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Rewilding reshapes gut microbiomes and parasite exposure in European bison: a 17-month release from Wilder Blean

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ABSTRACT

Reintroductions can restore lost ecological processes, but managers require practical health indicators to track the acclimation of released animals. We longitudinally profiled the gut microbiomes of European bison (*European bison bonasus*) released to the Wilder Blean area (Kent, UK), sampling three adult females before and after release, and a post-release male and calf. Using V3-V4 16S rRNA gene sequencing, we quantified alpha- and beta-diversity, identified differentially abundant taxa, and screened faeces for *Cryptosporidium*, *Enterocytozoon bieneusi* and *Blastocystis*. Post-release, adult microbiomes shifted significantly (PERMANOVA $P = 0.001$) and consistently across all examined animals. Calf microbiome profiles transitioned from early-life communities to an adult-like state concurrent with weaning. Parasite screening via separate PCR and qPCR showed that *Cryptosporidium* positivity declined in females from 36% pre-release to 13% post-release, whereas *E. bieneusi* emerged only after release (~10% of samples), with multiple genotypes detected. These patterns are consistent with dietary and environmental turnover following release, and they highlight opportunities for using microbiome and parasite metrics as complementary, non-invasive indicators of rewilding progress. We recommend reporting simple, management-relevant indicators, archiving sequence data, and documenting soft-release design and supplementary feeding info to aid interpretation. Integrating routine faecal microbiome and parasite monitoring into rewilding programmes can support adaptive management, inform supplementary feeding decisions, and strengthen biosecurity risk assessments.

1. Introduction

Like many industrialised nations, the UK has witnessed a precipitous decline in biodiversity over the past century. The 2023 State of Nature Report (SON), a comprehensive assessment of UK biodiversity, underscores this trend. Since 1970, the average decline has been 19% across 735 monitored species, with approximately 300 species experiencing moderate to substantial population reductions (≥50%). Vascular plant species have not been spared, with 54% showing decreased distributions between 1970 and 2019 (State of Nature 2023, n.d.)

Numerous rewilding initiatives have been implemented across the UK to combat this biodiversity loss. The Wilder Blean project in Kent exemplifies these efforts, aiming to restore ecosystem functions and promote biodiversity by reintroducing keystone species, such as the European bison (*European bison bonasus*). Efforts like these to restore biodiversity via the introduction of species, draw inspiration from the notable ecological recovery observed at Yellowstone National Park (US) following the reintroduction of grey wolves, which triggered beneficial trophic cascades affecting multiple species and habitat recovery (Boyce, 2018; Ripple & Beschta, 2012). The emergence of rewilding as a tool to help restore functioning ecosystems has gained considerable traction over the past decade (Lorimer et al., 2015;Carver et al., 2021; Egoh et al., 2021;Manning et al., 2024). Large herbivores play a pivotal role in such interventions, and considerable progress has been made in understanding their role in, for example, acting as surrogates for extinct species (Schowanek et al., 2021). An example of such extinct animal surrogacy in the UK is the steppe bison. Initially present in the UK until its extinction around 8,000 years ago, the steppe bison, *Bison priscus*,

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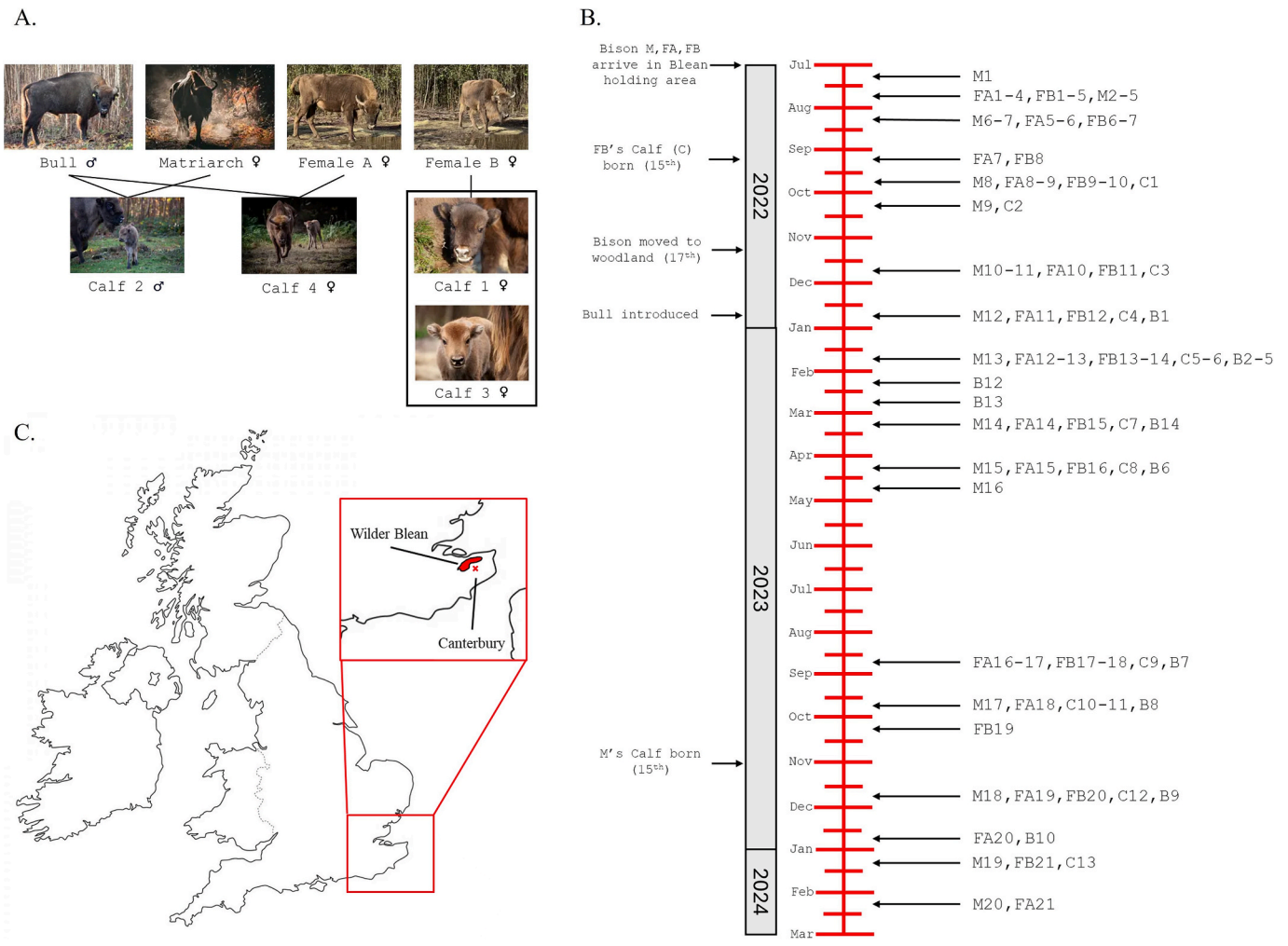

Fig. 1. Details of the European bison releases to the Blean woodland. A). Family dynamics of the European bison herd, containing all members/relationships as of March 2025. B). timeline from July 2022 to March 2024, containing the dates at which each member of the herd was sampled. C). Location of the Wilder Blean woodland release site.

shares a close genetic lineage with the European bison released into the Blean woodlands. These European bison were introduced as 'ecosystem engineers', whose natural behaviours such as grazing, wallowing, and soil disturbance have been demonstrated to enhance plant and arthropod diversity significantly (Nickell et al., 2018; Collins et al., 1998) As well as structuring plant and arthropod communities (Van Klink & WallisDeVries, 2018); habitat restoration (Smit et al., 2015) and mitigating climate change (Olofsson & Post, 2018). Less attention has been paid to the implications of releasing herbivores on the microbiomes of the animals themselves and the habitats they are intended to manage. This is despite the fact microbiome rewilding may have a significant impact on food security, human health and well-being as well as functioning ecosystems (Mills et al., 2017; Raaijmakers & Kiers, 2022; Contos et al., 2025). Tracking the development of microbiome communities in rewilded animals as they transition from captivity to the wild may therefore be important to understanding implications for both animal health and welfare as well as ecosystem function (Bornbusch et al., 2022; Korpita et al., 2023; Gao et al., 2025).

Their gut microbiomes profoundly influence the health and adaptive capacity of reintroduced populations. In ruminants, such as European bison, the gut and rumen microbiomes are crucial for digesting their fibrous, plant-based diets and maintaining overall health and disease resistance (Cammack et al., 2018). However, the study of microbiomes in conservation biology is still in its early stages, with the potential to provide crucial insights into the health, nutrition, and environmental adaptation of wildlife. Current research into the microbiomes of European bison is limited, with few studies employing contemporary metagenomic approaches. For example, a recent study on multiple herbivores (including European bison) highlighted a microbiome dominated by bacteria from the classes Firmicutes, Bacteroidota, Proteobacteria, and Verrucomicrobiota, which play significant roles in nutrient absorption and pathogen resistance (Sun et al., 2024). Comparable diet- and season-driven microbiome patterns have been reported across other large herbivores, including North American bison, muskoxen and yak, and provide a useful comparative framework for interpreting microbiome turnover following release (Bergmann et al., 2015; Bird et al., 2019; Andersen-Ranberg et al., 2018; Cui et al., 2023). The study herein aims to delve deeper into the role of the microbiome in the success of rewilding efforts by monitoring the shifts in gut microbial communities of European bison during and after their release into the Wilder Blean area. By examining the microbiome dynamics and screening for prevalent microscopic eukaryotic ruminant parasites, we seek to assess the health impacts of releases and enhance our understanding of how microbiomes contribute to the conservation and management of such populations. Insights gained could inform future rewilding projects and highlight the critical role of microbiome studies in the broader field of conservation biology.

2. Methods

2.1. Experimental design

A herd of European bison (*Bison bonasus*) consisting of a female 'Matriarch', two females and a calf [the calf (calf 1) of female B (FB) was born on 9th of September 2022 (this calf is referred to as 'calf' throughout the study] were moved from their original location (Female A and B are from Fota Wildlife Park, Ireland and the Matriarch from the Highland Wildlife Park, Scotland) on the 8th of July 2022, to a corral facility in the Wilder Blean in Kent (Fig. 1A/B). The European bison were initially held in a corral at Wildwood Park in Blean, measuring ~ 1,500 m2, with a water trough, hay rack, and woodchip floor. This corral was used initially for settling, introductions, quarantine, and required testing. The animals were then moved to a 'soft release' area, ~5ha, with native broadleaf trees and a pond (the herd were sampled regularly whilst in the corral and soft release area). The herd were then released into the Wilder Blean woodland on November 17, 2022. A male European bison bull was introduced to the herd on December 23, 2022, from a breeding centre at Tierpark Sababurg in Germany. The matriarch gave birth to a calf on November 15, 2023 (this calf (calf 2), was not monitored during the study). The final Wilder Blean woodland area consisted of approximately 50 Ha of mixed broadleaf woodland and a conifer plantation. Due to the high stocking density (animals per area), and the calf being born out of season (resulting in poor condition of the mother), a regimen of supplementary feeding occurred during this time. The European bison's diet was supplemented with sugar beet molasses, which was transitioned into cattle feed (Duffield cattle feed) during the winter of 2023 (24/11/23) till the end of the study period (ongoing since the end of the study period as well). Human interaction occurred in the form of 'bison rangers' sampling the animals. During their time in pre-release and after their release into the woodland, they were monitored, and their stool was collected, allowing for a comparison of their gut microbiome before and after release (Fig. 1C). The Matriarch was 18 years old, Females A and B were both 4 years old, and the bull (at the time of introduction) was also 4 years old.

2.2. Sample collection

Samples were collected by 'bison rangers' irregularly, depending on when the European bison could be located and when they voluntarily visited the rangers' facilities for supplementary feeding. Faecal samples were collected from each individual animal if possible. Samples were then immediately frozen at −20 °C until DNA extraction. Sample counts are shown in Table 1.

2.3. DNA extraction

Using the PureLink™ Microbiome DNA Purification Kit (Invitrogen) following manufacturer's guidelines, DNA was extracted using 0.4 g of thawed European bison stool. The extracted DNA was then stored at −20 °C.

2.4. 16S amplicon sequencing

High-throughput amplicon sequencing was performed on the V3-V4 region of the 16S SSU r*RNA* gene by Biomarkers Technologies (BMKGENE). Amplification was done on the Illumina NovaSeq platform utilising the 515F (GTGCCAGCMGCCGCGGTAA) and 907R (CCGTCAATTCCTTTGAGTTT) primer set. A paired end 2x 250 bp sequencing process was used. The raw reads were then provided; these had been purified and demultiplexed, and adaptors had been removed from the sequences.

Table 1
Table showing individual animals samples were collected from and the number of samples taken from each animal.

Animal	Matriarch	Female A	Female B	Bull	Calf
Collected samples	20	20	21	10	13

2.5. Data preparation

The raw reads provided by BMKGENE were processed into taxa for analysis using the LotuS2 software (Özkurt et al., 2022). LotuS2 was used to classify the reads into ASVs with the following settings/software: Firstly, chimaeras and unsuitable reads (stunted or fusions) were removed using minimap2 (Li, 2018). Suitable reads were then clustered into ASVs using the Divisive Amplicon Denoising Algorithm 2 (DADA2) (Callahan et al., 2016). ASVs were compared against the European bison reference genome (GCA_963879515.1_ETH_BisBon1_genomic) to identify contaminated sequences, 6/7706 ASVs were identified as contaminants and removed. The taxonomy assignment of the ASVs was performed with BLAST (Altschul et al., 1990) against the GreenGenes2 (GG2) database (DeSantis et al., 2006). This database was selected as it is a 16S rRNA database that has already been checked for chimaeras and contaminated sequences.

2.6. Data analysis and visualisation

All data visualisation was performed using RStudio version 4.2.3. ASV counts were first rarefied to the lowest total read count (47,843 reads) to avoid data errors potentially caused by uneven sequencing depths. Rarefaction was performed by randomly subsampling each sample to 47,843 reads. This subsampling was repeated 100 times to generate separate datasets. The mean (rounded to the nearest whole number) read count of each ASV (within each sample) was then calculated from the subsampled dataset. This 'true' rarefaction avoids the concerns raised over subsampling alone whilst preserving community compositions (McMurdie & Holmes, 2014)(Schloss, 2024). Post-rarefaction data was analysed for diversity using diversity metrics/equations; Shannon, Simpson, and Chao1 diversity, as well as true richness (observed taxa). Changes in diversity scores were analysed using ANOVA (Girden, 1992) or Kruskal-Wallis (Kruskal & Wallis, 1952) tests (dependant on data normality (determined via Shapiro-Wilks tests (Shapiro & Wilk, 1965)) Normal distribution: ANOVA, Non-normal distribution: Kruskal-Wallis). Principle coordinate/component analysis (PCoA/PCA) (Gower, 2014) was performed using Bray-Curtis (Bray & Curtis, 1957) and Binary Jaccard (Jaccard, P. (1901)) distance matrices (from the rarefied data), and statistical analysis of sample distribution was performed using PERMANOVA and Adonis2 (M. J. Anderson, 2017). linear discriminant analysis was used. Microbiome Multivariable Associations with Linear Models 3 (MaAsLin3) (Nickols et al., 2024) was utilised to look at taxa which were strong discriminants between the two timepoint groups (pre and post-release). The Spearmans rank test was used to identify significant correlations between taxa identified as significant by Maaslin3 and parasite prevalence.

2.7. Molecular detection (Parasite detection)

In this study, nested PCR and qPCR assays were employed to screen samples for the presence of *Blastocystis* sp., *Cryptosporidium* spp., and *Enterocytozoon bieneusi*. The small subunit ribosomal RNA (SSU rRNA) gene was amplified for the identification of Cryptosporidium spp., while the gp60 gene was targeted for *Cryptosporidium* subtyping. For *Blastocystis* spp., the SSU rRNA gene was amplified, and the internal transcribed spacer (ITS) region was targeted for *E. bieneusi* detection. Reaction conditions varied according to the parasite species and the genetic marker employed (Supplementary Table 2; Supplementary Fig. 5).

Quantitative PCR (qPCR) was also used to amplify a fragment of the *Blastocystis* SSU rRNA gene. Positive and negative controls were

included in all PCR and qPCR runs to ensure reliability and accuracy. Amplified PCR products were purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. All molecular procedures were conducted in accordance with previously published protocols (Maxamhud et al., 2023; Pinto et al., 2021; Jinatham et al., 2021; Buckholt et al., 2002; Muadica et al., 2020).

3. Results

CAP (constrained analysis) revealed that of the three considered variables (Release status, Timepoint and Animal of origin), Release status and timepoint variables had a significant impact on the observed variance (Release status: $r^2 = 0.73$, p-value = 0.002, Timepoint: $r^2 = 0.52$, p-value = 0.001, Animal of origin: $r^2 = 0.042$, p-value = 0.166). However, as variance inflation factor analysis (VIF), found Release status and Timepoint to be strongly colinear (GVIF^(1/(2*Df)) = 4.12) timepoint was not considered as a covariate for this analysis.

Stool samples were collected from the initial European bison herd (the matriarch and two females, A and B) during the pre-release period (from July 2022 until mid-November 2022) and then irregularly after their release into the Wilder Blean area, spanning 17 months (September 2022 to February 2024). Using *16S rRNA* gene sequencing, we characterised how the gut microbiome changed in these European bison over time. Fig. 2 shows the 10 most abundant taxa within in each animal over the time course of sample collection, aggregated to the genus level. All three adult females (The matriarch and females A/B) shared a very similar microbiome composition dominated by a few taxa—most notably by two unclassified genera of the families *Oscillospiraceae* and *Bacteroidaceae*. Upon release from the 'soft release' area into the true woodland (Timepoints associated with release: Matriarch T4, Female A T5 and Female B T6), subtle but consistent shifts were observed in other taxa (whilst *Oscillospiraceae* and *Bacteroidaceae* remained broadly consistent): the relative abundance of the *Flavobacteriaceae* (unclassified) decreased to almost absence in both female A and the Matriarch and across all 3 females. Observed also, was an increase in the genera *Ruminococcus_E* and *Lachnospiraceae* (unclassified) (inconsistently) post-release.

Although the bull was introduced later (with no pre-release samples available), its post-release microbiome exhibited the same key patterns over time (Fig. 2). The bull's gut community was dominated by the same core taxa as the females and it showed an increasing trend in relative abundance of *Ruminococcus_E*, as well as an unclassified member of *Butyricioccaceae*.

In contrast, the calf, born in the wild with no captive baseline. initially harboured a very different microbiome from the adults (Fig. 2). The first two samples collected soon after birth were dominated by an unclassified member of the order *Christensenellales*. However, within a few months, the calf's profile shifted to closely resemble the adult European bison microbiome: by later sampling points, the unclassified members of *Oscillospiraceae* and *Bacteroidaceae* became dominant, The change to resemble the adult microbiome aligned with the complete weaning of the calf onto a forage diet, which occurred late September 2023 (T12 and T13 are fully post weaning).

At the class level (Supplementary Fig. 1), communities were consistently dominated by *Clostridia* and *Bacteroidia*, which is typical for

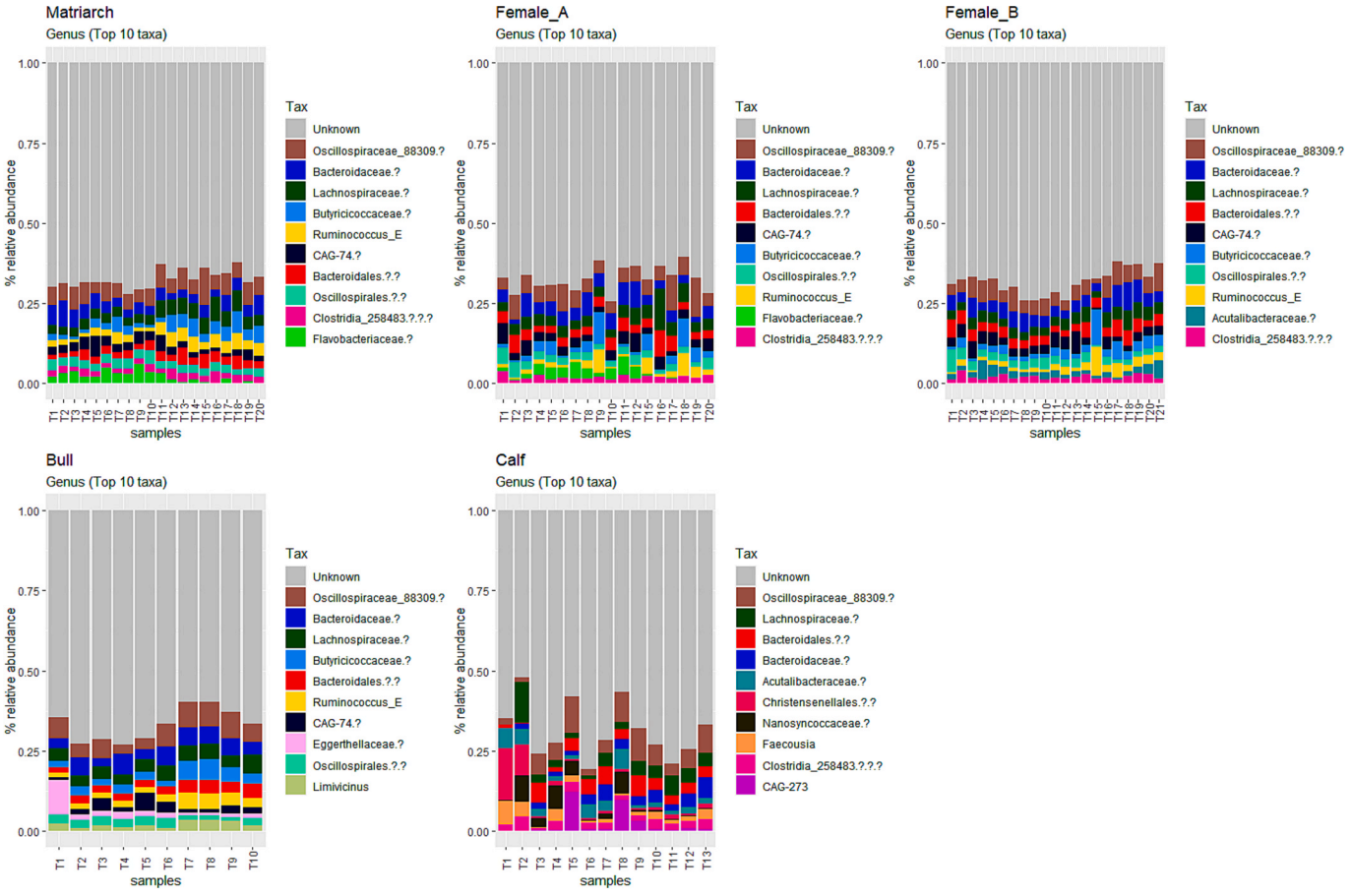

Fig. 2. Compositional plots comparing changes in the gut microbiome of the matriarch (A), Female European bison A (B), Female European bison B (C), Bull (D) and Calf (E) (born during the study) across the duration of the study. Taxa shown made up > 1% of the read counts in their respective samples; abundance is shown as relative abundance. All plots show taxa aggregated to the genus level.

grazing ungulates and reflects a fibre-fermenting microbiome. To address the suggestion that ratios of key groups can indicate forage energy harvest and dysbiosis, we examined the relative balance of fermentation-associated classes (*Clostridia* and *Bacteroidia*) against *Proteobacteria*-associated classes (including *Gammaproteobacteria*). Across individuals, the dominant fermentation classes remained stable, while Proteobacteria-associated classes showed a modest decline after release, which is directionally consistent with reduced dysbiosis risk rather than a post-release bloom. Because amplicon data are compositional, we interpret these patterns as shifts in relative community structure rather than absolute biomass (Gloor et al., 2017). At a higher taxonomic level, the class level compositions of the animals (Supplementary Fig. 1) were highly consistent, with *Clostridia*, *Bacteroidia* and *Bacilli* dominating throughout the time course. Post-release however, a decreasing trend in *Coriobacteriia*, and to a lesser degree, *Alphaproteobacteria*.

As displaying, only the 10 most abundant taxa is not comprehensive, quantification of the gut microbiome's diversity change post-release, was done using several alpha-diversity metrics. Diversity was compared in the three female European bison that had both pre- and post-release samples (the bull and calf lacked pre-release data). Four metrics of diversity were used for each animal's pre- versus post-release group of samples: Shannon diversity (which reflects both richness and

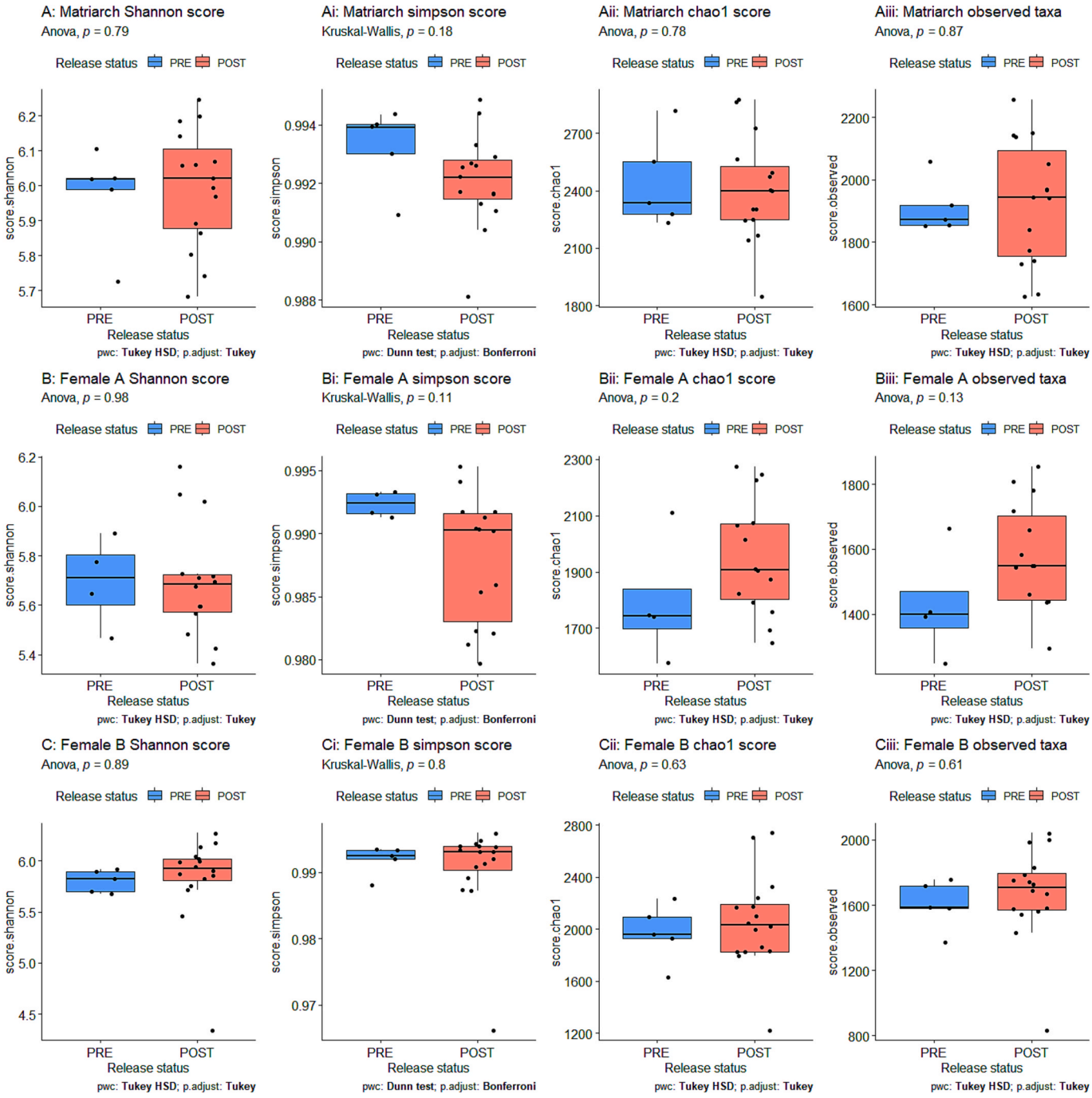

Fig. 3. Boxplots comparing the changes in the microbiome diversity of the three female European bison (matriarch, Female A and Female B) between samples Pre-release (blue) and POST-release (red). Plots show the average diversity score for four diversity metrics, Shannon, Simpson, Chao1 and observed taxa (richness). To compare the statistical significance of the changes, Kruskal-Wallis H-test + Dunn's test (Bonferroni p-adjust method) or ANOVA + Tukey-HSD test were used. Kruskal Wallis/ANOVA scores of > 0.05 indicate no significant differences between the groups.

evenness), Gini-Simpson diversity (weighted toward dominant taxa, referred to as Simpson), Chao1 (an estimator of total richness including rare taxa), and observed taxon count (actual richness). Two of the three females exhibited higher Shannon diversity in their post-release samples than in their pre-release samples, and the total number of observed taxa/Chao1 score was increased in all three animals (Fig. 3A, B, C). Gini-Simpson scores showed a slight decrease after release in the matriarch and female A but increased in female B. These patterns suggest a trend toward a more diverse microbiome after release (with more overall taxa but little reduction in the dominance of a handful of taxa). Notably, none of the differences were statistically significant. An ANOVA/Kruskal-Wallis test confirmed that the changes in diversity metrics between the pre- and post-release groups did not reach significance for any measure ($P > 0.05$).

Examining diversity dynamics over time (rather than grouping samples by pre/post) revealed considerable temporal fluctuations in each animal's microbiome diversity (Supplementary Fig. 3). Female B's diversity metrics remained relatively stable throughout most of the study (with only a transient drop at two mid-point samples). In contrast, Female A and the matriarch showed pronounced oscillations in all four-diversity metrics). By the final sample collection, each of the three females had diversity scores that were comparable to or exceeded their baseline values (with the sole exception that the matriarch's Simpson and Shannon index remained lower than its starting value). The observed patterns suggests that release was followed by a short-term disturbance in the gut microbiome (reflected by early diversity dips), after which the microbiome diversity recovered and even increased in diversity in most cases/metrics.

The bull and calf showed distinct trajectories of diversity. In the bull, alpha-diversity values varied over time but tended to increase toward later sampling points (Supplementary Fig. 4), indicating considerable disruption of gut microbiome diversity as the animal acclimatised to the wild habitat. The calf's microbiome diversity increased steadily across all metrics from the time of birth onward (Supplementary Fig. 4). As the young European bison matured in the wild environment, its gut microbiome became progressively more diverse, approaching the diversity levels observed in the adults.

To evaluate differences in overall community composition (beta-diversity), principal coordinate/component analysis (PCoA/PCA) was performed. Bray–Curtis dissimilarity (Fig. 4A) and binary Jaccard (Fig. 4B) matrices were utilised. Bray-Curtis PCoA comparisons of all sample groups (pre-release, post-release (of the three females) and of the bull and calf) revealed a clear temporal pattern. Samples collected later in the timeline clustered separately from earlier samples along the primary axis of variation (MDS1). Jaccard dissimilarity was also used to compare changes in taxon presence (rather than abundance), with the Jaccard matrix PCoA revealing a near-identical trend (Fig. 4B). For example, the bull's final samples (B10–B13) were positioned right of the ordination plot relative to the bull's initial samples, and a similar rightward shift over time was evident for the three females. While the calf samples showed a trend towards the latter samples of the other animals. The final clustering position of the calf samples (C10-13) was identical to that of the latter samples from the other animals. PERMANOVA testing of both Bray-Curtis and Jaccard matrices confirmed that there were highly significant overall differences among the four groups (pre-release, post-release, bull, and calf; $P = 0.001$). Indicating that the microbiome composition was significantly influenced by release status. Principle component analysis (PCA)(figure 4C), plotted using Hellinger-transformed data, showed the same trend with latter and earlier timepoints grouping separately. Loading scores, show the strongest 5 loading taxa, with increased *Lachnospiraceae* (unclassified) abundance correlating with the latter timepoints and *Oscillospiraceae* (unclassified), *Oscillospirales* (unclassified), *Bacteroidaceae* (unclassified) and *CAG-74* (unclassified) all correlated with the earlier timepoint cluster.

Pairwise Adonis tests on both Bray-Curtis and Jaccard matrices (Supplementary Table 1) further underscored these patterns. There was no significant difference between the post-release female group and the bull samples (Adonis P-value Bray-Curtis: 0.345, Jaccard: 0.185). Importantly, the pre-release vs. post-release comparison was significant (Adonis P-value Bray-Curtis: 0.007, Jaccard: 0.001). Moreover, all comparisons involving the calf versus an adult group were significant (the calf's microbiome differed from the pre-release, post-release, and bull groups with $P = 0.001/0.001$, $0.001/0.001$, and $0.01/0.011$, respectively). These results indicate that the major drivers of microbiome variation in this study were the release status (captive vs. wild) and the juvenility of the animal, with the calf's gut community being significantly distinct from that of the mature European bison.

To pinpoint the specific microbial taxa underlying the observed compositional shifts, linear discriminant analysis was performed on the pre- and post-release samples. The MaAsLin3 tool was applied to perform a stringent, multivariable analysis of taxon differences. Earlier CAP analysis confirmed release status as the primary and only variable used in MaAsLin3 analysis. Controlling for individual identity (changes exclusive to individuals) and adjusting for multiple comparisons, MaAsLin3 identified 51 unique taxa, whose abundance or prevalence (either decrease or increase) was significantly associated with release status (Fig. 5). Of the identified

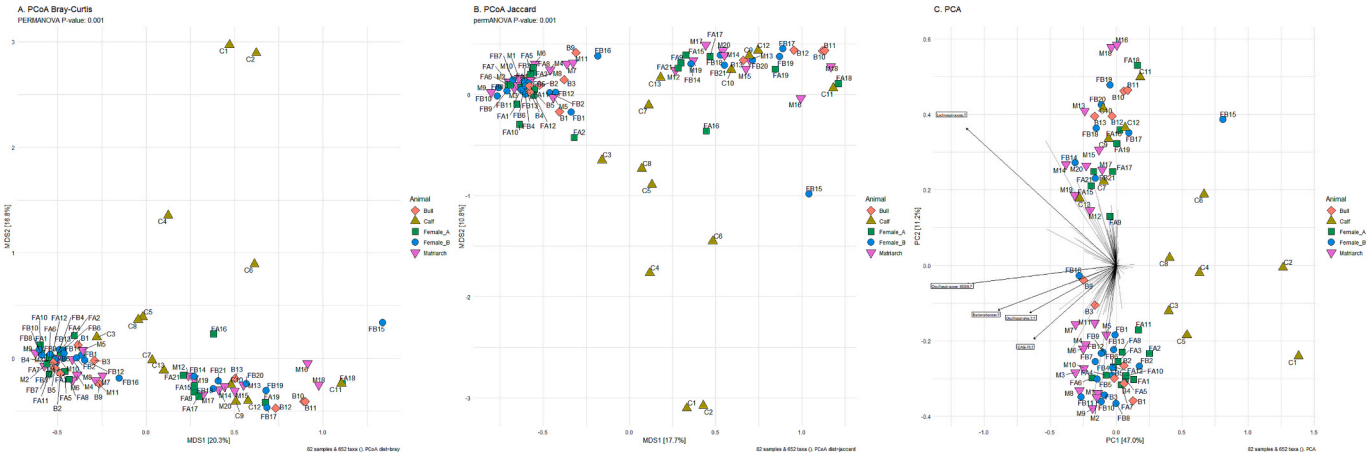

Fig. 4. Principal Coordinate analysis (PCoA) plots. Plots show the positions of the Bray-Curtis dissimilarity matrix (Euclidean distances) for each sample. The PRE (blue) and POST (red) release groups are indicated by colour, and animals are indicated by shape. Fig. 4A shows the positions of all animals (including Bull and Calf, indicated by respective colours), Fig. 4B shows the positions of samples from the female European bison (Matriarch, Female A and Female B). Statistical comparison of changes between the positions of samples group centrons was conducted using PERMANOVA. The PERMANOVA P-value of < 0.05 for both PCA plots indicates significant difference between the position of the PRE/POST centrons (as well as centrons for the BULL and CALF groups).

taxa, 24 had significant association between increasing abundance and 27 with decreasing abundance and the post-release sample status. The 24 taxa whose abundance was significantly positively associated with the post release status were; *Corynebacterium*, *Victivallaceae* (unclassified), *Synergistaceae* (unclassified), *Carnobacteriaceae* (unclassified), *Clostridioides_A*, *Dermatophilaceae_390796* (unclassified), *X46067*, *Sodaliphilus*, *TANB77* (unclassified), *Planococcaceae* (unclassified), *RUG762*, *RUG12438*, *Borkfalkia*, *Anaerovoracaceae* (unclassified), *Anaerotignaceae* (unclassified), *Phocaeicola_A_858004*, *Pseudomonadales_660879* (unclassified), *UBA2658*, *Bacillales_B_302584* (unclassified), *Fibrobacterales* (unclassified), *Limivicinus*, *Ruminococcus_E*, *Peribacillus_301373*, *CAG-274* (unclassified). The taxa whose increased abundance was negatively associated with the post-release status were; *CAG-239* (unclassified), *Janthinobacterium_571526*, *Burkholderiaceae_A_574934* (unclassified), *RUG115*, *Limosilactobacillus*, *QAKW01*, *RUG13615*, *UBA1547* (unclassified), *Lenti.01* (unclassified), *Pararheinheimera*, *Oscillospirales* (unclassified), *CAG-382* (unclassified), *Ligilactobacillus*, *Stenotrophomonas_A_615274*, *Anaerotruncus*, *Xylanivirgaceae* (unclassified), *Eubacterium_G*, *RUG410*, *CAG-74* (unclassified), *Sellimonas*, *CAG-508* (unclassified), *Soleaferrea*, *Anaerobutyricum*, *Anaerotignum_189125*, *Erysipelotrichaceae* (unclassified), *Nanosyncoccaceae* (unclassified), *UBA1381* (unclassified).

Because gut health and conservation outcomes are also influenced by pathogens, we screened the European bison faecal samples for common gastrointestinal parasites to determine if release affected their infection status. In particular, we tested for Cryptosporidium (a protozoan parasite) and Enterocytozoon bieneusi (a microsporidian parasite), which are known to infect cattle and wildlife, as well as for the protist Blastocystis (often considered a commensal or opportunistic parasite in animal guts), Blastocystis was screened for via qPCR. According to PCR and sequencing results (Supplementary Table 2), Cryptosporidium was detected in a total of 12 samples across the study and E. bieneusi in 13 samples. Three distinct Cryptosporidium species were identified among the positives: predominantly C. struthonis (found in 9 of the 12 positive cases), with C. parvum and C. bovis present in a few instances; one additional *Cryptosporidium*-positive sample could not be resolved to species. All the microsporidia-positive samples were confirmed as E. bieneusi, with a remarkable diversity of genotypes: eight different genotypes were observed (LW1, IV, K, C, KIN, GX, JLD-2, and JX41), the most frequent being genotype IV and genotype K (each detected in three samples).

Infection by E. bieneusi appeared in all animals at some point, indicating that every European bison in the herd (including the bull and the calf) tested positive for this parasite at least once during the monitoring period (Supplementary Table 2). The bull, in particular, had consecutive positive tests for E. bieneusi over four sampling points (B11–B14), although the infecting genotype varied over time. Cryptosporidium infections, in contrast, were observed only in the three adult females: each of the females had at least one positive Cryptosporidium result, and the matriarch was the most frequently infected individual. Notably, neither the bull nor the calf ever yielded a Cryptosporidium-positive sample during the study (Supplementary Table 2).

Comparing the pre- and post-release periods for the adult females revealed a marked change in parasite occurrence after the European bison were released (Fig. 6). During the pre-release phase, 36% of the samples from the female European bison were positive for Cryptosporidium, whereas this proportion dropped to 13% in the post-release period (Fig. 6A-B). Moreover, while all Cryptosporidium infections detected before release were due to C. struthonis, the few cases that occurred after release included additional species (C. bovis and C. parvum). The pattern for E. bieneusi was the opposite: none of the pre-release samples contained this microsporidian, yet approximately 10% of post-release samples from the females were positive for E. bieneusi (Fig. 6C-D), encompassing five distinct genotypes. *Blastocystis* detection was observed to be negatively correlated with release status, 100% of samples pre-release were positive for *Blastocystis*, this number fell to 64% post-release (Fig. 6E-F). Thus, release coincided with a reduced incidence of Cryptosporidium infection in the herd but also with the first appearance of E. bieneusi infections, as well as a reduction in *Blastocystis*. Highlighting how the transition to a wild environment can alter the spectrum of gut microorganisms—including both beneficial microbes and pathogenic parasites—in released animals.

Lastly, having identified the presence of various eukaryotic organisms and taxa correlated with the post-release status, a spearman's rank correlation test was performed to investigate if the presence of the various parasites and eukaryotic organisms (*Blastocystis*) correlated with any of the taxa that significantly increased or decreased in abundance post release. Shown in Fig. 7, the significant Benjamini-Hochberg adjusted P-values of the Spearman's rank correlations are shown as "+", a total of 34 significant comparisons were present (Supplementary Table 3), the vast majority of which were with *Blastocystis*. A single taxa *Soleaferrea*, was significantly correlated with *Cryptosporidium struthionis*., *Janthinobacterium*, *CAG-382* (unclassified), *RUG410*, *Soleaferrea*, *Eubacterium*, *Burkholderiaceae*(unclassified), *Nanosyncoccaceae*(unclassified), *Ligilactobacillus*, *Anaerotruncus*, *Erysipelotrichaceae*(unclassified), *Stenotrophomonas*, *Peribacillus*, *Soleaferrea*, *CAG.239*(unclassified), *QAKW01*, *CAG.74*(unclassified), *Anaerotignum*, *Xylanivirgaceae*(unclassified), and *Oscillospirales*(unclassified), were all significantly positively associated with *Blastocystis* presence. *Fibrobacterales*(unclassified), *Bacillales* (unclassified), *RUG12438*, *Sodaliphilus*, *Corynebacterium*, *Carnobacteriaceae* (unclassified), *Dermatophilaceae*, (unclassified), *Borkfalkia*, *Clostridioides*, *Anaerotignaceae* (unclassified), *Ruminococcus*, *Pseudomonadales* (unclassified), *UBA2658* and *Synergistaceae* (unclassified) were all significantly negatively associated with *Blastocystis*.

4. Discussion

The release of European bison into the Wilder Blean area represents a significant milestone in conservation efforts, offering a unique opportunity to study the microbiome dynamics associated with rewilding projects. This study has provided critical insights into how the microbiome of released species adapts and stabilises in a new ecological setting, emphasising the potential of microbiome research to significantly support conservation biology. Previous research on the effects of environmental changes on animal microbiomes is sparse but work on the skin microbiomes of amphibians (of crucial importance to amphibian health) has been conducted. Rewilded captive populations of frogs were observed to develop improved antifungal capabilities in their skin microbiomes, and to exhibit a 'restoration' of the microbiome observed in wild populations (Kueneman et al., 2022). However, thus far, there has been no work on how large herbivores such as European bison are affected by a release program like the one performed at Wildwood.

Our findings reveal substantial shifts in gut microbiome composition and diversity, highlighting the European bison's gut microbiota's adaptability to new environmental conditions. It must be noted that 'increases' and 'decreases' in taxa do not correlate directly to changes in the absolute count of said taxa within the animal. This is due to the compositional nature of amplicon data (Gloor et al., 2017). However, from these microbiome 'snapshots' changes in composition can still be observed.

Despite the lack of studies specifically focusing on 'rewilded' species, there is a considerable amount of microbiome studies comparing the effects of diet (grazing vs fodder/feed) and seasonality (two aspects likely to affect the European bison during the Wildwood release) on the closest relatives of the European bison, the American bison, the Yak (*Bos grunniens*), Muskox and the domestic cow (*Bos taurus*) (Wang et al., 2018; Grange et al., 2018). It has been shown that the microbiome of the American bison shifts subtly with the seasons and is dominated by *Bacteroidetes*, *Cyanobacteria*, *Euryarcheota*, *Firmicutes*, *Proteobacteria*,

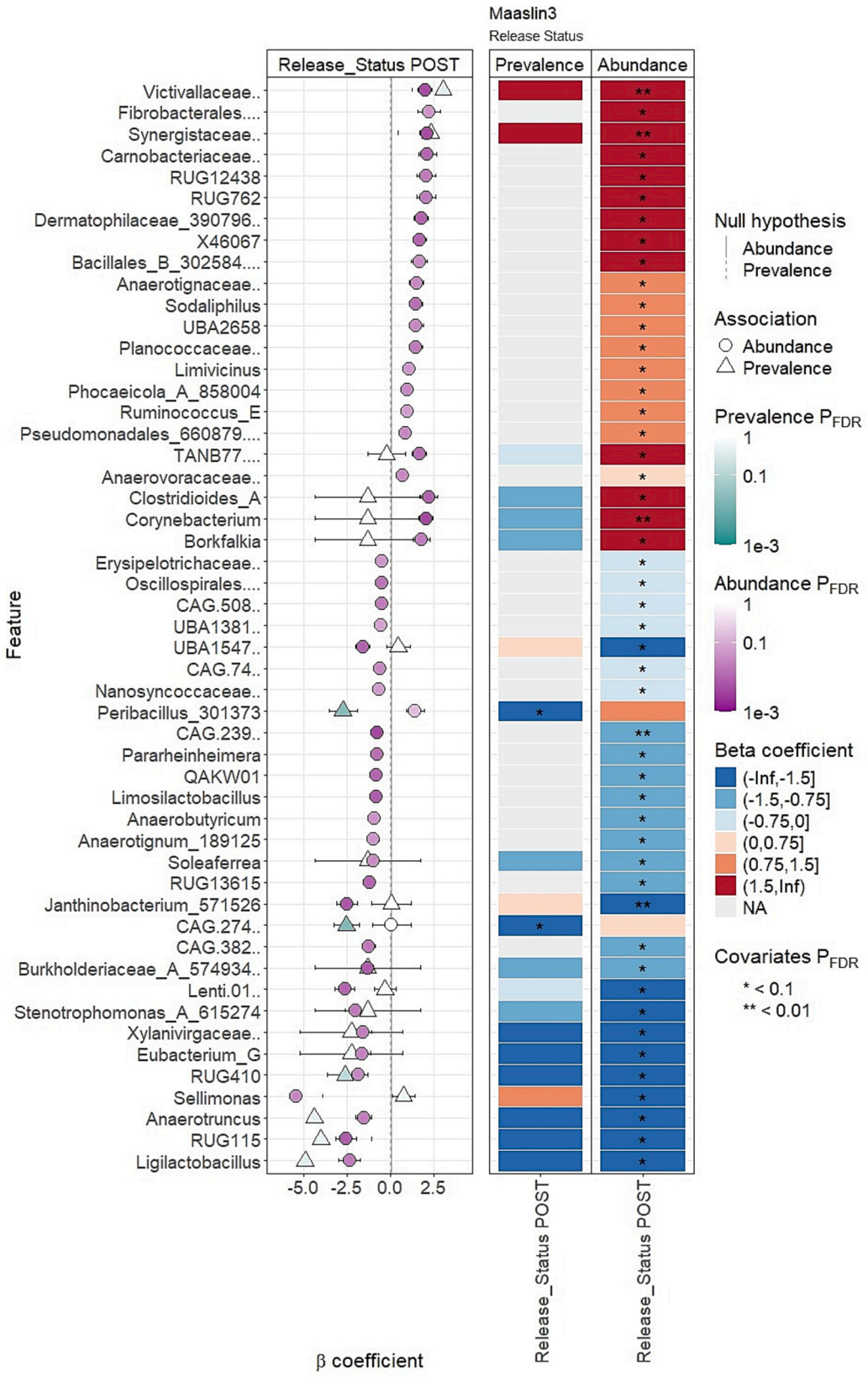

Fig. 5. Linear discriminant analysis conducted on the 16S sequencing data to determine discriminant 'biomarker' taxa for the different statuses of release. Due to the lack of release status for the Bull and Calf the analysis is conducted exclusively on the female European bison (female A, B and Matriarch). A). LEfSe analysis, taxa shown are considered significantly discriminant taxa for either samples taken PRE-release (blue) or POST-release (red), taxa with an LDA score >/<2 are considered significant. B). MaAslin3 analysis on release status (PRE-release used as reference for analysis). Taxa shown all had significantly decreased or increased abundance (circles)/prevalence(triangles) in samples taken from the European bison POST-release.

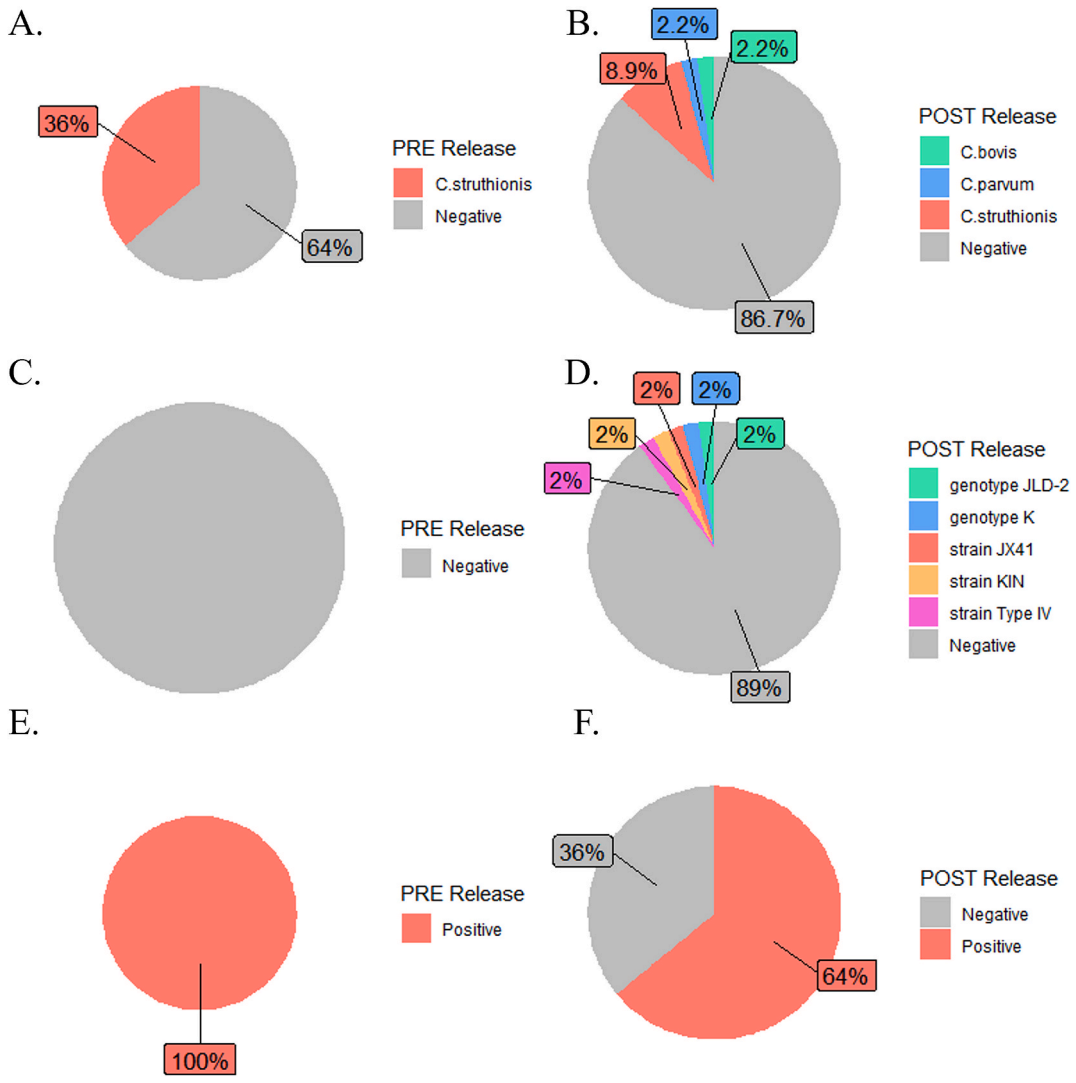

Fig. 6. Eukaryotic presence in the European bison samples from female A-B and Matriarch, determined by PCR, is shown as a percentage of the total samples. A-B, *Cryptosporidium* presence in samples pre-release (A.) and post-release (B.). C-D, *Enterocytozoon* presence in samples pre-release (C.) and post-release (D.). E-F, *Blastocystis* presence in samples pre-release (E.) and post-release (F.).

Tenericutes and *Verrucomicrobiota* (Bergmann et al., 2015). This aligns closely with our findings (supplementary figure 2), with all of these phyla being observed in the European bison (alongside *Actinobacteriota*, *Spirochaetota* and *Patescibacteria*) and observing same subtle changes in these phyla over the time course of monitoring. A similar pattern of a drop in Firmicutes prior to the winter months was observed in the phylum level composition data.

Furthermore, changes in the microbiome of yaks and American bison due to diet has also been well documented, comparing diets aligning with captive animals (grain/fodder etc.) and diets aligning with 'wild' animals (forage/browsing). Wild yaks show increased abundance of the genus *Ruminococcus* and the Phylum *Firmicutes* (Shah et al., 2024), and American bison fed a finishing diet of grain as opposed to forage had higher abundances of the class *Gammaproteobacteria* and again the Phylum *Firmicutes* (Bergmann, 2017). This would suggest that a diet more closely matching that of wild European bison (increased forage) would increase/decrease these taxa in the European bison. This trend is somewhat observed in our data, with a similar drop in *Gammaproteobacteria* over the time course post-release. Of the 51 taxa identified (by Maaslin3) as significantly discriminant taxa, none of the post-release status associated taxa belonged to *Gammaproteobacteria*, while four taxa from *Gammaproteobacteria* (*Pararheinheimera, Burkholderiaceae, Janthinobacterium, Stenotrophomonas* and) were all negatively associated with the post-release status.

There is overlap in the literature between taxa observed to be significantly positively associated with the post-release status (by Maaslin3), and taxa corelated with American bison, yaks and cattle (alongside other ruminants) that are allowed to graze/wild compared with concentrate/fodder fed 'captive' counterparts. *Planococcaceae* was more abundant in wood bison (*bison bison athabascae*) as opposed to plains bison (*bison bison bison*)(Grant et al., 2025), mimicking the environment of the post-release European bison more closely. Moreover, *Ruminococcus* and its family *Ruminococcacae* were found to be a core part of the American bison microbiome, and in higher abundance in the faeces of pasture fed vs grain fed animals (however, the opposite trend was observed in rumen samples). This pattern was also observed in wild grazing yaks vs those fed in captive conditions (Fresno Rueda et al., 2023; Shah et al., 2024). Incorporating data on non-bovid ruminants, *Phocaeicola* was significantly lower in captive vs wild reindeer (Zhao et al., 2024). *Pseudomonadales* was also found to be more common in grazing sheep vs those fed in indoor feedlots (Cui et al., 2023).

Alongside taxa positively associated with post-release and 'wild' and 'wild' adjacent diet ruminants, multiple taxa negatively associated with the post-release status are known to be positively correlated with fodder

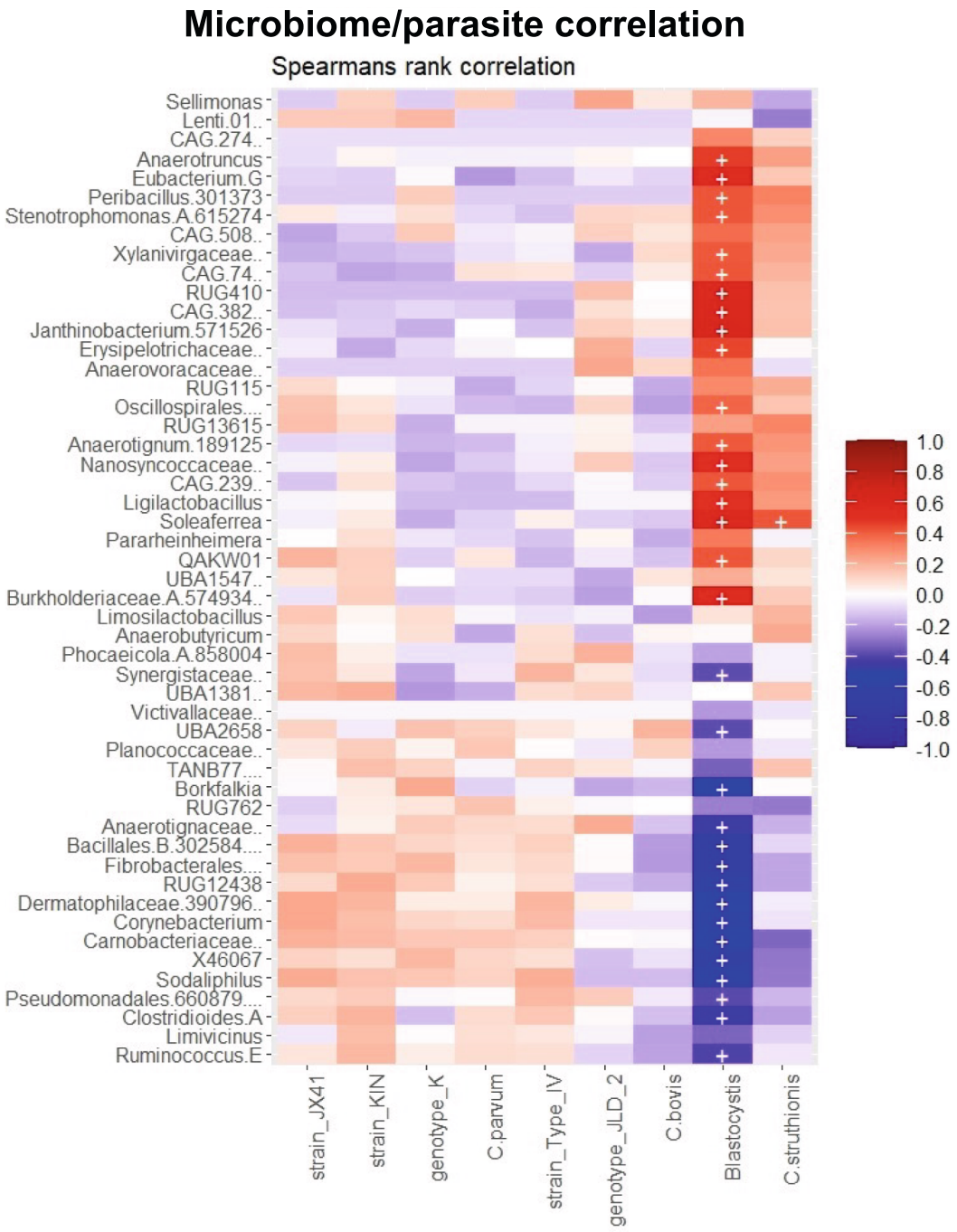

Fig. 7. Spearman rank correlation analysis between Linearly discriminant taxa (Maaslin3) (microbiota) and protozoal abundances. The heatmap shown shows correlations, positive (red) and negative (blue). Correlations with a + had significant Benjamini-Hochberg adjusted p-values (<0.05).

feeding/captive ruminants. *Erysipelotrichaceae* and *Oscillospirales* were both correlated with housed vs free range yaks (*Oscillospirales* being significantly increased and *Erysipelotrichaceae* making up the top 10 most abundant taxa in the housed yaks vs the free-range grazing yaks). *Eubacterium* was also found to be dominant in housed vs free range yaks (H. Wang et al., 2025). This literature supports that the changes in diet and environment (woodland browsing) observed in the European bison released into wildwood align with microbiome changes associated with ruminants in a 'wild' environment, arguably returning to a more 'wild'/ free range state.

Alongside the taxa correlated with wild/free range ruminants, there was also an increase in taxonomic groups known to contain pathogens, positively associated with the post-release status. The family *Dermatophilaceae* (unclassified), genera *Clostridoides* and *Corynebacterium* all contain within their genus known ruminant pathogens (Hurst, 2018; Ribeiro et al., 2022; Domenis et al., 2018; Limmathurotsakul et al., 2012). However, this does not mean the taxa associated with the post release status are pathogenic, for example, *Corynebacterium* and *Burkholderiaceae* are also known ruminant skin and gut commensals (Speck et al., 2006; C. J. Anderson et al., 2021). This may potentially suggest

that the release to the woodland may expose the European bison to more pathogenic microbes/encourage their increased abundance. On the other hand, despite the positive association of known pathogens with the post-release status, both the genera *Stenotrophomonas* and *Janthinobacterium* contain known pathogens of ruminants (Hurst, 2018). Also, *CAG-74* was associated with lower weight and residual feed intake in yaks (H. Wang et al., 2024). The reduction in these taxa post-release may suggest some pathogenic microbe containing taxa decrease in abundance in the post-release status, along with microbes known to negatively affect closely related ungulates. Moreover, the role of parasites and their impact on the health of released populations cannot be underestimated. The presence of parasites such as Cryptosporidium and Enterocytozoon bieneusi highlights the complex challenges associated with translocation and release programs. The fluctuation in parasitic load, with a noted decrease in Cryptosporidium and Blastocystis prevalence post-release and the emergence of E. bieneusi only after the European bison were released into the wild, underscores the dynamic nature of health risks faced by released animals.

The significant correlations between *Blastocystis* (and *C. struthionis*) and various taxa identified by Maaslin3 as significantly associated with the post-release status, indicates the role these protozoa play in affecting the microbiome of European bison during their release. Previous studies have confirmed that European bison possess a medium-level (when compared with American bison and other ruminants) protozoal population (Kišidayová et al., 2021). Alongside protozoa such as ciliates, *Blastocystis* subtypes (ST) ST1, ST3, ST5, ST7 have all been identified in bison at low levels (5.6%) (Kaczmarek et al., 2021). These European bison live in a 'wild' environment (the Białowieża Primeval Forest), therefore, the reduction in *Blastocystis* seen in the females' post-release (from 100% to 64%), may indicate a return to *Blastocystis's* 'wild' levels in European bison. Further supporting this idea many of the strongest positive correlations between taxa and *Blastocystis* (*Janthinobacterium*, *Clostridum* and *CAG-382*) were all significantly negatively associated with the post-release status. Possibly indicating a shift in both protozoan and bacterial populations to a more 'wild' states as seen in the Białowieża Primeval Forest European bison population.

These findings necessitate the development of robust health-monitoring frameworks to detect and mitigate the impact of such pathogens. Understanding the relationship between released animals and their risk of parasite exposure is paramount for future rewilding projects to prevent failures, such as the failed reintroduction of Caribou in the United States due to meningeal worm exposure (R. C. Anderson, 1972), and to prevent the spread of zoonotic diseases like *Microsporidia*. Understanding the interactions between parasites and host microbiome (such as identifying correlations between specific taxa and the presence of pathogen protozoa) could also lead to breakthroughs in managing disease outbreaks in wild populations, with microbiome changes already being linked to parasite infections in humans and mice (Zhou et al., 2023; Hu et al., 2020).

This study sets the stage for future research aimed at unravelling the complex interactions between translocated or released animals, their microbiomes, and ecosystem health. Further investigations could explore the longitudinal stability of the microbiome changes observed, assess the impact of these changes on the reproductive and survival rates of released populations, and extend these studies to other species and ecosystems to generalise the findings. Additionally, exploring the functional roles of the microbiome in nutrient cycling, disease resistance, and stress responses could provide deeper insights into the ecological impacts of large herbivore release.

Ultimately, integrating microbiome research into conservation strategies presents a promising avenue for enhancing the success of wildlife reintroductions/releases. By continuously monitoring microbiome dynamics and adapting management practices accordingly, conservationists can better support the health and ecological integration of reintroduced species, leading to more resilient and sustainable ecosystems. The insights gained from this study not only contribute to our understanding of ecological restoration but also highlight the potential for microbiome studies to revolutionize conservation practices, paving the way for a new era in ecological management and conservation biology.

4.1. Limitations

This study comes with its own limitations. First, inference is constrained by the small number of individuals tracked (three adult females before and after release, and one male and one calf post-release) and by the observational design without a matched control herd; effect sizes should therefore be interpreted cautiously. Second, seasonality and management interventions coincided with the release (soft-release and periods of supplementary feeding), making it difficult to fully disentangle diet and seasonal effects from rewilding per se. furthermore, the necessity of supplementary feeding may blur the lines of clean 'release' into a wild environment. Third, 16S rRNA amplicon profiling yields relative (compositional) data with limited taxonomic resolution; it cannot discriminate strains or directly infer function, and absolute abundances were not measured. Fourth, parasite detection relied on faecal DNA screening and is subject to intermittent shedding and imperfect sensitivity; we did not quantify oocyst/spore load or viability, so prevalence estimates may be conservative. Fifth, we lacked concurrent environmental sampling (e.g., water, pasture, soil) and physiological covariates (e.g., body condition, stress biomarkers), which would help attribute exposure pathways and health relevance, the time series spans 17 months at a single site; multi-site, multi-year replication and shotgun metagenomics/metabolomics would strengthen generality and mechanistic interpretation. In addition, we did not undertake predictive functional profiling from 16S data; while useful in some systems, it remains an indirect proxy and would not be robust here without validation against metagenomics or metabolomics. Finally, ratio-based summaries can be sensitive to compositional effects; we therefore focus on multivariate community shifts and higher-level compositional patterns rather than treating single ratios as diagnostic.

5. Conclusions

This pioneering study on the European bison released into the Wilder Blean area has provided groundbreaking insights into the role of the microbiome in the success of wildlife conservation efforts. The significant shifts in microbiome composition and diversity observed highlight the adaptability of the European bison's gut microbiome to new environmental conditions and align with previous research on captive vs wild populations of closely related ungulates, underscoring the potential for microbiome studies to inform and enhance conservation practices.

Our findings suggest that the microbiome could serve as a valuable indicator of the health and adaptability of translocated and reintroduced species. The identification of specific microbiome biomarkers post-release offers a promising tool for monitoring and managing released populations effectively. These biomarkers could help conservationists track the success of rewilding efforts and make informed decisions to ensure the health and sustainability of these populations. Furthermore, the observed impact of parasitic infections highlights the need for comprehensive health monitoring frameworks that integrate microbiome analysis to manage disease risks in reintroduced populations effectively. The dynamic nature of these parasitic threats highlights the ongoing challenges faced by conservation programs and the necessity for continuous research and adaptation of management strategies. Looking ahead, there is a clear need for longitudinal studies to track the long-term stability and functional impacts of microbiome changes in animal populations subjected to translocations and releases into new

habitats. Such studies could provide deeper insights into the ecological roles of microbiomes in supporting the health, reproduction, and survival of released species. Additionally, expanding this research to include other species and ecosystems would help to generalise these findings and refine rewilding practices across different conservation contexts.

In conclusion, integrating microbiome research into wildlife conservation represents a novel and promising approach to ensuring the success of translocation and release programs. By continuing to explore the complex interactions between released animals, their microbiomes, and their new environments, we can enhance our ability to restore and sustain biodiversity in natural habitats. The pioneering work presented in this study not only contributes valuable knowledge to the field of conservation biology but also sets a precedent for the role of advanced biological research in ecological restoration efforts worldwide.

6. Ethics and permits

All procedures complied with relevant institutional and national guidelines. Insert permit/license numbers (e.g., UK Home Office/ DEFRA/Local Wildlife Authority) and approval body details here.

CRediT authorship contribution statement

William JS Edwards: Writing – original draft, Methodology, Investigation, Formal analysis. **Yaseen Majid Salman Al-Adilee:** Writing – review & editing, Methodology, Investigation, Data curation. **Constance Denoyelle:** Writing – review & editing, Methodology, Investigation, Data curation. **Hannah Mackins:** Writing – review & editing, Resources, Investigation. **Richard A. Griffiths:** Writing – review & editing, Validation, Supervision, Project administration. **Anastasios D. Tsaousis:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jnc.2026.127304. Supplementary figures and tables containing higher taxonomic level and temporal representations of alpha diversity data, as well as supplementary tables containing p-value scores for beta diversity and Spearmans rank analysis.

Data availability

Data will be made available on request.

Sequence reads will be deposited in the European Nucleotide Archive / NCBI SRA; accession(s) will be provided upon acceptance. Derived data can be accessed from the SRA accession number: PRJNA1353861.

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