Do faecal samples represent the primary site of microbial plant fermentation in hindgut fermenters?

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Research Article

Abstract

Background

Most herbivorous mammals have symbiotic microbes living in their gastrointestinal tracts that help with harvesting energy from recalcitrant plant fibre. The bulk of research into these microorganisms has focused on samples collected from faeces, representing the distal region of the gastrointestinal (GI) tract. However, the GI tract in herbivorous mammals is typically long and complex, containing different regions with distinct physico-chemical properties that can structure resident microbial communities. Therefore, to determine how representative faeces are to other regions of the GI tract in hindgut-fermenting mammals, we characterised the microbial biogeography of the GI tract of two species of wombats.

Results

Using 16S rRNA gene sequencing, our results show that GI microbial communities of wombats are structured by GI region. For both the bare-nosed wombat (*Vombatus ursinus*) and the southern hairy-nosed wombat (*Lasiorhinus latifrons*), we observed a trend of increasing microbial diversity from stomach to distal colon. The microbial composition in the first proximal colon region was more similar between wombat species than the corresponding distal colon region in the same species. We found several microbial genera that were differentially abundant between the first proximal colon and distal colon regions. Surprisingly, only 99 (10.6%) and 204 (18.7%) amplicon sequence variants (ASVs) were shared between the first proximal colon region and the distal colon region for the bare-nosed and southern hairy-nosed wombat, respectively.

Conclusion

These results suggest that microbial communities in the first proximal colon region—the putative site of primary plant fermentation in wombats—are distinct from the distal colon, and that faecal samples have limitations in capturing the diversity of these communities. While faeces are still a valuable and effective means of characterising the distal colon microbiota, future work seeking to better understand how GI microbes impact the energy economy of wombats (and potentially other hindgut-fermenting mammals) may need to take gut biogeography into account.

Background

Gut functionality and health relies on a diverse community of microbes inhabiting the entire gastrointestinal tract. These microbes are instrumental in digesting food, producing nutrients for the host, and as a potential first barrier to invading pathogens (reviewed in [1, 2]). In herbivores specifically, gut microbes break down cellulose and other plant cellular components so they may be used as nutrients by the animal [3], and can detoxify plant defense compounds [4]. Without these microbes there would be little to no nutritional value in much of the plant matter herbivorous mammals are known to consume and thrive on. In ruminants, the bulk of microbial digestion and fermentation occurs in the foregut (a

specialized, multi chambered stomach) where nutrients are broken down by rumen microbiota and made ready for absorption through the small intestine. However, in herbivores without a foregut, the microbial breakdown of plant matter into usable nutrients and energy occurs in the latter parts of the gut—principally the cecum and colon [5]. In the case of wombats, a marsupial which relies on hindgut fermentation, > 60% of energy is thought to be derived from microbial fermentation in the colon [6]. The diversity and composition of microbial communities within the gut are known to be site-specific [7]. However, faeces are most commonly used in microbiome studies, which may not represent the full diversity of microbes along the GI tract. As a major goal of gut microbiome research is to understand how microbes influence host health and ecology, efforts should be made to better characterize the site-specific nature of microbes along the GI tract and determine how representative faecal samples are.

The diversity and composition of microbes throughout the mammalian GI tract remains largely under characterized, with most previous work done on well-studied placental mammals (horses, pigs, mice, humans). Such studies in horses, pigs, and humans found subtle but significant differences in microbial communities between the cecum, different sections of the colon, and faeces [8–14]. In mice, larger differences were observed between cecum, distal colon, and faeces microbial communities [15, 16]. How representative faecal samples are to other parts of the GI tract is likely host species specific, resulting from variations in GI morphology and function. Yet our understanding of these differences outside of domesticated placental mammals remains unstudied.

Wombats represent a useful herbivorous hindgut fermenting group to contribute to knowledge on microbial community differences along the GI tract. As marsupials, they are a valuable taxonomic group to add to a growing body of GI tract microbiota research. As grazers, wombats are also of comparative value to horses and thereby allow questions of similarity across functionally similar groups of mammals. Bare-nosed (*Vombatus ursinus*, hereafter BNW) and Southern hairy-nosed (*Lasiorhinus latifrons*, hereafter SHNW) wombats represent two of the three extant wombat species and are also in the same sub-order as the koala (diprotodontia). The BNW and SHNWs are large (20–35 kg), fossorial, metabolically depressed, nocturnal, and largely solitary marsupials found allopatrically across large areas of southeastern and southern Australia [17]. These two wombat species also represent temperate and arid adapted grazing diets, respectively [17, 18], and are thought to have shared a common ancestor ~ 8 million years ago [19]. The SHNW has a comparatively longer distal colon, with the BNW possessing a wider digestive tract [20]. Previous research measuring short chain fatty acids indicates that most plant fermentation occurs in the proximal colon of both wombat species rather than in other regions of the GI tract [6]. To date, gut microbiota research in wombats has focused on faecal samples obtained from captive [21] and wild wombats [22, 23], with nothing known about how microbial communities are structured throughout the GI tract, and how representative faeces are to other GI regions.

We hypothesized that the proximal colon would harbor a distinct microbial community to that of the rest of the gastrointestinal tract, including the distal colon in these wombat species. Therefore, we investigated the microbial community changes along the gastrointestinal tract of a wild BNW and SHNW.

We also aimed to determine whether distal colon microbial communities are representative of the gut microbial community at different sites along the gastrointestinal tract in these two wombat species.

Methods

Sample collection

Sample collection occurred during necropsies on two deceased wild wombats, one BNW and one SHNW. A 17.1 kg female BNW was hit by a vehicle in the New Norfolk area, Tasmania, Australia and taken to Bonorong Wildlife Sanctuary. Injuries to this wombat were too severe for rehabilitation, and it was euthanized on 13th November 2019. Immediately following euthanasia the carcass was placed in a -20°C freezer at the University of Tasmania, Hobart. The SHNW was captured near Swan Reach in the Murraylands of South Australia, approximately 100 km north-east of Adelaide, as part of an ecological study on seasonal reproduction and breeding, but then died later of an upper respiratory tract infection. The carcass was driven to Adelaide and put into a -20°C freezer within 2 hours of death.

Prior to necropsy, carcasses were put in a 4°C refrigerator and defrosted for ~ 60 h. The entire digestive systems were then removed from the abdominal cavity and the functional sections of the GI tract were labeled as per [20]. Samples of the digesta were collected in duplicate in the SHNW and in triplicate in the BNW, these included samples from the stomach, small intestine, proximal colon, and distal colon (Fig. 1A/B). While faecal samples could not be collected from the euthanized animals, the distal colon samples collected resembled faeces, being similar in size, shape, and moisture content. To avoid cross-contamination between sites the digesta was squeezed directly into individual sterile containers. Samples were then stored at -20°C prior to molecular work.

DNA extraction and 16S rRNA gene library preparation

DNA extraction was performed using the QIAamp® Fast DNA stool mini kit from QIAGEN according to the manufacturer's protocol (Qiagen Pty. limited Victoria, Australia). Duplicate samples from each GI region for both wombat species were processed. BNW samples were extracted at the University of Tasmania, and SHNW samples were extracted at the University of Adelaide. BNW DNA extracts were then shipped frozen to the University of Adelaide for library preparation. Barcoded V4 region 16S rRNA gene amplicons were generated using primers from [24] (forward primer 515F: AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGTG-TGCCAGCMGCCGCGGTAA and barcoded reverse primer

806R: CAAGCAGAAGACGGCAT-ACGAGATnnnnnnnnnnnnnnAGTCAGTCAGCCGGACTACHVGGGTW TCTAAT). The PCR reactions were prepared in a pre-PCR laboratory in a 5% sodium hypochlorite-cleaned and UV irradiated hood. Single reactions [25] of 2.5 µL X10 HiFi buffer, 0.1 µL Platinum™ Taq DNA Polymerase (ThermoFisher), 19.2 µL dH2O, 0.2 µL 100 mM dNTP mix, 0.5 µL each of 10 µM forward and reverse primer and 1 µL input DNA. The DNA was amplified using an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 1 min, elongation at

68°C for 90 s, with final adenylation for 10 min at 68°C [26]. Gel electrophoresis was performed on PCR reactions on a 3.5% agarose gel to ensure samples contained amplicons of the desired length (~ 390 bp). For each sample, 1 μL PCR amplified DNA was mixed into 199 μL Qubit® working solution (diluted Qubit® dsDNA HS Reagent 1:200 in Qubit® dsDNA HS Buffer) and quantified using a Qubit® 2.0 Fluorometer. Samples were then pooled equimolar and cleaned using AxyPrep™ (Axygen) following the manufacturer's instructions. The final pool was quantified and quality checked using an Agilent TapeStation. DNA sequencing was performed on an Illumina MiSeq (v2, 2 × 150 bp) at SAHMRI (South Australian Health and Medical Research Institute).

Bioinformatic analyses

QIIME2 [27] and R v4.0.2 [28] were used to perform the bioinformatic analyses and create figures. Reproducible code is publicly available at the following GitHub repository (https://github.com/EisenRa/2021_Wombat_GI_tract_16S). Forward reads were denoised using deblur [29] in QIIME2. Representative sequences were assigned taxonomy using the QIIME2 feature-classifier plugin [30] on the pre-trained SILVA [31] 138 V4 region classifier. A phylogenetic tree was created using the SATé-enabled phylogenetic placement (SEPP) technique [32] using the fragment-insertion QIIME2 plugin. Data was then imported into rStudio into a phyloseq [33] object using qiime2R (https://github.com/jbisanz/qiime2R), and manipulated with dplyr (https://github.com/tidyverse/dplyr), tidyr (https://github.com/tidyverse/tidyr/), and the microbiome package [34]. For all sample type comparisons, the table was rarefied at a depth of 21,710, for colon-only comparisons the table was rarefied to 47,301. ASVs that were found in only 1 sample were removed. Alpha and beta diversity measures (including UniFrac [35]) were calculated in R using phyloseq, and figures were plotted using ggplot2 [36]. Compositional analysis of the community was done using ANCOM-II in R (ANCOM v2.1 [37] in R v.4.0.3). Analysis was undertaken using an ANCOM - adjusted for covariates model which accounts for the covariation expected at the species/animal level. Analysis at the ASV level was performed and then an analysis at a rarefied genus level was performed for the proximal colon and distal colon samples only. For the venn and euler diagrams, ASVs needed more than 2 reads assigned at a given site (between two replicates) to be considered detected, and were then plotted using ggVennDiagram (https://github.com/gaospecial/ggVennDiagram).

Results

DNA sequencing of the 31 gastrointestinal digesta samples resulted in 4,923,858 (mean of 158,834 ± 76,389) forward reads (R1), which were denoised using deblur into 2,524 amplicon sequence variants (ASVs).

Microbial biogeography through the digestive tract of two wombat species

The stomach and small intestine of hindgut fermenting mammals are harsh environments for microbes to survive, typified by extremely low pH and various digestive enzymes and bile salts respectively. The colon by contrast is a more habitable region for complex gut microbial communities to thrive, and where

the bulk of microbial fermentation of plant material is thought to occur. To test if these different gut regions influenced the microbial communities, we measured microbial richness (the number of ASVs) of duplicate luminal samples taken throughout the GI tract of each wombat species. Microbial richness tended to increase through the length of the digestive tract for both wombat species (Fig. 1). Stomach and small intestine samples had the lowest ASV richness (~ 100–200), followed by the first two proximal colon samples (PC1/PC2: ~400–500), with the latter proximal colon and distal colon samples exhibiting the highest ASV richness (~ 500–800).

Like richness, the types of microorganisms living in a site and how they are structured (composition) can also be influenced by the environmental factors of that site. To test this, we calculated the microbial compositions of these distinct sites using both the abundance weighted and unweighted UniFrac distance metrics. Using both methods, the major differences observed in microbial composition were associated with GI tract site not wombat species, with stomach, small intestine colon, and colon samples clustering across the axis of most variation (Axis 1: Fig. 2A/B). Colon samples from the different wombat species were separated across axis 2 for the unweighted UniFrac analysis (Fig. 2A), whereas axis 2 of the abundance-weighted analysis further separated proximal and distal colon samples (Fig. 2B). Taking into account colon samples only, species-specific differences in abundance-weighted microbial composition between colon samples were observed across axis 2 (SI Fig. 1B). Interestingly, the microbial composition of the first proximal colon samples of different wombat species are more similar to each other than they are to the distal colon samples of the same species (Fig. 2B, SI Fig. 2B).

We next investigated what types of microbes (the taxonomic composition) were present in the different GI samples for both wombat species. Consistent with differences we observed in the ordination analyses, the stomach and small intestine samples were taxonomically distinct to the colon samples for both wombat species (Fig. 3). At the phylum level, these samples tended to be dominated by Proteobacteria, Firmicutes, and Fusobacteriota (SI Fig. 3), with the dominant families being Pasteurellaceae (59% in BNW-ST, 56% in SHNW-ST, 43% in SHNW-PSI), Peptostreptococcaceae (67% in BNW-SI), and Fusobacteriaceae (81% in SHNW-DSI) (Fig. 3). In contrast, the colon samples for both wombat species were dominated by the Bacteroidota, Firmicutes, and Spirochaetota phyla (SI Fig. 3). We also observed taxonomic differences between proximal and distal colon samples for both the wombat species (Fig. 3). The first proximal colon samples were dominated by Prevotellaceae (~ 40% relative abundance), and Fibrobacteraceae (~ 10% for the BNW), whereas distal colon samples contained higher proportions of Spirochaetaceae, Rikenellaceae, and WCHB1-41. These results indicate that microbial diversity and composition vary throughout the GI tract of two hindgut fermenting species, and that the start of the proximal colon–the putative primary site of fermentation in these wombat species–is distinct from the distal colon.

Microbial differences between proximal and distal colon sites

We next focused on characterising microbial community compositional differences between the proximal and distal colons for both species. As there were many ASVs that were classified to the same taxa we

collapsed the ASV table to the genus-level, and ran ANCOM-II to identify genera that were differentially abundant between different regions of the colon (i.e. PC1, PC2, DC, etc.). Of the 255 genera classified in the dataset, ANCOM-II identified 84 that were significantly differentially abundant throughout the colon when the W-statistic threshold for rejecting the null was at 70% (Fig. 4) (SI Table 1). Taxa that had significantly higher abundance in PC1/2 vs. DC include: *Prevotella* (W = 245), Prevotellaceae_*UCG-001* (W = 241), *Bacteroides* (W = 239), and Bacteroidales_RF16_group (W = 231). Taxa with a higher relative abundance in DC vs. PC1/2 include: Bacteroidales_BS11_gut_group (W = 250), Bacteroidales_*UCG-001* (W = 243), WCHB1-41 (W = 242), and Izemoplasmatales (W = 226).

To test how representative the microbial community of faeces are to the first proximal colon site, we measured how many ASVs were shared between PC1 and the last DC sample for each wombat species. Surprisingly, only 99 (10.6%) and 204 (18.7%) ASVs were shared between PC1 and DC samples for the BNW and SHNW, respectively (Fig. 5). See (SI Fig. 3) for a cross-species comparison. The ASVs that were unique to the BNW PC1 and DC sites accounted for 25% and 64% of the relative abundance in those sites, respectively. Likewise, the ASVs that were unique to the SHNW PC1 and DC sites accounted for 32% and 67%, respectively. These results indicate that the first proximal colon and distal colon sites harbour distinct communities of microbes.

Discussion

Mammalian gut microbial communities play vital roles in the harvesting of energy and nutrients [1]. Due to ease of sampling, most previous mammalian gut microbiota research has focused on faecal samples as proxies for the gut microbiota. However, the mammalian GI tract contains a series of distinct microbial growth conditions that can structure the diversity and composition of resident microbial communities [7]. Outside of model or domesticated placental mammal species there has been little work in characterising how GI microbial communities are structured along GI tracts, and how representative faeces are of other GI regions. Our work here fills this gap for two species of free-living, large, hindgut-fermenting marsupials —the BNW and SHNW. We found that GI region seemingly structured the microbial communities along the GI tract of these wombat species, and that the first proximal colon region—the putative primary site of fermentation—was highly dissimilar to the distal colon. Our findings also indicate that faecal samples collected for wombats may not be representative of the primary site of fermentation. Future studies seeking to understand the roles that GI microbial communities play in host energy acquisition and health should consider the microbial biogeography of the mammalian GI tract.

Previous physiological work in the 90s' by Barboza and Hume suggested that the bulk of microbial fermentation in the BNW and SHNW occurs in the proximal colon, specifically highlighting the first proximal colon region (PC1) [6, 20]. Our study builds on this work by characterising the microbial communities present at distinct regions along the GI tract of the same two species of wombats. A major finding was that the microbial communities present in PC1 were more similar between wombat species, than to subsequent colon region samples from the same species. PC1 in both wombat species was dominated by the family Prevotellaceae (~ 40% relative abundance), a family that contains members with

extensive carbohydrate/fiber fermentation capabilities [38, 39]. All other GI sites contained lower levels of Prevotellaceae (< 5% relative abundance). In addition, PC1 for both wombat species contained significantly higher levels of bacteria classified to the genus *Bacteroides*, which are known to possess a diverse suite of carbohydrate-active enzymes [40]. PC1 of the BNW had relatively high levels (~ 10% relative abundance) of the genus *Fibrobacter* (~ 0.3% in the SHNW PC1), which have known fiber-degrading capacity and have been found in various placental foregut- and hindgut-fermenting herbivores [41]. Taken together, these distinct microbial compositions in PC1 of both wombat species compared to subsequent colon regions lend support to Barboza and Hume's previous work indicating that PC1 is likely the primary site of microbial fermentation in these wombat species.

Given the major microbial compositional differences observed between PC1 and distal colon samples, we sought to determine how many PC1-associated microbes would be detected by sampling faeces, the most commonly used sample type in mammalian gut microbiota research. Surprisingly, only a very small proportion of PC1 ASVs could be detected in distal colon samples (10.6% and 18.7% in the BNW and SHNW, respectively). This suggests that seeking to understand microbial functions at the putative primary site of fermentation through faecal samples may not be feasible. This is not to say that faecal samples are not useful, as previous gut microbiome research on captive [21] and wild wombats [22, 23] have yielded insights into wombat digestion and ecology. Eisenhofer et al. used faecal samples from both captive and wild SHNWs to show that captivity has a large influence on the faecal microbiota of SHNWs. They were also able to identify population-specific microbial signatures, and found a correlation between habitat type (degraded vs. intact) and faecal microbiota composition in SHNWs [22]. Shiffman et al. used shotgun metagenomic sequencing on faecal samples collected from a captive SHNW, and found numerous microbial genes involved in plant degradation and urea recycling [21]. Interestingly, they made note that they could not detect bacteria from the phylum *Fibrobacterota*, which are commonly found in herbivorous mammals [41]. We identified *Fibrobacterota* in both wombat species, with a higher relative abundance in the BNW, particularly in the proximal colon. Overall, our results suggest that faeces may not be a representative sample type for the putative primary site of microbial fermentation in wombats. We propose that future research seeking to understand the roles that GI microbes play in wombat (or other mammal) digestion consider this, and aim to study other regions of the GI tract where possible.

Our study is not without limitations. The opportunistic nature of sample collection from fresh necropsied animals limited this study to only one animal for each wombat species. This hampered our ability to compare microbial differences between wombat species, as the differences observed may be related to individual-level variation. However, the consistency observed between the biological replicates and in overall trends for both species of wombat support our findings relating to PC1/faecal microbial differences and the level of representability of the faeces to PC1—despite the ecological differences between wombat species and the estimated ~ 8 million years since they last shared a common ancestor [19]. Another limitation is that we could not distinguish DNA from living or dead cells, and it is possible that the differences between GI sites is greater than measured in our study due to dead cells/relic DNA moving from site to site and inflating similarity. Future studies with larger sample sizes will enable more

detailed microbial comparisons between wombat species, and improve estimates of within-animal microbial differences along the GI tract.

The findings of our study have spawned several promising avenues of future research. Using shotgun metagenomics to compare and contrast the microbial functions present between proximal and distal colons could allow for a greater understanding of the roles GI microbes play in wombat digestion and health. The strain-level information obtained from shotgun metagenomics would also allow for greater tracking of microbes along the GI tract, and help disentangle issues relating to the over splitting of microbes due to intragenomic heterogeneity in 16S rRNA genes [42]. Future, larger sample size comparisons between wombat species could also seek to identify microbes that are shared between wombat species, and consistently found within species. Any such 'core' microbes that are still shared between wombat species despite ~ 8 million years of separation could indicate that they are important for host health. In our study the distal colon exhibited the greatest microbial differentiation between wombat species. Such interspecies microbial differences in the latter parts of the colon could be due to GI morphological/physiological differences between wombat species [20], and relate to differences in the ecology of the two wombat species. Further larger-scale comparisons between BNW (mesic living) and SHNWs (arid/semi-arid living) could offer insights into the potential roles that GI microbes play in the arid and temperate adaptability of mammals in Australia.

Conclusions

Our study suggests that microbial communities in the first proximal colon region—the putative site of primary plant fermentation in wombats—are distinct from the distal colon, and that faecal samples have limitations in capturing the diversity of these communities. While faeces are still a valuable and effective means of characterising the distal colon microbiota, future work seeking to better understand how GI microbes impact the energy economy of wombats (and potentially other hindgut-fermenting mammals) may need to take gut biogeography into account.

Abbreviations

ANCOM: Analysis of Composition of Microbiomes

ASV: Amplicon Sequence Variant

BNW: Bare-Nosed Wombat

DC: Distal Colon

DSI: Distal Small Intestine

GI: Gastrointestinal

PC: Proximal Colon

PCoA: Principal Coordinates Analysis

PCR: Polymerase Chain Reaction

PSI: Proximal Small Intestine

SI: Small Intestine

SHNW: Southern Hairy-Nosed Wombat

ST: Stomach

Declarations
Contributions

All authors initiated and conceived the project. ED, SC, and BP collected the BNW samples. DT and RE collected the SHNW samples. ED and RE performed the molecular work, bioinformatic and statistical analysis. All authors wrote, edited, and approved the final manuscript.

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Ethics declarations

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Competing interests

The authors declare that they have no competing interests.

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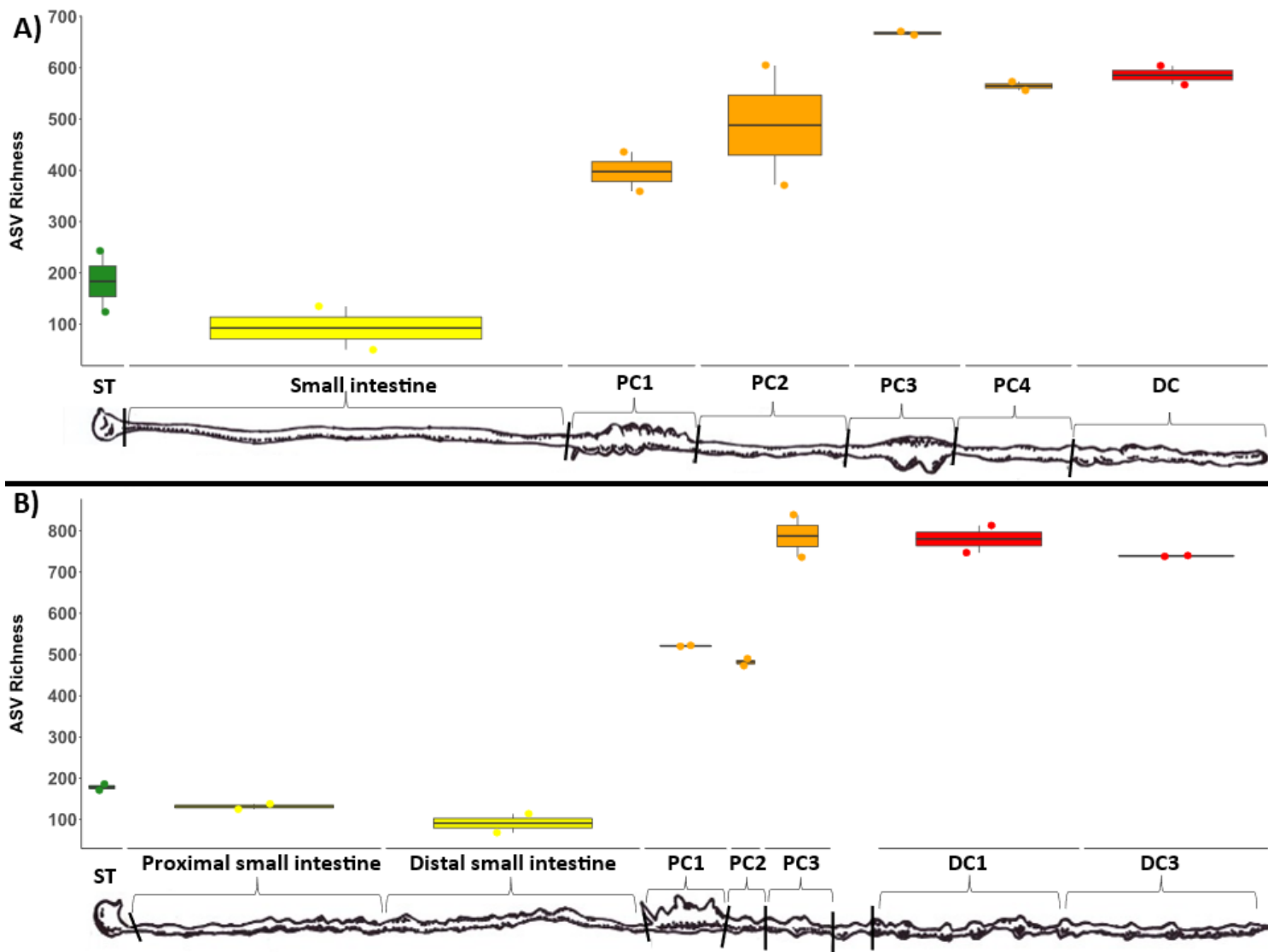
Figures

Figure 1

| Microbial diversity (ASV richness) of samples collected throughout the wombat digestive tract, ordered from start to end. ST = stomach, SI = small intestine, PSI = proximal small intestine, DSI = distal small intestine, PC = proximal colon, DC = distal colon. Two technical replicates were collected and processed for each site. A) bare-nosed wombat. B) southern hairy-nosed wombat. Digestive tract drawings were adapted from Barboza and Hume 1992.
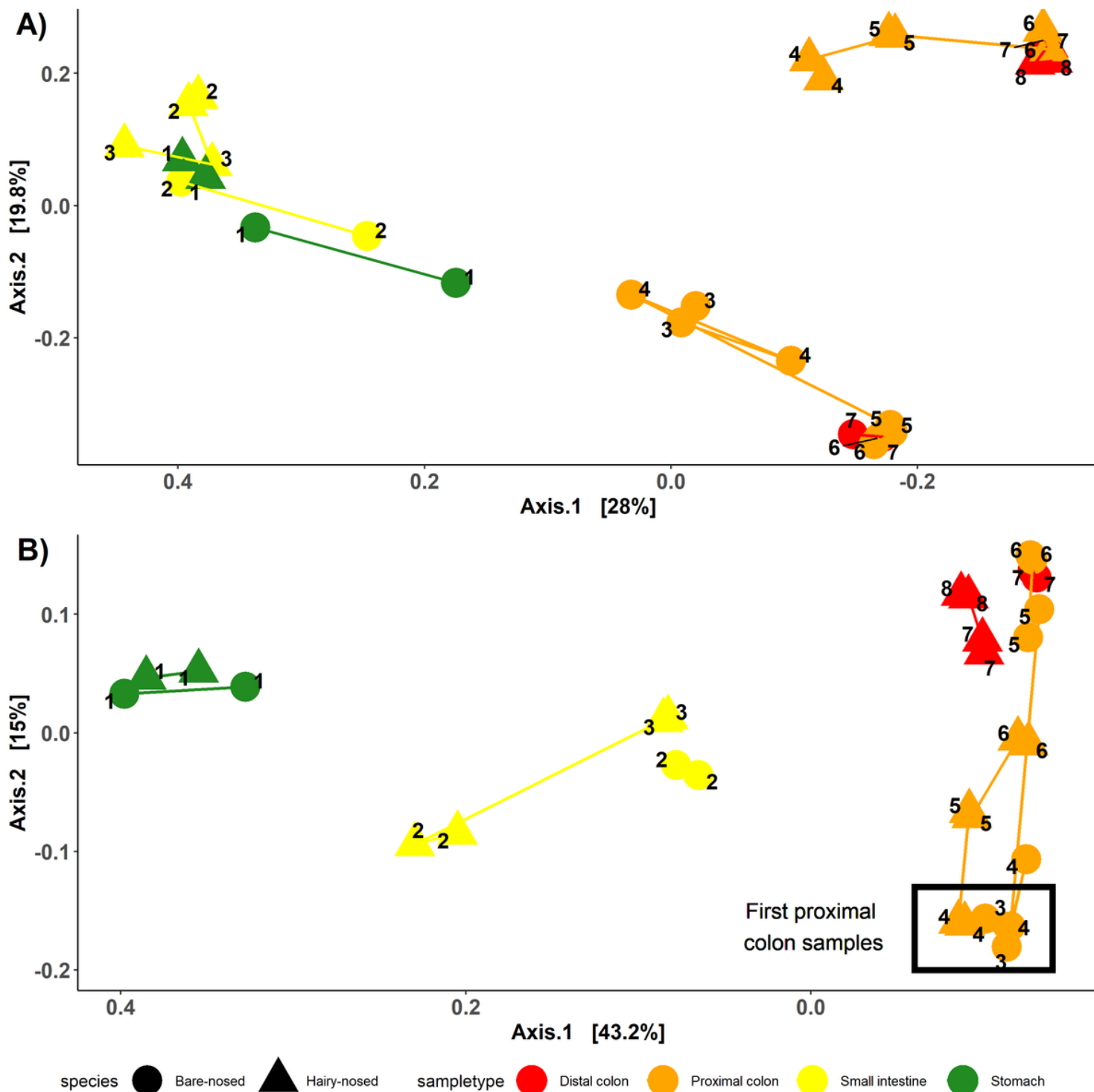
Figure 2

Microbial composition differs throughout the wombat digestive tract. A) PCoA of unweighted UniFrac distances and B) PCoA of weighted UniFrac distances. Samples are coloured by sample type, and shaped by host species. Numbers indicate order in which samples occur in the digestive tract. Lines connect samples from the same sample type.
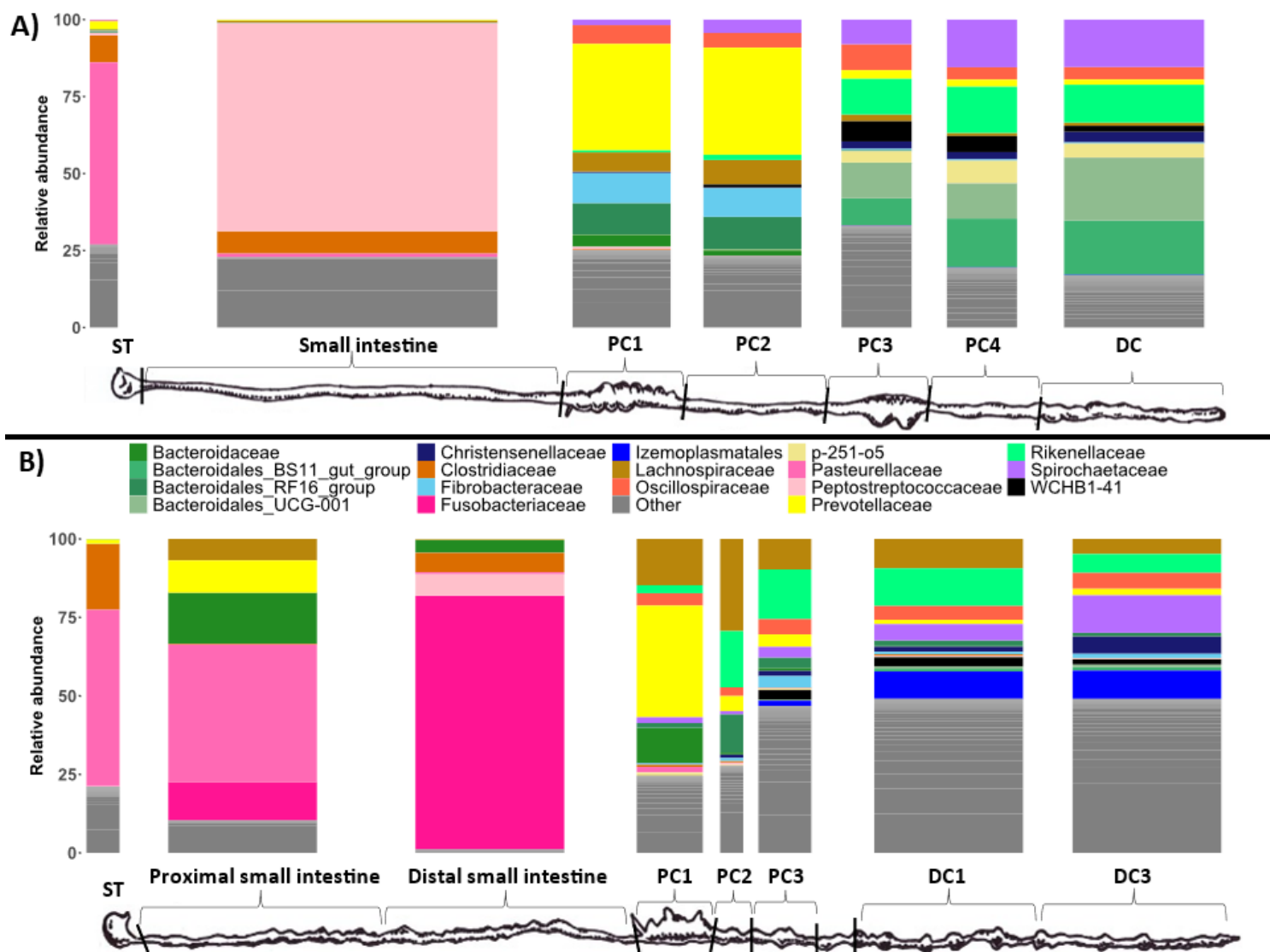
Figure 3

Taxonomic composition of the different sample types at the family level. Only the top 20 most abundant families are displayed for clarity (these families account for 81.17% of reads). Replicate samples were merged per sample site. A) bare-nosed wombat. B) southern hairy-nosed wombat. The widths of the bars are scaled to the length of the GI region.
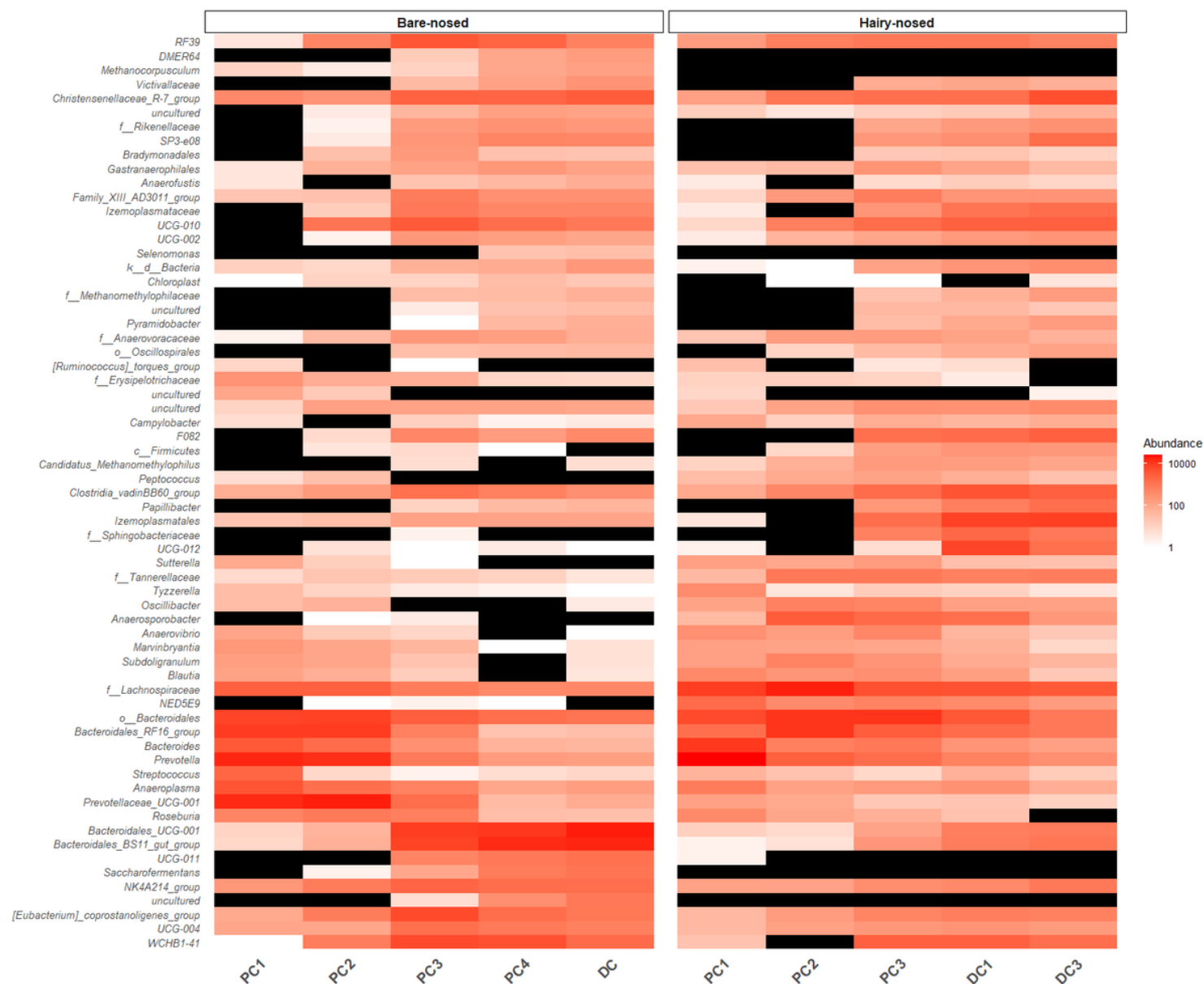
Figure 4

Heat map of differentially abundant taxa (0.7 threshold) identified in the ANCOM-II analysis.
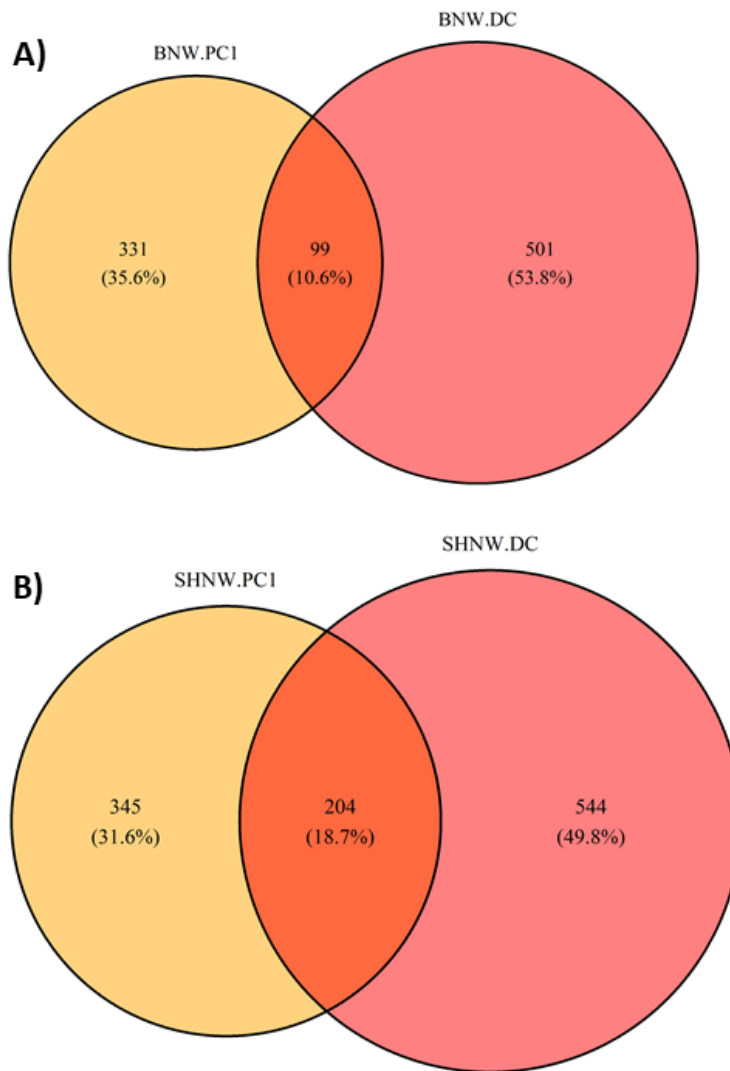
Figure 5

Euler diagram of amplicon sequence variants (ASVs) shared between proximal colon 1 (PC1) and last distal colon site (DC) for A) bare-nosed wombat (BNW) and B) southern hairy-nosed wombat (SHNW). Percentages represent the proportion of ASVs specific to a given area.

Supplementary Files

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- SupplementaryFiguresandTables.docx