Variation in the microbiome of the urogenital tract of koalas (*Phascolarctos cinereus*) with and without 'wet bottom'

Alistair R. Legione[1, *], Jemima Amery-Gale[1], Michael Lynch[2], Leesa Haynes[3], James R. Gilkerson[4], Joanne M. Devlin[1, #] and Fiona M. Sansom[1, #]

[1]Asia Pacific Centre for Animal Health, The University of Melbourne, Parkville, Victoria 3052, Australia

[2]Veterinary Department, Melbourne Zoo, Parkville, Victoria 3052, Australia

[3]Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Werribee 3030, Victoria, Australia

[4]Centre for Equine Infectious Diseases, The University of Melbourne, Parkville, Victoria 3052, Australia

[*]Corresponding author (email: alegione@student.unimelb.edu.au)

[#]These authors contributed equally

Abstract

Koalas (*Phascolarctos cinereus*) are iconic Australian marsupials currently threatened by several processes. Infectious reproductive tract disease, caused by *Chlamydia pecorum,* and koala retrovirus infection are considered key drivers of population decline. The clinical sign of 'wet bottom', a staining of the rump associated with urinary incontinence, is often linked to chlamydial urogenital tract infections. But, wet bottom has been recorded in koalas free of *C. pecorum*, suggesting other causative agents in those individuals. Current understanding of the bacterial community of the koala urogenital tract is limited. We used 16S rRNA diversity profiling to investigate the microbiome of the urogenital tract of ten female koalas. Five presented with wet bottom and five were clinically normal. We detected thirteen phyla across the ten samples, with Firmicutes occurring at the highest relative abundance. The order Lactobacillales comprised 70.3% of the reads from all samples. After normalising reads using DESeq2 and testing for significant differences, there were 25 operational taxonomic units more commonly found in one group over the other. This study provides an essential foundation for future investigations of both the normal microflora of the koala urogenital tract, and better understanding of the causes of koala urogenital tract disease.

Introduction

The koala (*Phascolarctos cinereus*) is an iconic marsupial species endemic to Australia. Northern koala populations, in the states of Queensland and New South Wales, are currently declining due to impacts from both disease and increased urbanisation. Two significant pathogens of koalas, *Chlamydia pecorum* and koala retrovirus (KoRV), have been the focus of koala infectious disease investigation since their respective discoveries. KoRV is currently undergoing endogenisation into the genome across the koala population in Australia[1]. KoRV has been detected in all northern koalas sampled[2], and has been associated with a large number of clinical signs of disease[3]. *Chlamydia pecorum* infection causes ocular and urogenital infections and can lead to blindness and infertility in koalas, greatly impacting population fecundity and survivability[4,5]. *C. pecorum* is commonly associated with the clinical sign known as 'wet bottom' or 'dirty tail'[6]. This staining or scalding of the rump is associated with cystitis due to *C. pecorum* infection in some popultations[7], but recently samples from a large number of koalas from Victorian populations with mild wet bottom were negative via qPCR for *C. pecorum*[8]. In particular, koalas in a population considered at the time to be free of *C. pecorum*[9] had a similar prevalence and severity of wet bottom to populations where *C. pecorum* occurred in more than 35% of the population. Further analysis demonstrated that whilst wet bottom could be significantly linked to the detection of *C. pecorum* infection in male Victorian koalas, this relationship did not exist in females[10]. It may be that some other as yet unidentified organism is causing these mild clinical signs of disease in koalas, however to date there has not been extensive research to determine the normal flora of the koala urogenital tract, making it difficult to use traditional microbiological techniques to detect species of interest. Modern sequencing technology, specifically 16S rRNA biodiversity profiling, was used to improve our understanding of the microbiome of the urogenital tract of koalas, and to compare the microbiome of koalas with and without mild wet bottom.

Methods

Sample Collection and initial screening

Samples used in this study were urogenital swabs, from female koalas, stored in RLT buffer (Qiagen) taken from an archive of koala samples collected in 2011 from French Island, Victoria, Australia (38°21’0” S, 145°22’12” E). Koala samples were collected under general anaesthetic by veterinarians and trained field assistants during routine population management exercises and clinical health of koalas was recorded at the time. Sample collection was approved by the University of Melbourne Faculty of Veterinary Science Animal Ethics Committee, application ID:1011687.1. Koala specific data collected included body condition score[8], age (based on tooth wear)[5] and wet bottom score[11]. After screening all samples for *Chlamydia* spp. using a previously described qPCR[12,13], we selected ten samples from female koalas where no *C. pecorum* was detected. We used five samples collected from koalas showing no clinical signs of urogenital disease and five samples collected from koalas that showed clinical signs of wet bottom (Table 1). As no blood samples were available from the koalas used in this study urogenital swabs were utilised for KoRV screening using qPCR protocols previously described[14].

Amplification and sequencing

DNA extraction and amplification from the swab samples was performed commercially by The Australian Genome Research Facility (Australia). Sequencing was performed on the Illumina MiSeq platform. Variable domains three and four of bacterial 16S rRNA were amplified using primers 341F (5’ CCTAYGGGRBGCASCAG 3’) and 806R (5’ GGACTACNNGGGTATCTAAT 3’).

Quality filtering and OTU assignment

Quality filtering and operational taxonomic unit (OTU) assignment was undertaken using a mixture of scripts and algorithms available in the programs USEARCH[15] and QIIME 1.9.1 (Quantitative Insights Into Microbial Ecology)[16]. The resulting paired-end reads for each swab sample were merged using USEARCH script **fastq_mergepairs**. Primers were then trimmed using seqtk[17] and reads were filtered for quality using USEARCH script **fastq_filter**, utilising an expected error cut off of 1, rather than PHRED quality score[18]. Paired reads which were shorter than 400 bp were discarded. Unique reads within the entire sample set were assigned OTUs using the USEARCH algorithms **derep_fulllength** and **cluster_otus**[19], with a minimum identity of 97% for clustering. Singletons were excluded from analysis due to the high likelihood that they contain errors, as per USEARCH **cluster_otus** manual[20]. The merged reads from each swab sample, including the previously excluded singletons were matched with the produced OTUs using USEARCH script **usearch_global**, with a threshold of 97% identity to group a read into specific OTU. The taxonomy of each OTU was determined by using the QIIME script **assign_taxonomy.py** in conjunction with the Greengenes taxonomy database[21]. Chloroplast and mitochondrial OTUs were removed from the dataset using the QIIME script **filter_taxa_from_otu_table.py**.

Read normalisation and analysis

Read data was assessed using three different methods. Relative abundance was utilised to compare basic phylum presence in each sample. Rarefaction of reads was undertaken, using **multiple_rarefactions.py** QIIME script, to assess alpha and beta diversity at a set read level. Negative-binomial normalisation of reads, using DESeq2[22] as recommended by McMurdie and Holmes [23], was performed using the QIIME script **normalize_table.py**. Alpha-diversity metrics assessed were species richness, Chao1[24], phylogenetic distance and Shannon's diversity[25], using reads sampled to a depth equalling the sample with the fewest reads (rounded down to the nearest 5,000 reads). Non-parametric comparisons of alpha diversity between the two sample groups (wet bottom present or absent) were undertaken with the **compare_alpha_diversity.py** QIIME script, with 10,000 permutations. Beta-diversity was assessed using the **beta_diversity_through_plots.py** QIIME script, in which both unweighted and weighted UniFrac distances[26] were assessed. Bray-Curtis dissimilarity[27] between samples was also assessed. 3D PCoA plots were drawn within the script using EMPeror software[28]. Distance and dissimilarity metrics were used to compare the microbial communities between the two groups by utilising the permutational ANOVA (PERMANOVA) method within the **compare_categories.py** QIIME script, with 10,000 permutations. Statistical comparisons of the differential abundance of OTUs between koalas with and without wet bottom utilised DESeq2 within the QIIME script **differential_abundance.py**. These comparisons aimed to determine OTUs which were over-represented in either group. Statistically significant results were based on P-values < 0.05, and were adjusted for false discovery[29] within the script. The analyses listed above were repeated to compare koalas from which KoRV provirus was or was not detected.

Results

Clinical status of koalas

Of the five koalas with wet bottom, the median wet bottom clinical score was 3 (ranging from 2 – 4). The five clinically healthy animals all had wet bottom clinical scores of 0. The median body condition score of all koalas included in this study was 3 (ranging from 2 – 4). The mean weight for koalas was 7.4 kg (± 1.01 standard deviation [S.D.]) (Table 1). All koalas were negative for *C. pecorum*. Three koalas were positive for KoRV provirus, two in the group of koalas without wet bottom (Table 1).

Analysis and processing of sequencing data

A total of 2,295,607 paired reads were obtained across the ten samples, with a mean read number of (229,560.7 ± 40,522 S.D.). The GC content of the reads was 51.8%. After quality filtering and discarding merged sequences shorter than 400, the total number of reads OTU clustering was 1,946,587, with a mean read number of 194,658.7 (S.D. ± 29,951.1) per sample (Table 1).

The filtered reads were clustered into 261 OTUs, 7 of which were either chloroplasts or mitochondria and were subsequently removed. For comparison, the same filtering and clustering methodology was run without the removal of singletons, which resulted in the clustering of reads into 592 OTUs.

Phylum presence and relative abundance

In total, 13 phyla were detected in the ten samples (Table 1), with Firmicutes occurring at the highest relative abundance (77.61%). Just over a third of the OTUs were classified as Firmicutes (95/254), followed by Proteobacteria (59/254) and the Bacteroidetes (35/254). When samples were split into the two groups, koalas without wet bottom had 89.3% of reads classified as Firmicutes, followed by those which could not be assigned using the 97% cut-off (5.2%) and Actinobacteria (3.5%). Koalas with wet bottom had 68.2% Firmicutes. The next two most prevalent phyla were Proteobacteria (12.5%) and Bacteroidetes (12.2%). Deferribacteres were detected in only one sample (Koala 70, wet bottom present) and Acidobacteria were only detected in two (one clinically normal koala and one displaying wet bottom). Armatimonadetes was detected in three koalas without wet bottom, but in none of the five diseased koalas. These three phyla where detected at the lowest relative abundance across the ten samples.

Richness and diversity

Species richness within each sample, using absolute abundance, is described in Table 1. Across the ten samples sequenced, the mean number of OTUs in each sample (with singletons, chloroplasts and mitochondria removed) was 80.0 (ranging from 55 to 126). After rarefaction to 160,000 reads, the mean OTUs of the two groups were 80.0 (S.D. ± 9.62) and 75.93 (S.D. ± 24.61) for koalas with wet bottom and without wet bottom, respectively. Assessing absolute reads, the median Shannon's diversity of these samples was 2.48 (ranging from 1.08 to 4.09) and the median diversity in koalas with and without wet bottom was comparable (Kruskal-Wallis test; $H = 0.53$, d.f. = 1, $P = 0.465$). At 160,000 reads alpha diversity metrics for samples from koalas with or without wet bottom were comparable. This included OTU abundance ($P = 0.81$), Chao1 ($P = 0.83$), phylogenetic distance ($P = 0.71$) and Shannon's diversity ($P = 0.86$) (Supplementary materials S1). Results detailing presence/absence for all OTUs detected in koala urogenital samples is recorded in supplementary materials S2.

Fewer than half of the OTUs detected across the two sample groups were shared between them (112/254) (Figure 1). At a read depth of 160,000 there was a significant difference between the microbial communities in koalas with wet bottom compared to those without, based on the results of PERMANOVA using Bray-Curtis dissimilarity ($F = 4.92$, $P = 0.019$) and unweighted (qualitative) UniFrac distances ($F = 1.62$, $P = 0.031$). There was no significant difference detected when using weighted (quantitative) UniFrac distances ($F = 1.51$, $P = 0.061$). The 2D and 3D principle coordinate analysis graphs comparing koalas with and without wet bottom are shown in supplementary materials S3 and S4. There were no significant differences in either alpha or beta diversity metrics when comparing koalas with or without KoRV provirus detected.

Comparisons between samples using DESeq2 normalised reads

Negative binomial normalisation of reads from each sample using DESeq2 still resulted in Firmicutes as the most dominant phylum across all samples. This was followed by Proteobacteria and Bacteroidetes (Figure 2). Overall there were 25 OTUs with significant ($P < 0.05$) over-representation or under-representation in wet bottom affected koalas, in comparison to clinically normal koalas, based on these normalised read counts (Table 2). Of those OTUs, when assessing absolute read count, six occurred only in koalas with wet bottom, whilst eight occurred only in koalas without wet bottom (Table 2). There were no significant differences between abundances of normalised OTUs between koalas with or without KoRV provirus detected.

Discussion

Previous assessment of the koala microbiome has focused on the unique digestive system of koalas comparing either two free ranging animals from northern populations[30] or two captive koalas in Europe[31], from which the ocular microbiome was also assessed. This study is the first investigation of the microbiome of the urogenital tract of the female koala using modern high-throughput techniques, and only the second to assess the urogenital tract of a marsupial, with the Tammar wallaby investigated previously using terminal restriction fragment length polymorphism analysis[32]. The majority of reads in our sample set were classified in the order Lactobacillus. This dominance of Firmicutes mirrors what has been seen in the human vaginal microbiome[33]. In humans, the acidic pH of the genital tract is maintained by these lactic acid producing bacteria, which in turn is thought to play a role in preventing pathogenic infection[34]. It appears from our sample set that koalas have a different family within the

Lactobacillus, possibly performing a similar role. The most common family within our classified OTUs, in terms of either relative or normalised read abundance, was *Aerococcaceae*, whilst in humans the *Lactobacilli* dominate the reproductive tract. Within the *Aerococcaceae*, the genera *Aerococcus* and *Facklamia* were both represented in the top four most abundant OTUs. For all four significantly differentially abundant *Aerococcus* spp. OTUs, the same OTU could be detected in at least 4/5 (80%) of the converse sample group in absolute reads. For example, OTU 4, an *Aerococcus* spp. whose representative sequence had 91.8% identity to *Aerococcus urinae* occurred in all ten koala samples, but was present in significantly higher quantities in clinically normal koalas after normalisation. Whether specific *Aerococcus* spp. that are over or under-represented are an important factor in terms of disease presence requires further investigation. The production of hydrogen peroxide by commensal *Lactobacillus* is thought to play a role in reducing the successful establishment of sexually transmitted diseases in humans[35,36], and it has been shown that *Aerococcus* spp. are involved in hydrogen peroxide production[37,38]. In humans *Aerococcus* spp. have also been associated with disease, including the aforementioned *Aerococcus urinae*, which can cause urinary tract infections[39] and septicaemia[40]. Investigations into the urinary microbiome of women with and without ‘urgency urinary incontinence’ found that *Aerococcus* spp. were detected more frequently in cases where disease was present[41]. In our study, the four *Aerococcus* spp. OTUs that had significantly different normalised abundance were evenly split, with two having higher abundance in koalas with wet bottom and two in koalas without wet bottom. The role of organisms within this family as opportunistic pathogens in koalas cannot be ruled out.

The *Aerococcus* were the most common genus amongst those OTUs with significant differential abundance after normalisation using DESeq2. The representative sequences of these four OTUs did not match known species within the *Aerococcus* genus with an identity greater than 97%, suggesting that these represent novel species. This is not unexpected, as the culture of organisms from the koala urogenital tract has been limited to only a small number of studies, with the majority focused on diagnosing what was later deemed to be chlamydial infection[42-44]. Efforts in culturing novel bacteria from koalas have focused primarily on its unusual gut microbiome[45], owing to its diet of eucalyptus leaves, as well as the microbial flora in the pouch[46]. Of the OTUs that were classified to species level in our study, one was classified as *Lonepinella koalarum*, which was first isolated from the faeces of a captive koala[47]. In our koalas, *L. koalarum* was present in 6/10 samples and occurred at a relatively low abundance in the majority, ranging from 1 to 3139 absolute reads (median of 1) (Supplementary material S2). Whilst it is possible for a species to occupy multiple body sites, it could also suggest that our samples contain minor contaminants from the intestinal tract. The anatomy of the koala, with the cloaca through which the urogenital tract is accessed also containing the rectal opening, means that such contaminants are difficult to avoid. Future studies of the urogenital tract microbiome would benefit from either taking control samples from the rectum of the koala being sampled, or inverting the cloaca so that the urogenital opening is more easily accessible, as described previously for the tammar wallaby[32]. In that study, approximately a quarter of phylotypes (26/96) were detected in both the urogenital and rectal samples, suggesting that bacteria occupying multiple sites in marsupials is not unusual.

Whilst there did not appear to be any strong clustering on our 2D or 3D PCoA plots, comparisons of the beta-diversity between groups highlighted that the makeup of the communities was significantly different when assessing Bray-Curtis dissimilarity and unweighted UniFrac distances. These metrics assess presence/absence of OTUs between groups, with UniFrac also considering phylogenetic distance between OTUs present. The finding that weighted UniFrac distances, which considers the abundance of OTUs, were comparable between groups suggests that there was no clustering due to OTU prevalence.

The average number of OTUs detected in our samples is difficult to compare to other publications investigating koala microbiomes. This is both due to the impact that sample site differences would have on OTUs present, as well as the method of OTU classification used. For instance, previous research on the koala intestinal microbiome used QIIME for analysis of 454 pyrosequencing reads[30] and detected 1855 OTUs, after removal of chimeras and singletons, from caecum, colon, and faecal samples. Similarly, an Illumina based study of microbiomes from ocular, oral, rectal and faecal samples from two captive koalas found OTU numbers ranging between 597 to 3,592, with a median of 1,456[31]. The average raw read numbers per sample assessed in these projects ranged from 12,831 (454 pyrosequencing) to 323,030 (Illumina). Our own average raw reads per sample were within that range (229,561), suggesting that the OTU differences between our studies are either associated with the sample site (urogenital versus digestive tract) or clustering methodology used. We employed UPARSE due to its demonstrated ability to correctly identify OTUs in a mock community and minimise spurious OTUs[19].

It could be argued that the skewed relative abundance of Proteobacteria and Bacteroidetes in the samples from koala 49 and 70, respectively, could be a result of swab contamination with faecal material, which would impact diversity inferences. The human microbiome project identified that reads from stool samples were predominately from the Bacteroidetes phylum[48], and the most recent assessment of the koala rectal microbiome found these two phyla to be the most abundant in samples taken from both koalas assessed[31]. The representative sequence from the OTU with the highest relative abundance from koala 49 matched *Escherichia coli* with 100% identity (429 bp) (GenBank Accession Number: KY305421). *E. coli* is an organism commonly found in the gut of mammals. It can also cause urinary tract disease through opportunistic infections in species such as humans[49], cats and dogs[50]. It is possible that the *E. coli* detected here is responsible for a urinary tract infection, resulting in "wet bottom", rather than simply resulting from faecal contamination. Early investigations into reproductive tract disease in koalas isolated *E. coli* from the uterine horns[51] and from purulent exudate[52] of koalas suffering pyometritis. Increased sample sizes, as well as samples from different anatomical regions of the same individuals would allow elucidation of the role of *E. coli* as a causative agent of urogenital disease. The most abundant OTU in the sample from koala 70 had a 92.2% nucleotide identity to *Tannerella forsythia* (424 bp) (GenBank Accession Number: JN713185). This organism is more commonly considered an oral pathogen in humans, but has been isolated from women suffering from bacterial vaginosis[53]. An organism related to this pathogen, represented by this OTU, could also be causing wet bottom clinical signs in koalas, but more data is required to truly assess its impact.

The other family of significant interest are the Tissierellaceae, within the order Clostridiales. The four OTUs classified as Tissierellaceae with a significant differential abundance, three in the genus *Peptoniphilus*, all occurred in higher normalised quantities in koalas with wet bottom present. Interestingly, only one of these four OTUs was detected at all in the group of koalas without wet bottom, and only from the reads of one koala within this group. The *Peptoniphilus*, previously part of the genus *Peptostreptococcus*[54] within the family Peptostreptococcaceae (also in the order Clostridiales), have been associated with inflammatory diseases in other species. This includes mastitis in cattle[55] and pelvic inflammatory disease in humans[56]. Organisms in this genus are fastidious anaerobes[54] and therefore potentially overlooked in culture based methods of investigating urogenital tract pathogens.

Our sample size is larger than previous studies of koala microbiomes, which have incorporated at most two individuals, yet it is substantially smaller than many studies in human medicine which include hundreds of samples[57]. Our samples were opportunistically collected during population management exercises, and chosen from our sample archive due to the absence of *C. pecorum* from the French Island koala population at the time of testing[9]. Whilst *C. pecorum* was subsequently determined to be present in this population[13], no koalas used in this project were positive via a *Chlamydia* spp. PCR. Importantly, no koalas used in this study were found to have reads classified within the Chlamydiae phylum after taxonomic assignment of OTUs.

We have recently shown that koalas with wet bottom are almost twice as likely to be infected with KoRV[58]. This, in combination with the knowledge that *C. pecorum* was not associated with wet bottom in female Victorian koalas, led us to hypothesise that mild wet bottom could be associated with opportunistic urinary tract infections arising as a result of KoRV-induced immunosuppression. As no blood samples were obtained from the koalas utilised in this study, we did not have an accurate means of testing for KoRV provirus in our individuals. Previous studies have validated the use of faecal samples for KoRV testing[59], but it is unlikely that urogenital swabs accurately reflect the true frequency of KoRV in our sample set, particularly as the virus has not entered the germline in Victorian koalas[1]. To more rigorously assess the hypothesis that KoRV might induce wet bottom in koalas through immunosuppression and opportunistic infection, a broader study using individuals of known KoRV status would be required.

Disturbance of the normal vaginal flora in humans, such as in cases of bacterial vaginosis, is a risk factor associated with infection by retroviruses (such as human immunodeficiency virus) and *Chlamydia trachomatis*[60]. Our study provides useful data as to what bacteria could be expected in a clinically normal koala's urogenital tract. This will allow for broader, more detailed studies on the impact of the koala urogenital microbiome on KoRV and *C. pecorum* infections, and vice versa. Future studies would benefit from a greater sample size and a more diverse array of sampled regions both within a single state, and across the country. It would be interesting to follow the same individuals over time to determine if mating and breeding impact the microbiome of the urogenital tract, as occurs in humans[61]. However, animal welfare issues regarding recapturing wild koalas multiple times may make this unfeasible. Additionally, as our study focused solely on female koalas, a follow up survey of the microbiome of the male reproductive tract would be enlightening. Finally, targeted studies assessing the prevalence of organisms associated with wet bottom would increase our understanding of organisms potentially impacting koala populations and could in turn assist with conservation of this iconic species.

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Author Contributions

ARL conceived the project, processed and analysed the data and drafted the manuscript. JAG collected samples used, collected clinical information from sampled koalas and revised the manuscript. ML refined the project and revised the manuscript. LH collected clinical information from koalas and revised the manuscript. JG refined the project and revised the manuscript. JMD conceived and refined the project and drafted the manuscript. FMS conceived and refined the project and drafted the manuscript.

Additional Information

All reads used in the project are available through the NCBI BioProject ID: PRJNA359726. The authors declare that there are no competing financial interests in relation to this research.

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Table 1. Koala metrics and relative abundance data from ten samples submitted for 16S rRNA amplicon sequencing. All koalas were female and sampled from French Island, Victoria, Australia in 2011.

Koala/Sample name	K1	K2	K3	K4	K5	K31	K49	K55	K59	K70
Weight (kg)	6.0	7.4	6.4	7.0	8.0	7.9	7.8	7.3	7.1	9.7
Wet bottom score*	0	0	0	0	0	2	3	3	4	3
Body condition score^	4	4	3	4	3	3	4	3	2	3
Tooth wear class+	II	IVa	III	I	IVb	III	III	IVa	IVc	IVb
KoRV detected	No	Yes	Yes	No	No	No	No	Yes	No	No
Total reads used	225868	178678	169576	203062	166906	162343	177452	216270	192105	254327
Total OTUs	93	66	86	89	74	55	61	74	76	126
Phyla#										
Acidobacteria	-	-	-	-	< 0.01%	-	-	-	-	0.01%
Actinobacteria	5.47%	9.06%	2.92%	0.17%	0.03%	3.27%	0.66%	1.50%	0.30%	0.19%
Armatimonadetes	< 0.01%	< 0.01%	-	-	< 0.01%	-	-	-	-	-
Bacteroidetes	0.57%	0.05%	2.14%	1.72%	0.21%	0.33%	0.05%	9.05%	1.00%	50.53%
Cyanobacteria	< 0.01%	-	< 0.01%	-	-	-	-	-	-	0.02%
Deferribacteres	-	-	-	-	-	-	-	-	-	< 0.01%
Firmicutes	92.92%	89.57%	85.67%	79.17%	98.92%	80.35%	40.92%	84.88%	95.65%	39.09%
Fusobacteria	0.02%	< 0.01%	< 0.01%	0.07%	< 0.01%	< 0.01%	-	< 0.01%	0.02%	1.09%
Planctomycetes	-	-	< 0.01%	-	0.01%	-	-	-	< 0.01%	0.80%
Proteobacteria	0.24%	0.15%	1.66%	1.51%	0.45%	0.23%	56.90%	0.19%	2.37%	2.70%
Synergistetes	0.08%	0.02%	0.30%	0.31%	0.01%	-	-	< 0.01%	0.02%	4.35%
TM7	0.02%	0.50%	0.21%	-	< 0.01%	1.38%	0.05%	2.86%	< 0.01%	0.02%
Verrucomicrobia	< 0.01%	< 0.01%	< 0.01%	-	0.02%	< 0.01%	-	-	0.01%	0.69%
Unassigned	0.69%	0.65%	7.07%	17.04%	0.34%	14.44%	1.42%	1.52%	0.61%	0.52%

* Wet bottom score ranges from 0 (absent) to 10 (most severe)[11]

^ Body condition score ranges from 1 (low/poor condition) to 5 (high/over conditioned). A score of 3 is considered standard[8]

+ Tooth wear class can be used to estimate koala age and ranges from I (young) to VIII (old)[5]

Phyla assigned using QIIME[16] script **assign_taxonomy.py** utilising Greengenes[21] curated 16S rRNA library

Table 2. Significant operational taxonomic units (OTU) assessed using DESeq2[22], ordered from lowest to highest adjusted *P* value. Representative sequences were compared to NCBI nucleotide database using MegaBLAST[62], excluding 'uncultured organisms'

OTU ID	Adjusted *P* value	Higher abundance group*	OTU present in samples/n		NCBI Mega BLAST ^	Nucleotide Identity (%)^	Accession number^
			WB absent	WB present			
38	< 0.001	WB present	0/5	5/5	*Peptoniphilus indolicus*	96.8	NR_117566
21	< 0.001	WB present	1/5	5/5	*Peptoniphilus asaccharolyticus*	100	KP944181
47	< 0.001	WB present	0/5	3/5	*Levyella massiliensis*	100	NR_133039
51	< 0.001	WB present	0/5	3/5	*Peptoniphilus lacrimalis*	100	KM624632
65	0.001	WB present	1/5	2/5	*Sutterellaceae bacterium*	99.5	LK054638
86	0.003	WB absent	3/5	0/5	*Bacteroides thetaiotaomicron*	100	KU234409
75	0.004	WB absent	2/5	0/5	*Clostridium* sp.	96.5	AB622820
4	0.004	WB absent	5/5	5/5	*Lactobacillales* bacterium	92.8	HQ115584
70	0.005	WB absent	2/5	0/5	*Clostridium neopropionicum*	94.6	JQ897394
73	0.005	WB present	0/5	2/5	*Alistipes onderdonkii*	93.6	NR_113151
69	0.005	WB absent	2/5	0/5	*Lachnospiraceae* bacterium	95.3	EU728729
2	0.006	WB absent	5/5	5/5	*Trichococcus* sp.	94.2	KU533824
94	0.007	WB absent	2/5	1/5	*Rhizobiales* sp.	100	KJ016001
95	0.013	WB absent	2/5	0/5	*Rhizobium leguminosarum*	100	KX346599
103	0.019	WB absent	2/5	0/5	*Piscinibacter aquaticus*	88.6	NR_114061
106	0.019	WB absent	3/5	0/5	*Burkholderia cenocepacia*	100	KU749979
109	0.019	WB present	0/5	2/5	*Peptostreptococcus anaerobius*	94.1	NR_042847
148	0.019	WB present	0/5	2/5	*Trichococcus* sp.	87.5	KU533824
159	0.019	WB present	2/5	4/5	*Abiotrophia defectiva*	87.9	JF803600
114	0.019	WB absent	2/5	1/5	*Massilia* sp.	99.8	JF279920
113	0.019	WB absent	3/5	0/5	*Agrobacterium tumefaciens*	100	KU955329
1	0.030	WB present	5/5	5/5	*Aerococcus viridans*	95.1	KC699123
105	0.035	WB present	4/5	5/5	*Aerococcus sanguinicola*	93.0	LC145565
250	0.038	WB present	1/5	2/5	*Hippea* sp.	79.5	FR754504

90	0.038	WB present	1/5	2/5	*Olsenella scatoligenes*	97.8	NR_134781

[*] OTU was detected with significantly higher normalised read counts in koalas with (WB present) or without (WB absent) wet bottom

[^] Organism with the lowest e-value detected using a MegaBLAST search of the NCBI nucleotide database, the nucleotide identity compared to the representative sequence, and the accession number of the hit

Supplementary Material 1. Alpha diversity metrics for microbial communities in the urogenital tract of koalas with and without wet bottom. All metrics assessed at a depth of 160,000 reads, with 100 permutations. *P* values comparing categories are non-parametric t-tests using 10,000 Monte Carlo permutations.

	Wet bottom absent						Wet bottom present						*P* value
	Koala 1	**Koala 2**	**Koala 3**	**Koala 4**	**Koala 5**	**Mean (± SD)**	**Koala 31**	**Koala 49**	**Koala 55**	**Koala 59**	**Koala 70**	**Mean (± SD)**	
Shannon	2.58	2.74	2.97	3.08	1.08	2.49 (± 0.73)	2.37	1.44	2.30	1.81	4.09	2.40 (± 0.91)	0.86
Chao1	97.09	84.91	91.46	92.46	87.57	90.70 (± 4.19)	58.69	76.39	91.54	87.42	127.94	88.39 (± 22.81)	0.83
Richness	88.77	64.11	85.40	88.03	73.70	80.00 (± 9.62)	54.91	59.24	69.23	72.87	123.39	75.93 (± 24.61)	0.81
PD whole tree	9.13	7.03	8.91	7.70	7.93	8.14 (± 0.78)	6.48	6.52	7.81	7.83	10.44	7.98 (± 1.44)	0.71

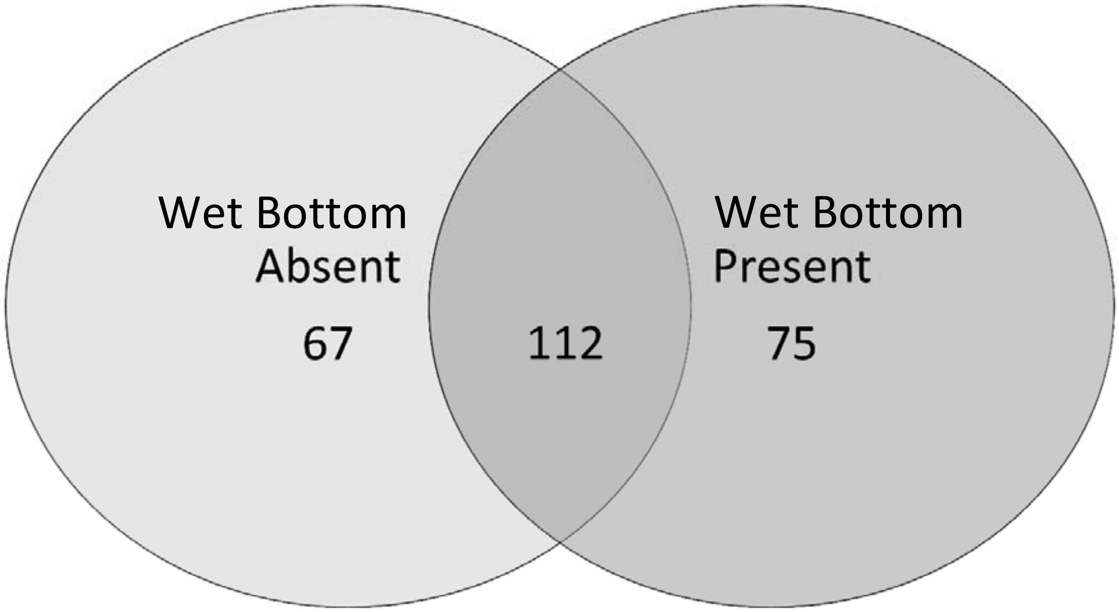


Figure 1. Venn diagram of the total operational taxonomic units (OTUs) detected in koalas with or without wet bottom. Overlap does not scale with OTU number.

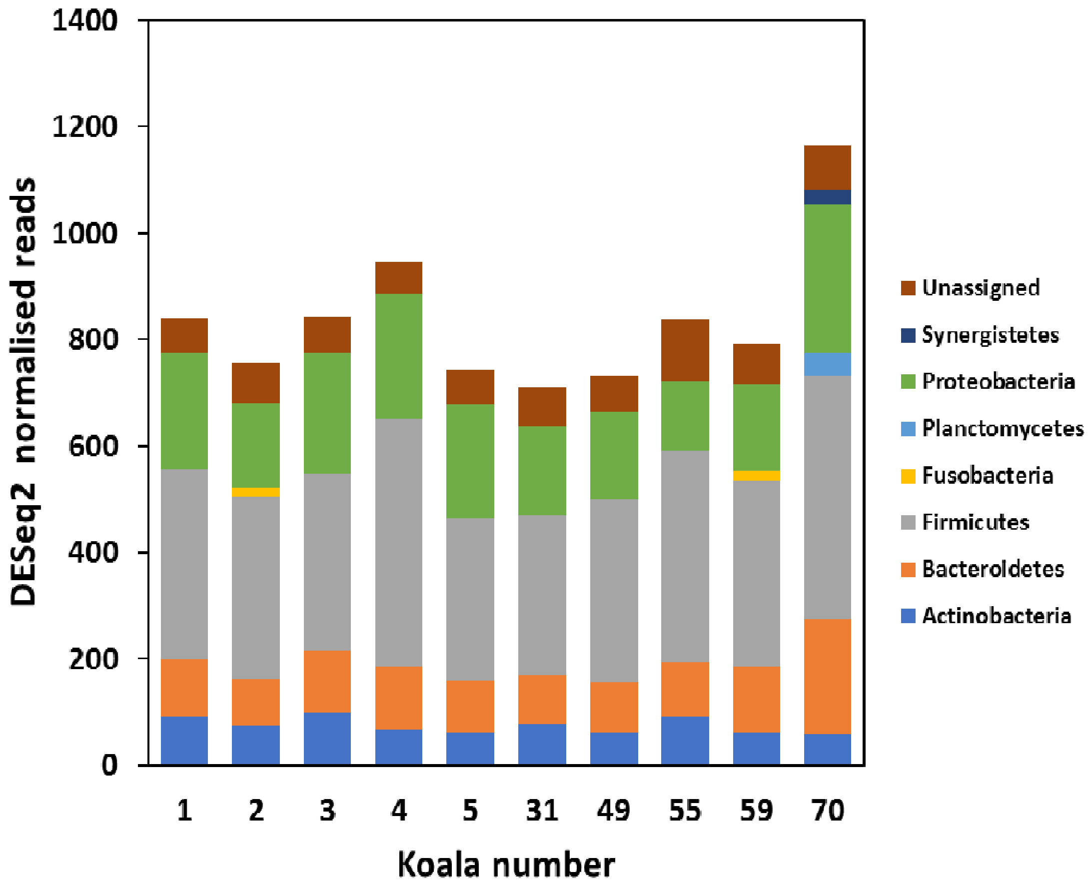
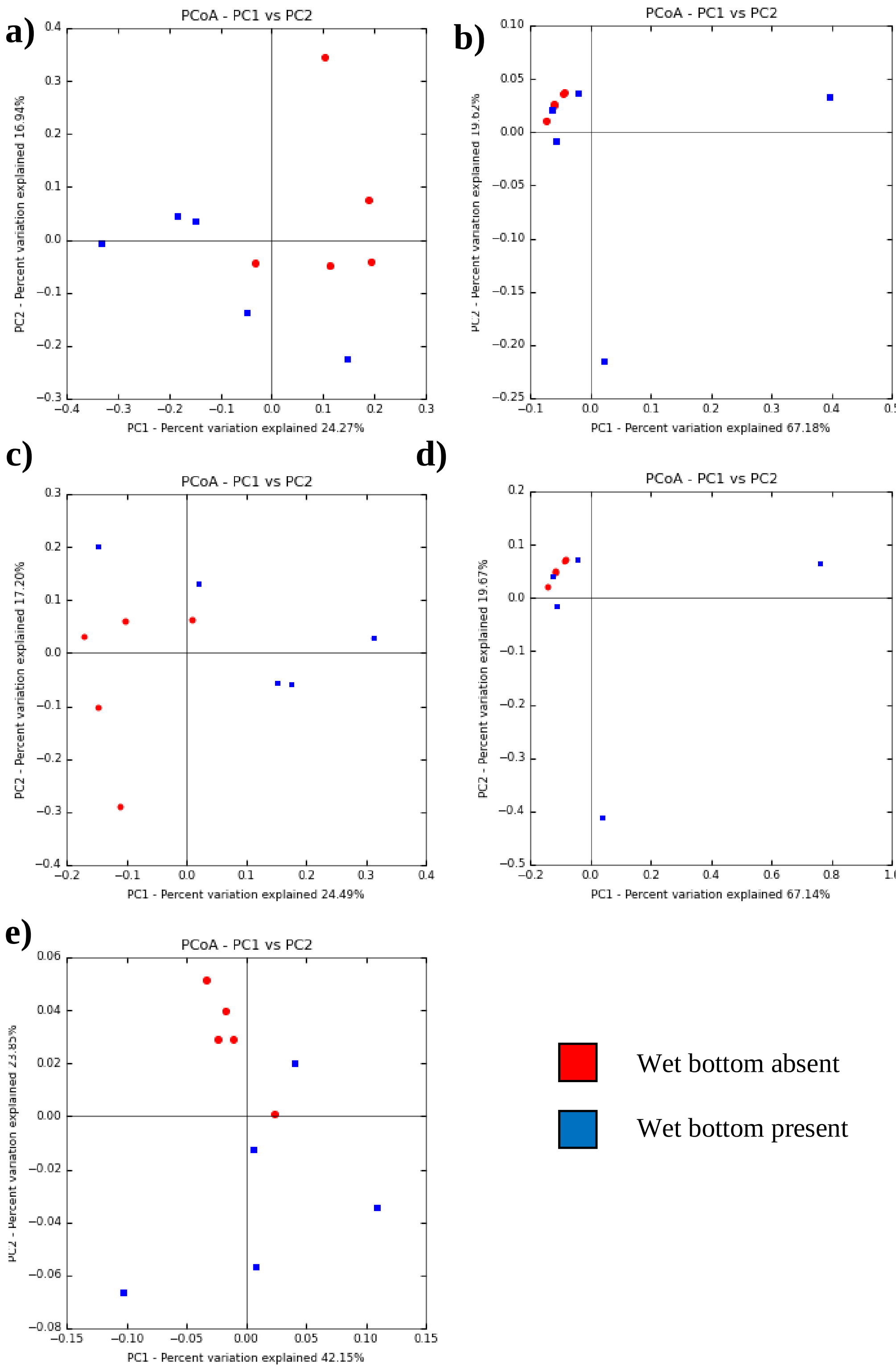


Figure 2. DESeq2 normalised read counts of phyla detected in koala urogenital swab samples. Reads were characterised into taxanomic groups using QIIME[16], utilising Greengenes[21] as a reference database.

Supplementary Material 3. 2D PCoA plots of koala samples, with and without wet bottom, using a) unweighted UniFrac distances of rarefracted reads, b) weighted UniFrac distances of rarefracted reads, c) jackknifed unweighted UniFrac distances to 160,000 reads, d) jackknifed weighted UniFrac distances to 160,000 reads, e) weighted UniFrac distances of normalised reads



Supplementary Material 4. 3D PCoA plots of koala samples, with and without wet bottom, using a) unweighted UniFrac distances of rarefracted reads, b) weighted UniFrac distances of rarefracted reads, c) jackknifed unweighted UniFrac distances to 160,000 reads, d) jackknifed weighted UniFrac distances to 160,000 reads, e) weighted UniFrac distances of normalised reads