamnosus* GG treatment. PLoS ONE 8(1):e53028.

Cardona, S., A. Eck, M. Cassellas, M. Gallart, C. Alastrue, J. Dore, F. Azpiroz, J. Roca, F. Guarner, and C. Manichanh. 2012. Storage conditions of intestinal microbiota matter in metagenomic analysis. BMC Microbiology 12:158.

Carroll, I. M., T. Ringel-Kulka, J. P. Siddle, T. R. Klaenhammer, and Y. Ringel. 2012. Characterization of the fecal microbiota using high-throughput sequencing reveals a stable microbial community during storage. PLoS ONE 7(10):e46953.

Choo, J. M., L. E. Leong, and G. B. Rogers. 2015. Sample storage conditions significantly influence faecal microbiome profiles. Scientific Reports 5:16350.

de la Cuesta-Zuluaga, J., N. T. Mueller, V. Corrales-Agudelo, E. P. Velásquez-Mejía, J. A. Carmona, J. M. Abad, and J. S. Escobar. 2017. Metformin is associated with higher relative abundance of mucin-degrading *Akkermansia muciniphila* and several short-chain fatty acid–producing microbiota in the gut. Diabetes Care 40:54–62.

DNA Genotek. 2019. PERFORMAbiome·GUT | PB-200. <https://www.dnagenotek.com/ROW/products/collection-animals/performabiome/PB-200.html>. Accessed 5 Oct 2021.

Emslie, R. 2020. *Ceratotherium simum*. The International Union for Conservation of Nature Red List of Threatened Species 2020. e.T4185A45813880

Emslie, R., K. Adcock, and H. Hansen. 1995. Fine tuning rhino management group age class system. Rhino Management Group 1–17. <http://www.rhinoresourcecenter.com/index.php?s=1%26act=refs%26CODE=ref_detail%26id=1165240732>. Accessed 20 Jul 2020.

Ericsson, A. C., P. J. Johnson, L. M. Gieche, C. Zobrist, K. Bucy, K. S. Townsend, L. M. Martin, and A. M. LaCarrubba. 2021. The influence of diet change and oral metformin on blood glucose regulation and the fecal microbiota of healthy horses. Animals 11(4):976.

Fouhy, F., J. Deane, M. C. Rea, Ó. O'Sullivan, R. P. Ross, G. O'Callaghan, B. J. Plant, and C. Stanton. 2015. The effects of freezing on faecal microbiota as determined using MiSeq sequencing and culture-based investigations. PLoS ONE 10(3):e0119355.

Griffith, G. E., J. M. Omernik, J. Comstock, M. P. Schafale, W. H. McNab, D. R. Lenat, and T. F. MacPherson. 2002. Ecoregions of North Carolina. Western Ecology Division, National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency, Durham, North Carolina, USA.

Hale, V. L., C. L. Tan, R. Knight, and K. R. Amato. 2015. Effect of preservation method on spider monkey (*Ateles geoffroyi*) fecal microbiota over 8 weeks. Journal of Microbiological Methods 113:16–26.

Illumina. 2013. 16S metagenomic sequencing library preparation. <https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf>. Accessed 5 Oct 2021.

International Air Transport Association. 2021. Dangerous Goods Regulations (IATA—Resolution 618 Attachment "A"): Effective 1 January-31 December 2022. IATA, Montreal, Quebec, Canada.

Kandel, S., S. Sripiboon, P. Jenjaroenpun, D. W. Ussery, I. Nookaew, M. S. Robeson, and T. Wongsurawat. 2020. 16S rRNA gene amplicon profiling of baby and adult captive elephants in Thailand. Microbiology Resource Announcements 9(24):e00248–20.

Klindworth, A., E. Pruesse, T. Schweer, J. Peplies, C. Quast, M. Horn, and F. O. Glöckner. 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Research 41(1):e1.

Landis, E. A., A. M. Oliverio, E. A. McKenney, L. M. Nichols, N. Kfoury, M. Biango-Daniels, L. K. Shell, A. A. Madden, L. Shapiro, S. Sakunala, et al. 2021. The diversity and function of sourdough starter microbiomes. Elife 10:e61644.

Leff, J. W. 2017. mctoolsr: Microbial Community Data Analysis Tools. R package version 0.1.1.2. <https://github.com/leffj/mctoolsr>. Accessed 2 Jan 2021.

Lin, C. Y., T. W. L. Cross, E. Doukhanine, and K. S. Swanson. 2020. An ambient temperature collection and stabilization strategy for canine microbiota studies. Scientific Reports 10:13383.

Luo, G. J., R. Kiese, B. Wolf, and K. Butterbach-Bahl. 2013. Effects of soil temperature and moisture on methane uptake and nitrous oxide emissions across 3 different ecosystem types. Biogeosciences 10:3205–3219.

Mead, J. G. 2009. G—Gastrointestinal Tract. Pages 472–477 *in* W. F. Perrin, B. Würsig, and J. G. M. Thewissen, editors. Encyclopedia of Marine Mammals (Second Edition). Academic Press, London, UK.

Metrione, L. C., and A. Eyres. 2014. Rhino Husbandry Manual. International Rhino Foundation, Fort Worth, Texas, USA.

Moissl-Eichinger, C., M. Pausan, J. Taffner, G. Berg, C. Bang, and R. A. Schmitz. 2018. Archaea are interactive components of complex microbiomes. Trends in Microbiology 26:70–85.

Moossavi, S., P. A. Engen, R. Ghanbari, S. J. Green, A. Naqib, F. Bishehsari, S. Merat, H. Poustchi, A. Keshavarzian, and R. Malekzadeh. 2019. Assessment of the impact of different fecal storage protocols on the microbiota diversity and composition: a pilot study. BMC Microbiology 19:145.

National Climatic Data Center. 2022. National centers for environmental information. <https://www.ncdc.noaa.gov>. Accessed 7 Sep 2022.

Oksanen, J., R. Kindt, and B. O'Hara. 2019. Vegan: community ecology package. R package version 2. 5–6. <https://CRAN.R-project.org/package=vegan>. Accessed 2 Jan 2021.

R Core Team. 2022. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Roth, T. L., A. Switzer, M. Watanabe-Chailland, E. M. Bik, D. A. Relman, L. E. Romick-Rosendale, and N. J. Ollberding. 2019. Reduced gut microbiome diversity and metabolome differences in rhinoceros species at risk for iron overload disorder. Frontiers in Microbiology 4(10):2291.

Saito, K., S. Koido, T. Odamaki, M. Kajihara, K. Kato, S. Horiuchi, S. Adachi, H. Arakawa, S. Yoshida, T. Akasu, et al. 2019. Metagenomic analyses of the gut microbiota associated with colorectal adenoma. PLoS ONE 14(2):e0212406.

Segata, N., J. Izard, L. Waldron, D. Gevers, L. Miropolsky, W. S. Garrett, and C. Huttenhower. 2011. Metagenomic biomarker discovery and explanation. Genome Biology 12:R60.

Smith, H. F., R. E. Fisher, M. L. Everett, A. D. Thomas, R. Randal Bollinger, and W. Parker. 2009. Comparative anatomy and phylogenetic distribution of the mammalian cecal appendix. Journal of Evolutionary Biology 22: 1984–1999.

Song, S. J., A. Amir, J. L. Metcalf, K. R. Amato, Z. Z. Xu, G. Humphrey, and R. Knight. 2016. Preservation methods differ in fecal microbiome stability, affecting suitability for field studies. mSystems 1:e00021–16.

Steinmetz, K., S. Murphy, O. Ó. Cadhla, J. Barnett, and L. Mirimin. 2021. Enabling pinniped conservation by means of non-invasive genetic population analysis. Conservation Genetics Resources 13:131–142.

Swaisgood, R. R., D. M. Dickman, and A. M. White. 2006. A captive population in crisis: testing hypotheses for reproductive failure in captive-born southern white rhinoceros females. Biological Conservation 129:468–476.

Tedjo, D. I., D. M. A. E. Jonkers, P. H. Savelkoul, A. A. Masclee, N. van Best, M. J. Pierik, and J. Penders. 2015. The effect of sampling and storage on the fecal microbiota composition in healthy and diseased subjects. PLoS ONE 10(5):e0126685.

Tubbs, C. W., L. A. Moley, J. A. Ivy, L. C. Metrione, S. LaClaire, R. G. Felton, B. S. Durrant, and M. R. Milnes. 2016. Estrogenicity of captive southern white rhinoceros diets and their association with fertility. General and Comparative Endocrinology 238:32–38.

Vlčková, K., J. Mrázek, J. Kopečný, and K. J. Petrželková. 2012. Evaluation of different storage methods to characterize the fecal bacterial communities of captive western lowland gorillas (*Gorilla gorilla gorilla*). Journal of Microbiological Methods 91:45–51.

Williams, C. L., A. R. Ybarra, A. N. Meredith, B. S. Durrant, and C. W. Tubbs. 2019. Gut microbiota and phytoestrogen-associated infertility in southern white rhinoceros. mBio 10:e00311–19.

Wood, J., E. Koutsos, C. J. Kendall, L. J. Minter, T. N. Tollefson, and K. A. Heugten. 2020. Analyses of African elephant (*Loxodonta africana*) diet with various browse and pellet inclusion levels. Zoo Biology 39:37–50.

Wu, W. K., C. C. Chen, S. Panyod, R. A. Chen, M. S. Wu, L. Y. Sheen, and S. C. Chang. 2019. Optimization of fecal sample processing for microbiome study—The journey from bathroom to bench. Journal of the Formosan Medical Association 118:545–555.

Yanez-Montalvo, A., O. Gaona, B. Águila, N. Arias-Domínguez, L. I. Falcón, and J. Pérez-Flores. 2021. *Tapirus bairdii*-associated fecal microbiome from a critical conservation area: Calakmul, México. Current Microbiology 78: 2648–2659.

Zhang, B., M. Brock, C. Arana, C. Dende, L. Hooper, and P. Raj. 2020. Impact of bead-beating intensity on microbiome recovery in mouse and human stool: optimization of DNA extraction. <https://www.biorxiv.org/content/10.1101/2020.06.15.151753v1>. Accessed 5 Nov 2021.

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SUPPORTING INFORMATION

Additional supporting material may be found in the online version of this article at the publisher's website.

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