Intrinsic and Extrinsic Factors Shape Cane Toad Gut Bacteria Across an Expanding Invasive Range

A thesis submitted to The University of Adelaide in fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

The cane toad (*Rhinella marina*) is one of the most successful invasive species worldwide. Since their introduction to Queensland in the 1930's, Australian cane toads have expanded westward and now are present in Western Australia. My thesis examines the gut bacteria in Australian cane toads to determine how environmental factors (e.g., diet, climate) and intrinsic factors of hosts (e.g., genetics, body size, parasite infection) interact to maintain and influence the composition and stability of intestinal bacteria. I first investigated sampling methodologies to determine whether non-lethal (cloacal and faecal) sampling accurately represent gut bacteria. I found that cloacal swabs are better proxies for large intestinal bacteria than faeces in toads. I then tested whether behaviours associated with invasion are correlated with intestinal bacterial community assemblage and function. Behaviours thought to be linked to invasion ability differ in toads from the extreme ends of this range. Although behaviour has been linked to gut bacteria in other taxa, cane toad gut bacteria has not been investigated. I characterised gut bacteria composition and behaviour of wild-sampled cane toads across their northern Australian range and found significant difference in bacterial community and predicted functions between Western Australia and Queensland cane toads, based on 16S rRNA sequencing. Environmental factors including Isothermality, Annual Mean Temperature and the presence of co-introduced lungworms (*Rhabdias pheudosphaerocephala*) best explained bacterial community assemblage. These same factors, in addition to certain behaviours linked to invasion ability (righting reflex time and the presence of righting reflex movements) best explained bacterial function. I then used Next Generation Sequencing to characterize and compare cane toad genetic (single nucleotide polymorphism), epigenetic (DNA methylation), and gut bacteria differences across populations. I found no significant association between host heterozygosity and gut bacterial diversity within individuals. However, I did find that pairwise genetic diversity was positively associated with pairwise

epigenetic diversity. Interestingly, the positive correlation between pairwise epigenetic diversity and bacteria diversity was greater in pairs with lower genetic diversity. Finally, I examined cane toad diet (taxonomy of stomach contents) and found that the presence of plant matter in cane toads' stomachs was associated with gut bacteria variation, but that gut bacteria was not significantly associated with the main component of cane toad diet (insects). My thesis provides important methodological advances in the study of amphibian gut bacteria and suggests that in cane toads, gut bacteria variation is strongly linked to lungworm infection and to DNA methylation. These results highlight possible mechanisms through which cane toads could increase the plasticity of their response to novel environments encountered during invasion.

DECLARATION

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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LIST OF PUBLICATIONS

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Contributions: Designed the experiment, collected and analysed data, wrote the paper as corresponding author

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LIST OF RESEARCH CONFERENCE PRESENTATIONS

Conference presentations

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LIST OF ABBREVIATIONS/ACRONYMS

ASV, amplicon sequence variant

core, range-core

dbRDA, distance-based Redundancy Analysis

DNA, Deoxyribonucleic Acid

front, invasion-front

GLM, Generalized Linear Models

HL, Homozygosity by Locus

LMMs, Linear Mixed Models

MHC, Major Histocompatibility class

MS222, tricaine methanesulfonate

msGBS, methylation-sensitive Genotype By Sequencing

NGS, Next-Generation Sequencing

nMDS, non-metric Multidimensional Scaling

OTUs, Operational Taxonomic Units

PCoA, Principal Coordinate Analysis

perMANOVA, permutational multivariate analysis of variance

PICRUST, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States

QIIME, Quantitative Insight Into Microbial Ecology

QLD, Queensland

rRNA, ribosomal Ribonucleic Acid

SCFAs, short chain fatty acids

SD, Standard Deviation

SNP, Single Nucleotide Polymorphism

SUL, Snout-Urostyle Length

SVL, Snout-Vent Length

WA, Western Australia

CHAPTER 1: Introduction and literature review

1.1Introduction

Invasive species have negative environmental and economic impacts worldwide. Their management may be improved by clarifying the factors that contribute to their invasion success. Dispersal ability is a key factor in invasive species' success, and this can be enhanced by behavioural traits, such as boldness, exploration and aggressiveness, which encourage the exploitation of new habitats and resources (Duckworth & Badyaev, 2007; Fraser, Gilliam, Daley, Le, & Skalski, 2001; Gruber, Brown, Whiting, & Shine, 2017a; Sih & Bell, 2008). While encountering novel environments, there are many potential pathways that can result in changes to behaviour, including selection, epigenetic variability, health status, and endobiome. In my thesis I will focus on the endobiome, especially the gut bacteria, as a factor that may contribute to invasion success, and the intrinsic and extrinsic factors that shape gut bacterial communities.

Gut microbiome research is a rapidly growing field of study, and it is becoming clear that variation in gut microbial assemblages plays an important role in host health and behaviour (Diaz Heijtz et al., 2011; Schretter, 2019; Vuong, Yano, Fung, & Hsiao, 2017). This draws attention to the potential role that manipulation of the gut microbiome may have in species conservation and management (Bahrndorff, Alemu, Alemneh, & Lund Nielsen, 2016; Jiménez & Sommer, 2017; Trevelline, Fontaine, Hartup, & Kohl, 2019). However, the majority of evidence regarding the associations between intestinal microbiota and host behaviour are found in human and mouse/rat models (Vuong et al., 2017). Little is known about the gut microbiome of invasive species and the influence of gut microbiota on species' invasion ability. A few studies have showed that there are differences in gut microbiota between invasive and native populations in fish (Ye, Amberg, Chapman, Gaikowski, & Liu,

2014) and toad (Wagener, Mohanty, & Measey, 2020). The latter study found that toads from the invasive range had decreased physiological performance when given faecal microbial transplants from native range toads. These findings suggest that there might be common traits in gut microbiome that links to invasion success.

Studying the impact of gut microbiome on an invasive species can be challenging. The interactions between host and gut microbial community are complex and have been identified in many potential pathways, known as gut-microbiota-brain axis (Reardon, 2014; P. A. Smith, 2015). In natural environments, individuals normally present large amounts of gut microbiota variation, due to a range of intrinsic and extrinsic factors (Benson et al., 2010; Spor, Koren, & Ley, 2011). To study whether gut microbiome plays an important role in species' invasion success, it is useful to know whether microbiome is associated with the host phenotypes that enhance invasion and whether microbiomes from across the invasion trajectory respond differentially to intrinsic and extrinsic factors.

The cane toad (*Rhinella marina*) is one of the most notorious invasive species in Australia and its invasion speed has increased as it has expanded westward following introduction (from ~15 km to 60 km per annum; (C. M. Hudson, McCurry, Lundgren, McHenry, & Shine, 2016; Shine, 2012). Distinctive changes in morphology, physiology and behaviour have been documented between the dispersive cane toads from the Western Australian ("invasion-front") and those in the Queensland ("range-core") (Lindström, Brown, Sisson, Phillips, & Shine, 2013; Rollins, Richardson, & Shine, 2015). Invasion-front toads presented invasion-enhancing phenotypes (morphology and behaviour) compared to range-core toads (Gruber, Brown, et al., 2017a; Gruber, Brown, Whiting, & Shine, 2017b; Gruber, Whiting, Brown, & Shine, 2017; C. M. Hudson et al., 2016; Phillips, Brown, Webb, & Shine, 2006). Because of these clear changes found across the range, Australian cane toads are an excellent model to study whether gut microbiota plays a role in this invasion. Prior to my thesis, the gut microbiome of Australian cane toads was unstudied. Therefore, I aimed to determine best

practices for non-lethal sampling, determine whether toad gut bacterial communities differ across the range, identify important intrinsic and extrinsic factors in this system, and identify inter-relationships between these factors and gut bacterial communities across the cane toad's Australian range.

1.2 Literature review

The literature review covers the impact of endobiome (parasites and gut microbiome) on host behaviours, the factors (intrinsic and extrinsic) that contribute to gut microbiome variation, the methods for studying gut microbiome, and background information about cane toads.

1.2.1 Endobiome and behaviour

Parasites and gut microbiota, known as the endobiome, largely share their habitats and interact to influence hosts (Leung, Graham, & Knowles, 2018; Mejia et al., 2020; Ramírez-Carrillo et al., 2020). Understanding their respective roles in driving host behaviour is needed, as is a clearer understanding of how parasites might mediate or exacerbate the effects of the gut microbiome. Further, studying changes in microbial function in conjunction with behavioural assays can illuminate the mechanisms underlying observed effects.

1.2.1.1 Parasites and host behaviour

Pathogens and parasites (for example, in brain or gut) have been shown to modify host behaviour in a manner that improves the probability of parasite transmission and survival (Gegear, Otterstatter, & Thomson, 2006; House, Vyas, & Sapolsky, 2011; Poulin, 2010). There are wide range of parasites that can effectively manipulate an animal's behaviour. One notable pathogen that affects host behaviour is a baculovirus that causes infected caterpillars to display light-dependent climbing behaviour, which exposes them to predators and facilitates the virus' lifecycle (van Houte, van Oers, Han, Vlak, & Ros, 2014). Another

example comes from a fungus (*Ophiocor dyceps unilaterali*,) that produces ‘zombie’ ants, which attach to leaves near the forest floor, where fungal development is optimal (Hughes et al., 2011). Another well-known example of a parasite that manipulates host behaviour is *Toxoplasma gondii*, which requires cats as a final host in order to sexually reproduce; infected mice are attracted to, rather than repelled by cat odours (House et al., 2011). Macro-parasites can also manipulate host behaviours. For instance, nematomorph worms induce terrestrial insects to commit suicide in water so that the worms can complete their lifecycle and reproduce in the water (F. Thomas et al., 2002).

Parasites are common in human and animals and may occur in otherwise healthy individuals. For example, gut parasites (*Blastocystis* and *Dientamoeba*) appear in healthy individuals due to their low pathogenicity (Stensvold & van der Giezen, 2018). These parasites can potentially be indicators or active manipulators of gut microbial structure and function (Stensvold & van der Giezen, 2018), suggesting that parasite presence and abundance may need to be considered in gut bacteria studies.

1.2.1.2 Gut microbe and host behaviour

Intestinal microbiota contains the major proportion of the host microbiota (H. X. Wang & Wang, 2016) and up to 98% of the intestinal microbiota are bacteria, while the rest is comprised of fungi, viruses, and protists (Qiu et al., 2015). Accumulating evidence indicates that variation in gut microbial assemblages can significantly affect the health and behaviour of the host (C. Mu, Yang, & Zhu, 2016; Stilling, Dinan, & Cryan, 2014). Specifically, intestinal microbiota have been associated with host behaviour in human and mouse/rat models (Diaz Heijtz et al., 2011; Messaoudi et al., 2011; Neufeld, Kang, Bienenstock, & Foster, 2011; Sudo et al., 2004). Differences in gut microbiota between invasive and native species has been observed: analysis of the hindgut microbial communities of invasive Asian carp and native American fish differ, with the order *Bacteroidales*, the genus *Bacillariophyta*

and the genus *Clostridium* being significantly more abundant in native than in invasive fish (Ye et al., 2014). Moreover, a recent study found gut microbiome in invasive guttural toads (*Sclerophrys gutturalis*) differed compositionally, phylogenetically and functionally from its source population (Wagener et al., 2020). Furthermore, they found the transplantation of faeces from native range toads decreased physiological performance (shorter travel distance and lower performance speeds) in toads from the invasive ranges in Cape Town. These results suggest that finding the common traits of gut bacteria linked to enhancing-dispersal behaviour may help us to define the role of gut bacteria during invasion.

1.2.1.3 Mechanisms of how gut microbe affecting host behaviour

Gut microbiota is thought to be able to affect host behaviours through the gut-microbiota-brain axis (Reardon, 2014; P. A. Smith, 2015). The interaction between a host and its gut microbiota has many potential pathways. Firstly, the enteric nervous system is directly connected to the central nervous system through the vagus nerve (Forsythe, Bienenstock, & Kunze, 2014). Further, intestinal microbes produce metabolic precursors to hormones and neurotransmitters or directly produce the active metabolites themselves (Lyte, 2014; Sharon et al., 2014). It has also been hypothesized that gut microbial communities can alter their host's behaviours through the epigenetic regulation of the host's genes, which affects host gene expression and thus host phenotype without change the host's DNA sequence. For example, gut-microbial products can affect the chromatin state within brain cells and cause changes in gene transcription (Stilling et al., 2014). Furthermore, the gut microbiome has been linked to differential expression of host brain microRNAs (which have the potential to regulate host's genes) and have been implicated in the onset of anxiety- and fear-related behaviours (A. E. Hoban et al., 2018; A. E. Hoban et al., 2017). However, the interactions between gut microbiota and host epigenetic changes are poorly understood. In invasive species that are more likely to experience novel environmental stressors, and thus potentially more

environmentally induced changes to their epigenome, this relationship may be even more important but is virtually unexplored.

Due to the broad taxonomic range of gut microbiota, the mechanisms underlying their impacts on host behaviour can be very diverse. Studying their association in combination with host epigenetics (e.g. DNA methylation) or gene expression could help to identify mechanistic pathways (i.e. affected genes provide candidates that can be further investigated).

1.2.2 Factors that affect gut microbiota

Gut microbiota consists of a complex and dynamic community. It is well-established that intrinsic and extrinsic factors interact to maintain and affect the composition and stability of a host's gut microbiota (Benson et al., 2010; Marques et al., 2010; C. Mu et al., 2016; Penders et al., 2006; Spor et al., 2011; G. D. Wu et al., 2011). Investigating how gut microbiota respond to these factors, and how this might change across an invasive range, is important to understanding the potential of gut microbiota to drive the expansion of a species following introduction.

1.2.2.1 Intrinsic factors

Intrinsic factors, including genetics, age, sex, and physical status are important drivers of gut microbiota variation (Kers et al., 2018; Maslowski & Mackay, 2011; Spor et al., 2011; Yukgehnaish et al., 2020). Using a number of approaches, host genetics have been shown to impact gut microbiota: by comparing microbiota across host phylogenetic groups (Nelson, Rogers, Carlini, & Brown, 2013; Youngblut et al., 2019) or across host genotypes of same species (Benson et al., 2010; Griffiths et al., 2018; Macke, Callens, De Meester, & Decaestecker, 2017), or by key health-related genetic factors (Kozik, Nakatsu, Chun, & Jones-Hall, 2017; Matsuki et al., 2016). Young and aged populations show differences in gut microbiota, which is linked to age-related health status (Maynard & Weinkove, 2018). For

example, gut microbiota of aged mice are associated with inflammatory disease, particularly gastrointestinal and liver disorders, as well as anxiety-like behaviours (K. A. Scott et al., 2017). The impact of sex on gut microbiota have been linked to sex hormones (Yoon & Kim, 2021). For example, gonadectomy and hormone replacement had clear effects on mice gut microbial composition, suggesting possible mechanism of the sex impacting gut microbiota (Org et al., 2016). Physical status, such as body weight (Chai, Dong, Chen, & Wang, 2018) and health status (Videvall, Strandh, Engelbrecht, Cloete, & Cornwallis, 2018), can also significantly affect gut microbiota variation. This evidence indicates that host intrinsic factors need to be examined in studies of gut bacteria.

1.2.2.2 Extrinsic factors

Extrinsic environmental factors such as habitat and diet also can influence the composition and variation of gut microbiota (Bletz et al., 2016; Carmody et al., 2015; Marques et al., 2010). For example, environmental characteristics including elevation (H. Li, Zhou, Zhu, Huang, & Qu, 2019), temperature (Hylander & Repasky, 2019; Kohl & Yahn, 2016; Tong, Cui, Hu, et al., 2020), season (Maurice et al., 2015; Tong et al., 2019; Tong, Hu, Du, Bie, & Wang, 2020), agricultural activity (Chang, Huang, Lin, Huang, & Liao, 2016; Huang, Chang, Huang, Gao, & Liao, 2017), and the presence of chemical pollution (D. Mu et al., 2018) have been linked to gut microbial variation. A number of studies have compared variation in gut microbiota across environment types. For example, the gut microbiota of fire salamander (*Salamandra salamandra*) larvae differed depending on whether they were sampled from ponds or streams (Bletz et al., 2016). Translocating animals to a different habitats has been shown to shift their gut microbiota to resemble that of residents of the new habitat. Further, gut microbiomes can be similar across phylogenetically distinct, but sympatric species: in amphibians (such as *Fejervarya limnocharis* and *Babina adenopleura* inhabiting both

farmlands and forests), environmental conditions (in both species) influenced bacterial diversity, which was higher in farmland populations (Huang et al., 2017).

In some species, diet is a key environmental factor that affects gut microbiota composition (Youngblut et al., 2019). The changes observed in gut microbiota between populations living in different habitats are likely to be influenced by variation in the host diets (K. P. Scott, Gratz, Sheridan, Flint, & Duncan, 2013; Zmora, Suez, & Elinav, 2019). The majority of previous studies on the impact of diet on gut microbiota in human or mice have used artificial diets to investigate a single nutritional component (Khan et al., 2020; Makki, Deehan, Walter, & Bäckhed, 2018; C. Zhang et al., 2013). These controlled diet studies provide insights regarding the impact of specific aspects of an organism's diet on gut microbiota, but they do not account for feeding behaviours under natural conditions (Baxter et al., 2015; H. Li et al., 2016). Wild animals are more likely to eat a wide range of different foods based on prey availability; additionally, they also inadvertently consume biotic and non-biotic items while ingesting their intended prey. Host gut microbiota can be affected by not only prey species or nutrient components they normally consume, but also the availability of food resources, food diversity, and random food items they consume.

In summary, both extrinsic and intrinsic factors are observed to alter gut microbiota variation. There are a broad range of factors in shaping gut microbiome across different taxonomic groups and the majority of evidence comes from studies of human or model animals in captive environments. Examination of these factors in natural environments is more challenging but is essential for understanding the impact of gut microbiota on a broad range of ecological contexts, including invasion.

1.2.3 Methods for studying gut microbiota

Gut microbiota is located in the host's intestine. To estimate the gut microbiota profile, direct access to the intestinal content may require the host to be sacrificed. However, the

identification of causality between microbial community assemblages and phenotypic traits of hosts requires the use of experimental interventions (e.g., antibiotic administration, faecal microbiota transfer, co-housing, and cross-fostering or rederivation) (Ericsson & Franklin, 2015). Therefore, robust, non-lethal methods are needed to effectively study gut microbiota, which provide accurate information to enable the assessment of the intestinal microbiota before and after interventions.

Gut microbiota research is a fast-growing field. The advanced methodologies for processing samples and profiling gut microbial community enable researchers to explore gut microbiota in larger range of wild animals. Next I will discuss the latest information about non-lethal sampling, gut content analysis and data analysis pipelines.

1.2.3.1 Non-lethal sampling

Faecal samples and cloacal swabs are two commonly used non-lethal sampling methods; however, the accuracy with which each sample represents the intestinal microbiota is often untested (Bassis et al., 2017). In different hosts, these methods may differ in their representation of the intestinal microbiota, yielding distinct taxonomic compositions. In birds, faecal samples have been shown to provide a more accurate assessment of the large intestinal (colon) microbiota than cloacal samples (Videvall et al., 2018). In lizards, faecal samples were very similar to the large intestinal (hindgut) microbiota, yet were less representative of the stomach or small intestinal microbiota (Kohl et al., 2017); in bats, the faecal sample microbiota did not provide an accurate representation of large intestinal microbiota (Ingala et al., 2018). Faeces has been demonstrated to be a robust proxy for the gut microbiota in Asiatic toad tadpoles (X. Song, Song, Song, Zeng, & Shi, 2018). Because of these contrasting results across species, it is necessary to compare microbial profiles between different non-lethal sample types to those taken directly from intestine to determine the most suitable non-lethal sampling method.

1.2.3.2 Methodologies for analysing samples

There are two categories of gut microbiome studies, namely culture-dependent and culture-independent approaches. Culture-dependent techniques, based on selective culturing, followed by morphological, biochemical, and physiological assays, provides in-depth information about the physiology of particular bacteria (Gong & Yang, 2012). However, there are limitations to this method: (1) only 10-60% of gut bacteria can be cultured under laboratory conditions, and (2) studying gut microbial diversity is difficult with this approach because they cannot simulate the interactions of bacteria and the complex natural gut environment (Gong & Yang, 2012; Zoetendal, Collier, Koike, Mackie, & Gaskins, 2004). DNA-based culture-independent methods have been developed to overcome the above-mentioned drawbacks of culture-dependent techniques, namely PCR (polymerase chain reaction) based DNA profiling, quantitative PCR (Q-PCR), fluorescent in situ hybridization (FISH), flow cytometry, DNA sequencing and DNA microarray (Gong & Yang, 2012). There are also limitations to culture-independent approaches: (1) these approaches do not discriminate alive from dead bacteria (Soejima et al., 2008), (2) for PCR-based approaches, bias can be generated during the PCR amplification step (Acinas, Sarma-Rupavtarm, Klepac-Ceraj, & Polz, 2005; Sidstedt, Rådström, & Hedman, 2020), (3) although whole metagenomics approaches (e.g., shotgun) can overcome PCR bias, this approach requires high sequencing coverage (Pereira-Marques et al., 2019) and is currently limited to quality-checked reference genome databases, especially for non-model host gut microbiome (Hiergeist, Gläsner, Reischl, & Gessner, 2015). Using a mock community, containing multiple fully characterized species, as a positive control can assist in the identification of biases in the chosen protocols and techniques (Jumpstart Consortium Human Microbiome Project Data Generation Working Group, 2012). Next-Generation Sequencing (NGS), is the most commonly DNA-based approach used for the study of microbial communities.

However, it does require extensive bioinformatics analysis to handle the large data sets generated.

The analysis of 16S ribosomal RNA data is a common approach to study bacterial communities (Human Microbiome Project Consortium, 2012; McCafferty et al., 2013; Weisburg, Barns, Pelletier, & Lane, 1991), which incorporates the selective usage of different hypervariable regions (v1-v9) in this gene (Bukin et al., 2019; M. Kim, Morrison, & Yu, 2011; Tremblay et al., 2015; Yang, Wang, & Qian, 2016). 16S rRNA gene V3-4 region amplicon sequencing via the Illumina Miseq platform showed a close relationship with shotgun sequencing data (Whon et al., 2018) and has been widely used in gut microbiota research (Dorsaz et al., 2020; M. Guo et al., 2020; Liu, Li, Guo, Liang, & Wang, 2018; Ma, Qin, Hao, Shi, & Fu, 2020). Moreover, the databases containing taxonomically identified 16S rRNA gene sequences have dramatically increased the number of available entries, represented as four taxonomic classifications: Greengenes, silva, ribosomal database project (RDP) or NCBI (Balvočiūtė & Huson, 2017; Breitwieser, Lu, & Salzberg, 2019). The combined advancement in DNA sequencing techniques and improved databases is enabling a better understanding of gut microbial communities.

1.2.3.3 Bioinformatics analysis

The open-source pipeline used in Quantitative Insight Into Microbial Ecology (QIIME) processes data from raw sequences to various downstream data formats and combines publication-quality statistical analyses, such as taxonomy assignment (referring to Greengenes/SILVA database) and microbial biodiversity analysis (richness, relative abundance, and alpha and beta diversity matrixes) and visualization of results (Caporaso et al., 2010; Qin et al., 2010). The use of Amplicon sequence variants (ASVs) has been proven to be better at resolving fine-scale variation and producing more accurate results than the operational taxonomic units (OTUs) method. ASVs are used to characterize taxonomic and

phylogenetic structure in microbial communities, which is generated by DADA2 (B. J. Callahan et al., 2016) and implemented in open-source QIIME2 (Bolyen et al., 2019). R packages (data2, phyloseq, DESeq2, ggplot2, structSSI and vegan) can also be used to analyse microbiome data from raw reads for community analyses by filtering, statistical analysis and visualization (B. J. Callahan, Sankaran, Fukuyama, McMurdie, & Holmes, 2016). Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUST) was developed to predict the functional composition of a metagenome using marker gene data and a database of reference genomes (Langille et al., 2013). This analysis aims to address our sparse knowledge of bacterial functions. Recently, PICRUST2 was developed to provide more accurate functional prediction, which provides interoperability with ASV and increases database of gene families and reference genomes (Douglas et al., 2020).

In summary, an evaluation experiment investigating non-lethal sampling methods would be an indispensable step before studying causal relationship between gut microbiome and host dispersal-enhanced behaviours. With the decreased cost, more robust databases, and established bioinformatics workflows, 16S rRNA sequencing provides a promising method to investigate gut microbial community for achieving this research aim.

1.2.4 Cane toads (*Rhinella marina*)

To better assess the impact of gut microbiota on species invasion, I have studied the invasive cane toad in Australia. Here I present relevant background information on the cane toad's introduction and invasion history, biological characteristics, invasion-related behaviours, endobiome (e.g. internal parasites), and potential factors that may contribute to gut microbiota variation in this species.

1.2.4.1 Cane toads in Australia

Cane toads are native to Central and tropical South America (Crossland & Alford, 1998) and, after introduction to Australia 1935 by the Queensland sugar cane industry as a means of controlling pest beetles, unexpectedly became one of the most invasive species in Australia. In approximately 85 years, toads have spread from the original sites of introduction in Queensland to Western Australia and their rate of invasion has accelerated from around 15 km to 60 km per annum (Alford, Brown, Schwarzkopf, Phillips, & Shine, 2009; Gruber, Brown, et al., 2017a; C. M. Hudson et al., 2016; B. L. Phillips, Brown, Greenlees, Webb, & Shine, 2007).

Cane toads possess life history traits that enable successful invasion. Adults breed in static water bodies and produce egg clutches containing more than 30,000 eggs each time, which quickly develop into free-swimming larvae (within 3-4 days). Tadpoles metamorphose into metamorphs (or toadlets) in approximately 16 days, resulting in high population densities (DeVore, Crossland, & Shine, 2021) and rapidly reach sexual maturity (around 90mm snout-vent length) at about one year post-metamorphosis (Zug & Zug, 1979).

Since their introduction, micro-evolutionary changes have occurred in both cane toads and Australian animals that prey on toads (e.g., frog-eating snakes) (Shine, 2012). Cane toads have been threatening native predator species in the Northern Territory, Queensland and Western Australia because they rapidly reach high densities in toads' invaded areas and compete with local native species for resources (Shine, 2010). At all life stages, Cane toads are highly toxic to most predator species, including birds, other frogs, reptiles and mammals (Crossland & Alford, 1998; Hayes, Crossland, Hagman, Capon, & Shine, 2009).

In summary, cane toads' expansion across Australia has impacted native species, disturbing natural environments. Knowledge of the factors and underlying mechanisms that contribute

their invasion success could advance the management of this notorious invader, and thus protect native Australia species.

1.2.4.2 Invasion-related behaviour

Differences in dispersal enhancing behavioural traits have been found between invasion-front and range-core toads in Australia. Cane toads from the range-front are more exploratory, exhibit a bolder behavioural phenotype and are more likely to take risks in novel environments than conspecifics from the range-core (Gruber, Brown, et al., 2017b, 2017a; Gruber, Whiting, et al., 2017). Although all cane toads are nomadic, those at the invasion-front leave their retreat-sites more frequently and often travel longer distances (G. P. Brown, Phillips, Webb, & Shine, 2006; B. L. Phillips et al., 2007); they also move for more hours each day and are more likely to use straight paths (Alford et al., 2009).

Many abiotic and biotic factors influence dispersal behaviour in cane toads (Kearney et al., 2008; L. Pizzatto & Shine, 2008; Urban, Phillips, Skelly, & Shine, 2008). For example, the major drivers of metamorph distribution were found to be temperature, moisture level, body size, density, and cannibalism pressure (Child, Phillips, & Shine, 2008). It has been suggested that drivers of the increase in dispersal ability seen in invasion-front toads may be both environmental and intrinsic (Alford et al., 2009; B. L. Phillips, Brown, Travis, & Shine, 2008; Urban et al., 2008). Environments characterized by high temperatures, heterogeneous topography, low elevation, dense road networks, and high patch connectivity were found to be associated with increased invasion speed of toads (Urban et al., 2008). Besides the impact of environments, some newly developed physiological characteristics in invasion-front toads also contribute to increased dispersal rate. For example, invasion-front toads have developed wider forelimbs, narrower hindlimbs, and more compact skulls, reflecting a movement style of increased bounding with multiple short hops in quick succession that may be better suited to sustained long-distance travel (C. M. Hudson et al., 2016). Transcriptome analyses

identified stronger responses to environmental stressors (upregulated cellular repair) in invasion-front toads compared to those from the range-core (Rollins et al., 2015), which may assist invasion-front toads' exploration of new environments. Despite these evolving traits that may positively affect dispersal ability, there are some trade-offs between increased dispersal and health. For instance, invasion-front toads have suppressed immunocompetence and are able to maintain a rapid rate of dispersal by suppressing sickness behaviours such as reduced activity (D. Llewellyn, Brown, Thompson, & Shine, 2011; D. Llewellyn, Thompson, Brown, Phillips, & Shine, 2012). Moreover, toads with dispersal-enhancing traits (longer legs, narrower heads) have reduced investment in reproduction (e.g. lower gonad mass; (C. Kelehear & Shine, 2020).

Invasion-front cane toads have developed dispersal enhancing behavioural traits as compared to the those from range-core. There are many different factors that have been linked to these observed differences. Despite this deep understanding of cane toad ecology, there remains a lack of effective control of this invasive anuran. Gut microbiota, as a potential impacting factor on host behaviour, has not been investigated prior to this thesis, but may provide valuable insights for managing this invader.

1.2.4.3 Cane toad endobiome

Cane toads living in Australia lack many parasites common in the native-range; the most commonly reported microparasite in Australian toads is a nematode lungworm (*Rhabdias pseudosphaerocephala*, Dubey & Shine, 2008). Lungworms invade anurans through their skin or alimentary tract and then subsequently migrate to their lungs (C. Kelehear, Brown, & Shine, 2011). Cane toad lungworms were co-introduced to Australia with toads and occur throughout the toads' native range (Dubey & Shine, 2008; L. Pizzatto, Kelehear, Dubey, Barton, & Shine, 2012). This parasite is widespread among Australia cane toads, occurring in over 80% of toads, except in populations close to the invasion-front (Barton, 1998; C.

Kelehear et al., 2011; B. L. Phillips et al., 2010). Lungworms are less frequently present in larger toads (Barton, 1998), and the wet season (main breeding season) is the peak transmission period (Barton, 1998). Infection causes impaired locomotor ability, reduced prey intake, lowered growth rates and reduced viability of toads (C. Kelehear, Webb, & Shine, 2009; C. Kelehear et al., 2011).

Besides knowledge of lungworm infection, there is scant information about other components of the endobiome in cane toads. However, the skin microbiome has been studied. Interestingly, toads' skin microbiota appear to protect them from infective larval lungworms (Christian et al., 2021). This finding suggests that studying the holobiont (the host and those species living in or on the host) could clarify our understanding of how toads interact with their environment. However, it also would be beneficial to understand the role of gut bacterial in this species.

1.2.4.4 Information about intrinsic factors that may impact cane toad gut microbiome

As demonstrated in other species, host factors (e.g., genetics, sex, age, and physical status) should be taken into account as potential factors impacting cane toad gut microbiome. There are genetic differences between cane toad populations from either end of the Australian range (Selechnik, Richardson, Shine, DeVore, et al., 2019). These differences may affect cane toad behaviour indirectly, by causing modifications to gut microbiota that impact behaviour. DNA methylation also differs between range-core and invasion-front toads (Sarma et al., 2020), and this could be driven (at least in part) by gut microbiome. In amphibians, life stage can contribute to gut microbiota variation (Fontaine, Mineo, & Kohl, 2021; Tong, Cui, Hu, et al., 2020; M. Zhang et al., 2018). Focusing on one life stage (e.g. adult), can remove the complexities introduced by the impact of different life stages. Similarly, in a range of taxa including frogs, microbial profiles differ with sex (Kozik et al., 2017; Org et al., 2016; Pereira, Bandeira, Fonseca, & Cunha, 2020). Adult cane toads can be readily sexed using

external morphological characteristics (males possesses nuptial pads on the thumbs, rugose dorsal skin and yellow coloration), vocalization (males can be observed calling or producing release calls upon handling, C. M. Hudson, Brown, & Shine, 2016), and direct examination of their gonads (C. Kelehear et al., 2011). This enables initial investigations of adult toad microbiome to focus on a single sex, eliminating this source of variation. Finally, body size and body weight are widely reported to be associated with gut microbiota (Angelakis, 2017; Chai et al., 2018; J. Fan et al., 2019), which may be reflective of diet. The impact of all of these intrinsic factors need to be studied in cane toads to better understand their gut microbiota and, ultimately, its potential role in this invasion.

1.2.4.5 Information about extrinsic factors that may impact cane toad gut microbiome

As a range expanding population, cane toads have been exposed to different habitats as they have crossed Australia. Cane toads prefer open spaces, grasslands or anthropogenically disturbed habitats (Zug & Zug, 1979). Their current range in Australia encompasses varied environments that are not ideal habitat for cane toads, such as arid deserts and high cool mountains (G. P. Brown, Kelehear, & Shine, 2011; Rollins et al., 2015). Cane toads can adjust their thermal tolerance rapidly after encountering low temperature habitats, which allows them to invade cold montane areas (S. McCann, Greenlees, Newell, & Shine, 2014; S. M. McCann, Kosmala, Greenlees, & Shine, 2018). Despite their tolerance to a broad range of temperatures, they cannot tolerate extreme water loss, so the dry season and the resulting desiccation is considered to be a major mortality factor in Australia (Zug & Zug, 1979). For this reason, cane toads often congregate at waterholes in drying riverbeds or watered lawns to hydrate (G. P. Brown, Kelehear, Shilton, Phillips, & Shine, 2015) and are generally active in the evening (Zug & Zug, 1979). Selectively choosing sampling sites where represent dry and humid environments at similar latitudes from both invasion-front (Western Australia) and

range-core (Queensland) would reduce some of the bias introduced by habitat variability. A record of local climate data can also be used to evaluate the impact of different habitats.

Cane toads eat a wide variety of prey, mainly arthropods (Shine, 2010). In the Philippines, cane toads were recorded to prey on skinks, which highlights toads' generalist and carnivorous diet (Jabon et al., 2019). Both adults and juvenile toads can take a primarily cannibalistic diet, especially during dry weather spells which reduce the availability of alternative (invertebrate) prey (L. Pizzatto & Shine, 2008). Tadpoles have also been observed eating dead conspecifics (Sarma, pers. comm.). However, little is known about the diet of wild cane toads in Australia. The expansive range of toads in Australia encompasses a wide variety of habitats, which is likely to result in differences in prey availability. Therefore, studying diet composition in wild toads may be important to tease apart the factors influencing gut microbiota.

1.2.5 Summary of literature review

Both parasites and gut microbes can impact host behaviour and the underlying mechanisms of their impact are diverse due to their broad range of taxonomy. Evidence shows associations between parasites and gut microbes, both of which can influence host behaviour, and suggests that they may interact. Although there are limited studies on the gut microbiota of invasive species, there are observed difference in gut microbial community between invasive and native populations. These findings suggest that gut microbiota may play an important role in driving changes in invasive behaviour, thus enhancing species invasion. The mechanisms underlying how gut microbiome affects host behaviour through the gut-brain axis are complex and under-explored. However, studying the association between host DNA methylation and gut microbiota may provide valuable insights into these mechanisms.

A large number of intrinsic and extrinsic factors have been reported to be associated with gut microbiota variation and most of the evidence is from studies of human and other model

species. The contribution of each factor may vary across taxa, so it is valuable to investigate these broadly. However, to avoid confounding results and reduce the complexity (and associated cost) of studies, it may be important to control factors such as life stage, sex, or environment.

As with most fields of study, a careful choice of methods is key to advancing the study of gut microbiomes. For example, identifying robust, non-lethal sampling methodologies is essential for studying the causal relationships between gut microbiota and host behaviours. These methodologies should be validated before use. Prior to this thesis, non-lethal sampling of gut microbiome has not been validated in adult amphibians. The method chosen to characterise gut microbial communities will determine both the cost and the amount of data produced. A next-generation sequencing approach, based on 16S rRNA V3-4 region incorporated with developed bioinformatics pipelines (QIIME2, PICRUST2 and various R packages), can be an effective method to study gut microbial profiles.

1.2.6 Research questions and objectives of the thesis

The overall aims of my thesis are to characterise drivers of gut bacterial variation in invasive cane toads and, by using a comparative approach between range-core versus invasion-front toads, determine whether any of these correlations are associated with invasion ability in this iconic species. Specifically, I aimed to:

(1) Evaluate effective non-invasive sampling methods of gut bacteria in this species
(2) Compare intestinal bacterial communities between range-core and invasion-front toads in Australia
(3) Study the important extrinsic (e.g. environmental) and intrinsic factors (e.g. host traits) that may interact to maintain and influence the composition and stability of intestinal bacteria

(4) Analyse behaviour associated with invasion and determine if these behaviours are correlated with intestinal bacterial composition

1.2.7 Overview of experimental data chapters

1.2.7.1 Chapter 2

In this chapter, I compared the bacterial profiles of faeces and cloacal swabs, to those obtained from extracted gut content samples from the small and large intestine of adult cane toads. I found that cloacal swabs are better proxies for large intestinal microbiome than faeces in toads. This manuscript has been published in *Molecular Ecology Resources* (Zhou, Nelson, Rodriguez Lopez, Sarma, et al., 2020). These findings will enable future manipulative experiments to study causational relationships between gut bacteria and host phenotype.

1.2.7.2 Chapter 3

In this chapter, I compared gut bacteria of wild-caught cane toads from the range-core to that of the invasion-front (Figure 1.1). During sample collection, I conducted baseline behavioural assays, health condition assays and other phenotypic traits, to determine whether there was a relationship between gut bacterial community assemblage and cane toad phenotypes previously associated with range expansion. I also collected parasites infection data (gut parasites, lungworms) and used previously collected climate data to investigate how environment might affect gut bacteria. I found significant difference in bacterial community and predicted functions between invasion-front and range-core toads. The combination of isothermality, annual mean temperature, and the presence of lungworms explained the greatest variation (12.0%) in bacterial community. The combination of isothermality, annual mean temperature, the presence of lungworms, righting reflex time, and the presence of righting reflex movements explained the greatest variation (26.0%) in bacterial functions. The manuscript is published as preprint (Zhou, Nelson, Rodriguez Lopez, Zhou, et al., 2020).

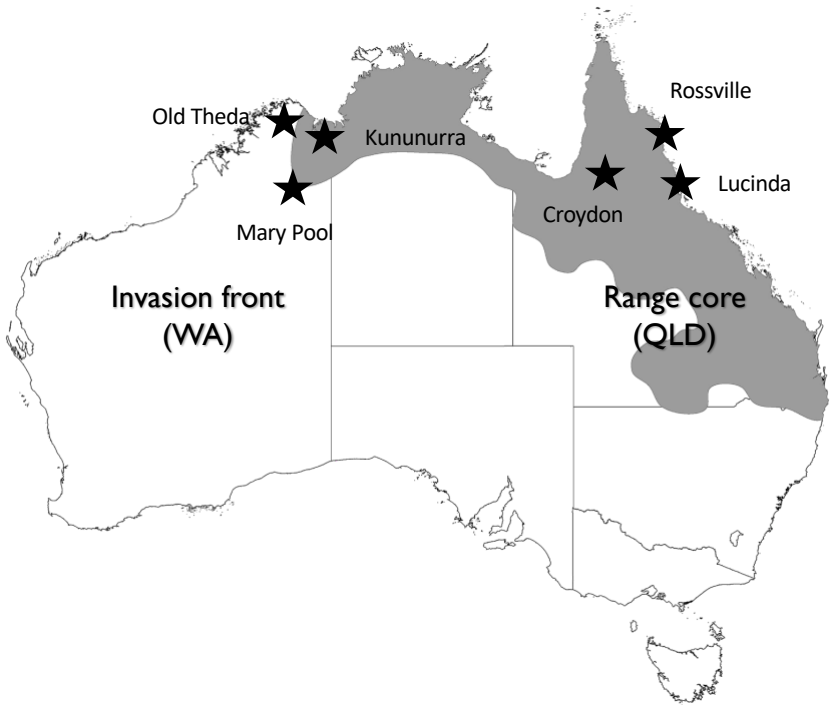

Figure 1.1 Sampling localities.

Dark grey region is the geographic distribution of the cane toad in Australia. Map adapted from Selechnik et al., (2017). Three sites near the invasion-front (Kununurra 15.776566° S, 128.744293° E, Old Theda 14.790795° S, 126.497624° E, Mary Pool 18.72528° S, 126.870096° E. n=30, collected November 2018) and three sites near the range-core (Rossville 15.697069° S, 145.254385° E, Croydon 18.207536° S, 142.245702° E, Lucinda 18.530149° S, 146.331264° E. n=30, collected December 2018).

1.2.7.3 Chapter 4

In chapter 4, I examined whether cane toad genotype (single nucleotide polymorphism: SNP) and epigenetic (DNA methylation) profiles were associated with the differences observed in toad gut bacteria. I processed methylation-sensitive genotype by sequencing (msGBS) DNA from blood samples for DNA methylation profiling and SNP calling. I then calculated their association with bacterial alpha and beta diversity. I found no significant association between host heterozygosity and gut bacterial diversity within individuals. However, I did find that pairwise genetic diversity was positively associated with pairwise epigenetic diversity. Interestingly, the positive correlation I identified between pairwise epigenetic diversity and bacteria diversity was greater in pairs with lower genetic differentiation.

1.2.7.4 Chapter 5

In the final data chapter, I examined the relationship between cane toad diet (taxonomy of stomach contents) and its association with gut bacteria. Stomach contents included plants, non-organic matters, and the majority of the diet consisted of animals (mainly insects, like termites and ants). Even though I did not find a difference in alpha diversity of animal food items within each toad's diet, I did observe a significant difference in beta diversity of those items between invasion-front and range-core toads. I found that the presence of plant matter in cane toads' stomachs was associated with gut bacterial variation, but gut bacteria was not associated with the main component of cane toad's diet.

CHAPTER 2: A comparison of non-lethal sampling methods for amphibian gut bacteria analyses

This chapter is published in *Molecular Ecology Resources*:

Zhou, J., Nelson, T. M., Rodriguez Lopez, C., Sarma, R. R., Zhou, S. J., & Rollins, L. A. (2020). A comparison of nonlethal sampling methods for amphibian gut microbiome analyses. *Molecular Ecology Resources*. doi:10.1111/1755-0998.13139

Statement of Authorship

Title of Paper	A comparison of non-lethal sampling methods for amphibian gut microbiome analyses
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Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	20/4/21

Please cut and paste additional co-author panels here as required.

Abstract

Non-invasive sampling methods for studying intestinal microbiome are widely applied in studies of endangered species and in those conducting temporal monitoring during manipulative experiments. Although existing studies show that non-invasive sampling methods among different taxa vary in their accuracy, to date, no studies have been published comparing non-lethal sampling methods in adult amphibians. In this study, I compare microbiomes from two non-invasive sample types (faeces and cloacal swabs) to that of the large intestine in adult cane toads, *Rhinella marina*. I used 16S rRNA sequencing to investigate how bacterial communities change along the digestive tract and which non-lethal sampling method better represents large intestinal bacteria. I found that cane toads' intestinal bacteria was dominated by Bacteroidetes, Proteobacteria, Firmicutes and, interestingly, I also saw a high proportion of Fusobacteria, which has previously been associated with marine species and changes in frog immunity. The large and small intestine of cane toads had a similar bacterial composition, but the large intestine showed higher diversity. My results indicate that cloacal swabs were more similar to large intestine samples than were faecal samples, and small intestine samples were significantly different from both non-lethal sample types. This study provides valuable information for future investigations of the cane toad gut bacteria and validates the use of cloacal swabs as a non-lethal method to study changes in the large intestine bacteria. These data provide insights for future studies requiring non-lethal sampling of amphibian gut bacteria.

Keywords: amphibian, cane toad, gut bacteria, Illumina, 16S rRNA

Introduction

Accumulating evidence indicates that variation in gut microbial assemblages can significantly affect the host phenotype (C. Mu et al., 2016; Stilling et al., 2014). However, the majority of the work investigating this idea has been descriptive in nature and limited to the identification of correlative associations between microbial community assemblages and phenotypic traits of hosts. The identification of causality requires the use of experimental interventions (e.g., antibiotic administration, faecal microbiota transfer, co-housing, and cross-fostering or rederivation) (Ericsson & Franklin, 2015) that enable the comparison of the phenotypic trait of interest in response to manipulations of intestinal microbiota in the same individuals across time. Therefore, non-lethal methods are needed to further this field of research by providing representative information to enable the assessment of the intestinal microbiota before and after interventions.

Faecal samples and cloacal swabs are two commonly used non-lethal sampling methods; however, the accuracy with which each sample represents the intestinal microbiota is often untested (Bassis et al., 2017). In different hosts, these methods may differ in their representation of the intestinal microbiota, yielding distinct compositions of bacterial taxa. In birds, faecal samples have been shown to provide a more accurate assessment of the large intestinal (colon) microbiota than cloacal samples (Videvall et al., 2018). In another study of birds testing only swabs, the authors found that cloacal swab samples shared similar microbial species with large intestinal (caecal) samples, but displayed different relative abundances (Stanley, Geier, Chen, Hughes, & Moore, 2015). In lizards, faecal samples were very similar to the large intestinal (hindgut) microbiota, yet were less representative of the stomach or small intestinal microbiota (Kohl et al., 2017); in bats, the faecal sample microbiota did not provide an accurate representation of large intestinal microbiota (Ingala et al., 2018). Faeces has been demonstrated to be a robust proxy for the gut microbiota in tadpoles (X. Song et al., 2018). In summary, different species show different patterns regarding the comparison of the

large intestinal microbiota to different non-lethal sampling methods and there are no studies in adult amphibians.

Cane toads are one of the most successfully invasive species globally. In Australia, cane toads have spread from the original sites of introduction in Queensland (introduced in 1935), westward to Western Australia and southward to New South Wales (Alford et al., 2009; Gruber, Brown, et al., 2017a), with increasing annual spreading rates from 10-15 to 55-60km per annum (Tingley et al., 2017; Urban et al., 2008). This increasing invasion speed may be caused by environmental factors as well as through the development of dispersal-related traits following their introduction (C. M. Hudson, Brown, & Shine, 2017; Urban et al., 2008). Morphological and physiological traits underlie this accelerated expansion, yet it is also clear that behavioural traits have evolved across this invasion; western cane toads exhibit bolder behaviour than eastern toads (Gruber, Brown, et al., 2017a). Notably, these changes have occurred despite low genetic diversity in Australian toads (Lillie, Shine, & Belov, 2014; Selechnik, Richardson, Shine, DeVore, et al., 2019; Slade & Moritz, 1998). Environmental and intrinsic factors (e.g., genetic components) that may affect behavioural traits have been previously studied (Rollins et al., 2015; Selechnik, Richardson, Shine, Brown, & Rollins, 2019; Urban et al., 2008). Although there is direct evidence of the impact of host gut microbiome on adaptation in novel environments (Hauffe & Barelli, 2019), gut microbiota has never been characterized in cane toads; therefore, the impact of gut microbiota on the changes in invasive behaviour across the invasion range has not been investigated.

In order to explore the intestinal bacteria in cane toads, it is essential to answer the following questions: (1) what bacteria are found inside the intestinal tract? (2) which non-destructive sampling method (cloaca or faeces) better represents the intestinal bacteria? and (3) which host factors contribute to the identified differences in the intestinal bacteria? In order to answer the questions, I used 16S rRNA sequencing to characterize bacterial communities from samples of the large intestine, small intestine, faeces and cloacal swabs. Because it is

well known that microbial communities are affected by host sex and body size (related to differences in diet and age) (Kozik et al., 2017; Muegge et al., 2011), I also examined whether bacterial microbiota varies with sex and body size in cane toads. These finding will inform future gut bacterial studies of adult amphibians.

Materials and methods

Sample collection

Eighteen adult cane toads were sampled from a captive breeding colony. These individuals were originally collected from wild populations located in Mary Pool, Western Australia (WA; nine males) and Port Douglas, Queensland (QLD; three males, six females) in April 2018. Individuals were sexed by external morphological characteristics (males possesses nuptial pads on the thumbs, rugose dorsal skin and yellow colouration), and vocalizations (males can be observed calling or producing release calls upon handling) (C. M. Hudson et al., 2016). All individuals were held in captivity for one week before being injected with leuprorelin acetate to stimulate breeding (for another experiment). For the following three months, animals were housed in sex-specific and sampling location-specific outdoor bins (1,165 x 1,165 x 780mm) fitted with insect-attracting lights and water sprinklers (bin A = males from the WA, bin B = females from the QLD, bin C = males from the QLD). During this period, toads fed on local insects attracted to lights installed over their bins.

After three months in captivity, body weight and body length (SUL = snout-urostyle length and SVL = snout-vent length) were measured and then toads were placed in individual 1L containers with 1mm water in the bottom, fed commercial crickets and housed overnight. The next morning, faecal samples were collected from containers and toads were euthanized using the injection of MS-222 (Sigma Aldrich). Then cloacal samples were collected with swabs, and samples were collected from large intestinal and small intestine immediately after death

to avoid potential differences due to the time of collection. Tools and work surfaces were sterilised between each sample. Faecal and cloacal samples were collected using sterile cotton applicators (FLOQSwabs, Copan Diagnostics Inc.); all large intestine and small intestine samples were collected by squeezing entire contents of each part of intestine separately into clean tubes (free of detectable RNase, DNase, DNA and PCR inhibitors, Scientific Specialties, Inc. SSIbio). All samples were preserved in 95% ethanol (S. J. Song et al., 2016) and stored at 4°C before shipping back to the laboratory. Sterile cotton applicators were used to sample air above the lab bench before and after sampling, which helps to characterize any contaminants present in the sample collection process (environmental controls) (Eisenhofer et al., 2019).

DNA extraction and 16S amplicon library preparation

Prior to DNA extraction, individual ethanol preserved samples were homogenised by thoroughly mixing them, and then dehydrated using a SpeedVac Concentrator (Thermo Scientific SAVANT DNA 120) at medium temperature for 50 mins. Total DNA was extracted using the DNeasy PowerSoil kit (Qiagen) following the manufacturer's protocol. DNA concentration was estimated using a Qubit^{TM} ds DNA HS Assay kit (ThermoFisher Scientific) on an Invitrogen Qubit 4 Fluorometer.

16S rRNA amplicon libraries were prepared following guidelines for the Illumina MiSeq System. Zymo isolated DNA (D6305) was used as community positive controls and MilliQ water as a PCR negative control to determine contamination during the library preparation process (Eisenhofer et al., 2019). A total of 76 DNA samples (18 faeces samples, 18 cloaca samples, 18 large intestine samples, 18 small intestine samples, 2 environmental controls, 1 community positive control and 1 PCR negative control) were used for Next Generation Sequencing library preparation. The hypervariable (V3-V4) region of the 16S rRNA gene from each sample using primers 341F (5'-

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and 785R (5'-**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GACTACHVGGGTATCTAATCC-3') was amplified (Herlemann et al., 2011) (llumina's overhang transposase adapter sequence shown in bold). Briefly, the initial PCR was set up in 25 ul reactions using 2.5 ul (>1.0 ng/ul) of input DNA, 0.75 ul (10 uM) forward and reverse primers, as well as 12.5 ul of EconoTaq® PLUS and EconoTaq PLUS GREEN 2X Master Mixes (Lucigen). The PCR protocol included: an initial denaturation at 94 °C for 2 mins, 30 cycles of 94 °C (30 sec), 55 °C (30 sec), and 72 °C (40 sec), and a final extension at 72 °C for 7 mins. PCR products were cleaned with Zymo DNA clean and concentrator (Zymo Research). Amplification success and product concentration was determined using a Fragment Analyzer with dsDNA Reagent kit (35-1500 bp) (DNF-910) (Agilent Technologies, Inc.). Cleaned amplicons were sent to the Ramaciotti Centre for Genomics (University of New South Wales, Kensington, Sydney), where all libraries were standardized for DNA concentration, indexed using a Nextera XT DNA library preparation kit (Illumina®-Nextera™), and sequenced on the Illumina MiSeq platform targeting 2x300bp paired-end sequence reads.

Data analysis

Demultiplexed FASTQ data were downloaded from Illumina's BaseSpace cloud storage, and amplicon sequence variants (ASVs) were created using the open-source QIIME2 pipeline (Bolyen et al., 2018). Demultiplexed sequence counts from samples and the positive control ranged between 82,108 and 216,977; the counts of environmental controls and the PCR negative control ranged between 24,644 and 58,785. The DADA2 pipeline (B. J. Callahan et al., 2016), implemented in QIIME2, was used to filter and trim the first 20 bases from each read and truncate sequences to 220 bases. The remaining sequences were dereplicated, then forward/reverse reads were merged, chimeras were removed, and finally ASVs were generated for downstream analysis (B. J. Callahan et al., 2016). ASVs provide finer resolution

of amplicon sequences resulting in more novel bacterial taxa than OTUs (operational taxonomic units) which are dependent on assignment to a reference database and clustering to retain taxa that meet an arbitrary similarity cut-off (usually 97%) (B. J. Callahan, McMurdie, & Holmes, 2017). After quality filtering, reads from samples and the positive control ranged between 65,184 and 167,363 counts; the reads from environmental controls and the PCR negative control ranged between 10,867 and 26,754 counts. The taxonomic assignment of ASVs was performed using Greengenes version 13_8 (DeSantis et al., 2006).

Data were pruned to remove representatives classified to Archaea (N = 2), Chloroplast (N = 4), and 53 unassigned ASVs implemented in the package 'phyloseq' in the R statistical program version 1.26.1 (McMurdie & Holmes, 2013). ASVs with abundance less than four were removed, which makes the logged counts per sample more evenly distributed (Figure S2.1). The remaining 5,298 taxa were classified to the Kingdom Bacteria with 69.03% assigned to phylum level. Relative abundance of bacteria (abundance >2%) in different sample types were visualised, and classified to the phylum and genus level.

Observed ASVs (DeSantis et al., 2006) and evenness (Pielou, 1966) indices were calculated through QIIME2 for all samples, including environmental controls, the PCR negative control and the community positive control. To compare the alpha diversity (within sample) of the communities between different sampling methods, Shannon Index (Shannon, 1948) was calculated through QIIME2, which accounts for both abundance and evenness of the taxa present. Boxplots of alpha diversity indices, generated using the *boxplot* command in base R (R Core Team, 2020), displayed asymmetrical boxes across the medians. Also, some data were potential outliers and were not normally distributed ($p < 0.001$) according to a Shapiro-wilk test to assess multivariate normality using RVAideMemoire package in R (Herve, 2018). Therefore, nonparametric tests (Wilcoxon signed-rank test) were used for pairwise comparisons of medians, using the command *wilcox.test* (Bauer, 1972) in base R to compare alpha diversity between different sampling methods.

For beta diversity, a Hellinger transformation implemented in the package “microbiome” in R (Valverde, Makhalanyane, & Cowan, 2014) was used and then Bray Curtis dissimilarity matrix was calculated and visualized using Principle Coordinate Analysis (PCoA) plots using commands from the package “Phyloseq” (McMurdie & Holmes, 2013). The *adonis2* command from the package “Vegan” in R (Oksanen et al., 2019) was used to perform permutational multivariate analysis of variance (perMANOVA) to check whether the microbial communities of each sample type were significantly different and to identify differences between individual toads. The command *betadisper* in the package “Vegan” in R was used to check the homogeneity of group variances, an assumption of perMANOVA. After finding significant differences between sample types, pairwise comparisons were made between groups using the command *pairwise.perm.manova* function in “RVAideMemoire” package with the Wilks test (Nath & Pavur, 1985) and corrections for multiple testing were conducted using the Benjamini & Yekutieli (2001) ("BY") procedure (Yekutieli & Benjamini, 2001). To identify the significant differences in ASVs between groups, differential abundance testing was performed using the function *DESeq* in the package “DESeq2” (Love, Huber, & Anders, 2014). This included comparisons between non-lethal samples (faeces and cloaca) and intestine samples (large intestine and small intestine samples) to identify taxa that differed significantly and only the 69.03% taxa that assigned to phylum level were counted.

To identify correlation between host characteristics, SUL, SVL, body weight and sex, with the bacterial community, distance-based redundancy analysis (dbRDA) was conducted. dbRDA performs constrained ordination directly on a distance or dissimilarity matrix with the function *capscale* in the “Vegan” package in R. Correlation analyses were conducted using between each pair of host characteristics (SUL, SVL, body weight and sex). A Bray Curtis dissimilarity matrix of ASVs was ordinated and the results were analysed using redundancy analysis with constraining variables that was not highly correlated to estimate their

explanatory proportion. Results for all statistical tests were considered significant where p-values < 0.05.

Results

Data quality analysis

The number of distinct ASVs and the diversity from abundant data in the negative controls (environment and PCR) were lower compared to the samples. The number of observed species in PCR (55) and environmental controls (mean = 36 ± 2.8) were lower when compared to toad samples (mean = 257 ± 121.2), which indicates minimal contamination in our sampling, DNA extraction and library preparation processes. Sample blanks have the highest evenness (Pielou's evenness) when compared with experimental samples (see Table S2.1).

The number of observed species (N = 28) in the community positive control was low compared to experimental samples (mean N = 257 ± 121.2). The six genera used as microbial community standards constituted 99.8% of our sequenced community positive control, as shown in Figure S2.2, indicating minimal PCR bias in our library preparation process.

Within sample microbiota composition and diversity

Among all samples, the dominant phyla present in samples were from the Bacteroidetes (35.55 ± 18.8%), Proteobacteria (26.91 ± 15.0%), Firmicutes (24.03 ± 17.1%), and Fusobacteria (11.55 ± 10.3%), which accounted for 98.13% of assigned phyla. The dominant phyla were consistently present in each of the four sample types (Figure S2.3). The toad large intestine had a greater abundance of Bacteroidetes (Figure S2.4A), where the small intestine contained more Firmicutes (Figure S2.4B). At the lower taxonomic level, the genera *Bacteroides*, *Cetobacterium*, *Plesiomonas*, *Clostridium* and *Epulopiscium* were consistently

present in each of the four sample types with different ratios (Figure 2.1A). Large intestine samples tended to have more *Bacteroides* and *Epulopiscium* (Figure S2.4C), but less *Clostridium* than small intestine sample (Figure S2.4D).

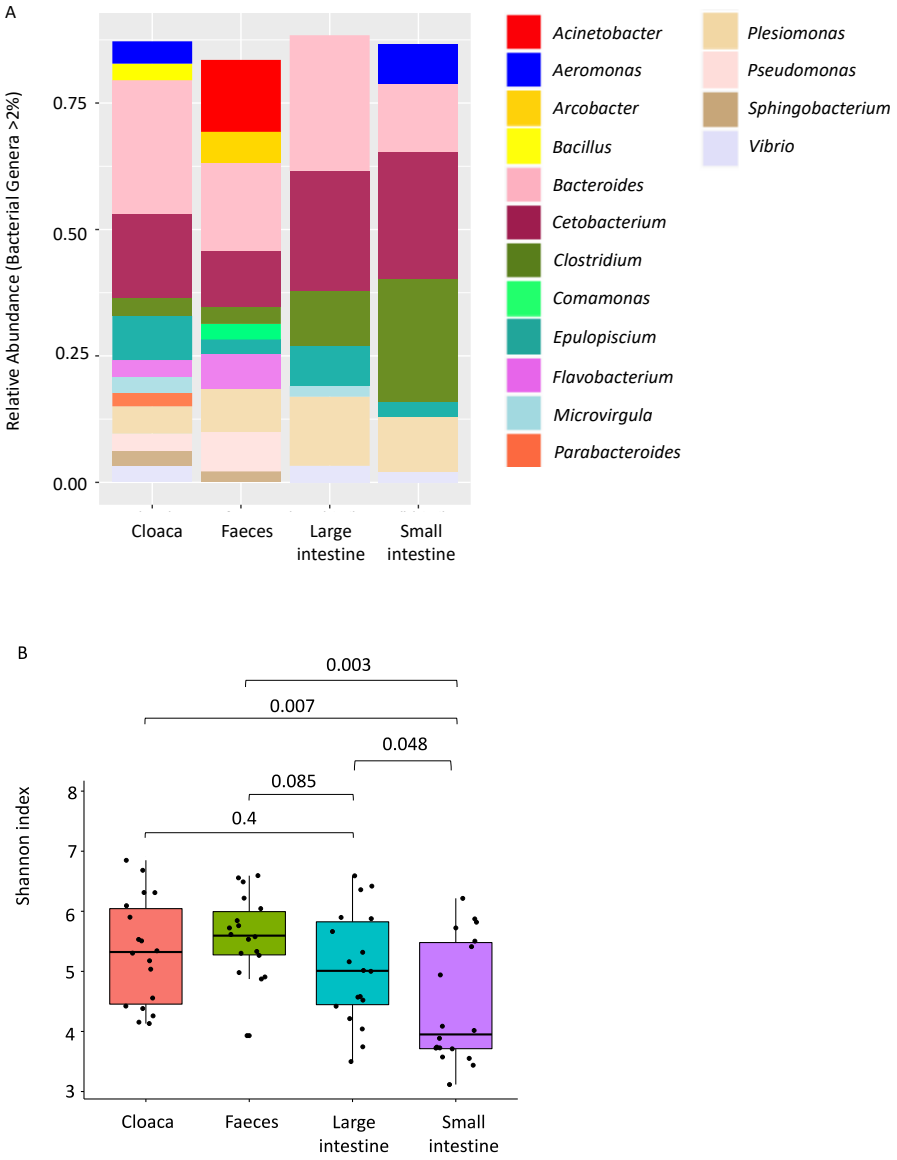

Figure 2. 1 Bacterial community composition and alpha diversity of different samples types.

Relative abundance bar plots display genera (> 2%) (A). Boxplots show the Shannon index (B). Pairwise testing between non-lethal sample groups (cloaca, faeces) and intestine sample groups (small intestine and large intestine), and between small intestine and large intestine samples were conducted using Wilcoxon signed-rank test with p-values indicated.

There were no significant compositional differences between non-lethal sampling methods (cloaca and faeces) and large intestinal samples according to observed species and evenness

(Figure S2.5A, S2.5B). Compared to the large intestine, cloaca samples had a similar abundance of dominant phyla, with a higher abundance of Proteobacteria and reduced abundance of Fusobacteria. However, faecal samples had a higher abundance of Proteobacteria and lower abundance of Firmicutes and Fusobacteria (Figure S2.3). Compared to the small intestine, cloacal samples had similar abundance of dominant phyla too, with a higher abundance of Bacteroidetes and reduced abundance of the other three phyla. Faecal samples had a higher abundance of Bacteroidetes and Proteobacteria and lower abundance of Firmicutes and Fusobacteria (Figure S2.3). Wilcoxon signed-rank test, for pairwise comparisons of medians of observed ASVs showed that large intestinal samples did not differ significantly from small intestine ($p = 0.95$), faeces ($p = 0.09$), cloacal samples ($p = 0.17$); small intestinal samples did not differ significantly from faeces ($p = 0.15$), cloacal samples ($p = 0.35$) (Figure S2.5A). Pairwise comparison of species evenness showed large intestinal samples were not different from cloacal ($p = 0.79$)and faecal samples ($p = 0.14$) (Figure S2.5B). However, evenness of the small intestine was significantly different from samples from the large intestine ($p = 0.006$), cloaca ($p = 0.004$) and faeces ($p < 0.001$).

I characterized differences in bacterial community (alpha diversity) with the Shannon index. The pairwise comparison of the Shannon index showed that neither the cloacal ($p = 0.4$) or faeces ($p = 0.085$) samples had significantly different bacterial communities when compared to samples of the large intestine (Figure 2.1B). Small intestine samples had significantly different bacterial communities than those taken from the large intestine ($p = 0.048$), cloaca ($p = 0.007$) and faeces ($p = 0.003$).

Microbial community comparison between sample type

I used Bray Curtis dissimilarity matrix to calculate beta diversity and used perMANOVA to test the dissimilarities in microbial community composition among cloacal, faecal and intestinal samples. The dissimilarities in community composition were clustered by sample

types (Figure 2.2; perMANOVA: F = 3.003, p < 0.001). Significant result was checked for homogeneity of variance (F = 1.0619, p = 0.37). The further pairwise comparison between communities showed that cloacal samples were similar in microbial community composition to large intestine samples (Wilcox, p = 0.50) and samples from the small intestine had similar bacterial community composition to those from the large intestine (p = 0.57). The cloacal bacterial community was significantly different from that of the small intestine (p = 0.03). The faecal bacterial community was significantly different from those of the cloaca, the large intestine and the small intestine (Wilcox, p = 0.01, p < 0.001 and p < 0.001, respectively).

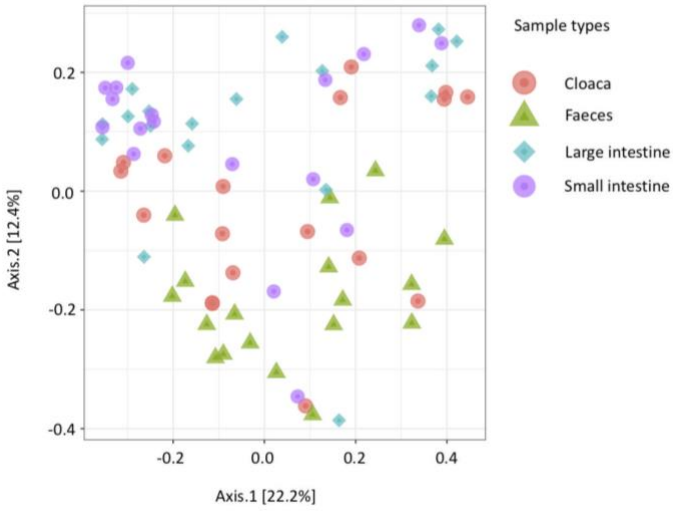

Figure 2. 2 Beta diversity by sample type.

Principle coordinate analysis plot of Bray Curtis distances of cloaca, faeces, large intestine and small intestine samples from 18 cane toad individuals.

I performed differential abundance testing between faecal and large intestine samples (Figure 2.3A), between cloacal and large intestine samples (Figure 2.3B), between faecal and small intestine samples (Figure 2.3C) and between cloacal and small intestine samples (Figure 2.3D) to identify ASVs that differed significantly (p=0.05). The figures show the significant differences between each pair of sample types as the log fold change of a taxa in one sample type compared to the control sample type. In this case, I set taxa abundance of large (or small) intestine as the control and the ASVs with significant abundance were shown in the figures:

the taxa with higher abundance in large intestine shown at the right, the taxa with higher abundance in other sample types shown at the left. Among the 69.03% taxa that assigned to phylum level, 125 ASVs in faecal samples and 60 ASVs in cloacal samples were significantly different from large intestine samples (Table S2.2, S2.3), and 116 ASVs in faecal samples and 64 ASVs in cloacal samples were significantly differed from small intestine samples (Table S2.4, S2.5). I then compared the taxa that differed significantly between non-lethal samples (cloacal and faecal samples) to large intestine samples. A large number of ASVs in the phylum of Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria were significantly more abundant in both faecal and the cloacal samples, including the classes Sphingobacteriia (all genus *Sphingobacterium*), Gammaproteobacteria (mainly genera *Acinetobacter* and *Pseudomonas*), Flavobacteriia (mainly family Flavobacteriaceae), Betaproteobacteria (mainly family Comamonadaceae), Bacilli (mainly family Bacillaceae), Alphaproteobacteria (mainly family Caulobacteraceae), Actinobacteria (mainly families Microbacteriaceae and Nocardiaceae), and [Saprospirae](family Chitinophagaceae). Both cloacal and faecal samples displayed lower $\log_2$ fold abundances in the class Clostridia (family Clostridiaceae). The comparison between faecal and large intestine samples showed lower abundances of some taxa from the classes Bacteroidia (family Rikenellaceae) and Bacilli that was not seen in the comparison between cloacal samples and those from the large intestine. Further, in the class Thermomicrobia, one taxa were less abundant in in faecal samples as compared to those of the large intestine but such lowered abundances were not seen in cloacal samples. Faecal samples had more taxa with higher abundance than large intestine in the class Epsilonproteobacteria (genus *Arcobacter*) than did cloacal samples.

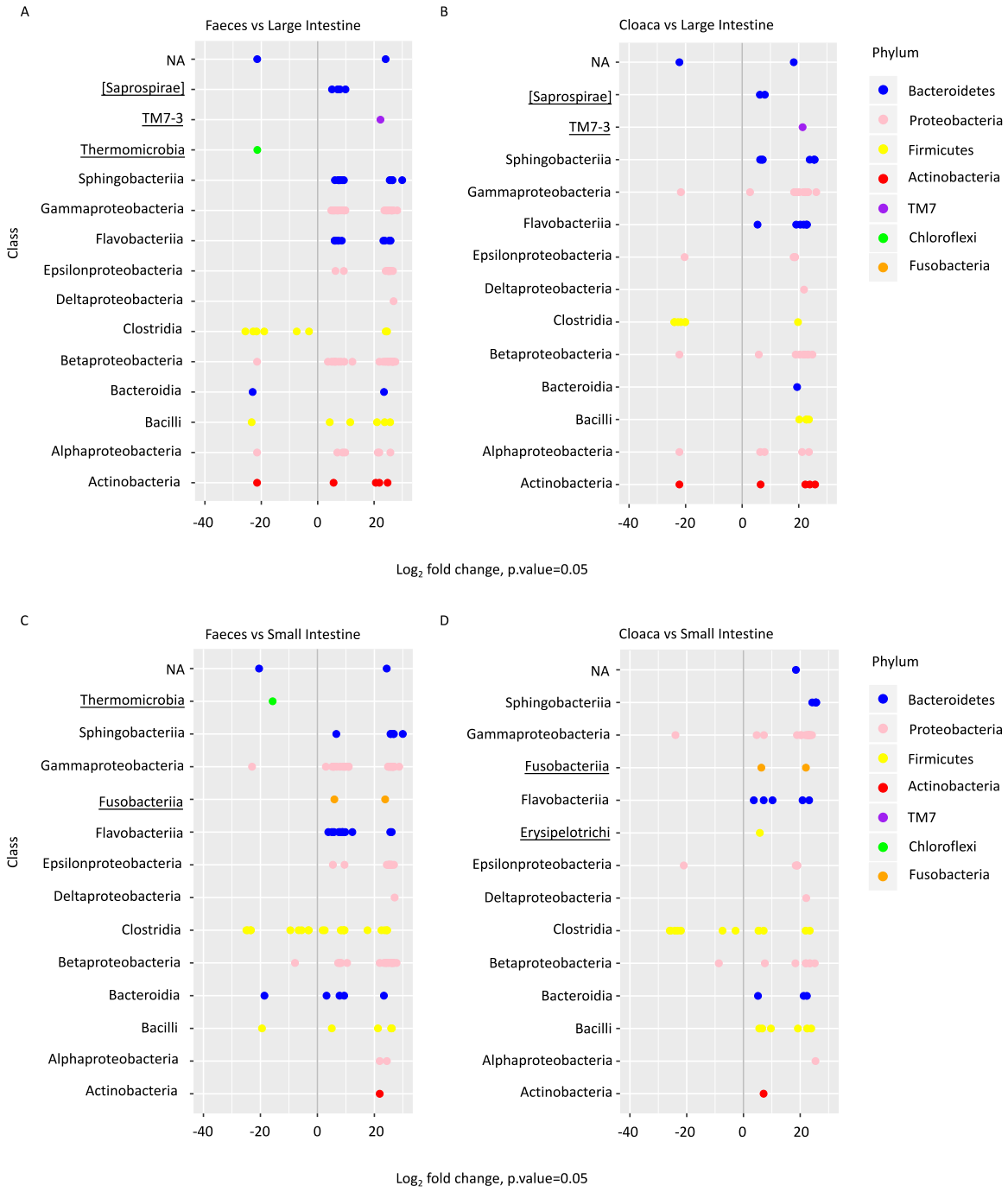

Figure 2. 3 Significantly different bacterial taxa between sample types in cane toads.

Dot plots display the significant difference between ASVs in faeces and large intestine samples (A), cloacal and large intestine samples (B), faeces and small intestine samples (C), and, cloacal and small intestine samples (D). Significant differences were identified between sample types via differential abundance testing based on a negative binomial distribution. The dots represent the average log-2 fold change (x axis) abundance of bacterial taxa classified to the taxonomic level of class (y axis) and coloured by taxonomic level of phylum, where Actinobacteria = “red”, Bacteroidetes = “blue”, Chloroflexi = “green”, Firmicutes = “yellow”, Proteobacteria = “pink”, TM7 = “purple”, and Fusobacteria = “orange”. Positive log_2 fold changes signify increased ASV abundance in either faeces or cloaca, and negative log_2 fold changes display increased abundance in large intestine or small intestine. Family name in bracket is proposed taxonomy by Greengene. NA indicates the ASVs could not match to a known bacterial class and may represent novel taxa. The taxa underlined are the class that do

not have significantly different taxa from both large intestine and small intestine when comparing to non-lethal samples.

Host factors associated with microbial community assemblage

I found significant bacterial dissimilarity between individuals (Figure S2.6) (perMANOVA: $F = 4.5254$, $p < 0.001$) and there was no significant difference in the homogeneity of variance ($F = 0.4103$, $p = 0.98$). I hypothesized that host factors, such as sex, body weight, and body length (Table S2.6), would impact microbial communities. Cane toad SUL, SVL and body weight were correlated (> 0.80). I found sex was significantly associated with microbial variation (Figure 2.4A). Because all female toads in this study were from eastern Australia and male toads were from both regions, I then looked at the bacterial difference between male and female toads in eastern toads to exclude the influence of sampling location. Since I was more interested in large intestine samples, and cloacal samples were found to be better at representing the large intestinal microbial community, I plotted the PCoA of eastern toads in both large intestinal and cloacal samples. I found that the bacterial communities were grouped by sex in both sample types (Figure 2.4B), with increased relative abundance of genera *Bacteroides*, *Comamonas*, *Flavobacterium*, *Microvirgula*, *Parabacteroides*, *Pseudomonas* and decreased relative abundance of *Cetobacterium, Clostridium*, *Epulopiscium*, *Plesiomonas, Vibrio* in female toads (Figure S2.7). The combination of sex and body weight explained the greatest variation (14.7%) in gut bacteria through model selection (Figure 2.4A).

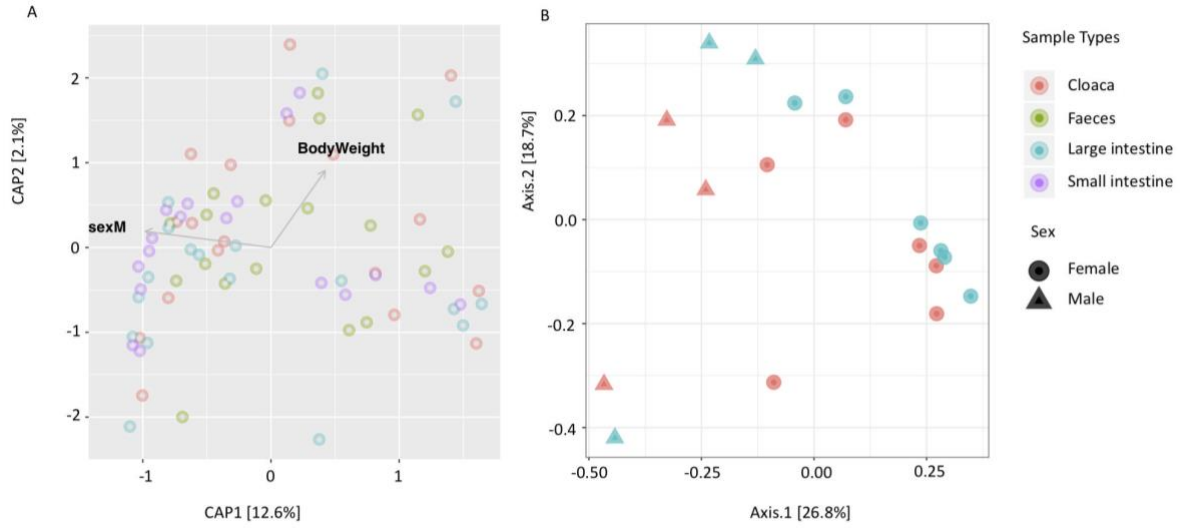

Figure 2. 4 Main host factors that affects differentiation of cane toads large intestinal and cloacal bacteria among individuals.

CAP (capscale) plot displays the combination of variables that explained the greatest variation in the bacteria through model selection, where cloacal = red, faeces = green, large intestine = blue and small intestine = purple (A). The variables implemented in the final model were sex and body weight, which explained 14.7% of variation in the microbiota. Principle coordinate analysis plot of Bray Curtis dissimilarity matrix of the large intestinal and cloaca samples from 9 eastern Australian cane toads, where cloacal = red, large intestine = blue, female = circle, and male = triangle (B).

Discussion

This is the first study to compare non-lethal sampling for the study of gut bacteria in adult amphibians, which is important information when repeated sampling or non-lethal sampling is needed. Further, this is the first study to characterise cane toad intestinal bacteria, which is fundamental for understanding the adaptive potential of behaviours that may be influenced by microbiota across the Australian invasion. Here, I characterised intestinal tract microbial composition and diversity, explored the similarities and differences in the bacterial community between the non-destructive sampling methods, and examined the potential impact of host factors that may contribute to the differences in intestinal bacteria. My data show that in cane toad, cloacal samples are more representative of the large intestinal bacteria than are faecal samples. This finding is likely to be useful to other studies of adult amphibian

gut microbiome. Similar to other studies of gut microbiome across taxa, I found that both sex and body weight were important factors accounting for inter-individual variation in gut bacterial community assemblage. However, further work is needed to identify the effect of metamorphic transition in cane toad gut microbiomes since previous studies have shown that this is a factor regulating the composition of the microbiome in amphibians (Demircan et al., 2018).

I found that the cane toad's intestinal microbiota were dominated by four phyla: Bacteroidetes, Proteobacteria, Firmicutes, and Fusobacteria. The first three taxonomic groups are commonly identified in the intestine of frogs and toads (Chang et al., 2016; Huang et al., 2017; Kohl, Amaya, Passement, Dearing, & McCue, 2014; Wiebler, Kohl, Lee, & Costanzo, 2018). On the contrary, although Fusobacteria have also been found as dominant phyla in the intestinal microbiota of Asiatic toad (Chai et al., 2018), it has not been reported as a dominant phyla in other frogs and toads (Chang et al., 2016; Huang et al., 2017; Kohl et al., 2014; Wiebler et al., 2018). Proteobacteria, Bacteroidetes and Firmicutes are generally identified in the intestines of terrestrial mammals, marine taxa and amphibians while Fusobacteria are commonly found in marine species (e.g., fish: Larsen, Mohammed, & Arias, 2014; Nelson et al., 2013). The cane toad's microbiota showed similarity to both amniotes and fish, although the adult frog microbial community was previously found to be more similar to amniotes rather than fish (Kohl, Cary, Karasov, & Dearing, 2013). The Proteobacteria genus *Plesiomonas* were found consistently in cane toads' intestine, which is widely found in different hosts and nature (especially aquatic environments, J. A. Santos, Rodríguez-Calleja, Otero, & García-López, 2015). The Bacteroidetes genus *Bacteroides* and Firmicutes genus *Clostridium* were also found consistently in the cane toads' intestine, which were reported in other studies of amphibian intestinal microbiota (e.g., slimy salamander (Plethodon glutinous): Okelley, Blair, & Murdock, 2010; and frogs: Chang et al., 2016; Okelley et al., 2010; Wiebler et al., 2018). Another Firmicutes, genus *Epulopiscium*, were also abundantly

existing in my samples. However, it is interesting to note the presence of *Epulopiscium* spp, which are large bacteria and the most well-known species, *Epulopiscium fishlsoni*, has a unique symbiotic relationship with the tropical marine, herbivorous brown surgeonfish and has not been observed elsewhere (Angert, Clements, & Pace, 1993; Clements, Sutton, & Choat, 1989; Fishelson, Montgomery, & A. Myrberg, 1985). Fusobacteria are anaerobic gram-negative bacilli (Bennett & Eley, 1993) that ferment carbohydrates and produce butyrate (Potrykus, Mahaney, White, & Bearne, 2007; van Gylswyk, 1980). Butyrate or butyric acid is a short chain fatty acid with known positive effects on the control of enteric pathogens and gut health (Bedford & Gong, 2018). The most abundant Fusobacteria genus in our samples were *Cetobacterium*, which has been found in both mammals and fish (Finegold et al., 2003; Tsuchiya, Sakata, & Sugita, 2008). Interestingly, the *Cetobacterium* were observed in the gut of juvenile Cuban tree frogs and were positively correlated with parasite resistance in adults (Knutie, Wilkinson, Kohl, & Rohr, 2017). The *Cetobacterium* may contribute to the host immune response by producing vitamin B_{12} (cobalamin) (Degnan, Taga, & Goodman, 2014; Yoshii, Hosomi, Sawane, & Kunisawa, 2019).

I found that the cane toad's large and small intestines were similar in terms of abundances of phyla and observed species. However, bacterial communities in the large intestine have greater diversity (richness and evenness) than small intestine. This could be due to slower intestinal motility and longer transit time in large intestine which increases the probability of microbial colonization (Berg, 1996; Hillman, Lu, Yao, & Nakatsu, 2017). Large intestine samples tended to have more *Bacteroides* and less *Clostridium* than those from the small intestine, which has also been observed in mice (Onishi et al., 2017). Both genera contain anaerobic bacteria (Wells & Wilkins, 1996; Wexler, 2007) and both contain commensals and pathogens; interestingly, these taxa have also been implicated in the maintenance of host gut physiology, including SCFA production (Lopetuso, Scaldaferri, Petito, & Gasbarrini, 2013; Wexler, 2007). *Bacteroides* are important in fermenting soluble carbohydrates in the human

large intestine (P. Louis, Scott, Duncan, & Flint, n.d.). The genus *Clostridium*, a Firmicutes, performs most of their metabolic functions through the release of butyrate that is essential as fuel for colonocytes and maintains gut homeostasis (Lopetuso et al., 2013).

I found that cloacal samples are better than faecal samples for representing the large intestinal bacteria of cane toads. There were no significant compositional differences between cloacal, faecal and large intestine samples with respect to relative abundances of phyla and alpha diversity (observed species, evenness and Shannon index). Shannon's index accounts for both abundance and evenness of the taxa present; therefore, it is unsurprising that this metric (Figure 2.1B) showed similar patterns of the observed species (Figure S2.5A) and evenness plots (Figure S2.5B) among different sample types. However, the beta diversity results showed that cloaca samples were not significantly different to the large intestine bacteria, while faecal samples were significantly different to the large intestine bacteria. Also, I identified more significantly different ASVs in faecal samples (125 ASVs) than cloacal samples (60 ASVs) compared to large intestine samples.

One important variable driving the observed differences between faeces and large intestine microbiota could be that faeces had been deposited overnight and collected in the morning. This exposure to aerobic conditions could have a significant effect on their microbial profiles as discussed below. Previous studies in humans have showed that faecal and rectal swab microbiota from the same individual were similar (Bassis et al., 2017). However, collecting "clean" and fresh faeces samples from humans is relatively easy; it is much more difficult to accomplish this in wild animals, such as cane toads. Interestingly, opposite results were found in birds: faeces samples were significantly better than cloacal swabs in representing the colon bacterial community of juvenile ostriches (Videvall et al., 2018). As mentioned above, the representativeness of large intestine microbiota from different non-lethal sampling types varies between species. My results highlight the importance of validating non-lethal sampling methods for each taxonomic group. It is, however, important to highlight that literature

describing the changes in post-mortem gut microbiome composition in hot-blooded animals identifies time since death as a factor for gut microbiome changes (Brooks, 2016). Although no obvious variation is observed immediately post-mortem in the rectum samples in mice and human (DeBruyn & Hauther, 2017; J. Guo et al., 2016), clear changes can be found as fast as 5 minutes after death (Heimesaat et al., 2012). One factor affecting these changes is the loss of body temperature that occurs after death in hot-blooded animals. Even though there is no study on the effect of time since death on the gut microbiome of cold-blooded organisms, it is unlikely that loss of body temperature has a significant effect on microbiome composition when samples are collected immediately after death.

In my results, the majority of ASVs that were found to be significantly more abundant in both faecal and cloacal samples as compared to the large intestine were aerobic, except for a few ASVs that are facultatively anaerobic or commonly found in diverse environments. Moreover, faecal samples had more aerobic taxa than cloacal samples. This is indicative of a rapid depletion of anerobic taxa in both types of non-lethal sampling approaches, especially faecal samples, that should be taken into account in future studies. For example, the class Clostridia (family Clostridiaceae) from which I found fewer ASVs in both cloacal and faecal samples than in large intestine samples is, unsurprisingly, anaerobic and actively involved in energy metabolism with Enterobacteriaceae (Wiegel, Tanner, & Rainey, 2006; Wüst, Horn, & Drake, 2011). Compared to cloacal samples, faecal samples had lower abundance of family Rikenellaceae and this family had been found in increased abundance in healthy humans than in non-alcoholic fatty liver disease patients (Jiang et al., 2015).

Sex was a major contributor to the differences in the intestinal microbial community of cane toads in this study and body weight was less influential. Both of these factors were found to influence intestinal microbiota in humans (Borgo et al., 2018). Sex hormones were found to mediate the changes in mice intestinal microbiota composition (Bray Curtis distances) by gonadectomy and testosterone hormone replacement (Org et al., 2016). Also, Org et al.

(2016), showed that hormonal changes and gender differences strongly affected bile acid profiles, which is prominent in response to a high-fat/high-sugar diet and have been shown to affect gut microbiota (Islam et al., 2011; T. Li & Chiang, 2015). While it is possible that the interaction of diet and sex may be important in microbiome studies of amphibians because male toads spend more time near water than females, the animals used in this study were housed in a common environment with access to the same diet. However, it is important to note that the intestinal microbiota has previously been strongly associated with long-term diets in humans (G. D. Wu et al., 2011).

Conclusion

This study provides important information about non-lethal sampling of gut bacteria in adult amphibians and broadens our scant knowledge of amphibians' intestinal bacteria. Further, I show that cloacal samples are a better choice than faecal samples for the accurate characterisation of cane toad intestinal microbiota. Having a reliable non-invasive method will allow the same animal to be sampled repeatedly across time, enabling manipulative experiments to investigate the role of gut bacteria in influencing behaviours important to invasion. This capability opens an entirely new dimension to the field of invasion ecology.

1

CHAPTER 3: Association between host behaviour and gut bacteria across an expanding range of an invasive anuran

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Overall percentage (%)	90%		
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Abstract

Invasive species cause negative environmental and economic impacts worldwide. Their management may be improved by clarifying the role of behaviour in advancing invasions. Gut microbial communities are known to affect behaviour of wild populations, but their impact on behaviour underlying invasiveness remains unexplored. Invasive populations of the cane toad (*Rhinella marina*) in Australia have expanded across the continent and exhibit variation in behavioural traits along their expansion trajectory, making this an ideal system to investigate the relationship between gut microbes and behaviours. I collected wild female toads from both ends of their Australian range (Queensland: n = 30; Western Australia: n = 30) and conducted simple tests on behavioural traits previously associated with invasion ability. I investigated the relationships between toad gut bacteria, behavioural traits and the presence and intensity of co-introduced lungworms (*Rhabdias pseudosphaerocephala*), which are known to affect toad thermal and hydration preferences and defecation behaviour. Based on 16S rRNA sequencing data, I found that gut bacteria in cane toad colons were dominated by the phyla Bacteroidetes, Proteobacteria, Firmicutes, Verrucomicrobia, and Fusobacteria. I found significant differences in microbiota composition (p-value < 0.001) between regions and in predicted microbial functional groups (p-value = 0.002). Behavioural traits were associated with bacterial functional variation, but not bacterial compositional variation. However, lungworm occurrence was strongly associated with variation in both bacterial composition and bacterial functions. These results support the "holobiont concept" (investigating the assemblage associated with a host) to fully understand drivers of invasion and highlight the need for experimental manipulations to detect causal relationships between gut bacteria, parasites and host behaviour.

Introduction

An animal's behaviour can be influenced by the bacterial microbiome within its intestines. To understand this, scientists have experimentally manipulated the microbiome in host species. For example, gut microbiota transfer has been found to modify exploratory behaviour of germ-free mice to resemble that of the donors (Bercik et al., 2011), and to influence emotional reactivity in Japanese quail (*Coturnix japonica*; Kraimi et al., 2019). Further, altering gut microbiota by treatment with a sterile diet or with antibiotics changed chemical social cues and triggered aggressive behaviour in leaf-cutting ants (*Acromyrmex echinatior*; Teseo et al., 2019). Remarkably, host behavioural changes can be due to only an individual bacterial species. Mono-colonization with one dominant species, *Lactobacillus brevis*, can decrease walking speed and daily activity of germ-free or antibiotic-treated fruit flies (*Drosophila melanogaster*; Schretter et al., 2018). Dietary administration with the probiotic *Lactobacillus rhamnosus* IMC 501 to zebrafish (*Danio rerio*) caused shoaling behaviour to be more uniform and exploratory than that of the control groups (Borrelli et al., 2016) and administration of *Bifidobacteria* reduced stress-related behaviour of BALB/c mice (Savignac, Kiely, Dinan, & Cryan, 2014). The gut microbiota can contribute to host adaptability (Hauffe & Barelli, 2019) through mating choices (Sharon et al., 2010) and food resource usage (Vogel et al., 2017). For example, gut microbiota influenced foraging decisions in fruit flies (*Drosophila melanogaster*; Wong et al., 2017) and were associated with different behavioural tasks, such as foraging, food processing and nursing in honey bees (Jones et al., 2018).

Empirical evidence for the role of intestinal microbiota on host behaviour is increasing, yet to date have focused primarily on human or domesticated models. Studying wild models in-situ can elucidate the microbial functional value of this biological phenomenon, particularly how it relates to host fitness and subsequent ecological or evolutionary processes (Davidson, Raulo, & Knowles, 2020). This also allows us to understand the impact of microbes on host invasive behaviour (Stuart, Shine, & Brown, 2019), particularly when encountering novel

environmental stressors (Sampson & Mazmanian, 2015). This idea recently has been tested in the guttural toad (*Sclerophrys gutturalis*) in south Africa (Wagener et al., 2020). Invasive toads' gut microbiota were found to be distinct from that of native toads, and toads inoculated with invasive gut microbiomes had significantly higher physiological performance compared to toads inoculated with the native gut microbiome (Wagener et al., 2020). However, further evidence regarding the impact of gut microbial variation between populations with different invasion characters is needed to clarify these relationships.

Host behaviour appears to be more closely linked to the microbiota of the colon rather than that of the small intestine. From a mechanistic perspective, it is known that the microbial community in the colon can ferment complex carbohydrates, such as dietary fibre, into short chain fatty acids (SCFAs) that are known to have neuroactive properties (Dinan, Stilling, Stanton, & Cryan, 2015). Certain microbial species in the colon can cause changes to brain metabolites: in pigs, *Ruminococcus* spp. can influence brain N-acetylaspartate through the mediation of serum cortisol (Mudd, Berding, Wang, Donovan, & Dilger, 2017). In the cane toad, reactive nerve cell bodies were observed to be common in the submucosa of colon and less common in small intestine (Z. S. Li, Furness, Young, & Campbell, 1992). In addition to these explicit links to behaviour, there is evidence that colon microbiota may have a greater effect on hosts than microbiota in other parts of the digestive system. The 'stationary phase' is the point at which the maintenance of the bacterial population size reaches equilibrium. Following regular nutrient provisioning, microbial communities replicate quickly and reach the stationary phase after only 20 minutes in the colon as compared to more than 2.2 hours in other organs of the digestive system. Microbiota also have longer transit times in the colon (10-60 hours versus less than 3 hours in all other digestive organs; Fetissov, 2017), increasing their opportunity to impact their host.

Extrinsic environmental factors such as habitat and diet can influence the composition and variation of gut microbiota (Bletz et al., 2016; Carmody et al., 2015; Marques et al., 2010).

Amphibians are good models to study these changes because they utilize both aquatic and terrestrial habitats and their skin is permeable, making them highly sensitive to environmental variation (Hopkins, 2007). For example, the gut microbiota of fire salamander (*S. salamandra*) larvae differs depending on whether they live in ponds or streams (Bletz et al., 2016). Translocating animals to the opposite habitat type shifts their gut microbiota to resemble that of residents. Further, gut microbiomes can be similar across phylogenetically distinct, but sympatric amphibian species (such as *Fejervarya limnocharis* and *Babina adenopleura* inhabiting both farmlands and forests) and mirror environmental conditions (in both species bacterial diversity was higher in farmland populations; (Huang et al., 2017). Because diet is a key environmental factor affecting gut microbial community assemblages, these changes are likely to be influenced by differences in diet across environments (K. P. Scott et al., 2013; Zmora et al., 2019).

Intrinsic factors such as genetics, age, and sex also can interact to maintain and shape the host's gut microbiota community (Kozik et al., 2017; Org et al., 2016; J. Wang et al., 2016). In mice, significantly different microbiota are observed in narrow age ranges, such as between 4-5 weeks and 6-7 weeks (Kozik et al., 2017). Males and females can also possess different microbiota (in mice: Kozik et al., 2017, and cane toads: Zhou, Nelson, Rodriguez Lopez, Sarma, et al., 2020). Further, host health is associated with gut microbiota, and the presence of health metrics (such as intestinal parasites occurrence) can be predicted by studying gut microbial composition. For example, *Blastocystis* colonization is negatively associated with *Bacteroides* (Stensvold & van der Giezen, 2018). Because parasites are also known to impact host behaviours (Herbison, 2017; Klein, 2003), it is important to consider interactions of parasites and gut bacteria host behavioural variation. In summary, both environmental factors and host factors should be considered when studying intestinal microbiota variation.

To reduce the impact of biological invasions, we need to study the factors that enable successful invaders. Invasive cane toads in Australia are an ideal system for studying how

parasite load, microbiome and behaviour interact to facilitate invasion. Toads were introduced to Queensland in 1935 and have been wildly successfully colonizing Australia. They display significant differences across their Australian distribution in behaviours likely to be related to increased capacity for dispersal ability; invasion front toads are bolder and more exploratory (Gruber, Brown, et al., 2017a; Stuart et al., 2019). Toads from the invasion-front also differ from range-core toads in their load of the co-introduced parasitic lungworm, *Rhabdias pseudosphaerocephala*. Interestingly, lungworm infection affects toads' thermal and hydration preferences and defecation behaviour (Finnerty, Shine, & Brown, 2018).

Drivers of variation in invasion-related behaviours have been studied in this species, including genetics, morphology, habitat, diet, prior experience and parasites (G. P. Brown, Kelehear, Pizzatto, & Shine, 2016; Child et al., 2008; C. M. Hudson et al., 2017; Selechnik, Richardson, Shine, DeVore, et al., 2019; Stuart et al., 2019). However, there have been no investigations of gut microbiota as a potential driver of behavioural shifts across an invasive range in any species. A range of differences in dispersal-related morphology and performance metrics also have been shown: for example, wild-caught invasion-front toads have longer legs and toads with longer legs move greater distances (B. L. Phillips et al., 2006) and toad morphology and performance are linked (C. M. Hudson, Vidal-García, Murray, & Shine, 2020), endurance is greater in invasion-front adult toads held in captivity (Llewelyn, Phillips, Alford, Schwarzkopf, & Shine, 2010), invasion-front toads move in a more consistent direction than those from the range-core (G. P. Brown, Phillips, & Shine, 2014). However, the relationships between these traits are complex and may be affected by factors such as sex (C. M. Hudson et al., 2020) or rearing environment (Stuart et al., 2019). Understanding how microbiota may contribute to invasion success may be key to successful management of species such as the cane toad.

Here, I investigated the intestinal bacteria of invasive cane toads in Australia to determine whether there were significant differences in the identity or relative composition of gut

microbiota in cane toads from range-core (i.e. the introduction site) versus invasion-front populations. I predicted that extrinsic and intrinsic factors would impact bacterial communities, including environmental conditions and host parasite prevalence and intensity. I also investigated how bacterial variation correlated with cane toad behaviour. Given my knowledge of differences in cane toad behaviour across the Australian range, I predicted that the gut bacteria of animals captured from the invasion-front populations would differ to that of the range-core populations, and that this would be correlated with their behaviour. I discuss the potential implications of these impacts on invasion ability in this iconic invader.

Methodology

Study species, sample collection and behavioural assays

Cane toads are large anurans (measuring up to 23 cm, 1.25 kg) introduced into Australia in 1935 as a biocontrol for pests of sugar cane crops (Shine, 2010). They are actively invading northern Australia and critically threaten many native predator species who are fatally poisoned when they attempt to eat them (Shine, 2010). I hand captured 60 wild adult females from three sites at the invasion front edge in Western Australia ('invasion-front': Kununurra 15.776566° S, 128.744293° E, Old Theda 14.790795° S, 126.497624° E, Mary Pool 18.72528° S, 126.870096° E. N=30, collected November 2018) and three sites in Queensland (QLD) ('range-core', Rossville 15.697069° S, 145.254385° E, Croydon 18.207536° S, 142.245702° E, Lucinda 18.530149° S, 146.331264° E. N=30, collected December 2018) for this study (Figure 1.1). The University of Adelaide Animal Ethics Committee approved all animals that had been used in this research (approval number: S-2018-056).

I conducted brief behavioural assays while collecting toads based on the methodologies described in a common-garden experiment on this species (Stuart et al., 2019) including: (1) struggle score (number of arm or leg kicks after being picked up until toad remains still for 5

seconds and struggle likelihood) and (2) righting behaviour (within two minutes after the toad is placed on its dorsal side: time to right itself, number of limb kicks ('righting effort'), and righting effort likelihood). In that common-garden experiment, righting time was correlated with speed ($r^2 = 0.09$, $p = 0.004$) and stamina ($r^2 = 0.07$, $p = 0.01$), and righting effort was correlated with speed ($r^2 = 0.05$, $p = 0.02$) after body size correction (Stuart, pers. comm.); these data demonstrate the relevance of the simple assays used here to traits more clearly linked to invasion potential in this species. After behavioural assays were conducted, I placed the animals into individual, moist, calico bags and weighed, measured (snout urostyle length; SUL) and euthanised them by injecting tricaine methanesulfonate (MS222) buffered with bicarbonate of soda to balance pH. After euthanasia, I used a clean surface and clean tools to dissect each animal. From each animal, I preserved 0.3cm of colon near the cloaca (including gut content) in 95% ethanol. I also collected data on two toad parasites: an encysted gut parasites (*Physalopterinae*: physalopterine larvae; C. Kelehear & Jones, 2010) and the co-introduced toad lungworm, which passes into the toad gut as eggs and then hatches into larvae in the gut (M. R. Baker, 1979). I examined the gut for evidence of the former. To investigate the latter, I dissected and inverted the lungs (right lobe when facing the ventral side) and counted lungworms. Body size and parasite data are hereafter collectively referred to as 'host characteristics'.

Analysing morphological and ecological data

I compared host characteristics, host behavioural traits and environmental factors between range-core and invasion-front toads. Within each of these data sets, I conducted correlation tests to exclude highly corrected variables for downstream analyses. Because body length (SUL) and weight were significantly correlated, I only included SUL in further analyses. For host characteristics and behavioural traits, I used SUL as a covariate in generalized linear models (GLM). I compared count and occurrence data (i.e. absence = 0, presence = 1) for host parasite infection and behavioural traits. To analyse occurrence data, I used GLM with

logistic regression (R Core Team, 2020) and with negative binomial regression *glm.nb* in the "MASS" package (Venables & Ripley, 2002) to analyse over-dispersed count data, when the conditional variance exceeded the conditional mean. I obtained environmental data for 19 bioclimatic variables for each sampling site from WorldClim (Table S3.1, Fick & Hijmans, 2017). I analysed these data using the Shapiro-Wilk test to assess multivariate normality using "RVAideMemoire" package (Herve, 2018) and then used a nonparametric Wilcoxon signed rank test to compare the median of the difference between groups because the data were not normally distributed.

DNA extraction, library preparation and sequencing

I extracted microbial DNA from colon contents following the manufacturer's protocol for the DNeasy PowerSoil kit (Qiagen) and prepared 16S rRNA amplicon libraries by following guidelines for the Illumina MiSeq System. I used Zymo isolated DNA (D6305) as a community positive control and MilliQ water as a PCR negative control to identify environmental contamination during the library preparation process (Eisenhofer et al., 2019). Libraries were prepared based on the hypervariable (V3-V4) region of the 16S rRNA gene from each sample using primers 341F (5' – **TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CCTACGGGNGGCWGCAG-3') and 785R (5'-**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GACTACHVGGGTATCTAATCC-3') (Herlemann et al., 2011). The library preparation details have been reported previously (Zhou, Nelson, Rodriguez Lopez, Sarma, et al., 2020). The libraries were sequenced at the Ramaciotti Centre for Genomics (University of New South Wales, Kensington, Sydney) on the Illumina MiSeq platform targeting 2x300bp paired-end sequence reads.

Identifying and classifying bacteria community

I downloaded demultiplexed FASTQ data from Illumina's BaseSpace cloud storage, then created an amplicon sequence variants (ASVs) abundance table using the open-source QIIME2 pipeline (Bolyen et al., 2018). Demultiplexed sequence counts from samples and the positive control ranged between 138,989 and 305,662; the count of the PCR negative control was 67,506. I used the DADA2 pipeline (B. J. Callahan et al., 2016), implemented in QIIME2, to filter and trim the first 20 bases from each read and truncate sequences to 200 bases. I dereplicated the remaining sequences, then merged forward/reverse reads, removed chimeras, and generated ASVs for downstream analysis (B. J. Callahan et al., 2016). After quality filtering, reads from colon samples and the positive control ranged between 103,245 and 245,059 counts; the PCR negative control yielded 6,727 reads. I performed the taxonomic assignment of ASVs using Greengenes version 13_8 (DeSantis et al., 2006). I pruned data to remove representatives classified to Archaea (N = 28), chloroplast (N = 17), mitochondria (N = 186), and 151 unassigned ("kingdom") ASVs implemented in the package 'phyloseq' (McMurdie & Holmes, 2013). I also removed the ASVs with prevalence of less than four, which makes the logged counts per sample more evenly distributed. The remaining 9,878 taxa were classified to the Kingdom Bacteria with 62.62% assigned to phylum level and 39.65% assigned to family level.

Characterizing diversity and microbial function of bacteria communities

I calculated observed ASVs (DeSantis et al., 2006), evenness (Pielou, 1966), and Shannon (Shannon, 1948) indices through QIIME2 for alpha diversity (a measure of diversity within individuals). I used a Wilcoxon signed-rank test to compare Shannon Index of toad microbiomes from different locations/sites and used a GLM model to check the correlation between Shannon Index and locations/sites with consideration of SUL as a covariate.

In order to explore the bacterial taxa that may contribute to the observed differences in community, I calculated the Core50 gut community (Bletz et al., 2016) as follows: the ASV

table was filtered to include only the ASVs present in a minimum of 50% of individual toads from each site. This calculation was performed separately for three sites from invasion-front toads: Kununurra (gut Core50: n = 111 ASVs), Old Theda (gut Core50: n = 118 ASVs), and Mary Pool (gut Core50: n = 129 ASVs); three sites from range-core toads: Rossville (gut Core50: n = 148 ASVs), Croydon (gut Core50: n = 86 ASVs), and Lucinda (gut Core50: n = 117 ASVs). Then I subsequently compiled filtered ASVs of six sites to avoid excluding ASVs that may be specific to only one site. In combination, the gut Core50 contained 325 unique ASVs. I visualized relative abundance of bacteria (abundance >2%) in different sample types, classified to the phylum and family level.

To compare microbial communities between samples, I used a Hellinger transformation implemented in the package "microbiome" (Valverde et al., 2014) in R and then calculated and generated a weighted UniFrac dissimilarity matrix, which was visualized using Principal Coordinate Analysis (PCoA) plots using commands from the package "Phyloseq" (McMurdie & Holmes, 2013). I used the *adonis* command from the package "Vegan" to perform permutational multivariate analysis of variance (perMANOVA) to check whether the microbial communities of toads from each location were significantly different. I used the command *betadisper* in the package "Vegan" (Oksanen et al., 2019) to check the homogeneity of group variances, an assumption of perMANOVA. After finding significant differences between invasion-front and range-core toads, I performed pairwise comparisons between six sites from both locations using the command *pairwise.perm.manova* function in "RVAideMemoire" package with the Wilks Lambda (Nath & Pavur, 1985) and corrections for multiple testing (Herve, 2018) were conducted using the Hochberg procedure (Hochberg, 1988).

I used PICRUST2 (Douglas et al., 2019) to predict microbial functions for Core50 ASVs and generated pathway abundances considering the taxonomic contributions of ASVs. I removed all pathways with less than 0 prevalence and 474 out of 484 pathways remained for analysis.

Then I used a Hellinger transformation and calculated and plotted Bray Curtis dissimilarity matrix through package "Phyloseq". I performed perMANOVA to check whether the pathway abundance of toads from each location and site were significantly different and used the command *betadisper* to check the homogeneity of group variances.

Correlating intrinsic and extrinsic factors with host bacteria

To identify the association of individual host characteristics, host behavioral traits and environmental factors with bacterial community and predicted microbial functions, I conducted statistical analysis using the function envfit in the package "Vegan" on Bray Curtis dissimilarities. Bray Curtis dissimilarities were visualized with non-metric multidimensional scaling (nMDS) plots. Host characteristics and behavioral traits were fitted to the ordination plots using the function envfit. Environmental variables that were significantly associated with microbiota taxonomy and microbial function were also fitted to the ordination plots using the function envfit and smooth surfaces of the same environmental factors were added on the nMDS plot with the ordisurf function in package "Vegan".

After analysing all variables individually, I also conducted distance-based redundancy analysis (dbRDA) combining all host characteristics and behavioral traits to identify relationships between variables with respect to their impact on gut microbiome. dbRDA performs constrained ordination directly on a distance or dissimilarity matrix with the function *capscale* in the "Vegan" package in R. I checked the correlation between each pair of traits. Bray Curtis dissimilarity matrixes of ASVs and predicted microbial functions were ordinated and the results were analyzed using redundancy analysis with constraining variables that were not highly correlated (correlation coefficient < 0.85 and > -0.85) to estimate their explanatory proportion. I did not include environmental factors in the *capscale* analysis because these data are not independent (toads from the same site have the same environmental factors).

Identifying differences in bacterial taxa and their predicted bacterial functions in range-core versus range front toads

To identify the significant differences in ASVs and predicted microbial functions between range-core and invasion-front toads, I performed differential abundance testing using the function *DESeq* in the package "DESeq2" (Love et al., 2014). I report significant differences as the log fold change of a taxa in range-core toads versus those from the invasion-front. I set ASVs and microbial functions abundance in invasion-front toads as the control group. Results for all statistical tests were considered significant where p-values < 0.05.

Results

Morphological characteristics and parasite load in range-core versus invasion-front toads

The SUL (body length) of wild-caught invasion-front toads was longer than that of range-core toads (front: mean = 103.51 mm, SD = 9.42; core: mean = 96.24 mm, SD = 12.5; t = -2.543, df = 53.897, p-value = 0.014). There were no significant differences in lungworm and gut parasite prevalence or intensity between the range-core and invasion-front toads we collected (Tables 3.1, S3.2): in all sampled toads, I report the number of lungworms (mean = 2.56, SD = 5.37), the occurrence of lungworms (mean = 0.52, SD = 0.50), the number of gut parasites (mean = 3.26, SD = 5.35) and occurrence of gut parasites (mean = 0.5, SD = 0.51).

Table 3. 1 Comparison of host characteristics, behavioural traits and environmental factors between range-core and invasion-front toads.

Variables		**wilcox.test**	**GLM (SUL as covariate)**		
			glm.nb	**glm**	**binomial**
Host characteristics	Body length (SUL)	_	_	0.01099 *	_

	Lungworms	_	0.8600	_	_
	Occurrences of lungworms	_	_	_	0.07783 .
	Gut parasites	_	0.817578	_	_
	Occurrences of gut parasites	_	_	_	0.604488
Behavioural traits	Struggle score	_	0.002196 **	_	_
	Struggle likelihood	_	_	_	0.008477 **
	Righting effort	_	0.3203	_	_
	Righting effort likelihood	_	_	_	0.03569 *
	Righting time	_	0.5067	_	_
Environmental factors	Longitude	1.596e-11***	_	_	_
	Latitude	0.458	_	_	_
	Annual Mean Temperature	1.608e-07 ***	_	_	_
	Mean Diurnal Range	0.0001836 ***	_	_	_
	Isothermality	0.4515	_	_	_
	Temperature Seasonality	0.02501 *	_	_	_
	Max Temperature of Warmest Month	1.608e-07 ***	_	_	_

Min Temperature of Coldest Month	0.458	_	_	_
Temperature Annual Range	0.0001836 ***	_	_	_
Mean Temperature of Wettest Quarter	1.608e-07 ***	_	_	_
Mean Temperature of Driest Quarter	0.02099 *	_	_	_
Mean Temperature of Warmest Quarter	1.608e-07 ***	_	_	_
Mean Temperature of Coldest Quarter	0.0001836 ***	_	_	_
Annual Precipitation	1.608e-07 ***	_	_	_
Precipitation of Wettest Month	1.608e-07 ***	_	_	_
Precipitation of Driest Month	8.069e-12 ***	_	_	_
Precipitation Seasonality	0.02501 *			
Precipitation of Wettest Quarter	1.608e-07 ***	_	_	_
Precipitation of Driest Quarter	5.222e-06 ***	_	_	_
Precipitation of Warmest Quarter	1.596e-11 ***	_	_	_

	Precipitation of Coldest Quarter	5.222e-06 ***	_	_	_

Note

Negative binomial regression (glm.nb) was used for over-dispersed count data, that is when the conditional variance exceeds the conditional mean. Shapiro-wilk test to assess multivariate normality using RVAideMemoire package in R (Herve, 2018). If it is below 0.05, the data significantly deviate from a normal distribution. Wilcox.test used for the data that is not normally distributed and ttest for the data that is normally distributed. Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1. SUL: snout urostyle length (body length).

Behavioural traits of range-core versus invasion-front toads

Range-core toads were found to have higher struggle scores when compared with invasion-front toads (Table 3.1, S3.2). I found range-core toads were more likely to struggle than invasion-front toads (core: mean = 0.83, SD = 0.37; front: mean = 0.27, SD = 0.43; p-value = 0.008) and, in those who did struggle, the number of struggle movements was significantly higher for range-core toads than for invasion-front toads (core: mean = 2.63, SD = 2.52; front: mean = 2.1, SD = 4.54; p-value = 0.002). The righting effort likelihood was higher for range-core toads than for invasion-front toads (core: mean = 0.73, SD = 0.45; front: mean = 0.5, SD = 0.51; p-value = 0.036) (Tables 3.1, S3.2). The righting effort and righting time did not differ between range-core and invasion-front toads (Table 3.1, S3.2): across all sampled toads, I report the righting effort (mean = 2.2, SD = 3.56) and righting time (mean = 18.88sec, SD = 41.32).

Environmental conditions experienced by range-core and invasion-front toads

Cane toads at the invasion-front experienced significantly hotter (six temperature measurements, see Table 3.1, S3.2 for statistics) and dryer (seven precipitation measurements, see Table 3.1, S3.2 for statistics) conditions year-round than those in the range-core. Moreover, cane toads at the invasion-front experienced more changeable weather than those

in range-core (three temperature variation measurement and one precipitation variation measurement, see Table 3.1, S3.2 for statistics). However, one temperature measurement (min temperature of coldest month: mean = 14.65, SD = 1.77) and one temperature variation measurement (isothermality; higher isothermality refers to more variable temperature: mean = 55.17, SD = 2.29) were not different in both environments included here (Table 3.1).

DNA sequence data quality

The number of observed species in the PCR negative control (N = 36) was much lower compared to that of toad colon samples (mean = 336, SD = 137.8), which indicated minimal contamination throughout the library preparation processes (Table S3.3). The number of observed species (N = 19) in the community positive control was also low compared to experimental samples. The eight species used as microbial community standards constituted more than 99.8% of ASVs from our sequenced community positive control, as shown in Figure S3.1, indicating minimal PCR bias in our library preparation process.

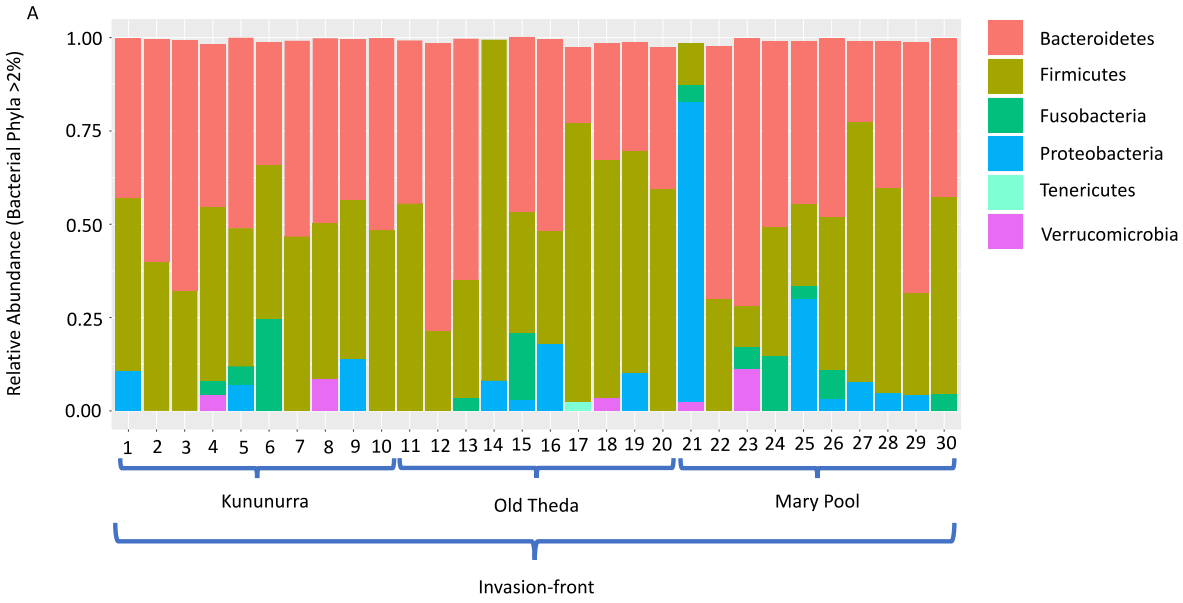
Comparison of bacterial composition and diversity with total gut bacteria

The alpha diversity (diversity within individual toads) was not significantly different between range-core and invasion-front toads: observed species (*wilcox.test*, p-value = 0.631), Pielou's evenness (*wilcox.test*, p-value = 0.307), Shannon (*wilcox.test*, p-value = 0.230, Figure S3.2A). Lucinda had the highest Shannon diversity, followed by Old Theda, Mary Pool, Rossville, Croydon and Kununurra (Figure S3.2B). Shannon diversity in individual samples was not correlated with measured behavioural traits (*glm*, covariate = SUL, p-values > 0.050).

A permutational MANOVA test was conducted on a weighted UniFrac dissimilarity matrix and showed that total bacterial communities were not significantly different between range-core and invasion-front toads (R^2 = 0.013, F = 0.743, p-value = 0.490).

Relative abundance of Core50 bacteria in large intestine

A total of 89.23% ASVs were assigned to the level of phylum and dominant phyla (average abundance > 2%) were Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, and Verrucomicrobia (Figure S3.3, Table S3.4). Across all individuals, we found that: (1) the most dominant phyla were Firmicutes (average abundance = 47.89%, SD = 19.25%) and Bacteroidetes (average abundance = 39.05%, SD = 19.26%) (Table S3.4); (2) the phylum Bacteroidetes were consistently present in most individuals, except for two toads (Toad 14, 21) from the invasion-front and two toads (Toad 43, 48) from the range-core (Figure 3.1); and (3) there was one invasion-front toad (Toad 21) that showed a higher abundance of Proteobacteria (77.35%) when compared with other individuals (average abundance = 5.13%, SD = 7.99%).



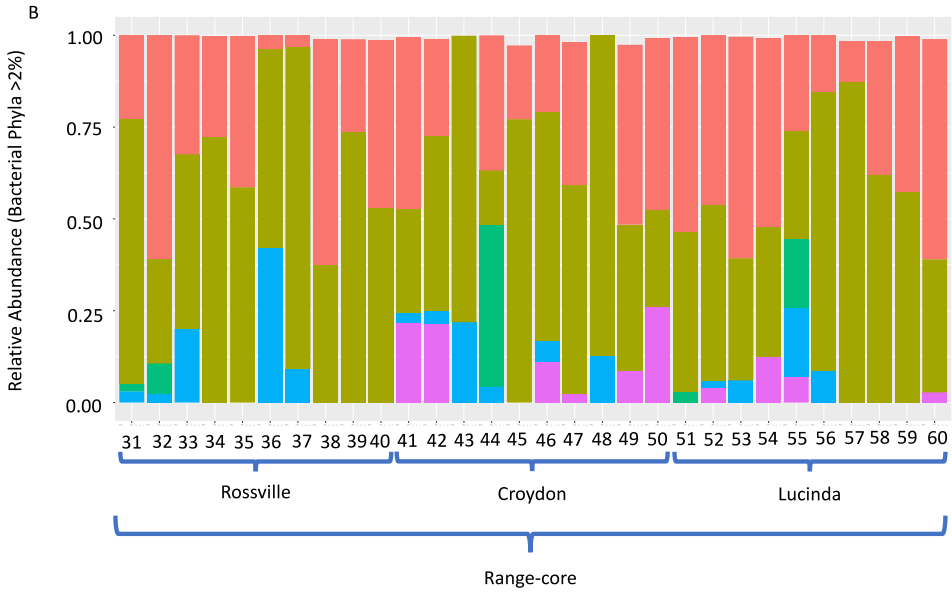


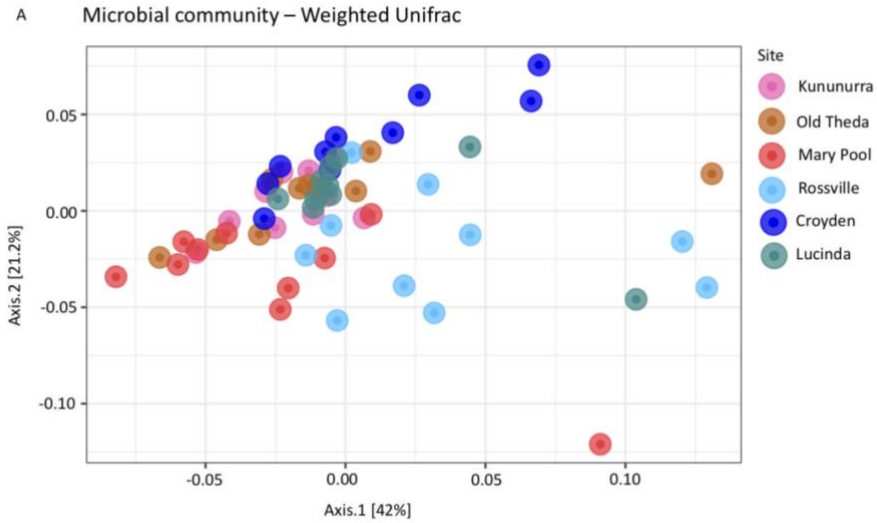
Figure 3. 1 Core bacterial community composition of different individual toads. Relative abundance plot shows phyla (>2%).

A total of 70.77% ASVs were assigned to the family level and dominant families (average abundance > 2%) included Bacteroidaceae, Lachnospiraceae, Porphyromonadaceae, Clostridiaceae, Enterobacteriaceae, Erysipelotrichaceae, Fusobacteriaceae, Bacillaceae, Ruminococcaceae, Verrucomicrobiaceae, Turicibacteraceae, and Veillonellaceae (Figure S3.4, Table S3.5). Toad 21, who had a high abundance of Proteobacteria, also had a higher abundance of Enterobacteriaceae (77.34%) when compared with other individuals (average abundance = 4.55%, SD = 7.86%) (Figure S3.5).

A total of 40.62% ASVs were assigned to the genus level and dominant genera (average abundance > 2%) included *Bacteroides*, *Parabacteroides*, *Clostridium*, *Epulopiscium*, *Akkermansia*, *Turicibacter*, *Bacillus* (Figure S3.6, Table S3.6).

Comparison of bacterial community taxonomy and bacterial function between individuals with Core50 gut bacteria

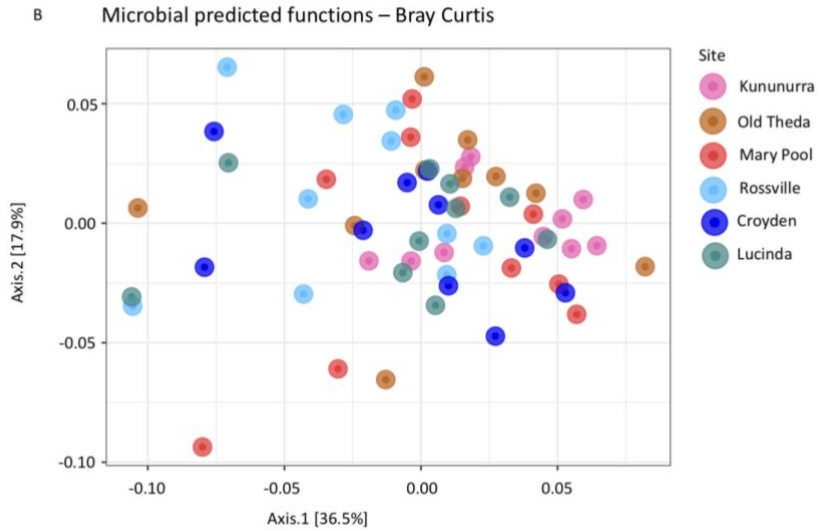
Bacterial taxonomic communities were significantly different between locations (Figure 3.2A, R^2 = 0.095, F = 6.092, p-value < 0.001). Bacterial communities were marginally different between most sites (p-values < 0.050), except that Old Theda was not significantly different from Kununurra, Mary Pool, Croydon and Lucinda (Figure 3.2A). Among 230 ASVs that assigned to family level, there were 124 ASVs that significantly differed between range-core and invasion-front toads' colon (Table S3.7). The number of significantly different ASVs in each phylum were: Bacteroidetes (60 ASVs), Firmicutes (55 ASVs), Proteobacteria (7 ASVs), Actinobacteria (1 ASVs), Verrucomicrobia (1 ASV) (Table S3.7, Figure 3.3A).



Pairwise comparisons using permutation MANOVAs on a weighted UniFrac distance matrix of Core50 gut bacteria

	Kununurra	Old Theda	Mary Pool	Rossville	Croydon
Old Theda	0.0608	-	-	-	-
Mary Pool	0.0045	0.0584	-	-	-
Rossville	0.0011	0.0340	0.0175	-	-
Croydon	0.0020	0.0608	0.0011	0.0011	-
Lucinda	0.0011	0.1352	0.0011	0.0324	0.0175

P value adjustment method: Hochberg (1988)



Pairwise comparisons using permutation MANOVAs on a Bray Curtis distance matrix of Core50 bacterial functions

	Kununurra	Old Theda	Mary Pool	Rossville	Croydon
Old Theda	0.298	-	-	-	-
Mary Pool	0.298	0.298	-	-	-
Rossville	0.009	0.298	0.298	-	-
Croydon	0.105	0.298	0.298	0.298	-
Lucinda	0.046	0.298	0.298	0.298	0.298

P value adjustment method: Hochberg (1988)

Figure 3. 2 Beta diversity by locations and sites.

Principle coordinate analysis plot of weighted UniFrac distance of bacterial community (R^2 = 0.095, F = 6.092, p-value < 0.001) (A) and Bray Curtis distance of predicted functional groups (R^2 = 0.064, F = 4.110 , p-value= 0.002) (B) from 60 cane toad individuals of the invasion-front (Kununurra, Old Theda, and Mary Pool) and the range-core (Rossville, Croydon, and Lucinda). Pairwise comparisons were conducted on both distance matrixes using permutation MANOVAs. P values were adjusted with Hochberg method.

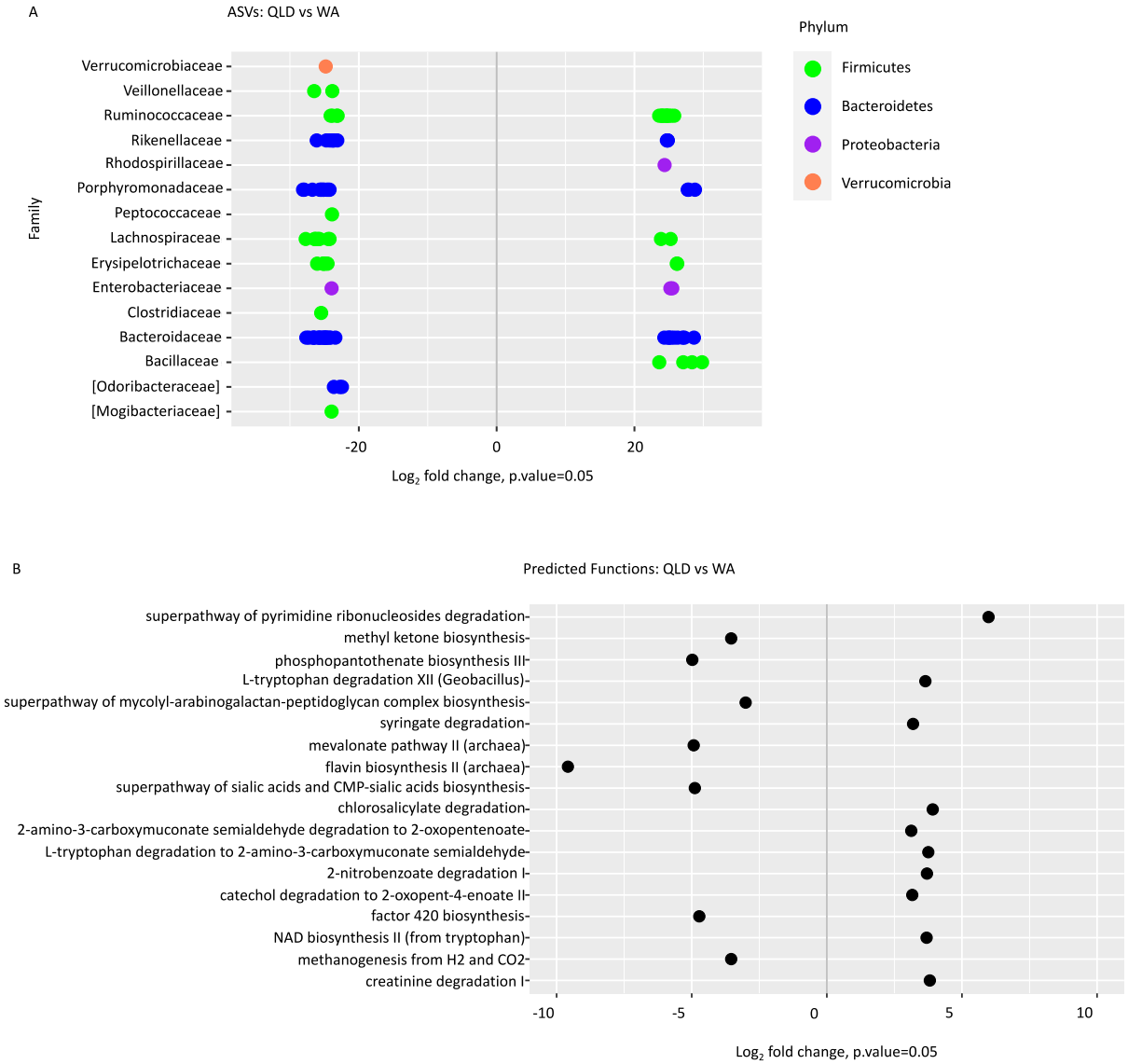


Figure 3. 3 Significantly different bacterial taxa and predicted functions between range-core (QLD) and invasion-front (WA) toads' colon.

Significant differences were identified between locations via differential abundance testing based on a negative binomial distribution. The dots represent the average log-2 fold change (x axis) abundance and positive log_2 fold changes signify increased abundance in range-core, and negative log_2 fold changes display increased abundance in invasion-front. Bacterial taxa (A) were classified to the taxonomic level of family (y axis) and coloured by taxonomic level of phylum. Family name in bracket is proposed taxonomy by Greengenes. Only ASVs that could be matched to a known bacterial family and with a log2FoldChange value higher than 20 or lower than -20 are presented. Predicted functions (B) with a log2FoldChange value higher than 3 or lower than -3 are presented.

Among the identified 474 predicted bacterial functions, I found significant differences

between invasion-front and range-core toads (Figure 3.2B; R^2 = 0.064, F = 4.110, p-value =

0.002). Pairwise tests between sampling sites indicated that toads from Kununurra had significantly different bacterial functions to toads from Rossville (p-value = 0.009) and Lucinda (p-value = 0.046), and the other sites were not significantly different (p-values > 0.050) (Figure 3.2B). The top six most abundant pathways were pentose phosphate pathway (non-oxidative branch), adenosine deoxyribonucleotides de novo biosynthesis II, guanosine deoxyribonucleotides de novo biosynthesis II, pyruvate fermentation to isobutanol (engineered), Calvin-Benson-Bassham cycle, and adenine and adenosine salvage III (Figure 3.3). There were 84 out of 474 microbialfunctions that were significantly different between invasion-front and range-core toads (Table S8, Figure 3.3B). Range-core toads have significantly higher abundance of microbial function in superpathway of pyrimidine ribonucleosides degradation (log2FoldChange = 5.98) and lower abundance of bacterial functions in phosphopantothenate biosynthesis III (log2FoldChange = -4.98), superpathway of sialic acids and CMP-sialic acids biosynthesis (log2FoldChange = -4.89) and factor 420 biosynthesis (log2FoldChange = -4.72) than invasion-front toads (Table S8, Figure 3.3B). Out of the top 30 abundant bacterial functions, range-core have significantly lower abundance of bacterial function in urate biosynthesis/inosine 5'-phosphate degradation (log2FoldChange = -0.10) than invasion-front toads (Figure 3.4, Table S3.8).

Figure 3. 4 Heatmap for top 30 functional group abundance.

Heatmap indicates the top 30 functional groups in the intestinal samples from range-core and invasion-front toads. Abundance indicates the raw count of functional groups inferred from taxonomic 16S sequences using PICRUSt where light blue is high abundance and dark blue is lower abundance. Functional pathways that differ significantly between range-core and invasion-front toads are highlighted in bold.

Associations between host, environment and intestinal bacteria

I assessed which host or environmental factors are strongly associated with gut bacterial taxonomy and bacterial function. When comparing the association between individual host characteristics and behaviours with gut bacterial variation, only the occurrence of lungworms was significantly associated with the bacterial taxonomic community (R^2 = 0.128, p-value = 0.023) (Tables S3.9, S3.10; Figure S3.7A, B). Among the 21 environmental factors investigated here, longitude (R^2 = 0.194, p-value = 0.001), isothermality (R^2 = 0.193, p-value = 0.002), and mean temperature of driest quarter (R^2 = 0.146, p-value = 0.015) were strongly associated with bacterial taxonomic community. The mean temperature of driest quarter (R^2 = 0.187, p-value = 0.004) and isothermality (R^2 = 0.175, p-value = 0.007) were also strongly associated with bacterial functions (Table S3.11). Because longitude is a proxy for sampling region, we only present mean temperature of driest quarter and isothermality in figure S3.7C,

D. Host characteristics and behaviours are independent measurements, yet environmental factors are the same for all 10 toads from each site. The variable isothermality was identified as having a possible correlation with gut bacterial taxonomy and function at the range of 54.5 – 57.5 (Figures S3.7C, D). The contour lines of mean temperature of driest quarter show less linear relationship than it does for isothermality with gut microbial taxa, which is further supported by envfit results (Figure S3.7C). The contour lines of mean temperature of driest quarter show the strongest linear relationship with gut microbial functions (Figure S3.7D). However, the deviance explained by this ordisurf model is the lowest (18.3%) compared to other three ordisurf models (isothermality x gut bacterial taxa, isothermality x gut microbial function, and mean temperature of driest quarter x microbial taxa).

I calculated correlations between the factors that were significantly associated with gut bacterial taxonomy and function as: (1) the occurrence of lungworms is correlated with isothermality (t = 2.3234, df = 58, p-value = 0.02369, coefficient = 0.292, method = "pearson"), (2) the occurrence of lungworms is not correlated with mean temperature of the driest quarter (t = 1.0534, df = 58, p-value = 0.2965, coefficient = 0.137), (3) isothermality is highly correlated with mean temperature (t = 8.3496, df = 58, p-value = 1.594e-11, coefficient = 0.739).

I used a redundancy analysis to assess the association of all host variables. The best model which was associated with the most variation in the gut bacterial composition included only the occurrence of lungworms (AIC = 178.58), whereas the best model which was associated with the most variation in predicted gut bacterial functions included righting effort likelihood (AIC = 53.613), the occurrence of lungworms (AIC = 54.297) and righting time (AIC = 56.912). The combination of these three factors was associated with 17.8% of variation in the predicted gut bacterial functions (Figure 3.5).

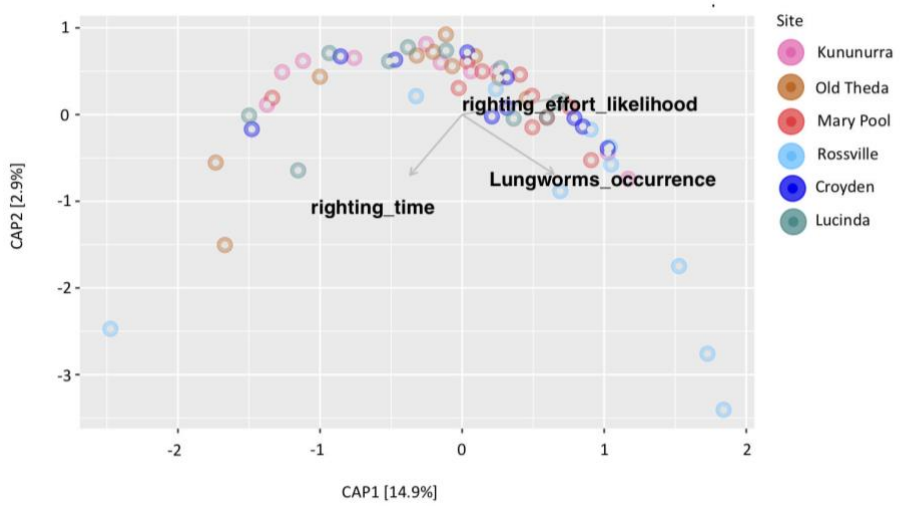


Figure 3. 5 Main variables that affect predicted function differentiation among individuals.

CAP (*capscale*) plot displays the combination of variables that explained the greatest variation in the predicted functions through model selection, using 60 cane toad individuals from the invasion-front (Kununurra, Old Theda, and Mary Pool) and the range-core (Rossville, Croydon, and Lucinda). The final model explained 17.8% of variation in the microbial predicted functions, which includes righting effort likelihood (AIC = 53.613), occurrence of lungworms (AIC = 54.297) and righting time (AIC = 56.912) explained the greatest variation.

Discussion

My research is the first of its kind to investigate how the gut bacteria of an invasive species changes: 1) as a function of intrinsic (host) and extrinsic (environmental) factors, 2) with invasion context (established resident versus invading populations), and 3) importantly, with relation to aspects of animal behaviour that influence dispersal, and thus wider invasion success. There is growing acknowledgement that microbiota influences animal behaviour in diverse ways (Davidson, Cooke, Johnson, & Quinn, 2018; Davidson, Raulo, et al., 2020) yet until now, the links between microbiota and behaviours that influence invasiveness have not been widely studied. I found that whilst the alpha diversity in microbiota was similar in the invasion-front and range-core individuals, there were significant differences in both the composition and predicted microbial function of gut microbiota in toad populations across the Australian invasive range. Predicted microbial functions better explain cane toad righting

behaviour than does microbial composition. Interestingly, the presence of the co-introduced parasitic lungworm and environmental factors related to temperature could contribute to the variation of both gut microbial community composition and microbial functions, highlighting the importance of studying how these factors interact.

Geographic divergence in host characteristics and behaviors

Because invasion-front toads were larger than range-core toads, we included SUL as a covariate for the statistical analyses in this study. Neither counts nor presence of parasites (lungworm and gut) differed across the range, even though parasites are likely to be absent from invasion-front toads (B. L. Phillips et al., 2010).

Range-core toads were more likely to struggle and, in those that did struggle, the number of struggle movements was higher for range-core toads. Range-core toads also were more likely to attempt to right themselves when turned over. Invasion-front toads are more reluctant to flee in simulated predation trials (C M Hudson et al., 2017). Dampened stress responses can be related to more exploratory behaviour (Golla, Østby, & Kermen, 2020), and to greater dispersal ability (Gruber, Brown, et al., 2017a). Rearing conditions also affect righting behaviour (Stuart et al., 2019). The lower likelihood of righting effort in invasion-front toads also might be linked to higher incidence of spinal arthritis (G. P. Brown, Shilton, Phillips, & Shine, 2007).

Bacterial taxa

Four phyla (Firmicutes, Bacteroidetes, Proteobacteria, and Fusobacteria) were found to be dominant (Mean > 2%) in toad samples from both ends of the range, similar to results of a previous study of cane toads from both of these regions held in captivity in a common facility (Zhou, Nelson, Rodriguez Lopez, Sarma, et al., 2020). The previous three phyla are commonly observed as dominant in the intestine of frogs and toads (Chang et al., 2016; Kohl et al., 2014; Wiebler et al., 2018), which suggests these three phyla may play important roles for maintaining the health of toads, and that these taxa are not dependent on particular host or environmental conditions. Even though not described as dominant phyla, Fusobacteria were

found in the intestine of other frogs and toads (Chai et al., 2018; Kohl et al., 2014). Fusobacteria are dominant phyla in the fish intestine (Colombo, Scalvenzi, Benlamara, & Pollet, 2015; Kohl et al., 2014) and Fusobacteria (*Cetobacterium*) were previously reported to be associated with changes in immunity of Cuban tree frogs (Knutie et al., 2017). Interestingly, I found a higher abundance of the phylum Verrucomicrobia in range-core toads than in toads from the invasion-front (core: mean = 3.98%, SD = 7.39%; front: mean = 1.29%, SD = 2.62%) (Table S4, Figure S3). Species within the phylum Verrucomicrobia are commonly found in the intestinal microbiota of frogs or toads (Jiménez & Sommer, 2017; J. Li et al., 2019) and one dominant genus within this phylum in anurans is *Akkermansia* (Chai et al., 2018; Kohl et al., 2013), which is also identified in range-core wild-caught toads (Figure S3.6, Table S3.6). One particular species (*A. muciniphila*) is common in the human intestinal tract, specializing in the degradation of intestinal mucins (Derrien, Collado, Ben-Amor, Salminen, & de Vos, 2008) and therefore often considered a to be associated with a 'healthy' metabolic status in humans (Dao et al., 2016). The reduction in members of the phylum Verrucomicrobia in invasion-front toads could be a result of an altered habitat or different available dietary options. Previously, members of the phylum Verrucomicrobia were not found to be dominant in the gut of captive toads from the invasion-front and range-core (Zhou, Nelson, Rodriguez Lopez, Sarma, et al., 2020). In the study of cane toad skin microbiota, a higher proportions of bacteria with antifungal properties (fungal pathogen: *Batrachochytrium dendrobatidis*-inhibitory) were detected in toads from range-core than those from invasion-front. These bacteria are known to be shaped by site-specific pathogen pressures (Weitzman et al., 2019). Interestingly, even though cane toads eat their skins, the 14 core *Batrachochytrium dendrobatidis*-inhibitory bacteria found on cane toad skin were not observed in its core gut bacteria, except family Enterobacteriaceae (*Bufotyphonius*-inhibitory_9).

I found significant differences in the beta diversity of gut bacterial composition between range-core and invasion-front toads even though their dominant phyla and alpha diversity were similar. ASVs in the family Veillonellaceae were found to be significantly higher in invasion-front toads, compared to range-core toads (Figure 3.3A). ASVs in this family have been found to influence host metabolic regulation. For example, in an experiment that altered air temperature for Brandt's voles (*Lasiopodomys brandtii*); in colder temperatures, voles which huddled had a higher abundance of Veillonellaceae as well as higher concentrations of short-chain fatty acids (SCFAs) in their intestines when compared with non-huddling voles (X. Y. Zhang et al., 2018). This family is known to produce SCFAs, such as propionic acid (Gamage et al., 2017; Petra Louis & Flint, 2017), and this has been linked to increased locomotor activity (R. H. Thomas et al., 2012). The relationship between the bacterial members of this family and host metabolic regulation, suggests that invasion-front toads may use the SCFAs to fuel their invasion by producing SCFAs, such as propionic acid and increasing locomotor activity (Llewelyn et al., 2010). ASVs from another family Clostridiaceae (Hugenholtz et al., 2018) that belongs to SCFA-producing bacteria were also higher in invasion-front toads than those from the range-core. Furthermore, the family Veillonellaceae may be associated with host sociality. A significant reduction of Veillonellaceae has been observed in children with Autism Spectrum Disorder, often known for desiring social isolation, when compared to a neurotypical group of children (Kang et al., 2013). Higher abundance of Veillonellaceae in invasion-front toads could foster their "bolder" personality, retaining a higher propensity for exploration and risk-taking (González-Bernal, Brown, & Shine, 2014; Gruber, Brown, et al., 2017a).

Several other ASVs that differed across the cane toad's range have previously been related to behaviour. ASVs from family Peptococcaceae were found to be significantly higher in invasion-front toads than in range-core ones (Figure 3.3A). Peptococcaceae have been found to be related with host neurotransmitter (noradrenaline linking visual awareness to external

world events, (Gelbard-Sagiv, Magidov, Sharon, Hendler, & Nir, 2018): for example, Peptococcaceae in the caecum showed a significantly positive correlation with noradrenaline levels in mice (Houlden et al., 2016). ASVs from family Bacillaceae were found to be significantly lower in invasion-front toads than range-core (Figure 3.3A). Bacillaceae have been found to be related to host anxiety: for example, this species was found in higher abundance in methamphetamine-treated rats versus the control (Ning, Gong, Xie, & Ma, 2017) and in higher abundance in excercised mice versus sedentary group (Choi et al., 2013). Higher abundance of Bacillaceae could contribute to higher anxiety-like behaviours thus heightening the stress response (Ning et al., 2017) and may be correlated with a decreased exploratory behaviour in new environments (Golla et al., 2020). In summary, invasion-front toads possessed gut bacterial communities that in other studies have been associated with SCFAs production and neurotransmitters, suggesting the potential to increase locomotor ability, visual awareness to external world events and propensity for exploration and risk-taking. In comparison, range-core toads possessed taxa are associated with higher anxiety-like behaviour, suggesting decreased propensity to explore. However, all the above-mentioned evidence supporting the association between gut bacterial taxa and host behaviours result from examinations of human or other animals rather than cane toads. Therefore, further manipulative experiments are needed to investigate these relationships in this species.

Predicted bacterial functions

Variation in the predicted gut bacterial functional groups observed between locations and sites was less obvious than in community composition (Figure 3.2). Other studies have found similar results, suggesting that microbial function is more likely to be preserved than composition (e.g. in fire salamanders, Bletz et al., 2016). This could result from different gut microbiota harbouring similar microbial functions and may contribute to increasing resilience and persistence of microbial functional stability of gut microbiota (Bletz et al., 2016; Huang et al., 2017; Lozupone, Stombaugh, Gordon, Jansson, & Knight, 2012). For invasive species,

this can also contribute to its adaptive potential in novel environments. I found significant differences in bacterial functions between range-core and invasion-front toads even though their most abundant bacterial functions were similar. There are significant differences in bacterial functional pathways that contribute to the microbe food sources and metabolism. As for microbe food sources, invasion-front toads have significantly lower abundance of bacterial function in the superpathway of pyrimidine ribonucleosides degradation. This pyrimidine ribonucleosides degradation provides nitrogen source for microbes (West, 1996) and plays an important role in perturbations in the uridine monophosphate (UMP) biosynthetic pathways. This allows the bacterial cell to sense signals such as starvation, nucleic acids degradation, and availability of exogenous pyrimidines, and to adapt the production of the extracellular matrix to the changing environmental conditions (Garavaglia, Rossi, & Landini, 2012). This may be related to the disappearance of Verrucomicrobia as a dominant taxon. As for microbe metabolism, invasion-front toads have a higher abundance of microbial functions in factor 420 biosynthesis; cofactor 420 is critical to bacterial metabolism and mediates a variety of important redox transformations involved in bacterial persistence, antibiotic biosynthesis, pro-drug activation and methanogenesis (Bashiri et al., 2019). Moreover, there are significant differences in bacterial functional pathways that contribute to host health. Invasion-front toads have higher abundance of bacterial functions that are beneficial to general host health and immunity: (1) phosphopantothenate biosynthesis, which is the first step for the bacteria production of coenzyme A (CoA), an indispensable enzyme cofactor for all living organisms (Sibon & Strauss, 2016); and (2) superpathway of sialic acids biosynthesis, which generates sialic acids that play multifarious roles in immunity including acting as host receptors and pathogen decoys for viruses and bacteria (Varki & Gagneux, 2012) and are especially critical for preventing neural tissue damage (Liao, Klaus, & Neumann, 2020).

Despite this higher abundance of bacterial functions that could enable invasion-front toads to expand their range, there were some indications that these toads may also face some health

challenges. Invasion-front toad gut bacterial communities have significantly higher abundance of urate biosynthesis function (urate biosynthesis/inosine 5'-phosphate degradation, the only significantly different one out of the top 30 abundant functions), which affects serum urate levels (Sinnott-Armstrong, Naqvi, Rivas, & Pritchard, 2020). Excessively high levels of urate can result in the formation of needle-like crystals of urate in the joints (gout). This may be related to the finding that invasion-front cane toads have a higher incidence of severe spinal arthritis, thought to be caused by high levels of movement in combination with morphological features specific to toads from that population (G. P. Brown et al., 2007). Emerging research in humans has highlighted the association of the gut microbiome on inflammation and the development of musculoskeletal disorders such as arthritis which has a significant impact on mobility (Szychlinska, Di Rosa, Castorina, Mobasheri, & Musumeci, 2019). Based on the interesting findings of the predicted bacterial functions from the literature, I recommend a metagenomics study with deeper sequencing depth to confirm the importance of bacterial function that significantly different between invasion-front and range-core toads.

Associations between gut bacteria, environmental isothermaliy and host lungworm

Environmental isothermality was positively associated with cane toad lungworm prevalence, and both factors could contribute to changes in the cane toads' gut bacteria. It is interesting that even though environmental isothermality and lungworm infection intensity are not significantly different between range-core and invasion-front toads, environmental isothermality and occurrence of lungworms are significantly associated with bacterial taxonomy and predicted function. Environmental temperature can alter microbial taxonomic community and predicted microbial function, resulting in increased phenotypic plasticity and persistence in harsh conditions (Fontaine & Kohl, 2020). For example, gut microbiota of invasion bullfrog tadpoles responds more rapidly to environmental temperature than non-invasive green frogs (Fontaine & Kohl, 2020). Environmental temperature alters the digestive performance and gut microbiota of red-backed salamanders (*Plethodon cinereus*: Fontaine,

Novarro, & Kohl, 2018) and in a study of western clawed frogs (*Xenopus tropicalis*) the authors observed a higher prevalence of Verrucomicrobia in the gut of frogs inhabiting a warmer environment, and an opposite pattern in Proteobacteria (J. Li et al., 2019). The effects of temperature on Verrucomicrobia, Proteobacteria, and Akkermansia observed in that study was consistent with previous studies in mammals (J. Li et al., 2019).

Pathogens and parasites impact the composition of the host microbiota and have also been shown to modify host behaviour in a manner that improves their probability of transmission and survival (Gegear et al., 2006; House et al., 2011; Poulin, 2010). Lungworms affected cane toad locomotor performance and reduced host endurance, presumably because of the reduced oxygen supply from infected lungs (L. Pizzatto & Shine, 2012). Lungworms are also known to alter a cane toad's thermal preference and can manipulate the timing and location of defecation, thereby enhancing lungworm egg production and larvae survival (Finnerty et al., 2018). Lungworms lag behind their host on the invasion front by 2-3 years (B. L. Phillips et al., 2010) and affect righting behaviour (prolongs righting time; (Finnerty, 2017). In the current study, although I collected invasion-front toads in areas where toads had been present for less than one year, I found no difference in lungworm presence or intensity between the invasion-front and range-core toads, nor did I find differences in righting times between these populations. In combination, these data suggest a need for further studies to determine how isothermality and lungworm occurrence may affect cane toad gut bacteria variation, and whether this influences toad behaviour.

Associations between gut bacteria and host righting reflex behaviour

Interestingly, behaviours including righting effort likelihood and righting time were not associated with the gut bacterial taxonomic composition but were associated with predicted bacterial functions. It is possible that multiple identified bacteria share the same function or that one taxa contributes to multiple functions, which might obscure the relationship between

behaviour and taxonomic composition. Nevertheless, these relationships I found between gut bacterial function and righting behaviours may be related to toads' health and/or rearing conditions. A dampened stress response (lower level of corticosterone) in invasion-front toads exposed to stressors (G. P. Brown et al., 2015) could be a result of higher abundance of microbial functions that are beneficial to general host health and immunity, especially the superpathway of sialic acids biosynthesis, which generates sialic acids that are especially critical for preventing neural tissue damage (Liao et al., 2020). Further, invasion-front toads respond less to stressful stimuli and are more reluctant to flee than those from range-core toads in simulated predation trials, exhibiting "bold" anti-predator responses when exposed to novel predators (C. M. Hudson et al., 2017). Studies showed that dampened stress responses are related to higher propensity for exploratory behaviour (Golla et al., 2020), which has been linked to dispersal ability (Gruber, Brown, et al., 2017a). Rearing conditions also have been shown to affect righting behaviour: juvenile toads whose parents were collected on the invasion-front that were raised in high exercise regimes in a common-garden setting righted themselves more quickly than those whose parents were collected from the range core, whereas the opposite trend was seen in controls (Stuart et al., 2019). It is also possible that the lower likelihood of righting effort I found in invasion-front toads could be caused by the higher incidence of severe spinal arthritis (G. P. Brown et al., 2007) described above, which could be related to the higher abundance of bacterial functions (urate biosynthesis function) that contribute to the formation of needle-like crystals of urate in the joints (Sinnott-Armstrong et al., 2020). Although manipulative studies are needed to clarify causal relationships between stress responses, proactive behaviours, and gut microbial functions, my results indicate that behaviour and toad gut bacterial functions are related, suggesting that gut microbiome should be considered as a potentially important driver of invasion.

In this chapter, I used PICRUSt as the primary bacterial functional prediction tool, based on 16S rRNA data from a wide range of species (P. Fan et al., 2020; Martínez-Mota, Kohl, Orr,

& Denise Dearing, 2020; Xiang et al., 2020). Recently, Sun et al., 2020 reported the limitation of using PICRUSt, PICRUSt2 and Tax4Fun for microbial function prediction in non-human samples (Sun, Jones, & Fodor, 2020). Owing to the predictive nature of PICRUSt and its use here with a non-model host, metagenomics will be an important future research direction for verifying my results related to gut bacterial function.

Conclusion

In this study, I observed significant differences in the bacterial community and predicted bacterial functions between range-core and invasion-front Australian cane toad gut microbiota. Both environmental factors (isothermality and mean temperature of driest quarter) and host factors (occurrence of lungworms) were significantly associated with these differences. Also, I found that behavioural traits were not associated with the gut bacterial taxonomy, but were associated with their predicted bacterial functions. In model organisms, the gut microbiome has been shown to impact host metabolic activity, brain function and the pathogenesis of disease. My research demonstrates that the cane toad gut bacteria are linked to behaviours that may be important to invasion, providing a new perspective of species invasions. Further, the relationship between lungworm infection and shifts in gut bacteria that are presented here, and previous evidence that lungworm infection affects behaviour (Finnerty et al. 2018) suggest a need to study these factors in conjunction. Manipulative experiments (e.g. faecal transplants, parasite infection) coupled with longer term behavioural measurements would be useful to clarify these relationships and how they may relate to invasion ability in this species.

CHAPTER 4: Genetic similarity enhances the strength of the relationship between gut bacteria and host DNA methylation

This chapter is available as preprint:

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Publication Details	Jia Zhou, Shao Jia Zhou, Lee Ann Rollins, Carlos Rodriguez Lopez This is an experimental manuscript about identifying the correlation of gut microbial profile, host SNPs and DNA methylation profile by using wild cane toads across Australian ranges.

Principal Author

Name of Principal Author (Candidate)	Jia Zhou		
Contribution to the Paper	Designed the experiment, collected and analysed data, wrote the paper as corresponding author		
Overall percentage (%)	95%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Please cut and paste additional co-author panels here as required.

Abstract

Factors such as host age, sex, diet, health status and genotype constitute the environmental envelope shaping microbial communities in the host's gut. It has also been proposed that gut microbiota may be influenced by host epigenetics. Although the relationship between the host's genotype/epigenotype and its associated microbiota has been the focus of a number of recent studies, the relative importance of these interactions and their biological relevance are still poorly understood. In this chapter, I used methylation-sensitive genotyping by sequencing and 16S rRNA gene sequencing to genotype, epigenotype and characterize the gut bacterial communities of free-living invasive cane toads (*Rhinella marina*) from the species' Australian range-core (three sites) and the invasion-front (three sites). I then tested the possible effects that genotype and epigenotype could be asserting on the individuals gut microbiome. My results indicate that the genotypes, epigenotypes and gut bacteria of the range-core and the invasion-front are significantly different. Additionally, I did find a positive association between host pairwise genetic distance and host pairwise epigenetic distance. Although the analysis of the gut bacterial community diversity and genetic diversity within individual, and of host pairwise genetic distance and the pairwise distance of their gut bacterial communities showed no significant association, I did identify a positive relationship between host pairwise epigenetic distance and pairwise distance of their gut bacterial communities. Interestingly, this association increased as genetic differentiation decreased. This finding may suggest that in range-expanding populations where individuals are often genetically similar, the interaction of gut bacteria and host epigenetic status may provide a mechanism through which invaders increase the plasticity of their response to novel environments, potentially increasing their invasion success.

Introduction

Gut microbiota can play a key role in host adaptation by determining hosts' phenotype (Alberdi, Aizpurua, Bohmann, Zepeda-Mendoza, & Gilbert, 2016). Concurrently, host factors such as age, sex and health status contribute to the environmental envelope shaping gut microbial communities (Pereira et al., 2020; Tong, Cui, Du, et al., 2020). In addition to these factors, host genetic diversity also may be an important determinant of host-microbial relationships. For example, host heterozygosity (within-individual genetic variation) has been positively associated with individual fitness and adaptive potential (Mainguy, Côté, & Coltman, 2009; Velando, Barros, & Moran, 2015). Such heterozygosity-fitness correlations have been widely studied, including in the context of disease/parasite resistance, and host body mass, reproductive performance and survival (Brambilla, Keller, Bassano, & Grossen, 2018; Coltman, Pilkington, Smith, & Pemberton, 1999; Luikart, Pilgrim, Visty, Ezenwa, & Schwartz, 2008; Mainguy et al., 2009; Penn, Damjanovich, & Potts, 2002; Velando et al., 2015). In gut microbial studies, alpha diversity (microbial diversity within individual hosts) also has been associated with increased host fitness (e.g. resistance to parasites and disease; (Estaki et al., 2016; Kreisinger, Bastien, Hauffe, Marchesi, & Perkins, 2015; Suzuki, 2017). These results suggest a positive correlation between host genetic diversity and microbial alpha diversity. However, a negative relationship between these metrics has been found in at least one species (fur seals; Grosser et al., 2019), indicating that further analysis of these relationships in a broader range of taxa would allow a better understanding of how the host's genetic diversity affects gut microbial community diversity.

In addition to the degree of host genetic diversity within an individual, the patterns of genetic variation across the genome warrant investigation with respect to interactions with the host's microbial community. Even though gut microbiota are largely acquired from the environment (Alberdi et al., 2016), this community also can be shaped by host genotype (Blekhman et al., 2015; Goodrich et al., 2014, 2016). Particular host genotypes have accounted for substantial

differences in microbial community composition and diversity; for example, in humans, microbiota variation was driven by immunity-related host genotype (Blekhman et al., 2015). This suggests that there could be a heritable component to gut microbial composition, thus genetically similar hosts may share similar gut bacteria. Microbiome composition of desert bighorn sheep (*Ovis canadensis nelsoni*) diverged in accordance with both landscape-scale environmental and host population characteristics (Couch et al., 2020). Stickleback gut microbiota variation across populations was associated with host genotype more than with environmental factors (C. C. R. Smith, Snowberg, Gregory Caporaso, Knight, & Bolnick, 2015). Conversely, host genetic effects were much weaker than the environment in shaping human gut microbiota (Rothschild et al., 2018). Collectedly, these results indicate that the relative strength of host genetic versus environmental influence on gut microbiota may be species-specific, and that these relationships need to be examined further before general conclusions can be drawn.

Gut microbiota may also interact with host epigenotype, providing a mechanism through which gut microbial communities can affect host health and adaptation (Krautkramer et al., 2016; Stilling et al., 2014). For example, clear associations between bacterial composition and DNA methylation profiles have been identified in relation to body weight and metabolism regulation (Cuevas-Sierra, Ramos-Lopez, Riezu-Boj, Milagro, & Martinez, 2019; Kumar et al., 2014). Additionally, gut microbiota guides and/or facilitates epigenetic development of intestinal stem cells during the postnatal period and may influence lifelong gut health (Yu et al., 2015). At the same time, host epigenetic status may affect gut microbiota: DNA methylation in intestinal tissue is known to contribute to the regulation of genes involved in cell proliferation, anti-bacteria metabolite production, anti-inflammation and play a critical role in re-establishing gut homeostasis in mice (Ansari et al., 2020; J. Wu et al., 2020). These results suggest that hosts that possess similar gut bacteria may also have similar DNA

methylation profiles. It is important to examine the interaction between gut bacteria and host DNA methylation, and that this relationship can be bi-directional.

There also exists a clear influence of genotype on an individual's epigenotype (Bell et al., 2011; Dubin et al., 2015). For example, genetic variation can contribute to the transgenerational heritability of DNA methylation in human (McRae et al., 2014). Genetic effects are also known to be stronger than the effects of manipulating DNA methylation in cane toads (Sarma et al., 2020). In addition to the relationship between an individual's genotype and epigenotype, it has been suggested that, at the population level, an increased variability in DNA methylation may occur in populations with low genetic variation, as compared to populations with higher genetic variation. In particular, this has been discussed in the context of expanding range-edge populations of invasive species, and it has been hypothesised that this may facilitate adaptation to new environments by creating phenotypic diversity (Ardura, Zaiko, Morán, Planes, & Garcia-Vazquez, 2017; Carja et al., 2017; Hawes et al., 2018; Sheldon, Schrey, Andrew, Ragsdale, & Griffith, 2018).

Although these relationships between gut microbiota, host genotype and host epigenotype (e.g. DNA methylome) have been examined in human and domesticated animals (Ansari et al., 2020; Cuevas-Sierra et al., 2019; I. David, Canario, Combes, & Demars, 2019; Goodrich et al., 2016; Ryan et al., 2020; F. Z. Xu et al., 2020), little of this research has been conducted in non-model species. Further, the relevance of these relationships to invasion success is virtually unexplored. Here, I use the iconic invasive cane toad to conduct the first characterisation of these relationships in an amphibian and to determine whether these relationships change when comparing samples collected across an expanding invasive range. Although gut bacterial communities and toad genetics have been previously characterised across Australia, their relationship to each other has not been studied. I found significant differences between the gut bacteria of range-core and invasion-front cane toads in chapter 3 (Zhou, Nelson, Rodriguez Lopez, Zhou, et al., 2020). Host genetics also differ across the

range: population structure has been identified across Australia and genetic diversity is reduced at the range edge (Selechnik, Richardson, Shine, DeVore, et al., 2019). Moreover, substantial shifts in gene expression in spleen and muscle tissue were identified between invasion-front and range-core toads (Rollins et al., 2015; Selechnik, Richardson, Shine, Brown, et al., 2019). To date, there have been no investigations of DNA methylation in wild-collected toads in Australia.

In this chapter, I investigated whether the variation observed in cane toad gut bacteria across Australia is mediated by host genetics, and whether gut bacterial communities are correlated with host DNA methylation. Specifically, I answered the following questions: (1) Is there a positive correlation between host genetic diversity and microbial alpha diversity? (2) Is genetic diversity negatively correlated with DNA methylation diversity? (3) Are genetically similar toads sharing similar gut bacteria? (4) Do cane toads that possess similar gut bacteria also have similar DNA methylation profiles?"

Materials and Methodology

Animal materials

I hand-captured 60 wild adult female cane toads from three sites in the Australian invasion-front and three sites in the range-core (Figure 1.1) and euthanized them by injecting tricaine methanesulfonate (MS222) buffered with bicarbonate of soda. I collected blood and colon content by heart puncture and colon dissection respectively, and preserved these samples in 95% ethanol. Samples were frozen at -20 °C for storage until I conducted DNA extractions. The University of Adelaide Animal Ethics Committee approved the collection and use of animals for in this research (approval number: S-2018-056).

Blood Genomic DNA extraction and Methylation-sensitive genotype by sequencing

I extracted genomic DNA from blood using a PureGene Tissue Kit (QIAGEN), following the manufacturer's protocols. I performed msGBS on blood DNA as described by Kitimu et al. (Kitimu et al., 2015). In addition to the 60 genomic DNA samples, I included a water blank to account for environmental contamination introduced during sequencing library preparation. I used two enzymes, *EcoRI* (cutsite: GAATTC) and *HpaII* (cutsite: CCGG), to generate restriction products. Enzymatic restrictions were performed in a 16 µl mix containing: 1.6 µl Cut Smart Buffer, 0.32 µl *EcoRI*-HF (NEB #R0101 (20,000 units/ml)), 0.64 µl *HpaII* (NEB #R0171S (10,000 units/ml)), and 13.4 µl DNA (10ng/µl). The enzyme digestion reaction was conducted at 37 °C for 2 h and then 65 °C for 20 min for enzyme inactivation.

A set of barcoded adapters with an *HpaII* overhang and a common Y adapter with an *EcoRI* overhang (Table S4.1) were used for the ligation reaction. Working stocks of barcoded (0.02 µM) and common Y adapters (3 µM) were prepared in advance as described by Poland et al., (2012). The 32 µl ligation reaction was carried out by adding 0.08 µl T4 Ligase (200 U, NEB) and 3.2 µl T4 Ligase buffer (10X, NEB), 8.72 µl water and 4 µl of the working adapter stock to the 16 µl restriction products. Ligation mixes were incubated at 22 °C for 2 h and 65 °C for 20 min. I removed unused adapters and restriction/ligation products smaller than 100bp using AMPure XP magnetic beads (x0.9 bead/reaction volume to volume ratio). The clean-up products were used for PCR amplification. Each 25 µl PCR consisted of 10ul digested/ligated DNA library (< 1,000 ng), 12.5 µl of Q5 MasterMix (Q5 High-Fidelity 2X Master Mix), 2 µl forward and reverse primers @10 µM (Table S1) and 0.5 µl of water. Reactions were performed at 98 ^{o}C for 30 sec, 12 cycles for (98 ^{o}C for 30 sec, 62 ^{o}C for 20 sec, 72 ^{o}C for 30 sec) and 72 ^{o}C for 5 min. PCR product concentrations were estimated using NanoDrop One^{c} spectrophotometer. Samples were then equimolarly mixed into a single pool. The resultant pool was then split into four subsamples. Fragments below 100 bp and above 600 bp were removed using a magnetic beads and double size selection (x1 bead/reaction volume to volume ratio followed by x0.55 bead/reaction volume to volume ratio). All four size selected

fractions were then pooled and quality checked using a Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and a Fragment Analyzer (Agilent, Santa Clara, CA, USA). Sequencing was performed using HiSeq 4000 150bp PE at Novogene Corporation Inc (Sacramento, CA, USA).

Bacterial DNA isolation and amplicon sequencing

I extracted bacterial DNA from colon content using the DNeasy PowerSoil kit (Qiagen), following the manufacturer's protocols. I performed 16S rRNA gene amplicon sequencing on DNA samples by following guidelines for the Illumina MiSeq System. I included 60 colon DNA extracts, one Zymo isolated DNA standard (D6305, community positive control) and one water blank (PCR negative control). I prepared libraries based on the hypervariable (V3-V4) region of the 16S rRNA gene using primers 341F (5' – **TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CCTACGGGNGGCWGCAG-3') and 785R (5'-**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GACTACHVGGGTATCTAATCC-3') (Herlemann et al., 2011). Sequencing was conducted on the Illumina MiSeq platform at the Ramaciotti Centre for Genomics (University of New South Wales, Kensington, Sydney).

Data analysis

DNA Methylation profiling

I demultiplexed sequencing data with GBSX v1.3 (Herten, Hestand, Vermeesch, & Van Houdt, 2015) and checked quality using FastQC v0.11.4 (Andrews, 2010). I trimmed data using AdapterRemoval v2.2.1 (Schubert, Lindgreen, & Orlando, 2016) and aligned trimmed data to the cane toad reference genome (*Rhinella marina* PRJEB24695; Edwards et al., 2018) using HISAT2 v2.1.0 (D. Kim, Langmead, & Salzberg, 2015). The water blank had very low

QC-passed reads (637 reads) and showed low contamination in library preparation and sequencing process. Non-control samples presented an average of 23,330,961 (+/- 21,763,581) QC passed reads, with a mean GC content of 45.96% (+/- 1.95%) and a mean mapping efficiency of 78.21% (+/- 1.11%). Samples presenting less than 5,000,000 reads were removed from further analysis, resulting in the inclusion of 55 cane toad samples. I used SAMtools (H. Li et al., 2009) to sort and index bam files and then loaded them into Rstudio (R Core Team, 2020). I estimated the methylation status of the sequenced loci using "msgbsR" v1.12.0, an R package developed specifically for msGBS data analysis (Mayne et al., 2018). After removing loci not yielding reads in more than 40% of the toad samples and less than one count per million (CPM) in at least 60% of toads using "edgeR" v3.30.3 (Robinson, McCarthy, & Smyth, 2010) in R, a total 165,858 loci were kept for further analysis.

SNPs profiling

I used BCFtools v1.9 (H. Li et al., 2009) for SNP calling. I used VCFtools v0.1.15 (Danecek et al., 2011) filtering to only keep variants that have been successfully genotyped in 60% of individuals, a minimum quality score of 30, and a minor allele count of 3. These were then imported as a vcf output file into Tassel v5.0 (Bradbury et al., 2007). Here, only SNPs with at least 0.05 minor allele frequency (Suzuki et al., 2019) were kept (i.e., 38,140 SNPs). For duplicate positions, only the first SNP record was retained and the final SNPs dataset included 38,129 SNPs.

Bacterial community profiling

I processed raw 16S rRNA gene sequencing data to create an amplicon sequence variants (ASVs) list using QIIME2 v2020.8 (Bolyen et al., 2018). In summary, sequences were filtered by trimming the first 20 bases and truncating each read to 200 bases (based on sequence base quality score), dereplicating, then merging forward/reverse reads, removing

chimeras, and finally generating ASVs for downstream analysis through the DADA2 pipeline, implemented in QIIME2 (B. J. Callahan et al., 2016). In this ASVs table, reads from colon samples and the positive control ranged between 103,245 and 245,059 counts; the PCR negative control yielded 6,727 reads. I used Greengenes version 13_8 to assign taxonomy to the ASVs (DeSantis et al., 2006).

I imported ASVs into the R package "phyloseq" (McMurdie & Holmes, 2013) to remove representatives classified to Archaea (n = 28), chloroplast (n = 17), mitochondria (n = 186), and 151 unassigned ("kingdom") ASVs. I also removed the ASVs with prevalence of less than four, which makes the logged counts per sample more evenly distributed. The remaining 9,878 taxa were classified to the kingdom with 62.62% assigned to phylum level and 39.65% assigned to family level. I imported the pruned ASVs into QIIME2 and calculated observed ASVs (DeSantis et al., 2006), evenness (Pielou, 1966), and Shannon (Shannon, 1948) indices for bacterial alpha diversity (a measure of microbial diversity within individual host).

I calculated Core50 gut community (Bletz et al., 2016) by filtering ASVs and keeping only those presented in a minimum of 50% of individual toads from each site. This calculation was performed separately for three sites from invasion-front toads: Kununurra (gut Core50: n = 111 ASVs), Old Theda (gut Core50: n = 118 ASVs), and Mary Pool (gut Core50: n = 129 ASVs); three sites from range-core toads: Rossville (gut Core50: n = 148 ASVs), Croydon (gut Core50: n = 86 ASVs), and Lucinda (gut Core50: n = 117 ASVs). I then compiled filtered ASVs of the six sites to avoid excluding ASVs that may be specific to only one site. In combination, the gut Core50 contained 325 unique ASVs, which I used for analysis of beta diversity.

Association analysis of heterozygosity and gut bacterial diversity within individuals

Since host heterozygosity and homozygosity matrixes are highly correlated (Chapman, Nakagawa, Coltman, Slate, & Sheldon, 2009; Charpentier, Boulet, & Drea, 2008), I chose

Homozygosity by locus (HL, (Aparicio, Ortego, & Cordero, 2006) as a metric of diversity within individuals. I used the R package "Genhet" v3.1 (Coulon, 2010) to calculate HL. Similarly, because the gut bacterial alpha diversity matric (Shannon) is highly correlated with other matrices (observed ASVs and evenness: $R^2 \geq 0.8$), I used Shannon diversity to estimate diversity within individuals. I examined the relationship between host heterozygosity and bacterial alpha diversity using the *lmer* function in the R package "lme4" v1.1-23 (Bates, Maechler, Bolker, & Walker, 2015) to run linear mixed models (LMMs) by setting alpha diversity as the response and heterozygosity as the fixed effect, and collection site as a random effect. The linear mixed model dispersion and residuals were checked with DHARMa v0.3.3.0 (F. Hartig, 2019).

Estimation of host genetic, host DNA methylation and gut bacterial diversity and differentiation

I used the R package "phyloseq" v1.32.0 (McMurdie & Holmes, 2013) to calculate a Bray Curtis pairwise distance matrices for SNP data, bacterial taxa abundance, and methylation abundance (per locus). Before calculating Bray Curtis distances, I used a Hellinger transformation (Legendre & Gallagher, 2001) implemented in the package "microbiome" (Valverde et al., 2014) in R for bacteria and methylation abundance data, which converted absolute abundance to relative abundance. For SNP data, I used TASSEL v5.0 to convert vcf file genotype information into the probability that an allele selected at random at a site is the major allele (e.g. homozygous for major allele = 1.0, homozygous for minor allele = 0.0, and heterozygous genotype = 0.5). I used PCoA analysis though R package "phyloseq" v1.32.0 to visualize data, which is not very sensitive to the influence of double-zeros in the ordination analysis. To compare the diversity of genetic, DNA methylation and gut microbiota between invasion-front and range-core, we calculated the mean and standard deviation (SD) of Bray Curtis distances.

I used the *adonis* command from the package "Vegan" to perform permutational multivariate analysis of variance (perMANOVA) to check whether the cane toad genotype, DNA methylation profile, and bacterial communities from each region were significantly different. I used the command *betadisper* in the package "Vegan" (Oksanen et al., 2019) to check the homogeneity of group variances, an assumption of perMANOVA. After finding significant differences between invasion-front and range-core toads, I performed pairwise comparisons between the six sampling sites using the command *pairwise.perm.manova* function in "RVAideMemoire" package with the Wilks Lambda (Nath & Pavur, 1985) and corrected for multiple testing (Herve, 2018) using the Hochberg procedure (Hochberg, 1988).

To investigate relationships between host genotype, host DNA methylation and gut bacteria, I used two methods. First, I used a partial Mantel test implemented in the function mantel.partial in the R package "vegan" to compare Bray Curtis distance matrices, while controlling for the effect of geographic distance. After that, I examined the interactions among these three Bray Curtis distance matrices. Then I used LMMs to compare pairwise Bray Curtis distance matrices, accounting for geographic distance (rescaled) and population (invasion-front or range-core) as fixed factors, and individual toad ID as a random factor. I selected the models based on AIC and BIC values and checked their dispersion and residual plots. Each pairwise distance included two individuals: i and j. Each was used in two models: a model with bacterial distance as the response ($Bacteria\_dist_{ij} \sim Genotype\_dist_{ij} * Methylation\_Dist_{ij} + scale(Geographic\ Distance_{ij}) + Population_i + Population_j + (1|SampleID_i) + (1|SampleID_j)$); and a model with DNA methylation distance as the response ($Methylation\_Dist_{ij} \sim Genotype\_dist_{ij} * Bacteria\_dist_{ij} + scale(Geographic\ Distance_{ij}) + Population_i + Population_j + (1|SampleID_i) + (1|SampleID_j)$). I then used "emmeans" v1.5.4 (V. Lenth et al., 2021) and "ggplot2" v3.3.2 (Wickham, 2016) packages in R to visualize the output of the models with an interaction between gut bacterial, host DNA methylation and host genetic distances. To examine the interactions, I visualized how gut bacterial and host

DNA methylation varied across different genetic distance classes. I used three representative values to present an infinite set of values with which to fix the continuous genetic distances (UCLA: Statistical Consulting Group, n.d.). The three representative values of host genetic distances were the mean level of genetic distance, one standard deviation above the mean level of genetic distance, and one standard deviation below the mean level of genetic distance. The slope of the relationship between gut bacteria and host DNA methylation was estimated based on these distance classes, which is a modified version of spotlight analysis (Aiken & West, 1991).

Results

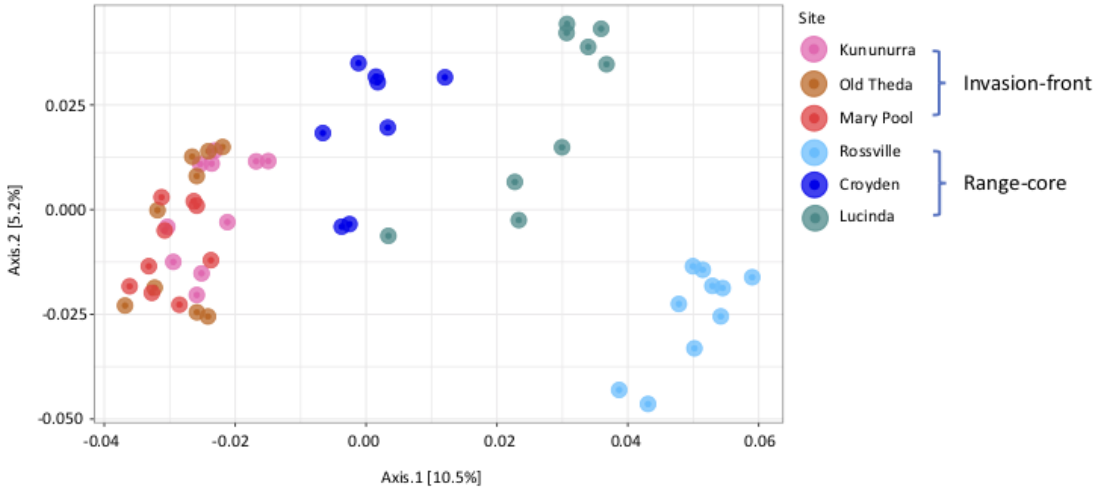
Association of host heterozygosity and gut bacterial diversity within individuals

I investigated whether there was a relationship between individual heterozygosity index (HL) and gut bacterial alpha diversity (Shannon's diversity Index). In all samples, the mean of HL is 0.49 (SD = 0.03) and the mean of Shannon's index was 6.00 (SD = 0.92). LMM results indicated that these measures were not correlated (df = 52.716, t-value = 1.444, p-value = 0.16, accounting for sampling site as a random effect).

Estimation of host genetic, host DNA methylation and gut bacterial diversity and differentiation

PCoA plots indicated that host genotype, methylation profiles and gut bacteria clustered according to their provenance and illustrated the amount of diversity within invasion-front and range-core populations (Figure 4.1 plots A, B, C). Comparison of Bray Curtis values from SNP data indicated that range-core toads had significantly higher levels of genetic differentiation than those from the invasion front (invasion front: mean = 0.126, SD = 0.009; range-core: mean = 0.140, SD = 0.010; t test: t = 19.569, df = 710.3, p-value < 0.01; Figure 4.1, Table 4.1). However, range-core and invasion-front toads had similar levels of DNA

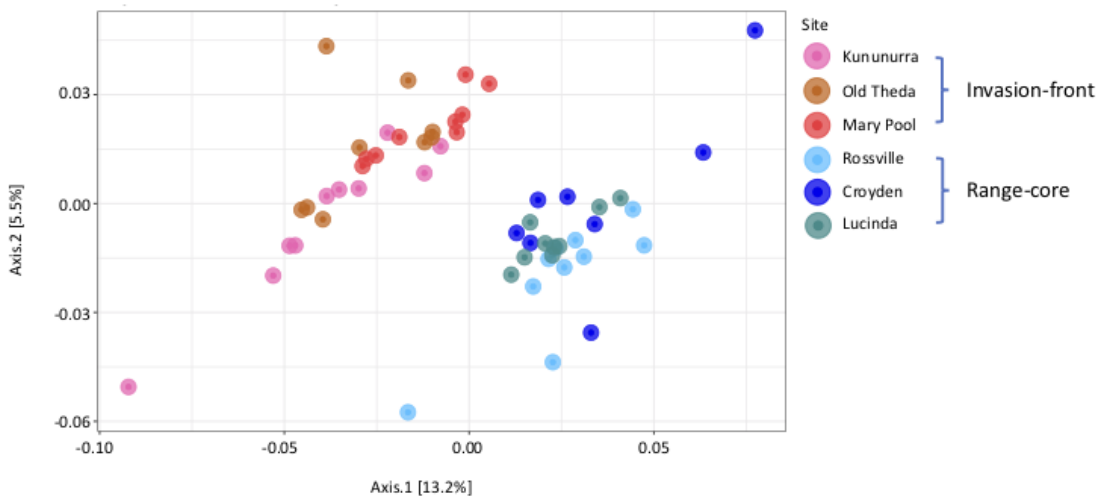
methylation differentiation (t test: t = 0.232, df = 723.42, p-value = 0.82, Table 4.1) and gut bacteria (t test: t = 1.708, df = 723.19, p-value = 0.09, Table 4.1).



Pairwise comparisons using permutation MANOVAs on a Bray Curtis distance matrix of SNPs. Bold font indicates significant comparisons.

	Kununurra	**Old Theda**	**Mary Pool**	**Rossville**	**Croydon**
Old Theda	0.069	-	-	-	-
Mary Pool	**0.001**	0.368	-	-	-
Rossville	**0.001**	**0.001**	**0.001**	-	-
Croydon	**0.001**	**0.001**	**0.001**	**0.001**	-
Lucinda	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**

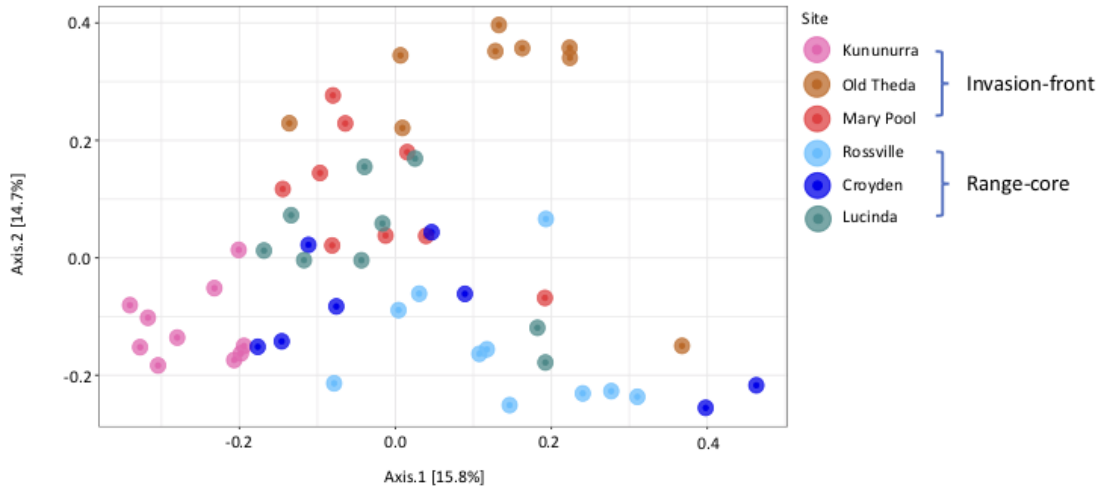
P value adjustment method: Hochberg (1988)



Pairwise comparisons using permutation MANOVAs on a Bray Curtis distance matrix of DNA methylation. Bold font indicates significant comparisons.

	Kununurra	Old Theda	Mary Pool	Rossville	Croydon
Old Theda	0.063	-	-	-	-
Mary Pool	**0.002**	0.063	-	-	-
Rossville	**0.001**	**0.001**	**0.001**	-	-
Croydon	**0.001**	**0.001**	**0.001**	**0.001**	-
Lucinda	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**

P value adjustment method: Hochberg (1988)



Pairwise comparisons using permutation MANOVAs on a Bray Curtis distance matrix of Core50 gut bacteria. Bold font indicates significant comparisons.

	Kununurra	Old Theda	Mary Pool	Rossville	Croydon
Old Theda	**2e-04**	-	-	-	-
Mary Pool	**2e-04**	**2e-04**	-	-	-
Rossville	**2e-04**	**2e-04**	**2e-04**	-	-
Croydon	**2e-04**	**2e-04**	**2e-04**	**2e-04**	-
Lucinda	**2e-04**	**2e-04**	**2e-04**	**2e-04**	**2e-04**

P value adjustment method: Hochberg (1988)

Figure 4. 1 Principle coordinate analysis plot of host SNPs, DNA methylation profile and gut bacteria profiles based on Bray Curtis distance matrices.

Differentiation between the invasion-front (Kununurra, Old Theda, and Mary Pool) and the range-core (Rossville, Croydon, and Lucinda): (A) SNPs, (B) DNA methylation profile, (C), bacterial community. Pairwise comparisons were conducted using permutation MANOVAs. P values were adjusted with Hochberg method (Tables).

Table 4. 1 The mean values (M) and standard deviation (SD) of Bray Curtis pairwise distances for genetic, DNA methylation and gut bacteria from invasion-front and range-core cane toads.

Bray Curtis pairwise distances												
Invasion-front						**Range-core**					
Genetic		**Methylation**		**Bacteria**		**Genetic**		**Methylation**		**Bacteria**	
M	**SD**	**M**	**SD**	**M**	**SD**	**M**	**SD**	**M**	**SD**	**M**	**SD**
0.126	0.009	0.121	0.022	0.640	0.132	0.140	0.010	0.122	0.019	0.656	0.114

Genetic, DNA methylation and gut bacterial community differentiation were calculated between invasion-front and range-core (Figure 4.1). I found significant differences between invasion-front and range-core in host SNPs (adonis2: R^2 = 0.089, F = 5.162, p-value < 0.001; betadisper: F-value = 23.337, p-value < 0.001), host DNA methylation (adonis2: R^2 = 0.104, F = 6.120, p-value < 0.001; betadisper: F-value = 0.005, p-value = 0.942) and gut bacteria (adonis2: R^2 = 0.099, F = 5.830, p-value < 0.001; betadisper: F-value = 0.226, p-value = 0.636). Pairwise comparisons between sampling sites differed across all three comparisons

(e.g. genetic, DNA methylation and gut bacteria), except that host genotype and methylation profiles of the toads from Old Theda were similar to the toads from Kununurra and Mary Pool (p-values > 0.05, tables in Figure 4.1).

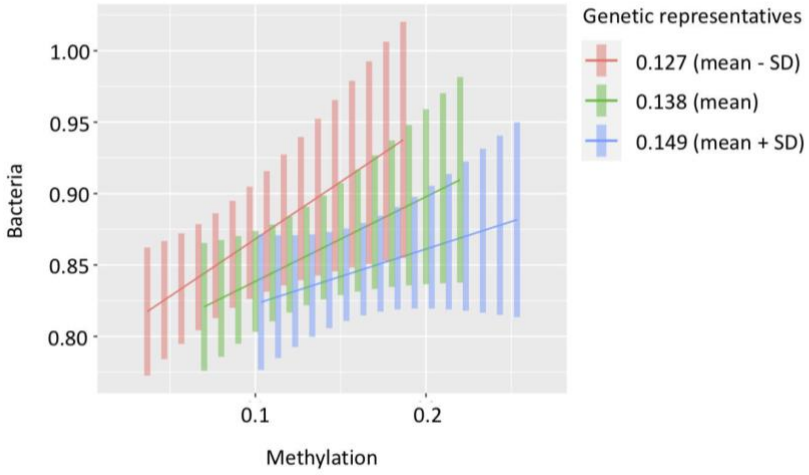
Genetically similar toads did not share similar gut bacteria (Mantel r = 0.0788, p-value = 0.116, Table 4.2). On the contrary, toads with similar DNA methylation profiles shared similar gut microbial composition (Mantel r = 0.1553, p-value = 0.03, Table 4.2). Host genotype and host DNA methylation distance were positively associated (Mantel r = 0.654, p-value = 0.001, Table 4.2).

Table 4. 2 Partial mantel test of Bray Curtis distances (genetic, DNA methylation and gut bacteria), controlling for geographic distance.

Predictor variables	**Response variables**	**Accounted for Geographic distance (mantel partial)**	
		Spearman correlation	
		Mantel r	**P-value**
Genetic distance	**Bacteria distance**	0.0788	0.116
Genetic distance	**Methylation distance**	0.654	0.001
Methylation distance	**Bacteria distance**	0.1553	0.026

LMM analysis with gut microbial distance as the response variable indicated that gut bacterial differentiation was affected by host DNA methylation differentiation (df = 1461.645, t-value = 2.505, p-value = 0.01), and the interaction of host genetic distance with host methylation distance (df = 1441.646, t-value = -2.155, p-value = 0.03; Figure 4.2). The observed relationship between gut bacterial distance and host DNA methylation distance was stronger in cane toad pairs that were more genetically similar (Figure 4.2). When host DNA methylation distance was used as the response variable, LMM analysis indicated that DNA methylation distance was not affected by gut bacterial distance (df = 1390, t-value = 0.598, p-

value = 0.55; Figure 4.3). DNA methylation distance was significantly associated with genetic distance (df = 1390, t-value = 5.734, p-value < 0.001; Figure 4.3). There was no interaction between these relationships and population (invasion-front and range-core).

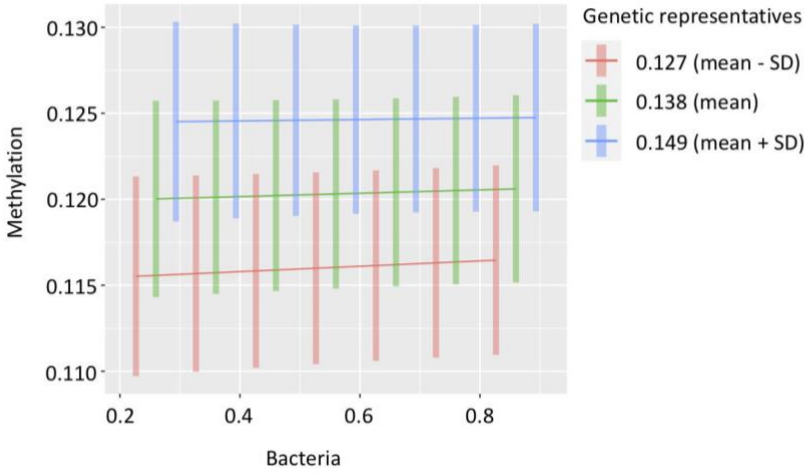


AIC = -3372.482, BIC = -3319.45						
Random effects						
Groups	**Name**	**Variance**	**Std.Dev.**			
SampleID$_i$	(Intercept)	0.001879	0.04335			
SampleID$_j$	(Intercept)	0.004906	0.07005			
Residual		0.004862	0.06973			
Number of obs: 1485, groups: c1, 54; c2, 54						
Fixed effects:						
	Estimate	**Std.Error**	**df**	**t value**	**Pr(>|t|)**	
(Intercept)	0.58232	0.14258	1474.21264	4.084	4.66E-05	***
genetics	1.62221	1.01523	1453.39647	1.598	0.1103	
methylation	3.20298	1.27862	1461.64514	2.505	0.0124	*
scale(geographic)	0.21469	0.01275	1446.59752	16.838	< 2e-16	***
Population$_i$	-0.37168	0.0286	533.77127	-12.997	< 2e-16	***
Population$_j$	0.31752	0.03024	232.814	10.501	< 2e-16	***
genetics:methylation	-18.90622	8.77433	1441.64551	-2.155	0.0313	*
Signif. codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1**						
Correlation of Fixed Effects:						
	(Intr)	**genetics**	**methylation**	**scale(geographic)**	**Population$_i$**	**Population$_j$**
genetics	-0.957					
methylation	-0.881	0.829				
scale(geographic)	0.179	-0.127	0.134			

Population$_i$	-0.175	0.128	-0.123	-0.884		
Population$_j$	0.084	-0.097	0.044	0.727	-0.663	
genetics:methylation	0.895	-0.905	-0.970	-0.100	0.07	-0.009

Figure 4. 2 LMM on the Bray Curtis pairwise distances: $Bacteria\_dist_{ij} \sim Genetics\_dist_{ij} * Methylation\_Dist_{ij} + scale(Geographic\ Distance_{ij}) + Population_i + Population_j + (1|SampleID_i) + (1|SampleID_j)$.

Bacterial distance was used as the response variable and genetic distance, DNA methylation distance, geographic distance (rescaled), and population (invasion-front or range-core) were used as fixed factors. Sample ID was used as random factor. Three representative values represent an infinite set of values with which to fix the continuous genetic distances (UCLA: Statistical Consulting Group, n.d.): the mean level of genetic distance, one standard deviation above the mean level of genetic distance, and one standard deviation below the mean level of genetic distance.



AIC = -11590.97 , BIC = -11537.94						
Random effects						
Groups	**Name**	**Variance**	**Std.Dev.**			
SampleID$_i$	(Intercept)	2.01E-04	0.01419			
SampleID$_j$	(Intercept)	1.74E-04	0.01318			
Residual		1.52E-05	0.0039			
Number of obs: 1485, groups: c1, 54; c2, 54						
Fixed effects:						
	Estimate	**Std.Error**	**df**	**t value**	**Pr(>|t|)**	
(Intercept)	6.01E-02	1.09E-02	1.41E+03	5.544	3.52E-08	***
genetics	4.22E-01	7.36E-02	1.39E+03	5.734	1.20E-08	***
bacteria	8.29E-03	1.39E-02	1.39E+03	0.598	0.54962	
scale(geographic)	-0.001968	8.18E-04	1.39E+03	-2.405	0.01632	*

Population$_i$	1.35E-02	4.19E-03	7.08E+01	3.208	0.00201	**
Population$_j$	-1.062e-02	3.87E-03	6.87E+01	-2.743	0.00775	**
genetics:bacteria	-5.297e-02	1.03E-01	1.39E+03	-0.513	0.60835	
Signif. codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1**						
Correlation of Fixed Effects:						
	(Intr)	**genetics**	**bacteria**	**scale(geographic)**	**Population$_i$**	**Population$_j$**
genetics	-0.926					
bacteria	-0.874	0.946				
scale(geographic)	0.375	-0.279	-0.179			
Population$_i$	-0.307	0.099	0.064	-0.378		
Population$_j$	-0.049	-0.070	-0.054	0.353	-0.137	
genetics:bacteria	0.866	-0.951	-0.994	0.138	-0.050	0.041

Figure 4. 3 LMM on the Bray Curtis pairwise distances: Methylation_Distij ~ Genetics_distij * Bacteria_distij + scale(Geographic Distanceij) + Populationi + Populationj + (1|SampleIDi) + (1|SampleIDj).

DNA methylation distance was used as response variable and genetic distance, bacterial distance, geographic distance (rescaled), and population (invasion-front or range-core) were used as fixed factors. Sample ID was used as random factor. Three representative values represent an infinite set of values with which to fix the continuous genetic distances (UCLA: Statistical Consulting Group, n.d.): the mean level of genetic distance, one standard deviation above the mean level of genetic distance, and one standard deviation below the mean level of genetic distance.

Discussion

In this chapter, I explored the relationship between host genotype, host epigenotype, and gut bacteria. I found that these relationships do not differ in range-core toads versus toads from the expanding invasion-front in Australia. Through the use of next-generation sequencing, I found no relationship between a host's genetic diversity and the diversity of its gut bacteria within individual. Additionally, I found that while genetic differentiation was positively related to differentiation of DNA methylation, there did not appear to be a relationship between the diversity of these two measures. I also found that pairwise differentiation between cane toad gut bacteria was associated with pairwise differentiation between host

DNA methylation, and this association was stronger in pairs that were more genetically similar.

There is a growing interest in how host or environmental factors contribute to gut microbiota variation, and how this may impact host phenotype (Kreznar et al., 2017; Ussar et al., 2015; C. Zhang et al., 2010). However, there are few studies that have investigated the relationship between host heterozygosity and gut bacteria. Because both host heterozygosity (Mainguy et al., 2009; Velando et al., 2015) and gut bacterial diversity (Estaki et al., 2016; Kreisinger et al., 2015) have been reported to be positively related to individual fitness, I predicted that hosts with higher levels of heterozygosity would have more diverse gut bacteria. Interestingly, this relationship was investigated in fur seals and that study found that an individual's heterozygosity (calculated with microsatellite data) was negatively associated with its microbial diversity (Grosser et al., 2019). The authors of that study proposed that higher quality individuals (who have greater heterozygosity) should be more effective at suppressing nonbeneficial microbes, thus having less diverse microbiota (Grosser et al., 2019). A negative relationship between these metrics was also found a study of sticklebacks, where individuals with greater heterozygosity at the MHC (Major Histocompatibility class II) had less diverse gut microbiota (Bolnick et al., 2014). In this chapter, I found no relationship between host individual heterozygosity and bacterial diversity in cane toads. The hypotheses in all of these studies depend on a positive relationship between heterozygosity and fitness. However, the validity of studies of heterozygosity-fitness correlations where small numbers of markers (e.g. microsatellites) have been used has been challenged because the correlation between estimated heterozygosity and true genome-wide heterozygosity is weak (Dewoody & Dewoody, 2005; Forstmeier, Schielzeth, Mueller, Ellegren, & Kempenaers, 2012). The SNP data set used here to calculate heterozygosity was large (>38,000 SNPs) and, thus, may provide a more accurate picture of these relationships.

There could be a heritable influence on gut microbial composition, mediated by host genotype (Blekhman et al., 2015; Goodrich et al., 2014, 2016). To investigate this in toads, I tested whether genetic similarity between hosts was related to similarity of their gut bacteria. I found no significant association between these metrics. Results of other studies investigating this question are mixed. In chickens, host genetics played a minor role in shaping the gut microbiota (Wen et al., 2019). However, in wild house mice, gut microbiota dissimilarity was significantly correlated with both host genetic distance and body mass index, but not significantly associated with other factors, including diet, climate and geographic distance (Suzuki et al., 2019). Gut microbiota was also found to be significantly correlated with host genetics in fish and amphibians (Griffiths et al., 2018; U. Webster, Consuegra, Hitchings, & G. de Leaniz, 2018). Because gut microbiota can be affected by a wide variety of host and environmental factors, it seems likely the relationship between host genetics and gut bacteria is complex and may vary depending on the strength of other host and environmental factors. Nevertheless, the amphibian gut microbiome has been identified to have weaker signal of cophylogeny than that of mammals (Youngblut et al., 2019). Amphibians are one of very few vertebrates that undergo metamorphosis, and this process can cause significant changes in gut bacterial communities, presumably related to physiological and environmental changes to the host (Kohl et al., 2013). Additionally, hibernating amphibians (non-feeding) exhibit a decrease in bacterial population and an increase in ureolytic capacity, compared to active (feeding) ones, which could result from urea hydrolysis by gut bacteria in hibernating animals (Wiebler et al., 2018). Therefore, further studies on the relationship of amphibian species and their gut bacteria are needed, especially those that consider the impacts of metamorphosis, hibernation and the urea recycling process.

Gut microbiota can cause heritable phenotype changes through epigenetic modification of host DNA (Grieneisen, Muehlbauer, & Blekhman, 2020; Krautkramer et al., 2016; Stilling et al., 2014) and DNA methylation in intestinal tissue is known to play a critical role in re-

establishing gut homeostasis (Ansari et al., 2020; J. Wu et al., 2020), suggesting this may be a bi-directional relationship. The observed changes in some phenotypic traits (e.g. behaviour, see Chapter 3) in cane toads across Australia have been linked to their gut bacteria (Zhou, Nelson, Rodriguez Lopez, Zhou, et al., 2020). It is possible that this association could be mediated through shifts in DNA methylation in toads. Moreover, in this chapter, I found that both DNA methylation and gut bacteria were significantly different between different sampling localities, and that differentiation of host DNA methylation was positively related to differentiation of gut bacteria between pairs of individuals. Because the direction of the relationship between host DNA methylation and gut bacterial community is unknown, I ran separate LMMs using each of these metrics as the dependent variable. Interestingly, when gut bacterial distance was used as the dependent variable, its relationship to DNA methylation distance was stronger in pairs of individuals that were more genetically similar (Figure 2). This dynamic suggests that in populations with more genetically similar individuals (e.g. invasion-front populations), the relationship between DNA methylation and gut bacteria also may be stronger. Interestingly, when DNA methylation distance was used as the dependent variable, there was no significant relationship with gut bacterial distance (Figure 3). The strong influence of host genotype on DNA methylation may mask any potential influence of gut bacteria on DNA methylation.

The strengthened relationship between gut bacteria and DNA methylation in cane toads that are genetically similar could facilitate cane toad adaptation to novel environments in Australia. First, gut bacterial variation caused by environmental factors (e.g. food resources) could alter host DNA methylation, leading to beneficial phenotypic changes that increase host fitness (Grieneisen et al., 2020; Krautkramer et al., 2016; Stilling et al., 2014). Second, environmental factors could alter host DNA methylation, which could affect the host's ability to use local microbes or to maintain a balanced gut bacteria by supressing nonbeneficial microbes (Ansari et al., 2020; J. Wu et al., 2020). In order to study the causal relationship in

this system, fecal transplantation and methylation manipulation experiments may illuminate the underlying mechanisms.

During invasions, increased variation in host DNA methylation could be compensatory for low genetic diversity, and facilitate adaptation to novel environments by creating phenotypic diversity (Ardura et al., 2017; Carja et al., 2017; Hawes et al., 2018; Sheldon et al., 2018). This is an intriguing idea, and could explain the multitude of phenotype shifts seen in toads as they have spread across Australia (Rollins et al., 2015), despite their low genetic diversity, especially at the invasion-front (Lillie et al., 2014; Selechnik, Richardson, Shine, DeVore, et al., 2019). In this chapter, I found that although genetic diversity differed across the Australian range, DNA methylation patterns did not, suggesting that no such relationship exists in this invasion. Similarly, in a study of invasive house sparrows (*Passer domesticus*) in Australia, no compensatory relationship between genetic diversity and DNA methylation diversity also was detected (Sheldon et al., 2018). In human populations, diversity of DNA methylation mirrored genetic diversity (Carja et al., 2017). Together, this evidence suggests that further research is needed to understand whether these factors interact to promote phenotypic variation on invasion fronts and, if so, whether the strength of this relationship depends on the degree of influence that the genome exerts on the epigenome for a given species.

Conclusion

My results demonstrate that gut bacterial community differentiation of invasive cane toads in Australia is positively correlated with individual DNA methylation profile changes, and this is accentuated when genetic differentiation is low. DNA methylation variation is similar across the invasion, whereas genetic diversity decreases on the invasion front, suggesting no relationship between the diversity of these metrics. However, genetic differentiation and DNA methylation differentiation have a strong, positive association suggesting that genetic

composition determines DNA methylation in this species. These findings provide insights into the dynamics between host genotype, epigenotype and gut bacteria in this iconic invasive amphibian. Moreover, this study draws our attention to the complexity of these relationships and how they may shift over an expanding invasion.

CHAPTER 5: Minor not major dietary components are associated with gut bacteria in an Australian wild invasive amphibian

This chapter is in preparation for submission with the following co-authors: Ryan Shofner, Carlos Rodriguez Lopez, Shao Jia Zhou, Lee Ann Rollins.

Statement of Authorship

Title of Paper	Minor not major dietary components are associated with gut bacteria in an Australian wild invasive amphibian
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Jia Zhou, Ryan Shofner, Carlos Rodriguez Lopez, Shao Jia Zhou, Lee Ann Rollins This is an experimental manuscript about characterizing diet profiles for cane toad from two localities, invasion-front and range-core in Australia and studying the relationship between gut microbial community and the diversity of prey taxa, as well as specific prey relative abundance.

Principal Author

Name of Principal Author (Candidate)	Jia Zhou		
Contribution to the Paper	Designed the experiment, collected and analysed data, wrote the paper as corresponding author		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	12 April 2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Ryan Shofner		
Contribution to the Paper	Classified the taxonomy of diet content and commented on the manuscript		
Signature		Date	21 April 2021

Name of Co-Author	Carlos Rodriguez Lopez		
Contribution to the Paper	Supervised experimental design and project development, helped in data interpretation and commented on the manuscript.		
Signature		Date	20/4/21

Name of Co-Author	Shao Jia Zhou		
Contribution to the Paper	Supervised experimental design and project development, helped in data interpretation and commented on the manuscript.		
Signature		Date	21/4/2021

Name of Co-Author	Lee Ann Rollins		
Contribution to the Paper	Supervised experimental design and project development, helped in data interpretation and commented on the manuscript.		
Signature		Date	20/4/21

Please cut and paste additional co-author panels here as required.

Abstract

Diet is known to be a key factor affecting gut microbial community assemblages in many species, but little is known about the impact of diet composition on gut bacteria in wild invasive species expanding across novel environments. Here I investigate whether diet affects the gut bacteria of an invasive species cane toad (*Rhinella marina*) in Australia. Toads feeding behaviours are likely to be opportunistic and they are likely to be encountering different resources in new environments as they expand across Australia. To study cane toad diet, I collected 60 toads from a total of six sampling sites evenly distributed across invasion-front and range-core areas in Australia. I characterised diet using microscopic observation of stomach contents and used Next-Generation Sequencing to characterize the toads' gut bacterial profiles. I found animals, plants, and non-organic matter in toads' stomachs and the most common dietary components were animals (mainly insects, like termites and ants). I did not find differences in alpha diversity of prey items within each toad's diet, but I did observe differences in beta diversity of those items between invasion-front and range-core toads. Also, I found that more invasion-front toads have plant matter present in their stomach than those from the range-core. I found that the presence of plant matter was associated with gut bacterial variation, but gut bacteria was not significantly associated with the main dietary component (insects). The latter may be explained by toads' opportunistic diet, which is likely to be beneficial for their adaptation to new environments and, subsequently, their invasion success.

Introduction

Understanding how host and environmental factors determine the composition and diversity of gut microbiota is an important step to understand host health and function (Marques et al., 2010; Org et al., 2016; Ussar et al., 2015; Zmora et al., 2019). Diet is known to be a key

factor affecting gut microbial community assemblages (Carmody et al., 2015; Pérez-Cobas et al., 2015; C. Zhang et al., 2010). Thus, the changes observed in gut microbiota between populations living in different habitats are likely to be influenced by variation in the host's diet (K. P. Scott et al., 2013; Zmora et al., 2019). The diet can affect gut microbiota through: (1) serving as a source of gut microbiota, and (2) altering gut microbial community and its metabolites through dietary macronutrients like carbohydrates, proteins and fats (Carmody et al., 2015; P. Fan, Liu, Song, Chen, & Ma, 2017; K. P. Scott et al., 2013).

The majority of studies on the impact of diet on gut microbiota have used artificial diets to investigate single nutrient component (Khan et al., 2020; Makki et al., 2018; C. Zhang et al., 2013). These controlled diet studies provide insights on the impact of specific aspects of an organism's diet on gut microbiota, but they do not account for feeding behaviours under natural conditions (Baxter et al., 2015; Huan Li et al., 2016). Domesticated species tended to exhibit stronger diet-microbiome linkages and greater turnover in diet and microbiome due to seasonal changes than wildlife (Kartzinel, Hsing, Musili, Brown, & Pringle, 2019). Wild animals are more likely to eat a wide range of different foods based on their availability. This may be more pronounced in invasive species who are likely to encounter novel prey as they expand their range, which could lead the relationship between diet and microbiome change in different populations. An absence of a connection between diet and gut microbiome may be an advantage for such species. Therefore, studying the interaction of diet and bacteria in such systems will provide crucial information for understanding the relationship between diet and gut bacteria, and how this might affect invasion.

The ideal system to study these relationships is an invasive population that is rapidly expanding its range, because it enables the investigation of closely related individuals across different environments. One such species is the cane toads (*Rhinella marina*), which is rapidly expanding its invasive range across Australia. Cane toads have successfully spread throughout Australia for nearly 90 years since their introduction to Queensland in 1935. Their

current range now extends from north-eastern New South Wales up to eastern Queensland, and across the Northern Territory to northern Western Australia. This expansive range encompasses a wide variety of habitats, which might be expected to be linked to shifts in prey availability. Like with many anurans, cane toad diet is difficult to observe directly because they are opportunistic feeders, eating almost anything of appropriate size and accessibility (Shine, 2010). Instead of tracking diet directly, previous studies of toad diet have been limited to quantification of changes in body mass over time or the presence and size of abdominal fat bodies, which may serve as a proxy for diet (G. P. Brown et al., 2015; G. P. Brown et al., 2013). As diet generalist, they eat a wide range of taxa and also inadvertently consume additional biotic and non-biotic items while ingesting their intended prey (Jabon et al., 2019). This is important to consider because, while gut microbiome can be affected by prey species, it can also be affected by the availability of food resources, food diversity, and random food items the host consumes. Studying cane toad diet composition not only helps us to understand their dietary habits and how it interacts with their gut microbiome, but also provides insight to the impact of cane toads on their currently occupied ecological habitats, thus enabling better evaluation and management of this invasive species.

Methods of studying animal diet composition include visual observation (direct observation of feeding, or by microscopic examination), prey-specific antibodies, plant alkane fingerprints, stable isotopes, and recent DNA-based approaches such as DNA profiling through amplification of the gut contents using general or group-specific primers, TGGE or DGGE (temperature or denaturing gradient gel electrophoresis), and DNA metabarcoding (De Barba et al., 2014; Pompanon et al., 2012; Symondson, 2002). Currently, the most common methods used to study anuran diet is microscopic observation of stomach contents following euthanasia (Apayor-Ynot, Tan, Lim, Delima-Baron, & B. Mohagan, 2017; Chang et al., 2016; Heise-Pavlov & Longway, 2011) or non-lethal stomach flushing (Chowdhary et al., 2016; Park, Lee, & Cho, 2018). Insect prey items are normally classified to order level (Chang et al.,

2016; Heise-Pavlov & Longway, 2011) and the identified prey items are measured by relative abundance (R. Baker, Buckland, & Sheaves, 2014).

To clarify the relationship between diet and gut microbiota in cane toads, I characterised diet profiles for cane toads from Australian invasion-front and range-core localities and studied the relationship between gut bacterial community and the diversity of prey taxa, as well as specific prey relative abundance.

Methods

Animal materials

In this study, I used 60 wild adult female cane toads from three sampling sites in Western Australia ('invasion-front', N = 30) and three sampling sites in Queensland (QLD) ('range-core', N = 30, Figure 1.1). All animals used in this study were approved by the University of Adelaide Animal Ethics Committee (approval number: S-2018-056). I collected stomach and colon contents from these animals upon euthanasia. I preserved collected samples in 95% ethanol at 4 °C while in the field and the frozen at -20 °C for storage until DNA extractions.

Stomach content characterization conducted by Dr. Ryan Shofner

A sterile surgical scalpel was used to make a longitudinal incision in the stomach from the esophageal sphincter to the pyloric sphincter. Sterile forceps were used to spread the walls of the stomach and remove the contents, which were placed into a sterile 50 mL falcon tube containing 15 – 20 mL 95% ethanol for storage. The stomach was also dunked repeatedly into the ethanol to dislodge any remaining contents. Contents were transferred to a 90 mm x 14 mm sterile petri dish and examined under a Leica M205 C stereomicroscope. Contents were counted and classified as inorganic or organic, with organic matter being classified as either plant or animal. Animals were identified to order, and invertebrates were identified to family.

All countable animal taxa were classified into 53 families. The presence of plant, non-organic matter (sand and pebble) and unidentifiable animal/plant matter was noted. The finalised dietary matrix was used for downstream analysis using the R package "phyloseq" v1.32.0 (McMurdie & Holmes, 2013).

Intestinal bacteria profile characterization

I extracted bacterial DNA of colon contents using DNeasy PowerSoil kit (Qiagen), following the manufacturer's protocols. I performed 16S rRNA amplicon sequencing on microbial DNA samples by following guidelines for the Illumina MiSeq System. I sequenced 62 samples in total, including 60 colon microbial DNA extracts, one Zymo isolated DNA sample (D6305, community positive control) and one water blank (PCR negative control). I prepared libraries based on the hypervariable (V3-V4) region of the 16S rRNA gene using primers 341F (5' – **TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CCTACGGGNGGCWGCAG-3') and 785R (5'-**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GACTACHVGGGTATCTAATCC-3') (Herlemann et al., 2011). Sequencing was performed using the Illumina MiSeq platform at the Ramaciotti Centre for Genomics (University of New South Wales, Kensington, Sydney).

Bacteria community identification and classification

I processed raw 16S rRNA sequence data and generated amplicon sequence variants (ASVs) using the QIIME2 pipeline (Bolyen et al., 2018). In summary, sequences were filtered by trimming the first 20 bases and truncating each read to 200 bases, dereplicating, then merging forward/reverse reads, removing chimeras, and finally generating ASVs for downstream analysis using the DADA2 pipeline, implemented in QIIME2 (B. J. Callahan et al., 2016). In this ASVs table, reads from colon samples and the positive control ranged between 103,245

and 245,059 counts; the PCR negative control yielded 6,727 reads. I used Greengenes version 13_8 to assign taxonomy to the ASVs (DeSantis et al., 2006).

I imported ASVs into the R package "phyloseq" v1.32.0 to remove representatives classified to Archaea (N = 28), chloroplast (N = 17), mitochondria (N = 186), and 151 unassigned ("kingdom") ASVs. I also removed the ASVs with a prevalence of less than four, which makes the logged counts per sample more evenly distributed. The remaining 9,878 taxa were classified to the Kingdom Bacteria with 62.62% assigned to phylum level and 39.65% assigned to family level.

Diet content comparison between invasion-front and range-core toads

I first compared the occurrence of plants in toad stomachs between invasion-front and range-core samples. I used Shapiro-Wilk test to check data normality using "RVAideMemoire" v0.9-77 package in R (Hervé, 2020) and then used a Wilcoxon rank sum test (<stats>::<wilcox.test>, (Hollander & A. Wolfe, 1999) to test for significance because the Shapiro-Wilk test indicated that the data were not normally distributed.

For the table of countable animal taxa, I calculated alpha diversity (observed taxa, Shannon and Chao1) using the function *estimate_richness* through the "phyloseq" package. I used the Shapiro-Wilk test to assess multivariate normality with p-values below 0.05, indicating the data significantly deviated from a normal distribution. In cases where data was not normally distributed, I used the Wilcoxon rank sum test to compare the alpha diversity between invasion-front and range-core toads. I used relative proportion of prey items by percentage frequency of occurrence (R. Baker et al., 2014; Chang et al., 2016) to view different taxonomic classification and also for downstream analyses.

I used the "phyloseq" package to calculate the Euclidean distance pairwise distance matrix to compare beta diversity between individuals' diet, and visualized this relationship using a

PCoA plot. I used a Hellinger transformation (Legendre & Gallagher, 2001) for the diet content table before calculating the distance matrix, which makes the logged counts per sample more normally distributed (Figure S5.1). I performed a perMANOVA comparison between range-core and invasion-front populations using function *adonis2* in R package "vegan" v2.5-6 (Oksanen et al., 2019). I used function *betadisper* in the package "vegan" to check for data normality. I also did pairwise comparisons between sampling sites using the "RVAideMemoire" package using a Wilks Test and adjusted p-values using a Benjamin Hochberg adjustment.

I then individually compared seven dominant diet families (relative abundance >0.02) between regions (range-core vs. invasion-front) using a Wilcoxon rank sum test and sampling sites using a Kruskal-Wallis test (McKight & Najab, 2010). I conducted pairwise comparisons between each pair of sampling sites (Wilcoxon rank sum test, p-values adjusted; (Benjamini & Hochberg, 1995).

The impact of diet on gut microbial community

I imported the pruned ASVs into QIIME2 and calculated observed ASVs (DeSantis et al., 2006), evenness (Pielou, 1966), and Shannon (Shannon, 1948) indices for microbial alpha diversity (a measure of microbial diversity within individual host). I investigated whether diet alpha diversity was related to gut microbial alpha diversity. Since Shannon is highly correlated with other alpha diversity indexes, I only considered the relationship between Shannon diversity of diet and gut bacteria using linear mixed models (LMMs). I set gut bacterial alpha diversity as the response variable and diet alpha diversity as fixed effect, with sampling site used as a random effect. The linear mixed model dispersion and residuals were checked with "DHARMa" (F. Hartig, 2020).

To test whether toads with similar diet shared similar gut bacteria, I conducted a partial Mantel test using a Bray Curtis distance matrix of gut bacteria and a Euclidean distance

matrix of diet, controlling for the effect of geographic distance. I used "vegan" to calculate Bray Curtis pairwise distance matrix for microbial taxa abundance based on Hellinger transformed data.

To identify the association of individual diet factors (family level) with gut bacterial community, I conducted statistical analyses using the function *envfit* in the "vegan" package on Bray Curtis distances. I included alpha diversity of accountable diet content, occurrence of plant, occurrence of non-organic matter (sand and pebble), and relative abundance of each single taxa (family) individually as a factor.

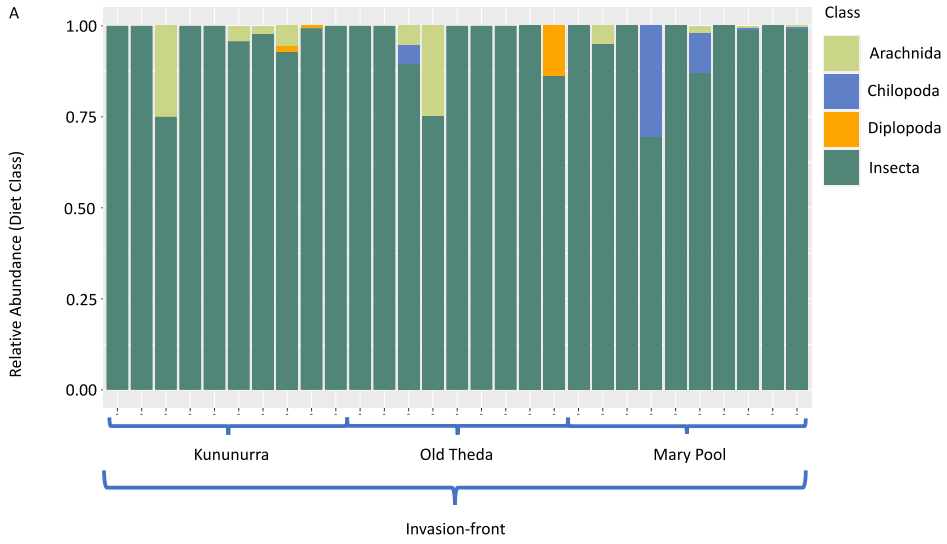
Results

Cane toad diet composition

I found that a wide variety of objects were ingested by toads, which comprised both animal and plant material, as well as non-organic matter (Table S5.1). Of the 60 toad stomachs examined, 54 contained identifiable animals. Forty-seven toads had plant matter present in their stomach content: 14 toads ate grass and three toads ate plant fruits. Eighteen toads had pebbles and 11 toads had sand present in their stomach. One toad had an empty stomach, and three other toads could not contribute to the accountable animal taxa classification: (1) one stomach was almost empty except two insect parts that could not be identified, (2) one stomach only contained sand and bits of unidentifiable organic matter, (3) one stomach only had unidentifiable insect matter and plant matter that could not be counted.

The majority of a cane toad's diet was comprised of animals, predominantly termites and ants. By removing plants, non-organic matter, and unidentifiable insect matter, I classified all accountable animal taxa to the family level. Insecta was the most dominant diet class in toads from all sampling sites. I observed invertebrates from four different classes in invasion-front toads: Diplopoda (millipedes) were found in three toads, Chilopoda (centipedes) were found

in five toads, Arachnida (spiders and mites) were found in ten toads, while Insecta were found in all twenty-nine toads (Figure 5.1A). I also observed invertebrates from four different classes in range-core toads: Gastropoda (snails) and Malacostraca (wood lice) were found in one toad respectively, Arachnida were found in five toads, and Insecta were found in twenty-six toads (Figure 5.1B). In total, I observed invertebrates from six different classes (Arachnida, Chilopoda, Diplopoda, Gastropoda, Insecta, and Malacostraca) in toads' diets (Figure 5.1C). I observed invertebrates from 18 different orders with 17 identified: ten orders were found dominant (relative abundance > 0.02) in individual toads from the invasion-front (Figure S5.2A) and 14 orders were found dominant in individual toads from the range-core (Figure S5.2B). The dominant orders within each site were Hymenoptera (ant, honey bee, and wasp), Blattodea (termite and cockroach), Coleoptera (beetle), Hemiptera (cicada nymph and various bugs), and Araneae (spider) (Figure S5.2C, Table S5.1).



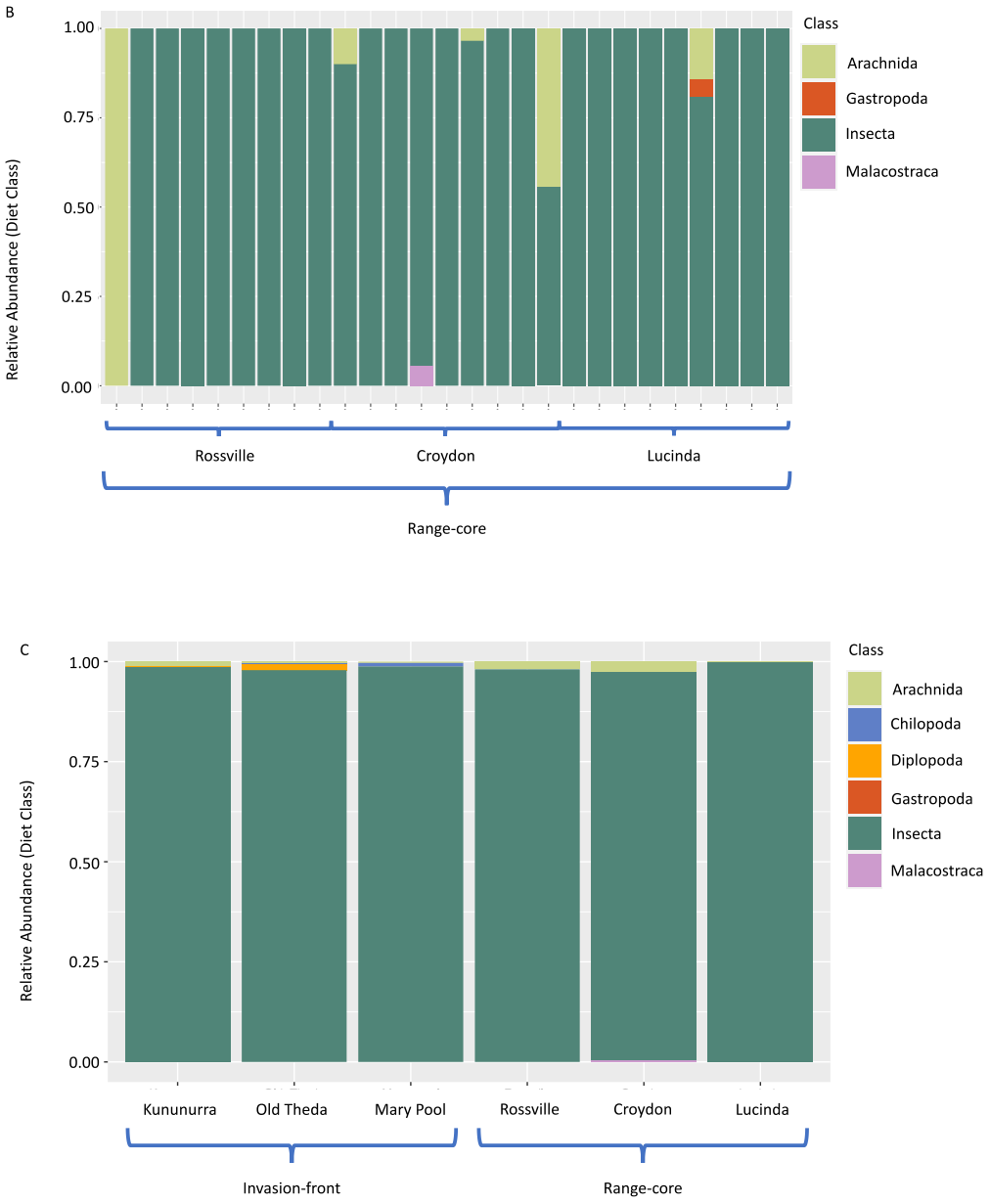
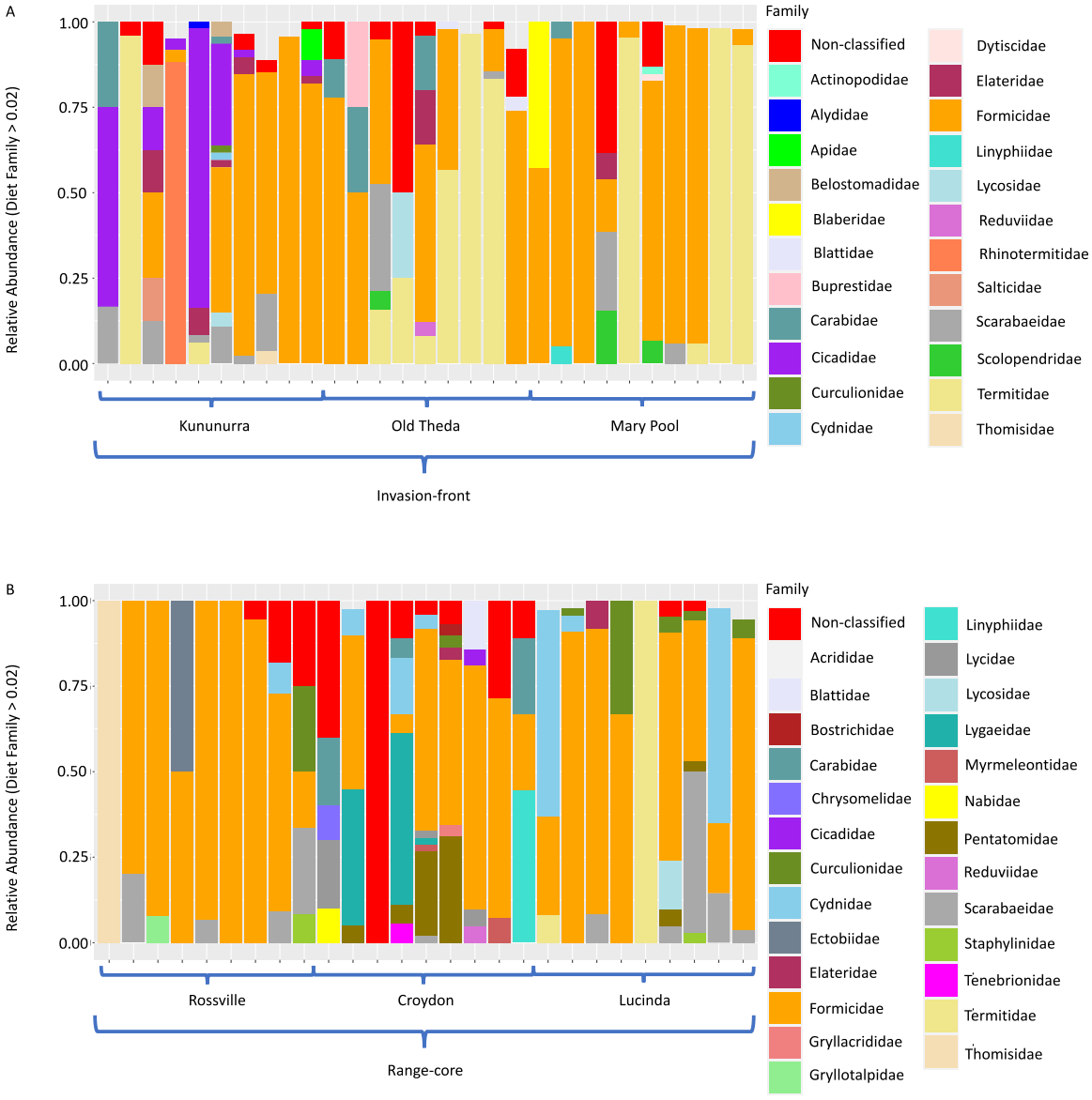


Figure 5. 1 Relative abundance of prey taxa composition in 56 cane toads at class level.

Finally, I observed 53 families with 37 identified: 24 families were found dominant in individual toads from the invasion-front (Figure 5.2A) and 27 families were found dominant in individual toads from the range-core (Figure 5.2B). The dominant families within each site were Termitidae (termite), Scarabaeidae (beetle), Rhinotermitidae (termite), Pentatomidae (stink bug), Lygaeidae (seed bug), Lycidae (beetle), Formicidae (ant), Elateridae (click beetle), Cydnidae (burrowing bug), Curculionidae (weevil), Cicadidae (cicada nymph), and Carabidae (ground beetle), (Figure 5.2C, Table S1). There were seven dominant families

across Australia: Formicidae (mean = 0.48, SD = 0.35), Termitidae (mean = 0.14, SD = 0.32), Scarabaeidae (mean = 0.05, SD = 0.09), Cicadidae (mean = 0.04, SD = 0.14), Cydnidae (mean = 0.03, SD = 0.12), Carabidae (mean = 0.02, SD = 0.06), Unclassified order Lepidoptera (mean = 0.02, SD = 0.08, Table 5.1).



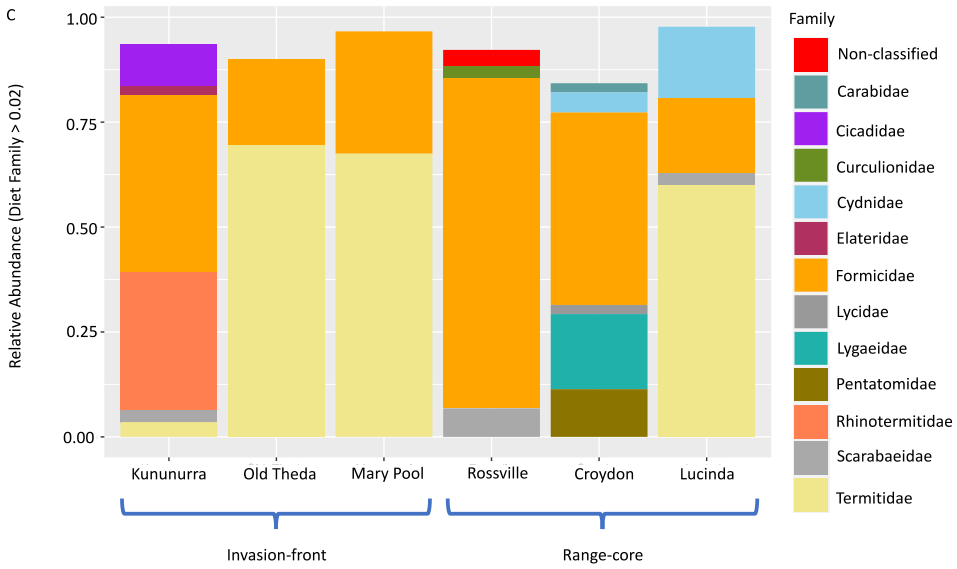


Figure 5. 2 Relative abundance of prey taxa composition in 56 cane toads at family level.

Table 5. 1 The mean and SD of dominant diet families relative abundance.

Relative abundance > 0.02.

	invasion-front		**range-core**		**Australia**	
	mean	SD	mean	SD	mean	SD
Formicidae	0.43	0.37	0.46	0.36	0.48	0.35
Termitidae	0.23	0.38	0.04	0.18	0.14	0.32
Scarabaeidae	0.04	0.08	0.05	0.10	0.05	0.09
Cicadidae	0.06	0.18	0.00	0.01	0.04	0.14
Cydnidae	0.00	0.00	0.06	0.16	0.03	0.12
Carabidae	0.03	0.07	0.02	0.05	0.02	0.06
Unclassified Order Lepidoptera	0.03	0.10	0.01	0.04	0.02	0.08

Diet differentiation between habitats

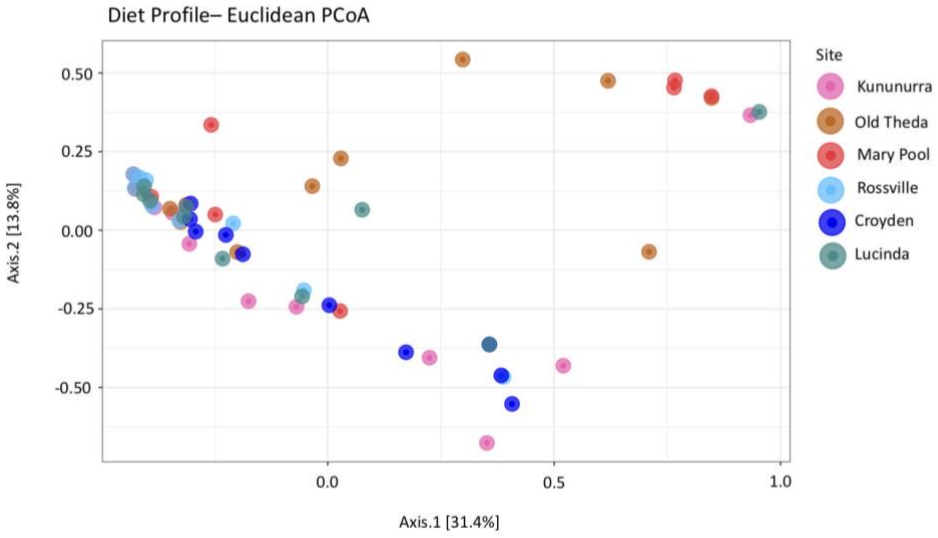
Invasion-front toads had more plant matter in their stomachs compared with range-core toads (invasion-front: mean = 0.90, SD = 0.31; range-core: mean = 0.67, SD = 0.48; W = 345, p-value = 0.03, Table 5.2). For the countable animal taxa, there was no difference in diet composition alpha diversity between range-core and invasion-front toads: observed taxa (mean = 4.32, SD = 2.58; W = 353.5, p-value = 0.15), Chao1 (mean = 6.10, SD = 5.13; W = 392.5, p-value = 0.40) and Shannon (mean = 0.73, SD = 0.54; W = 446, p-value = 0.96, Table 5.2).

Table 5. 2 Alpha diversity of prey taxa at family level and plant matter occurrence in diet content.

		invasion-front		**range-core**		**Australia**	
		mean	SD	mean	SD	mean	SD
Alpha diversity matrixes	observed taxa	4.8	2.5	3.83	2.61	4.32	2.58
	Chao1	6.28	4.3	5.91	5.91	6.10	5.13
	Shannon	0.73	0.52	0.73	0.58	0.73	0.54
Plant matter		0.90	0.31	0.67	0.48	0.78	0.42

I analysed at beta diversity of cane toad diets based on Euclidean distance. The perMANOVA test showed there were significant differences in diet composition between invasion-front and range-core toads (R2 = 0.045, p-value = 0.011, Figure 5.3). Diets of range-core toads were more closely clustered than those from invasion-front toads based on the PCoA plot (Figure 5.3). I then compared seven dominant diet families individually between invasion-front and range-core toads. I found three diet families were significantly different, namely Termitidae (invasion-front: mean = 0.23, SD = 0.38; range-core: mean = 0.04, SD = 0.18; Wilcoxon rank sum test: W = 303, p-value < 0.01), Cicadidae (invasion-front: mean = 0.06, SD = 0.18; range-core: mean = 0.00, SD = 0.01; Wilcoxon rank sum test: W = 330.5, p-value < 0.01), Cydnidae (invasion-front: mean = 0.00, SD = 0.00; range-core: mean = 0.06, SD = 0.16;

Wilcoxon rank sum test: W = 543.5, p-value = 0.02); and four diet families were not different, namely Formicidae (Wilcoxon rank sum test: W = 466.5, p-value = 0.81), Scarabaeidae (Wilcoxon rank sum test: W = 431, p-value = 0.76), Carabidae (Wilcoxon rank sum test: W = 378, p-value = 0.14), Unclassified order Lepidoptera (Wilcoxon rank sum test: W = 380, p-value = 0.13).



Association between diet content variation and locations: (diet content: Euclidean matrix)

Permutation test for adonis under reduced model					
Terms added sequentially (first to last)					
Permutation: free					
Number of permutations: 9999					
adonis2(formula = microbiota ~ Location, data = df, permutations = 9999)					
	Df	**SumOfSqs**	**R2**	**F**	**Pr(>F)**
Location	1	1.499	0.04486	2.7238	0.0106 *
Residual	58	31.910	0.95514		
Total	59	33.409	1.00000		
Signif. codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1**					

Pairwise comparisons using permutation MANOVAs on a Euclidean distance matrix of diet content

	Kununurra	**Old Theda**	**Mary Pool**	**Rossville**	**Croydon**
Old Theda	0.47	-	-	-	-
Mary Pool	0.47	0.66	-	-	-
Rossville	0.47	0.27	0.47	-	-
Croydon	0.29	0.33	0.33	0.47	-
Lucinda	0.47	0.47	0.66	0.66	0.47

P value adjustment method: Hochberg (1988)

Figure 5. 3 Diet prey item beta diversity by locations and sites.

Principle coordinate analysis plot of Euclidean distance of diet prey items, perMANOVA comparison between locations (invasion-front and range-core) and pairwise comparisons between sites (invasion-front: Kununurra, Old Theda, and Mary Pool; range-core: Rossville, Croydon, and Lucinda).

perMANOVA tests of diet composition based on beta diversity showed no significant difference between sampling sites (p-values > 0.05, Figure 5.3). I then compared the three dominant diet families where previous observed significantly different between invasion-front and range-core to see whether they were also different between sampling sites. I found only two diet families were different from each other, namely Termitidae (Kruskal-Wallis: p-value = 0.014, Figure S5.3A) and Cicadidae (Kruskal-Wallis: p-value = 1.1e-08, Figure S5.3B). Pairwise comparisons between every site showed that Termitidae relative abundance in toads from Old Theda were significantly higher than those from Rossville (Wilcoxon rank sum test, p-value = 0.04) and Croydon (Wilcoxon rank sum test, p-value = 0.04), but there were no differences between other pairs of sampling sites (Wilcoxon rank sum test, p-values > 0.05, Figure S5.3A). For Cicadidae, Kununurra toads had significantly higher abundance than all other sampling sites (Wilcoxon rank sum test, p-values < 0.01). There were no differences between other pairs of sampling sites (Wilcoxon rank sum test, p-values > 0.05, Figure S5.3B).

Despite there being differences in Cydnidae relative abundance between invasion-front and range-core toads, there were no differences between sampling sites (Kruskal-Wallis: p-value = 0.14, Figure S5.3C). Pairwise comparisons between sampling sites revealed no difference (Wilcoxon rank sum test, p-values > 0.05, Figure S5.3C).

The impact of diet on gut microbial community

Neither alpha nor beta diversity showed a strong correlation between diet composition (countable animal preys) and gut bacteria. LMM results indicated that diet alpha diversity and gut bacterial alpha diversity were not correlated (t-value = -0.93, p-value = 0.36, accounting for sampling site as random effect). The Spearman correlation in the partial Mantel test indicated that toads with similar diets do not share similar gut bacteria (Mantel r = -0.0085, p-value = 0.541).

Envfit results showed that cane toads' stomach content composition was not significantly associated with gut microbial community variation. The three alpha diversity indices of diet content were not correlated with gut microbial composition: observed diet taxa (r2 = 0.05, p-value = 0.25), Chao1 (r2 < 0.01, p-value = 0.87), and Shannon (r2 = 0.04, p-value = 0.40). Pebble and sand presence were also not correlated with gut microbial composition: pebble (r2 < 0.01, p-value = 0.97) and sand (r2 = 0.04, p-value = 0.35). However, plant matter occurrence was significantly correlated with gut microbial composition (r2 = 0.18, p-value = 0.01). Moreover, majority of the 53 observed diet families' relative abundance were not significantly associated with gut microbial community variation, except four diet taxa: Lycosidae (r2 = 0.14, p-value = 0.02), Blaberidae (r2 = 0.15, p-value < 0.01), Blattidae (r2 = 0.11, p-value < 0.05) and unclassified order Coleoptera (r2 = 0.10, p-value < 0.05) (details see Table S5.2).

Discussion

There is much discussion about the impact of diet on gut microbiota, however the majority of the evidence for the impact of diet on gut microbiota is mainly from studies of model species (e.g. human, (L. A. David et al., 2014; Zmora et al., 2019); and mice, (Magnusson et al., 2015) and from diet manipulations in laboratory environments (Davidson, Wiley, et al., 2020; Fülling et al., 2020; W. Li, Dowd, Scurlock, Acosta-Martinez, & Lyte, 2009). These controlled experimental systems do not allow us to actually study the relationship between diet and gut microbiota for

wild animals whose diet relies on prey availability. Here I investigated the impact of diet on gut microbiota in an invasive anuran across a wide range of natural habitats, and characterized Australian cane toad diet across their invasion ranges for the first time. I found cane toad stomach contents consist of a wide range of animals, plants, and non-organic matter, however the most common dietary components were insects. Invasion-front toads are more likely to have plant matter present in their stomach than those from the range-core and the presence of plant matter was associated with gut bacterial variation. Although I found difference in prey items between invasion-front and range-core toads, gut bacteria was not significantly associated with the main dietary component (insects). This indicates cane toad diet is opportunistic and highlights the potential weaker connection between diet and gut bacteria in this, and potentially other, opportunistic feeders.

Cane toad diet composition

Cane toads feed opportunistically on a variety of invertebrate prey species, including Arachnida (spiders and mites), Chilopoda (centipedes), Diplopoda (millipedes), Gastropoda (snails), Malacostraca (wood lice) and, primarily, a wide range of Insecta (Figure 5.1C, Table S5.1). For Insecta, we observed a high abundance of ants (family: Formicidae), followed by termites (family: Termitidae), beetles (family: Scarabaeidae), cicada nymphs (family: Cicadidae), various cockroaches (order: Blattodea) and various bugs (order: Hemiptera). Another study of cane toad diet from the range-core in Australia also found that ants are the primary prey, followed by beetles (Heise-Pavlov & Longway, 2011). Their results suggested that termites were not a major diet component for cane toads in the range-core. Interestingly, in this chapter, I also found range-core toads eat termites less frequently than toads on the invasion-front (Figure S5.2, Figure 5.4A), which may reflect differences in termite abundance. Termite biology may come into play, as some species observed in range-core toads' stomach tend to be highly subterranean, so may be less available for consumption. A diet study of cane toads in Philippines found a similar insect diet, including order Coleoptera,

Blattodea, Hemiptera, Hymenoptera but in different relative abundances, and ants were not the primary prey item (Apayor-Ynot et al., 2017). This agrees with the hypothesis that the prey of this invasive anuran largely reflects a snapshot of the invertebrate composition in that location at a specific point in time (Heise-Pavlov & Longway, 2011).

Toads from Kununurra had more Cicadidae (cicada nymph) in their stomachs than toads from any other sampling site. Since the emergence of cicadas happens in mass over a relatively short period of time (Williams & Simon, 1995), I imagined that the Kununurra toads consumed cicada nymphs opportunistically. I found more Cydnidae (burrowing bug) in range-core toads compared with invasion-front toads. Cydnidae are leaf litter specialists and are primarily recorded occurring in the forests of eastern Australia (the east coast, and especially in the Wet Tropics, SE Queensland, and the Gippsland in southern Victoria https://bie.ala.org.au/species/urn:lsid:biodiversity.org.au:afd.taxon:630d4409-7ca4-4ed1-af9f-d0886f1bc3bc). Interestingly, I also found some true bugs in toads' stomach and many true bugs utilize chemical defences to ward off predation by being distasteful (Krall, Bartelt, Lewis, & Whitman, 1999). Moreover, even though cane toads may not intentionally search for plants and non-organic matter to eat, the presence of those items in their stomach were consistent with the observed diet content of cane toads in Philippines (Apayor-Ynot et al., 2017). Interestingly, invasion-front toads were more likely to have plant matter present in their stomach compared with range-core toads. Cane toads are carnivorous and they eat plant matter unintentionally while preying on insects, so invasion-front toads may eat more plant matter coincidentally. Compared to the range-core (Queensland), invasion-front (Western Australia) sites have fewer water resources, so the toads are more likely to gather near water resources where vegetation normally grow. Together, this evidence supports the idea that cane toads are highly opportunistic feeders who eat according to food availability (Shine, 2010), which could be a crucial to their invasion success.

Correlation between gut bacteria and diet composition

Plant matter occurrence in toads' stomachs was significantly correlated with gut bacterial composition. Although plant materials may not be a direct food item of cane toads given that they are carnivorous (Apayor-Ynot et al., 2017), the consumed plant matter may still contribute significantly to their gut bacteria. Plants are known to affect gut microbial composition. For example, the presence of complex plant polysaccharides in ground squirrels were found to be correlated with abundance of many Firmicutes, which are the taxa that specialize on these substrates (Carey, Walters, & Knight, 2013). Diet manipulation in mice showed that switching from a low-fat, plant polysaccharide-rich diet to a high-fat, high-sugar diet shifted the structure of the microbiota dramatically (Turnbaugh, Ridaura, et al., 2009). This shifting of gut microbiota structure may also include the changes of the core microbiome that encodes metabolic traits related to processing of otherwise indigestible plant polysaccharides (Turnbaugh, Hamady, et al., 2009).

With respect to animal prey, the majority of the 53 observed diet families' relative abundance was not associated with gut microbial community variation, except for four diet taxa: Lycosidae (wolf spider), Blaberidae (Surinam cockroach), Blattidae (barred cockroach or woodland cockroach), and unclassified order Coleoptera (beetle). However, none of these taxa were dominant within sampling sites or across the range, and they did not differ between invasion-front and range core toads. These results suggest that gut bacteria variation is not correlated with majority of diet composition in cane toads in Australia. Similar results were found in a study on wild mice (Baxter et al., 2015), where the authors could not find the association between gut microbiota (16S rRNA gene sequences) and diet composition (18S rRNA gene sequences). Together, this study and our own suggest that in wild carnivorous or omnivorous animals, particularly invasive species with an opportunistic diet, there might be a weak correlation between diet taxonomy and gut bacteria. Interesting, there was a significant relationship between the invertebrate diet taxonomy and gut microbiome composition in individual wild white-faced capuchins (Mallott, Amato, Garber, & Malhi, 2018). These results indicate that the levels of

association between diet and gut microbiota vary in different species under natural environments. This might be explained by diet stability; species with highly consistent diets are more likely to have links between their diet and their gut microbiome. This is consistent with what we see in animals in captivity (Bolte et al., 2021; Fragiadakis et al., 2020) or animals with more specialist diet (Dill-McFarland, Weimer, Pauli, Peery, & Suen, 2016). In human or model animals, diet component variation can cause individual gut microbiota change temporarily (Leeming, Johnson, Spector, & Le Roy, 2019) and gut microbiota variation appears to compensate to the differences caused by diet (Amato et al., 2015). For invasive cane toads, a diminished connection between diet and gut bacteria could be an advantage when encountering novel environment during range expansion.

Limitation and suggestions for cane toad diet analysis

Microscopic observation of stomach content is a low-cost and straightforward approach. It has been widely used to study frog or toad diets. However, despite many of the toads' prey items having hard exoskeletons, a great deal of the contents had been partially or largely digested. This greatly complicated identification, and may have affected count data, because separate parts of the same prey item could potentially have been scored as multiple individuals. Therefore, we posit that molecular methods are needed for cane toad diet analysis, so that we can more accurately predict the taxa and relative abundance of plant matter and digested insect parts. Currently, molecular techniques have been used to study detailed analyses of prey consumed by a wide range of wild carnivorous or omnivorous animals, including fish (Jarman & Wilson, 2004), bird (Deagle et al., 2007), reptiles (D. S. Brown, Ebenezer, & Symondson, 2014; D. S. Brown, Jarman, & Symondson, 2012), and mammals (Clare, Barber, Sweeney, Hebert, & Fenton, 2011; Marshall et al., 2010). A study on a large omnivorous mammal, the brown bear, achieved a resolution of 60% taxa can be classified into genus and species level from Illumina Hiseq data (De Barba et al., 2014). Methods based on metabarcoding, multiplexing, and next-generation sequencing provide a

promising way to reveal the full spectrum of food items that comprise an omnivorous diet (De Barba et al., 2014). Moreover, in most diet studies based on the examination of DNA metabarcoding sequences, public or customized reference databases are available for the sequences to be accurately identified (Valentini, Pompanon, & Taberlet, 2009). However, there is no study used molecular methods for amphibian diet analysis and only one study used for reptile diet (D. S. Brown et al., 2014).

Neither microscopic observation of stomach content nor prey DNA examination will provide the information of diet nutritional levels. Nutritional levels have been largely linked to gut microbiota variation in a wide range of species (Carmody et al., 2015; Davidson, Wiley, et al., 2020; Pérez-Cobas et al., 2015; J. H. Wang et al., 2019). The macronutrient analysis might illuminate connections between diet and microbiome in Australian cane toad. Therefore, future research is needed to look at nutritional level of their diet to examine the functional outcomes of diet (Rana, Tiwari, Krishan, & Sharma, 2018; J. P. V. Santos et al., 2018).

Conclusion

In summary, Australian cane toads primarily consume termites and ants, but they also consume other animals, plants and non-organic matter. Their diet composition and diversity differ between invasion-front and range-core individuals, which may be largely caused by the availability of different food items. The plasticity of cane toad diet may assist this invader to adapt to various environments. Surprisingly, I found scant evidence for an association between cane toad diet composition and gut bacteria as compared to previous reports in other species: gut bacteria was not significantly associated with the main component of cane toads' diet (insects). I did find that the presence of plant matter in cane toads' diet was associated with gut bacterial variation. This highlights that diet may contribute less to gut bacteria of wild animals that have an opportunistic diet compared to those that are in controlled environments or have a specialist diet. Further studies based on metabarcoding or

macronutrient analysis are needed to better understand the contribution of diet to gut microbiota in this species.

CHAPTER 6: General discussion and conclusion

Species invasions can cause significant environmental and economic damage. The success of invasive populations can be enhanced by rapid changes in phenotypes, especially behavioural traits, during encountering new environments. Therefore, clarifying the factors and mechanisms that contribute to rapid adaptation may provide insightful information for invasive species management. Gut microbiota, as a potential factor to influence dispersal and invasion success through the enhancement of behavioural traits is under explored. I propose that cane toads, which show invasive behaviour differences across their invasion range in Australia, can be used as a model to further this field. My thesis is the first cohesive research project studying cane toad gut bacteria. Here, I pioneer the analysis of how gut bacteria may contribute to the dispersal-enhancing phenotypes observed in Australian invasion-front toads and determine which factors influence the composition of gut bacterial communities in cane toads.

The characterization of cane toads' intestinal bacteria profiles in captive and wild toads showed that dominant phyla are the similar. In wild and captive toads, only the proportion of Verrucomicrobia and Fusobacteria varied. This could be caused by captivity as a result of an altered habitat or by differences in the composition and variety of available dietary options. This section of my thesis was the first to test the suitability of non-lethal sampling methods to study amphibian gut bacteria. My results validated the usage of cloacal samples as a non-lethal method to study changes in the large intestine bacteria. These findings lay the groundwork for future gut microbiota manipulation experiments and contribute to our understanding of amphibian gut microbiota.

There is a growing literature on the effects of gut microbiome on host behaviour (Davidson, Raulo, et al., 2020; Johnson & Foster, 2018; Yuval, 2017). Cane toads exhibit behavioural

differences across their Australia range (Gruber, Brown, et al., 2017a), and these behaviours are believed to promote range expansion. However, prior this thesis, the relationship between gut microbiome and behaviour was unexplored. As a first step in this process, I examined whether differences in gut bacteria exist between wild invasion-front and range-core toads. I found differences in bacterial composition and predicted microbial functional groups across the toad's invasive range. Further, my results showed significant differences in morphology and behaviour between range-core and invasion-front toads, which are in agreement with previous investigations of these traits (G. P. Brown et al., 2007; Gruber, Brown, et al., 2017a). I found a strong relationship between the occurrence of the parasitic lungworm and gut bacterial composition and predicted function. Interestingly, although behavioural traits were not associated with differences in microbial composition, they were associated with microbial functional variation. Collectively, these results suggest that toad's gut bacteria is strongly associated with the animal's ecology and behaviour, which support the application of the "holobiont concept" (investigating the assemblage associated with a host) to fully understand the role of gut microbiota in driving geographic variation in behaviours that are important to invasion. I propose that further studies that include experimental manipulations (such as faecal transplants, and translocation of hosts among environments) coupled with longer term behavioural measurements will shed light on the causal relationships between gut microbiota and cane toad behaviour.

In order to understand the relationship between gut bacteria and intrinsic host factors, I investigated how gut bacterial communities interact with host genotype and epigenotype. While I did not find a relationship between host genotype and gut bacterial variation, I did find a positive relationship between host DNA methylation and gut bacteria. This relationship was greater in pairs of individuals that were more genetically similar. This indicates that the relationship between host DNA methylation and gut bacteria might be most important in invasion-front populations, which typically have low genetic diversity. I did not find a

negative association between host genetic and epigenetic diversity in cane toad, in contrast to the hypothesis that in invasive populations, epigenetic diversity may be compensatory when genetic diversity is low. In total, these findings advance our knowledge of the dynamics between host genotype, methylome, and gut bacteria in this iconic invasive amphibian.

There are other intrinsic factors that may influence gut bacteria, including sex, body size and age. In Chapter 2, I found that body size and sex explained the greatest variation in gut bacteria, highlighting the need to account for these factors in microbiome studies. However, this may be difficult in studies of some species that cannot be morphologically sexed until they reach maturity. For example, morphological determination of cane toad sex is not possible in early life stages. Since sex plays an important role in driving cane toad gut bacteria changes in adults, it may also affect toads at other life stages and, thus, it would be ideal if a molecular tool was developed to provide this information. In all of my research on toads, I only used adults, so the impact of age on gut bacteria variation in this species remains unknown. In other species of amphibians, the influence of age on the gut microbiome has primarily been studied by comparing differences across life stages (Fontaine et al., 2021; Tong, Cui, Hu, et al., 2020; M. Zhang et al., 2018). However, in order to assess the impacts of age on adult amphibians, a precise estimation of age would be ideal.

Currently, amphibian age is most often assessed using body length or weight, both which are strongly affected by environmental factors (Rozenblut & Ogielska, 2005). Methods such as skeletochronology analysis of bones (Rozenblut & Ogielska, 2005) and age-associated epigenetic marker analyses (Polanowski, Robbins, Chandler, & Jarman, 2014; Spiers et al., 2016) may be useful to precisely age adult amphibians. Skeletochronology has been widely used to estimate age in wild amphibian populations (Rozenblut & Ogielska, 2005; Sahoo & Kara, 2017; Sinsch, 2015; Sinsch & Dehling, 2017). However, skeletochronology, which requires access to long bones, is destructive. Age-related DNA methylation analysis, which can use blood samples, may be a better option if non-lethal sampling is required (Horvath et

al., 2012; Z. Xu & Taylor, 2014), but such methods are expensive and time-consuming. Currently, there is no reliable method to age adult cane toads, making the study of population dynamics challenging. Accurate cane toad age estimation methodologies would be beneficial for the study of this invasive population and enable the investigation of the impact of age on toad gut microbiota.

There are many extrinsic factors that can affect gut microbiome, including environmental variables and diet. My analysis of the contribution of environmental factors to bacterial composition indicated that local isothermality and mean temperature of driest quarter, were significantly associated with the variation of bacterial composition and predicted microbial functional groups. Interestingly, I found that gut bacterial composition was not significantly associated with the main component of cane toad's diet (insects), although the presence of plant matter was associated with gut bacterial variation. In this omnivorous animal, the weak association between insect taxonomy in toads' diet and their gut bacteria may have resulted from their opportunistic eating habits. Cane toads are highly opportunistic feeders who eat according to food availability (Shine, 2010), which could be a crucial to their invasion success. However, the taxonomy of stomach contents at a single time point may not adequately reflect toad diet. Further investigation into diet across longer time spans might yield a clearer picture of the relationship between diet and gut microbiota in this species. Altogether, these findings suggest that in wild animals, particularly invasive species with an opportunistic diet, there might be a weaker correlation between diet and gut bacteria than those observed in human and model animals, where much of current research has focused. Finally, assessing diet using approaches other than taxonomy of stomach contents may help to further unravel the contribution of diet to gut microbiota. Studies using metabarcoding (De Barba et al., 2014) may provide a more complete assessment of prey taxonomy. It may also be beneficial to consider nutritional analysis (i.e. protein, carbohydrate, fat content) to examine the functional outcomes of diet (Rana et al., 2018; J. P. V. Santos et al., 2018).

In summary, my thesis explores factors that shape gut bacteria in cane toads across their Australian invasive range and investigates mechanisms through which gut bacteria may promote the expansion of this invasion. Here I have clarified that in this species, sex, body size, lungworm presence, and host DNA methylation appear to shape the gut bacterial community. I also have illustrated shifts in cane toad gut bacteria across the Australian range and found behaviours (righting time and righting effort likelihood) are associated with predicted bacterial function. Toad gut bacteria are significantly associated with environmental temperature variability (isothermality), however, have a surprisingly low association with taxonomy of prey. Some of these factors appear to be interrelated, as explained below in mechanism (1). In conjunction with my findings of regional differences in gut bacteria, this may suggest that microbiome can promote invasion in this species through the following three mechanisms:

(1) gut bacteria may respond to the two positively correlated factors (lungworm prevalence and environmental temperature variability), further affecting host behaviours. It is interesting that even though environmental isothermality and lungworm occurrence are not significantly different between range-core and invasion-front toads, these two factors are significantly associated with bacterial taxonomy and predicted function. Environmental temperature can alter microbial taxonomic community and predicted microbial function in mammals and amphibians (J. Li et al., 2019), affecting host phenotypic plasticity and adaptation (Fontaine & Kohl, 2020; Fontaine et al., 2018). Lungworms are known to alter a cane toad's thermal preference and can manipulate the timing and location of defecation, thereby enhancing lungworm egg production and larvae survival (Finnerty et al., 2018). Lungworms also affected cane toad locomotor performance and reduced host endurance, presumably because of the reduced oxygen supply from infected lungs (L. Pizzatto & Shine, 2012). Furthermore, *C. elegans* are known to prefer specific bacterial foods (Abada et al., 2009), suggesting that lungworm larvae may also feed selectively on bacteria in the gut, generating differences in

bacterial communities between lungworm-infected toads versus non-infected conspecifics. Therefore, there might an interactive influence of environmental temperature and lungworm on gut bacteria and cane toad behaviour.

(2) gut bacteria can affect or respond to host DNA methylation changes, thus improving cane toad phenotypic plasticity and adaptivity in populations having more genetically similar individuals (e.g. invasion-front populations). Both DNA methylation and gut bacteria were significantly different between sampling localities, and that differentiation of host DNA methylation was positively related to differentiation of gut bacteria between pairs of individuals. Moreover, in populations with more genetically similar individuals, the relationship between DNA methylation and gut bacteria also may be stronger. The strengthened relationship between gut bacteria and DNA methylation in cane toads that are genetically similar could facilitate cane toad adaptation to novel environments in Australia. First, gut bacterial variation caused by environmental factors (e.g. temperature and diet) could alter host DNA methylation, leading to beneficial phenotypic changes that increase host fitness (Grieneisen et al., 2020; Krautkramer et al., 2016; Stilling et al., 2014). Second, environmental factors could alter host DNA methylation, which could affect the host's ability to use local microbes or to maintain a balanced gut bacteria by supressing nonbeneficial microbes (Ansari et al., 2020; J. Wu et al., 2020).

(3) gut bacteria are not directly correlated with prey taxonomy, thus improving cane toads' adaptation in various environments that may have novel food resources. Further, these results suggests that in wild carnivorous or omnivorous animals, particularly invasive species with an opportunistic diet, there might be a weak correlation between diet taxonomy and gut bacteria. This might be explained by diet stability; species with highly consistent diets are more likely to have links between their diet and their gut microbiome. This is consistent with what we see in animals in captivity (Bolte et al., 2021; Fragiadakis et al., 2020) or animals with more specialist diet (Dill-McFarland et al., 2016). For invasive cane toads, a diminished connection

between diet and gut bacteria could be an advantage when encountering novel environment during range expansion.

Invasion-front toads have accelerated their speed of range expansion (B. L. Phillips et al., 2006), and likely represent the most dispersive and potentially most invasive individuals in this population. However, the next crucial step requires manipulative experiments to determine whether the relationships identified here are causal or co-incidental. Understanding drivers of invasion success is key to our ability to effectively manage these populations and mitigate the damage they cause to introduced environments.

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APPENDICES

Appendix I Supplemental Tables

Table S2. 1 Alpha diversity metrics (observed ASVs, evenness and Shannon)

Sample ID	observed ASVs	Pielou evenness	Shannon	Sample ID	observed ASVs	Pielou evenness	Shannon
RmC001	272	0.661	5.343	**RmLI001**	120	0.662	4.571
RmC002	140	0.579	4.131	**RmLI002**	84	0.586	3.745
RmC003	255	0.663	5.303	**RmLI003**	136	0.595	4.214
RmC004	125	0.629	4.381	**RmLI004**	107	0.599	4.041
RmC005	142	0.581	4.154	**RmLI005**	176	0.614	4.581
RmC006	129	0.631	4.422	**RmLI006**	136	0.624	4.420
RmC007	241	0.696	5.507	**RmLI007**	235	0.719	5.663
RmC008	287	0.773	6.312	**RmLI008**	250	0.648	5.159
RmC009	201	0.557	4.259	**RmLI009**	100	0.527	3.499
RmC010	276	0.728	5.902	**RmLI010**	278	0.724	5.877
RmC011	331	0.544	4.556	**RmLI011**	369	0.588	5.015
RmC012	351	0.746	6.310	**RmLI012**	242	0.671	5.317
RmC013	264	0.644	5.177	**RmLI013**	130	0.644	4.522
RmC014	233	0.703	5.531	**RmLI014**	196	0.657	5.001
RmC015	399	0.773	6.682	**RmLI015**	335	0.786	6.590
RmC016	254	0.630	5.037	**RmLI016**	328	0.761	6.359
RmC017	435	0.695	6.092	**RmLI017**	388	0.746	6.419
RmC018	694	0.726	6.849	**RmLI018**	362	0.694	5.897
RmF001	264	0.688	5.533	**RmSI001**	132	0.580	4.087
RmF002	134	0.556	3.930	**RmSI002**	88	0.577	3.727
RmF003	192	0.657	4.980	**RmSI003**	124	0.559	3.886
RmF004	239	0.711	5.614	**RmSI004**	131	0.527	3.710
RmF005	244	0.668	5.301	**RmSI005**	89	0.577	3.739
RmF006	221	0.685	5.332	**RmSI006**	113	0.524	3.575
RmF007	264	0.726	5.844	**RmSI007**	157	0.487	3.552
RmF008	311	0.751	6.219	**RmSI008**	187	0.532	4.016
RmF009	232	0.670	5.266	**RmSI009**	134	0.441	3.116
RmF010	317	0.728	6.045	**RmSI010**	278	0.666	5.411
RmF011	295	0.598	4.906	**RmSI011**	281	0.677	5.503
RmF012	312	0.695	5.761	**RmSI012**	150	0.476	3.438
RmF013	217	0.628	4.873	**RmSI013**	345	0.442	3.724
RmF014	270	0.690	5.577	**RmSI014**	609	0.629	5.819

RmF015	449	0.744	6.556	**RmSI015**	470	0.645	5.723
RmF016	323	0.687	5.722	**RmSI016**	332	0.742	6.216
RmF017	481	0.728	6.490	**RmSI017**	315	0.595	4.941
RmF018	412	0.759	6.593	**RmSI018**	357	0.693	5.873
RmBa001	38	0.806	4.230	**Pos_control**	28	0.649	3.120
RmBa002	34	0.834	4.245	**Neg_control**	55	0.889	5.141

Table S2. 2 Differences in abundance of specific ASVs in the faeces versus large intestine.

ASVs	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Kingdom	Phylum	Class	Order	Family	Genus	Species
81c866fdb8d3c254e01dfb391096937e	1.887	-21.498	4.112	-5.228	1.7E-07	2.2E-06	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	mitochondria	NA	NA
3f659b1f9b53e71e31795f439d664ecd	8.060	23.526	3.210	7.330	2.3E-13	7.9E-12	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Macellibacteroides	fermentans
854c65fb139207cd8a98ff32a7ad7fa4	32.808	25.695	4.109	6.253	4.0E-10	7.9E-09	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	NA	NA
610af965b5d08af3b7d363b2096c6ea8	82.587	6.066	2.162	2.806	5.0E-03	3.9E-02	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	Chryseobacterium	NA
eaf3866e8291b6c954fc8b5f6270c781	79.320	6.009	2.083	2.885	3.9E-03	3.2E-02	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	Elizabethkingia	meningoseptica
9be05bd3848582a05c60e651191bdd2a	15.464	23.283	4.110	5.665	1.5E-08	2.2E-07	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	Elizabethkingia	meningoseptica
a16499d50611f338eae7f56722d918a3	23.386	25.331	4.109	6.165	7.1E-10	1.3E-08	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Myroides	NA
4e3ca547519c61ef5fce9caa876e91e6	11.734	23.810	3.470	6.861	6.8E-12	1.6E-10	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	NA
784715da4547a81d0a04377d9a3395f2	396.361	6.456	1.938	3.332	8.6E-04	8.1E-03	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	NA
ad5b88565858891941260e06393e33ae	70.889	25.849	3.010	8.588	8.9E-18	7.5E-16	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	NA

4a2ab6a21ca86e0dce99f6e5a716e0b8	61.486	5.076	1.656	3.064	2.2E-03	1.9E-02	Bacteria	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	Niabella	NA
b39673e25051b1e1805918111e893ce1	2.769	-21.454	4.112	-5.217	1.8E-07	2.3E-06	Bacteria	Bacteroidetes	NA	NA	NA	NA	NA
7b51f16122f70d75b7549145d3c0b6b0	154.424	9.877	1.675	5.895	3.8E-09	6.1E-08	Bacteria	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	NA	NA
e24d90b893e3d5447d3043dfa779c6dd	7.651	7.073	2.554	2.770	5.6E-03	4.2E-02	Bacteria	Bacteroidetes	[Saprospirae]	[Saprospirales]	NA	NA	NA
baf03aff00ef0962d4879e33921316ac	71.275	7.884	1.935	4.074	4.6E-05	5.2E-04	Bacteria	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	NA	NA
d7035b7ecb9a9f80177bb8ab39c7a689	10.255	24.100	3.873	6.222	4.9E-10	9.5E-09	Bacteria	Bacteroidetes	NA	NA	NA	NA	NA
740a47d290d00e0a95624955c3184721	13.935	8.556	2.427	3.526	4.2E-04	4.1E-03	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	Fluviicola	NA
10a94e36f634795b8f58b5511fb1e229	52.065	7.173	2.189	3.277	1.0E-03	9.6E-03	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	Fluviicola	NA
d1aeb438718074a56344c3cddc13fd24	940.034	7.394	1.168	6.329	2.5E-10	5.0E-09	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	NA	NA
9f4f5c3135ae0e4a2c735bb5ba47fde5	6.618	-23.074	4.110	-5.614	2.0E-08	2.9E-07	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	NA	NA
e30ad8b1ddf5ce4cd347d7bae50d7a49	32.533	9.305	2.204	4.222	2.4E-05	2.8E-04	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	faecium
3ecbd0ef00802867826610d291c00516	13.738	25.531	3.512	7.270	3.6E-13	1.1E-11	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	NA

76ecc203416862f614d54f64e22e28b0	20.373	29.977	3.483	8.608	7.5E-18	6.9E-16	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	NA
5c372f9692e53bd9ba1501c5c05e34a8	22.599	7.382	2.001	3.689	2.3E-04	2.3E-03	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	NA
4222943d364ac644b83025e99dd1652f	15.227	7.666	2.603	2.945	3.2E-03	2.7E-02	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	multivorum
cf8e285185051fe388d5ac012e06afaf	11.876	7.286	2.669	2.730	6.3E-03	4.7E-02	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	NA
b32671d9fa84d40d5e464bbb684d45ed	596.206	6.099	1.443	4.227	2.4E-05	2.7E-04	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	mizutaii
a231b1b0a07c0dbd7622a779ec7f6fdc	60.849	25.895	2.674	9.683	3.6E-22	1.1E-19	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	mizutaii
87779f938855a9c148ea299e6d1865d3	79.460	26.453	2.271	11.650	2.3E-31	2.1E-28	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	NA
3b5e11a04e554cd082958ee45c6c7541	76.559	26.527	4.109	6.456	1.1E-10	2.2E-09	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	NA
74503093aa965a82e296233791a46992a	294.049	8.702	1.770	4.916	8.8E-07	1.1E-05	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	NA
a643d51923fd7727d8db063addd02e62	370.237	8.046	1.884	4.270	2.0E-05	2.3E-04	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	mizutaii
c6674f5f44332e72e753a048741fabb1	12.107	25.251	3.772	6.694	2.2E-11	4.9E-10	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	NA	NA	NA
0ca8603fb15c8a91f5631960cc1cd4fb	22.869	26.712	4.109	6.501	8.0E-11	1.7E-09	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	NA	NA
4b9201a6f859b16b62273591900a5a03	21.643	9.278	3.002	3.090	2.0E-03	1.7E-02	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	NA

b93d7bee43264347cfddf75aca306960	10.181	24.122	3.354	7.192	6.4E-13	1.9E-11	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	NA
57bef08ec8fa1afa0723c6c128d59d0a	2444.459	6.315	1.175	5.373	7.8E-08	1.1E-06	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	cryaerophilus
d3d1613e8880cf6f877e41e341c68ba8	48.592	25.789	3.303	7.809	5.8E-15	2.4E-13	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	cryaerophilus
7753dc875cd2015e75759bd9fac451da	19.221	25.980	3.282	7.915	2.5E-15	1.1E-13	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	NA	NA	NA
13411d3e4548d3eb7350b626bc80af3f	18.224	24.910	4.038	6.169	6.9E-10	1.3E-08	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	NA
9a56472d58b4f738a076bf3acbc25218	27.347	25.271	3.004	8.411	4.1E-17	2.4E-15	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	NA	NA	NA
dd8f1357cfe8ed3c16e1e4e1aaa2ccf2	13.984	24.959	4.109	6.074	1.3E-09	2.2E-08	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	NA
db8912ee629be7155bf1275ed6f8ee87	8.062	22.271	4.111	5.417	6.1E-08	8.8E-07	Bacteria	TM7	TM7-3	NA	NA	NA	NA
1a30d4905482e4439aba81b553069468	1.887	-21.498	4.112	-5.228	1.7E-07	2.2E-06	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	NA	NA
f7be86a4767b5db9c61dbe357b911084	13.708	20.602	3.529	5.837	5.3E-09	8.3E-08	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	NA
e7c0f7f07af48d7018a5c92675f747e4	73.414	24.790	3.310	7.490	6.9E-14	2.5E-12	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	NA	NA	NA
627c882680046cf7d82174612a307fcc	1.127	21.857	4.118	5.308	1.1E-07	1.5E-06	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	Rhodococcus	fascians

e144f7969262be2a5734244cbf299bc6	116.352	5.642	1.902	2.967	3.0E-03	2.5E-02	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Aeromicrobium	NA
0fda6c6dfb592a2148b1a8b38dff001f	388.083	4.931	1.304	3.781	1.6E-04	1.7E-03	Bacteria	Proteobacteria	Betaproteobacteria	NA	NA	NA	NA
8e37210789665725cba38fe5d1918802	35.363	25.943	3.561	7.285	3.2E-13	1.0E-11	Bacteria	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Aquaspirillum	serpens
bc370bed8d29ede400799a287689541d	64.089	12.310	2.641	4.661	3.1E-06	3.8E-05	Bacteria	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Aquaspirillum	serpens
084ec0dd717edcb782d54908e200360b	44.954	6.215	2.233	2.783	5.4E-03	4.1E-02	Bacteria	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	NA	NA
c9f72e7ebb4ec3a0c097ee9ca8ae50ab	30.433	9.568	3.195	2.995	2.7E-03	2.3E-02	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	NA	NA
e441207041cdb74f4af2d698206a02fb	18.633	23.658	3.518	6.725	1.8E-11	4.1E-10	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Wohlfahrtiimonas	NA
6755df6b0f787a99458aa76221a3f763	38.815	26.590	3.114	8.537	1.4E-17	9.1E-16	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Aquabacterium	NA
323a287b2a8c16911ca6c6548f755490	19.753	9.481	2.538	3.735	1.9E-04	2.0E-03	Bacteria	Proteobacteria	Betaproteobacteria	NA	NA	NA	NA
a549d898240e6567ddc5b0df2e856678	1.127	21.857	4.118	5.308	1.1E-07	1.5E-06	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Propionivibrio	NA
73d72408715669afd448dc53fe5321a0	100.443	26.842	2.929	9.166	4.9E-20	7.6E-18	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	NA	NA
420d3fe56e2ac49ce40872a6679ec16f	37.566	6.968	2.167	3.215	1.3E-03	1.2E-02	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	NA	NA	NA
26b921305cae586606ba962e728a5223	1.127	21.857	4.118	5.308	1.1E-07	1.5E-06	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	NA	NA

2f06fe30e3730ec6220c58d463391f55	77.858	26.916	2.576	10.449	1.5E-25	6.9E-23	Bacteria	Proteobacteria	Deltaproteobacteria	Bdellovibrionales	Bacteriovoracaceae	Peredibacter	starrii
0930562a9275395e2773534c2e9b5585	35.851	-7.438	2.415	-3.080	2.1E-03	1.8E-02	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	NA
eb1ef5162c595c6c58dfb1ef998a46d0	8.144	-18.974	4.118	-4.607	4.1E-06	4.9E-05	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NA	NA
56fbb77cd4ff0e99c3f339d60f34bdbf	80.599	-25.643	2.997	-8.556	1.2E-17	8.3E-16	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	NA
de9583e172af6b53a3813403b3ea939d	17.329	24.610	3.284	7.493	6.8E-14	2.5E-12	Bacteria	Firmicutes	Clostridia	Clostridiales	NA	NA	NA
fe2a6d33153ce8e6d6f3de41864c9372	30.486	23.771	3.372	7.050	1.8E-12	4.8E-11	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus	NA
6367d5639a8fd5ffdd5f26aaa3407e63	35.340	11.551	3.486	3.314	9.2E-04	8.5E-03	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus	boronitolerans
b24016ff03e7da66f3407920d36554e6	6.272	23.854	4.110	5.804	6.5E-09	1.0E-07	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA
c6b930e0a5f22f9a4edb5aebd0ca4b9a	1.887	-21.498	4.112	-5.228	1.7E-07	2.2E-06	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA
d68f6edf4fd42cbbb429763ebfa7115c	37.677	24.942	3.276	7.613	2.7E-14	1.0E-12	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA
d4c15acd802bc5b13a4a4f4b75c11471	14.499	23.198	2.897	8.008	1.2E-15	5.7E-14	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA

ef4d84fdccee408ff39bd31bbb9d711f	7.457	24.365	4.110	5.928	3.1E-09	5.3E-08	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA
d9afa483d90f3c90b2ac7a443af2f9c0	17.271	25.411	4.109	6.184	6.3E-10	1.2E-08	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Giesbergeria	NA
c32cf5c54cb6e9d0ce614eaba7a7c5ba	26.083	25.029	3.517	7.117	1.1E-12	3.1E-11	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA
0c592092a1ddb00c46ef8303e0fc18d9	150.220	7.431	2.191	3.391	7.0E-04	6.6E-03	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas	NA
efff8366ae01fbabd82b0f370aa90487	29.809	26.415	3.823	6.910	4.8E-12	1.2E-10	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA
2571eabc30fb309a1ac650a72a221e70	868.648	5.613	1.501	3.739	1.9E-04	2.0E-03	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA
1a5445779a9243320b8f7caab087c0a6	727.385	6.393	1.713	3.732	1.9E-04	2.0E-03	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA
efbbf1faf333f02625e8b1185c2922dd	122.579	27.553	3.072	8.970	3.0E-19	3.9E-17	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA
b2900ec7030b97299cb25e5b15c7538c	89.372	26.729	2.881	9.278	1.7E-20	3.2E-18	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax	caeni
e121b9489c7d832c515a4a9178c3ff0b	2124.321	3.630	1.316	2.758	5.8E-03	4.4E-02	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas	NA
f3307f9aa4af0caa71b3db83c549c00a	42.347	8.276	2.383	3.473	5.1E-04	4.9E-03	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax	NA
a184c23c576ec0de4342c06f1987c9cf	856.891	-3.062	1.101	-2.780	5.4E-03	4.1E-02	Bacteria	Firmicutes	Clostridia	Clostridiales	NA	NA	NA

1b2b04dee8b598e369d2906ca754a8f8	6.086	-21.675	3.564	-6.082	1.2E-09	2.1E-08	Bacteria	Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]	Anaerovorax	NA
eb2a7bbf48baeeb9172f41a4bba3b817	9.306	-22.877	2.970	-7.703	1.3E-14	5.4E-13	Bacteria	Firmicutes	Clostridia	Clostridiales	NA	NA	NA
61981f62d08d858332280d45351bd211	5.005	24.134	4.111	5.871	4.3E-09	6.9E-08	Bacteria	Firmicutes	Clostridia	Clostridiales	NA	NA	NA
c1d278a3dea30c9bc378cf85b107d3a7	2.595	-21.414	4.112	-5.208	1.9E-07	2.4E-06	Bacteria	Chloroflexi	Thermomicrobia	JG30-KF-CM45	NA	NA	NA
74f7726be03488357d59073773cd8f93	154.165	8.865	1.558	5.688	1.3E-08	2.0E-07	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	NA	NA
2933e2bea3bc0a580df9616f700ac5c5	47.220	25.791	2.989	8.630	6.2E-18	6.4E-16	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	NA	NA
80a23fd8a244066efa7063ec9b9f68d1	67.399	-23.420	3.313	-7.069	1.6E-12	4.3E-11	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	NA	NA
9befdf2cf9cf203d191c2971f1408d01	19.767	25.704	3.528	7.287	3.2E-13	1.0E-11	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	NA
b31bc2f24f3bb2a7869cc541316823f4	585.465	4.280	1.483	2.886	3.9E-03	3.2E-02	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	NA
f24b5ab27ed82cbe8e641f1719c0d44d	9.092	21.026	4.116	5.108	3.3E-07	4.1E-06	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	NA
aef972b38f3b85220b771167e101fe36	14.862	21.322	3.251	6.558	5.4E-11	1.2E-09	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rhodobacter	NA

be71f065772bdecc8b688a016385bae7	41.692	9.765	2.570	3.800	1.4E-04	1.6E-03	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	NA	NA
69d2ab76f962a4d382e6c2d649211f70	787.698	4.836	1.310	3.691	2.2E-04	2.3E-03	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella	NA
da250fd6da0b540153bff16e816857da	17.422	24.237	4.109	5.898	3.7E-09	6.1E-08	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	NA	NA	NA
99ae79babc8bff89ec120d8105deef4c	1106.872	5.805	1.577	3.682	2.3E-04	2.3E-03	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
50ab78850a8ea7a16461d8a9bc5fa01e	158.150	6.859	2.448	2.802	5.1E-03	3.9E-02	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	johnsonii
19a3330b72bca172782965a8fb9f29cc	298.146	8.272	2.135	3.875	1.1E-04	1.2E-03	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
6037d904e7f5b0c345dec4c584a29a13	76.010	26.236	3.775	6.950	3.7E-12	9.2E-11	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
6b207aeb680ce920df015bea804ba576	163.812	6.433	2.224	2.892	3.8E-03	3.1E-02	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
54bdc8263c64c084ace5ddcb79648435	573.322	7.396	1.573	4.703	2.6E-06	3.1E-05	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
c0bf0288f4487de723ec913ead27ad4f	76.423	26.810	3.268	8.204	2.3E-16	1.2E-14	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
03bd8d255f5779c1382705802dcef3c8	69.442	9.115	2.488	3.664	2.5E-04	2.5E-03	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
fccdc0e0da4adf6a2ab114c0af2e7645	46.555	26.238	3.603	7.282	3.3E-13	1.0E-11	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
86b089c805a0509bba9ad29d8984c58a	51.843	7.489	2.025	3.698	2.2E-04	2.2E-03	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	NA	NA

154eba8ce650878a7e294d90c3c060ec	106.270	5.795	2.037	2.845	4.4E-03	3.5E-02	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	guillouiae
1305048188284f16ad5feafe65daf98e	20.874	24.249	4.109	5.901	3.6E-09	6.1E-08	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
5a63b759dd051d8f72341d639f9a7526	156.006	5.378	1.661	3.237	1.2E-03	1.1E-02	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
90326ded16ffad8daee3618dddb2e2a2	31.881	9.841	2.711	3.630	2.8E-04	2.8E-03	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
61253335a3d7ca9d7d3ef98cef5e41b2	108.970	5.995	1.932	3.104	1.9E-03	1.7E-02	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
ac178114bb17c82c85fb0e7c9d07c699	2266.471	5.190	1.330	3.903	9.5E-05	1.1E-03	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
70ce739bab38b88f373201703926216c	28.956	9.621	2.984	3.224	1.3E-03	1.1E-02	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	NA	NA
1d66d89f395f71592c81649e9ab9b8bd	51.503	26.134	2.771	9.432	4.0E-21	9.3E-19	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	NA	NA
852fe06fea622fb11cf98143376791e6	18.956	6.871	2.449	2.806	5.0E-03	3.9E-02	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	cuatrocienegasensis
2511787484d0c7b8e9791bac60cb531f	18.547	24.588	2.952	8.328	8.2E-17	4.5E-15	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	NA
754d5f63a21a2f313965a90cc6640ac9	63.999	26.303	3.067	8.577	9.7E-18	7.5E-16	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	NA
9490cfd72171fc2f50b8b7b00593454b	291.193	28.216	3.552	7.943	2.0E-15	9.2E-14	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	NA
af7f8cfcaae55e0e7ff05b442f0fe41e	89.072	7.081	2.472	2.864	4.2E-03	3.3E-02	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	NA

bc607eff3a3e950019c5be9cd039e72d	2074.628	5.176	1.591	3.253	1.1E-03	1.0E-02	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	NA
155d4e8473b04bea5ac96e80f7742328	19.764	24.857	3.561	6.979	3.0E-12	7.6E-11	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	NA
8021101d3b324ccf53a4abdefbaa054a	32.560	25.535	2.933	8.707	3.1E-18	3.6E-16	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	[Chromatiaceae]	Alishewanella	NA
d39c0d110aab036bc826e065e37066d6	16.965	25.112	3.794	6.620	3.6E-11	8.0E-10	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	[Chromatiaceae]	Alishewanella	NA
4c9fc51eade85fb7d7520e32690c1428	34.381	26.292	3.086	8.519	1.6E-17	1.0E-15	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	[Chromatiaceae]	Rheinheimera	NA

Table S2. 3 Differences in abundance of specific ASVs in the cloaca versus large intestine.

ASVs	**baseMean**	**log2FoldChange**	**lfcSE**	**stat**	**pvalue**	**padj**	**Kingdom**	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**
81c866fdb8d3c254e01dfb391096937e	1.887	-22.177	4.112	-5.393	6.9E-08	3.3E-06	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	mitochondria	NA	NA
3f659b1f9b53e71e31795f439d664ecd	8.060	19.447	3.223	6.034	1.6E-09	1.3E-07	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Macellibacteroides	fermentans
854c65fb139207cd8a98ff32a7ad7fa4	32.808	22.726	4.111	5.529	3.2E-08	2.0E-06	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	NA	NA
9be05bd3848582a05c60e651191bdd2a	15.464	21.761	4.112	5.293	1.2E-07	5.6E-06	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	Elizabethkingia	meningoseptica
a16499d50611f338eae7f56722d918a3	23.386	20.572	4.117	4.997	5.8E-07	2.5E-05	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Myroides	NA

4e3ca547519c61ef5fce9caa876e91e6	11.734	19.117	3.484	5.487	4.1E-08	2.3E-06	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	NA
ad5b88565858891941260e06393e33ae	70.889	22.934	3.011	7.616	2.6E-14	7.6E-12	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	NA
4a2ab6a21ca86e0dce99f6e5a716e0b8	61.486	6.277	1.655	3.792	1.5E-04	5.4E-03	Bacteria	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	Niabella	NA
b39673e25051b1e1805918111e893ce1	2.769	-22.175	4.112	-5.393	6.9E-08	3.3E-06	Bacteria	Bacteroidetes	NA	NA	NA	NA	NA
7b51f16122f70d75b7549145d3c0b6b0	154.424	8.031	1.676	4.792	1.7E-06	6.8E-05	Bacteria	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	NA	NA
d7035b7ecb9a9f80177bb8ab39c7a689	10.255	18.245	3.892	4.688	2.8E-06	1.1E-04	Bacteria	Bacteroidetes	NA	NA	NA	NA	NA
d1aeb438718074a56344c3cddc13fd24	940.034	5.449	1.168	4.664	3.1E-06	1.2E-04	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	NA	NA
76ecc203416862f614d54f64e22e28b0	20.373	25.652	3.491	7.349	2.0E-13	3.3E-11	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	NA
5c372f9692e53bd9ba1501c5c05e34a8	22.599	7.157	2.001	3.577	3.5E-04	1.2E-02	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	NA
b32671d9fa84d40d5e464bbb684d45ed	596.206	6.348	1.443	4.401	1.1E-05	4.2E-04	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	mizutaii
a231b1b0a07c0dbd7622a779ec7f6fdc	60.849	25.532	2.674	9.547	1.3E-21	1.0E-18	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	mizutaii
87779f938855a9c148ea299e6d1865d3	79.460	25.354	2.271	11.165	6.0E-29	1.4E-25	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	NA

3b5e11a04e554cd082958ee45c6c7541	76.559	23.899	4.109	5.816	6.0E-09	4.4E-07	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	NA
7450309aa965a82e296233791a46992a	294.049	6.687	1.770	3.778	1.6E-04	5.6E-03	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	NA
a643d51923fd7727d8db063addd02e62	370.237	7.296	1.885	3.872	1.1E-04	4.0E-03	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	mizutaii
b93d7bee43264347cfddf75aca306960	10.181	18.623	3.373	5.521	3.4E-08	2.0E-06	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	NA
9950d38e7195046ee4a669b68edec4ce	21.101	-20.343	3.385	-6.009	1.9E-09	1.5E-07	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	NA
13411d3e4548d3eb7350b626bc80af3f	18.224	18.199	4.057	4.486	7.2E-06	2.8E-04	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	NA
9a56472d58b4f738a076bf3acbc25218	27.347	18.756	3.024	6.203	5.5E-10	5.3E-08	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	NA	NA	NA
db8912ee629be7155bf1275ed6f8ee87	8.062	21.394	4.112	5.202	2.0E-07	8.6E-06	Bacteria	TM7	TM7-3	NA	NA	NA	NA
1a30d4905482e4439aba81b553069468	1.887	-22.177	4.112	-5.393	6.9E-08	3.3E-06	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	NA	NA
f7be86a4767b5db9c61dbe357b911084	13.708	23.943	3.520	6.802	1.0E-11	1.2E-09	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	NA
76c9abaa7eee34d87bb8152e600111af	2.062	22.330	4.112	5.430	5.6E-08	3.0E-06	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	NA	NA	NA
e7c0f7f07af48d7018a5c92675f747e4	73.414	25.809	3.309	7.799	6.3E-15	2.1E-12	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	NA	NA	NA

e144f7969262be2a5734244cbf299bc6	116.352	6.528	1.901	3.433	6.0E-04	2.0E-02	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Aeromicrobium	NA
8e37210789665725cba38fe5d1918802	35.363	21.689	3.565	6.083	1.2E-09	1.0E-07	Bacteria	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Aquaspirillum	serpens
e441207041cdb74f4af2d698206a02fb	18.633	18.375	3.531	5.204	2.0E-07	8.6E-06	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Wohlfahrtiimonas	NA
73d72408715669afd448dc53fe5321a0	100.443	22.111	2.931	7.543	4.6E-14	1.0E-11	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	NA	NA
420d3fe56e2ac49ce40872a6679ec16f	37.566	7.938	2.166	3.664	2.5E-04	8.7E-03	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	NA	NA	NA
2f06fe30e3730ec6220c58d463391f55	77.858	21.892	2.581	8.483	2.2E-17	1.3E-14	Bacteria	Proteobacteria	Deltaproteobacteria	Bdellovibrionales	Bacteriovoracaceae	Peredibacter	starrii
0930562a9275395e2773534c2e9b5585	35.851	-23.908	2.415	-9.899	4.2E-23	4.9E-20	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	NA
eb1ef5162c595c6c58dfb1ef998a46d0	8.144	-20.139	4.118	-4.890	1.0E-06	4.2E-05	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NA	NA
9cff10d3d52e024200041cdb101e7543	8.128	-22.706	4.111	-5.523	3.3E-08	2.0E-06	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	NA
de9583e172af6b53a3813403b3ea939d	17.329	19.759	3.296	5.994	2.0E-09	1.6E-07	Bacteria	Firmicutes	Clostridia	Clostridiales	NA	NA	NA
fe2a6d33153ce8e6d6f3de41864c9372	30.486	20.208	3.375	5.987	2.1E-09	1.6E-07	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus	NA

c6b930e0a5f22f9a4edb5aebd0ca4b9a	1.887	-22.177	4.112	-5.393	6.9E-08	3.3E-06	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA
d68f6edf4fd42cbbb429763ebfa7115c	37.677	24.845	3.276	7.583	3.4E-14	8.6E-12	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA
d4c15acd802bc5b13a4a4f4b75c11471	14.499	23.468	2.896	8.103	5.3E-16	2.5E-13	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA
c32cf5c54cb6e9d0ce614eaba7a7c5ba	26.083	18.938	3.532	5.362	8.2E-08	3.9E-06	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA
2571eabc30fb309a1ac650a72a221e70	868.648	5.882	1.501	3.918	8.9E-05	3.3E-03	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA
efbbf1faf333f02625e8b1185c2922dd	122.579	23.153	3.073	7.534	4.9E-14	1.0E-11	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA
b2900ec7030b97299cb25e5b15c7538c	89.372	20.335	2.891	7.034	2.0E-12	2.4E-10	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax	caeni
ab5b3fa62e7f8c099cafedb4c33baa19	11.611	-20.117	3.704	-5.431	5.6E-08	3.0E-06	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Epulopiscium	NA
2459b9194d0b569745cfd62ec393aaed	1.451	-21.703	4.113	-5.277	1.3E-07	5.9E-06	Bacteria	Firmicutes	Clostridia	Clostridiales	NA	NA	NA
eb2a7bbf48baeeb9172f41a4bba3b817	9.306	-23.971	2.970	-8.071	7.0E-16	2.7E-13	Bacteria	Firmicutes	Clostridia	Clostridiales	NA	NA	NA
74f7726be03488357d59073773cd8f93	154.165	6.364	1.559	4.082	4.5E-05	1.7E-03	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	NA	NA

2933e2bea3bc0a580df9616f700ac5c5	47.220	21.212	2.994	7.085	1.4E-12	1.8E-10	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	NA	NA
02c7163e4f851814ac57883e60cda22a	2.197	22.685	4.112	5.517	3.5E-08	2.0E-06	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	NA	NA
511a107e49072880015e928336a03b75	2.484	22.778	4.112	5.540	3.0E-08	1.9E-06	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	NA	NA
f24b5ab27ed82cbe8e641f1719c0d44d	9.092	23.636	4.110	5.751	8.8E-09	6.2E-07	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	NA
aef972b38f3b85220b771167e101fe36	14.862	23.612	3.246	7.275	3.5E-13	5.3E-11	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rhodobacter	NA
be5216f480170d46ce50802fa30103ec	909.283	2.788	0.797	3.496	4.7E-04	1.6E-02	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA	NA
da250fd6da0b540153bff16e816857da	17.422	22.303	4.111	5.426	5.8E-08	3.0E-06	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	NA	NA	NA
6037d904e7f5b0c345dec4c584a29a13	76.010	21.656	3.778	5.732	9.9E-09	6.7E-07	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
c0bf0288f4487de723ec913ead27ad4f	76.423	23.353	3.269	7.143	9.1E-13	1.2E-10	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
fccdc0e0da4adf6a2ab114c0af2e7645	46.555	22.462	3.605	6.230	4.7E-10	4.7E-08	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
1305048188284f16ad5feafe65daf98e	20.874	22.853	4.110	5.560	2.7E-08	1.8E-06	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
bfdc9ed3b15aee63ea5ea094986cc77d	39.156	-21.670	3.580	-6.053	1.4E-09	1.2E-07	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA

1d66d89f395f71592c81649e9ab9b8bd	51.503	20.135	2.783	7.235	4.7E-13	6.7E-11	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	NA	NA
2511787484d0c7b8e9791bac60cb531f	18.547	19.768	2.965	6.667	2.6E-11	2.9E-09	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	NA
754d5f63a21a2f313965a90cc6640ac9	63.999	23.016	3.068	7.502	6.3E-14	1.2E-11	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	NA
9490cfd72171fc2f50b8b7b00593454b	291.193	26.208	3.552	7.377	1.6E-13	2.9E-11	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	NA
155d4e8473b04bea5ac96e80f7742328	19.764	22.065	3.564	6.191	6.0E-10	5.5E-08	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	NA
8021101d3b324ccf53a4abdefbaa054a	32.560	19.314	2.949	6.549	5.8E-11	6.1E-09	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	[Chromatiaceae]	Alishewanella	NA

Table S2. 4 Differences in abundance of specific ASVs in the faeces versus small intestine.

ASVs	**baseMean**	**log2FoldChange**	**lfcSE**	**stat**	**pvalue**	**padj**	**Kingdom**	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**
3f659b1f9b53e71e31795f439d664ecd	8.060	9.416	3.210	2.934	3.4E-03	2.9E-02	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Macellibacteroides	fermentans
00a9489561825a4c5f530c0bb930b18f	9.689	23.363	3.282	7.119	1.1E-12	3.1E-11	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	NA	NA	NA
854c65fb139207cd8a98ff32a7ad7fa4	32.808	26.115	4.109	6.356	2.1E-10	4.0E-09	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	NA	NA
723c9cde286df58f2f72ed0215d307dc	15.239	9.766	3.146	3.104	1.9E-03	1.8E-02	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	Cloacibacterium	NA

16564cfc259d63314a3c737cd465117e	205.426	3.832	1.311	2.924	3.5E-03	2.9E-02	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	NA	NA
eaf3866e8291b6c954fc8b5f6270c781	79.320	7.504	2.089	3.591	3.3E-04	3.6E-03	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	Elizabethkingia	meningoseptica
a16499d50611f338eae7f56722d918a3	23.386	25.579	4.109	6.225	4.8E-10	8.8E-09	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Myroides	NA
9576e2b36f5be49471de161c203dc800	58.834	12.284	2.790	4.403	1.1E-05	1.4E-04	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	NA
d5db028332860fd56499e9a408f3f926	218.213	5.835	1.830	3.189	1.4E-03	1.4E-02	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	NA	NA
784715da4547a81d0a04377d9a3395f2	396.361	8.012	1.942	4.125	3.7E-05	4.5E-04	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	NA
b39673e25051b1e1805918111e893ce1	2.769	-20.475	4.114	-4.977	6.5E-07	9.4E-06	Bacteria	Bacteroidetes	NA	NA	NA	NA	NA
418ccc7cbd2386cb462f30746b6f48f3	7632.126	3.215	1.070	3.006	2.6E-03	2.3E-02	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
95f95221bbdb22f2d8acf66306afa400	15.690	8.815	2.840	3.104	1.9E-03	1.8E-02	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	columnare
d7035b7ecb9a9f80177bb8ab39c7a689	10.255	24.339	3.873	6.284	3.3E-10	6.3E-09	Bacteria	Bacteroidetes	NA	NA	NA	NA	NA
740a47d290d00e0a95624955c3184721	13.935	8.803	2.426	3.628	2.9E-04	3.2E-03	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	Fluviicola	NA
d1aeb438718074a56344c3cddc13fd24	940.034	5.099	1.164	4.379	1.2E-05	1.6E-04	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	NA	NA

9f4f5c3135ae0e4a2c735bb5ba47fde5	6.618	-18.590	4.137	-4.494	7.0E-06	9.5E-05	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	NA	NA
3ecbd0ef00802867826610d291c00516	13.738	25.678	3.512	7.312	2.6E-13	8.4E-12	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	NA
76ecc203416862f614d54f64e22e28b0	20.373	29.989	3.483	8.611	7.2E-18	4.8E-16	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	NA
a231b1b0a07c0dbd7622a779ec7f6fdc	60.849	25.785	2.674	9.642	5.3E-22	8.3E-20	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	mizutaii
87779f938855a9c148ea299e6d1865d3	79.460	26.655	2.271	11.739	8.1E-32	3.7E-29	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	NA
3b5e11a04e554cd082958ee45c6c7541	76.559	26.861	4.109	6.537	6.3E-11	1.3E-09	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	NA
74503099aa965a82e296233791a46992a	294.049	6.649	1.759	3.781	1.6E-04	1.8E-03	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	NA
c6674f5f44332e72e753a048741fabb1	12.107	25.189	3.772	6.677	2.4E-11	5.4E-10	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	NA	NA	NA
0ca8603fb15c8a91f5631960cc1cd4fb	22.869	26.885	4.109	6.543	6.0E-11	1.3E-09	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	NA	NA
4b9201a6f859b16b62273591900a5a03	21.643	9.526	3.002	3.173	1.5E-03	1.4E-02	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	NA
b93d7bee43264347cfddf75aca306960	10.181	24.382	3.354	7.270	3.6E-13	1.1E-11	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	NA
57bef08ec8fa1afa0723c6c128d59d0a	2444.459	5.440	1.175	4.630	3.6E-06	5.1E-05	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	cryaerophilus

d3d1613e8880cf6f877e41e341c68ba8	48.592	25.917	3.303	7.847	4.2E-15	1.9E-13	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	cryaerophilus
7753dc875cd2015e75759bd9fac451da	19.221	26.086	3.282	7.947	1.9E-15	8.8E-14	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	NA	NA	NA
13411d3e4548d3eb7350b626bc80af3f	18.224	25.145	4.038	6.227	4.8E-10	8.8E-09	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	NA
9a56472d58b4f738a076bf3acbc25218	27.347	25.543	3.004	8.502	1.9E-17	1.1E-15	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	NA	NA	NA
dd8f1357cfe8ed3c16e1e4e1aaa2ccf2	13.984	25.053	4.109	6.097	1.1E-09	1.9E-08	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	NA
f16e128d2050fb24c42d534278e9d2b7	7.437	7.785	2.692	2.892	3.8E-03	3.2E-02	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
627c882680046cf7d82174612a307fcc	1.127	21.894	4.118	5.317	1.1E-07	1.6E-06	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	Rhodococcus	fascians
bc07279d050a885ed0edb768f6fae6b7	10.858	7.895	2.644	2.986	2.8E-03	2.4E-02	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	NA
8e37210789665725cba38fe5d1918802	35.363	26.235	3.561	7.367	1.7E-13	6.2E-12	Bacteria	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Aquaspirillum	serpens
bc370bed8d29ede400799a287689541d	64.089	8.361	2.614	3.198	1.4E-03	1.4E-02	Bacteria	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Aquaspirillum	serpens
17e0b566d185ed85dbbb96626d7391d9	17.443	24.120	3.317	7.272	3.6E-13	1.1E-11	Bacteria	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Microvirgula	NA
32f844e217771d72fe9f7f47619bf0df	24.336	-7.872	2.252	-3.495	4.7E-04	5.1E-03	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Ralstonia	NA

38646b9ab2c8fbf2820bec3967a3a24b	27.255	25.136	3.507	7.168	7.6E-13	2.2E-11	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Pseudoxanthomonas	NA
6755df6b0f787a99458aa76221a3f763	38.815	26.891	3.114	8.634	5.9E-18	4.6E-16	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Aquabacterium	NA
323a287b2a8c16911ca6c6548f755490	19.753	7.762	2.524	3.075	2.1E-03	1.9E-02	Bacteria	Proteobacteria	Betaproteobacteria	NA	NA	NA	NA
a549d898240e6567ddc5b0df2e856678	1.127	21.894	4.118	5.317	1.1E-07	1.6E-06	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Propionivibrio	NA
b00c723cb5db6172e1ffba8b9c04d3ac	44.271	25.967	2.910	8.922	4.6E-19	4.2E-17	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Dechloromonas	fungiphilus
420d3fe56e2ac49ce40872a6679ec16f	37.566	24.409	2.187	11.159	6.5E-29	2.0E-26	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	NA	NA	NA
26b921305cae586606ba962e728a5223	1.127	21.894	4.118	5.317	1.1E-07	1.6E-06	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	NA	NA
2f06fe30e3730ec6220c58d463391f55	77.858	27.121	2.576	10.529	6.4E-26	1.2E-23	Bacteria	Proteobacteria	Deltaproteobacteria	Bdellovibrionales	Bacteriovoracaceae	Peredibacter	starrii
a4029ba43f26f4cb196c8fe877c99d4e	11.998	-6.572	2.242	-2.931	3.4E-03	2.9E-02	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NA	NA
04c557eddc311760dd28dce415077fad	109.634	5.960	1.923	3.100	1.9E-03	1.8E-02	Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium	NA
c5dc7413a652cff91990588926e0b643	40.565	-5.552	1.805	-3.075	2.1E-03	1.9E-02	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NA	NA

95cfb042cf74eb896e5422a3ee343dd6	5.136	-23.360	4.110	-5.684	1.3E-08	2.1E-07	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NA	NA
0930562a9275395e2773534c2e9b5585	35.851	-9.508	2.414	-3.939	8.2E-05	9.6E-04	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	NA
eb1ef5162c595c6c58dfb1ef998a46d0	8.144	-23.428	4.109	-5.701	1.2E-08	1.9E-07	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NA	NA
b898aa61ce6a979b9c46fb66cfccefea	22.927	-24.913	3.571	-6.977	3.0E-12	7.8E-11	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NA	NA
5305560aa419b3c756c9501c6c1e6a51	25.007	8.221	2.318	3.546	3.9E-04	4.3E-03	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	NA
385264a8c659a97ee870c9d17b2f1d7a	201.383	1.834	0.556	3.299	9.7E-04	9.7E-03	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NA	NA
56fbb77cd4ff0e99c3f339d60f34bdbf	80.599	-24.495	2.997	-8.172	3.0E-16	1.6E-14	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	NA
de9583e172af6b53a3813403b3ea939d	17.329	9.153	3.282	2.789	5.3E-03	4.3E-02	Bacteria	Firmicutes	Clostridia	Clostridiales	NA	NA	NA
fe2a6d33153ce8e6d6f3de41864c9372	30.486	25.982	3.372	7.706	1.3E-14	5.4E-13	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus	NA
6367d5639a8fd5ffdd5f26aaa3407e63	35.340	25.943	3.486	7.443	9.8E-14	3.8E-12	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus	boronitolerans

b24016ff03e7da66f3407920d36554e6	6.272	23.904	4.110	5.816	6.0E-09	9.8E-08	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA
d68f6edf4fd42cbbb429763ebfa7115c	37.677	25.232	3.276	7.701	1.3E-14	5.4E-13	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA
ef4d84fdccee408ff39bd31bbb9d711f	7.457	24.613	4.110	5.988	2.1E-09	3.6E-08	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA
d9afa483d90f3c90b2ac7a443af2f9c0	17.271	26.164	4.109	6.367	1.9E-10	3.8E-09	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Giesbergeria	NA
7933be9b8e6280235b8aa916e2cfc2fd	8.229	23.340	3.353	6.961	3.4E-12	8.5E-11	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA
efff8366ae01fbabd82b0f370aa90487	29.809	26.696	3.823	6.984	2.9E-12	7.6E-11	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA
2571eabc30fb309a1ac650a72a221e70	868.648	7.269	1.505	4.831	1.4E-06	1.9E-05	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA
efbbf1faf333f02625e8b1185c2922dd	122.579	27.816	3.072	9.056	1.3E-19	1.4E-17	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA
f3307f9aa4af0caa71b3db83c549c00a	42.347	10.429	2.400	4.345	1.4E-05	1.8E-04	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax	NA
bef527884805834618fddd6de3bf280d	11.638	22.509	3.344	6.730	1.7E-11	3.8E-10	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Epulopiscium	NA
ab5b3fa62e7f8c099cafedb4c33baa19	11.611	24.495	3.696	6.627	3.4E-11	7.4E-10	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Epulopiscium	NA
dd420316f447eee7b7cbfce79358e873	24.278	9.525	2.375	4.010	6.1E-05	7.2E-04	Bacteria	Firmicutes	Clostridia	Clostridiales	NA	NA	NA

a184c23c576ec0de4342c06f1987c9cf	856.891	-3.041	1.101	-2.761	5.8E-03	4.7E-02	Bacteria	Firmicutes	Clostridia	Clostridiales	NA	NA	NA
2459b9194d0b569745cfd62ec393aaed	1.451	17.606	4.135	4.258	2.1E-05	2.6E-04	Bacteria	Firmicutes	Clostridia	Clostridiales	NA	NA	NA
6e28471e01c31d6b1803c9c7b4424d89	16.894	8.484	2.819	3.010	2.6E-03	2.3E-02	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	NA	NA
e80e520363fc36a89486c3b8a98b0e04	22.876	9.476	2.584	3.668	2.4E-04	2.8E-03	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	NA	NA
4e8173c58f1a05dc45488cadb3e965d2	31.281	23.575	3.502	6.731	1.7E-11	3.8E-10	Bacteria	Firmicutes	Clostridia	Clostridiales	NA	NA	NA
61981f62d08d858332280d45351bd211	5.005	24.366	4.111	5.927	3.1E-09	5.2E-08	Bacteria	Firmicutes	Clostridia	Clostridiales	NA	NA	NA
c1d278a3dea30c9bc378cf85b107d3a7	2.595	-15.714	4.137	-3.799	1.5E-04	1.7E-03	Bacteria	Chloroflexi	Thermomicrobia	JG30-KF-CM45	NA	NA	NA
ddd0aa2ab9c3dd94309ae3c089e4aaaa	94.099	2.563	0.753	3.405	6.6E-04	6.9E-03	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	NA
a784c5917816ca2e86ea323fe4038fd4	11.169	23.804	3.449	6.901	5.2E-12	1.3E-10	Bacteria	Fusobacteriia	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Cetobacterium	NA
80a23fd8a244066efa7063ec9b9f68d1	67.399	-19.458	3.321	-5.858	4.7E-09	7.7E-08	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	NA	NA
9befdf2cf9cf203d191c2971f1408d01	19.767	26.136	3.528	7.409	1.3E-13	4.7E-12	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	NA

b31bc2f24f3bb2a7869cc5413 16823f4	585.465	5.056	1.4 84	3.40 7	6.6E-04	6.9E-03	Bacteri a	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	NA
f24b5ab27ed82cbe8e641f171 9c0d44d	9.092	21.275	4.1 16	5.16 8	2.4E-07	3.5E-06	Bacteri a	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	NA
ee8b0f9779cddb57200003b2 9b97dd7c	7.578	-22.948	3.5 40	-6.48 2	9.0E-11	1.8E-09	Bacteri a	Proteobact eria	Gammaproteoba cteria	Enterobacteri ales	Enterobacteriac eae	NA	NA
da250fd6da0b540153bff16e8 16857da	17.422	24.814	4.1 09	6.03 8	1.6E-09	2.7E-08	Bacteri a	Proteobact eria	Gammaproteoba cteria	Alteromonada les	NA	NA	NA
6685ad260979277f7d47f795c 7e4f124	521.211	2.991	1.0 74	2.78 5	5.4E-03	4.4E-02	Bacteri a	Proteobact eria	Gammaproteoba cteria	Enterobacteri ales	Enterobacteriac eae	Plesiomonas	shigelloides
50ab78850a8ea7a16461d8a9 bc5fa01e	158.150	7.931	2.4 52	3.23 5	1.2E-03	1.2E-02	Bacteri a	Proteobact eria	Gammaproteoba cteria	Pseudomonad ales	Moraxellaceae	Acinetobacter	johnsonii
a1497928637e05bf2e2a4d6fe f54f500	80.272	9.808	2.3 09	4.24 8	2.2E-05	2.7E-04	Bacteri a	Proteobact eria	Gammaproteoba cteria	Pseudomonad ales	Moraxellaceae	Acinetobacter	johnsonii
51064dc3da92d9ffdda0aa69c 29dc626	70.777	26.570	2.4 24	10.9 61	5.9E-28	1.4E-25	Bacteri a	Proteobact eria	Gammaproteoba cteria	Pseudomonad ales	Moraxellaceae	Acinetobacter	schindleri
19a3330b72bca172782965a8f b9f29cc	298.146	28.743	2.1 79	13.1 92	9.8E-40	9.1E-37	Bacteri a	Proteobact eria	Gammaproteoba cteria	Pseudomonad ales	Moraxellaceae	Acinetobacter	NA
6b207aeb680ce920df015bea8 04ba576	163.812	10.933	2.2 52	4.85 4	1.2E-06	1.7E-05	Bacteri a	Proteobact eria	Gammaproteoba cteria	Pseudomonad ales	Moraxellaceae	Acinetobacter	NA
54bdc8263c64c084ace5ddcb 79648435	573.322	5.234	1.5 69	3.33 7	8.5E-04	8.5E-03	Bacteri a	Proteobact eria	Gammaproteoba cteria	Pseudomonad ales	Moraxellaceae	Acinetobacter	NA
c0bf0288f4487de723ec913ea d27ad4f	76.423	27.016	3.2 68	8.26 7	1.4E-16	7.5E-15	Bacteri a	Proteobact eria	Gammaproteoba cteria	Pseudomonad ales	Moraxellaceae	Acinetobacter	NA

fccdc0e0da4adf6a2ab114c0af2e7645	46.555	26.424	3.603	7.334	2.2E-13	7.7E-12	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
5a63b759dd051d8f72341d639f9a7526	156.006	5.828	1.662	3.507	4.5E-04	4.9E-03	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
4d2ab2217ae1d674e06a395f034615da	31.596	25.627	3.192	8.028	9.9E-16	4.8E-14	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	lwoffii
90326ded16ffad8daee3618dddb2e2a2	31.881	25.798	2.714	9.505	2.0E-21	2.3E-19	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
ac178114bb17c82c85fb0e7c9d07c699	2266.471	5.522	1.330	4.152	3.3E-05	4.0E-04	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
bfdc9ed3b15aee63ea5ea094986cc77d	39.156	26.148	3.575	7.313	2.6E-13	8.4E-12	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
70ce739bab38b88f373201703926216c	28.956	25.558	2.987	8.557	1.2E-17	7.1E-16	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	NA	NA
1d66d89f395f71592c81649e9ab9b8bd	51.503	26.438	2.771	9.542	1.4E-21	1.9E-19	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	NA	NA
852fe06fea622fb11cf98143376791e6	18.956	10.961	2.472	4.434	9.2E-06	1.2E-04	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	cuatrocienegasensis
2511787484d0c7b8e9791bac60cb531f	18.547	9.327	2.950	3.162	1.6E-03	1.5E-02	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	NA
b91493359 18dcee4f7a6e18310993d65	2988.763	5.822	1.364	4.267	2.0E-05	2.5E-04	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	NA
754d5f63a21a2f313965a90cc6640ac9	63.999	26.815	3.067	8.744	2.3E-18	1.9E-16	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	NA
9490cfd72171fc2f50b8b7b00593454b	291.193	10.978	3.537	3.104	1.9E-03	1.8E-02	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	NA

af7f8cfcaae55e0e7ff05b442f0fe41e	89.072	6.785	2.471	2.746	6.0E-03	4.8E-02	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	NA
155d4e8473b04bea5ac96e80f7742328	19.764	25.090	3.561	7.045	1.9E-12	5.1E-11	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	NA
8021101d3b324ccf53a4abdefbaa054a	32.560	9.883	2.929	3.374	7.4E-04	7.5E-03	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	[Chromatiaceae]	Alishewanella	NA
d39c0d110aab036bc826e065e37066d6	16.965	25.945	3.794	6.839	8.0E-12	1.9E-10	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	[Chromatiaceae]	Alishewanella	NA
4c9fc51eade85fb7d7520e32690c1428	34.381	26.585	3.086	8.614	7.1E-18	4.8E-16	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	[Chromatiaceae]	Rheinheimera	NA
083982c2bdd5d279e64b1bc501a632a9	18.072	8.890	2.928	3.036	2.4E-03	2.1E-02	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Providencia	NA
88cb6f84baff08af68723268859bf0c9	1883.473	-3.172	0.936	-3.388	7.0E-04	7.3E-03	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	NA

Table S2. 5 Differences in abundance of specific ASVs in the cloaca versus small intestine.

ASVs	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Kingdom	Phylum	Class	Order	Family	Genus	Species
00a9489561825a4c5f530c0bb930b18f	9.689	22.344	3.283	6.807	1.0E-11	2.9E-10	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	NA	NA	NA
854c65fb139207cd8a98ff32a7ad7fa4	32.808	23.146	4.111	5.631	1.8E-08	2.7E-07	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	NA	NA

16564cfc259d63314a3c737cd465117e	205.426	3.636	1.311	2.774	5.5E-03	4.8E-02	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	NA	NA
eaf3866e8291b6c954fc8b5f6270c781	79.320	7.128	2.089	3.411	6.5E-04	6.9E-03	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	Elizabethkingia	meningoseptica
a16499d50611f338eae7f56722d918a3	23.386	20.821	4.117	5.057	4.3E-07	5.5E-06	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Myroides	NA
9576e2b36f5be49471de161c203dc800	58.834	10.239	2.791	3.669	2.4E-04	2.7E-03	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	NA
834fccc419149174d300899013838a4f	161.084	5.120	1.530	3.346	8.2E-04	8.6E-03	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
d7035b7ecb9a9f80177bb8ab39c7a689	10.255	18.484	3.892	4.749	2.0E-06	2.5E-05	Bacteria	Bacteroidetes	NA	NA	NA	NA	NA
9f4f5c3135ae0e4a2c735bb5ba47fde5	6.618	21.222	4.114	5.158	2.5E-07	3.3E-06	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	NA	NA
76ecc203416862f614d54f64e22e28b0	20.373	25.664	3.491	7.352	1.9E-13	8.8E-12	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	NA
a231b1b0a07c0dbd7622a779ec7f6fdc	60.849	25.422	2.674	9.506	2.0E-21	2.0E-19	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	mizutaii
87779f938855a9c148ea299e6d1865d3	79.460	25.555	2.271	11.254	2.2E-29	6.2E-27	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	NA
3b5e11a04e554cd082958ee45c6c7541	76.559	24.232	4.109	5.897	3.7E-09	6.4E-08	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	NA
b93d7bee43264347cfddf75aca306960	10.181	18.883	3.373	5.598	2.2E-08	3.2E-07	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	NA
9950d38e7195046ee4a669b68edec4ce	21.101	-21.030	3.388	-6.208	5.4E-10	9.9E-09	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	NA

13411d3e4548d3eb7350b626bc80af3f	18.224	18.434	4.057	4.544	5.5E-06	6.5E-05	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	NA
9a56472d58b4f738a076bf3acbc25218	27.347	19.028	3.024	6.293	3.1E-10	6.9E-09	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	NA	NA	NA
ad70a9687ad360a08c486fd54a9e2952	88.965	7.094	2.445	2.901	3.7E-03	3.3E-02	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	NA	NA	NA
8e37210789665725cba38fe5d1918802	35.363	21.981	3.565	6.165	7.0E-10	1.3E-08	Bacteria	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Aquaspirillum	serpens
17e0b566d185ed85dbbb96626d7391d9	17.443	23.361	3.317	7.042	1.9E-12	6.2E-11	Bacteria	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Microvirgula	NA
32f844e217771d72fe9f7f476119bf0df	24.336	-8.687	2.254	-3.853	1.2E-04	1.3E-03	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Ralstonia	NA
38646b9ab2c8fbf2820bec3967a3a24b	27.255	23.072	3.508	6.577	4.8E-11	1.2E-09	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Pseudoxanthomonas	NA
b00c723cb5db6172e1ffba8b9c04d3ac	44.271	18.306	2.935	6.238	4.4E-10	8.5E-09	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Dechloromonas	fungiphilus
420d3fe56e2ac49ce40872a6679ec16f	37.566	25.378	2.186	11.607	3.8E-31	2.1E-28	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	NA	NA	NA
2f06fe30e3730ec6220c58d463391f55	77.858	22.098	2.581	8.563	1.1E-17	8.7E-16	Bacteria	Proteobacteria	Deltaproteobacteria	Bdellovibrionales	Bacteriovoracaceae	Peredibacter	starrii
a4029ba43f26f4cb196c8fe877c99d4e	11.998	-7.387	2.245	-3.291	1.0E-03	1.0E-02	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NA	NA
04c557eddc311760dd28dce415077fad	109.634	6.304	1.923	3.279	1.0E-03	1.0E-02	Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium	NA
95cfb042cf74eb896e5422a3ee343dd6	5.136	-23.232	4.110	-5.653	1.6E-08	2.4E-07	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NA	NA

0930562a9275395e2773534c2e9b5585	35.851	-25.946	2.414	-10.748	6.0E-27	8.4E-25	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	NA
eb1ef5162c595c6c58dfb1ef998a46d0	8.144	-24.169	4.109	-5.881	4.1E-09	6.8E-08	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NA	NA
b898aa61ce6a979b9c46fb66cfccefea	22.927	-25.649	3.571	-7.183	6.8E-13	2.4E-11	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NA	NA
5305560aa419b3c756c9501c6c1e6a51	25.007	7.126	2.320	3.072	2.1E-03	2.0E-02	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	NA
9cff10d3d52e024200041cdb101e7543	8.128	-22.029	4.112	-5.357	8.4E-08	1.1E-06	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	NA
e85e07989197f8f56ae3ed6cf3efca45	112.811	5.758	1.885	3.055	2.3E-03	2.1E-02	Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	NA	NA
fe2a6d33153ce8e6d6f3de41864c9372	30.486	22.418	3.375	6.642	3.1E-11	8.2E-10	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus	NA
6367d5639a8fd5ffdd5f26aaa3407e63	35.340	19.190	3.501	5.481	4.2E-08	5.9E-07	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus	boronitolerans
d68f6edf4fd42cbbb429763ebfa7115c	37.677	25.135	3.276	7.672	1.7E-14	1.1E-12	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA
7933be9b8e6280235b8aa916e2cfc2fd	8.229	21.951	3.355	6.543	6.0E-11	1.5E-09	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA
2571eabc30fb309a1ac650a72a221e70	868.648	7.538	1.505	5.010	5.4E-07	6.9E-06	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA
efbbf1faf333f02625e8b1185c2922dd	122.579	23.417	3.073	7.620	2.5E-14	1.4E-12	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	NA

cd2369ac303469289f259aad2a11a480	310.758	5.372	1.620	3.316	9.1E-04	9.4E-03	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Epulopiscium	NA
bef527884805834618fddd6de3bf280d	11.638	23.356	3.342	6.988	2.8E-12	8.6E-11	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Epulopiscium	NA
4e8173c58f1a05dc45488cadb3e965d2	31.281	21.894	3.504	6.248	4.2E-10	8.3E-09	Bacteria	Firmicutes	Clostridia	Clostridiales	NA	NA	NA
5b610c592b8e350e8776132c9b74a6f3	59.031	5.321	1.868	2.848	4.4E-03	3.9E-02	Bacteria	Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]	Anaerovorax	NA
a784c5917816ca2e86ea323fe4038fd4	11.169	22.001	3.451	6.374	1.8E-10	4.2E-09	Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Cetobacterium	NA
c2a68404d1aeadd97fc35fb5a8a0f8ca	191.032	5.567	1.840	3.026	2.5E-03	2.3E-02	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	NA	NA
e8e4773a299cd1bb28f8e4c7b37979b2	227.552	9.681	2.031	4.766	1.9E-06	2.3E-05	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	NA	NA
8bf15e0e2f038dbf87e886a8a5732dcc	372.473	6.609	1.631	4.053	5.1E-05	5.8E-04	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	NA	NA
f24b5ab27ed82cbe8e641f1719c0d44d	9.092	23.883	4.110	5.812	6.2E-09	9.8E-08	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	NA
ee8b0f9779cddb57200003b29b97dd7c	7.578	-23.957	3.540	-6.767	1.3E-11	3.6E-10	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA	NA
da250fd6da0b540153bff16e816857da	17.422	22.881	4.111	5.566	2.6E-08	3.7E-07	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	NA	NA	NA
51064dc3da92d9ffdda0aa69c29dc626	70.777	23.045	2.426	9.499	2.1E-21	2.0E-19	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	schindleri
19a3330b72bca172782965a8fb9f29cc	298.146	24.135	2.180	11.072	1.7E-28	3.2E-26	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA

6b207aeb680ce920df015bea804ba576	163.812	7.194	2.254	3.192	1.4E-03	1.4E-02	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
c0bf0288f4487de723ec913ead27ad4f	76.423	23.560	3.269	7.207	5.7E-13	2.1E-11	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
fccdc0e0da4adf6a2ab114c0af2e7645	46.555	22.647	3.605	6.282	3.3E-10	7.2E-09	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
4d2ab2217ae1d674e06a395f034615da	31.596	18.869	3.210	5.878	4.2E-09	6.8E-08	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	lwoffii
90326ded16ffad8daee3618dddb2e2a2	31.881	20.060	2.729	7.351	2.0E-13	8.8E-12	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	NA
70ce739bab38b88f373201703926216c	28.956	21.820	2.991	7.295	3.0E-13	1.2E-11	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	NA	NA
1d66d89f395f71592c81649e9ab9b8bd	51.503	20.439	2.783	7.344	2.1E-13	8.8E-12	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	NA	NA
b9149335918dcee4f7a6e18310993d65	2988.763	4.659	1.364	3.415	6.4E-04	6.9E-03	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	NA
754d5f63a21a2f313965a90cc6640ac9	63.999	23.529	3.068	7.669	1.7E-14	1.1E-12	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	NA
155d4e8473b04bea5ac96e80f7742328	19.764	22.298	3.564	6.256	3.9E-10	8.1E-09	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	NA
88cb6f84baff08af68723268859bf0c9	1883.473	-2.791	0.936	-2.981	2.9E-03	2.6E-02	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	NA

Table S2. 6 Host characteristics of 18 cane toad individuals.

The body length of toads were measured as SVL (snout-vent-length) and SUL (snout-urostyle length).

Toad ID	SVL(mm)	SUL (mm)	Body weight (g)	Sex
1	112.8	103.6	104.36	M
2	94.7	86.7	59.42	M
3	104	98.2	104.57	M
4	96.2	93	70.45	M
5	105	98	107.25	M
6	101	92.8	104.9	M
7	105	97.7	109.34	M
8	100	95	106.16	M
9	114	107	109.92	M
10	102.4	95.3	102.79	F
11	105	98.2	111.6	M
12	111.6	108.3	142.59	M
13	101	97.7	90.61	M
14	98	95.7	96.09	F
15	119	111.4	165.67	F
16	103	98	98.95	F
17	101.4	97.9	105.67	F
18	108	99.3	109.67	F

Table S2. 7 Model selection of the most influential host factors causing the changes to gut bacteria.

Start: AIC = 229.53				
Distance ~1				
	Df	**AIC**	**F**	**Pr(>F)**
+ sex	1	222.12	9.7708	0.005 **
+ body weight	1	228.59	2.9166	0.005 **
<none>		229.53		
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1				
Step: AIC=222.12				
distance ~ sex				
	Df	**AIC**	**F**	**Pr(>F)**
+ body weight	1	222.04	2.025	0.005 **

<none>		222.12		
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1				
Step: AIC=222.04				
distance ~ sex + body weight				

Table S3. 1 Bioclim variables

Variables	Description
BIO1	Annual Mean Temperature
BIO2	Mean Diurnal Range (Mean of monthly: max temp - min temp)
BIO3	Isothermality (BIO2/BIO7) (×100)
BIO4	Temperature Seasonality (standard deviation ×100)
BIO5	Max Temperature of Warmest Month
BIO6	Min Temperature of Coldest Month
BIO7	Temperature Annual Range (BIO5-BIO6)
BIO8	Mean Temperature of Wettest Quarter
BIO9	Mean Temperature of Driest Quarter
BIO10	Mean Temperature of Warmest Quarter
BIO11	Mean Temperature of Coldest Quarter
BIO12	Annual Precipitation
BIO13	Precipitation of Wettest Month
BIO14	Precipitation of Driest Month
BIO15	Precipitation Seasonality (Coefficient of Variation)
BIO16	Precipitation of Wettest Quarter
BIO17	Precipitation of Driest Quarter
BIO18	Precipitation of Warmest Quarter
BIO19	Precipitation of Coldest Quarter

Table S3. 2 Mean and SD of host characteristics, behavioural traits and environmental factors used in this study

variables			**Range-core**		**Invasion-front**		**Australia**	
			mean	**SD**	**mean**	**SD**	**mean**	**SD**
Host characteristics	**Morphological data**	**SUL**	96.24	12.50	103.51	9.42	99.88	11.57
		Body weight	98.90	38.19	143.87	49.63	121.38	49.41
	Parasite load	**Lungworms**	2.33	3.01	2.97	7.02	2.65	5.37
		Occurrence of lungworms	0.60	0.50	0.43	0.50	0.52	0.50
		Gut parasites	1.55	3.53	4.40	6.07	3.26	5.35

		Occurrence of gut parasites	0.35	0.49	0.60	0.50	0.50	0.51
Behavioural traits		**Struggle scores**	2.63	2.53	2.10	4.54	2.37	3.65
		Struggle likelihood	0.84	0.37	0.28	0.43	0.57	0.50
		Righting effort	2.20	2.20	2.20	4.57	2.20	3.56
		Righting effort likelihood	0.73	0.45	0.50	0.51	0.62	0.49
		Righting time (sec)	24.83	47.36	12.93	34.03	18.88	41.32
Environmental factors	**GPS**	**long**	144.61	1.76	127.37	1.00	135.99	8.81
		lat	-17.48	1.29	-16.43	1.70	-16.95	1.59
	Temperature	**AnnualMeanTemp**	24.93	1.35	27.57	0.97	26.25	1.77
		MaxTempofWarmestMonth	33.47	3.29	37.80	1.95	35.63	3.46
		MinTempofColdestMonth	14.63	1.35	14.67	2.14	14.65	1.77
		MeanTempofWettestQuarter	27.33	1.38	30.13	1.33	28.73	1.95
		MeanTempofDriestQuarter	21.80	0.87	22.57	1.46	22.18	1.26
		MeanTempofWarmestQuarter	27.97	1.89	31.03	1.32	29.50	2.24
		MeanTempofColdestQuarter	20.90	0.83	22.57	1.46	21.73	1.45
	Temperature variation	**MeanDiurnalRange**	10.53	2.26	12.77	1.63	11.65	2.25
		Isothermality	55.67	2.54	54.67	1.92	55.17	2.29
		TempSeasonality	27.74	5.51	33.48	8.02	30.61	7.41
		TempAnnualRange	18.83	4.34	23.13	3.66	20.98	4.53
	precipitation	**AnnualPrecipitation**	1594.67	637.97	799.67	359.85	1197.17	651.45
		PrecipitationofWettestMonth	368.00	109.88	212.33	83.73	290.17	124.66
		PrecipitationofDriestMonth	19.67	13.50	0.33	0.48	10.00	13.59
		PrecipitationofWettestQuarter	1000.33	349.80	552.33	248.39	776.33	376.16
		PrecipitationofDriestQuarter	66.00	45.72	9.00	5.19	37.50	43.21
		PrecipitationofWarmestQuarter	803.00	319.97	235.33	48.40	519.17	365.24
		PrecipitationofColdestQuarter	80.67	55.40	9.00	5.19	44.83	53.18
	precipitation variation	**PrecipitationSeasonality**	101.00	15.25	114.67	2.09	107.83	12.80

Table S3. 3 Non-phylogenetic alpha diversity metrics (observed ASVs, evenness and Shannon)

SampleID	**before pruning**			**after pruning**		
	Pielou evenness	**observed ASVs**	**Shannon**	**Pielou evenness**	**observed ASVs**	**Shannon**
RmC001	0.801	275	6.493	0.775	338	6.513
RmC002	0.719	307	5.937	0.681	421	5.936
RmC003	0.647	281	5.262	0.629	353	5.320

RmC004	0.733	285	5.977	0.699	384	6.002
RmC005	0.661	151	4.786	0.635	198	4.841
RmC006	0.533	249	4.245	0.486	459	4.297
RmC007	0.621	212	4.796	0.582	307	4.805
RmC008	0.574	205	4.404	0.542	273	4.384
RmC009	0.739	258	5.918	0.714	320	5.938
RmC010	0.753	169	5.571	0.711	231	5.581
RmC011	0.784	428	6.856	0.764	538	6.930
RmC012	0.729	299	5.999	0.683	455	6.034
RmC013	0.763	311	6.316	0.720	450	6.345
RmC014	0.480	241	3.801	0.438	418	3.811
RmC015	0.662	97	4.371	0.623	136	4.415
RmC016	0.767	339	6.446	0.739	410	6.417
RmC017	0.707	369	6.032	0.687	476	6.108
RmC018	0.759	461	6.718	0.740	593	6.820
RmC019	0.785	478	6.990	0.764	591	7.036
RmC020	0.781	439	6.853	0.756	559	6.897
RmC021	0.478	621	4.437	0.428	1230	4.392
RmC022	0.754	456	6.663	0.713	637	6.643
RmC023	0.738	329	6.172	0.704	450	6.203
RmC024	0.784	265	6.310	0.762	314	6.322
RmC025	0.770	423	6.717	0.746	530	6.747
RmC026	0.704	264	5.660	0.667	364	5.675
RmC027	0.674	221	5.249	0.633	321	5.270
RmC028	0.610	370	5.207	0.579	543	5.256
RmC029	0.782	349	6.603	0.746	492	6.669
RmC030	0.735	502	6.590	0.695	779	6.677
RmC031	0.810	593	7.462	0.785	753	7.498
RmC032	0.731	324	6.096	0.705	467	6.252
RmC033	0.738	374	6.309	0.689	564	6.293
RmC034	0.607	303	5.006	0.548	586	5.036
RmC035	0.707	419	6.161	0.641	830	6.218
RmC036	0.542	187	4.087	0.481	347	4.057
RmC037	0.610	335	5.115	0.550	637	5.123
RmC038	0.794	420	6.917	0.743	676	6.983
RmC039	0.731	357	6.199	0.695	507	6.247
RmC040	0.775	309	6.412	0.734	444	6.454
RmC041	0.781	254	6.237	0.751	325	6.268

RmC042	0.723	310	5.984	0.699	394	6.025
RmC043	0.624	85	3.999	0.551	158	4.021
RmC044	0.699	242	5.537	0.667	332	5.587
RmC045	0.789	472	7.007	0.761	651	7.116
RmC046	0.677	178	5.058	0.641	263	5.153
RmC047	0.820	563	7.493	0.794	751	7.586
RmC048	0.651	121	4.504	0.592	200	4.522
RmC049	0.727	181	5.451	0.689	246	5.476
RmC050	0.728	290	5.958	0.698	382	5.986
RmC051	0.763	340	6.416	0.723	486	6.456
RmC052	0.753	449	6.633	0.727	583	6.679
RmC053	0.764	452	6.737	0.725	637	6.754
RmC054	0.784	303	6.460	0.747	405	6.468
RmC055	0.740	268	5.969	0.699	385	6.007
RmC056	0.722	202	5.527	0.691	254	5.522
RmC057	0.745	821	7.210	0.725	1046	7.269
RmC058	0.822	538	7.461	0.798	699	7.540
RmC059	0.796	536	7.219	0.768	721	7.291
RmC060	0.748	276	6.066	0.727	325	6.065
Neg_control	0.683	36	3.534			
Pos_control	0.725	19	3.079			

*** Data were pruned to remove representatives classified to Archaea (N = 28), chloroplast (N = 17), mitochondria (N = 186), and 151 unassigned ("Kingdom") ASVs**

Table S3. 4 Relative abundance at phylum level

Phylum	Mean (W)	SD (W)	Mean (E)	SD (E)	Mean (W+E)	SD (W+E)
Firmicutes	42.98%	17.85%	52.79%	19.64%	47.89%	19.25%
Bacteroidetes	44.79%	18.38%	33.31%	18.67%	39.05%	19.26%
Proteobacteria	6.87%	14.90%	5.81%	9.04%	6.34%	12.23%
Fusobacteria	3.38%	5.87%	2.71%	8.59%	3.04%	7.30%
Verrucomicrobia	1.29%	2.62%	3.98%	7.39%	2.63%	5.66%
NA	0.54%	0.95%	1.38%	4.34%	0.96%	3.14%
Tenericutes	0.12%	0.44%	0.02%	0.08%	0.07%	0.32%
Actinobacteria	0.03%	0.05%	0.00%	0.00%	0.02%	0.04%
OD1	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%

* Mean (W) means the relative abundance of each taxa within 30 invasion-front toads. Mean (E) means from 30 range-core toads. Mean (W+E) means from all 60 toads.

Table S3. 5 Relative abundance at family level.

Family	Mean (W)	SD (W)	Mean (E)	SD (E)	Mean (W+E)	SD (W+E)
NA	26.68%	13.60%	26.68%	15.27%	26.68%	14.34%
Bacteroidaceae	18.61%	10.34%	14.84%	9.66%	16.73%	10.10%
Lachnospiraceae	12.72%	9.88%	16.82%	11.59%	14.77%	10.88%
Porphyromonadaceae	8.72%	6.93%	6.85%	6.82%	7.79%	6.88%
Clostridiaceae	5.54%	5.87%	6.08%	7.14%	5.81%	6.48%
Enterobacteriaceae	6.83%	14.91%	4.71%	8.87%	5.77%	12.21%
Erysipelotrichaceae	4.21%	4.23%	2.50%	2.53%	3.35%	3.56%
Fusobacteriaceae	3.38%	5.87%	2.71%	8.59%	3.04%	7.30%
Bacillaceae	2.03%	10.10%	3.89%	5.72%	2.96%	8.19%
Ruminococcaceae	1.61%	1.69%	3.89%	3.45%	2.75%	2.93%
Verrucomicrobiaceae	1.29%	2.62%	3.98%	7.39%	2.63%	5.66%
Turicibacteraceae	1.95%	2.84%	2.81%	3.77%	2.38%	3.34%
Veillonellaceae	3.44%	4.03%	0.93%	2.01%	2.19%	3.40%
Rikenellaceae	1.74%	3.17%	1.16%	2.26%	1.45%	2.75%
Peptostreptococcaceae	0.40%	0.51%	0.59%	1.24%	0.49%	0.95%
Rhodospirillaceae	0.00%	0.00%	0.88%	3.53%	0.44%	2.52%
Peptococcaceae	0.20%	0.35%	0.20%	0.53%	0.20%	0.45%
[Odoribacteraceae]	0.40%	0.94%	0.00%	0.00%	0.20%	0.69%
[Mogibacteriaceae]	0.14%	0.21%	0.24%	0.42%	0.19%	0.33%
Desulfovibrionaceae	0.03%	0.15%	0.21%	0.44%	0.12%	0.34%
Christensenellaceae	0.05%	0.16%	0.00%	0.00%	0.03%	0.11%
Coriobacteriaceae	0.03%	0.05%	0.00%	0.00%	0.02%	0.04%
Mycoplasmataceae	0.00%	0.00%	0.02%	0.08%	0.01%	0.05%

* Mean (W) means the relative abundance of each taxa within 30 invasion-front toads. Mean (E) means from 30 range-core toads. Mean (W+E) means from all 60 toads.

Table S3. 6 Relative abundance at genus level.

Genera	Mean (W)	SD (W)	Mean (E)	SD (E)	Mean (W+E)	SD (W+E)
NA	62.20%	12.28%	57.53%	11.96%	59.87%	12.25%
Bacteroides	18.05%	10.19%	14.31%	9.44%	16.18%	9.92%
Parabacteroides	5.09%	5.98%	5.06%	5.56%	5.08%	5.73%
Clostridium	4.02%	5.52%	3.65%	4.32%	3.84%	4.92%
Epulopiscium	1.40%	5.05%	4.48%	11.20%	2.94%	8.75%
Akkermansia	1.29%	2.62%	3.98%	7.39%	2.63%	5.66%
Turicibacter	1.95%	2.84%	2.81%	3.77%	2.38%	3.34%

Bacillus	2.03%	10.10%	2.53%	4.31%	2.28%	7.71%
Oscillospira	0.65%	0.68%	2.20%	2.17%	1.42%	1.77%
Cetobacterium	0.31%	0.93%	1.64%	7.23%	0.98%	5.15%
Coprobacillus	0.76%	1.88%	0.66%	1.12%	0.71%	1.54%
cc_115	0.58%	1.29%	0.17%	0.40%	0.37%	0.97%
[Eubacterium]	0.56%	1.01%	0.19%	0.71%	0.37%	0.88%
Odoribacter	0.40%	0.94%	0.00%	0.00%	0.20%	0.69%
Anaerotruncus	0.06%	0.12%	0.31%	0.40%	0.18%	0.32%
Phascolarctobacterium	0.21%	0.47%	0.07%	0.23%	0.14%	0.38%
Bilophila	0.03%	0.15%	0.21%	0.44%	0.12%	0.34%
AF12	0.20%	0.36%	0.00%	0.00%	0.10%	0.27%
Anaerorhabdus	0.00%	0.00%	0.12%	0.35%	0.06%	0.25%
PW3	0.08%	0.23%	0.00%	0.00%	0.04%	0.17%
Ruminococcus	0.02%	0.07%	0.06%	0.12%	0.04%	0.10%
Anaerofilum	0.05%	0.14%	0.00%	0.00%	0.03%	0.10%
rc4-4	0.03%	0.10%	0.00%	0.00%	0.01%	0.07%
Mycoplasma	0.00%	0.00%	0.02%	0.08%	0.01%	0.05%
Blautia	0.00%	0.00%	0.01%	0.03%	0.01%	0.02%
Plesiomonas	0.00%	0.00%	0.01%	0.03%	0.00%	0.02%

* Mean (W) means the relative abundance of each taxa within 30 invasion-front toads. Mean (E) means from 30 range-core toads. Mean (W+E) means from all 60 toads.

Table S3. 7 Differences in abundance of specific ASVs in large intestine from range-core cane toads versus invasion-front cane toads.

Only included the ones that classified to family level.

ASVs	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Kingdom	Phylum	Class	Order	Family	Genus	Species
6c58d45d4b8ff8cdecd8e9d1fb4c5a69	1487.697	-3.250	1.037	-3.134	1.7E-03	3.7E-03	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA	NA
7557c00fea8f70259b8904b3b3d76880	895.763	2.531	0.993	2.550	1.1E-02	2.2E-02	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Epulopiscium	NA
cbdb93196f42014d5b0ab3ab42c17f88	527.943	3.495	1.155	3.026	2.5E-03	5.3E-03	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA	NA
6419daf56085a3de7da0ea88506dc9fd	764.777	1.842	0.642	2.870	4.1E-03	8.6E-03	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	NA
a267538570c1388d346a727261fe91be	172.240	-24.844	1.694	-14.662	1.1E-48	1.4E-47	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
0162cd21d987db84a48ea32e8cce0eb7	56.217	-25.854	2.426	-10.657	1.6E-26	1.1E-25	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
e46a7bcff7461ee34f3b6b0d69df6d19	631.347	-2.136	0.956	-2.235	2.5E-02	4.8E-02	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
e5cb7a6bb59f52f361cf3760b9f9ff4f	104.997	-26.595	2.275	-11.689	1.5E-31	1.4E-30	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA

80222d96e87adadb78c4b491e56a576b	421.000	-2.742	1.232	-2.225	2.6E-02	4.8E-02	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	NA	NA
492e18555f4701f64bc74822c9b95b9a	105.234	-26.732	2.671	-10.008	1.4E-23	6.9E-23	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	NA	NA
86290c20aa914c92a60c3b1928ef8552	84.244	-26.423	2.656	-9.950	2.5E-23	1.1E-22	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	NA
8c7ded70bf5271c289232d20c8565f34	151.400	-27.231	1.391	-19.583	2.1E-85	1.2E-83	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	ovatus
a4a53a3ed7fc3c92875b6d43d0f59a1b	233.009	-27.716	2.735	-10.133	3.9E-24	2.1E-23	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	NA
780e6a8bc3c4294880771d06eb0d14cf	25.306	-24.247	2.595	-9.343	9.4E-21	2.9E-20	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	NA
6902008369ce59136e20a96f2acd5e8b	60.760	-25.130	1.437	-17.491	1.7E-68	4.3E-67	Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	cc_115	NA
a34ef14f8bbed084541dbbc7738bbcf6	81.138	-26.363	1.535	-17.179	3.8E-66	7.3E-65	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	NA
7b8c080caae57d2f824112c9a7fc3a6a	53.462	-25.778	2.069	-12.460	1.2E-35	1.4E-34	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
ef7cc9c06b4d9a3393bcb9e79db07c61	20.440	-24.444	2.583	-9.464	3.0E-21	9.5E-21	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	NA

54e630043d77fd8382fa7f3b6ed035fa	13.532	-22.453	2.910	-7.716	1.2E-14	2.8E-14	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	[Odoribacteraceae]	Odoribacter	NA
b5b8abbd375f4b9fcdc0af60d2777c99	30.332	-24.987	2.378	-10.509	7.9E-26	4.8E-25	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
e1daff26a7f4ebcdd446769acc793647	26.210	-24.542	2.205	-11.128	9.2E-29	7.5E-28	Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	[Eubacterium]	dolichum
b10a19c6f57b7ef748a93f0a36046246	53.135	-25.591	1.476	-17.341	2.3E-67	4.8E-66	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	uniformis
3af5a570a8cbca4da5182555f5b8a257	6.010	-6.051	2.631	-2.300	2.1E-02	4.1E-02	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	NA	NA
3fbd34428a142daa4c75dc669aa7d8ce	69.508	-25.872	2.643	-9.787	1.3E-22	5.3E-22	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	NA
60a58022cb997004475c70088245e826	4.819	-5.733	2.390	-2.399	1.6E-02	3.2E-02	Bacteria	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	NA	NA
ed7f5ceb297ad51ce5945e0f55a98027	14.344	-23.948	2.860	-8.373	5.6E-17	1.3E-16	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA	NA
4d05437a5f453059f791e488873c77e4	69.534	-26.157	2.640	-9.907	3.9E-23	1.7E-22	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	NA
39c8a7e2acbaab44f409f03f267e754b	546.170	5.515	1.027	5.371	7.8E-08	1.7E-07	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	NA
44f67bdfbd628b322769918ba839915a	248.837	-27.923	1.423	-19.620	1.0E-85	8.0E-84	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	NA

8ee379f05f6903bf6d292d30a9fd4395	96.668	-26.609	2.254	-11.807	3.6E-32	3.6E-31	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
174d67bd393815c98bb437be24448376	277.450	-28.094	1.527	-18.404	1.2E-75	4.0E-74	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	distasonis
afb88164af28994054d9be391f1ad7af	82.816	-26.401	2.439	-10.824	2.7E-27	2.0E-26	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
2b7a7d24b576f24d679fa5c353f0f7f3	26.501	-24.806	2.589	-9.580	9.7E-22	3.5E-21	Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Akkermansia	NA
b7a2c1afa54353b8946b9d7c115c7a44	189.563	4.089	1.632	2.506	1.2E-02	2.4E-02	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	NA	NA
8f53eb1f0dd7db73f3995c22fe4f2388	64.676	-26.055	2.421	-10.760	5.3E-27	3.8E-26	Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	NA	NA
5d7b261edd80dc99e0c4100e8db8cfdb	198.905	-27.622	1.505	-18.351	3.2E-75	9.2E-74	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	ovatus
c8f8bb35d044c738a35039ae87bae185	11.424	-23.621	2.586	-9.136	6.5E-20	1.8E-19	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	[Odoribacteraceae]	Odoribacter	NA
d081db749cdc490e77383b1059f85d8f	50.480	-25.709	2.422	-10.613	2.6E-26	1.8E-25	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Epulopiscium	NA

b908275e7b7716ca1c353d1c7b844483	67.899	-26.111	1.618	-16.135	1.5E-58	2.4E-57	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	AF12	NA
2ee89c589341947cb387326928eb95e5	35.058	-25.192	2.199	-11.457	2.2E-30	2.0E-29	Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	NA	NA
590ca5ec1fa06013677ae3d8337043f3	36.909	-24.294	2.870	-8.466	2.5E-17	6.2E-17	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	NA	NA
e991093fcfddadae8044626080e8f7c6	51.056	-25.469	1.697	-15.008	6.5E-51	8.8E-50	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	NA
a1ba648cceb09bd2f407b369f3e64b55	38.587	-24.619	2.611	-9.428	4.2E-21	1.3E-20	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
adc1e93b602d5cba53afff8f74915944	76.552	-24.446	1.624	-15.052	3.3E-51	4.8E-50	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	NA
f16dc2d38b6d9adf50e8870184112060	24.228	-24.676	2.597	-9.502	2.1E-21	6.9E-21	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	NA
e39d5eb9eb23d683073ca0dbbad1a672	38.087	-25.313	2.393	-10.576	3.8E-26	2.5E-25	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
04e5b39fb640a01af7d62efa921adf13	7.078	-6.288	2.362	-2.662	7.8E-03	1.6E-02	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	NA
74f47914a81d27d19d24fda1ba90542a	24.910	-24.720	2.592	-9.539	1.4E-21	5.0E-21	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	PW3	NA

045d65d76b036254cf0e59ec1717e221	18.821	-23.120	2.588	-8.934	4.1E-19	1.1E-18	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	NA	NA
3b8ffd49ee49e9dd639b32985c8ec6fd	8.374	-23.192	2.603	-8.909	5.1E-19	1.3E-18	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	NA	NA
519b011405b6f41ac7727b8eb1f1b5b4	15.799	-24.084	2.593	-9.289	1.6E-20	4.7E-20	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Anaerofilum	NA
0936ab029dadd9cd26863bc1b3b0c2f2	11.643	-23.650	2.372	-9.971	2.0E-23	9.6E-23	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	NA	NA
ddf9de7466cd35a6d2af7858afec335c	40.417	-25.398	2.604	-9.755	1.8E-22	7.0E-22	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	NA	NA
2a3d09d2028de2b116bd1cc7986e6ca6	5.716	-5.979	2.637	-2.267	2.3E-02	4.4E-02	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	NA	NA
c2972dcd4d3380ce07afc32b4d56732c	25.773	-24.297	2.596	-9.358	8.2E-21	2.5E-20	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
bcfce8cc1f1d41d4ca4291dc70ba36d4	7.713	-23.077	2.610	-8.841	9.5E-19	2.4E-18	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	NA
a84952a043073200cd76472c25259e0a	9.382	-22.779	2.596	-8.775	1.7E-18	4.3E-18	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	[Odoribacteraceae]	Odoribacter	NA
5dab6fb4a24683c16a17a1e865db6f3b	14.446	-23.955	2.362	-10.142	3.6E-24	2.0E-23	Bacteria	Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]	NA	NA
49d79c5f2707995019dbb234ac26f5ce	13.774	-23.893	2.586	-9.241	2.4E-20	7.1E-20	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	NA	NA
648b48ced2889e428f20630130d56c62	15.776	-7.445	1.621	-4.594	4.3E-06	9.6E-06	Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	NA	NA

6c5022573f8f973316205803e3059526	11.567	-23.649	2.592	-9.123	7.3E-20	2.0E-19	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	NA	NA
a6f3f75daa5b4b143aefea057c3b0340	5.848	-6.012	2.641	-2.276	2.3E-02	4.4E-02	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	NA	NA
539b882160c167bb0e9be429075a4ea9	13.083	-23.821	2.585	-9.216	3.1E-20	8.8E-20	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	NA	NA
adcee925b55ce9e5ace0c30909d39f37	7.374	-6.346	2.622	-2.420	1.6E-02	3.1E-02	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	NA	NA
177eb3f471db964278cb7b238f78a184	836.171	6.493	1.187	5.470	4.5E-08	1.0E-07	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Epulopiscium	NA
f9bede316aab2d20a7f9fb07840e565d	32.180	-25.073	2.622	-9.563	1.1E-21	4.1E-21	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
009da521e089ef304d1f66c8b04e28ea	32.882	-25.103	2.387	-10.516	7.3E-26	4.5E-25	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	distasonis
fe086df68487400843a44fb4e7ab9f16	15.184	-24.027	2.910	-8.257	1.5E-16	3.5E-16	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	NA	NA
8dd58bf584f5a402ff021b724147e395	17.603	-24.238	2.027	-11.954	6.2E-33	6.7E-32	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	NA
f2663b93e6b7afbc782d5134276f0fdd	52.178	-25.508	2.415	-10.564	4.4E-26	2.8E-25	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	distasonis
1724290d764f85a42c9db7d7cb939846	60.986	5.546	1.240	4.471	7.8E-06	1.7E-05	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Bilophila	NA

04411f2511ea9f914f865deb3927832e	34.979	-25.121	2.600	-9.661	4.4E-22	1.7E-21	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	NA
47546847c4397857f887fff788f08f60	13.313	-23.846	2.366	-10.077	7.0E-24	3.6E-23	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	NA	NA
1a9aa68607bc39a575a4b7c81be479c0	88.182	-26.481	2.097	-12.629	1.5E-36	1.8E-35	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium	NA
a01dd3da1aafce98d380fee8b94331be	49.548	-25.679	2.631	-9.762	1.6E-22	6.6E-22	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	NA
605564c34134416f446b516ae52d0179	29.076	-24.894	2.201	-11.309	1.2E-29	1.1E-28	Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	NA	NA
16a88166845e1d751f122c0dcca98a5b	9.769	-23.406	2.595	-9.020	1.9E-19	5.0E-19	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	ovatus
64991db060643dc3bdfb8c0fdf51e6e3	13.321	-23.841	2.362	-10.096	5.8E-24	3.0E-23	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	NA	NA
62bc67a80f888b27125bc2690bf3ee51	49.278	-24.095	2.626	-9.176	4.5E-20	1.3E-19	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	ovatus
da0b19878792e922e6b3f6ccc84f77c5	24.955	-24.713	2.187	-11.300	1.3E-29	1.1E-28	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	fragilis
b9409976332c20ab631937981f61654b	6.271	-6.113	2.163	-2.827	4.7E-03	9.7E-03	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA

a2397f0089a72cfa1395121f94fc49eb	17.390	-7.581	2.622	-2.891	3.8E-03	8.1E-03	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	NA
93d1b46d5dc3e1619eea42b1bff80f05	651.062	29.816	1.909	15.616	5.6E-55	8.6E-54	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	NA	NA
b9a467ef0617f4d815fa015fb0a2ffd4	88.833	26.980	2.909	9.273	1.8E-20	5.3E-20	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	fragilis
6904cbd6783c02626a2aba468a5640f3	275.743	28.641	1.283	22.319	##########	##########	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
a51279dcacf45d515b7eaacc70b802a4	308.968	28.784	1.529	18.827	4.5E-79	2.1E-77	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	distasonis
be967ee151c8743bda1042cadc340ed5	203.438	27.890	2.516	11.084	1.5E-28	1.2E-27	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	NA
210d4d637761db63f3f16c30ee407979	49.483	26.289	2.649	9.923	3.3E-23	1.5E-22	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	NA	NA
339e1c8994f7fbc0a23010fe97bce89d	15.545	24.708	2.606	9.480	2.6E-21	8.4E-21	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	NA	NA
75c0ea2b7d539613e42a006091e3f56b	142.138	27.733	1.490	18.612	2.6E-77	9.9E-76	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	distasonis
1692e6cc71419edc280c6c756ba9e0af	224.980	28.362	1.157	24.517	##########	##########	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	NA
fd304167835f6234bae704afa06f4614	47.280	26.218	2.624	9.991	1.7E-23	8.0E-23	Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Anaerorhabdus	furcosa
95cc31da651c0d1d75c6a86ac1159f26	88.364	27.081	1.549	17.480	2.0E-68	4.7E-67	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	NA
4aa5cfe66220aa950fbd6db3361a3a86	44.608	26.145	2.624	9.962	2.2E-23	1.0E-22	Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	NA	NA

f7ded0075eb755c68df28bc168423431	46.193	8.931	1.400	6.379	1.8E-10	4.1E-10	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
eb29bde72a89e554fd3871a4c957a6dd	61.146	25.323	2.431	10.417	2.1E-25	1.2E-24	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	ovatus
9f6285cb058aa80b10a2a2ddae126f79	68.520	9.499	1.124	8.449	2.9E-17	7.1E-17	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	NA
226d5e071abccfc78b7782c435775a73	36.171	24.333	2.392	10.172	2.6E-24	1.5E-23	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
7a2bb48b8b3e3e6a46d949d0992af1a6	19.531	25.015	2.386	10.486	1.0E-25	5.9E-25	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	NA
fb42aec635a286734175e40ced11d984	8.315	23.853	2.611	9.137	6.4E-20	1.8E-19	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	NA
324cd8f38431f705cb73a98d824808dd	5.821	5.942	2.634	2.256	2.4E-02	4.5E-02	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Anaerotruncus	NA
37d1958bb40faa4f43062c658209c5f9	6.393	6.077	2.391	2.542	1.1E-02	2.2E-02	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	NA
5f3dc837177b94869a0aff87c5714c43	22.672	25.218	2.865	8.801	1.4E-18	3.4E-18	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA	NA
fe8896769cf56f04c74c8d954ba40f46	28.131	25.496	2.602	9.797	1.2E-22	4.8E-22	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA	NA
27b633e38e3296a88295f474cbe3b0a8	15.458	24.689	2.589	9.537	1.5E-21	5.0E-21	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	NA
2e507d213cc3c9d5a8f671095253f35c	36.719	25.336	1.541	16.446	8.9E-61	1.6E-59	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	NA
0db2ae9cddc3b7aa17cbd7b31385af59	34.505	25.781	2.386	10.806	3.2E-27	2.4E-26	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	NA

314fa069eccc1d6240d8af01638d5b41	6.889	23.594	2.910	8.107	5.2E-16	1.2E-15	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	NA	NA
b0b496c9204914b969eafc30901252ed	17.328	24.847	2.594	9.579	9.8E-22	3.5E-21	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	NA	NA
0f179b8bfed9e2286ea5f352e27b157e	17.755	24.889	2.592	9.603	7.8E-22	2.9E-21	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	fragilis
3c064bab12d667f909b0272d9faefaad	8.650	6.511	1.516	4.296	1.7E-05	3.8E-05	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	subterminale
bcd408786a42363c89b7950ad357a38c	12.417	24.377	2.910	8.378	5.4E-17	1.3E-16	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	NA	NA
a8e25c5d1456422d37addd96abcbb11b	100.317	27.251	2.291	11.897	1.2E-32	1.3E-31	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
927c28b2c30481130d754bd0c4f50b66	23.278	25.258	2.600	9.716	2.6E-22	1.0E-21	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	NA
4a15ae0b8939617699a4c1f30262992d	43.607	25.037	2.887	8.672	4.2E-18	1.0E-17	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
005555dabe243422d4d02f4404ab0bcc	35.081	25.761	2.619	9.835	7.9E-23	3.4E-22	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	NA
a71e588587585eeccb10b72cd277e3d1	10.543	24.164	2.595	9.312	1.3E-20	3.8E-20	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Anaerotruncus	NA
205652d10f1305824452dc4031a8a515	22.071	24.747	2.593	9.543	1.4E-21	4.8E-21	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	NA
a8415a87a5673fb528660c8b990cfb40	7.006	23.604	2.632	8.968	3.0E-19	8.0E-19	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	NA	NA
b5c52517ccb11e5fab20a6e4e367efc2	16.701	24.801	2.588	9.583	9.4E-22	3.5E-21	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	NA	NA

93bbe1e0eda851951753f40d62553f82	6.396	6.078	2.380	2.553	1.1E-02	2.2E-02	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	NA
2dbe579fb8ea4ed05be478d8d23cdeab	12.384	24.040	2.602	9.240	2.5E-20	7.1E-20	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	NA
91127a840605abfe8cf4a1bc1bc501b4	8.821	23.935	2.382	10.050	9.2E-24	4.6E-23	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	NA	NA
3fa4268f5769ec9ca10bd42eec388a02	13.714	24.526	2.588	9.476	2.6E-21	8.5E-21	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	NA

Table S3. 8 Differences in abundance of specific predicted functions in large intestinal bacteria from range-core cane toads versus invasion-front cane toads.

Pathway shorten names	**predicted functional pathways**	**baseMean**	**log2FoldChange**	**lfcSE**	**stat**	**pvalue**	**padj**
AEROBACTINSYN-PWY	**aerobactin biosynthesis**	263.716	-2.211	0.543	-4.070	4.7E-05	6.0E-04
ANAEROFRUCAT-PWY	**homolactic fermentation**	43046.792	-0.110	0.035	-3.180	1.5E-03	1.2E-02
ARGDEG-PWY	**superpathway of L-arginine, putrescine, and 4-aminobutanoate degradation**	896.212	1.533	0.567	2.705	6.8E-03	3.9E-02
CENTFERM-PWY	**pyruvate fermentation to butanoate**	10961.088	0.514	0.184	2.796	5.2E-03	3.1E-02
CRNFORCAT-PWY	**creatinine degradation I**	552.210	3.810	0.575	6.625	3.5E-11	1.7E-09

FOLSYN-PWY	**superpathway of tetrahydrofolate biosynthesis and salvage**	38619.204	-0.166	0.039	-4.272	1.9E-05	2.7E-04
GALLATE-DEGRADATION-I-PWY	**gallate degradation II**	241.732	2.376	0.536	4.434	9.2E-06	1.5E-04
GALLATE-DEGRADATION-II-PWY	**gallate degradation I**	242.098	2.363	0.527	4.487	7.2E-06	1.3E-04
GLUCONEO-PWY	**gluconeogenesis I**	46507.393	-0.094	0.030	-3.088	2.0E-03	1.5E-02
GLYCOLYSIS	**superpathway of glycolysis and Entner-Doudoroff**	46490.176	-0.129	0.032	-4.062	4.9E-05	6.0E-04
HCAMHPDEG-PWY	**3-phenylpropanoate and 3-(3-hydroxyphenyl)propanoate degradation to 2-oxopent-4-enoate**	228.844	2.278	0.560	4.066	4.8E-05	6.0E-04
LACTOSECAT-PWY	**lactose and galactose degradation I**	1003.482	-0.896	0.331	-2.710	6.7E-03	3.9E-02
METHANOGENESIS-PWY	**methanogenesis from H2 and CO2**	5.656	-3.540	0.725	-4.882	1.0E-06	2.2E-05
METHYLGALLATE-DEGRADATION-PWY	**methylgallate degradation**	300.947	2.369	0.535	4.426	9.6E-06	1.5E-04
NADSYN-PWY	**NAD biosynthesis II (from tryptophan)**	503.798	3.689	0.481	7.666	1.8E-14	1.2E-12
NAGLIPASYN-PWY	**lipid IVA biosynthesis**	20574.919	-0.510	0.191	-2.677	7.4E-03	4.0E-02
ORNARGDEG-PWY	**superpathway of L-arginine and L-ornithine degradation**	896.212	1.533	0.567	2.705	6.8E-03	3.9E-02
P108-PWY	**pyruvate fermentation to propanoate I**	32200.249	-0.303	0.113	-2.680	7.4E-03	4.0E-02

P164-PWY	**purine nucleobases degradation I (anaerobic)**	29501.263	0.377	0.108	3.483	5.0E-04	4.6E-03
P221-PWY	**octane oxidation**	536.493	-1.229	0.432	-2.846	4.4E-03	2.8E-02
P241-PWY	**coenzyme B biosynthesis**	4.218	-2.542	0.700	-3.633	2.8E-04	2.8E-03
P381-PWY	**adenosylcobalamin biosynthesis II (late cobalt incorporation)**	18.306	-2.832	0.634	-4.470	7.8E-06	1.3E-04
P42-PWY	**incomplete reductive TCA cycle**	44742.461	-0.269	0.085	-3.149	1.6E-03	1.3E-02
POLYISOPRENSYN-PWY	**polyisoprenoid biosynthesis (E. coli)**	30996.681	-0.282	0.077	-3.666	2.5E-04	2.5E-03
PWY-1541	**superpathway of taurine degradation**	173.948	1.788	0.579	3.088	2.0E-03	1.5E-02
PWY-3781	**aerobic respiration I (cytochrome c)**	10141.296	1.472	0.343	4.294	1.8E-05	2.5E-04
PWY-5088	**L-glutamate degradation VIII (to propanoate)**	201.648	-1.844	0.618	-2.986	2.8E-03	1.9E-02
PWY-5180	**toluene degradation I (aerobic) (via o-cresol)**	3794.583	1.475	0.284	5.189	2.1E-07	4.9E-06
PWY-5181	**toluene degradation III (aerobic) (via p-cresol)**	427.599	1.711	0.532	3.215	1.3E-03	1.1E-02
PWY-5182	**toluene degradation II (aerobic) (via 4-methylcatechol)**	3794.583	1.475	0.284	5.189	2.1E-07	4.9E-06
PWY-5198	**factor 420 biosynthesis**	2.754	-4.721	1.226	-3.852	1.2E-04	1.3E-03

PWY-5266	**p-cymene degradation**	2312.737	1.444	0.370	3.896	9.8E-05	1.1E-03
PWY-5273	**p-cumate degradation**	2312.737	1.444	0.370	3.896	9.8E-05	1.1E-03
PWY-5415	**catechol degradation I (meta-cleavage pathway)**	1829.794	1.291	0.370	3.486	4.9E-04	4.6E-03
PWY-5419	**catechol degradation to 2-oxopent-4-enoate II**	714.726	3.160	0.410	7.713	1.2E-14	1.1E-12
PWY-5420	**catechol degradation II (meta-cleavage pathway)**	937.214	2.957	0.382	7.732	1.1E-14	1.1E-12
PWY-5484	**glycolysis II (from fructose 6-phosphate)**	40147.094	-0.178	0.044	-4.018	5.9E-05	7.1E-04
PWY-5507	**adenosylcobalamin biosynthesis I (early cobalt insertion)**	4640.006	1.915	0.369	5.185	2.2E-07	4.9E-06
PWY-5647	**2-nitrobenzoate degradation I**	246.527	3.704	0.474	7.807	5.9E-15	1.1E-12
PWY-5651	**L-tryptophan degradation to 2-amino-3-carboxymuconate semialdehyde**	328.831	3.754	0.483	7.779	7.3E-15	1.1E-12
PWY-5654	**2-amino-3-carboxymuconate semialdehyde degradation to 2-oxopentenoate**	296.221	3.122	0.448	6.971	3.1E-12	1.7E-10
PWY-5659	**GDP-mannose biosynthesis**	38965.581	-0.166	0.062	-2.683	7.3E-03	4.0E-02
PWY-5695	**urate biosynthesis/inosine 5'-phosphate degradation**	50771.204	-0.095	0.037	-2.605	9.2E-03	4.8E-02
PWY-5823	**superpathway of CDP-glucose-derived O-antigen building blocks biosynthesis**	166.483	-1.632	0.562	-2.906	3.7E-03	2.4E-02

PWY-6071	**superpathway of phenylethylamine degradation**	733.055	1.855	0.552	3.362	7.7E-04	6.8E-03
PWY-6107	**chlorosalicylate degradation**	61.228	3.917	0.500	7.830	4.9E-15	1.1E-12
PWY-6123	**inosine-5'-phosphate biosynthesis I**	45852.669	-0.111	0.043	-2.594	9.5E-03	4.9E-02
PWY-6145	**superpathway of sialic acids and CMP-sialic acids biosynthesis**	2.478	-4.887	1.865	-2.620	8.8E-03	4.7E-02
PWY-6147	**6-hydroxymethyl-dihydropterin diphosphate biosynthesis I**	34107.142	-0.219	0.073	-2.997	2.7E-03	1.9E-02
PWY-6167	**flavin biosynthesis II (archaea)**	11.160	-9.583	1.805	-5.310	1.1E-07	3.2E-06
PWY-6174	**mevalonate pathway II (archaea)**	3.427	-4.929	1.051	-4.688	2.8E-06	5.4E-05
PWY-6185	**4-methylcatechol degradation (ortho cleavage)**	283.925	1.667	0.543	3.070	2.1E-03	1.5E-02
PWY-6339	**syringate degradation**	412.943	3.189	0.560	5.696	1.2E-08	4.1E-07
PWY-6397	**mycolyl-arabinogalactan-peptidoglycan complex biosynthesis**	0.355	-2.020	0.534	-3.786	1.5E-04	1.6E-03
PWY-6404	**superpathway of mycolyl-arabinogalactan-peptidoglycan complex biosynthesis**	0.941	-3.007	0.595	-5.054	4.3E-07	9.4E-06
PWY-6467	**Kdo transfer to lipid IVA III (Chlamydia)**	16777.842	-0.523	0.184	-2.840	4.5E-03	2.8E-02
PWY-6505	**L-tryptophan degradation XII (Geobacillus)**	402.798	3.646	0.476	7.655	1.9E-14	1.2E-12

PWY-6562	**norspermidine biosynthesis**	1212.200	1.321	0.451	2.931	3.4E-03	2.3E-02
PWY-6590	**superpathway of Clostridium acetobutylicum acidogenic fermentation**	12954.282	0.493	0.168	2.932	3.4E-03	2.3E-02
PWY-6608	**guanosine nucleotides degradation III**	29572.896	0.302	0.093	3.244	1.2E-03	1.0E-02
PWY-6612	**superpathway of tetrahydrofolate biosynthesis**	34378.395	-0.186	0.041	-4.534	5.8E-06	1.1E-04
PWY-6629	**superpathway of L-tryptophan biosynthesis**	2082.939	-1.565	0.570	-2.747	6.0E-03	3.5E-02
PWY-6641	**superpathway of sulfolactate degradation**	725.382	2.570	0.433	5.936	2.9E-09	1.1E-07
PWY-6654	**phosphopantothenate biosynthesis III**	4.455	-4.981	1.521	-3.275	1.1E-03	9.2E-03
PWY-6690	**cinnamate and 3-hydroxycinnamate degradation to 2-oxopent-4-enoate**	228.844	2.278	0.560	4.066	4.8E-05	6.0E-04
PWY-6700	**queuosine biosynthesis**	26862.916	-0.323	0.093	-3.483	5.0E-04	4.6E-03
PWY-6703	**preQ0 biosynthesis**	22139.674	-0.338	0.105	-3.214	1.3E-03	1.1E-02
PWY-6728	**methylaspartate cycle**	42.338	-1.660	0.542	-3.065	2.2E-03	1.6E-02
PWY-6944	**androstenedione degradation**	5.711	-2.453	0.625	-3.927	8.6E-05	1.0E-03
PWY-7003	**glycerol degradation to butanol**	28255.622	0.239	0.092	2.608	9.1E-03	4.8E-02

PWY-7007	**methyl ketone biosynthesis**	13.891	-3.542	0.674	-5.258	1.5E-07	4.0E-06
PWY-7209	**superpathway of pyrimidine ribonucleosides degradation**	96.089	5.981	1.108	5.399	6.7E-08	2.1E-06
PWY-722	**nicotinate degradation I**	12.977	2.792	0.454	6.145	8.0E-10	3.2E-08
PWY-7323	**superpathway of GDP-mannose-derived O-antigen building blocks biosynthesis**	27105.052	-0.344	0.120	-2.853	4.3E-03	2.8E-02
PWY-7373	**superpathway of demethylmenaquinol-6 biosynthesis II**	1.518	-2.418	0.842	-2.871	4.1E-03	2.7E-02
PWY-7376	**cob(II)yrinate a,c-diamide biosynthesis II (late cobalt incorporation)**	10.773	-2.896	0.661	-4.384	1.2E-05	1.7E-04
PWY-7527	**L-methionine salvage cycle III**	557.896	1.147	0.407	2.814	4.9E-03	3.0E-02
PWY-7539	**6-hydroxymethyl-dihydropterin diphosphate biosynthesis III (Chlamydia)**	35516.097	-0.216	0.068	-3.153	1.6E-03	1.3E-02
PWY-7616	**methanol oxidation to carbon dioxide**	103.706	2.952	0.450	6.559	5.4E-11	2.4E-09
PWY0-1277	**3-phenylpropanoate and 3-(3-hydroxyphenyl)propanoate degradation**	573.344	1.881	0.524	3.592	3.3E-04	3.2E-03
PWY0-321	**phenylacetate degradation I (aerobic)**	764.834	1.898	0.558	3.399	6.8E-04	6.1E-03
REDCITCYC	**TCA cycle VIII (helicobacter)**	11272.754	0.886	0.196	4.509	6.5E-06	1.2E-04
RHAMCAT-PWY	**L-rhamnose degradation I**	19246.146	-0.349	0.111	-3.138	1.7E-03	1.3E-02

THISYN-PWY	**superpathway of thiamin diphosphate biosynthesis I**	27259.032	-0.303	0.109	-2.774	5.5E-03	3.3E-02

Table S3. 9 Nonmetric multidimensional scaling (nMDS) analysis of the relationship between host factors and bacterial community (top) and bacterial predicted function (bottom) using Bray Curtis distances.

The association between single host factor and microbial community using *envfit* function ("Vegan" R package)					
Host factor	**MDS1**	**MDS2**	**r2**	**Pr(>r)**	
SUL	0.17025	0.98540	0.0707	0.119	
BodyWeight	-0.00862	0.99996	0.0509	0.231	
lung_worms	0.25370	-0.96728	0.0198	0.585	
lungworms_Y_N	0.45275	-0.89164	0.1279	0.023	*
The association between single host factor and predicted microbial function using *envfit* function ("Vegan" R package)					
Host factor	**MDS1**	**MDS2**	**r2**	**Pr(>r)**	
SUL	0.68034	0.73290	0.0207	0.556	
BodyWeight	0.60978	0.79257	0.0405	0.311	
lungworms	-0.79064	0.61228	0.0066	0.839	
lungworms_Y_N	-0.82739	-0.56163	0.0586	0.187	
Signif. codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1**					
Number of permutations: 999					

Table S3. 10 Nonmetric multidimensional scaling (nMDS) analysis of the relationship between behaviour and bacterial community (top) and bacterial predicted function (bottom) using Bray Curtis distances

The association between single behavioural trait and microbial community using *envfit* function ("Vegan" R package)				
Behavioural trait	**MDS1**	**MDS2**	**r2**	**Pr(>r)**
Struggle score	-0.81432	0.58041	0.0435	0.268
Struggle likelihood	-0.88029	-0.47443	0.0206	0.555
Righting effort	-0.12366	0.99232	0.0745	0.121
Righting effort likelihood	0.49917	0.8665	0.0255	0.474
Righting time	0.93284	0.36028	0.0615	0.174
The association between single behavioural trait and predicted microbial function using *envfit* function ("Vegan" R package)				
Behavioural trait	**MDS1**	**MDS2**	**r2**	**Pr(>r)**
Struggle score	0.37562	0.92677	0.0505	0.221
Struggle likelihood	0.18289	0.98313	0.0167	0.606
Righting effort	0.5709	0.82102	0.0701	0.141
Righting effort likelihood	0.39866	0.9171	0.0353	0.362

Righting time	-0.95578	-0.29409	0.0041	0.894
Number of permutations: 999				

Table S3. 11 Nonmetric multidimensional scaling (nMDS) analysis of the relationship between environmental factors and bacterial community (top) and bacterial predicted function (bottom) using Bray Curtis distances

The association between single environmental factor and microbial community using *envfit* function ("Vegan" R package)					
Environmental factor	**MDS1**	**MDS2**	**r2**	**Pr(>r)**	
long	-0.06317	-0.99800	0.1939	0.001	***
lat	0.94788	-0.31862	0.0130	0.674	
AnnualMeanTemp	-0.98185	0.18966	0.0361	0.340	
MeanDiurnalRange	-0.71754	0.69652	0.0333	0.367	
Isothermality	0.33148	-0.94346	0.1933	0.002	**
TempSeasonality	-0.26606	0.96396	0.0595	0.178	
MaxTempofWarmestMonth	-0.82399	0.56661	0.0317	0.383	
MinTempofColdestMonth	0.13696	-0.99058	0.0633	0.166	
TempAnnualRange	-0.57041	0.82136	0.0455	0.273	
MeanTempofWettestQuarter	-0.63810	0.76996	0.0414	0.311	
MeanTempofDriestQuarter	-0.11629	-0.99322	0.1457	0.015	*
MeanTempofWarmestQuarter	-0.85730	0.51482	0.0399	0.321	
MeanTempofColdestQuarter	-0.71467	-0.69946	0.0517	0.212	
AnnualPrecipitation	0.32367	-0.94617	0.0102	0.729	
PrecipitationofWettestMonth	0.31669	-0.94853	0.0032	0.907	
PrecipitationofDriestMonth	0.11823	-0.99299	0.0427	0.279	
PrecipitationSeasonality	-0.51035	0.85997	0.0316	0.382	
PrecipitationofWettestQuarter	0.36969	-0.92916	0.0055	0.843	
PrecipitationofDriestQuarter	0.18911	-0.98196	0.0193	0.554	
PrecipitationofWarmestQuarter	0.21516	-0.97658	0.0507	0.228	
PrecipitationofColdestQuarter	0.26204	-0.96506	0.0304	0.404	
The association between single environmental factor and predicted microbial function using *envfit* function ("Vegan" R package)					
Environmental factor	**MDS1**	**MDS2**	**r2**	**Pr(>r)**	
long	-0.95617	0.29281	0.0429	0.294	
lat	-0.06142	-0.99811	0.0716	0.129	
AnnualMeanTemp	0.74494	-0.66713	0.0615	0.168	
MeanDiurnalRange	0.87809	0.47849	0.0366	0.349	
Isothermality	-0.34726	-0.93777	0.1746	0.007	**

TempSeasonality	0.54562	0.83803	0.0373	0.335	
MaxTempofWarmestMonth	0.99319	-0.11654	0.0393	0.320	
MinTempofColdestMonth	-0.17158	-0.98517	0.0920	0.067	.
TempAnnualRange	0.74970	0.66178	0.0437	0.288	
MeanTempofWettestQuarter	0.95123	-0.30849	0.0410	0.305	
MeanTempofDriestQuarter	-0.03452	-0.99940	0.1874	0.004	**
MeanTempofWarmestQuarter	0.95831	-0.28574	0.0466	0.251	
MeanTempofColdestQuarter	0.35550	-0.93468	0.0831	0.073	.
AnnualPrecipitation	-0.81159	0.58423	0.0213	0.562	
PrecipitationofWettestMonth	-0.49208	0.87055	0.0260	0.489	
PrecipitationofDriestMonth	-0.98383	0.17913	0.0322	0.392	
PrecipitationSeasonality	0.87755	0.47948	0.0415	0.297	
PrecipitationofWettestQuarter	-0.66226	0.74927	0.0206	0.570	
PrecipitationofDriestQuarter	-0.89062	0.45475	0.0272	0.464	
PrecipitationofWarmestQuarter	-0.96890	0.24744	0.0401	0.302	
PrecipitationofColdestQuarter	-0.98610	0.16618	0.0324	0.398	
Signif. codes: 0 ‘*’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1**					
Number of permutations: 999					

Table S4. 1 31 msGBS specific adaptors and one universal adaptor with their sequences.

31 specific adaptors						
Sample ID	**DNA Name**	**Sequence (5' - 3')**	**MW**	**μg/OD**	**GC%**	**Barcode_sequence**
RmWC001	HpaIIF.51:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTTAGAA	13147.65	30.55	50.00	CTTAGAA
RmWC002	HpaIIF.52:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACGTTGT	13141.65	30.32	52.38	ACGTTGT
RmWC003	HpaIIF.53:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGACCTA	13172.66	30.41	52.38	TGACCTA
RmWC004	HpaIIF.54:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGACAATT	13147.65	30.59	50.00	GACAATT
RmWC005	HpaIIF.55:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTGTTAT	13165.67	29.98	50.00	CTGTTAT
RmWC006	HpaIIF.56:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGTCGGA	13117.63	30.68	54.76	TGTCGGA
RmWC007	HpaIIF.57:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAAGCTT	13132.64	30.75	52.38	GAAGCTT
RmWC008	HpaIIF.58:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACCATGA	13163.65	30.66	52.38	ACCATGA
RmWC009	HpaIIF.59:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGTTAAT	13156.66	30.28	50.00	CGTTAAT
RmWC010	HpaIIF.60:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTCGGAAT	13445.85	30.51	51.16	TTCGGAAT
RmWC011	HpaIIF.61:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAGACTAA	13427.83	30.90	51.16	GAGACTAA
RmWC012	HpaIIF.62:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACACAGTA	13467.85	30.78	51.16	ACACAGTA
RmWC013	HpaIIF.63:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTTGTAAT	13469.87	30.11	48.84	CTTGTAAT
RmWC014	HpaIIF.64:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCGTGTTA	13454.86	30.21	51.16	TCGTGTTA
RmWC015	HpaIIF.65:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGAACGTT	13421.83	30.81	53.49	GGAACGTT
RmWC016	HpaIIF.66:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAACCGAAT	13467.85	30.81	51.16	AACCGAAT
RmWC017	HpaIIF.67:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTTGTGTA	13454.86	30.15	51.16	CTTGTGTA
RmWC018	HpaIIF.68:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGCTATAA	13460.86	30.36	48.84	TGCTATAA

RmWC019	HpaIIF.69:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAGAACAT	13427.83	31.00	51.16	GAGAACAT
RmWC020	HpaIIF.70:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACACGATT	13476.86	30.51	51.16	ACACGATT
RmWC021	HpaIIF.71:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGTCGAA	13445.85	30.52	51.16	TTGTCGAA
RmWC022	HpaIIF.72:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTGCTTA	13430.84	30.53	53.49	GGTGCTTA
RmWC023	HpaIIF.73:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAACATGTT	13460.86	30.40	48.84	AACATGTT
RmWC024	HpaIIF.74:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCACGCAT	13517.87	30.78	55.81	CCACGCAT
RmWC025	HpaIIF.75:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTGTAATA	13460.86	30.26	48.84	CTGTAATA
RmWC026	HpaIIF.76:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCTGTTAA	13469.87	30.15	48.84	TCTGTTAA
RmWC027	HpaIIF.77:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGAACAATA	13732.03	31.03	50.00	GGAACAATA
RmWC028	HpaIIF.78:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAACCTGCAT	13806.07	30.63	52.27	AACCTGCAT
RmWC029	HpaIIF.79:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGTACTTA	13783.08	29.96	47.73	TTGTACTTA
RmWC030	HpaIIF.80:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTGGTCAT	13720.03	30.68	54.55	GGTGGTCAT
RmWC031	HpaIIF.50:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAACGCTA	13163.65	30.66	52.38	AACGCTA
RmWC032	HpaIIF.51:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTTAGAA	13147.65	30.55	50.00	CTTAGAA
RmWC033	HpaIIF.52:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACGTTGT	13141.65	30.32	52.38	ACGTTGT
RmWC034	HpaIIF.53:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGACCTA	13172.66	30.41	52.38	TGACCTA
RmWC035	HpaIIF.54:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGACAATT	13147.65	30.59	50.00	GACAATT
RmWC036	HpaIIF.55:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTGTTAT	13165.67	29.98	50.00	CTGTTAT
RmWC037	HpaIIF.56:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGTCGGA	13117.63	30.68	54.76	TGTCGGA
RmWC038	HpaIIF.57:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAAGCTT	13132.64	30.75	52.38	GAAGCTT
RmWC039	HpaIIF.58:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACCATGA	13163.65	30.66	52.38	ACCATGA
RmWC040	HpaIIF.59:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGTTAAT	13156.66	30.28	50.00	CGTTAAT
RmWC041	HpaIIF.60:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTCGGAAT	13445.85	30.51	51.16	TTCGGAAT
RmWC042	HpaIIF.61:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAGACTAA	13427.83	30.90	51.16	GAGACTAA
RmWC043	HpaIIF.62:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACACAGTA	13467.85	30.78	51.16	ACACAGTA

RmWC044	HpaIIF.63:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTTGTAAT	13469.87	30.11	48.84	CTTGTAAT
RmWC045	HpaIIF.64:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCGTGTTA	13454.86	30.21	51.16	TCGTGTTA
RmWC046	HpaIIF.65:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGAACGTT	13421.83	30.81	53.49	GGAACGTT
RmWC047	HpaIIF.66:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAACCGAAT	13467.85	30.81	51.16	AACCGAAT
RmWC048	HpaIIF.67:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTTGTGTA	13454.86	30.15	51.16	CTTGTGTA
RmWC049	HpaIIF.68:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGCTATAA	13460.86	30.36	48.84	TGCTATAA
RmWC050	HpaIIF.69:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAGAACAT	13427.83	31.00	51.16	GAGAACAT
RmWC051	HpaIIF.70:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACACGATT	13476.86	30.51	51.16	ACACGATT
RmWC052	HpaIIF.71:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGTCGAA	13445.85	30.52	51.16	TTGTCGAA
RmWC053	HpaIIF.72:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTGCTTA	13430.84	30.53	53.49	GGTGCTTA
RmWC054	HpaIIF.73:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAACATGTT	13460.86	30.40	48.84	AACATGTT
RmWC055	HpaIIF.74:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCACGCAT	13517.87	30.78	55.81	CCACGCAT
RmWC056	HpaIIF.75:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTGTAATA	13460.86	30.26	48.84	CTGTAATA
RmWC057	HpaIIF.76:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCTGTTAA	13469.87	30.15	48.84	TCTGTTAA
RmWC058	HpaIIF.77:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGAACAATA	13732.03	31.03	50.00	GGAACAATA
RmWC059	HpaIIF.78:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAACCTGCAT	13806.07	30.63	52.27	AACCTGCAT
RmWC060	HpaIIF.79:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGTACTTA	13783.08	29.96	47.73	TTGTACTTA
WATER	HpaIIF.80:	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTGGTCAT	13720.03	30.68	54.55	GGTGGTCAT
one universal adaptor						
Sample ID	**DNA Name**	**Sequence (5' - 3')**				**Barcode_sequence**
All samples	EcoRI.F	AATTAGATCGGAAGAGCGGGGACTTTAAGC				AATTAGATCGGAAGAGCGGGGACTTTAAGC

Table S4. 2 Alpha diversity index and host individual heterozygosity.

ToadID	sites	Shannon	HL	ToadID	sites	Shannon	HL
RmC001	Kununurra	6.476	0.523	**RmC031**	Rossville	7.512	0.502
RmC002	Kununurra	5.881	0.481	**RmC032**	Rossville	6.105	0.512
RmC003	Kununurra	5.248	0.490	**RmC033**	Rossville	6.224	0.473
RmC004	Kununurra	5.949	0.480	**RmC034**	Rossville	4.964	0.469
RmC005	Kununurra	4.823	0.478	**RmC035**	Rossville	6.172	0.468
RmC006	Kununurra	4.303	0.485	**RmC036**	Rossville	4.121	0.487
RmC007	Kununurra	4.777	0.489	**RmC037**	Rossville	5.127	0.467
RmC008	Kununurra	4.352	0.501	**RmC038**	Rossville	6.977	0.474
RmC009	Kununurra	5.905	0.536	**RmC039**	Rossville	6.184	0.471
RmC010	Kununurra	5.576	0.498	**RmC040**	Rossville	6.388	0.471
RmC011	Old Theda	6.881	0.538	**RmC041**	Croydon	6.237	0.468
RmC012	Old Theda	6.043	0.511	**RmC042**	Croydon	5.978	0.502
RmC013	Old Theda	6.325	0.486	**RmC043**	Croydon	4.023	0.465
RmC014	Old Theda	3.872	0.482	**RmC044**	Croydon	5.559	0.487
RmC016	Old Theda	6.396	0.479	**RmC045**	Croydon	7.104	0.487
RmC017	Old Theda	6.028	0.530	**RmC047**	Croydon	7.523	0.460
RmC018	Old Theda	6.766	0.522	**RmC048**	Croydon	4.507	0.473
RmC019	Old Theda	6.979	0.550	**RmC049**	Croydon	5.504	0.465
RmC020	Old Theda	6.844	0.503	**RmC051**	Lucinda	6.394	0.470
RmC022	Mary pool	6.582	0.506	**RmC053**	Lucinda	6.707	0.463
RmC023	Mary Pool	6.159	0.478	**RmC054**	Lucinda	6.495	0.455
RmC024	Mary Pool	6.319	0.487	**RmC055**	Lucinda	6.014	0.451
RmC025	Mary Pool	6.706	0.499	**RmC056**	Lucinda	5.485	0.476
RmC026	Mary Pool	5.639	0.591	**RmC057**	Lucinda	7.175	0.585
RmC027	Mary Pool	5.227	0.527	**RmC058**	Lucinda	7.466	0.497
RmC028	Mary Pool	5.197	0.486	**RmC059**	Lucinda	7.246	0.460
RmC029	Mary Pool	6.641	0.524	**RmC060**	Lucinda	6.082	0.465
RmC030	Mary Pool	6.620	0.486				

Table S5. 1 Raw diet taxonomy data.

Individual	Site	Organic	Descriptor	Kingdom	Phylum	Class	Order	Family	Genus	item_count
001	Kununurra	Y	unidentifiable insect matter	Animal	Arthropoda	Insecta	-	-	-	-
001	Kununurra	Y	termite	Animal	Arthropoda	Insecta	Blattodea	Rhinotermitidae	-	-
001	Kununurra	Y	ground beetle	Animal	Arthropoda	Insecta	Coleoptera	Carabidae	-	3
001	Kununurra	Y	scarab beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	2
001	Kununurra	Y	cicada nymph - small	Animal	Arthropoda	Insecta	Hemiptera	Cicadidae	-	7
001	Kununurra	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
002	Kununurra	N	pebble <5mm	-	-	-	-	-	-	-
002	Kununurra	N	pebble >5mm	-	-	-	-	-	-	1
002	Kununurra	N	sand	-	-	-	-	-	-	-
002	Kununurra	Y	termite	Animal	Arthropoda	Insecta	Blattodea	Termitidae	-	24
002	Kununurra	Y	beetle - smooth black	Animal	Arthropoda	Insecta	Coleoptera	-	-	1
002	Kununurra	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
003	Kununurra	Y	spider	Animal	Arthropoda	Arachnida	Araneae	-	-	1
003	Kununurra	Y	jumping spider	Animal	Arthropoda	Arachnida	Araneae	Salticidae	-	1
003	Kununurra	Y	unidentifiable insect matter	Animal	Arthropoda	Insecta	-	-	-	-
003	Kununurra	Y	click beetle	Animal	Arthropoda	Insecta	Coleoptera	Elateridae	-	1
003	Kununurra	Y	leaf chafer beetle - large red	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	1
003	Kununurra	Y	giant water bug	Animal	Arthropoda	Insecta	Hemiptera	Belostomadidae	-	1
003	Kununurra	Y	cicada nymph	Animal	Arthropoda	Insecta	Hemiptera	Cicadidae	-	1
003	Kununurra	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	-	2
003	Kununurra	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
004	Kununurra	Y	unidentifiable insect matter	Animal	Arthropoda	Insecta	-	-	-	-
004	Kununurra	Y	barred cockroach	Animal	Arthropoda	Insecta	Blattodea	Blattidae	Cosmozosteria	2
004	Kununurra	Y	subterranian termite	Animal	Arthropoda	Insecta	Blattodea	Rhinotermitidae	-	250
004	Kununurra	Y	ground beetle	Animal	Arthropoda	Insecta	Coleoptera	Carabidae	-	2
004	Kununurra	Y	weevil	Animal	Arthropoda	Insecta	Coleoptera	Curculionidae	-	1
004	Kununurra	Y	click beetle	Animal	Arthropoda	Insecta	Coleoptera	Elateridae	-	1

004	Kununurra	Y	click beetle	Animal	Arthropoda	Insecta	Coleoptera	Elateridae	-	3
004	Kununurra	Y	cicada nymph	Animal	Arthropoda	Insecta	Hemiptera	Cicadidae	-	9
004	Kununurra	Y	stink bug	Animal	Arthropoda	Insecta	Hemiptera	Pentatomidae	-	5
004	Kununurra	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	8
004	Kununurra	Y	trapjaw ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Odontomachus	2
004	Kununurra	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
004	Kununurra	Y	grass	Plant	Spermatophyta	Equisetopsida	Poales	Poaceae	-	-
005	Kununurra	Y	unidentifiable insect matter	Animal	Arthropoda	Insecta	-	-	-	-
005	Kununurra	Y	arboreal termite	Animal	Arthropoda	Insecta	Blattodea	Termitidae	Nasutitermes	3
005	Kununurra	Y	click beetle	Animal	Arthropoda	Insecta	Coleoptera	Elateridae	-	4
005	Kununurra	Y	scarab beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	1
005	Kununurra	Y	Broad-headed bug	Animal	Arthropoda	Insecta	Hemiptera	Alydidae	-	1
005	Kununurra	Y	cicada nymph	Animal	Arthropoda	Insecta	Hemiptera	Cicadidae	-	40
005	Kununurra	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
006	Kununurra	Y	wolf spider	Animal	Arthropoda	Arachnida	Araneae	Lycosidae	-	2
006	Kununurra	Y	unidentifiable insect matter	Animal	Arthropoda	Insecta	-	-	-	-
006	Kununurra	Y	ground beetle	Animal	Arthropoda	Insecta	Coleoptera	Carabidae	-	1
006	Kununurra	Y	weevil	Animal	Arthropoda	Insecta	Coleoptera	Curculionidae	-	1
006	Kununurra	Y	click beetle	Animal	Arthropoda	Insecta	Coleoptera	Elateridae	-	1
006	Kununurra	Y	rounded scarab beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	5
006	Kununurra	Y	giant water bug	Animal	Arthropoda	Insecta	Hemiptera	Belostomadidae	Diplonychus	2
006	Kununurra	Y	cicada nymph – large	Animal	Arthropoda	Insecta	Hemiptera	Cicadidae	-	1
006	Kununurra	Y	cicada nymph	Animal	Arthropoda	Insecta	Hemiptera	Cicadidae	-	13
006	Kununurra	Y	burrowing bug	Animal	Arthropoda	Insecta	Hemiptera	Cydnidae	-	1
006	Kununurra	Y	bigheaded ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Pheidole	17
006	Kununurra	Y	spiny ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Polyrachis	3
006	Kununurra	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
006	Kununurra	Y	eucalyptus fruits	Plant	Tracheophyta	Eudicots	Myrtales	Myrtaceae	Eucalyptus	1
007	Kununurra	Y	spider	Animal	Arthropoda	Arachnida	Araneae	-	-	1

007	Kununurra	Y	huntsman	Animal	Arthropoda	Arachnida	Araneae	Sparassidae	-	1
007	Kununurra	Y	unidentifiable insect matter	Animal	Arthropoda	Insecta	-	-	-	-
007	Kununurra	Y	unidentifiable insect matter	Animal	Arthropoda	Insecta	-	-	-	-
007	Kununurra	Y	beetle	Animal	Arthropoda	Insecta	Coleoptera	-	-	1
007	Kununurra	Y	click beetle	Animal	Arthropoda	Insecta	Coleoptera	Elateridae	-	2
007	Kununurra	Y	click beetle	Animal	Arthropoda	Insecta	Coleoptera	Elateridae	-	1
007	Kununurra	Y	click beetle	Animal	Arthropoda	Insecta	Coleoptera	Elateridae	-	1
007	Kununurra	Y	scarab beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	1
007	Kununurra	Y	scarab beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	1
007	Kununurra	Y	earwig	Animal	Arthropoda	Insecta	Dermoptera	-	-	2
007	Kununurra	Y	earwig	Animal	Arthropoda	Insecta	Dermoptera	-	-	2
007	Kununurra	Y	cicada nymph	Animal	Arthropoda	Insecta	Hemiptera	Cicadidae	-	2
007	Kununurra	Y	cicada nymph	Animal	Arthropoda	Insecta	Hemiptera	Cicadidae	-	-
007	Kununurra	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	2
007	Kununurra	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	2
007	Kununurra	Y	green tree ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Oecophylla	28
007	Kununurra	Y	green tree ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Oecophylla	36
007	Kununurra	Y	bigheaded ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Pheidole	2
007	Kununurra	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
007	Kununurra	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
008	Kununurra	N	pebble >5mm	-	-	-	-	-	-	1
008	Kununurra	N	sand	-	-	-	-	-	-	-
008	Kununurra	Y	money spider	Animal	Arthropoda	Arachnida	Araneae	Linyphiidae	-	1
008	Kununurra	Y	crab spider	Animal	Arthropoda	Arachnida	Araneae	Thomisidae	-	2
008	Kununurra	Y	millipede	Animal	Arthropoda	Diplopoda	-	-	-	1
008	Kununurra	Y	beetle	Animal	Arthropoda	Insecta	Coleoptera	-	-	1
008	Kununurra	Y	click beetle	Animal	Arthropoda	Insecta	Coleoptera	Elateridae	-	1
008	Kununurra	Y	scarab beetle – large	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	1
008	Kununurra	Y	scarab beetle – rounded	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	8

008	Kununurra	Y	earwig	Animal	Arthropoda	Insecta	Dermoptera	-	-	1
008	Kununurra	Y	cicada nymph	Animal	Arthropoda	Insecta	Hemiptera	Cicadidae	-	1
008	Kununurra	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	-	1
008	Kununurra	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	6
008	Kununurra	Y	trapjaw ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Odontomachus	19
008	Kununurra	Y	green tree ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Oecophylla	2
008	Kununurra	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Pheidole	7
008	Kununurra	Y	caterpillar	Animal	Arthropoda	Insecta	Lepidoptera	-	-	1
008	Kununurra	Y	caterpillar	Animal	Arthropoda	Insecta	Lepidoptera	-	-	1
009	Kununurra	Y	millipede	Animal	Arthropoda	Diplopoda	-	-	-	1
009	Kununurra	Y	cockroach	Animal	Arthropoda	Insecta	Blattodea	-	-	1
009	Kununurra	Y	leaf chafer beetle - large red	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	1
009	Kununurra	Y	leaf chafer beetle - medium black	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	1
009	Kununurra	Y	earwig	Animal	Arthropoda	Insecta	Dermoptera	-	-	3
009	Kununurra	Y	cicada nymph - large	Animal	Arthropoda	Insecta	Hemiptera	Cicadidae	-	1
009	Kununurra	Y	green tree ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Oecophylla	150
009	Kununurra	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
010	Kununurra	Y	unidentifiable insect matter	Animal	Arthropoda	Insecta	-	-	-	-
010	Kununurra	Y	beetle - rows of scales	Animal	Arthropoda	Insecta	Coleoptera	-	-	-
010	Kununurra	Y	click beetle	Animal	Arthropoda	Insecta	Coleoptera	Elateridae	-	1
010	Kununurra	Y	cicada nymph - large	Animal	Arthropoda	Insecta	Hemiptera	Cicadidae	-	1
010	Kununurra	Y	cicada nymph - small	Animal	Arthropoda	Insecta	Hemiptera	Cicadidae	-	1
010	Kununurra	Y	European honeybee	Animal	Arthropoda	Insecta	Hymenoptera	Apidae	Apis	4
010	Kununurra	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	-	22
010	Kununurra	Y	green tree ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Oecophylla	14
010	Kununurra	Y	moth	Animal	Arthropoda	Insecta	Lepidoptera	-	-	1
010	Kununurra	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
011	Old Theda	Y	unidentifiable insect matter	Animal	Arthropoda	Insecta	-	-	-	-
011	Old Theda	Y	cockroach	Animal	Arthropoda	Insecta	Blattodea	-	-	-

011	Old Theda	Y	termite	Animal	Arthropoda	Insecta	Blattodea	Rhinotermitidae	-	-
011	Old Theda	Y	ground beetle	Animal	Arthropoda	Insecta	Coleoptera	Carabidae	-	1
011	Old Theda	Y	wasp	Animal	Arthropoda	Insecta	Hymenoptera	-	-	1
011	Old Theda	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	-	1
011	Old Theda	Y	meat ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Iridomyrmex	2
011	Old Theda	Y	bigheaded ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Pheidole	2
011	Old Theda	Y	spiny ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Polyrachis	2
011	Old Theda	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
011	Old Theda	Y	grass	Plant	Spermatophyta	Equisetopsida	Poales	Poaceae	-	-
012	Old Theda	Y	unidentifiable insect matter	Animal	-	-	-	-	-	-
013	Old Theda	Y	unidentifiable insect matter	Animal	-	-	-	-	-	-
013	Old Theda	Y	beetle	Animal	Arthropoda	Insecta	Coleoptera	-	-	-
013	Old Theda	Y	metallic wood-boring beetle	Animal	Arthropoda	Insecta	Coleoptera	Buprestidae	-	1
013	Old Theda	Y	ground beetle	Animal	Arthropoda	Insecta	Coleoptera	Carabidae	-	1
013	Old Theda	Y	bigheaded ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Pheidole	2
013	Old Theda	Y	grass	Plant	Spermatophyta	Equisetopsida	Poales	Poaceae	-	-
014	Old Theda	Y	unidentifiable insect matter	Animal	-	-	-	-	-	-
014	Old Theda	Y	spider	Animal	Arthropoda	Arachnida	Araneae	-	-	1
014	Old Theda	Y	centipede	Animal	Arthropoda	Chilopoda	Scolopendromorpha	Scolopendridae	Cormocephalus	1
014	Old Theda	Y	grass	Animal	Spermatophyta	Equisetopsida	Poales	Poaceae	-	-
014	Old Theda	Y	arboreal termite	Animal	Arthropoda	Insecta	Blattodea	Termitidae	Nasutitermes	3
014	Old Theda	Y	scarab beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	6
014	Old Theda	Y	meat ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Iridomyrmex	3
014	Old Theda	Y	trapjaw ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Odontomachus	5
014	Old Theda	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
015	Old Theda	Y	wolf spider	Animal	Arthropoda	Arachnida	Araneae	Lycosidae	-	1
015	Old Theda	Y	termite	Animal	Arthropoda	Insecta	Blattodea	Termitidae	-	1
015	Old Theda	Y	moth	Animal	Arthropoda	Insecta	Lepidoptera	-	-	1
015	Old Theda	Y	caterpillar	Animal	Arthropoda	Insecta	Lepidoptera	-	-	1

015	Old Theda	Y	grass	Plant	Spermatophyta	Equisetopsida	Poales	Poaceae	-	-
016	Old Theda	N	pebble >5mm	-	-	-	-	-	-	1
016	Old Theda	Y	unidentifiable insect matter	Animal	Arthropoda	Insecta	-	-	-	-
016	Old Theda	Y	termite	Animal	Arthropoda	Insecta	Blattodea	Termitidae	-	2
016	Old Theda	Y	ground beetle - green irridescent	Animal	Arthropoda	Insecta	Coleoptera	Carabidae	-	2
016	Old Theda	Y	ground beetle - rainbow irridescent	Animal	Arthropoda	Insecta	Coleoptera	Carabidae	-	1
016	Old Theda	Y	ground beetle - rugose	Animal	Arthropoda	Insecta	Coleoptera	Carabidae	-	1
016	Old Theda	Y	click beetle	Animal	Arthropoda	Insecta	Coleoptera	Elateridae	-	4
016	Old Theda	Y	assasin bug	Animal	Arthropoda	Insecta	Hemiptera	Reduviidae	-	1
016	Old Theda	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	3
016	Old Theda	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	3
016	Old Theda	Y	trapjaw ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Odontomachus	7
016	Old Theda	Y	caterpillar	Animal	Arthropoda	Insecta	Lepidoptera	-	-	1
016	Old Theda	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
017	Old Theda	Y	barred cockroach	Animal	Arthropoda	Insecta	Blattodea	Blattidae	Cosmozosteria	1
017	Old Theda	Y	arboreal termite	Animal	Arthropoda	Insecta	Blattodea	Termitidae	Nasutitermes	26
017	Old Theda	Y	trapjaw ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Odontomachus	19
017	Old Theda	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
018	Old Theda	N	pebble >5mm	-	-	-	-	-	-	2
018	Old Theda	Y	termite	Animal	Arthropoda	Insecta	Blattodea	Termitidae	-	250
018	Old Theda	Y	click beetle	Animal	Arthropoda	Insecta	Coleoptera	Elateridae	-	3
018	Old Theda	Y	darkling beetle	Animal	Arthropoda	Insecta	Coleoptera	Tenebrionidae	-	1
018	Old Theda	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	1
018	Old Theda	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	3
018	Old Theda	Y	caterpillar	Animal	Arthropoda	Insecta	Lepidoptera	-	-	1
018	Old Theda	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
018	Old Theda	Y	grass	Plant	Spermatophyta	Equisetopsida	Poales	Poaceae	-	-
019	Old Theda	Y	termite	Animal	Arthropoda	Insecta	Blattodea	Termitidae	-	40
019	Old Theda	Y	scarab beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	1

019	Old Theda	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	3
019	Old Theda	Y	metallic ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Rhytidoponera	3
019	Old Theda	Y	caterpillar	Animal	Arthropoda	Insecta	Lepidoptera	-	-	1
019	Old Theda	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
019	Old Theda	Y	grass	Plant	Spermatophyta	Equisetopsida	Poales	Poaceae	-	-
020	Old Theda	Y	unidentifiable insect matter	Animal	-	-	-	-	-	-
020	Old Theda	Y	round-backed millipede	Animal	Arthropoda	Diplopoda	Julida	-	-	7
020	Old Theda	Y	barred cockroach	Animal	Arthropoda	Insecta	Blattodea	Blattidae	Cosmozosteria	2
020	Old Theda	Y	beetle	Animal	Arthropoda	Insecta	Coleoptera	-	-	1
020	Old Theda	Y	ground beetle	Animal	Arthropoda	Insecta	Coleoptera	Carabidae	-	1
020	Old Theda	Y	scarab beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	1
020	Old Theda	Y	stink bug	Animal	Arthropoda	Insecta	Hemiptera	Pentatomidae	Poecilometis	1
020	Old Theda	Y	meat ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Iridomyrmex	23
020	Old Theda	Y	trapjaw ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Odontomachus	9
020	Old Theda	Y	green tree ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Oecophylla	5
020	Old Theda	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
021	Mary Pool	N	pebble >5mm	-	-	-	-	-	-	1
021	Mary Pool	Y	Surinam cockroach	Animal	Arthropoda	Insecta	Blattodea	Blaberidae	Pycnoscelus	3
021	Mary Pool	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	-	4
021	Mary Pool	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
021	Mary Pool	Y	grass	Plant	Spermatophyta	Equisetopsida	Poales	Poaceae	-	1
022	Mary Pool	N	sand	-	-	-	-	-	-	-
022	Mary Pool	Y	money spider	Animal	Arthropoda	Arachnida	Araneae	Linyphiidae	-	1
022	Mary Pool	Y	ground beetle	Animal	Arthropoda	Insecta	Coleoptera	Carabidae	-	1
022	Mary Pool	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	-	14
022	Mary Pool	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	4
022	Mary Pool	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
022	Mary Pool	Y	aster seed?	Plant	Tracheophyta	Eudicots	Asterales	Asteraceae	-	1
023	Mary Pool	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	1

024	Mary Pool	Y	soil centipede	Animal	Arthropoda	Chilopoda	Geophilomorpha	-	-	2
024	Mary Pool	Y	centipede	Animal	Arthropoda	Chilopoda	Scolopendromorpha	Scolopendridae	-	2
024	Mary Pool	Y	click beetle	Animal	Arthropoda	Insecta	Coleoptera	Elateridae	-	1
024	Mary Pool	Y	Christmas beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	1
024	Mary Pool	Y	scarab beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	2
024	Mary Pool	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	-	1
024	Mary Pool	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	1
024	Mary Pool	Y	caterpillar	Animal	Arthropoda	Insecta	Lepidoptera	-	-	3
024	Mary Pool	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
024	Mary Pool	Y	grass	Plant	Spermatophyta	Equisetopsida	Poales	Poaceae	-	-
025	Mary Pool	N	pebble <5mm	-	-	-	-	-	-	-
025	Mary Pool	N	pebble >5mm	-	-	-	-	-	-	1
025	Mary Pool	N	sand	-	-	-	-	-	-	-
025	Mary Pool	Y	termite	Animal	Arthropoda	Insecta	Blattodea	Termitidae	-	300
025	Mary Pool	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	-	7
025	Mary Pool	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	7
025	Mary Pool	Y	meat ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Iridomyrmex	1
025	Mary Pool	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
026	Mary Pool	N	pebble <5mm	-	-	-	-	-	-	-
026	Mary Pool	N	pebble >5mm	-	-	-	-	-	-	2
026	Mary Pool	N	sand	-	-	-	-	-	-	-
026	Mary Pool	Y	mouse spider	Animal	Arthropoda	Arachnida	Araneae	Actinopodidae	Missulena	1
026	Mary Pool	Y	soil centipede	Animal	Arthropoda	Chilopoda	Geophilomorpha	-	-	2
026	Mary Pool	Y	centipede	Animal	Arthropoda	Chilopoda	Scolopendromorpha	Scolopendridae	-	3
026	Mary Pool	Y	predacious diving beetle	Animal	Arthropoda	Insecta	Coleoptera	Dytiscidae	-	1
026	Mary Pool	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	-	11
026	Mary Pool	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	24
026	Mary Pool	Y	caterpillar	Animal	Arthropoda	Insecta	Lepidoptera	-	-	4
026	Mary Pool	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-

026	Mary Pool	Y	grass	Plant	Spermatophyta	Equisetopsida	Poales	Poaceae	-	-
027	Mary Pool	Y	click beetle	Animal	Arthropoda	Insecta	Coleoptera	Elateridae	-	1
027	Mary Pool	Y	Christmas beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	1
027	Mary Pool	Y	scarab beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	4
027	Mary Pool	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	-	70
027	Mary Pool	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	1
027	Mary Pool	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Rhytidoponera	9
027	Mary Pool	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
027	Mary Pool	Y	grass	Plant	Spermatophyta	Equisetopsida	Poales	Poaceae	-	-
028	Mary Pool	N	sand	-	-	-	-	-	-	-
028	Mary Pool	Y	Mygalomorph spider	Animal	Arthropoda	Arachnida	Araneae	-	-	1
028	Mary Pool	Y	mouse spider	Animal	Arthropoda	Arachnida	Araneae	Actinopodidae	Missulena	1
028	Mary Pool	Y	centipede	Animal	Arthropoda	Chilopoda	Scolopendromorpha	Scolopendridae	-	1
028	Mary Pool	Y	termite	Animal	Arthropoda	Insecta	Blattodea	Termitidae	-	15
028	Mary Pool	Y	weevil	Animal	Arthropoda	Insecta	Coleoptera	Curculionidae	-	1
028	Mary Pool	Y	click beetle	Animal	Arthropoda	Insecta	Coleoptera	Elateridae	-	1
028	Mary Pool	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	-	6
028	Mary Pool	Y	meat ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Iridomyrmex	15
028	Mary Pool	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Pheidole	200
028	Mary Pool	Y	spiny ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Polyrachis	12
028	Mary Pool	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
029	Mary Pool	N	sand	-	-	-	-	-	-	-
029	Mary Pool	Y	termite	Animal	Arthropoda	Insecta	Blattodea	Termitidae	-	350
029	Mary Pool	Y	scarab beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	1
029	Mary Pool	Y	stink bug	Animal	Arthropoda	Insecta	Hemiptera	Pentatomidae	Oncocoris	1
029	Mary Pool	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	2
029	Mary Pool	Y	meat ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Iridomyrmex	3
029	Mary Pool	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
029	Mary Pool	Y	grass	Plant	Spermatophyta	Equisetopsida	Poales	Poaceae	-	-

030	Mary Pool	N	sand	-	-	-	-	-	-	-
030	Mary Pool	Y	mouse spider	Animal	Arthropoda	Arachnida	Araneae	Actinopodidae	Missulena	1
030	Mary Pool	Y	centipede	Animal	Arthropoda	Chilopoda	Scolopendromorpha	Scolopendridae	-	2
030	Mary Pool	Y	termite	Animal	Arthropoda	Insecta	Blattodea	Termitidae	-	
030	Mary Pool	Y	termite	Animal	Arthropoda	Insecta	Blattodea	Termitidae	-	275
030	Mary Pool	Y	ground beetle	Animal	Arthropoda	Insecta	Coleoptera	Carabidae	-	1
030	Mary Pool	Y	weevil	Animal	Arthropoda	Insecta	Coleoptera	Curculionidae	-	1
030	Mary Pool	Y	click beetle	Animal	Arthropoda	Insecta	Coleoptera	Elateridae	-	1
030	Mary Pool	Y	darkling beetle	Animal	Arthropoda	Insecta	Coleoptera	Tenebrionidae	-	1
030	Mary Pool	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	2
030	Mary Pool	Y	spiny ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Polyrachis	11
030	Mary Pool	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
030	Mary Pool	Y	grass	Plant	Spermatophyta	Equisetopsida	Poales	Poaceae	-	-
031	Rossville	N	pebble <5mm	-	-	-	-	-	-	-
031	Rossville	Y	crab spider	Animal	Arthropoda	Arachnida	Araneae	Thomisidae	-	2
032	Rossville	Y	scarab beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	2
032	Rossville	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	-	1
032	Rossville	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	2
032	Rossville	Y	trapjaw ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Odontomachus	2
032	Rossville	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Rhytidoponera	3
032	Rossville	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
033	Rossville	Y	unidentifiable insect matter	Animal	Arthropoda	Insecta	-	-	-	-
033	Rossville	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	12
033	Rossville	Y	mole cricket	Animal	Arthropoda	Insecta	Orthoptera	Gryllotalpidae	-	1
035	Rossville	N	pebble <5mm	-	-	-	-	-	-	-
035	Rossville	Y	German cockroach	Animal	Arthropoda	Insecta	Blattodea	Ectobiidae	Blatella	1
035	Rossville	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	1
035	Rossville	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
035	Rossville	Y	grass	Plant	Spermatophyta	Equisetopsida	Poales	Poaceae	-	-

036	Rossville	Y	scarab beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	1
036	Rossville	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	13
036	Rossville	Y	trapjaw ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Odontomachus	1
036	Rossville	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
037	Rossville	N	pebble <5mm	-	-	-	-	-	-	-
037	Rossville	Y	unidentifiable insect matter	Animal	Arthropoda	Insecta	-	-	-	-
037	Rossville	Y	trapjaw ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Odontomachus	2
037	Rossville	Y	green tree ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Oecophylla	18
037	Rossville	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
038	Rossville	N	sand	-	-	-	-	-	-	-
038	Rossville	Y	unidentifiable insect matter	Animal	Arthropoda	Insecta	-	-	-	-
038	Rossville	Y	beetle	Animal	Arthropoda	Insecta	Coleoptera	-	-	1
038	Rossville	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	1
038	Rossville	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Pheidole	16
038	Rossville	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
039	Rossville	Y	unidentifiable insect matter	Animal	Arthropoda	Insecta	-	-	-	-
039	Rossville	Y	beetle	Animal	Arthropoda	Insecta	Coleoptera	-	-	1
039	Rossville	Y	scarab beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	1
039	Rossville	Y	earwig	Animal	Arthropoda	Insecta	Dermoptera	-	-	1
039	Rossville	Y	burrowing bug	Animal	Arthropoda	Insecta	Hemiptera	Cydnidae	-	1
039	Rossville	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	7
039	Rossville	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
040	Rossville	Y	unidentifiable insect matter	Animal	Arthropoda	Insecta	-	-	-	-
040	Rossville	Y	beetle	Animal	Arthropoda	Insecta	Coleoptera	-	-	1
040	Rossville	Y	beetle	Animal	Arthropoda	Insecta	Coleoptera	-	-	1
040	Rossville	Y	weevil	Animal	Arthropoda	Insecta	Coleoptera	Curculionidae	-	1
040	Rossville	Y	weevil	Animal	Arthropoda	Insecta	Coleoptera	Curculionidae	-	1
040	Rossville	Y	weevil	Animal	Arthropoda	Insecta	Coleoptera	Curculionidae	-	1
040	Rossville	Y	scarab beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	3

040	Rossville	Y	rove beetle	Animal	Arthropoda	Insecta	Coleoptera	Staphylinidae	-	1
040	Rossville	Y	true bug	Animal	Arthropoda	Insecta	Hemiptera	-	-	1
040	Rossville	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	1
040	Rossville	Y	trapjaw ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Odontomachus	1
040	Rossville	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
041	Croydon	N	sand	-	-	-	-	-	-	-
042	Croydon	N	pebble <5mm	-	-	-	-	-	-	3
042	Croydon	Y	mite	Animal	Arthropoda	Arachnida	Acari	-	-	1
042	Croydon	Y	ground beetle	Animal	Arthropoda	Insecta	Coleoptera	Carabidae	-	2
042	Croydon	Y	leaf beetle	Animal	Arthropoda	Insecta	Coleoptera	Chrysomelidae	-	1
042	Croydon	Y	net-winged beetle	Animal	Arthropoda	Insecta	Coleoptera	Lycidae	-	2
042	Croydon	Y	fly	Animal	Arthropoda	Insecta	Diptera	-	-	1
042	Croydon	Y	damsel bug	Animal	Arthropoda	Insecta	Hemiptera	Nabidae	-	1
042	Croydon	Y	moth	Animal	Arthropoda	Insecta	Lepidoptera	-	-	2
042	Croydon	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
043	Croydon	N	pebble <5mm	-	-	-	-	-	-	-
043	Croydon	Y	Surinam cockroach	Animal	Arthropoda	Insecta	Blattodea	Blaberidae	Pycnoscelus	1
043	Croydon	Y	net-winged beetle	Animal	Arthropoda	Insecta	Coleoptera	Lycidae	-	1
043	Croydon	Y	burrowing bug	Animal	Arthropoda	Insecta	Hemiptera	Cydnidae	-	6
043	Croydon	Y	seed bug	Animal	Arthropoda	Insecta	Hemiptera	Lygaeidae	-	4
043	Croydon	Y	seed bug head+thorax	Animal	Arthropoda	Insecta	Hemiptera	Lygaeidae	-	16
043	Croydon	Y	seed bug	Animal	Arthropoda	Insecta	Hemiptera	Lygaeidae	-	1
043	Croydon	Y	seed bug	Animal	Arthropoda	Insecta	Hemiptera	Lygaeidae	Graptostethus	9
043	Croydon	Y	seed bug	Animal	Arthropoda	Insecta	Hemiptera	Lygaeidae	Stenophyella	1
043	Croydon	Y	stink bug	Animal	Arthropoda	Insecta	Hemiptera	Pentatomidae	Cuspicona	4
043	Croydon	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	-	35
043	Croydon	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
044	Croydon	N	sand	-	-	-	-	-	-	-
044	Croydon	Y	wasp	Animal	Arthropoda	Insecta	Hymenoptera	-	-	1

044	Croydon	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
045	Croydon	Y	ground beetle	Animal	Arthropoda	Insecta	Coleoptera	Carabidae	-	1
045	Croydon	Y	darkling beetle	Animal	Arthropoda	Insecta	Coleoptera	Tenebrionidae	-	1
045	Croydon	Y	burrowing bug	Animal	Arthropoda	Insecta	Hemiptera	Cydnidae	-	3
045	Croydon	Y	seed bug	Animal	Arthropoda	Insecta	Hemiptera	Lygaeidae	Graptostethus	8
045	Croydon	Y	seed bug	Animal	Arthropoda	Insecta	Hemiptera	Lygaeidae	Stenophyella	1
045	Croydon	Y	stink bug	Animal	Arthropoda	Insecta	Hemiptera	Pentatomidae	Cuspicona	1
045	Croydon	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	-	1
045	Croydon	Y	moth	Animal	Arthropoda	Insecta	Lepidoptera	-	-	1
045	Croydon	Y	wood louse	Animal	Arthropoda	Malacostraca	Isopoda	-	-	1
046	Croydon	N	pebble <5mm	-	-	-	-	-	-	-
046	Croydon	N	pebble >5mm	-	-	-	-	-	-	1
046	Croydon	Y	beetle	Animal	Arthropoda	Insecta	Coleoptera	-	-	2
046	Croydon	Y	net-winged beetle	Animal	Arthropoda	Insecta	Coleoptera	Lycidae	-	1
046	Croydon	Y	scarab beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	1
046	Croydon	Y	burrowing bug	Animal	Arthropoda	Insecta	Hemiptera	Cydnidae	-	2
046	Croydon	Y	seed bug	Animal	Arthropoda	Insecta	Hemiptera	Lygaeidae	Graptostethus	1
046	Croydon	Y	stink bug	Animal	Arthropoda	Insecta	Hemiptera	Pentatomidae	Cuspicona	12
046	Croydon	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	-	29
046	Croydon	Y	antlion	Animal	Arthropoda	Insecta	Neuroptera	Myrmeleontidae	-	1
046	Croydon	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
047	Croydon	N	pebble >5mm	-	-	-	-	-	-	1
047	Croydon	Y	spider	Animal	Arthropoda	Arachnida	Araneae	-	-	1
047	Croydon	Y	auger beetle	Animal	Arthropoda	Insecta	Coleoptera	Bostrichidae	-	1
047	Croydon	Y	weevil	Animal	Arthropoda	Insecta	Coleoptera	Curculionidae	-	1
047	Croydon	Y	click beetle	Animal	Arthropoda	Insecta	Coleoptera	Elateridae	-	1
047	Croydon	Y	stink bug	Animal	Arthropoda	Insecta	Hemiptera	Pentatomidae	Oncocoris	9
047	Croydon	Y	other ants	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	-	11
047	Croydon	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	2

047	Croydon	Y	meat ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Iridomyrmex	1
047	Croydon	Y	raspy cricket	Animal	Arthropoda	Insecta	Orthoptera	Gryllacrididae	-	1
047	Croydon	Y	thrips	Animal	Arthropoda	Insecta	Thysanoptera	-	-	1
047	Croydon	Y	achene	Plant	Tracheophyta	Eudicots	-	-	-	12
048	Croydon	Y	woodland cockroach	Animal	Arthropoda	Insecta	Blattodea	Blattidae	Melanozosteria	3
048	Croydon	Y	net-winged beetle	Animal	Arthropoda	Insecta	Coleoptera	Lycidae	-	1
048	Croydon	Y	cicada nymph	Animal	Arthropoda	Insecta	Hemiptera	Cicadidae	-	1
048	Croydon	Y	emisine assassin bug	Animal	Arthropoda	Insecta	Hemiptera	Reduviidae	-	1
048	Croydon	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	-	15
048	Croydon	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
049	Croydon	N	pebble <5mm	-	-	-	-	-	-	6
049	Croydon	Y	beetle	Animal	Arthropoda	Insecta	Coleoptera	-	-	2
049	Croydon	Y	earwig	Animal	Arthropoda	Insecta	Dermoptera	-	-	1
049	Croydon	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	-	9
049	Croydon	Y	antlion	Animal	Arthropoda	Insecta	Neuroptera	Myrmeleontidae	-	1
049	Croydon	Y	grasshopper	Animal	Arthropoda	Insecta	Orthoptera	-	-	1
049	Croydon	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
049	Croydon	Y	grass	Plant	Spermatophyta	Equisetopsida	Poales	Poaceae	-	-
050	Croydon	Y	money spider	Animal	Arthropoda	Arachnida	Araneae	Linyphiidae	-	4
050	Croydon	Y	ground beetle	Animal	Arthropoda	Insecta	Coleoptera	Carabidae	-	1
050	Croydon	Y	ground beetle	Animal	Arthropoda	Insecta	Coleoptera	Carabidae	-	1
050	Croydon	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	-	1
050	Croydon	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	1
050	Croydon	Y	moth	Animal	Arthropoda	Insecta	Lepidoptera	-	-	1
051	Lucinda	Y	burrowing bug	Animal	Arthropoda	Insecta	Hemiptera	Cydnidae	-	150
051	Lucinda	Y	arboreal termite	Animal	Arthropoda	Insecta	Blattodea	Termitidae	Nasutitermes	20
051	Lucinda	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Pheidole	20
051	Lucinda	Y	earwig	Animal	Arthropoda	Insecta	Dermoptera	-	-	2
051	Lucinda	Y	Surinam cockroach	Animal	Arthropoda	Insecta	Blattodea	Blaberidae	Pycnoscelus	1

051	Lucinda	Y	scarab beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	1
051	Lucinda	Y	scarab beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	1
051	Lucinda	Y	ground beetle	Animal	Arthropoda	Insecta	Coleoptera	Carabidae	-	1
051	Lucinda	Y	spiny ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Polyrachis	1
051	Lucinda	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	-	50
051	Lucinda	Y	ground beetle	Animal	Arthropoda	Insecta	Coleoptera	Carabidae	-	1
051	Lucinda	Y	unidentifiable insect matter	Animal	Arthropoda	Insecta	-	-	-	-
051	Lucinda	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
052	Lucinda	Y	grasshopper	Animal	Arthropoda	Insecta	Orthoptera	Acrididae	-	1
052	Lucinda	Y	weevil	Animal	Arthropoda	Insecta	Coleoptera	Curculionidae	-	1
052	Lucinda	Y	burrowing bug	Animal	Arthropoda	Insecta	Hemiptera	Cydnidae	-	2
052	Lucinda	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Pheidole	40
052	Lucinda	Y	unidentifiable insect matter	Animal	-	Insecta	-	-	-	-
053	Lucinda	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	10
053	Lucinda	Y	scarab beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	1
053	Lucinda	Y	click beetle	Animal	Arthropoda	Insecta	Coleoptera	Elateridae	-	1
053	Lucinda	Y	unidentifiable insect matter	Animal	Arthropoda	Insecta	-	-	-	-
053	Lucinda	Y	unidentifiable insect matter	Animal	Arthropoda	Insecta	-	-	-	-
054	Lucinda	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	2
054	Lucinda	Y	weevil	Animal	Arthropoda	Insecta	Coleoptera	Curculionidae	-	1
054	Lucinda	Y	unidentifiable insect matter	Animal	Arthropoda	Insecta	-	-	-	-
054	Lucinda	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
054	Lucinda	N	pebble >5mm	-	-	-	-	-	-	-
055	Lucinda	Y	termite alate	Animal	Arthropoda	Insecta	Blattodea	Termitidae	-	700
056	Lucinda	Y	weevil	Animal	Arthropoda	Insecta	Coleoptera	Curculionidae	-	1
056	Lucinda	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	7
056	Lucinda	Y	stink bug	Animal	Arthropoda	Insecta	Hemiptera	Pentatomidae	Poecilometis	1
056	Lucinda	Y	wolf spider	Animal	Arthropoda	Arachnida	Araneae	Lycosidae	-	3
056	Lucinda	Y	scarab beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	1

056	Lucinda	Y	snail	Animal	Mollusca	Gastropoda	Stylommatophora	-	-	1
056	Lucinda	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	-	7
056	Lucinda	Y	unidentifiable insect matter	Animal	Arthropoda	Insecta	-	-	-	-
056	Lucinda	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
057	Lucinda	Y	unidentifiable insect matter	Animal	Arthropoda	Insecta	-	-	-	-
057	Lucinda	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
058	Lucinda	Y	scarab beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	11
058	Lucinda	Y	scarab beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	4
058	Lucinda	Y	scarab beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae		1
058	Lucinda	Y	spiny ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Polyrachis	1
058	Lucinda	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Pheidole	10
058	Lucinda	Y	green tree ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Oecophylla	1
058	Lucinda	Y	earwig	Animal	Arthropoda	Insecta	Dermoptera	-	-	1
058	Lucinda	Y	stink bug	Animal	Arthropoda	Insecta	Hemiptera	Pentatomidae	Poecilometis	1
058	Lucinda	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	2
058	Lucinda	Y	rove beetle	Animal	Arthropoda	Insecta	Coleoptera	Staphylinidae	-	1
058	Lucinda	Y	unidentifiable insect matter	Animal	Arthropoda	Insecta	-	-	-	-
058	Lucinda	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
058	Lucinda	N	pebble <5mm	-	-	-	-	-	-	2
058	Lucinda	Y	weevil	Animal	Arthropoda	Insecta	Coleoptera	Curculionidae	-	1
059	Lucinda	N	pebble >5mm	-	-	-	-	-	-	2
059	Lucinda	Y	burrowing bug	Animal	Arthropoda	Insecta	Hemiptera	Cydnidae	-	52
059	Lucinda	Y	scarab beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	12
059	Lucinda	Y	carpenter ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Camponotus	17
059	Lucinda	Y	stink bug	Animal	Arthropoda	Insecta	Hemiptera	Pentatomidae	Poecilometis	1
059	Lucinda	Y	jewel beetle	Animal	Arthropoda	Insecta	Coleoptera	Buprestidae	-	1
059	Lucinda	Y	unidentifiable insect matter	Animal	Arthropoda	Insecta	-	-	-	-
059	Lucinda	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-
060	Lucinda	Y	leaf chafer	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	1

060	Lucinda	Y	weevil	Animal	Arthropoda	Insecta	Coleoptera	Curculionidae	-	3
060	Lucinda	Y	click beetle	Animal	Arthropoda	Insecta	Coleoptera	Elateridae	-	1
060	Lucinda	Y	green tree ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Oecophylla	30
060	Lucinda	Y	beetle	Animal	Arthropoda	Insecta	Coleoptera	-	-	1
060	Lucinda	Y	scarab beetle	Animal	Arthropoda	Insecta	Coleoptera	Scarabaeidae	-	1
060	Lucinda	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	Pheidole	13
060	Lucinda	Y	ant	Animal	Arthropoda	Insecta	Hymenoptera	Formicidae	-	3
060	Lucinda	Y	unidentifiable insect matter	Animal	Arthropoda	Insecta	-	-	-	-
060	Lucinda	Y	true bug	Animal	Arthropoda	Insecta	Hemiptera	-	-	1
060	Lucinda	Y	unidentifiable plant matter	Plant	-	-	-	-	-	-

Table S5. 2 Nonmetric multidimensional scaling (nMDS) analysis of the relationship between host diet and bacterial community using Bray Curtis distances.

The association between diet alpha diversity and bacterial community using *envfit* function ("Vegan" R package)									
Host factor	**MDS1**	**MDS2**	**r2**	**Pr(>r)**					
Observed	-0.259	-0.966	0.053	0.25					
Chao1	-0.799	-0.602	0.005	0.873					
Shannon	0.009	-1.000	0.035	0.398					
The association between single diet composition and predicted bacterial function using *envfit* function ("Vegan" R package)									
Host factor	**MDS1**	**MDS2**	**r2**	**Pr(>r)**					
plant.matter	0.889	-0.458	0.179	0.01	**				
pebble	-0.580	-0.815	0.001	0.969					
sand	-0.668	-0.744	0.039	0.345					
						Diet Taxa information			
						Kingdom	Class	Order	Family
diet_taxa1	-0.980	-0.198	0.035	0.344		Animal	Arachnida	Acari	-
diet_taxa2	0.179	-0.984	0.004	0.893		Animal	Arachnida	Araneae	-
diet_taxa3	0.897	-0.443	0.007	0.84		Animal	Arachnida	Araneae	Actinopodidae
diet_taxa4	-0.833	-0.553	0.033	0.415		Animal	Arachnida	Araneae	Linyphiidae
diet_taxa5	0.785	0.619	0.137	0.017	*	Animal	Arachnida	Araneae	Lycosidae
diet_taxa6	-0.775	-0.631	0.019	0.659		Animal	Arachnida	Araneae	Salticidae
diet_taxa7	-0.041	-0.999	0.053	0.287		Animal	Arachnida	Araneae	Sparassidae
diet_taxa8	-0.110	0.994	0.078	0.125		Animal	Arachnida	Araneae	Thomisidae
diet_taxa9	-0.416	-0.909	0.008	0.82		Animal	Chilopoda	Geophilomorpha	-
diet_taxa10	0.601	-0.799	0.013	0.694		Animal	Chilopoda	Scolopendromorpha	Scolopendridae
diet_taxa11	-0.435	-0.900	0.073	0.139		Animal	Diplopoda	-	-
diet_taxa12	-0.043	0.999	0.027	0.488		Animal	Diplopoda	Julida	-
diet_taxa13	0.956	0.292	0.022	0.525		Animal	Gastropoda	Stylommatophora	-
diet_taxa14	0.643	-0.766	0.004	0.875		Animal	Insecta	Blattodea	
diet_taxa15	0.713	-0.701	0.150	0.003	**	Animal	Insecta	Blattodea	Blaberidae
diet_taxa16	0.669	0.743	0.111	0.048	*	Animal	Insecta	Blattodea	Blattidae
diet_taxa17	0.945	-0.326	0.013	0.716		Animal	Insecta	Blattodea	Ectobiidae
diet_taxa18	0.724	0.689	0.000	1		Animal	Insecta	Blattodea	Rhinotermitidae
diet_taxa19	-0.673	0.740	0.034	0.414		Animal	Insecta	Blattodea	Termitidae
diet_taxa20	-0.298	-0.955	0.104	0.048	*	Animal	Insecta	Coleoptera	-
diet_taxa21	-0.226	0.974	0.022	0.597		Animal	Insecta	Coleoptera	Bostrichidae
diet_taxa22	-0.709	-0.705	0.003	0.936		Animal	Insecta	Coleoptera	Buprestidae
diet_taxa23	-0.998	0.065	0.061	0.187		Animal	Insecta	Coleoptera	Carabidae
diet_taxa24	-0.980	-0.198	0.035	0.344		Animal	Insecta	Coleoptera	Chrysomelidae

diet_taxa25	-0.031	-1.000	0.019	0.592		Animal	Insecta	Coleoptera	Curculionidae
diet_taxa26	0.945	-0.326	0.004	0.925		Animal	Insecta	Coleoptera	Dytiscidae
diet_taxa27	-0.820	-0.572	0.050	0.24		Animal	Insecta	Coleoptera	Elateridae
diet_taxa28	-0.992	-0.126	0.013	0.686		Animal	Insecta	Coleoptera	Lycidae
diet_taxa29	0.203	-0.979	0.025	0.538		Animal	Insecta	Coleoptera	Scarabaeidae
diet_taxa30	-0.065	-0.998	0.014	0.684		Animal	Insecta	Coleoptera	Staphylinidae
diet_taxa31	-0.438	0.899	0.021	0.606		Animal	Insecta	Coleoptera	Tenebrionidae
diet_taxa32	-0.434	-0.901	0.043	0.319		Animal	Insecta	Dermoptera	-
diet_taxa33	-0.980	-0.198	0.035	0.344		Animal	Insecta	Diptera	-
diet_taxa34	0.015	-1.000	0.039	0.35		Animal	Insecta	Hemiptera	-
diet_taxa35	0.500	-0.866	0.007	0.848		Animal	Insecta	Hemiptera	Alydidae
diet_taxa36	-0.325	-0.946	0.024	0.543		Animal	Insecta	Hemiptera	Belostomadidae
diet_taxa37	0.279	-0.960	0.021	0.59		Animal	Insecta	Hemiptera	Cicadidae
diet_taxa38	-0.498	0.867	0.048	0.271		Animal	Insecta	Hemiptera	Cydnidae
diet_taxa39	0.812	0.584	0.029	0.485		Animal	Insecta	Hemiptera	Lygaeidae
diet_taxa40	-0.980	-0.198	0.035	0.344		Animal	Insecta	Hemiptera	Nabidae
diet_taxa41	-0.981	0.196	0.003	0.924		Animal	Insecta	Hemiptera	Pentatomidae
diet_taxa42	0.483	0.876	0.049	0.256		Animal	Insecta	Hemiptera	Reduviidae
diet_taxa43	-0.076	-0.997	0.020	0.606		Animal	Insecta	Hymenoptera	-
diet_taxa44	0.085	-0.996	0.045	0.29		Animal	Insecta	Hymenoptera	Apidae
diet_taxa45	0.905	-0.426	0.024	0.501		Animal	Insecta	Hymenoptera	Formicidae
diet_taxa46	0.423	0.906	0.031	0.45		Animal	Insecta	Lepidoptera	-
diet_taxa47	-0.460	-0.888	0.083	0.102		Animal	Insecta	Neuroptera	Myrmeleontidae
diet_taxa48	-0.487	-0.874	0.052	0.252		Animal	Insecta	Orthoptera	-
diet_taxa49	-0.440	0.898	0.061	0.25		Animal	Insecta	Orthoptera	Acrididae
diet_taxa50	0.797	0.605	0.006	0.854		Animal	Insecta	Orthoptera	Gryllotalpidae
diet_taxa51	-0.226	0.974	0.022	0.597		Animal	Insecta	Orthoptera	Gryllacrididae
diet_taxa52	-0.226	0.974	0.022	0.597		Animal	Insecta	Thysanoptera	-
diet_taxa53	-0.450	0.893	0.018	0.678		Animal	Malacostraca	Isopoda	-

Signif. codes: 0 ‘*’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1**

Number of permutations: 999

Appendix II Supplemental Figures

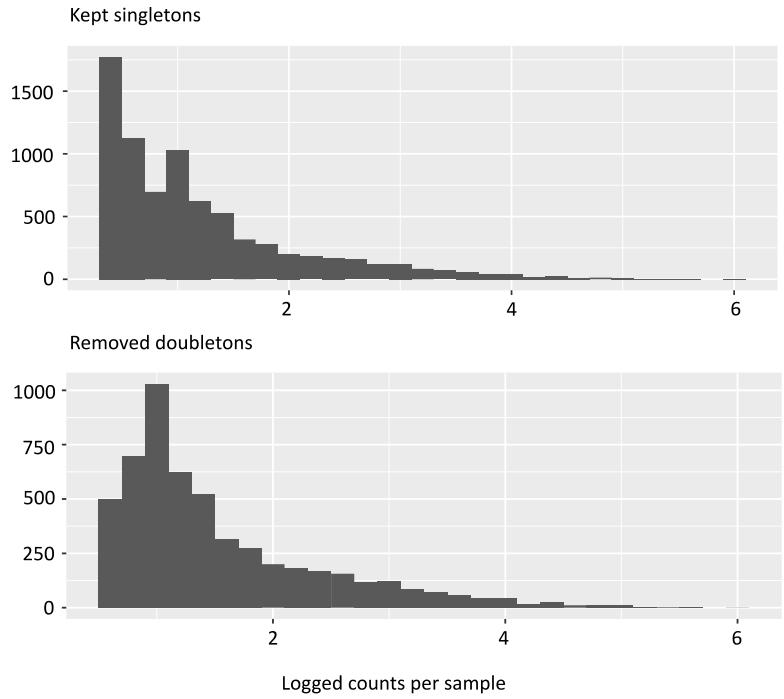


Figure S2. 1 Amplicon sequence variants (ASVs) pruning process comparison between removing the ASVs with less than 4 abundance with keeping singletons.

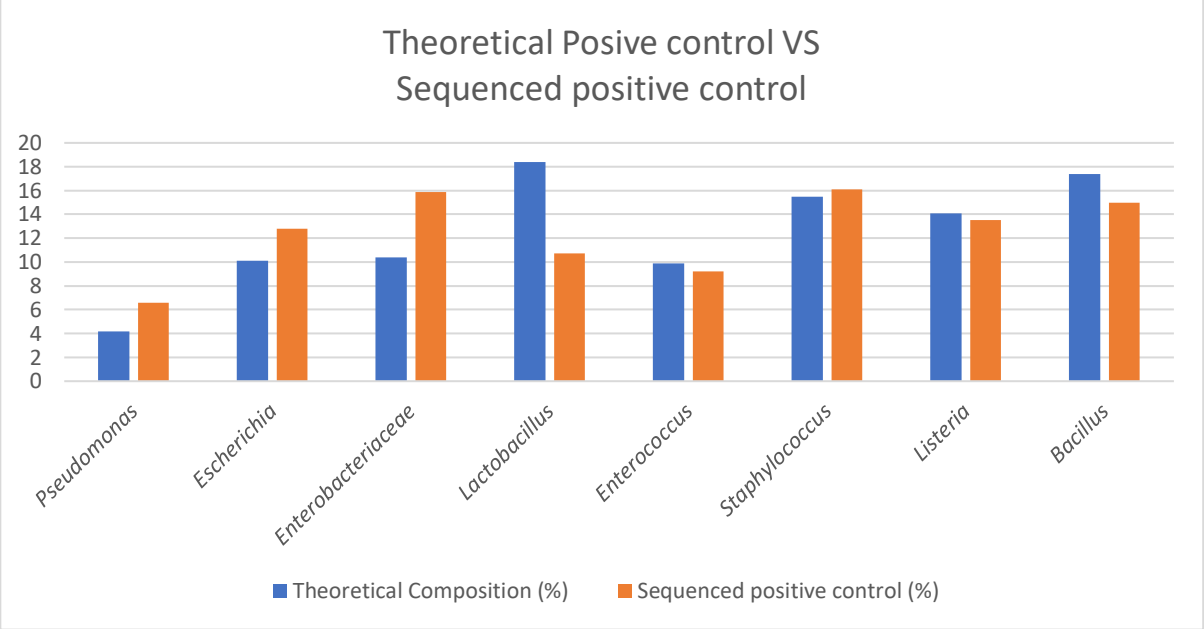


Figure S2. 2 The comparison of genera between theoretical community control and sequenced positive control.

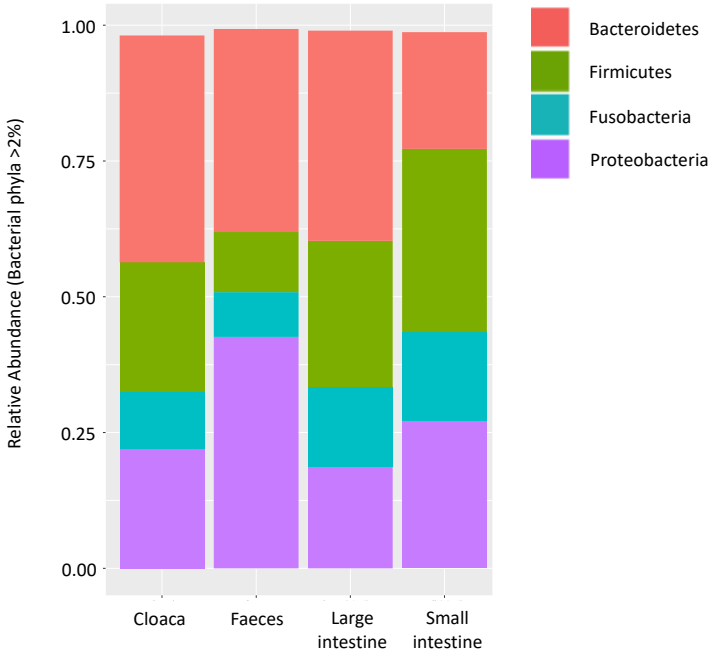
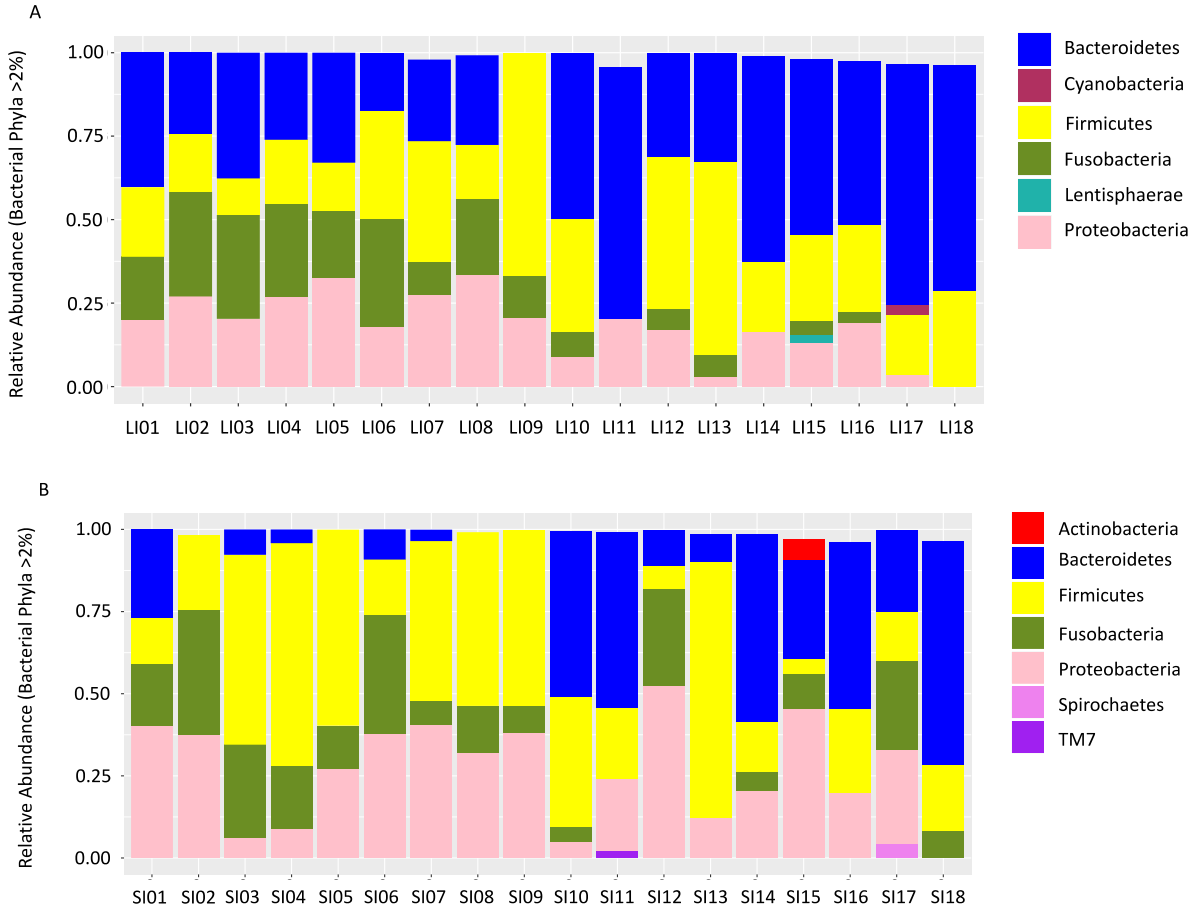


Figure S2. 3 Bacterial community composition of different samples types. Relative abundance plot shows phyla (>2%).



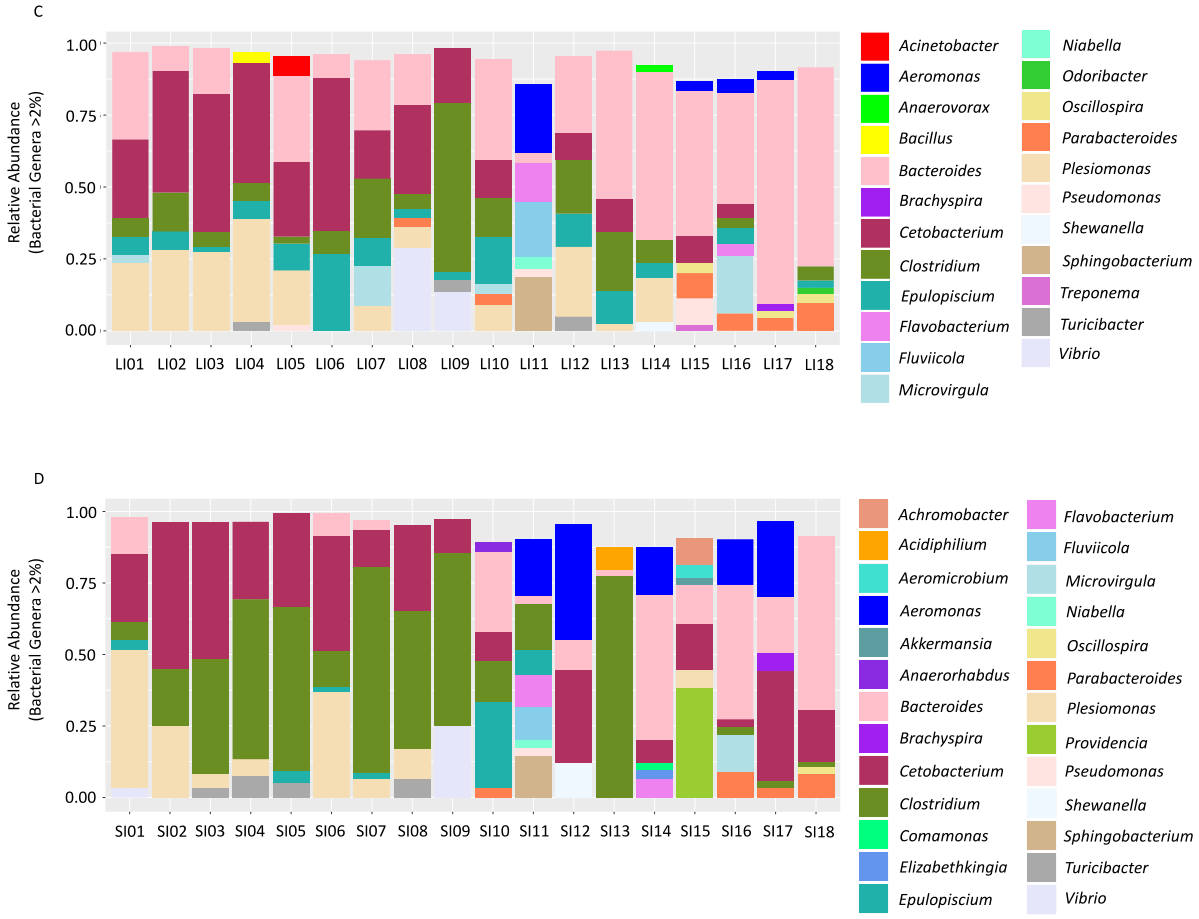


Figure S2. 4 Eighteen individual cane toads' intestinal bacterial community composition.

Relative abundance plot shows phyla (>2%) (A, B) and genera (>2%) (C, D) composition in large intestine (A, C) and small intestine (B, D).

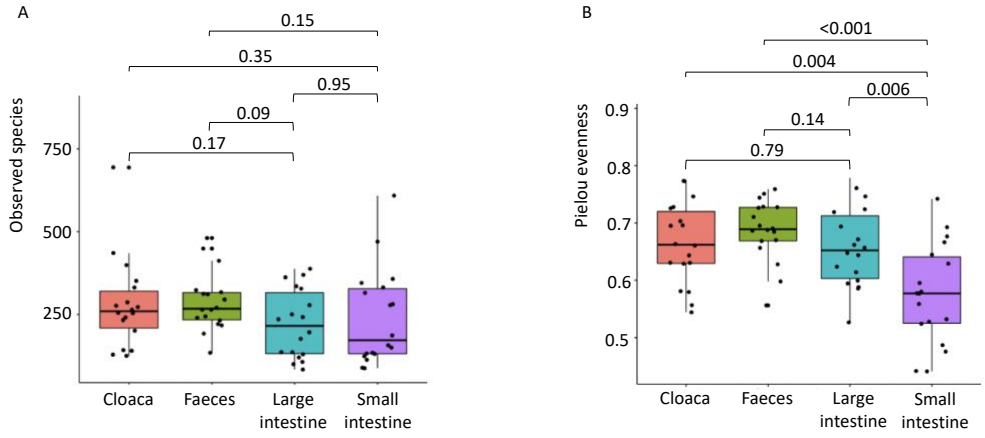


Figure S2. 5 Alpha diversity of different sample types.

Alpha diversity box plots showing observed species (A) and Pielou evenness (B). Pairwise testing between non-lethal sample groups (cloaca, faeces) and intestine sample groups (small intestine and large intestine), and between small intestine and large intestine samples were conducted using Wilcoxon signed-rank test with p-values indicated.

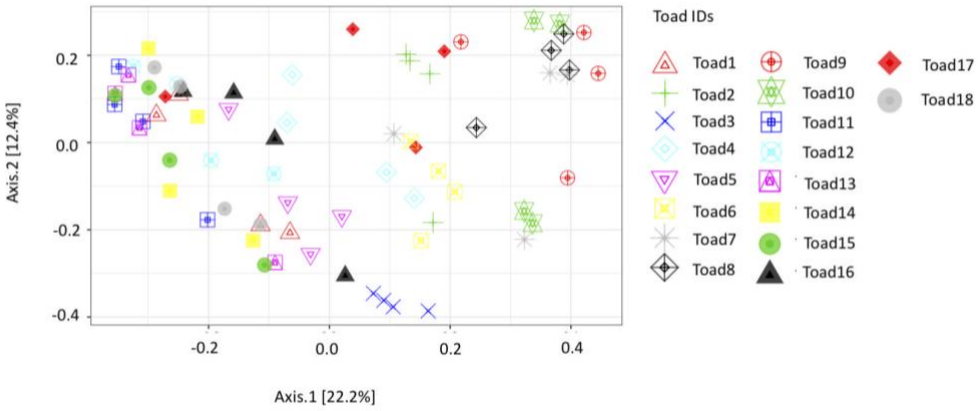


Figure S2. 6 Beta diversity by toad ID.

Principle coordinate analysis plot of Bray Curtis distances of 18 cane toad individuals, clustered by toad ID.

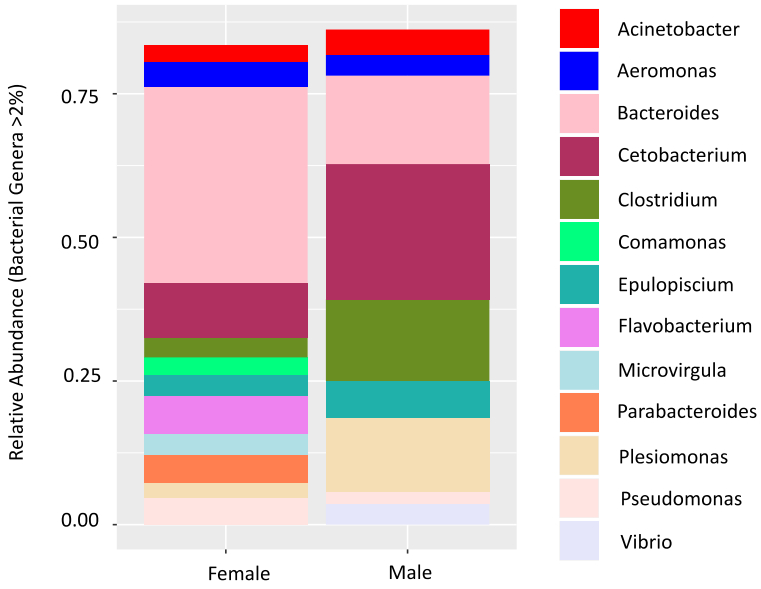


Figure S2. 7 Bacterial community composition in female and male toads.

Relative abundance bar plots display genera (>2%).

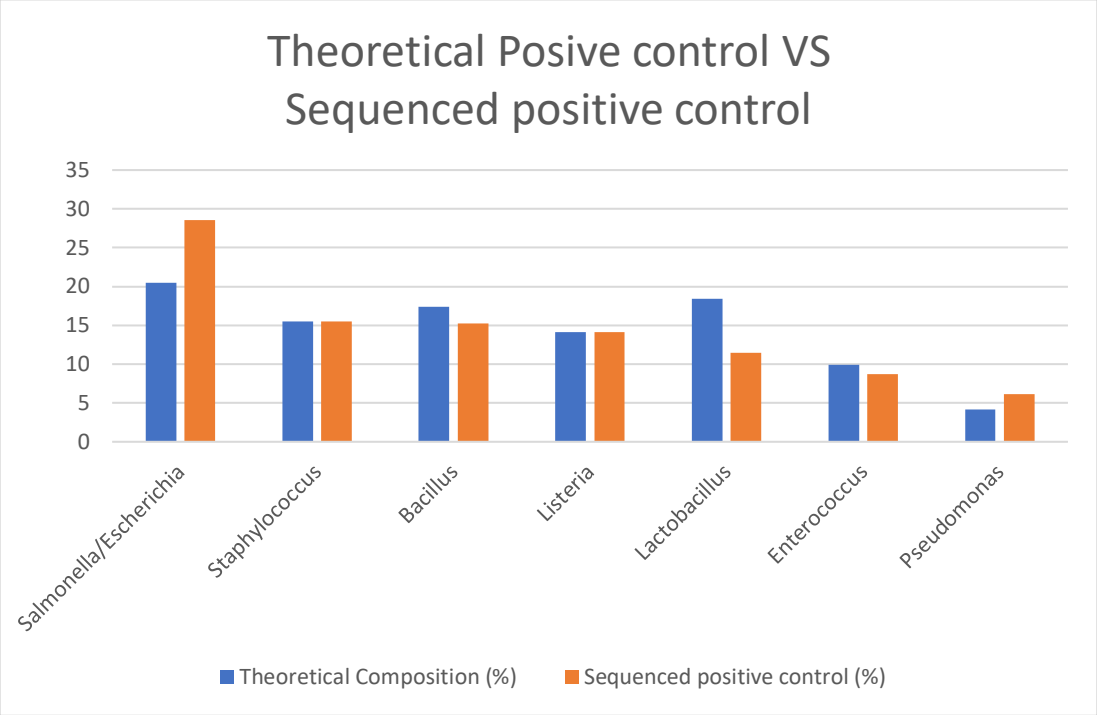
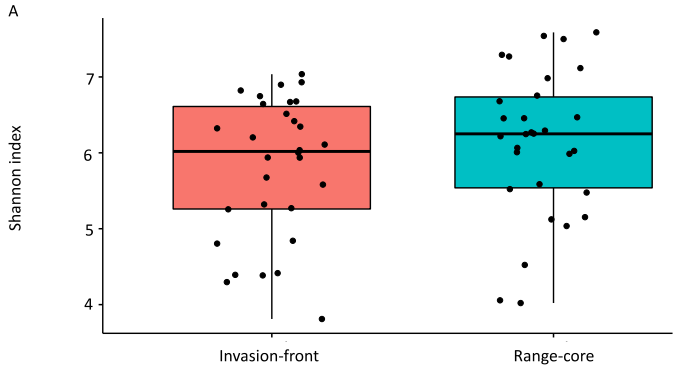


Figure S3. 1 The comparison of genera between theoretical community control and sequenced positive control.



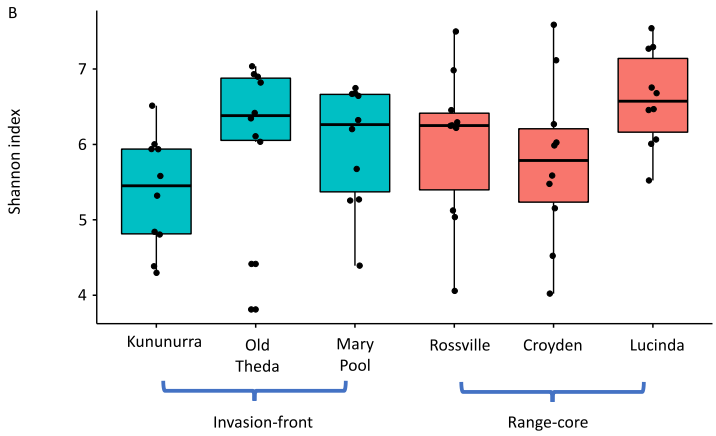


Figure S3. 2 Bacterial community Shannon diversity.

Boxplots show the Shannon index generated through QIIME2 with pruned data (Table S2) for locations (P = 0.23, Wilcoxon signed-rank test with p-values adjusted with Hochberg) (A) and sites (B).

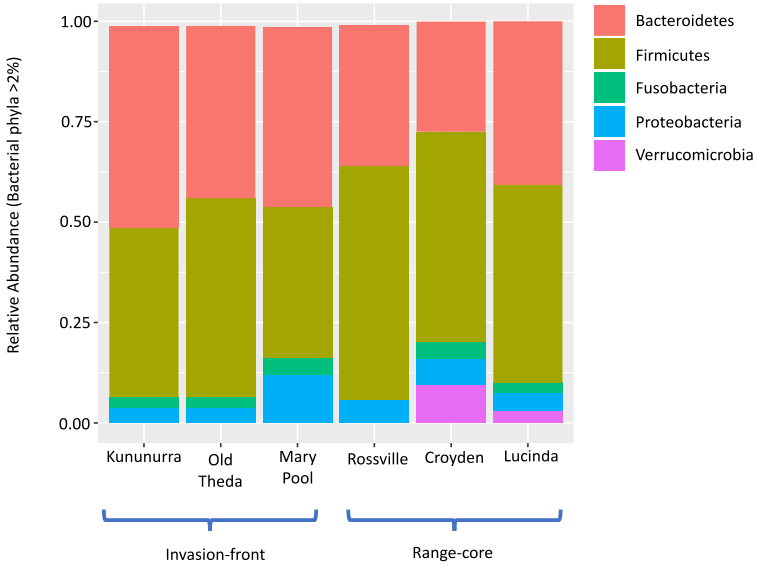


Figure S3. 3 Core bacterial community composition of different sites.

Relative abundance plot shows phyla (>2%).

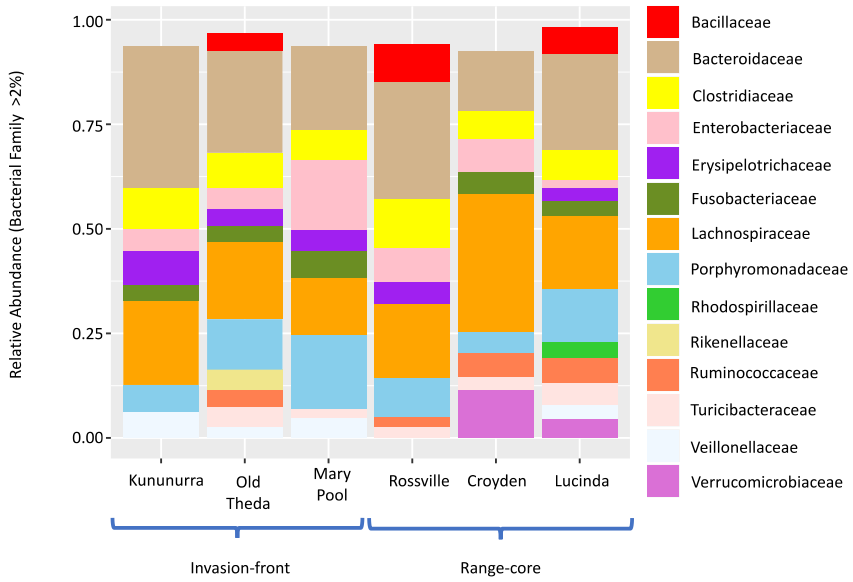
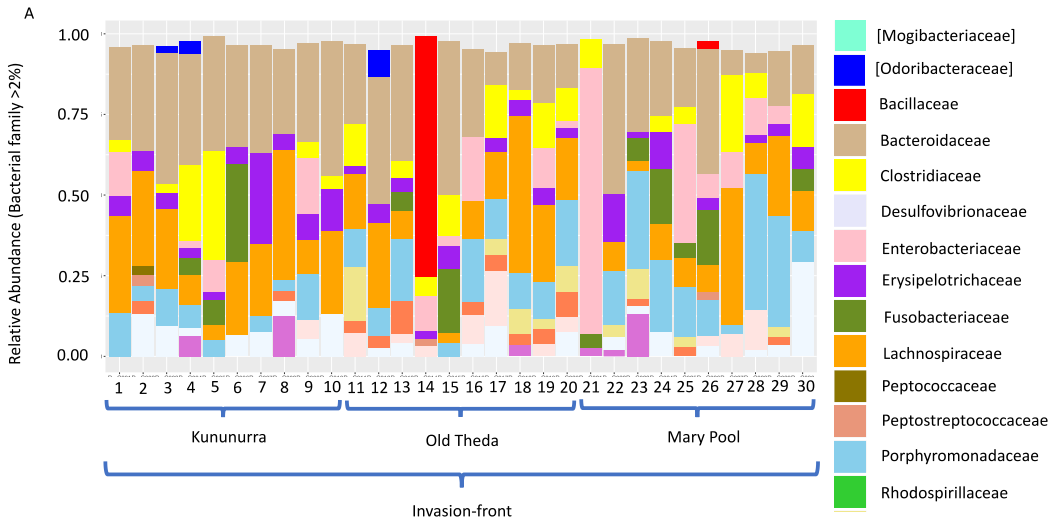


Figure S3. 4 Core bacterial community composition of different sites. Relative abundance bar plots display family (>2%).



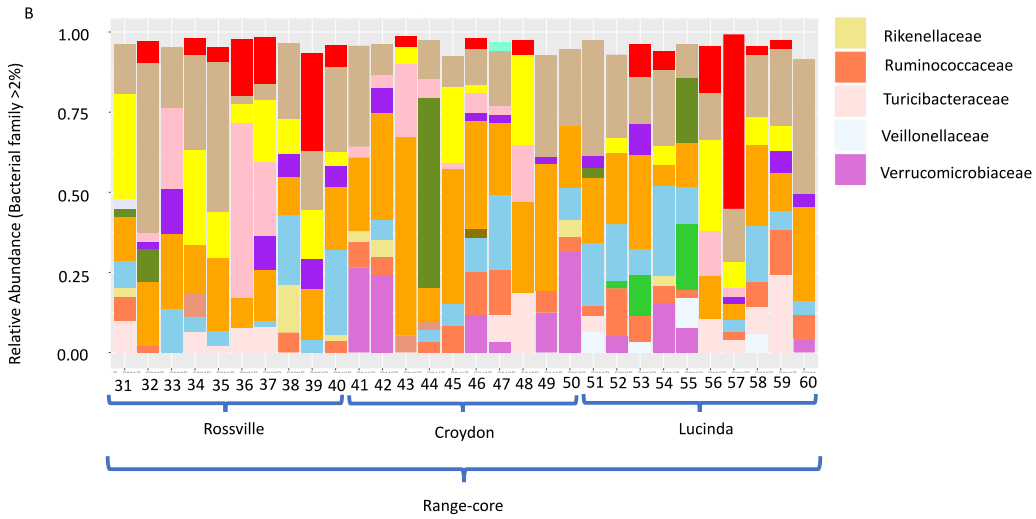


Figure S3. 5 Core bacterial community composition of different individual toads. Relative abundance plot shows family (>2%).

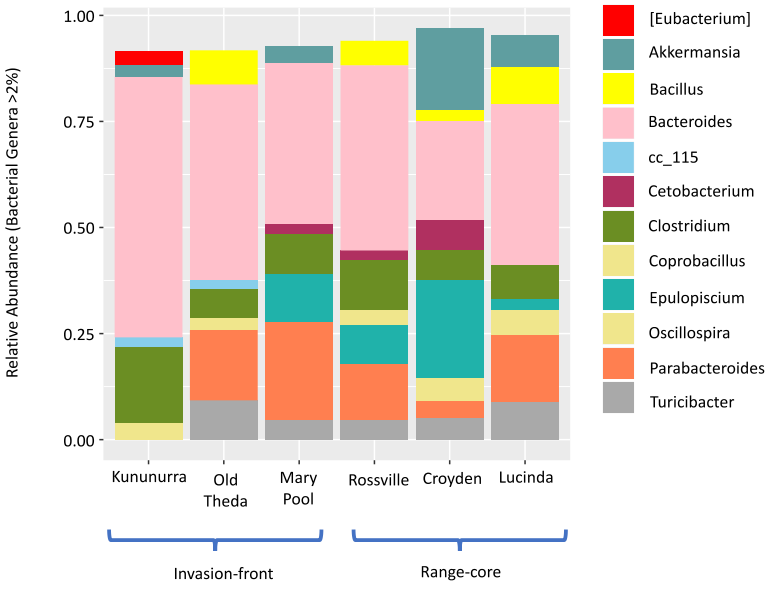
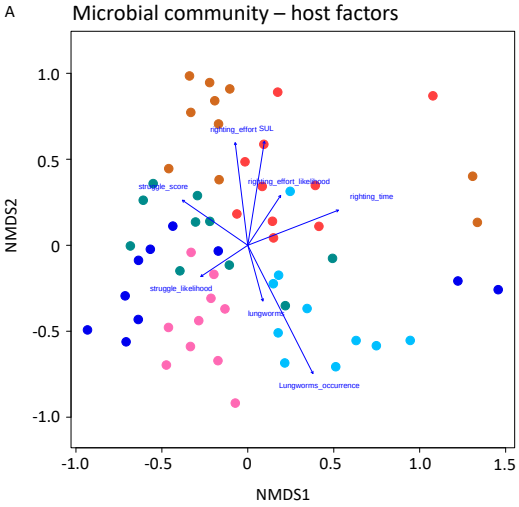
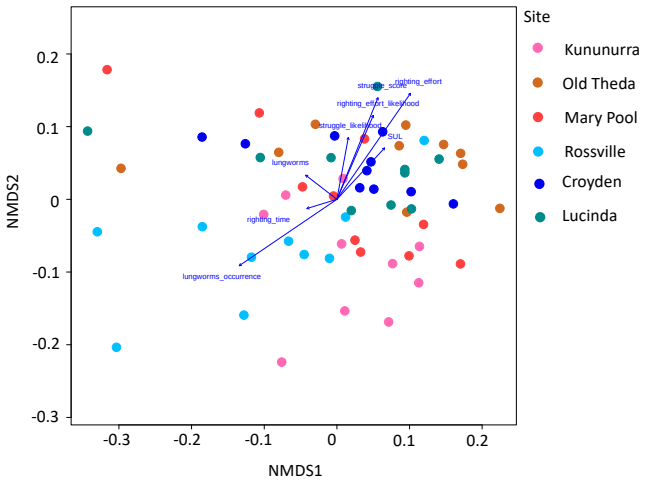


Figure S3. 6 Core bacterial community composition of different sites. Relative abundance bar plots display genera (>2%).

A



B



Envfit:

SUL: r2 = 0.071, p value = 0.119

Lungworms: r2 = 0.020, p value = 0.585

Occurrence of lungworms: r2 = 0.128, p value = 0.023 *

Struggle score: r2 = 0.044, p value = 0.268

Struggle likelihood: r2 = 0.021, p value = 0.555

Righting effort: r2 = 0.075, p value = 0.121

Righing effort likelihood: r2 = 0.026, p value = 0.474

Righting time: r2 = 0.062, p value = 0.174

Envfit:

SUL: r2 = 0.021, p value = 0.556

Lungworms: r2 = 0.007, p value = 0.839

Occurrence of lungworms: r2 = 0.059, p value = 0.187

Struggle score: r2 = 0.051, p value = 0.221

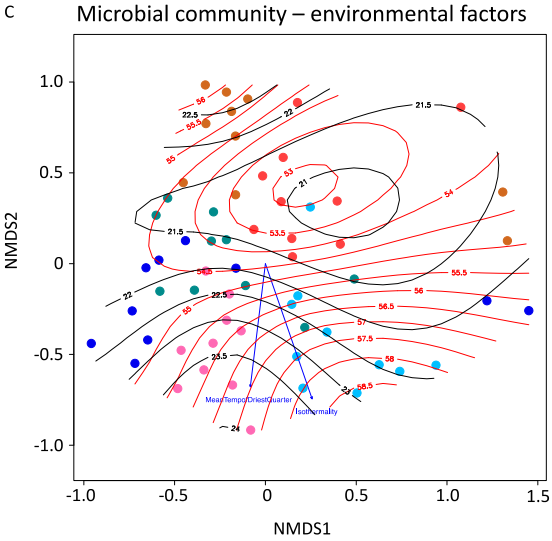
Struggle likelihood: r2 = 0.017, p value = 0.606

Righting effort: r2 = 0.070, p value = 0.141

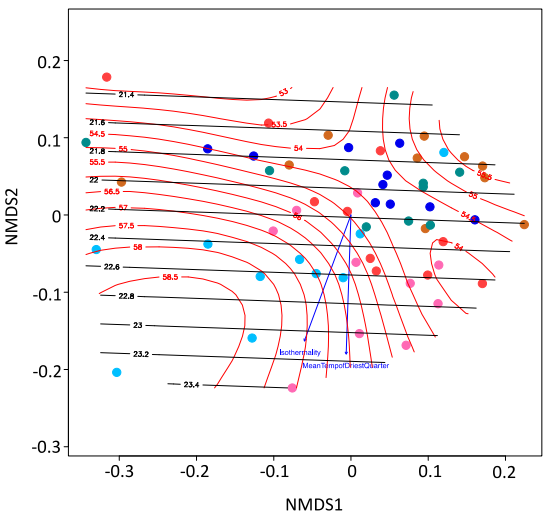
Righing effort likelihood: r2 = 0.035, p value = 0.362

Righting time: r2 = 0.004, p value = 0.894

C



D



Envfit:

Isothermality (r2 = 0.193, p value = 0.002 **)

Mean temp of driest quarter (r2 = 0.146, p value = 0.015 *)

Ordisurf:

Isothermality (r2 = 0.435, p value < 0.001 ***, deviance explained = 48.8%)

Mean temp of driest quarter (r2 = 0.438, p value < 0.001 ***, deviance explained = 49.5%)

Envfit:

Isothermality (r2 = 0.175, p value = 0.007 **)

Mean temp of driest quarter (r2 = 0.187, p value = 0.004 **)

Ordisurf:

Isothermality (r2 = 0.386, p value < 0.001 ***, deviance explained = 45.2%)

Mean temp of driest quarter (r2 = 0.159, p value = 0.003 ***, deviance explained = 18.3%)

Figure S3. 7 Main variables that affects cane toads' large intestinal microbial community and predicted functions differentiation among individuals.

Nonmetric multidimensional scaling (nMDS) based on Bray Curtis distance in the microbial (stress: 0.226) (A, C) and predicted functional profiles (stress = 0.162) (B, D). Dots represent 60 cane toad individuals from the invasion-front (Kununurra, Old Theda, and Mary Pool) and the range-core (Rossville, Croydon, and Lucinda). All host factors (host characteristics and behavioural traits) are shown in plots A and B. The environmental factors (isothermality and mean temperature of driest quarter) that significantly associated with taxa and functions are shown in plots C and D. These environmental factors also were fitted as smooth surfaces on the nMDS plot using the ordisurf function in package "Vegan". Isothermality (52 - 59) (red color contour lines) and mean temperature of driest quarter (20.7 - 24.2°C) (black color contour lines).

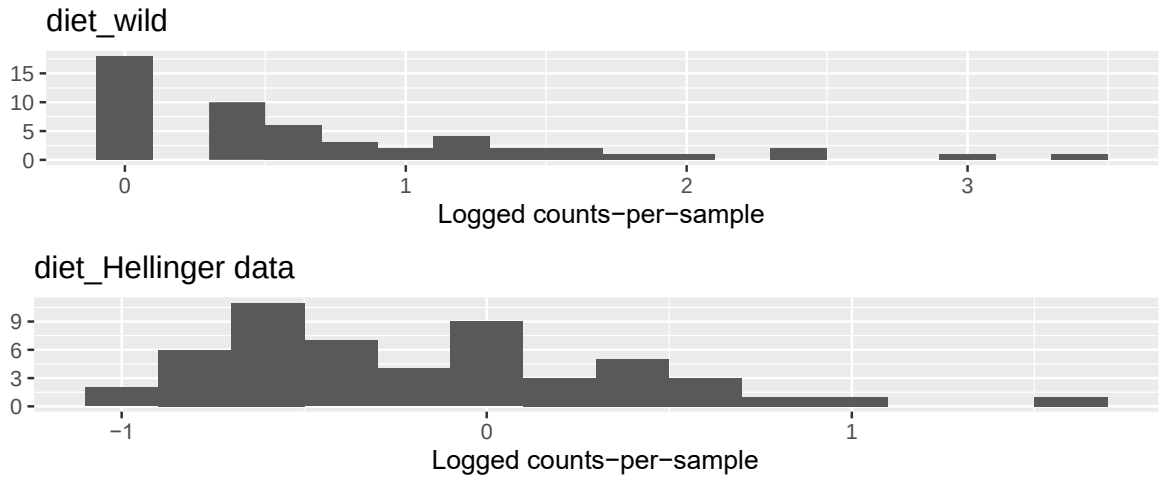
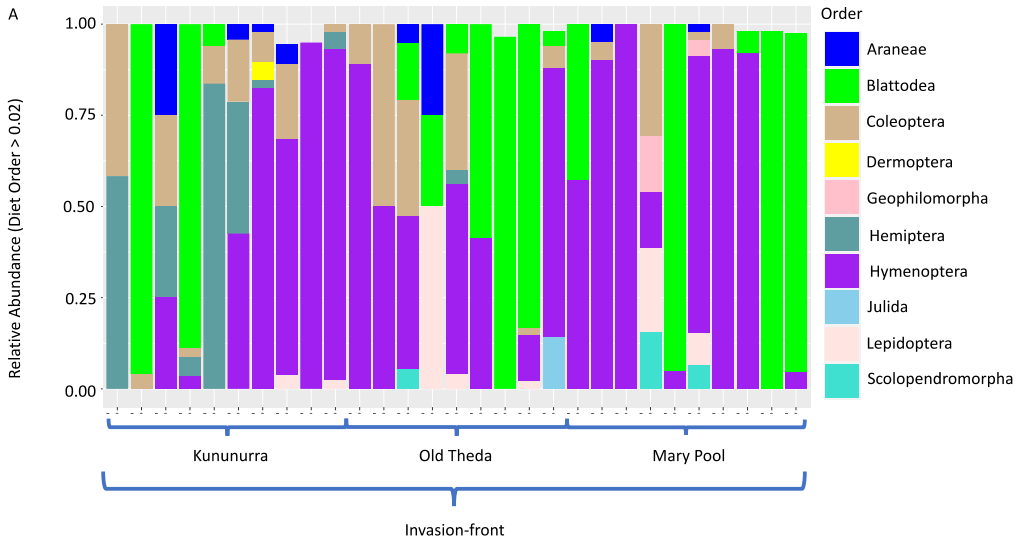


Figure S5. 1 Diet prey abundance data transformation process comparison between before Hellinger transform and after Hellinger transform.



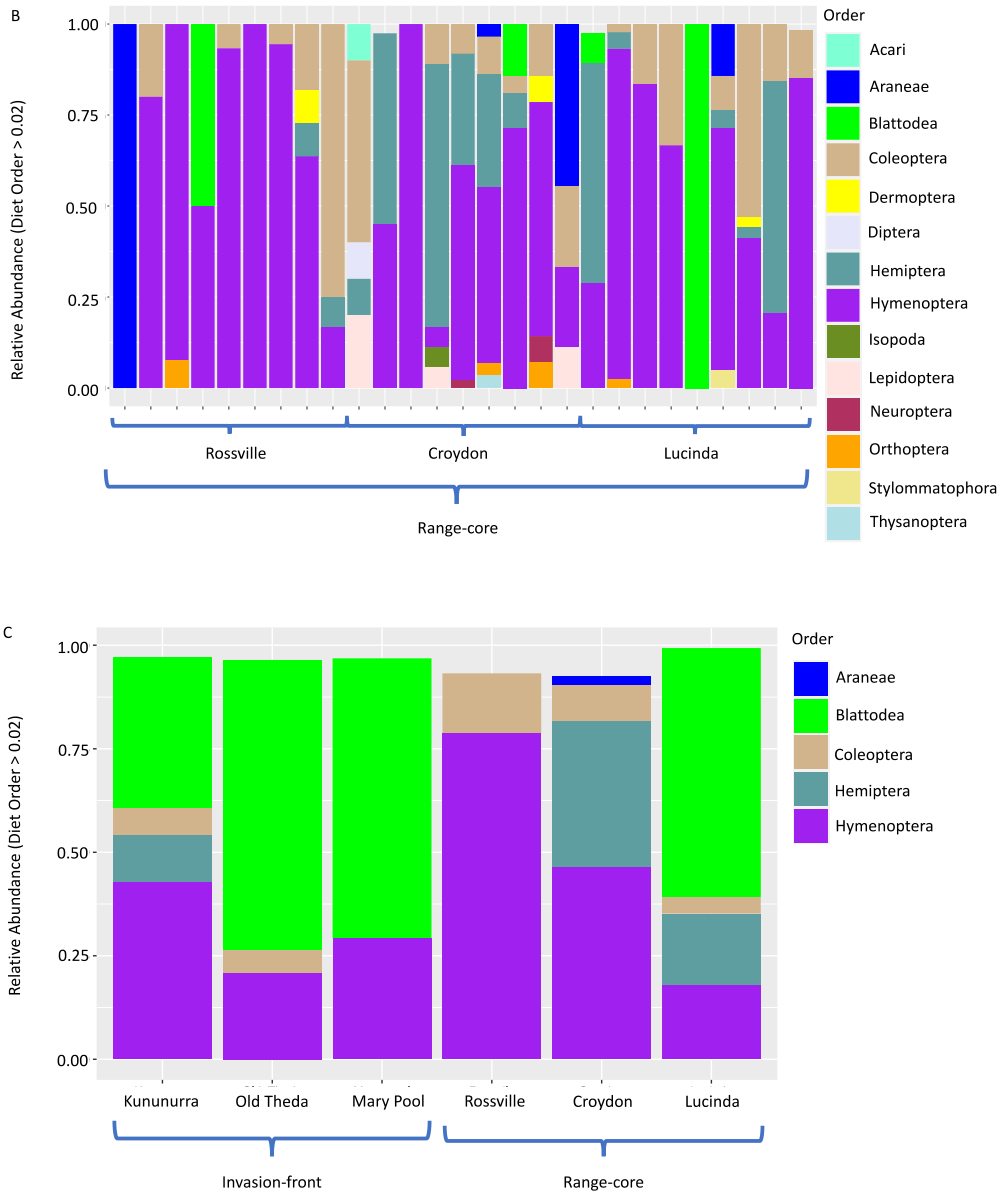
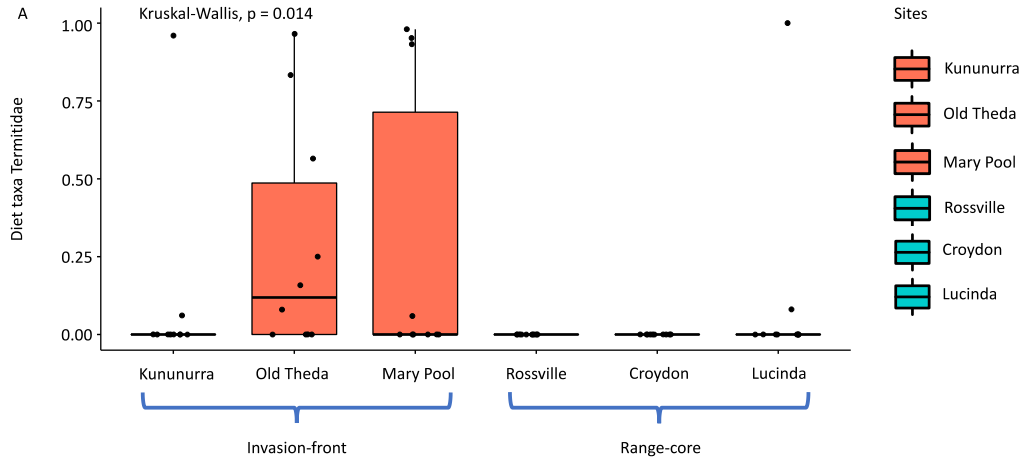


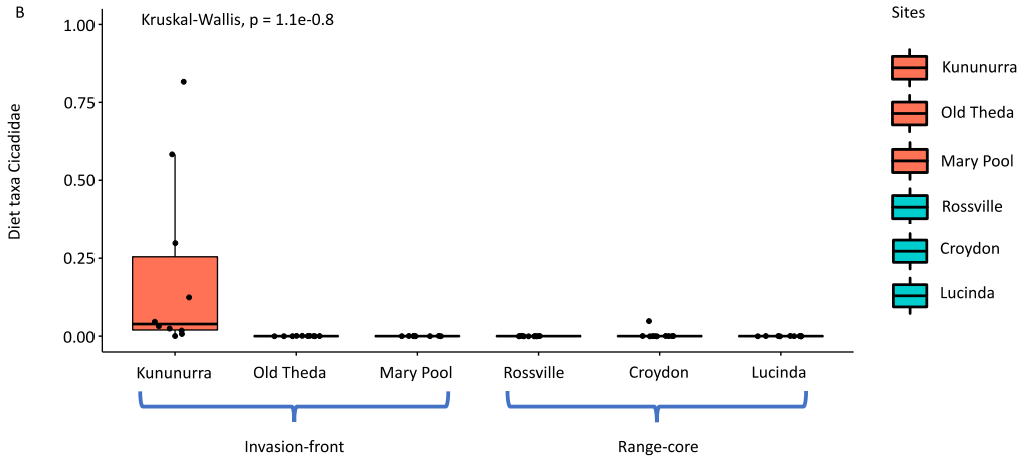
Figure S5. 2 Relative abundance of fifty-six cane toads' diet composition at order level.



Pairwise comparisons using Wilcoxon rank sum test with continuity correction

	Kununurra	Old Theda	Mary Pool	Rossville	Croydon
Old Theda	0.225	-	-	-	-
Mary Pool	0.478	0.707	-	-	-
Rossville	0.235	0.042*	0.122	-	-
Croydon	0.235	0.042*	0.122	-	-
Lucinda	0.957	0.235	0.500	0.235	0.235
Signif. codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1**					

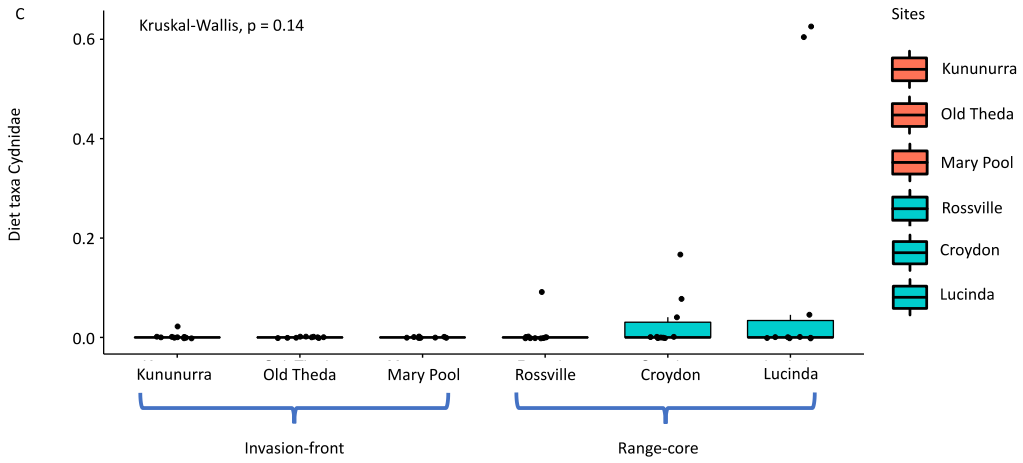
P value adjustment method: Benjamini & Hochberg (1995)



Pairwise comparisons using Wilcoxon rank sum test with continuity correction

	Kununurra	Old Theda	Mary Pool	Rossville	Croydon
Old Theda	0.0005***	-	-	-	-
Mary Pool	0.0005***	-	-	-	-
Rossville	0.0005***	-	-	-	-
Croydon	0.0029**	0.3681	0.3681	0.3681	-
Lucinda	0.0005***	-	-	-	0.3681
Signif. codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1**					

P value adjustment method: Benjamini & Hochberg (1995)



Pairwise comparisons using Wilcoxon rank sum test with continuity correction

	Kununurra	Old Theda	Mary Pool	Rossville	Croydon
Old Theda	0.43	-	-	-	-
Mary Pool	0.43	-	-	-	-
Rossville	1.00	0.43	0.43	-	-
Croydon	0.43	0.27	0.27	0.43	-
Lucinda	0.43	0.27	0.27	0.43	0.92
Signif. codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1**					

P value adjustment method: Benjamini & Hochberg (1995)

Figure S5. 3 Comparison of single taxon (family) between sites.

Kruskal-Wallis for multiple groups testing and Wilcoxon test for pairwise comparison between each two sites.