The Koala (*Phascolarctos cinereus*) faecal microbiome differs with diet in a wild koala population

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The koala and its gut microbes have evolved together to utilise a low nutrient, potentially toxic diet, composed almost exclusively of eucalypt foliage. We used the 16S rRNA gene to characterise the composition and diversity of faecal bacterial communities (n=32) from a wild koala population comprising individuals that predominately eat one of two different food species, one the strongly preferred and relatively nutritious *Eucalyptus viminalis* (subgenus *Symphyomyrtus*), the other comprising the less preferred and less digestible species *Eucalyptus obliqua* (subgenus *Eucalyptus*). Alpha diversity indices indicated consistently and significantly lower diversity (Shannon) and richness (Chao1) in koalas eating *E. viminalis*. We show a strong association between microbial community composition and host diet. The phyla Bacteroidetes and Firmicutes were codominant in all faecal microbiomes, with Cyanobacteria also codominant in some individuals; however, the *E. viminalis* diet produced communities dominated by the genera *Parabacteroides* and/or *Bacteroides* (Phylum Bacteroidetes), whereas the *E. obliqua*-associated diets were dominated by unidentified genera from the family Ruminococcaceae (phylum Firmicutes). We show that diet differences, even as apparently minor as that between two species within a single plant genus, can profoundly affect the gut microbiome of a specialist folivorous mammal, even amongst individuals in the same population. We identify key microbiota associated with each diet type and predict functions within the microbial community based on 80 previously identified *Parabacteroides* and Ruminococcaceae genomes.

Title page

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Running title: The koala (*Phascolarctos cinereus*) microbiome.

Originality-Significance Statement: This study involved a large cohort of wild (*in-situ*) koalas (n=32), to investigate the gut microbiome of an endangered species that is also a dietary specialist. *Significance:* We analysed and report diet data, which links diet to individual koala gut microbiomes (reported for the first time). Previous koala gut microbiome studies incorporated three koalas (two wild, one sick and one hit by vehicle, and one captive koala) and did not link the gut microbiome to diet eaten.

Summary

The koala and its gut microbes have evolved together to utilise a low nutrient, potentially toxic diet, composed almost exclusively of eucalypt foliage. We used the 16S rRNA gene to characterise the composition and diversity of faecal bacterial communities (n=32) from a wild koala population comprising individuals that predominately eat one of two different food species, one the strongly preferred and relatively nutritious *Eucalyptus viminalis* (subgenus *Symphyomyrtus*), the other comprising the less preferred and less digestible species *Eucalyptus obliqua* (subgenus *Eucalyptus*). Alpha diversity indices indicated consistently and significantly lower diversity (Shannon) and richness (Chao1) in koalas eating *E. viminalis*. We show a strong association between microbial community composition and host diet. The phyla Bacteroidetes and Firmicutes were codominant in all faecal microbiomes, with Cyanobacteria also codominant in some individuals; however, the *E. viminalis* diet produced communities dominated by the genera *Parabacteroides* and/or *Bacteroides* (Phylum Bacteroidetes), whereas the *E. obliqua*-associated diets were dominated by unidentified genera from the family Ruminococcaceae (phylum Firmicutes). We show that diet differences, even as apparently minor as that between two species within a single plant genus, can profoundly affect the gut microbiome of a specialist folivorous mammal, even amongst individuals in the same population. We identify key microbiota associated with each diet type and predict functions within the microbial community based on 80 previously identified *Parabacteroides* and Ruminococcaceae genomes.

Key words: Marsupial, Folivore, Microbiome, Dietary specialist, Plant secondary metabolites (PSMs), Formylated phloroglucinol compounds (FPCs), *Eucalyptus.*

Introduction

Our understanding of the contribution of the gut microbiome to digestive efficiency in vertebrate herbivores, particularly via fermentation and detoxification of low quality diets, is rapidly improving alongside improved technology for molecular sequencing (Flint and Bayer, 2008b; Kohl and Dearing, 2012; Miller *et al.,* 2014, Suzuki, 2017). Previous investigations into the microbiome have shown that gut microbes are essential to hosts and have identified key species that are thought to have co-evolved with their hosts and which normally maintain host homeostasis but can negatively impact the health and wellbeing of the host when disrupted through illness, antibiotic treatment or dietary dysbiosis (Clarke *et al.,* 2012). To identify the role of gut microbes in host health and wellbeing, many studies create dysbiosis on a large scale through administration of high doses of antibiotics (Kohl *et al.,* 2014d), substantial diet change e.g. from carnivorous to herbivorous (David *et al.,* 2014a), or through the addition or removal of toxic components of the diet (Kohl *et al.,* 2014d). Currently, research into the gut microbiomes of specialist folivores is neglected (Barker *et al.,* 2013; Alfano *et al.,* 2015; Barelli *et al.,* 2015).

True obligate dietary specialisation is rare in mammalian herbivores (Shipley *et al.,* 2009) and has been found to promote a highly-conserved but low-diversity gut microbiome in sloths (Dill-McFarland *et al.,* 2015). Dietary specialists often rely on plant species that produce potentially toxic plant secondary metabolites (PSMs) which have often evolved as chemical defences against herbivory and reduce consumption by deterrence or reduction of net nutritional benefit of food (Stupans *et al.,* 2001; Moore and Foley, 2005b; Provenza, 2006). However, gut microbes of specialists can sometimes also enhance tolerance of dietary PSMs, facilitating competition-free access to food sources (Kohl *et al.,* 2014d). For example, Kohl et al. (2014d) demonstrated how microbes facilitate the intake of toxic PSMs using two populations of the specialist desert wood rat (*Neotoma lepida*), one experienced and one naïve to feeding on creosote bush (*Larrea tridentata*), which produces a resin high in the toxic PSM nordihydroguaiaretic acid. Faecal transplantation of microbes from experienced to naïve wood rats increased the tolerance of the latter group to creosote PSMs and antibiotic disruption produced a naïve gut response in experienced wood rats.

The diet of the koala (*Phascolarctos cinereus*) is comprised almost exclusively of foliage from the eucalypt genera *Eucalyptus* and *Corymbia* (family Myrtaceae). However, eucalypts produce a

variety of PSMs including terpenes, cyanogenic glucosides, phenolics including condensed and hydrolysable tannins, formylated phloroglucinol compounds (FPCs) and unsubstituted B-ring flavanones (UBFs) which variously act as toxins, feeding deterrents and digestibility-reducers (Moore *et al.,* 2004a; Moore and Foley, 2005b; Marsh *et al.,* 2015) Concentrations and types of PSMs vary widely between eucalypt species, between individual trees and from region to region (Moore *et al.,* 2004b; Moore *et al.,* 2004c). Koalas living in regions with different eucalypt communities may therefore face differing nutritional and toxicological challenges (DeGabriel *et al.,* 2009b). Koala microbiomes have not previously been assessed in conjunction with diet quality, although linking the two may provide new insights. The 16S gene has previously been used to describe the microbial community structure of the wild (Barker *et al.,* 2013) and captive (Alfano *et al.,* 2015; Shiffman *et al.,* 2017) koala gut microbiome (Barker *et al.,* 2013; Alfano *et al.,* 2015; Shiffman *et al.,* 2017), finding it to be dominated by bacteria from the phyla Bacteroidetes and Firmicutes.

Background - Cape Otway

In southern Australia, some koala populations, usually associated with *Eucalyptus viminalis* (subgenus *Symphyomyrtus*), have repeatedly increased beyond the carrying capacity of their habitat, resulting in eventual population collapse and mass tree dieback (Martin, 1985a). At Cape Otway, Victoria (38°50’06” S, 143°30’25” E), koalas were reintroduced in the 1980s and the population rapidly increased, peaking at densities of up to 18 ha^{-1} in 2013, followed by a rapid population decline, or crash (Whisson *et al.,* 2016). High juvenile recruitment and low mortality of koalas contribute to this phenomenon, but the high nutritional quality of *E. viminalis*, a highly-preferred food tree which dominates areas experiencing overbrowsing, is a key factor. *Eucalyptus viminalis* possesses high foliar nitrogen (N) concentrations for a eucalypt and its tannins have a negligible impact on the nutritional availability of that N (DeGabriel *et al.,* 2008; Marsh *et al.,* 2014), although it also possesses high FPC concentrations that can limit koala feeding (Moore *et al.,* 2005). At Cape Otway, a minority of koalas use the generally non-preferred *Eucalyptus obliqua* (subgenus *Eucalyptus*), usually in forest patches without *E. viminalis*. Trees from the subgenus *Eucalyptus* do not produce FPCs (Eschler *et al.,* 2000), but in contrast to subgenus *Symphyomyrtus*, do possess UBFs, which possibly act to deter koalas as they do another eucalypt folivore, the common brushtail possum (*Trichosurus vulpecula*, Tucker *et al.,* 2010; Marsh *et al.,* 2015). On average, they also possess lower foliar available N (AvailN) concentrations, compared

with *Symphyomyrtus*, due to greater protein precipitation by tannins (Wallis *et al.,* 2010). These patterns present an opportunity to compare and contrast the gut microbial communities of koalas consuming two different eucalypt diets.

Here, we aim to: a) determine the impact of two eucalypt (*E. viminalis* and *E. obliqua*) diets on the abundance of dominant bacterial groups within the koala gut and; b) assess the diet quality of these two species. We hypothesised that the gut microbiome of koalas eating different eucalypt diets would differ in community composition. To test our hypothesis, we collected faecal material (pellets) from a single koala population that included individuals believed to consume two different eucalypt species, during the population peak in 2013 (n=14), and again, from different individuals, post population collapse in 2015 (n=18). We characterised the faecal microbial community as a proxy for gut microbial community composition (Amato *et al.,* 2013; David *et al.,* 2014a) using both terminal restriction fragment length polymorphism, and 16S amplicon sequencing. Diet composition was estimated by faecal plant wax analysis.

Experimental methods

Detailed experimental procedures can be found in the online version of this article under supporting information.

Study species

The koala is a specialist folivorous marsupial that is digestively and morphologically adapted to its challenging diet of eucalypt foliage. Koalas have an extended caecum and proximal colon which function as the major site of microbial fermentation (Cork and Warner, 1983a; Snipes *et al.,* 1993). In addition, they selectively retain solutes and small particulate matter in their hindgut for extended periods of up to 213 hours (Krockenberger and Hume, 2007). These adaptations increase the exposure of digesta to microbial fermentation, while reducing the amount of microbial protein lost in faecal matter. This concentrates the organisms involved in digestive processes within the hindgut, optimising nutrient extraction (Foley and Cork, 1992; Hume, 1993; Hume, 2005). Koala faecal material is expelled as hard, dry pellets.

147 *Faecal sample collection*

148 Faecal pellets were collected from a population of koalas at Cape Otway (38°50'06" S, 143°30'25"
149 E), Victoria, Australia in two collection years, the first from koalas (n=14) during February and
150 September 2013, and the second from koalas (n=18) during January 2015. The peak of the koala
151 population boom occurred at Cape Otway in 2013 and resulted in the eventual death by starvation
152 of hundreds or thousands of koalas (Whisson *et al.,* 2016). In 2013, most koalas in *E. viminalis*
153 patches had limited or no access to adult foliage, and subsisted on epicormic regrowth leaves, these
154 individuals may have experienced some degree of malnourishment. However, by 2015, koala
155 population density had declined, and samples were collected from areas with healthier *E. viminalis*
156 canopies containing a mixture of adult and epicormic foliage.

157 Faecal samples were collected from koalas (n=32) inhabiting forest patches locally dominated by
158 or exclusively containing either *E. viminalis* (n=19) or *E. obliqua* (n=13), and in most cases koalas
159 were observed feeding in the trees they were occupying. Koalas in *E. viminalis* forest at Cape
160 Otway occupy very small (0.9 to 1.0 ha, Whisson *et al.,* 2016) home ranges, allowing us to be
161 confident that *E. viminalis* was the dominant or only food source available for those areas, and
162 similarly in *E. obliqua* areas, *E. viminalis* was absent or entirely defoliated. In 2013, koalas were
163 present in high densities and signs of overbrowsing or severe defoliation were universal in *E.*
164 *viminalis* patches while koalas were much less abundant and overbrowsing was less apparent in *E.*
165 *obliqua* patches.

166 Mats were placed under koalas in trees and checked at intervals of no more than four hours
167 throughout the day. Fresh faecal pellets were counted and collected from mats, placed into zip-
168 lock bags and placed on ice until they could be transferred, within 2 hours, into a -20°C freezer for
169 storage. Studies into the impact of storage conditions on results of 16S ribosomal RNA (rRNA)
170 sequencing from faecal material have concluded that phylogenetic structure and community
171 diversity are not significantly impacted by either short-term storage at 4 °C or 20 °C (< 24 h), or
172 long-term storage at -20 °C or -80 °C (Carroll *et al.,* 2012, Lauber et al 2010).

173 Due to the non-invasive nature of faecal collection, faeces have been extensively used to study gut
174 microbial composition and ecology in humans and animals during both health and disease
175 (Turnbaugh *et al.,* 2009a; David *et al.,* 2014a; Dill-McFarland *et al.,* 2015; Lichtman *et al.,* 2015).
176 Previous studies have found differences in microbial community composition between

gastrointestinal and faecal samples (Stearns *et al.,* 2011; Alfano *et al.,* 2015). The use of faeces as a proxy for the gut microbial community has nonetheless revealed important and biologically meaningful findings in both humans and animals (Amato *et al.,* 2013; David *et al.,* 2014a) and is the only non-invasive method available for sample collection from wild animals.

Analysis of Bacterial community through rRNA gene sequencing and analysis

Amplification and sequencing of the V4 region of the bacterial 16S rRNA gene was undertaken using a previously established protocol and primers 515F and 806R from Caporaso *et al.,* (2011a). Paired-end 16S rRNA community sequencing was performed using the Illumina MiSeq® platform at the Ramaciotti Centre for Genomics (UNSW, Australia; 2013 koala faecal samples) and the Next-Generation Sequencing Facility at Western Sydney University (Richmond, Australia; 2015 koala faecal samples), using the same protocol, primers and Illumina platform. Analyses of sequence data were performed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline, version 1.8 (Caporaso *et al.,* 2010b). Sequences were quality-checked and low quality (< Q30) sequences were removed from further analysis. Sequences were aligned against the Greengenes 13_8-release database (DeSantis *et al.,* 2006) and potentially chimeric sequences (~4%) were removed using Chimera Slayer (Haas *et al.,* 2011). Sequences were aligned (PyNAST, Caporaso *et al.,* 2010a), and clustered (uclust, Edgar, 2010) into operational taxonomic units (OTUs) defined as sharing 97% sequence identity (hereafter, 'taxa'). Samples were rarefied to the smallest dataset consisting of 107 813 sequences, and alpha diversity measures including Chao1 (measure of species richness), and Shannon indexes (diversity) were calculated (Good, 1953).

Beta diversity and relative abundance of taxa were assessed using the phylogenetic distance-based measurement weighted and unweighted UniFrac (diversity, Lozupone *et al.,* 2011). Taxonomic identities at all level were assigned by default in QIIME using the Ribosomal Database Project (RDP, Wang *et al.,* 2007). PRIMER v 7.0.13 (Clarke, 1993) and PERMANOVA+ (Anderson, 2001) were used to conduct multivariate statistical analysis of the summarised OTU tables generated through QIIME as described for T-RFLP analysis (see supporting information for details). Multivariate statistical analysis of the weighted and unweighted UniFrac matrices were also conducted using PRIMER v 7.0.13 (Clarke, 1993) and PERMANOVA+ (Anderson, 2001) without data transformation, PERMANOVA models used for analysis of combined beta diversity and OTU tables, consisted of diet: fixed and collection: random, for analysis of the influence of

diet on the beta diversity and OTU tables from individual collection years, diet: fixed and koala: random respectively. PCoAs for the UniFrac metrices were generated in R studio using ggplots (Wickham, 2009; R Development Core Team, 2013). The variation in total microbial relative abundance across the samples was assessed using ANOVA and Tukey's post-hoc tests.

Cyanobacteria identity and chloroplast contamination assessment

To confirm identity of sequences identified as Cyanobacteria, we used the CLC genomics workbench software v 7.5 (CLC bio) to perform a basic local alignment search-nucleotide (BLASTn, Altschul *et al.,* 1990) analysis on the 16S rRNA gene sequences, and the previously identified rumen bacterium *YS2* 16S rRNA gene (accession number AF544207). To check for chloroplast contamination, a BLASTn analysis was performed on the 16S rRNA gene sequences, identified as Cyanobacteria *YS2*, and the 16S rRNA gene sequence from *Eucalyptus grandis* chloroplasts (accession number HM347959.1, Paiva *et al.,* 2011).

Leaf collection method

Eucalypts respond to severe defoliation by producing abundant epicormic growth, which is ontogenetically more similar to juvenile than to typical adult foliage. In the case of *E. viminalis*, epicormic foliage accounted for the majority of foliage available to koalas in 2013. Epicormic and adult *E. viminalis* (n=16) and *E. obliqua* (n=11) leaves were collected at Cape Otway in September 2013. Leaves were placed into labelled zip-lock bags and frozen at -20°C until processing. They were subsequently freeze-dried and ground to pass a 1 mm screen using a CT 193 Cyclotec™ Sample Mill (Foss, Mulgrave, Victoria, Australia).

Diet composition

The chemical composition of cuticular wax often differs among the foliage of different species of higher plants. Due to their ability to traverse the gastrointestinal tract relatively intact, *n*-alkanes are the most frequently used wax marker in diet composition studies (Dove and Mayes, 2005). Therefore, we used the *n*-alkane protocol described by Dove and Mayes (2005) to estimate diet composition. Analysis was performed on an Agilent 7890A gas chromatograph coupled with an Agilent 5975C MSD (Agilent Technologies Pty Ltd, Mulgrave, VIC, Australia; details provided in the supporting information). To estimate koala diet composition, 6 *n*-alkane peaks (C_{23}, C_{25}, C_{27}, C_{28}, C_{29} and C_{31}) were identified and quantified in leaf (n = 4 samples per species) and faecal

samples using the Agilent MSD Chemstation v E.02.02 software package (Agilent Technologies Pty Ltd). Estimates of diet composition were determined following the calculations protocol described by Dove and Mayes (2005).

Diet quality

Nitrogen, tannins and fibre are key determinants of herbivore diet quality. In the common brushtail possum *(Trichosurus vulpecula*), a generalist marsupial folivore that also eats *Eucalyptus*, a measure of available N (AvailN, DeGabriel *et al.,* 2009b) determined using an *in vitro* digestion, has successfully predicted reproductive success of free-living individuals (DeGabriel *et al.,* 2009a). We implemented the assay described by DeGabriel et al. (2008), which returns the following measures of foliar quality: *in-vitro* dry matter digestibility (DMD), total nitrogen concentration (N) and available, or digestible, N (AvailN). We analysed both epicormic (*E. viminalis* n=9 and *E. obliqua* n=5) and adult (*E. viminalis* n=7 and *E. obliqua* n=6) leaf samples. Total N concentrations for leaf material and their residues after digestion, were measured by a combustion method based on the Dumas method (Leco Corporation 2003) using the Leco C/N analyser (Leco Tru Mac® Corporation, Michigan, USA). Analysis of the *in-vitro* digestion data was conducted using PRIMER v 7.0.13 and PERMANOVA+ to assess overall differences in quality between the two diets, for assessment of the differences within and between diet variables including mean total N, mean DMD, mean N digestibility (Ndig) and mean AvailN, the Students *t*-test was used.

Results

Bacterial community rRNA gene sequencing

A total of 33,102,252 reads with 31,832,423 remaining post-sequence quality control were obtained from the 32 samples sequenced (see supporting information, Table S1). Faecal microbial communities of koalas eating *E. viminalis* were significantly less diverse (Shannon 4.60 ± 0.07 vs 5.30 ± 0.07; ANOVA P=0.001) and less taxonomically rich (Chao 8,100 ± 591 vs 10,314 ± 55; ANOVA P=0.001) than those of koalas eating *E. obliqua* (see supporting information, Table S2). We assessed whether differences between the faecal bacterial communities associated with the two diets were driven by community structure, i.e. differences in relative abundance of taxa (weighted

UniFrac, Lozupone *et al.,* 2011), or by altered presence / absence of microbial taxa (unweighted UniFrac) using the 16S rRNA genes retained after removal of chimeric sequences. These β-diversity matrices assess the extent of branch sharing on a master phylogenetic tree, weighting branches by the relative abundance of taxa (Lozupone *et al.,* 2011). The UniFrac PCA biplots revealed a clear separation based on the PC1 and PC2 axes of the weighted scatter plot, explaining 63% of the total variation between the two collections (Fig. 1a), reinforcing the observations from T-RFLP analysis (see supplementary information Fig. S1). The unweighted scatter explained 18% of the total variation (Fig. 1b). PERMANOVA analysis indicated no influence of collection year on the weighted UniFrac data (*Pseudo* F_1=0.44, PERMANOVA P=0.81), but a significant influence when we analysed the unweighted UniFrac data (*Pseudo* F_1=1.76, PERMANOVA P=0.01). When we analysed diet × collection years an influence was detected on community structure (weighted UniFrac, *Pseudo* F_2=10.07, PERMANOVA P=0.0001) and presence / absence of microbial taxa (unweighted UniFrac, *Pseudo* F_2=2.98, PERMANOVA P=0.0001).

PERMANOVA assessment of the weighted and unweighted UniFrac matrices from individual collection years (2013 and 2015), indicated that diet was a strong driver of both microbial community structure (relative abundance), and of microbial presence / absence during 2013 (weighted UniFrac, *Pseudo* F_1=5.88, PERMANOVA P=0.0001; unweighted UniFrac, *Pseudo* F_1=1.89, PERMANOVA P=0.0001). The influence of diet was also significant in 2015 (weighted UniFrac, *Pseudo* F_1=8.89, PERMANOVA P=0.0001; unweighted UniFrac, *Pseudo* F_1=2.76, PERMANOVA P=0.0001). PCoA analysis of the weighted and unweighted UniFrac matrices for the 2013 koala collection, showed a clear separation linked to diet, with 75% of total variation explained by PC1 (weighted UniFrac, Fig. 2a), while the unweighted UniFrac matrix also indicated an influence of diet, although the two axes explained less (30%) of the total variation (Fig. 2b). The 2015 koala collection scatters show a smaller separation occurring, with PC1 and PC2 of the weighted plot explaining 65% of the total separation (Fig. 3a). The first two principal components from the unweighted UniFrac matrix explained just 18% of the total variation for the 2015 koala collection (Fig. 3b). Similar patterns were observed during T-RFLP analysis of the two collection years (see supplementary information for details and Fig. S2a and S2b).

Analysis of phylum-level OTUs indicated a separation between diets for both 2013 and 2015 collections (2013, *Pseudo* F_1=65.47, PERMANOVA P=0.0001; 2015 *Pseudo* F_1=24.95, PERMANOVA $P < 0.0001$ respectively). In 2013, there was an almost three-fold increase in

relative abundance of Bacteroidetes in *E. viminalis* koalas, compared with an almost three-fold increase in relative abundance of Firmicutes in the *E. obliqua* koalas (Table 1). The same pattern in relative abundance was observed in the 2015 koala faecal microbiomes, although to a lesser extent (Table 1). There were also significant differences in the relative abundance of some phyla between collection years. For example, the phylum Synergistetes was more abundant in the faecal microbiomes of koalas eating both *E. viminalis* and *E. obliqua* in 2015 (Table 1). Cyanobacteria increased in relative abundance in the 2015 *E. viminalis* faecal microbiomes by 15 × that of the *E. viminalis* 2013 faecal microbiomes, where *E. obliqua* faecal microbiomes had an 75% increase in relative abundance of Cyanobacteria (Table 1). Interestingly, the difference in relative abundance of Cyanobacteria between the two diets reversed in 2015 (Table 1). BLASTn analysis of the sequences identified as Cyanobacteria order *YS2* confirmed that they are Cyanobacterial sequences (between 91 and 96% identity with the 16S rRNA gene from rumen bacterium *YS2* accession number AF544207) and not chloroplast contamination (between 74 and 88% identity with the 16S rRNA gene from *Eucalyptus grandis* chloroplast genome accession number HM347959.1 (Paiva *et al.,* 2011).

Analysis of genus-level OTUs also revealed a significant separation between koala diets in 2013 (*Pseudo* F_1=56.08, PERMANOVA P=0.001; see supporting information, Fig. S3a) and 2015 (*Pseudo* F_1=17.04, PERMANOVA P=0.001; see supporting information, Fig. S3b). In 2013, there were notable diet-associated differences in relative microbial abundance, in particular, the *E. viminalis* koala faecal microbiomes were dominated by *Parabacteroides* (52 ± 4; Fig. 4a; see supporting information, Table S3), while the faecal microbiomes of *E. obliqua* koalas were dominated by an unknown genus from the family Ruminococcaceae (45 ± 1; Fig. 4a; see supporting information, Table S3). BLASTn database searches (Altschul *et al.,* 1990) were unable to improve taxonomic resolution.

In 2015, the genus dominating faecal microbiomes of koalas eating *E. viminalis* was *Bacteroides* (25 ± 5; Fig. 4b; see supporting information, Table S3). The relative abundance was 3.5 × that seen in the 2013 *E. viminalis* koala faecal microbiomes, while the relative abundance of *Parabacteroides* was 42% lower in the 2015 *E. viminalis* koala faecal microbiomes compared with the 2013 (Fig. 4a & 4b; see supporting information, Table S3). In both 2013 and 2015, *E. obliqua* faecal microbiome communities were dominated by the family Ruminococcaceae (31 ± 4; Fig. 3a & 3b; see supporting information, Table S4). However, the 2015 *E. obliqua* faecal bacterial

communities had almost four × the *Parabacteroides* (19 ± 4), and half the *Bacteroides* relative abundances compared with 2013 *E. obliqua* faecal microbiome communities (Fig. 4a & 4b; see supporting information, Table S3). Other genera represented in both 2013 and 2015 faecal microbiomes at relative abundances from 3 to 13% include *Acidaminococcus*, *Akkermansia*, *Coprobacillus*, *Clostridium, Oscillospira* and *Ruminococcus*, (Fig. 4a & 4b; see supporting information, Table S3). Relative abundance of these OTUs showed differences within and / or between the two collection years and diet types, while the remaining genera did not show significant differences (Fig. 4a & 4b; see supporting information, Table S3).

Functional differences between previously identified and publicly available Parabacteroides and Ruminococcaceae genomes

Due to the dominance and significant changes in relative abundance of *Parabacteroides* and Ruminococcaceae we analysed 35 previously identified and publicly available, *Parabacteroides* and 45 Ruminococcaceae genomes and identified glycoside hydrolase (GH) families involved in the degradation of plant cell walls, starch and other components ranging from easily degraded to recalcitrant (see supporting information, Fig. S4). In general, we found that the *Parabacteroides* genomes (associated with *E. viminalis* diets) possessed more genes for oligosaccharide degradation than Ruminococcaceae genomes, while Ruminococcaceae genomes (more strongly associated with *E. obliqua* diets) possessed up to five × the number of enzymatic genes targeting the degradation of recalcitrant cellulose (see supporting information, Fig. S4). *Parabacteroides* genomes also have more genes from the GH67 and GH85 families, which are involved in the degradation of xylan and chitin, respectively. Interestingly, *Parabacteroides* genomes potentially have more genes associated with tannin degradation than Ruminococcaceae (Fig. S4). ATP-binding cassette (ABC) and phosphotransferase system (PTS) transporter genes were more common in Ruminococcaceae (average of 134 ABC and 18 PTS per genome, see supporting information, Table S5) than in *Parabacteroides* (82 ABC and 6 PTS, see supporting information, Table S5).

Diet composition

Because of the strong association observed between diet and microbiome, we analysed koala faecal pellets to confirm our expectation of diet composition (based upon koala location and tree

356 occupancy), using methods established to estimate composition of diets using the *n*-alkanes that
357 occur in the leaf cuticle of all plants as markers (Dove and Mayes, 2005).

358 Analysis of *n*-alkane markers in *E. viminalis* and *E. obliqua* leaf material showed significantly
359 different profiles between species, with *E. obliqua* cuticle dominated by the C_{27} chain-length *n*-
360 alkane (78 ± 3 of total alkanes) and *E. viminalis* dominated by C_{29} (91 ± 3). The relative abundance
361 of the C_{25} alkane was also 7 times greater in *E. obliqua* than *E. viminalis*. Koala faecal alkane
362 profiles clearly separated our two diet categories and largely confirmed our expectations about diet
363 composition based on tree canopy composition at koala pellet collection sites. In 2013, the *n*-
364 alkane method estimated that 8/9 "*E. viminalis"* koalas included > 80% *E. viminalis* in their diets,
365 and 5/5 "*E. obliqua*" koalas included > 80% E. *obliqua* (Fig. 5a). In 2015, 8/8 "*E. viminalis*" diets
366 were estimated to contain > 70% *E. viminalis*, and 6/7 "*E. obliqua*" diets > 50% *E. obliqua* (Fig.
367 5b).

368 *Diet quality*

369 Nutritional analysis of *E. viminalis* and *E. obliqua* epicormic and adult leaves indicated that overall
370 *E. viminalis* provided koalas with foliage of higher nutritional quality (i.e. greater *in vitro* dry
371 matter digestibility (DMD), available (or digestible) N (AvailN) and total foliar N concentrations;
372 *Pseudo* F_1=36.43 and PERMANOVA P=0.001) compared with E. *obliqua* foliage (Table 2). We
373 confirmed that adult *E. viminalis* foliage contained less recalcitrant fibre (i.e. hemicellulose, lignin
374 and cellulose) than adult *E. obliqua* foliage, using the neutral detergent fibre (NDF) assay (Van
375 Soest *et al.,* 1991; P=0.003, Table 2).

376

377 **Discussion**

378 Dramatic diet changes in humans and rodents e.g. switching from carnivorous to herbivorous diets,
379 can profoundly affect the microbial community structure in the gut (Turnbaugh and Gordon,
380 2009b; David *et al.,* 2014a). Here we show that equally dramatic effects can be produced, even
381 within a continuous animal population, by the relatively subtle differences between two different
382 *Eucalyptus* species. Bacterial communities associated with each of the two diets differed primarily
383 in the relative abundance of two phyla, Bacteroidetes and Firmicutes, rather than in presence and
384 absence of abundant taxa similar to changes seen in humans when undergoing major diet change

(David *et al.,* 2014a). Alpha diversity was significantly lower in terms of Shannon diversity and richness (Chao1) in koalas eating the nutritionally superior *E. viminalis*. Low microbial richness has been convincingly linked to higher feed efficiency in ruminants (Shabat *et al.,* 2016). In our study, lower diversity and richness is likely to be associated with a greater energy harvest from *E. viminalis* compared to *E. obliqua*. Efficient microbiomes are often less complex but more specialised, which creates higher availability of ecosystem goods, such as energy resources, to the host (De Groot *et al.,* 2002; Shabat *et al.,* 2016). In addition, the general rearrangement of taxa within the less diverse phyla Bacteroidetes and the more diverse Firmicutes (Parks *et al.,* 2018) in response to change in diet, could also contribute to lower richness and diversity in koalas eating *E. viminalis* compared to *E. obliqua*. Southern Australian koalas from *E. viminalis* forests and woodlands are unusual in specialising on a single eucalypt species, but such specialisation over many generations may have resulted in an equally unusual, specialised microbiome.

Associations between diet and the relative abundance of Bacteroidetes and Firmicutes have been linked to the functional potential of the gut microbiome in humans and animals (Muegge *et al.,* 2011). For instance, Bacteroidetes genomes encode GH enzymes targeting a wide variety of relatively easily degraded plant components including non-cellulosic polysaccharides, oligosaccharides and glycogen (Hooper *et al.,* 2002). In contrast, Firmicutes genomes encode GHs targeting cellulases and xylanases that would be beneficial in an environment dominated by recalcitrant fibre (Ben David *et al.,* 2015). High prevalence of Firmicutes and a diminished relative abundance of Bacteroidetes has been associated with adjustments of the microbiome to increased fibre intake and reduced protein consumption during transitions from animal to plant diets (David *et al.,* 2014a; Ben David *et al.,* 2015). Similarly, shifts in the Bacteroidetes: Firmicutes ratio in koalas eating *E. obliqua* could be due to the lower available protein and higher fibre content of *E. obliqua* as compared to *E. viminalis*.

We identified several further potential functional differences in microbiomes associated with diet in koalas. Koalas eating *E. viminalis* hosted bacterial communities dominated by *Parabacteroides*, the genomes of which usually encode multiple enzymes that sense, bind and metabolise a variety of oligosaccharides (Mahowald, 2010). These may represent a larger fraction of leaf material in *E. viminalis* foliage, which is 50% more digestible *in-vitro* than *E. obliqua,* which have more recalcitrant cell wall components compared with adult leaf foliage from *E. viminalis* (48 and 38% respectively). However, caution is required in interpretation of the NDF results as tannins are

known to inflate NDF measurements, leading to an overestimation of fibre (Makkar *et al.,* 1995). The microbiome associated with *E. obliqua* was dominated by the family Ruminococcaceae. Ruminococcaceae have smaller genomes than *Parabacteroides*, with fewer glycan-degrading enzymes, and are suited to the degradation of more varied dietary carbohydrates (Biddle *et al.,* 2013; Ben David *et al.,* 2015). They have more ABC and PTS transporters that may provide a competitive advantage over *Parabacteroides*, by facilitating faster bacterial uptake of sugars (Biddle *et al.,* 2013). The gut lumen of koalas eating *E. obliqua* would have higher concentrations of tannins and recalcitrant fibre, so while the observation of greater cellulose-degrading functionality associated with Ruminococcaceae is expected, the potentially greater tannin-degrading functionality of *Parabacteroides* is more surprising. Little is known of qualitative variation in tannin composition among eucalypt species.

Cyanobacteria (the order *YS2*) were observed at high relative abundance (up to 30%) in many of our samples, particularly in 2015 where the average relative abundance for koalas eating *E. viminalis* was 15%. Such high relative abundances are unprecedented in gastrointestinal microbiomes. Ley *et al.,* (2008a) reported an average relative abundance of 1% across mammals generally, although higher relative abundances have been reported e.g. 5% from a domestic cow (Bos *taurus*) and a capybara (*Hydrochoerus hydrochaeris*, Ley *et al.,* 2008a), 6.7% and 9% for individual koalas (Soo *et al.,* 2014; Shiffman *et al.,* 2017), and 4.7% and 4.5% for domestic rabbits and American pikas *(Oryctolagus cuniculus*, Zeng *et al.,* 2015; *Ochotona princeps*, Kohl *et al.,* 2017). Non-photosynthetic Cyanobacteria, now placed within the candidate phylum Melainabacteria (Di Rienzi *et al.,* 2013; Soo *et al.,* 2014), have only recently been recognized in gastrointestinal microbiomes, they are obligate anaerobic fermenters and syntrophic hydrogen producers which may benefit the host by the synthesis of vitamins B and K (Di Rienzi *et al.,* 2013), and which are enriched in Kyoto Encyclopedia of Genes and Genomes (KEGG) Ortholog groups specific to amino acid metabolism, relative to other cyanobacteria (Harel *et al.,* 2015).

The relatively low relative abundance of Proteobacteria (1%) in koala faeces from Cape Otway is less than that reported from faecal samples across mammals and herbivores generally (8.8% and 5%, Ley *et al.,* 2008a; Nelson *et al.,* 2013) and lower than detected in the folivorous two-and three-toed sloths (*Choloepus hoffmanni* and *Bradypus variegatus*, 60%; Dill-McFarland *et al.,* 2015). It is also substantially lower than that observed in zoo koalas by Shiffman *et al., (*2017) and Barker

446 *et al.,* (2013; 15% and 2 - 9% respectively), but consistent with the observations of Alfano *et al*.,
447 (2015).

448 Low-relative abundance microbes can also be functionally important in the gut microbiome (Qin
449 *et al.,* 2010). *Synergistes* is an anaerobic fermenter of some amino acids (Allison *et al.,* 2015), and
450 by fermenting the toxic amino acid, mimosine, in the forage legume, *Leucaena leucocephala*,
451 protects ruminant hosts from toxicosis (Allison *et al.,* 1992). *Synergistes* is also a member of a
452 consortium that can protect sheep from pyrrolizidine alkaloid toxicosis (Lodge-Ivey *et al.,* 2005;
453 Rattray and Craig, 2007) and can anaerobically degrade fluoracetate (Davis *et al.,* 2012). Shiffman
454 *et al.,* (2017) suggested that *Synergistaceae* may play as-yet unknown roles in addition to those
455 above, including the degradation of plant toxins from *Eucalyptus.* On the basis of its metabolic
456 potential and the relatively high abundance of *Synergistaceae* that they observed in koalas relative
457 to most other gut ecosystems, they also identified it as the most likely core specialised member of
458 the koala microbiota. However, this phylum occurred at lower concentrations in faecal
459 microbiomes from Cape Otway, particularly in 2013 where mean abundances were as low as
460 0.0004% (*E. obliqua*). This suggests that the key role of *Synergistaceae* in allowing the koala to
461 subsist on *Eucalyptus*, as proposed by Shiffman *et al.*, (2017), is either not essential at least for
462 some koala diets, or can be filled by other bacteria.

463 Another population previously identified by Shiffman *et al.,* (2017) as discriminating the koala
464 microbiota from the wombat microbiota is the family *S24-7* (phylum Bacteroidetes), which they
465 observed at a mean relative abundance greater than 10% in zoo koalas, and which they linked to
466 dietary specialisation in *Eucalyptus*. Ormerod *et al.,* (2016) detected two trophic guilds (α-glucan
467 and plant glucan) among *S24-7* population genomes isolated from a koala; these genomes were
468 remarkable for their large size relative to other *S24-7*. Shiffman *et al.* (2017) also detected a full
469 suite of ureolysis genes in an *S24-7* genome acccounting for ~8% of the faecal microbial
470 community. However, *S24-7* was not found amongst wild Cape Otway koalas, potentially
471 reinforcing the findings of Barker *et al.,* (2013), who detected S24-7 at only low abundance
472 (0.07%) in the caeca of two wild koalas, but not in their faecal pellets.

473 In common with other culture-independent investigations of the koala microbime (Alfano *et al.,*
474 2015; Barker *et al.,* 2013; Shiffman *et al.,* 2017), our study also found that tannin-protein complex
475 degrading bacteria (Enterobacteriaceae, Pasteurellaceae and Streptococcaceae) previously

476 cultured by Osawa and colleagues (Osawa 1990; Osawa 1992; Osawa et al., 1993; Osawa et al.,
477 1995) were rare and occurred at low abundance (4 and 1% for 2013 and 2015 collections
478 respectively) in wild koala faeces.

479 In addition to community differences associated with diet, differences were also apparent between
480 the two collection years. For instance, *Bacteroides* relative abundance was three × greater in *E.*
481 *viminalis* communities in 2015 than in 2013, while *Parabacteroides* relative abundance was lower
482 in *E. viminalis* but 47% greater in *E. obliqua* diets in 2015 compared to 2013. We speculate that
483 these differences might be explained by differences in food availability, leaf type and/or nutritional
484 status for *E. viminalis* koalas between 2013 and 2015. Therefore, observed differences in microbial
485 relative abundance between collection years might be explained by a return to a "normal diet" after
486 the peak of overbrowsing associated with peak koala population densities; this would be consistent
487 with the diet composition analysis, which suggested that all koalas were eating *E. viminalis* to
488 some extent in 2015. Periodic fasting through dietary restriction or seasonal hibernation has been
489 found to alter the microbial community structure in mammals (Clarke *et al.,* 2012). In particular,
490 other studies have observed increases in acetate-producing bacteria including *Akkermansia*
491 *muciniphila* during periods of fasting, and suggested that under these circumstances, host-derived
492 polysaccharides such as mucins are used as a substrate to produce short-chain fatty acids that
493 support the host (Carey *et al.,* 2013; Derrien *et al.,* 2008; Sonoyama *et al.,* 2009). The relative
494 abundance of *Akkermansia* was notably elevated (between 1 and 13%) in some of the koalas eating
495 *E. viminalis* in 2013, and this may indicate that these individuals were experiencing a shortage of
496 food. However, despite the differences in microbial relative abundance associated with collection
497 period, the gut microbial communities associated with the two different diets remained distinct
498 throughout.

499

500 **Conclusion**

501 Here we have shown that even a seemingly subtle dietary change can modulate the microbiome of
502 a specialist herbivore. Based on our results, we postulate that diet preferences and the availability
503 of resources will substantially impact the structure of gut microbial communities of koalas more
504 widely, with consequences for the efficiency of digestion of their complex diet. As strong regional
505 differences have been observed in the diet composition of koalas across their geographic range,

translocated and released koalas (e.g. after overpopulation, habitat degradation, or rehabilitation) and those treated with antibiotics may not have the same microbiome as natural populations. A priority area of research should be to determine whether the therapeutic or prophylactic alteration of koala microbiomes can assist management and welfare outcomes for koalas facing enforced dietary change. New conservation and management strategies could include the development of targeted inoculations, thereby facilitating an increased dietary breadth for koalas, as demonstrated by Kohl *et al.,* (2014d) and Kohl *et al.,* (2015) in the specialist desert woodrat.

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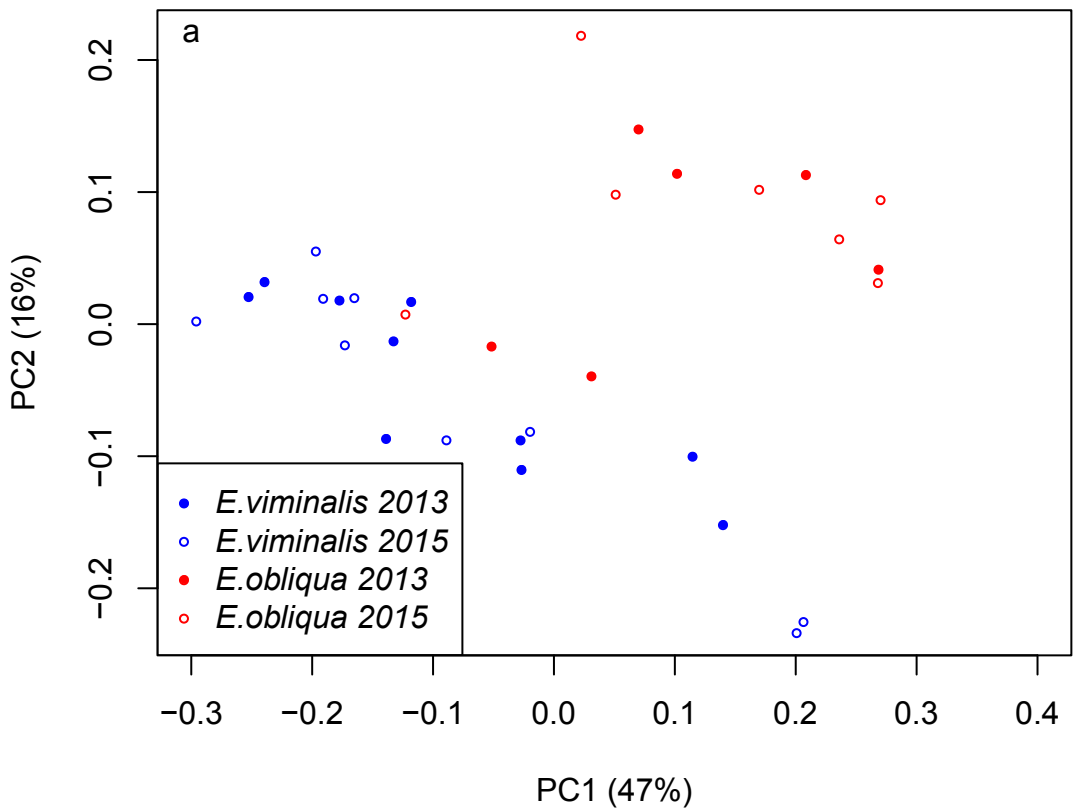
Figure 1(on next page)

PCoA of β diversity of koala faecal bacterial communities from Cape Otway.

Scatterplots from weighted (a) and unweighted (b) UniFrac matrices for the combined Cape Otway koala population with diets comprising *E. viminalis* and *E. obliqua* from 2013 and 2015.


a
PC2 (16%)
PC1 (47%)
E.viminalis 2013
E.viminalis 2015
E.obliqua 2013
E.obliqua 2015

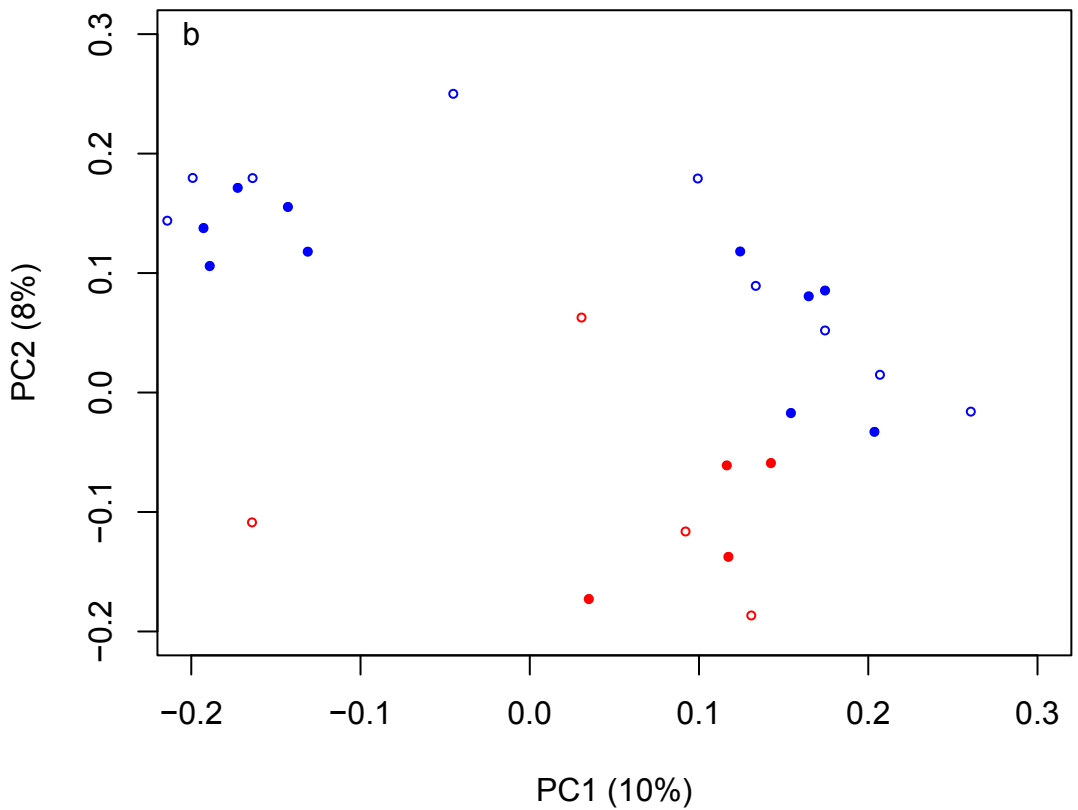
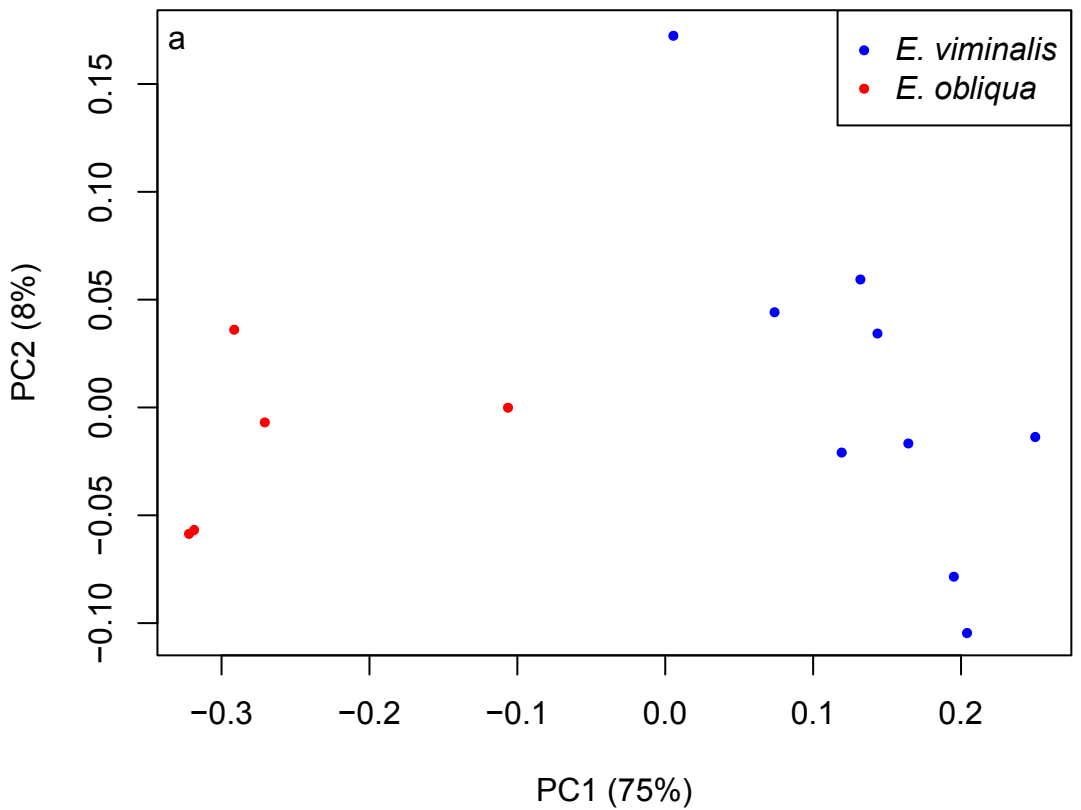

b
PC2 (8%)
PC1 (10%)

Figure 2(on next page)

PCoA of β diversity based on UniFrac matrices from the Cape Otway 2013 koala collection.

Scatterplots from weighted (a) and unweighted (b) UniFrac matrices for koalas with diets of *E. viminalis* and *E. obliqua* from the 2013 collection year.


a
E. viminalis
E. obliqua
PC2 (8%)
PC1 (75%)
−0.10
−0.05
0.00
0.05
0.10
0.15
−0.3
−0.2
−0.1
0.0
0.1
0.2

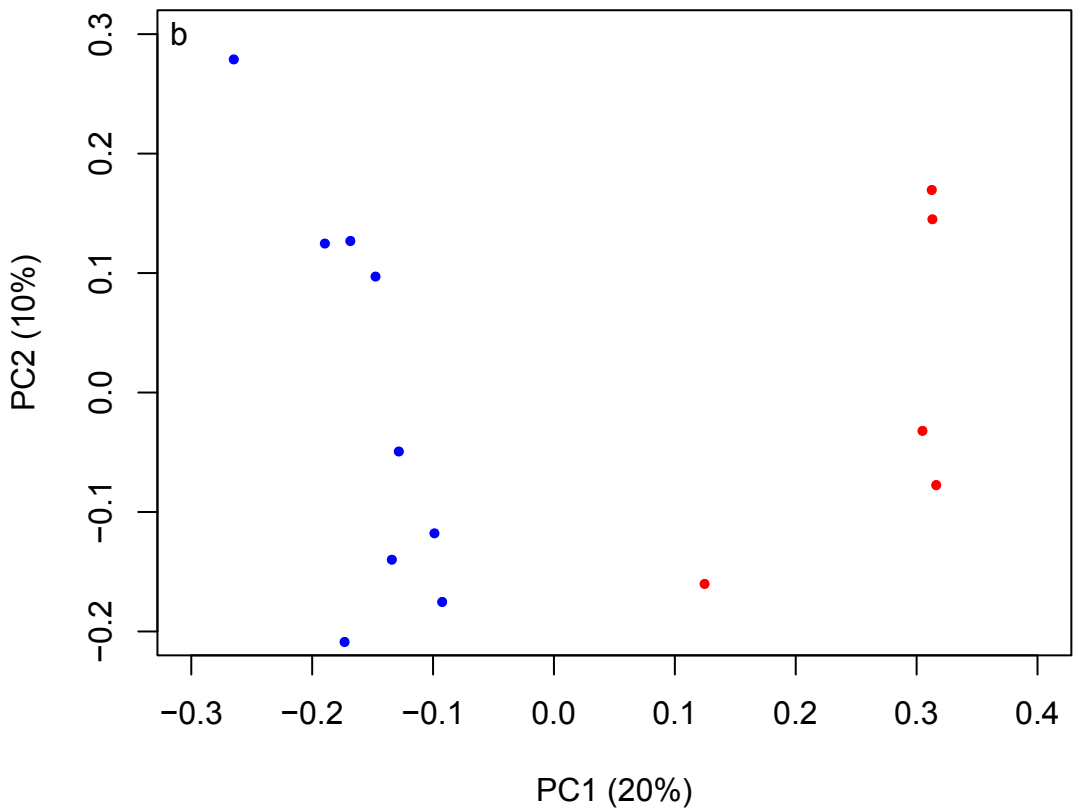
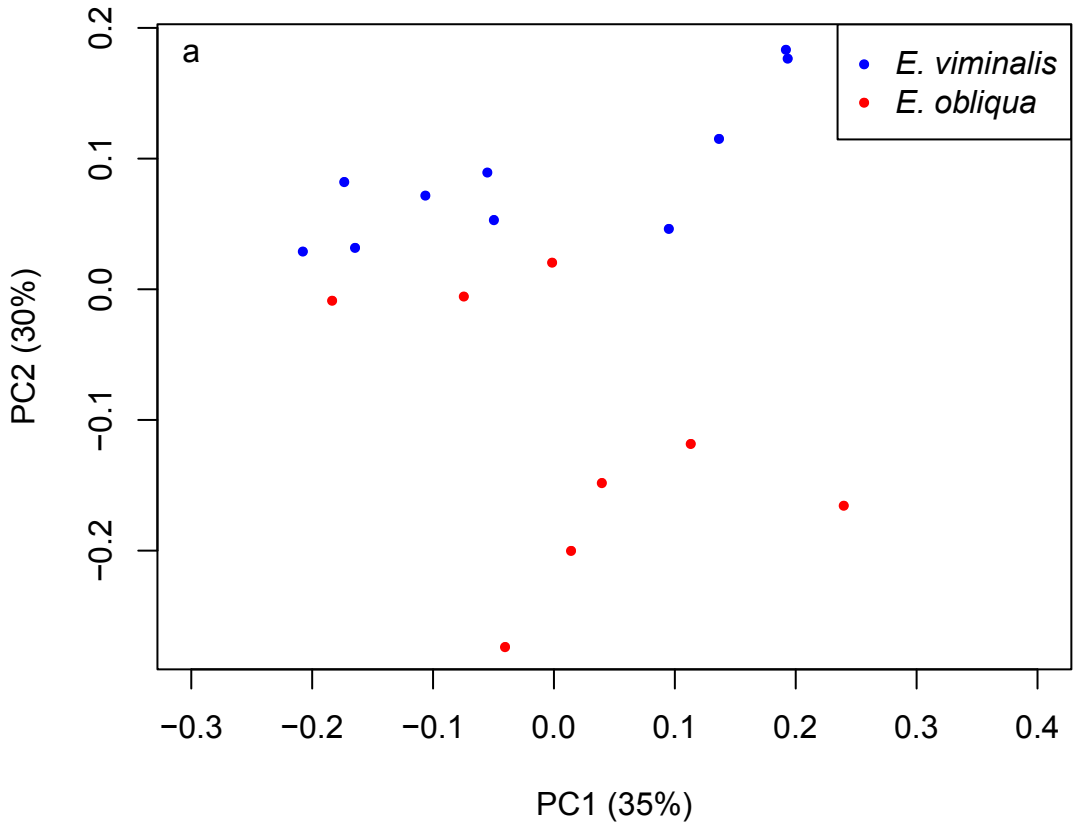

b
PC2 (10%)
PC1 (20%)
−0.2
−0.1
0.0
0.1
0.2
0.3
−0.3
−0.2
−0.1
0.0
0.1
0.2
0.3
0.4

Figure 3(on next page)

PCoA of β diversity based on UniFrac matrices from the Cape Otway 2015 koala collection.

Scatterplots from weighted (a) and unweighted (b) UniFrac matrices for koalas with diets comprising *E. viminalis* and *E. obliqua* from the 2015 collection year.


a
E. viminalis
E. obliqua
PC2 (30%)
PC1 (35%)
0.2
0.1
0.0
−0.1
−0.2
−0.3
−0.2
−0.1
0.0
0.1
0.2
0.3
0.4

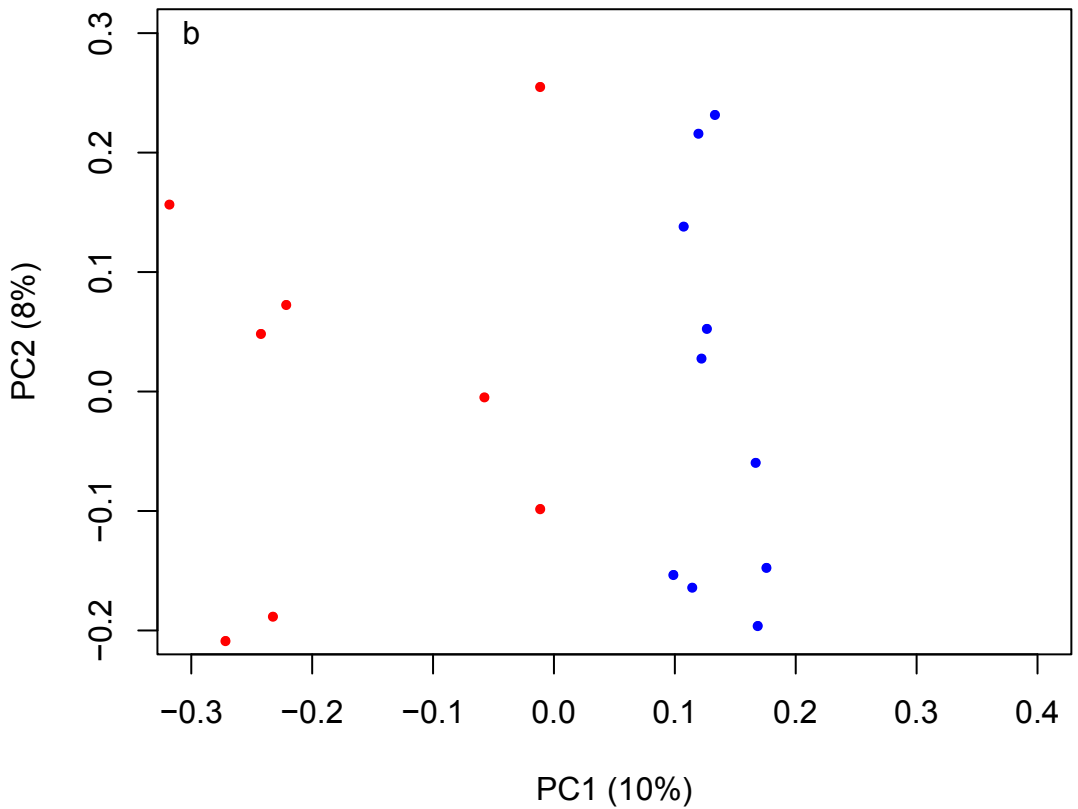

b
PC2 (8%)
PC1 (10%)
0.3
0.2
0.1
0.0
−0.1
−0.2
−0.3
−0.2
−0.1
0.0
0.1
0.2
0.3
0.4

Figure 4

Taxonomic bar charts of the most abundant genera in faecal microbiomes of koalas feeding from *E. viminalis* and *E. obliqua*

Taxonomic bar charts show the dominance of *Parabacteroides* and Ruminococcaceae in faecal microbiomes of koalas eating *E. viminalis* and *E. obliqua*, respectively, in 2013 (a), and the dominance of *Bacteroides, Parabacteroides* (*E. viminalis*) and Ruminococcaceae (*E. obliqua*) in 2015 (b).

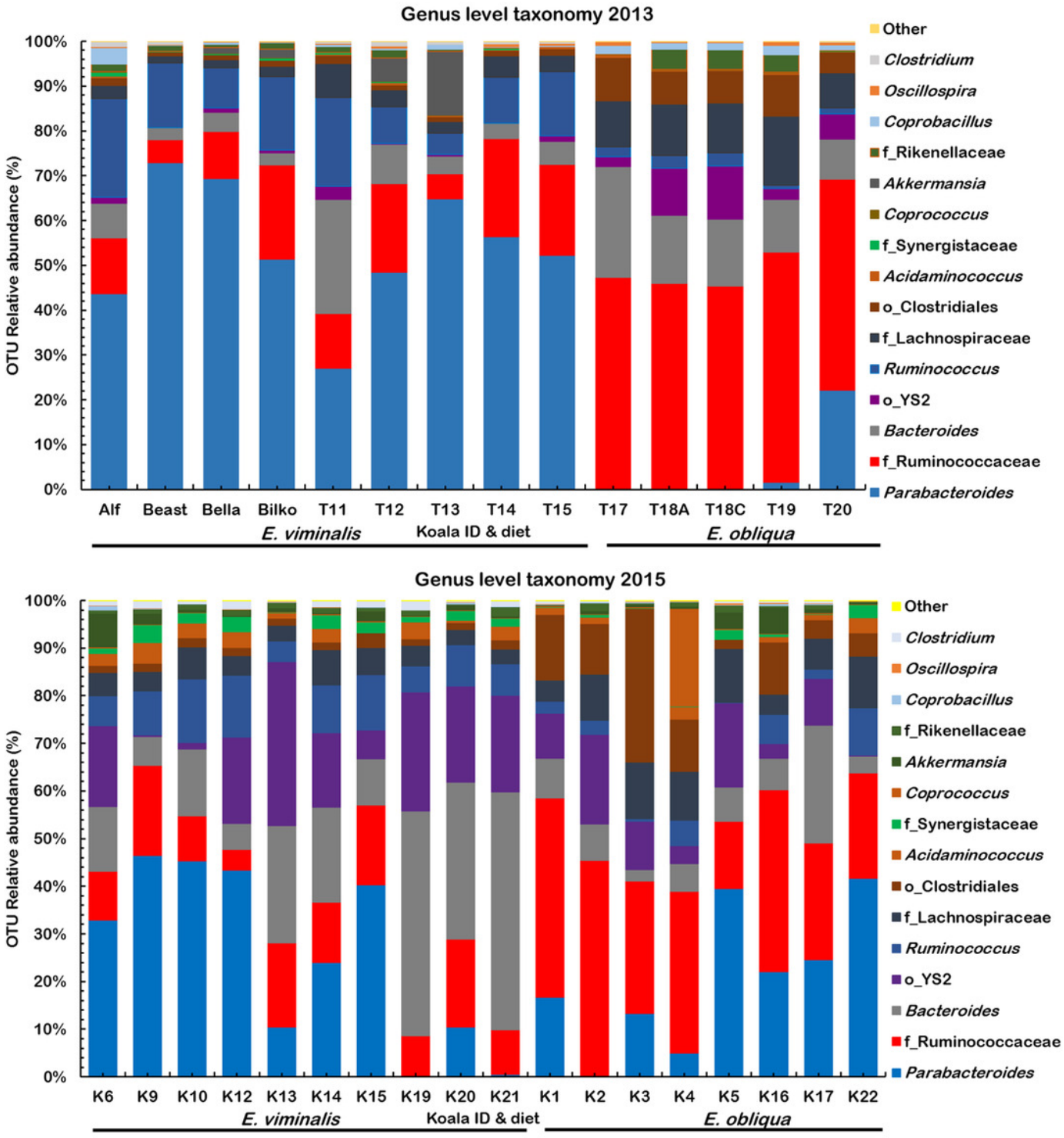

Genus level taxonomy 2013
OTU Relative abundance (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Alf
Beast
Bella
Bilko
T11
T12
T13
T14
T15
T17
T18A
T18C
T19
T20
E. viminalis
Koala ID & diet
E. obliqua
Other
Clostridium
Oscillospira
Coprobacillus
f_Rikenellaceae
Akkermansia
Coprococcus
f_Synergistaceae
Acidaminococcus
o_Clostridiales
f_Lachnospiraceae
Ruminococcus
o_YS2
Bacteroides
f_Ruminococcaceae
Parabacteroides
Genus level taxonomy 2015
OTU Relative abundance (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
K6
K9
K10
K12
K13
K14
K15
K19
K20
K21
K1
K2
K3
K4
K5
K16
K17
K22
E. viminalis
Koala ID & diet
E. obliqua
Other
Clostridium
Oscillospira
Coprobacillus
f_Rikenellaceae
Akkermansia
Coprococcus
f_Synergistaceae
Acidaminococcus
o_Clostridiales
f_Lachnospiraceae
Ruminococcus
o_YS2
Bacteroides
f_Ruminococcaceae
Parabacteroides

Figure 5

Estimated diet composition of koalas eating *E. viminalis* and *E. obliqua*.

Bar chart showing koala identity and percentage of diet species eaten for (**a**) 2013 and (**b**) 2015 koala faecal samples.

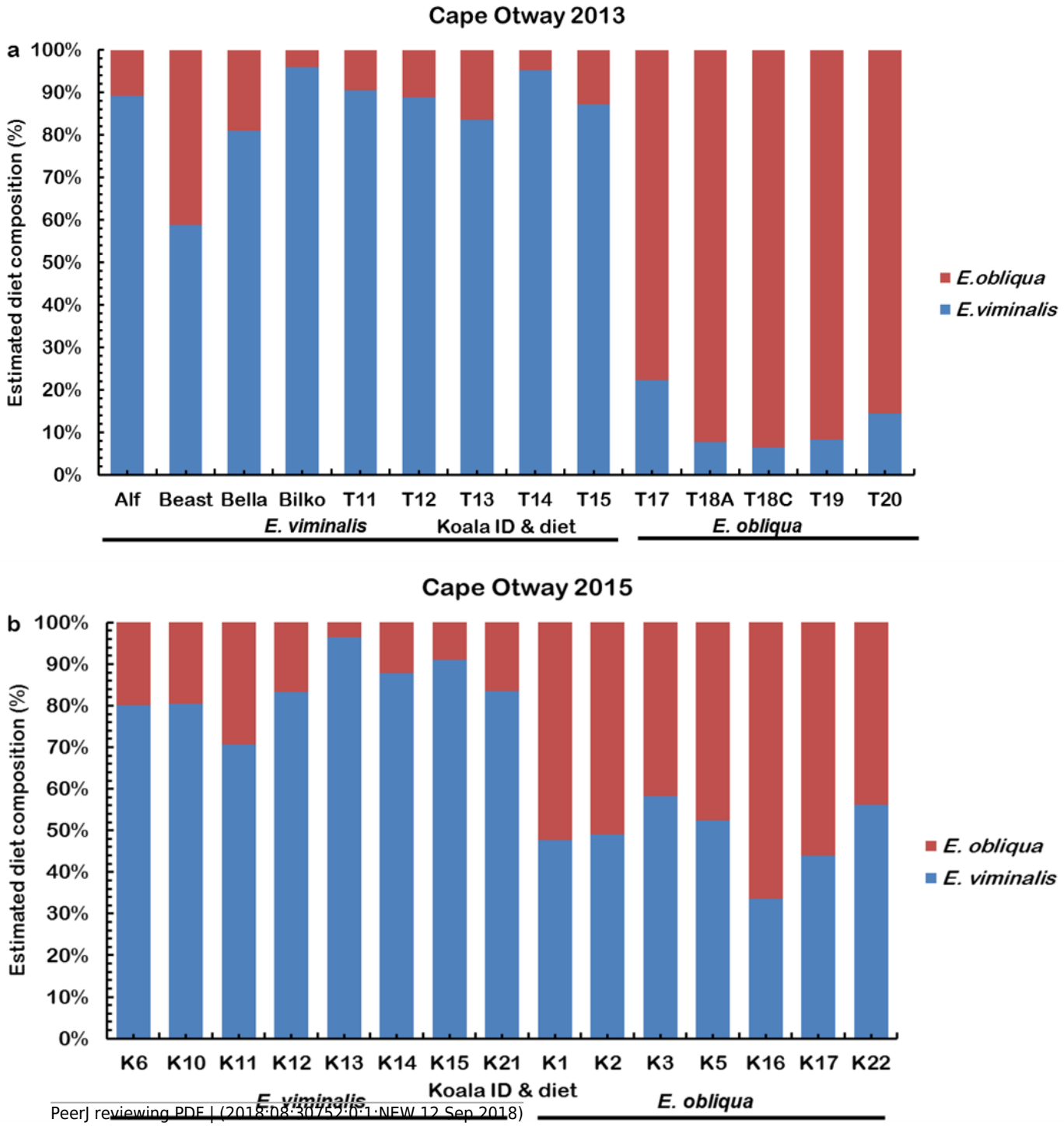


Table 1(on next page)

Relative abundance of several bacterial phyla in faeces of koalas eating *E. viminalis* and *E. obliqua* in 2013 and 2015.

footnote Values given as mean relative abundance ± standard error (SE) across diet and collection year. Across columns, mean followed by the same letter are not significantly different at $P<0.05$, using Tukey's test.

1

Frequency (%) ± SE				
Taxon-phylum	***E. viminalis* 2013**	***E. obliqua* 2013**	***E. viminalis* 2015**	***E. obliqua* 2015**
Bacteroidetes	61 ± 0 a	22 ± 0b	48 ± 0a	27 ± 0b
Firmicutes	34 ± 0 b	69 ± 0a	32 ± 0b	61 ± 0a
Cyanobacteria	1 ± 0b	6 ± 0ab	15 ± 0a	8 ± 0ab
Other	1 ± 0b	1 ± 0a	0.003 ± 0.001c	0.003 ± 0.001c
Proteobacteria	1 ± 0a	1 ± 0a	1 ± 0a	1 ± 0a
Synergistetes	0.003 ± 0.001b	0.0004 ± 0.0001b	2 ± 0a	1 ± 0b
Verrucomicrobia	2 ± 0a	0.00004 ± 0.00002a	1 ± 0a	1 ± 0a

2

Table 2(on next page)

Foliar nutritional quality of leaf material analysed through in-vitro digestion.

Ndig (N digestibility), N (total nitrogen), AvailN (Available nitrogen), DMD (dry matter digestibility) and NDF (neutral detergent fibre). *E. viminalis* (n=16) and *E. obliqua* (n=11).P values obtained by Student's *t* test assessing differences between same leaf ontogeny (i.e. epicormic or adult) of both eucalypts.

1

Mean foliar nutritional values % ± SE						
Leaf	***E. viminalis* epicormic**	***E. obliqua* epicormic**	**P**	***E. viminalis* adult**	***E. obliqua* adult**	**P**
Total N	2.03 ± 18	1.3 ± 0.4	0.04	2 ± 0.02	1.19 ± 3	0.05
Ndig	62 ± 11	6.3 ± 2.6	0.0001	66 ± 3	15 ± 5	0.0001
AvailN	1.30 ± 33	1 ± 0	0.001	1.14 ± 0.16	0.17 ± 0.06	0.001
DMD	54 ± 3	31 ± 2	0.0001	52 ± 0	35 ± 2	0.0001
NDF	-	-	-	38 ± 2	48 ± 1	0.003

2