

Pouch bacteria: an understudied and potentially important facet of marsupial reproduction

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ABSTRACT

Australia is home to a rich biodiversity of marsupials that are found nowhere else. Unfortunately, many of these species are currently threatened with extinction due to introduced feral predators and other anthropogenic factors. There is growing recognition that host-associated microorganisms can play important roles for animal health, with billions of dollars currently being invested into human gut microbiome research and the development of microbiome-based therapeutics to improve human health. Can microorganisms also be harnessed to stem the tide of marsupial extinctions? In this review, we provide an overview of some of the challenges facing Australia's marsupials, and our current understanding of the microbiology of the marsupial pouch. We also propose outstanding research questions pertaining to the marsupial pouch, which, if addressed, may provide actionable knowledge and novel microbial therapies that could help stem the tide of marsupial extinctions in Australia.

Introduction

The island continent of Australia is globally significant for its unique biodiversity, and is home to the most distinctive terrestrial mammal fauna on Earth. Australia is particularly notable for harbouring the world's richest diversity of extant marsupials, with over 200 species found only in Australia, across habitats ranging from arid deserts to tropical rainforests.¹ These include several iconic taxa such as kangaroos, koalas, and wombats, many of which hold great cultural significance to Australians.

Marsupials diverged from eutherian ('placental') mammals c. 160 Ma during the early Jurassic period,² with Australian lineages evolving largely in geographic isolation following separation from the Gondwanan supercontinent c. 40 Ma. Unsurprisingly given this divergence time, several anatomical and physiological differences exist between marsupials and eutherians – the most notable being reproductive strategy. Marsupials give birth after a short gestation period to undeveloped young (hereafter 'joeys'), which crawl toward and latch onto a teat located in a maternal pouch (marsupium) and continue development *ex utero* through lactation. This means that, unlike eutherian neonates, marsupial joeys are exposed to the external environment prior to developing a functional immune system and are thus highly vulnerable to microbial infections.³ Despite this risk, however, *ex utero* development is highly advantageous for survival in Australia's adverse and often unpredictable environments, as it allows for increased maternal control of reproductive effort during unfavourable conditions.⁴

Challenges facing Australia's marsupials

Despite harbouring much of the earth's mammalian diversity, Australia currently reports the highest mammalian extinction rate on earth, with 39 mammal species confirmed to have become extinct in the ~200 years since European colonisation.⁵ These extinctions represent >10% of all global mammal extinctions during this period, and amount to a major loss of global marsupial diversity.¹ With 52 Australian mammals currently listed as Endangered (incl. 9 Critically Endangered) and 58 listed as Vulnerable, further mammalian extinctions are likely in coming decades without substantial intervention.⁵ The primary drivers of population declines among Australian mammals are predation by introduced species such as feral cats (*Felis catus*) and foxes (*Vulpes vulpes*), and habitat loss due to extensive land clearing.¹ Several endangered marsupial species, including

quolls, koalas and Tasmanian devils, have also experienced significant population declines due to disease, the spread of which is exacerbated by increased habitat fragmentation.^{6–8} Additionally, Australia's mammals are vulnerable to increased frequency of extreme weather events such as bushfire, floods and drought resulting from climate change.⁹

Given the complexities associated with managing these threats, establishing healthy insurance populations *ex situ* through captive breeding is essential for preventing further mammal extinctions in Australia. These captive populations are an invaluable resource for reintroduction and repopulation programmes, as well as furthering research into threat and disease adaptation. Captive breeding is also beneficial for expanding and maintaining genetic diversity through the application of selective breeding and recent advances in artificial insemination.¹⁰ This is particularly useful for the genetic rescue of increasingly fragmented mammal populations by targeted translocation.¹¹

Despite these benefits, however, the successful management and breeding of endangered marsupials in captivity presents several challenges. For instance, the reproductive success of several species can be hindered by low fertility and conception rates. This can occur because of several factors, including breeding incompatibility among captive stock, behavioural or social shifts in captivity, and dietary changes.¹² Overall breeding outputs in some species are also affected by high rates of neonatal and juvenile mortality, which can occur because of infections, behavioural stress caused by environmental modification in captivity and other environmental factors that remain poorly understood.^{13–15} The issue of neonatal mortality during early development is of particular concern in captive koala colonies, where seasonal mortality rates among pouch young can exceed 50%, predominantly due to bacterial infections.¹³

Recent research into host–microbiome associations has shown that microbes play important roles in animal health and evolution.¹⁶ Monitoring and modulating the gut microbiome is being proposed as a tool for improving conservation outcomes for endangered animals.¹⁷ Comparatively less is known about host–microbiome associations in the context of the mammalian female reproductive tract (FRT; e.g. vagina, urogenital tract, milk, pouch), especially for endangered species. However, growing evidence suggests that FRT-associated microbial communities may play important roles in several host functions essential for reproductive and developmental success.¹⁸ Extending this logic to marsupials, we propose that microbial communities in the maternal pouch may represent an important and overlooked factor of successful reproduction (Fig. 1).

Pouch microbiology – what is known

To date, microbiological research into the marsupial pouch has received little attention, with fewer than ten studies being published since the first in 1972 by Yadav, Stanley and Waring on the quokka (*Setonix brachyurus*).¹⁹ This and the three other cultivation-dependent studies found substantial reductions in, or a complete lack of, culturable bacteria from koala, tammar wallaby, brushtail possum and quokka

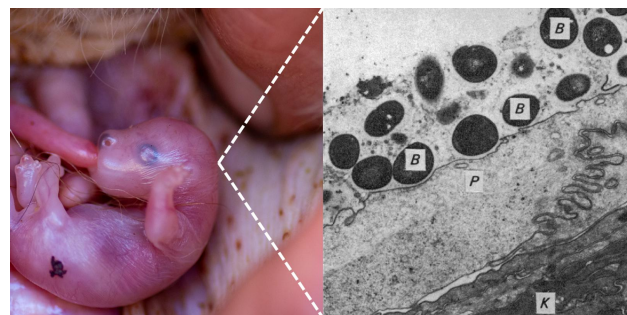


Fig. 1. (Left) A yellow-footed rock wallaby joey with fingernail for scale (source: Raphael Eisenhofer). (Right) Electron micrograph (12 000×) section from the skin of a newborn opossum from Krause *et al.*³ Bacteria (B) can clearly be seen coating the surface of the periderm (P). K is the forming keratinising layer of epidermis.

pouches prior to and immediately following birth.^{19–22} The first use of cultivation-independent techniques to study microbes in the marsupial pouch was in 2004 by Deakin and Cooper.²³ Using a mixture of cultivation-dependent and -independent methods on common brushtail possums (*Trichosurus vulpecula*), they found similar trends to the prior cultivation-dependent studies.²³ Similar trends were also observed in a cultivation-independent study on tammar wallaby (*Macropus eugenii*) pouches.²⁴ Two further cultivation-independent studies using high-throughput 16S rRNA gene sequencing on Tasmanian devil pouches (*Sarcophilus harrisii*) found differences in composition, but not diversity, between lactating and non-lactating females.^{25,26} Overall, the trend in these pouch microbiology studies is a shift in diversity or composition associated with the reproductive status of the host, generally with a reduction in bacterial diversity close to and immediately following joey birth.

However, it is increasingly recognised that DNA contamination can compromise 16S rRNA gene studies – particularly those targeting samples with low microbial biomass.²⁷ Recently, Weiss *et al.* applied a robust experimental framework, including the collection and sequencing of numerous negative control samples and the quantitative estimation of sample biomass using qPCR, to demonstrate that the southern hairy-nosed wombat (SHNW) (*Lasiorninus latifrons*) pouch does indeed harbour resident bacteria.²⁸ Weiss *et al.* analysed multiple sample types from 26 wild SHNWs to show that the pouch of reproductively active females is compositionally distinct from other body sites and is dominated by a handful of Gram-positive bacteria.²⁸ The closest match for three of the five dominant pouch bacteria were to pouch bacterial isolates from tammar wallabies,²⁴ with 16S rRNA gene sequence divergence consistent with the estimated divergence time between tammar wallabies and wombats – offering tantalising (albeit preliminary) evidence for co-speciation between pouch bacteria and marsupials.

Characterisation of pouch-associated microbial communities has also highlighted a potential link between bacterial composition and reproductive failure. This was demonstrated in a recent study (Maidment) of 38 captive koalas (*Phascolarctos cinereus*), where females who lost pouch young exhibited a significantly different pouch microbial compositional profile to females rearing to full term.

Interestingly, although both animal groups showed similar decreases in microbial richness between mating and parturition, the pouch microbiota of successful mothers re-diversified in the months following parturition, whereas unsuccessful mothers remained dominated by *Enterobacteriaceae* until loss of young occurred 5–7 months post-partum.²⁹ Taken together with similar findings in koalas by Osawa *et al.*²¹ and O’Callaghan,¹³ these recent findings add further weight to the hypothesis that dysbiosis of microbial communities in the marsupial pouch may be associated with neonatal mortality.

Unknowns and future directions

The past decade has seen a rapid accumulation of evidence demonstrating that the gut microbiome can play important roles in host health,¹⁶ and some have suggested that the gut microbiome should be considered for animal conservation.¹⁷ We wish to extend this idea and hypothesise that some pouch bacteria are beneficial to the reproductive success of marsupials. One beneficial function that pouch bacteria could bring to their hosts is the competitive exclusion of potentially harmful microorganisms from the pouch. The exact mechanisms are unknown, but bacteria are known to use a range of antagonistic tools to gain an advantage over heterospecific bacteria.³⁰ Such bacteria could have co-speciated with their marsupial hosts and are thus likely well adapted to surviving endogenous host antimicrobial defences. Perturbations of the ‘natural’ pouch microbiome could therefore have detrimental impacts to the reproductive success of marsupials by increased inflammation or joey mortality caused by opportunistic microorganisms. Such disruptions to the pouch microbiome could be caused by various factors present in captivity (which can affect the gut microbiome³¹), such as horizontal transfer of pathogens from humans, antibiotic treatment, or the lack of vertical transmission of pouch bacteria from mother to joey (in cases where an underdeveloped joey is rescued from a dead mother’s pouch).

Our current understanding of marsupial pouch microbiology is limited. More experiments are needed to confirm a link between pouch bacteria and the reproductive success of marsupials, and to identify factors that may influence the composition of the pouch microbiome across marsupials. Some key outstanding questions that we think should be addressed are:

1. Are captive pouch microbiomes different from wild?
2. For a given marsupial species, is there a ‘healthy’ pouch microbiome? If so, can diagnostic tests be developed to aid breeding programs?
3. Can pouch bacteria competitively exclude opportunistic pathogens? If so, by which mechanisms?
4. How do pouch bacteria evade endogenous host defences?
5. Can pouch bacteria influence the immunological profile of the pouch? If so, how?
6. Do pouch bacteria influence the development of the joey’s immune system? If so, what are the implications for the joey’s future health?

7. Have pouch bacteria been co-speciating with their marsupial hosts?
8. Can the pouch microbiome be manipulated by probiotics or pouch-microbiome transplants?

Such knowledge could be applied in a conservation management context, leading to the development of novel tools and therapies to improve the captive breeding success of marsupials and help stem the tide of marsupial extinctions in Australia.

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