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Spatiotemporal and Host Variations in Faecal Microbiomes of Hairy-Nosed Wombats (*Lasiorhinus* spp.)

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ABSTRACT

The gut microbiome can play a critical role in humans and wildlife, assisting with digestion, immunity and detoxification. This community is often adapted to the animal's ecosystem and fluctuates with seasonal shifts in diet. The gut microbiome can also be altered by anthropogenic effects such as invasive species creating increasingly homogenous landscapes. This in turn could reduce the overall diversity of the microbiome, which has been hypothesized to hinder adaptive potential. However, despite its probable importance, many species are still data deficient with regards to their specific gut microbiome. One data deficient species is the critically endangered northern hairy-nosed wombat (*Lasiorhinus krefftii*). Northern hairy-nosed wombats have undergone and slowly recovered from an extreme bottleneck over the last century, and the remaining populations live in increasingly homogeneous habitats dominated by invasive buffel grass (*Cenchrus ciliaris*). To fill in this gap we characterised the faecal microbiome of the northern-hairy-nosed wombat using 16S rRNA gene amplicon sequencing. To accomplish this, we collected fresh faecal samples from two populations across two seasons to generate a baseline profile of the microbiome as well as examine the effects of seasonality and geography. Additionally, we compared the faecal microbiome of the northern hairy-nosed wombat to the closest related species, the southern-hairy-nosed wombat (*Lasiorhinus latifrons*). From our results we determined that the microbiome of the northern hairy-nosed wombat changes from winter to spring and corresponding taxonomic shifts may be assisting the wombats with digestion during the dry months. Between the two wombat species we identified core taxa that are consistent with hindgut fermenting herbivores; however, differentially abundant taxa between the two species may indicate adaptations to the specific ecosystems they each inhabit.

1 | Introduction

The diverse assemblage of microorganisms that live throughout the digestive tract of many mammals is known as the gut microbiome. This community of microbes has been the focus of intense research in humans and animals and can contribute to pathogen defence (Spragge et al. 2023), improved nutrient extraction (Walter and Ley 2011), and training the immune system (Hooper et al. 2012; Schluter et al. 2020). However, significant changes to this community can lead to dysbiosis which has been suggested to contribute to illness in humans as well as translocated and captive animals (Schippa and Conte 2014; Williams et al. 2016; Westreich et al. 2019). Furthermore, recent reviews highlight the importance of the gut microbiome in wildlife conservation in relation to species reintroduction success, changes to microbiome function in captive animals, and the ability of species to adapt to environmental change (Trevelline et al. 2019; Dodd and Grueber 2021).

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The gut microbiome can be adapted to the specific diet and ecosystem that an animal inhabits (Brice et al. 2019; Rojas et al. 2021; Li et al. 2022). In herbivores, these microbial communities have evolved to help digest the varying types of plant material they encounter, from tree bark to ripe fruits (Price et al. 2012; Groussin et al. 2017; Rojas et al. 2021). This microbial workforce breaks down cellulose and other fibres to release nutrients otherwise unavailable to the host, and can neutralise potentially toxic compounds (Kohl et al. 2014, 2018; Shiffman et al. 2017; McKenney et al. 2018). The specificity and co-evolution of these microbial communities to their hosts, diets and ecosystem have allowed some herbivores to consume highly specific and potentially toxic diets. Two such cases are koalas (*Phascolarctos cinereus*) and desert woodrats (*Neotoma albigua*) (Kohl et al. 2018; Brice et al. 2019). While these assemblages of microorganisms might serve highly specialised roles, the communities are far from static.

Shifts in gut microbial communities can be caused by changes in diet, season, habitat, natural disaster, or captivity (Rojas et al. 2021; Perry et al. 2023; Ma et al. 2024). Gomez et al. (2016) found shifts in microbial communities associated with season based on availability of ripened fruits in gorilla species (*G. g. gorilla and G. b. beringei*). These changes coincided with the need to digest more fibrous unripe fruit during the winter months of November and December. At an even finer scale Brice et al. 2019, found that the gut microbiome of the koala changes based on which species of eucalypt they were foraging on. However, while these communities are malleable, they may not be capable of drastic shifts particularly when an herbivore has a more specialised diet. Eisenhofer et al. (2023) found that the microbiome of the koala remained stable over days to months which may impact their ability to adapt to large scale changes in their environment. This fact is concerning at a time when ecosystems are being rapidly altered, and human encroachment is increasing.

Human induced habitat change has been shown to affect the gut microbiomes of multiple wildlife species by altering the diets, reducing diversity, and introducing human or livestock associated microbes (Amato et al. 2013; Fackelmann et al. 2021; McManus et al. 2021; Moustafa et al. 2021; Heni et al. 2023). In the gut microbiome of Tome's spiny rats (*Proechimys semispinosus*) and black howler monkeys (*Aloutta pigra*), Fackelmann et al. 2021 and Amato et al. (2013) identified reduced alpha diversity and greater variation in beta diversity in animals living in highly disturbed and fragmented habitat. McManus et al. (2021) identified human and bovine associated microbes in the gut microbiomes of black-and-white ruffed lemurs (*Varecia variegata editorum*) suggesting a potential risk for zoonotic transfer. While the changes in microbial community suggest a degree of adaptive plasticity, the loss of microbial diversity and the limited ability to further change are significant points of concern. However, to understand if changes are deleterious, we first need to characterise the community and its natural fluctuations.

The northern hairy-nosed wombat (*Lasiorhinus krefftii*, NHNW) is a large (~32 kg), critically endangered fossorial marsupial that builds burrows in sandy soil of old river beds, and has a diverse diet that consists of native and introduced grasses (Johnson and Crossman 1991; Martin and Carver 2021; Casey et al. 2023). The historical range and habitat of the NHNW extended from central Queensland down to southern NSW (Martin and Carver 2021). However, the species suffered population extirpations in the late 19th and early 20th century resulting in a single surviving population at Epping Forest (central Queensland) by 1937 (Taylor et al. 1994; Horsup et al. 2021; Martin and Carver 2021) (Figure 1). By the early 1980s the species was reduced to ~30 individuals located at Epping Forest National Park (EFNP). Subsequent intensive management of the EFNP population has seen it recover to approximately 400 individuals, and allowed for the creation of two translocated populations at Richard Underwood Nature Refuge (RUNR, $n = 18$) and Duny Yumba National Park (formerly Powrunna State Forest) ($n = 37$, Department of Environment and Science 2025; Nothern hairy-nosed wombat recovery action plan 2022).

Wombats are hindgut fermenters and therefore rely heavily on the community of intestinal bacteria to extract nutrients from their diet (Barboza and Hume 1992; Sobek and Walker 2021; Camp et al. 2020; Casey et al. 2023, 2024). Recently Casey et al. (2023), characterised the diet of the NHNW wombat using DNA metabarcoding of scats and found it to be diverse with over 191 plant taxa that varied significantly based on site and season. However, most of these taxa were in low abundance and invasive buffel grass (*Cenchrus ciliaris*) was the major component of the diet across all study sites. They also found an increasing reliance on buffel grass since the first dietary studies more than 20 years ago (Woolnough and Foley 2002; Casey et al. 2023). To further assess the impacts of buffel grass Casey et al. (2024), examined the nutrient composition of the diet in northern hairy-nosed wombats and found that nutritional quality varied according to the season and site and that buffel grass may be an important source of food particularly in winter when resources are more scarce. However, the increasing reliance on buffel grass might be cause for concern due to its lower nitrogen values and the need to eat more to achieve the same nutritional levels, particularly as it rapidly outcompetes native grasses.

How habitat affects the faecal microbiome of wombats has been examined in the closest relative of the NHNW, the southern hairy-nosed wombat (*Lasiorhinus latifrons*, SHNW) (Eisenhofer et al. 2021). The SHNW is endemic to South Australia and Western Australia, and lives in semi-arid areas with hard calcrete soils (Figure 1) (Walker et al. 2006; Swinbourne et al. 2021). Eisenhofer et al. (2021) examined the faecal microbiome of the southern hairy-nosed wombat and compared its diversity and composition across different quality of habitats based on the amount of native grasses vs. introduced weeds. They found that the faecal microbiome had reduced diversity in the degraded habitat which could indicate a reduced fitness to future change, further emphasising the need to maintain diverse native grass assemblages. This is particularly relevant to a species like the NHNW that is living in an increasingly homogenous grassland which may be reducing the diversity of its gut microbial community. However, to date, no study has examined the faecal microbial community of the NHNW, or how the faecal microbiomes of different wombat species compare.

To this end, the aims of our study were to (1) characterise the faecal microbiome of the NHNW, (2) examine whether there is seasonal variation, (3) look for geographic variation between reserves and (4) compare the microbiome of the NHNW to SHNW (Eisenhofer et al. 2021). To accomplish this,

we collected fresh northern hairy-nosed wombat scats from Epping Forest National Park and Richard Underwood Nature Refuge across two seasons in 2022 and 2023 and characterised the microbial community with 16S rRNA gene amplicon sequencing.

2 | Materials and Methods

2.1 | Sample Locations and Collection

All samples were collected from two extant populations of northern hairy-nosed wombats at Epping Forest National Park in central Queensland and Richard Underwood Nature Refuge in southern Queensland (Figure 1). Samples were collected opportunistically from across each reserve. To explore seasonality of the wombat microbiome we collected 33 wombat faecal samples at Epping Forest National Park in September of 2022 (spring) and 53 in July 2023 (winter). To assess geographic influences, we collected an additional 30 fresh faecal samples from the translocated population at Richard Underwood Nature Refuge in July 2023 (Appendix S1). Approximately 15 NHNW had been translocated to RUNR in 2009 with a small number of additional translocations in subsequent years. Thus, wombats at RUNR had been resident for up to 15 years prior to sampling and they included a mix of translocated and locally born animals.

To minimise the microbial shift that occurs once an animal defecates, we collected all faecal samples at sunrise stopping by 10 am in spring (2022) and by 11 am in winter (2023). Before collection a faecal pile was examined and determined to be fresh by the shiny surface and tacky texture of one of the pellets. Visible urine stains were also used as an indication of freshness. All faecal samples were collected following the protocols outlined in Eisenhofer et al. 2021, which involved removing the internal part of the faecal pellet to mitigate surface contamination. Once the core was removed, we placed the sample in 1 mL of 95% molecular grade ethanol to preserve the microbial community (Song et al. 2016). All instruments were thoroughly cleaned between samples using 5% bleach followed by 70% ethanol. After collection samples were placed in a −20°C freezer at each reserve and stored on ice for transport back to a dedicated microbiome lab where they were stored at −20°C until DNA extraction.

2.2 | Sample Preparation

2.2.1 | DNA Extraction

All samples were extracted in a dedicated microbiome laboratory physically isolated from post-PCR areas. Within the lab we performed the DNA extractions in a dedicated Perspex hood that was UV decontaminated and wiped down with 3% bleach and 70% ethanol before and after each extraction. We performed a

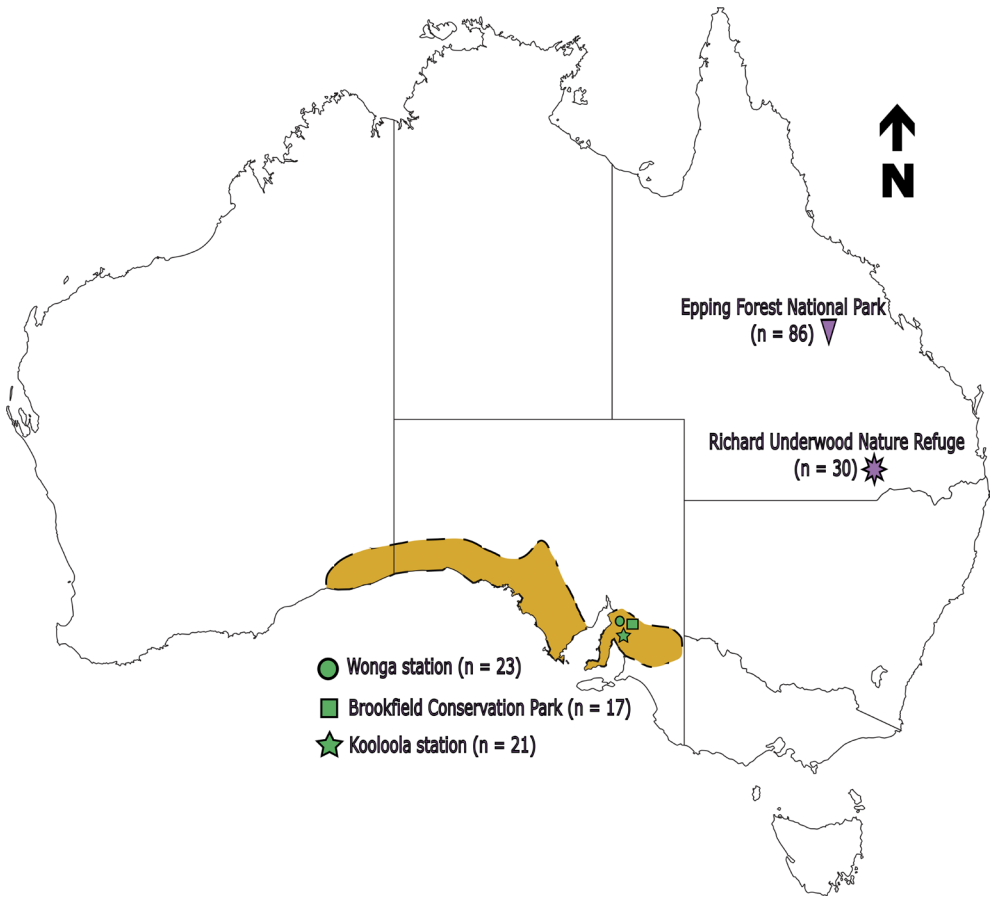


FIGURE 1 | Sampling locations for northern and southern hairy-nosed wombats. The two NHNW wombat populations are shown with a purple triangle (EFNP) and star (RUNR). The contemporary range of the SHNW is shown in brown and the three sampling locations from Eisenhofer et al. 2021 are shown with a green circle (Wonga station), square (Brookfield Conservation Park) and star (Kooloola station).

single DNA extraction on each sample using the Qiagen DNeasy PowerSoil Pro DNA extraction kit (Qiagen, Valencia, California, USA) following the manufacturer's instructions with minor modifications. To ensure proper mechanical lysis, samples were shaken for 10 min at maximum speed on a benchtop vortexer. All faecal samples were eluted twice using the same 100 μL of buffer C6 on the filter membrane. Each extraction included a blank to monitor for contamination. These extraction blanks were carried through library preparation and sequencing.

2.2.2 | Library Preparation and Sequencing

Sample PCRs were prepared in an amplicon free dedicated microbiome lab in a UV decontaminated Perspex hood that was cleaned in the same way as the DNA extraction. We amplified the V4 region of the bacterial 16S rRNA gene using primers 515F (AAT GATACGGCGACCACCGAGATCTACACTATGGTAATTGTG-TGCCAGCMGCCGCGGTAA) and a 12 bp barcoded reverse primer 806R (AGCAGAAGACGGCAT-ACGAGATnnnnnnnnn nnnAGTCAGTCAGCCGGAC-TACHVGGGTW TCTAAT) as in Eisenhofer et al. (2021) for comparability (Caporaso et al. 2011). Single 25 μL PCR reactions were performed for each sample following the recommendations from Marotz et al. (2019). PCR reactions included, 2.5 μL of 10X PCR buffer, 1 μL of 50 mM $MgCl_2$, 0.1 μL of 5 U/μL HiFi Taq polymerase, 0.2 μL 100 mM dNTP mix (25 mM each), 0.5 μL of 10 μM forward and reverse primers, 19.2 μL of dH_2O and 1 μL of gDNA. DNA was amplified with an initial denaturation of 94°C for 3 min followed by 35 cycles of 94°C for 45 s, 50°C for 1 min, 68°C for 90s and a final adenylation of 68°C for 10 min. Amplification was carried out on a Bio-Rad T100 Thermal cycler (Bio-Rad, Hercules, California, USA). To confirm successful amplification all samples were visualised on a 2% agarose gel using 2 μL of PCR product. Following visualisation samples were diluted 1:10 and 1 μL was quantified on a QuBit2 (Thermo Fisher Scientific Waltham MA, USA) fluorometer using the DNA high sensitivity kit. Following initial quantification, we combined the samples into two pools of equimolar concentrations. The pools were determined to be either a 'high' concentration containing samples with a concentration > 2.5 ng/μL or 'low' concentration containing samples with a concentration < 2.5 ng/μL. After pooling, we purified the samples using 1.1× ratio of AxyPrep beads (Axygen, Corning inc. Corning NY, USA) and eluted them in 30 μL of buffer EB (Qiagen, Valencia, California, USA). The pools were re-quantified on an Agilent Tapestation 4150 (Santa Clara, California, USA) to confirm they were free of dimers and small fragments. The 'low' concentration pool was added 'neat' as the dilution factor for final sequencing submission.

A single 5 nM library containing all samples was sequenced at the South Australian Genomics Centre on an Illumina MiSeq V2 2 × 150 bp flow cell producing ~15 million reads.

2.3 | Data Analysis

2.3.1 | Data Processing and Statistical Analysis

All samples were analysed on a High Performance Computing cluster using a conda virtual environment of QIIME2 v 2024.5 (Bolyen et al. 2019).

To examine the similarities and differences of wombat species gut microbiomes we downloaded and re-analysed the 16S amplicon data of southern hairy-nosed wombat faecal microbiome from Eisenhofer et al. (2021). Raw reads were imported into QIIME2 (Bolyen et al. 2019), using the import manifest methods, trimmed to 150 bp, and converted into features using deblur. Following that we mapped the features to a phylogenetic tree created using SATé-enabled phylogenetic placement (SEPP) with the fragment insertion module (Janssen et al. 2018). Any features that did not map to the tree were discarded. We rarefied all samples to 25 000 features, based on rarefaction curves and any samples that did not pass this threshold were discarded. To reduce the effects of low frequency features or features with low numbers of reads we also filtered our final table to ensure each feature was present in at least eight samples and had at least 500 reads associated with it. Lastly, we removed the joey, soil, captive and contaminated samples from Eisenhofer et al. (2021), as well as blanks from both studies.

We assigned taxonomic identities to the representative sequences using the feature classifier (naive bayesian) (Bokulich et al. 2018) and the SILVA 99% database v138.2 (Quast et al. 2013). We assigned all taxonomies to the family level to avoid over-classification.

We used the QIIME2 core diversity plug-in to examine the alpha and beta diversity of the NHNW across two seasons and reserves and to compare it to the SHNW. From this we calculated the observed features and Faith's phylogenetic diversity (PD) for each season, species and reserve. We examined the beta diversity using Bray–Curtis dissimilarity and a Permutational Multivariate Analysis of Variance (PERMANOVA) on weighted/unweighted UniFrac distances (Lozupone and Knight 2005). Bray–Curtis dissimilarity compares the differences between two sites based on the taxonomic counts, while UniFrac distance is a phylogenetic approach that uses the difference in unique branch lengths to measure the beta diversity. We used the permdisp plug-in within QIIME2 to determine if there was dispersion within season, species, and reserve that might be influencing the amount of variation detected with the PERMANOVA. We calculated the relative abundance of the bacterial families, any families that were less than 2% abundance were merged into 'other'. To test for differential abundance of microbes between season, reserve and species we used the ANCOM-BC plug-in (Lin and Peddada 2020). Lastly, we used the core-features plugin to determine which amplicon sequence variants (ASVs) were shared between seasons and species.

All plots were created using ggplot2 in RStudio (2025.05.1).

2.3.2 | Removal of Outliers

During our diversity analysis we determined four samples to be outliers within our data set. These clustered far from all the other samples in our diversity assessments and had significantly different taxonomic profiles to the rest of the samples; these profiles were not consistent with the extraction blanks. Based on this we removed them from further analysis (Appendix S1).

3 | Results

3.1 | Sequencing Stats

After DNA sequencing, we had ~15 million paired end reads corresponding to the 116 NHNW faecal samples. We combined these data with the raw data from the 61 wild SHNW faecal samples published in Eisenhofer et al. 2021 (BioProject ID PRJNA663974). After rarefaction, 23 NHNW and 10 SHNW samples were removed, as well as all the extraction and PCR blanks. We then removed any feature that wasn't present in at least 8 samples and at a frequency of 500. After merging the NHNW and SHNW data and filtering, we were left with ~12 million reads across 140 samples.

3.2 | Seasonal and Population Variation in the Northern Hairy-Nosed Wombat Faecal Microbiome

We found variation in the faecal microbiome of the NHNW associated with season. Samples collected in spring at EFNP had significantly higher phylogenetic diversity and richness compared to those collected in winter (Kruskal-Wallis tests of Faith's phylogenetic diversity and richness, p-values < 0.001, Figure 2A,C, Appendix S2). No statistically significant differences in alpha diversity were observed between EFNP and RUNR in winter (Kruskal–Wallis tests of Faith's phylogenetic diversity and richness, p-values > 0.05, Figure 2A,C, Appendix S2).

To further investigate the seasonal variation in microbial diversity we used PERMANOVA and permdisp on the weighted and unweighted UniFrac distances, and Bray–Curtis dissimilarity (999 permutations). We found statistically significant changes in microbial composition associated with season for all methods ($p = 0.001$, Appendices S3 and S5–S8). Additionally, statistical significance was also reached for microbial community differences between EFNP and RUNR during winter for all three metrics ($p = 0.001$, Figure 3). We used permdisp to determine if there was significant dispersion amongst our samples (Appendix S4). From this we observed non-significant results from the weighted UniFrac and Bray-Curtis metrics suggesting that dispersion between groups was not driving these findings (Appendix S4). However, when we examined the permdisp for unweighted UniFrac we found significant results comparing winter to spring at EFNP which indicates differences in dispersion between the groups may be influencing the

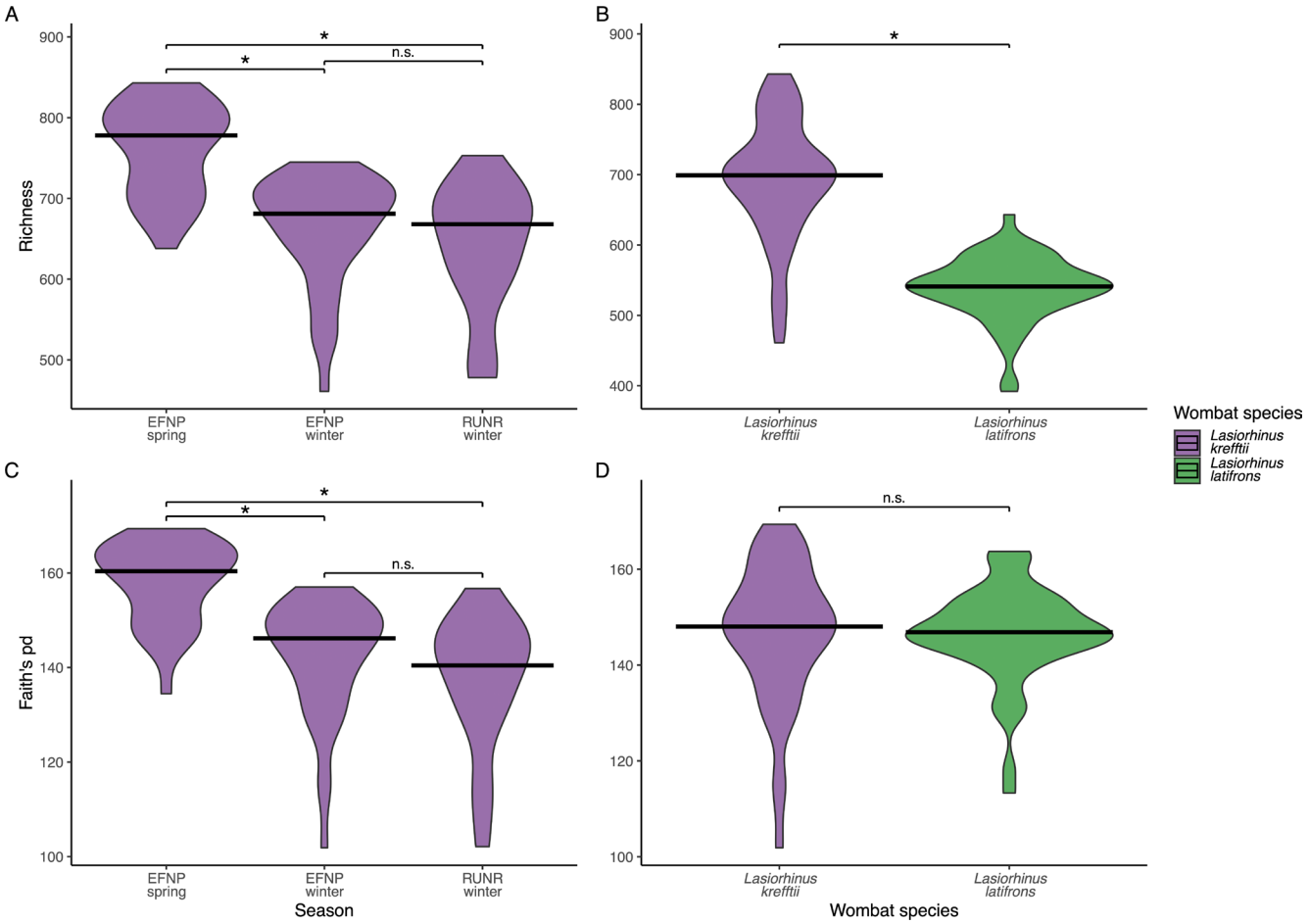


FIGURE 2 | Violin plots showing the richness and Faith's phylogenetic diversity between seasons and reserves in NHNW (A and C), and between species (B and D). The dark line shows the median; the star represents a significant difference and 'n.s.' indicates no significant difference. Significant differences were found between seasons in the NHNW using both metrics. Richness was significantly different between species and season but not reserve. Faith's PD was significant between season and reserve but not species.

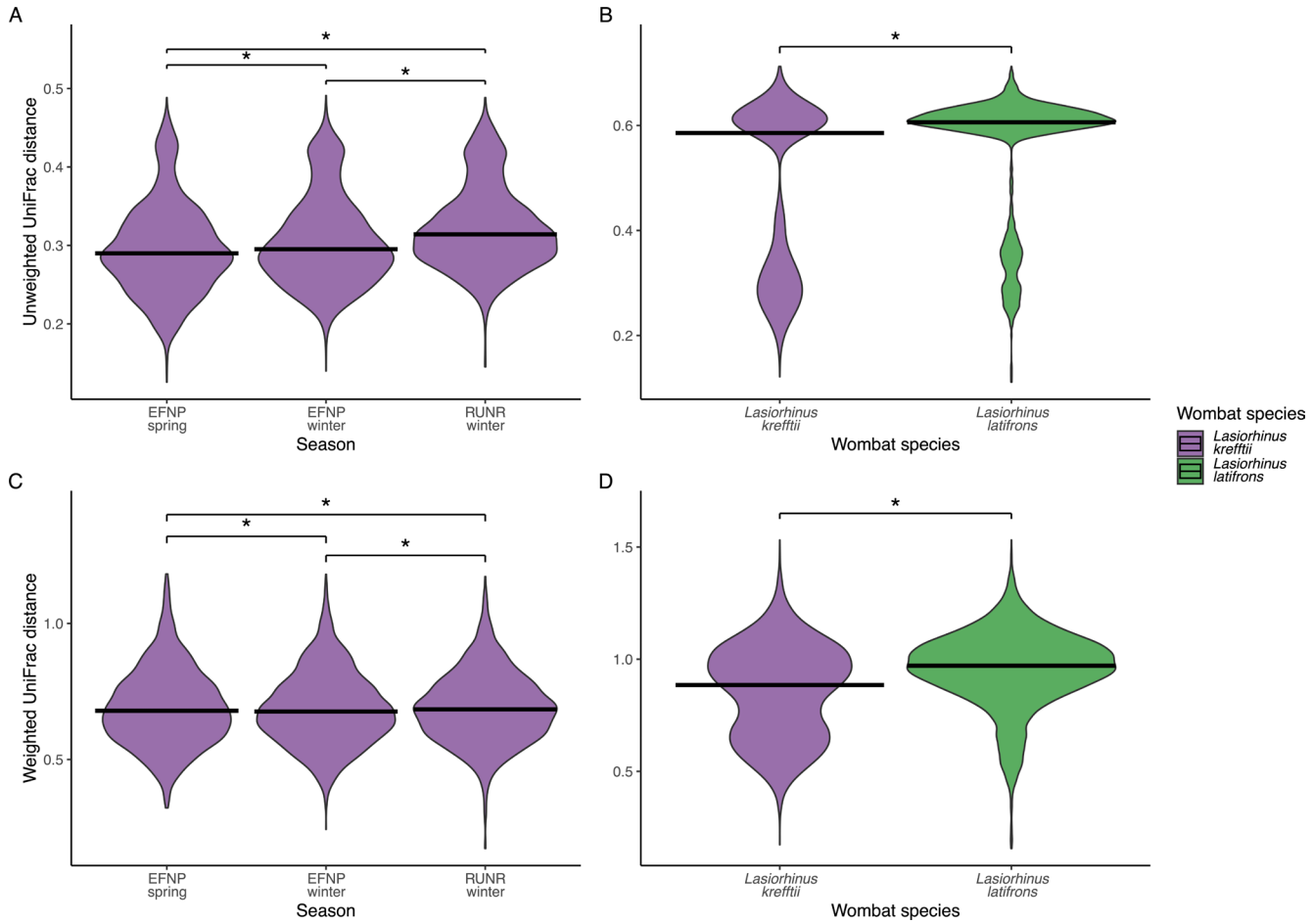


FIGURE 3 | Violin plots showing the results of a PERMANOVA comparing the weighted and unweighted UniFrac distance between seasons and reserves in NHNW (A and C) and species (B and D). There were significant differences between seasons, reserves, and species indicated by the star.

PERMANOVA (Appendix S4). Interestingly, the permdisp was non-significant for winter–winter comparisons between EFNP and RUNR suggesting a geographic influence on the beta diversity (Appendix S4).

To examine microbial community composition, we computed the mean relative abundances of the most frequently occurring taxa between the two reserves and two seasons. The most abundant bacterial family at EFNP was *Lachnospiraceae* at 13.5% and 16.9% in spring and winter, respectively. *Lachnospiraceae* was also the most abundant family at RUNR in winter (11.3%) (Figure 4A). The next most abundant families across both reserves and seasons (> 5%) were *Spirochaetaceae* and *Oscillospiraceae*. Next, we used ANCOM-BC to investigate differential abundances of the bacterial families between season and reserve. We found that from winter to spring at EFNP there were six bacterial families enriched with greater than 1 log fold change in relative abundance. The most highly enriched bacterial family from winter to spring at EFNP was *Coriobacteriales incertae sedis* (> 3 log fold) and the most depleted family was *Succinivibrionacea* (> 1.5 log fold) (Figure 5A). Furthermore, between the two reserves, we found four bacterial families that had > 1 log fold enrichment and four that had > 1 log fold depletion. Of these families the most enriched was *Paludibacteraceae* (> 1.5 log fold increase), while *Pirellulaceae* was the most depleted (~2.0 log fold decrease) (Figure 5B). We found 206 core features in 95% of the NHNW samples of which *Lachnospiraceae*, *Christensenellaceae* and *Clostridiaceae* were the most abundant (Figure 6).

3.3 | Microbial Community Differences Between Wombat Species

When we examined the alpha diversity between the two wombat species using richness, we found that the NHNW had higher alpha diversity than the SHNW (Kruskal Wallis test $p < 0.05$, Figure 2B). However, we found no significant difference in microbial phylogenetic diversity between the wombat species using Faith's PD (Figure 2D). We used PERMANOVA and permdisp on weighted and unweighted UniFrac distances, and Bray–Curtis dissimilarity to examine the beta diversity between species. From the PERMANOVA we found significant differences between the two species across all metrics ($p = 0.001$, Appendix S3) (Figure 3B). From the permdisp we found significant results between species for unweighted and weighted UniFrac distances as well as Bray Curtis ($p = 0.001$, $p = 0.001$, $p = 0.03$, Appendix S3). We also had significant results for all three tests using permdisp indicating a high level of sample variability. When we plotted these metrics using PCoA the differences between species accounted for 55.6% of the variation using unweighted UniFrac,

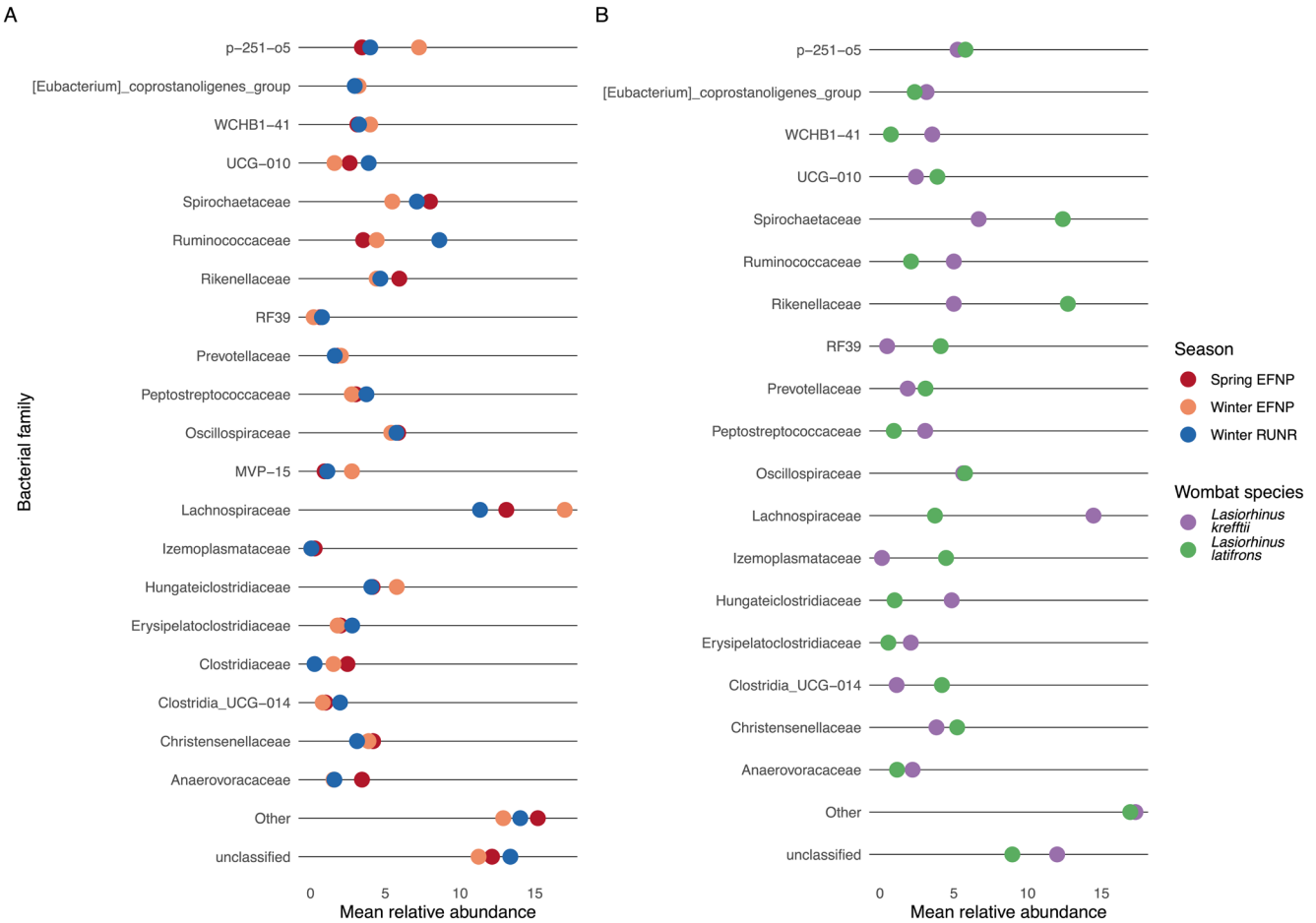


FIGURE 4 | Dot plots showing the mean relative abundance of bacterial families between seasons and reserves in NHNW (A) and between wombat species (B). Families that were under 2% abundance were pooled into the 'other' category.

30.81% using weighted UniFrac and 36.78% using Bray-Curtis (Appendices S9, S10, and S12).

3.4 | Taxonomic Differences in the Microbial Communities Between NHNW and SHNW

We compared the taxonomic profiles between species via the mean relative abundance as well as how the communities differed using ANCOM-BC. We found 18 bacterial families that were over 2% in relative abundance and shared in both species (Figure 4B). From this analysis we also found that the families *Spirochaetaceae* and *Rikenellaceae* were more abundant in SHNW than NHNW by ~10% while *Lachnospiraceae* was much more abundant in NHNW than SHNW. From the ANCOM-BC analysis we found nine bacterial families had greater than two log fold increases in NHNW compared to SHNW while 17 families had a greater than two log fold decrease from NHNW to SHNW (Figure 5C). The most enriched families in NHNW compared to SHNW (> 4 log fold increase) were *Sphingobacteriaceae* and *Izemoplasmataceae*, while the most depleted taxa (> 4 log fold) were Gamaproteobacteria, Burkholderiales and VadinHA21. Lastly, we computed the core features across 95% of our samples and found 42 ASVs that were present across both species. These were identified as 18 different families, the most abundant ones being *Ocillospiraceae*, *Lachnospiraceae* and *Christensenellaceae* (Figure 6). Both wombat species had unique members identified, six in NHNW and six in the SHNW (Figure 6).

4 | Discussion

Northern hairy-nosed wombats suffered an extreme population bottleneck followed by a slow recovery over the last 40 years, have experienced the arrival of an invasive grass species, and have been translocated to two new sites ~500 km to the south. To better understand how these changes may be impacting the wombats, we are the first to characterise the faecal microbiome of NHNWs across two sites and two seasons (winter and spring) and found temporal and spatial shifts in their microbial diversity. Additionally, this is the first study to compare the faecal microbiomes of two wombat species, the northern and southern hairy-nosed wombat, to better understand how their microbiomes may have adapted to their respective ecosystems.

4.1 | Seasonal and Geographic Variation in the NHNW

Samples collected in spring at EFNP had significantly higher alpha and beta diversity than the samples collected in winter

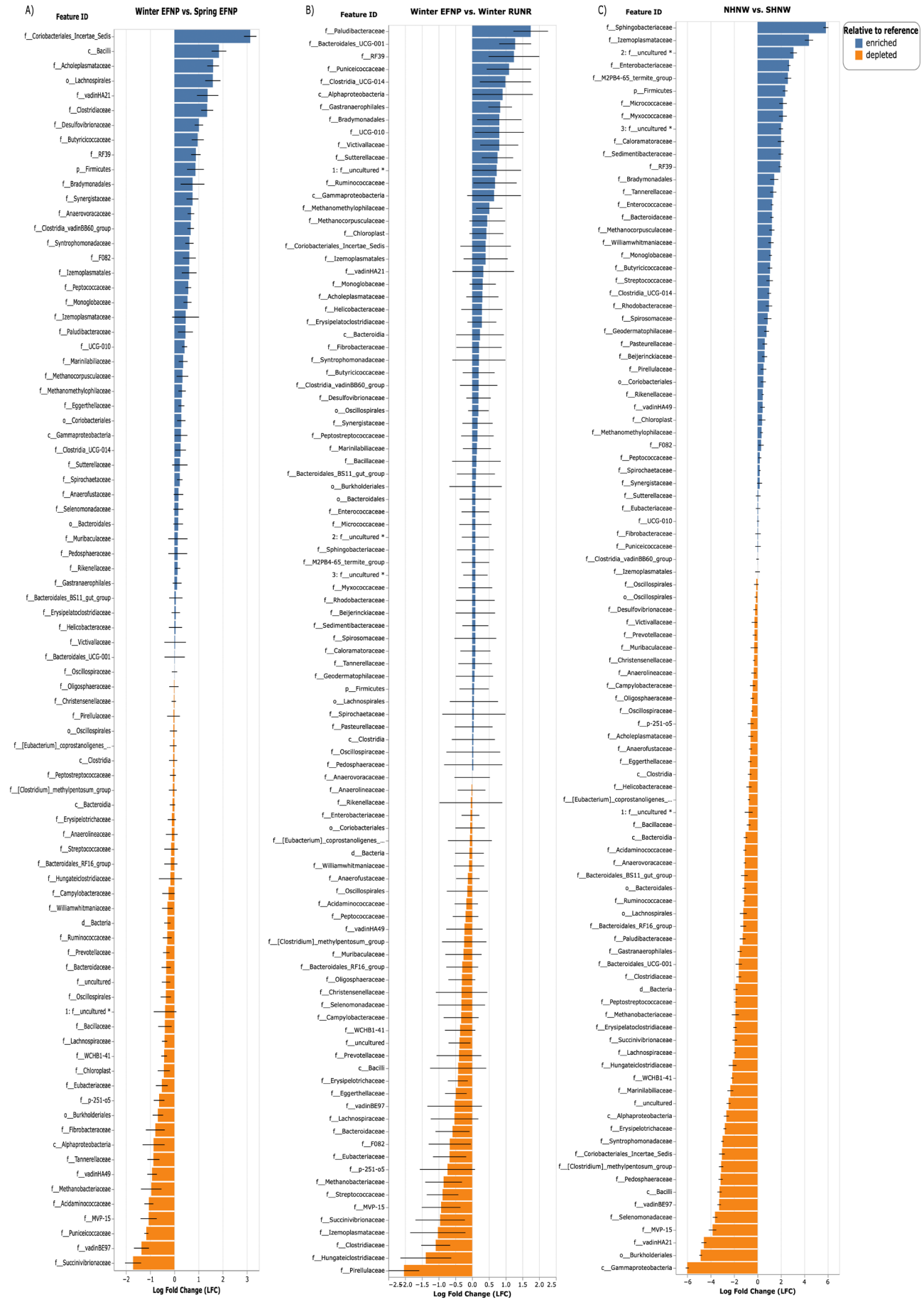


FIGURE 5 | Differential abundance of bacterial families between seasons (A), reserves (B), and species (C), calculated using ANCOM-BC.

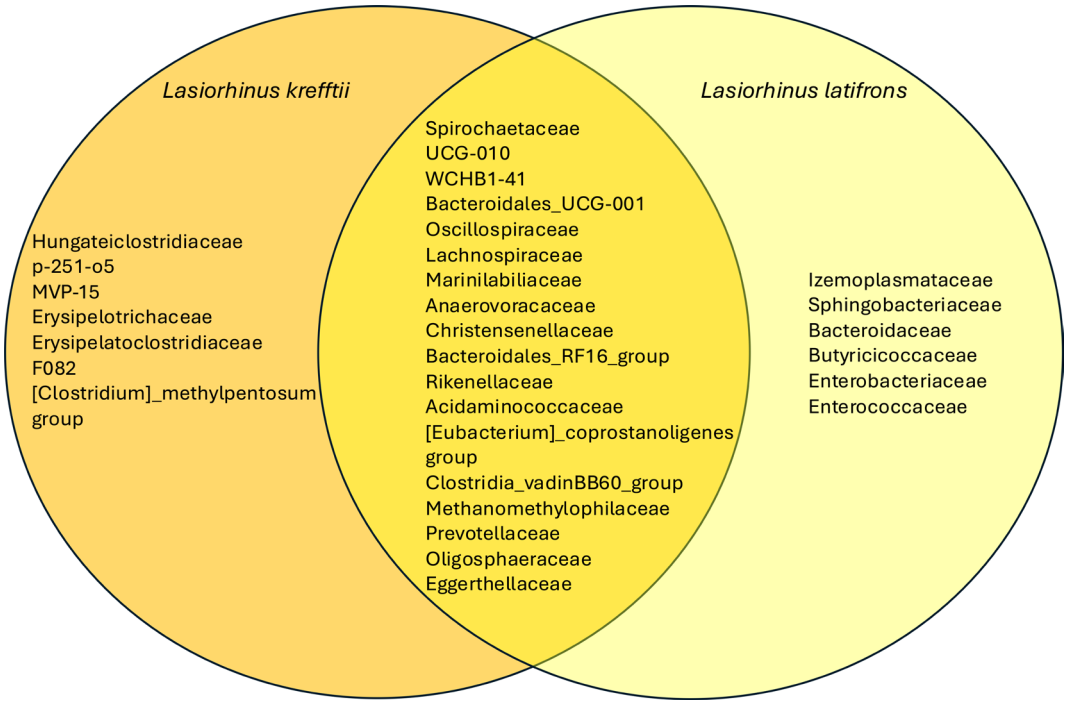


FIGURE 6 | The shared and unique bacterial families between both wombat species.

at both EFNP and RUNR. The change in microbial diversity within the season likely corresponds to changes in available food on the landscape. Woolnough (1998) and Flosser (1986) determined that the NHNW is a generalist browser that will consume what is available rather than targeting specific taxa. This was further supported by Casey et al. (2023) who also found seasonality within the diet that likely corresponded with availability though they did not perform surveys of forage availability. More specifically, the lower microbial diversity we identified corresponds with the increased abundance of buffel grass in the diet during the winter months. It is worth noting, however, that this association is based on only two seasons across two years, and no direct vegetation survey was conducted at the time of collection, so additional sampling and surveying could be conducted to further test this pattern.

The seasonal shift in microbial diversity due to changes in diet has been found in other species such as gorillas (Gomez et al. 2016), sika deer (*Cervus nippon*) (Ma et al. 2024), geladas (*Theropithecus gelada*) (Baniel et al. 2021), and Japanese macaques (*Macaca fuscata*) (Sawada et al. 2022). In the geladas, which are also hindgut fermenting and primarily rely on grasses for their diet, Baniel et al. 2021 found a shift between the types of fermenting and plant degrading taxa that might be assisting or adapting to the seasonal change of available food. More specifically, they found an increase in cellulolytic/fibrolytic bacteria during the wet season and an increase in starch degrading bacteria during the dry season. Similarly, we found an >1 log fold increase in *Lachnospiraceae* and *Bacteroidales_vadinHA21* from winter to spring, which is the height of the dry season. These two families have been attributed with cellulolytic/fibrolytic properties in mammalian guts (Salyers et al. 1977; Biddle et al. 2013; White et al. 2014). Thus, changes in the relative abundance of these taxa might be relevant to the extraction of nutrients from the dried grasses during the dry season (May to October). The most enriched bacterial family from winter to spring was *Coriobacteriales insertae sedis*. This family of bacteria has been associated with dietary polyphenol degradation in human gut and agricultural microbiome research (Amrit et al. 2023; Porter Starr et al. 2025). This may be an indication of change in pasture quality and available nutrients, particularly of available polyphenols which are important sources of antioxidants (Amrit et al. 2023). The largest decrease in bacterial families from winter to spring occurred in *Succinivibrionaceae*. Our collection time was September, which was before the summer rains in QLD and the landscape was drier. *Succinivibrionaceae* has been attributed to assisting to alleviate dietary stress in Tibetan macaques (*Macaca thibetana*) and may be contributing similarly in the wombats by aiding in breaking down more cellulose-rich woody plant parts (Sun et al. 2016). Based on the shifts in diversity that we see, it is likely that the microbiome of the northern hairy-nosed wombat is dynamic and shifts based on the seasonally available forage on the landscape.

Lastly, we identified geographical variation in the NHNW faecal microbiome between EFNP and RUNR. While the alpha diversity, observed features and Faith's PD were non-significant between the two reserves, the beta diversity was significantly different across all three methods tested. This suggests that the variation in dietary items that was found in Casey et al. (2023) between the two reserves is likely driving the community differences in the wombat gut microbiome.

4.2 | Similarities and Differences Between NHNW and SHNW Faecal Microbiomes

This is the first study to compare faecal microbiomes between two wombat species. From this we determined that the

community is consistent with other hind gut fermenting herbivores; however, there are key differences that suggest the microbial community is shaped by the diet of each wombat species. Examining the alpha diversity between the two species, we found similar levels of phylogenetic diversity. However, we found significantly higher richness in the NHNW. This finding stands out as it has been shown that reduced genetic diversity and increased inbreeding as a result of population bottlenecks reduces the diversity of the associated microbiome (Yuan et al. 2015; Wei et al. 2020; Ørsted et al. 2022). Considering that there were only ~30 NHNW left in the world 45 years ago, this degree of diversity compared to a non-bottlenecked relative is surprising. It also may suggest that the microbial diversity has recovered with the population at EFNP, which now stands at ~400 individuals. Further studies and sampling of translocated populations, which are managed bottlenecks, could test this hypothesis, as there are no pre-bottleneck microbiome samples (Horsup et al. 2021; Nothern hairy-nosed wombat recovery action plan 2022).

When we examined the beta diversity, we found large differences between the two wombat species. Our PCoAs showed 30%–55% of the variation driven by species along axis 1 (Appendix S8–S11). These results are consistent with other studies such as Rojas et al. (2021) that showed that gut microbiomes are species specific and the variation observed in closely related species is often driven by habitat and diet. We found a similar pattern in these two wombats, evidenced by the high degree of separation between their communities and the specific taxa that are enriched or depleted between.

When we compared the composition of each species' faecal microbiome, we found a core of 18 families that were shared across 95% of our samples. Many of these families, such as *Lachnospiraceae*, *Rumincococaceae*, and *Spirochaetaceae*, are common members of herbivore digestive tracts and aid in the breakdown of plant cell walls for secondary fermentation (Biddle et al. 2013; White et al. 2014). However, when we examined which families were most enriched and depleted between species, we found specific taxa that may be contributing to surviving in their respective habitats. Specifically, in the SHNW, the family *Sphingobacteriaceae* was the most highly enriched. The prevalence of this family in the guts of herbivores has been associated with assisting digestion of aromatic compounds found in shrubs, which might be more useful to the SHNW as their diet includes shrubs compared to the NHNW, which rely more exclusively on grasses (Camp et al. 2020; Sobek and Walker 2021; Casey et al. 2023; Cao et al. 2024). The most heavily depleted bacterial family in SHNW compared to NHNW was vadinHA21, a member of the order Bacteroidales. This order of bacteria is strongly associated with carbohydrate and SCFA breakdown, which might indicate the presence of higher levels of fibre and carbohydrates in NHNW; however, no direct comparison of nutritional value has been performed between the species (Salyers et al. 1977; Flint et al. 2012). While both species use the same digestive mechanism, the presence of these differentially expressed bacterial families highlights how the microbiome of these wombats has adapted to their specific diets and ecosystems.

4.3 | Conservation Implications

In this study we characterised the faecal microbiome of the NHNW and found significant shifts between seasons and reserves. This is the first study to examine the microbiome of this species and from it we determined that northern hairy-nosed wombats have a dynamic microbiome that shifts seasonally with what they are consuming on the landscape. As their diet becomes more homogeneous in winter, so does their microbiome. This information is critical for managers as the consistent increase in buffelgrass on both EFNP and RUNR may reduce the overall diversity of the microbiome and potentially reduce the wombats' ability to withstand large environmental or dietary shifts should they need to (Woolnough 1998; Casey et al. 2023; 2024). As was determined by Eisenhofer et al. (2021) SHNW inhabiting landscapes with native grasses had much greater diversity in microbial communities compared to habitats without native grasses. Due to this effect in closely related species, we recommend maintaining and promoting native grass diversity on the reserves as well as taking steps to mitigate the spread of buffel grass to promote a varied and robust microbiome.

4.4 | Future Directions and Considerations

While we were able to detect a significant shift in the microbiome between winter and spring that corresponded with changes in diet, we acknowledge the limitations of our sampling strategy. Namely that this study relies on a single time point from one spring and one winter sampling. Therefore, collecting samples from the wet season (November–March) and fall (April–June) over a single year would provide a better understanding of the microbial community shift in response to rain and increased plant productivity and diversity. Regarding our between-species comparison, it should be noted that the SHNW samples come from a particularly dry year and additional sampling should be carried out to understand if this profile is consistent under 'normal' precipitation conditions. Lastly, many of our associations are based on comparing results found in other host species due to the absence of publicly available wombat microbial genomes. We recommend future studies use shotgun metagenomic sequencing to generate a wombat-specific gut microbiome catalogue and examine its metabolic potential.

Author Contributions

Jeremy J. Austin: supervision, writing – review and editing, resources. **Raphael Eisenhofer:** supervision, writing – review and editing, methodology, validation. **Faith M. Walker:** writing – review and editing, supervision. **Colin Sobek:** conceptualization, investigation, writing – original draft, methodology, formal analysis, data curation, visualization, validation, funding acquisition, project administration.

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Ethics Statement

All faecal collection and sampling was performed under University of Adelaide Animal Ethics approval number S-2022-058 and Queensland Department of Environment and Science permit numbers P-PENPS-100425721, P-PTUKI-100425717 and P-PTUKI-100425728.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in Sequence Read Archive at https://www.ncbi.nlm.nih.gov/sra, reference number PRJNA1291405.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Appendix S1:** All samples used in this study. The † indicates the samples that were removed as outliers. **Appendix S2:** *p*-values for Kruskal–Wallis tests comparing alpha diversity between season, reserve and species. **Appendix S3:** *p*-values from PERMANOVA tests of beta diversity, for season, reserve and species. **Appendix S4:** *p*-values from permdisp tests of beta diversity, for season, reserve and species. **Appendix S5:** PCoA showing the comparison of unweighted UniFrac distances between EFNP and RUNR. **Appendix S6:** PCoA showing the comparison of weighted UniFrac distances between EFNP and RUNR. **Appendix S7:** PCoA showing the comparison of Jaccard similarity between EFNP and RUNR. **Appendix S8:** PCoA showing the comparison between EFNP and RUNR using Bray–Curtis dissimilarity. **Appendix S9:** PCoA sowing the comparison between NHNW and SNHW using unweighted UniFrac distances. **Appendix S10:** PCoA showing the comparison between NHNW and SHNW using weighted UniFrac distances. **Appendix S11:** PCoA showing the comparison between NHNW and SHNW using Jaccard similarity. **Appendix S12:** PCoA showing the comparison between NHNW and SHNW using Bray–Curtis dissimilarity.