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Microbial function is related to behavior of an invasive anuran — Source link

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The gut bacteria of an invasive amphibian respond to the dual challenges of range-expansion and parasite attack

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Abstract

Gut bacterial communities influence, and are influenced by, the behaviour and ecology of their hosts. Those interactions have been studied primarily in humans and model organisms, but we need field research to understand the relationship between an organism's gut bacteria and its ecological challenges, such as those imposed by rapid range expansion (as in invasive species) and the presence of host-manipulating parasites. Cane toads (*Rhinella marina*) provide an excellent model system in this respect, because the species' ongoing colonization of Australia has enforced major changes in phenotypic traits (including behaviour), and lungworm parasites (*Rhabdias pseudosphaerocephala*) modify host gut function in ways that enhance the viability of lungworm larvae. We collected female toads from across the species' invasive range and studied their morphology, behaviour, parasite infection status and gut bacterial community. Range-core *versus* range-edge toads differed in morphology, behaviour, gut bacterial composition and predicted gut bacterial function but did not differ in the occurrence of parasite infection nor in the intensity of infection. Toads infected with lungworms differed from uninfected conspecifics in gut bacterial composition and diversity. Our study demonstrates strong associations between gut bacterial community and host ecology and behaviour.

Introduction

The bacterial community within an organism's intestines can be strongly influenced by host behaviour and ecology, such as habitat selection and diet [1–5]. But that interaction runs both ways because gut bacteria can influence behaviour and ecology of the host. For example, transferring gut contents can modify the recipient host's behaviour (exploratory behaviour, *Mus musculus* [6]; emotional reactivity, *Coturnix japonica* [7]). Similarly, altering gut microbial communities by administering antibiotics or altering dietary composition triggered aggressive behaviour in leaf-cutting ants (*Acromyrmex echinatior* [8]). Remarkably, changes in only a single bacterial species within the gut can affect behaviour of the host (e.g., *Drosophila melanogaster* [9]; *Danio rerio* [10]; *M. musculus* [11]). Gut microbiota can also affect mating choices [12] and foraging [13–15].

To date, most evidence for effects of intestinal bacteria on host behaviour comes from studies on humans and "model organisms". To elucidate the functional significance of this phenomenon, we need to extend such studies to free-ranging animals, incorporating a wider range of taxa [16]. Invasive species offer good models for such research, because novel challenges in the invaded range create an opportunity to compare closely-related organisms exposed to profoundly different environments [17,18]. Host-parasite relationships also may be disrupted during biological invasions, due to processes such as "enemy release" (loss of co-evolved native pathogens from the native range [19]). Parasites can manipulate host behaviour and physiology in ways that enhance parasite fitness but reduce host fitness [20]. Thus, if the gut bacterial community provides a mechanism for such effects, parasite-infected individuals should exhibit different gut bacteria than uninfected conspecifics.

These ideas suggest two predictions: (i) that the gut bacterial community should differ between populations of an invasive species (e.g., between the range-core and the invasion-front); and (ii) that the gut bacterial community should differ between parasitized hosts and non-parasitized conspecifics. The colonization of Australia by cane toads (*Rhinella marina*) provides a robust opportunity to test these predictions. Since their release in north-eastern Australia in 1935, toads have dispersed into areas that are much hotter and more seasonally arid than in the native range or the initial release sites [21]; and toads have brought with them a native-range nematode lungworm (*Rhabdias pseudosphaerocephala*) that can have devastating impacts on host viability, and induces behavioural and physiological changes in the host [22]. Notably, infected toads produce copious watery faeces [22]; hence, we expect lungworm-infected toads to exhibit different gut bacterial communities than uninfected individuals.

Methodology

Study species, sample collection and behavioural assays

Cane toads are native to South America, and were introduced into Australia in 1935 as a biocontrol for pests of sugar cane crops [23]. As the toads spread through tropical Australia, they fatally poisoned many native predators [23]. Toads from range-core populations (eastern Australia) differ from invasion-front conspecifics (in north-western Australia) in phenotypic traits that confer increased dispersal ability, such as endurance [24], limb morphology [25], boldness and exploratory behaviour [17,26]. Toads from the invasion-front also have lower rates of infection of the co-

83 introduced parasitic lungworm [27]. Drivers of variation in invasion-related behaviours in this
84 species include genetics, morphology, habitat, diet, prior experience and parasites [17,28–31].
85 However, the possible role of gut bacteria as a potential driver of behavioural shifts across the
86 invasive range has not been studied.

87 We hand-captured 60 adult females from three sites on the invasion-front and three sites in the range-
88 core (Table S1). We conducted brief behavioural assays upon collection including: (i) struggle score
89 (number of kicks after being captured until toad remains still for 5 seconds) and struggle likelihood;
90 and (ii) righting effort (time to right itself, number of kicks within two minutes after toad is placed on
91 its dorsal side, and righting effort likelihood [17]. These measures are predictive of traits including
92 speed and stamina (K. Stuart, pers. comm.), suggesting that these simple assays may reveal a toad's
93 dispersal potential. We then placed animals into individual, moist, calico bags and weighed,
94 measured (snout urostyle length; SUL) and euthanised them by injecting tricaine methanesulfonate
95 (MS222) buffered with bicarbonate of soda.

96 We dissected the toads and scored the presence of two types of toad parasite: the gut-encysted
97 physalopterine larvae [32] and adult lungworms [22,33]. Lungworm larvae pass through the gut, but
98 are less easily detected and reliably counted than are adult lungworms. From each toad, we removed
99 0.3cm of colon near the cloaca (including gut contents) and preserved it in 95% ethanol (see
100 Supplementary Material for justification of sampling protocols).

101

102 ***Analyses***

103 We compared host morphology and behaviour between regions (range-core *versus* invasion-front),
104 and as a function of infection status (lungworm infected *versus* non-infected). Because body length
105 (SUL) and mass were correlated, we only included SUL as our measure of host morphology in
106 further analyses. We used a t-test to compare mean SUL between regions and infected/non-infected
107 toads. For associations between region or infection status with host characteristics or behavioural
108 traits, we used SUL as a covariate in generalized linear models (GLM). See Supplementary Material
109 for details of statistical analyses.

110

Laboratory methods and data pre-processing for characterizing gut bacterial community composition and predicted functions are described in Supplementary Material. Briefly, we calculated within-individual (alpha) bacterial diversity and between-site (beta) bacterial diversity. For the latter variable, we subset our data to include the Core50 gut community (Amplified Sequence Variants (ASVs) present in a minimum of 50% of toads from each site [2]). We predicted bacterial functions and generated pathway abundance based on Core50 ASVs. We compared bacterial composition and predicted function between regions, and between lungworm-infected and non-infected toads. We identified differences in individual ASVs and predicted bacterial functions between range-core and invasion-front toads and identified associations between host characteristics (including infection with parasites) with bacterial communities and predicted bacterial functions. Analyses were conducted in QIIME2 [34], PICRUST2 [35], and R packages in R version 4.0.2 [36].

Results

Ecological traits

Wild-caught invasion-front toads were larger than range-core toads (Tables S2, S3; mean SUL $t = 2.54$, $df = 53.90$, $p = 0.014$). Neither counts nor presence of parasites (lungworm and gut) differed significantly across the range (Table S2). Range-core toads were more likely to struggle (Tables S2, S3; $p = 0.008$, 95% CI: core [-0.174, 0.005], edge [-0.023, 0.199]) and, in those that did struggle, the number of struggle movements was higher for range-core toads (Tables S2, S3; $p = 0.002$, 95% CI: core [-0.057, 0.026], edge [0.046, 0.2]). Range-core toads also were more likely to attempt to right themselves ($p = 0.036$, 95% CI: core [-0.092, 0.04], edge [-0.006, 0.19]), but righting effort and righting time did not differ significantly between geographic regions (Tables S2, S3).

Because there were no significant differences in prevalence or intensity of lungworm between the range-core and invasion-front toads (Table S2), we combined samples to analyse correlates of lungworm infection. Infected toads were similar in SUL to non-infected toads (Tables S3, 4; $t = 0.86$, $df = 57.19$, $p = 0.393$), with no significant behavioural differences between the two groups (Table S3, 4).

Gut bacterial community composition and predicted bacterial function

Alpha diversity did not differ significantly between regions (Supplementary Material), but beta diversity of bacterial taxonomic communities differed between regions (Figures 1A, S1; $R^2 = 0.050$,

142 F = 3.050, p < 0.001) and sampling sites (Table S5; all p-values < 0.001). Among 230 ASVs that
143 were assigned to family level, the abundance of 124 ASVs differed between the colons of range-core
144 *versus* invasion-front toads (Table S6). The number of significantly different ASVs in each phylum
145 were: Bacteroidetes (60 ASVs), Firmicutes (55 ASVs), Proteobacteria (7 ASVs), Actinobacteria (1
146 ASVs), Verrucomicrobia (1 ASV) (Table S6, Figure 2A).
147
148 Among the identified 474 predicted bacterial functions, we found significant differences between
149 invasion-front and range-core toads (Figure 1B; R^2 = 0.064, F = 4.110, p-value= 0.002). Pairwise
150 tests between sampling sites indicated that Kununurra toads had different bacterial functions to
151 Rossville (p-value= 0.009) and Lucinda toads (p-value = 0.046), but no other sites differed
152 functionally from each other (Table S7; all other p-values > 0.05). In total, 84 predicted bacterial
153 functions differed between invasion-front and range-core toads (Table S8, Figure 2B). Range-core
154 toads had more abundant bacterial function in the superpathway of pyrimidine ribonucleosides
155 degradation (log2FoldChange = 5.98) and less abundant bacterial function in phosphopantothenate
156 biosynthesis III (log2FoldChange = -4.98), superpathway of sialic acids and CMP-sialic acids
157 biosynthesis (log2FoldChange = -4.89) and factor 420 biosynthesis (log2FoldChange = -4.72) than
158 did invasion-front toads (Table S8, Figure 2B). Among the 30 most abundant functions, range-core
159 toads had lower bacterial function in urate biosynthesis/inosine 5'-phosphate degradation
160 (log2FoldChange = -0.10) than did invasion-front toads (Figure 3, Table S8).
161
162 ***Associations between ecological traits and intestinal bacteria***
163 To assess correlates of gut bacterial composition and function, we compared characteristics of
164 individual hosts to bacterial variation. Only the occurrence of lungworms was significantly
165 associated with the bacterial composition (R^2 = 0.128, p = 0.02) (Table 1; Figure S2A, B).
166
167 In a redundancy analysis combining ecological traits measured here, the model that explained the
168 most variation in the bacterial community assemblage included only the occurrence of lungworms
169 (AIC = 178.58). The best model to explain variation in predicted bacterial functions included the
170 likelihood of righting (AIC = 53.613), the occurrence of lungworms (AIC = 54.297) and righting
171 time (AIC = 56.912). The combination of these three factors explained 17.8% of total variation in
172 predicted bacterial functions (Figure 4).
173

In explicit tests of whether bacterial community assemblages differed in infected *versus* non-infected toads, we found a significant association with lungworm occurrence (Table 2, p=0.005). Intensity of lungworm infection was not significantly associated with gut bacterial community but did have a significant interaction with region in this analysis (Table 2, p=0.04).

Discussion

Bacteria influence animal behaviour in diverse ways [16,37], but the ecological drivers of variation in gut bacterial composition remain largely unstudied. Our analyses of cane toads from two regions within their invasive range documents substantial variation in community assemblage and function of gut bacteria. Importantly, that variation was associated with two traits that we predicted to influence gut bacterial assemblages: invasion history and parasite infection. Interestingly, toad behaviour differed across the invasive range, and toad righting behaviour was associated with bacterial function but not with parasite infection.

Geographic divergence in gut bacteria

First, we consider the differences in gut bacteria between toads from the invasion-front and the range-core. Although these populations have been separated by less than a century, the toads have diverged remarkably in morphology, physiology and behaviour and much of that divergence is heritable [38,39]. Some of those shifts likely reflect evolutionary pressures for increased rates of dispersal, due to adaptive (natural selection) and non-adaptive (spatial sorting) mechanisms [40,41]. Other geographically variable aspects of toad phenotypes likely are responses to different climatic conditions in the newly-invaded regions (hot, seasonally arid) compared to the range-core (cooler, more equable climate) [42]. Similar geographic divergence has been reported for the microbiome on the toad's skin [43]. Our data illustrate that the invasion of Australia by cane toads has been accompanied by substantial divergence in gut bacterial communities. Alpha diversity in gut bacteria was similar in invasion-front and range-core individuals, but there were differences in both the gut bacteria composition and predicted bacterial function between toad populations across the species' Australian invasive range. Predicted bacterial functions better explained cane toad righting behaviour than did gut bacterial community composition. Intriguingly, similarity between gut bacterial communities between individuals within regions in Australia is related to the similarity of their host's epigenome, and this relationship is strengthened in populations where genetic diversity is lowest, such as on the invasion front [44]. Relationships between gut bacterial communities and their hosts

are complex, and that a clear understanding of these relationships requires careful consideration of numerous environmental, host and gut bacterial factors.

The diversity and composition of bacterial communities differed between range-core and invasion-front toads, despite an overall similarity in their dominant phyla and alpha diversity. ASVs in the family Veillonellaceae were higher at the invasion-front (Figure 2A). The abundance of this bacterial family may influence host metabolic regulation. For example, in Brandt's voles (*Lasiopodomys brandtii*) exposed to colder temperatures, voles which huddled had more Veillonellaceae and more short-chain fatty acids (SCFAs) in their intestines than did non-huddling voles [45]. This family produces SCFAs such as propionic acid [46,47], which can increase locomotor activity [48]. The link to host metabolic regulation suggests that invasion-front toads might fuel their invasion in this way [24]. ASVs from another family of SCFA-producing bacteria, Clostridiaceae [49], were also higher in invasion-front toads than those from the range-core. Furthermore, the family Veillonellaceae may be associated with host sociality. A reduction of Veillonellaceae has been observed in children with Autism Spectrum Disorder, often known for desiring social isolation [50]. Higher abundance of Veillonellaceae in invasion-front toads could foster their "bolder" personality, retaining a higher propensity for exploration and risk-taking [26,51].

Several other ASVs that differed across the toad's range also may affect behaviour. ASVs from the family Peptococcaceae, more common in invasion-front toads (Figure 2A), are related to host neurotransmitter levels (noradrenaline linking visual awareness to external world events [52]). For example, Peptococcaceae levels in the caecum are positively correlated with noradrenaline levels in mice [53]. ASVs from family Bacillaceae, lower in invasion-front toads (Figure 2A), might be related to host anxiety (e.g., abundant in methamphetamine-treated rats [54], and in exercised *versus* sedentary mice [55]). Abundant Bacillaceae might induce anxiety-like behaviours, thus intensifying the stress response [54] and decreasing exploratory behaviour in new environments [56]. In summary, invasion-front toads possessed gut bacterial communities that in other studies have been associated with SCFAs production and neurotransmitters. That pattern supports the idea that gut microbes in invasion-front toads may increase locomotor ability, alertness and propensity for exploration and risk-taking. In comparison, range-core toads possessed bacterial taxa that have been associated with anxiety, and a decreased propensity to explore.

Geographic variation was less obvious in the predicted bacterial functional groups than in community composition (Figure 1), consistent with the hypothesis that bacterial function is more conservative than taxonomic composition (e.g. in fire salamanders [2]). Different gut microbiota can have similar bacterial functions, increasing resilience and functional stability [2,3,57]. Despite this broad similarity, bacterial functions differed between range-core and invasion-front toads. These differences included those involved in functional pathways related to food sources and metabolism. Invasion-front toads had less bacterial function in the superpathway of pyrimidine ribonucleosides degradation, which provides a nitrogen source for microbes [58] and plays an important role in perturbations in the uridine monophosphate (UMP) biosynthetic pathways. This pathway allows the bacterial cell to sense signals such as starvation, nucleic acid degradation, and availability of exogenous pyrimidines, and to adapt the production of the extracellular matrix to changing environmental conditions [59]. This function might help to explain the disappearance of Verrucomicrobia as a dominant taxon. As for microbe metabolism, invasion-front toads have higher abundance of bacterial functions in factor 420 biosynthesis, critical to bacterial metabolism and mediating important redox transformations involved in bacterial persistence, antibiotic biosynthesis, pro-drug activation, and methanogenesis [60].

We also detected geographic variation in bacterial functional pathways that contribute to host health. Invasion-front toads exhibited bacterial functions beneficial to host health and immunity: (i) phosphopantothenate biosynthesis (involved in bacterial production of coenzyme A [61]); and (ii) superpathway of sialic acids biosynthesis (involved in immunity including acting as host receptors and pathogen decoys for viruses and bacteria [62] and especially critical for preventing neural tissue damage [63]). Despite this abundance of health-promoting bacterial functions, these toads may also face health challenges. Invasion-front toad bacteria had a 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 [64]. High levels of urate can result in the formation of needle-like crystals of urate in the joints (gout), perhaps related to severe spinal arthritis in invasion-front cane toads [65].

Associations between lungworms and host gut bacteria

Pathogens and parasites impact the composition of the host microbiota and can modify host behaviour in a manner that improves parasite transmission and survival [66–68]. Lungworms can affect cane toad locomotor performance and reduce host endurance, curtailing oxygen supply from

267 infected lungs [69]. Lungworms also can alter a cane toad's thermal preference and manipulate the
268 timing and location of defecation, thereby enhancing lungworm egg production and larval survival
269 [22]. Lungworms are reported to lag behind their host on the invasion-front by 2-3 years [27] and to
270 affect righting behaviour (prolongs righting time [70]). In the current study, although we collected
271 invasion-front toads in recently invaded areas, we found no difference in lungworm presence or
272 intensity between the invasion-front *versus* range-core toads, nor did we find behavioural differences
273 in lungworm-infected *versus* uninfected toads.
274
275 Infection by parasitic lungworms was associated with differences in gut bacteria. Here, the direction
276 of causation is less ambiguous than is the case for geographic variation in the gut bacteria. It seems
277 unlikely that a toad's bacteria affect its probability of carrying adult lungworms, although bacterial-
278 driven differences in habitat selection might create such a link. Instead, we suggest that the presence
279 of lungworms induces a shift in gut bacteria. Consistent with that hypothesis, experimental trials have
280 shown that lungworms modify gastric function in their hosts, changing the volume and consistency
281 of faeces produced in ways that enhance survival of larval lungworms [22]. Shifts in the microbiome
282 inside the gut might be either causes or consequences of that shift in gastric function. Moreover, *C.*
283 *elegans* are known to prefer specific bacterial foods [71], suggesting that lungworm larvae may also
284 feed selectively on bacteria in the gut, generating differences in bacterial communities between
285 lungworm-infected toads *versus* non-infected conspecifics. Additionally, gut bacteria may affect
286 lungworms via microbiome-induced shifts in host immunity [72].
287
288 ***Associations between host behaviours and gut bacteria***
289 Interestingly, behaviours including righting effort likelihood and righting time were associated more
290 closely with predicted gut bacterial functions than with bacterial taxonomic composition. Multiple
291 identified taxa may share the same bacterial function, or one taxon may contribute to multiple
292 bacterial functions, obscuring the relationship between host behaviour and bacterial taxonomic
293 composition. Nonetheless, these relationships we found between gut bacterial function and righting
294 behaviours may be related to toad health and/or rearing conditions. A dampened stress response
295 (lower corticosterone levels) in invasion-front toads [73] could result from higher abundance of
296 bacterial functions beneficial to host health and immunity, especially the superpathway of sialic acids
297 biosynthesis [63]. Further, invasion-front toads are more reluctant to flee in simulated predation trials
298 [74]. Dampened stress responses can be related to more exploratory behaviour [56], and to greater
299 dispersal ability [26]. Rearing conditions also affect righting behaviour [17]. Although manipulative

studies are needed to clarify causal relationships between stress responses, proactive behaviours, and gut bacterial functions, our results indicate that host behaviour and gut bacterial functions are related, suggesting that gut bacteria may be an important driver of invasion.

Our study has identified patterns rather than testing alternative hypotheses about underlying causal processes. To clarify causal mechanisms underlying the geographic divergence in gut bacteria across the toads' Australian range, future studies could use reciprocal transplantation to examine if (and how) their gut bacteria respond to novel environmental conditions. Breeding these animals, and raising their offspring under common-garden conditions, could reveal the degree to which a toad's gut bacteria is driven by host genetics *versus* their rearing conditions [75,76]. To clarify the hypothesis that changes in gut bacteria mediate the ability of lungworm parasites to modify host gut function, we could implant colon contents from infected into uninfected toads. In short, our discovery of strong associations between gut bacteria and important facets of toad ecology provides the opportunity to move to hypothesis-testing experimental studies.

Our research illustrates that during invasion, as a species expands across a novel and variable landscape, a complex relationship between host behaviour, its parasite community, and its microbiome may unfold. A clearer understanding of these relationships and how they influence the rate of expansion are key to understanding the role of the holobiont during invasion [77]. Such studies also will advance our understanding of co-evolution and may facilitate innovative approaches to invasive species management.

Ethics

Approved by University of Adelaide Animal Ethics Committee (S-2018-056).

Author Contributions

Designed research: JZ, TMN, CRL, SJZ, LAR; performed research: JZ, CRL, GWF, KS, LAR; analyzed data: JZ, TMN, CRL, SJZ, KS, LAR; drafted manuscript: JZ, RS, TMN, CRL, SJZ, GWF, KS, LAR.

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Data Availability Statement

Supplementary methods and results available online. Code available at: https://github.com/jiazhou0116/gut-microbiome-analyses-2. Sequence data are available in NCBI Sequence Read Archive (PRJNA670039). Raw ecological data available on Dryad (doi:10.5061/dryad.v15dv41tw).

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562 **Tables and Figures**

563 **Table 1a-d.** The association between a) single host factors and microbial community, b) single host
564 factors and predicted microbial function, c) single behavioural trait and microbial community and d)
565 single behavioural trait and predicted microbial function. Significant p-values denoted by an asterisk.

566

	MDS1	MDS2	r2	Pr(>r)
a. Host factor/microbial community				
SUL	0.170	0.985	0.071	0.119
BodyWeight	-0.009	1.000	0.051	0.231
Lungworms	0.254	-0.967	0.020	0.585
Occurrence of lungworms	0.453	-0.892	0.128	0.023*
b. Host factor/microbial function				
SUL	0.680	0.733	0.021	0.556
BodyWeight	0.610	0.793	0.041	0.311
Lungworms	-0.791	0.612	0.007	0.839
Occurrence of lungworms	-0.827	-0.562	0.059	0.187
c. Behavioural trait/microbial community				
Struggle score	-0.814	0.580	0.044	0.268
Struggle likelihood	-0.880	-0.474	0.021	0.555
Righting effort	-0.124	0.992	0.075	0.121
Righting effort likelihood	0.499	0.867	0.0255	0.474
Righting time	0.933	0.360	0.0615	0.174
d. Behavioural trait/microbial function				

Struggle score	0.376	0.927	0.051	0.221
Struggle likelihood	0.183	0.983	0.017	0.606
Righting effort	0.571	0.821	0.070	0.141
Righting effort likelihood	0.399	0.917	0.035	0.362
Righting time	-0.956	-0.294	0.004	0.894

567

568

569 **Table 2.** Association between gut microbiota variation and lungworm occurrence and intensity, based
570 on Bray Curtis dissimilarity values for microbial community assemblages.

	Df	SumOfSqs	R2	F	Pr(>F)
Location	1	1.314	0.091	6.026	<0.001***
Lungworm_occurrence	1	0.482	0.033	2.213	0.005**
Lungworms_intensity	1	0.150	0.010	0.688	0.861
Location:Lungworm_occurrence	1	0.331	0.023	1.520	0.075
Location:Lungworm_intensity	1	0.357	0.025	1.638	0.043*
Residual	54	11.774	0.817		
Total	59	14.409	1.000		

Significance codes: 0 '***' ≤0.001 '**' ≤0.01 '*' ≤0.05.

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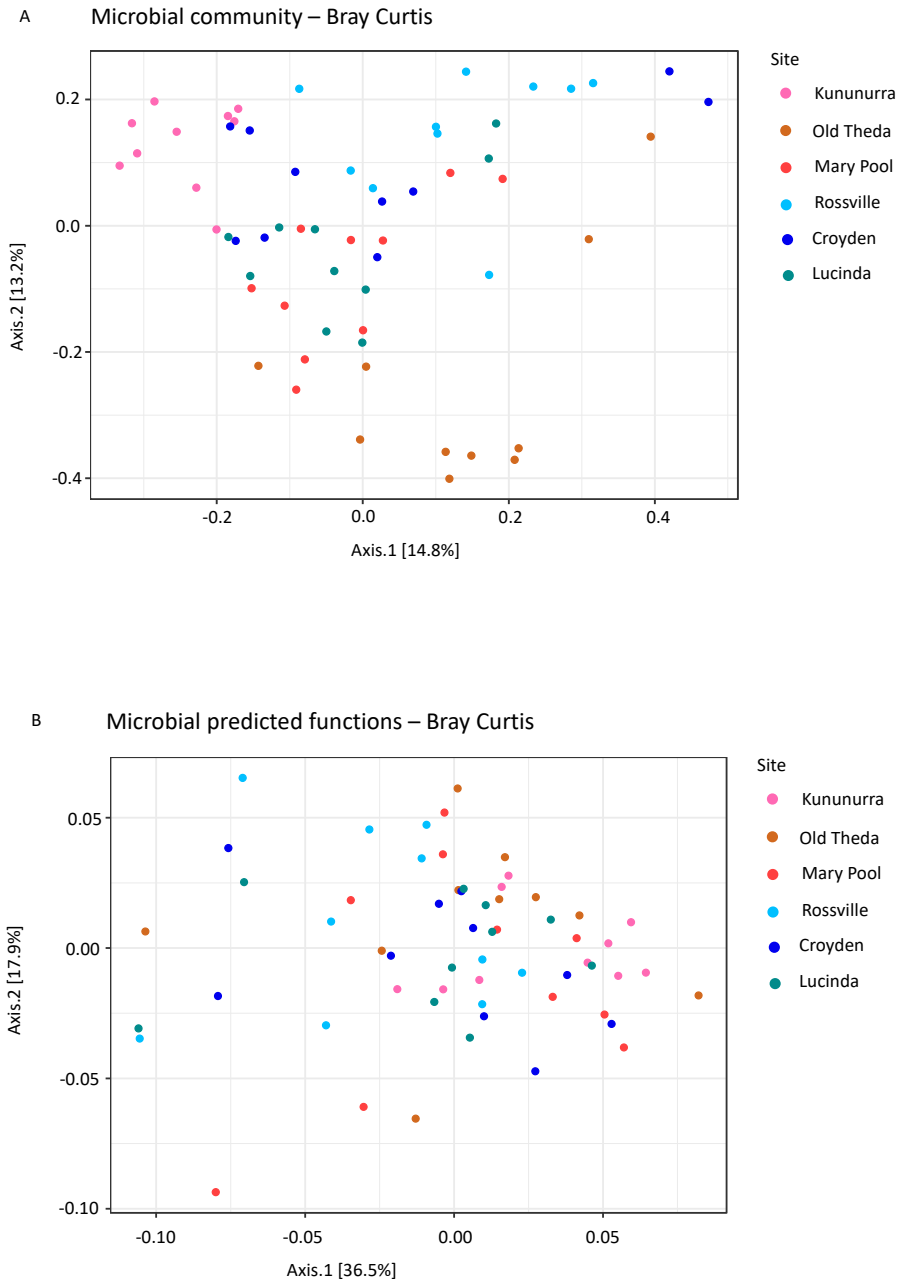

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578 **Figure 1. Beta diversity by location.** Principle coordinate analysis plot of Bray Curtis distance of
579 microbial community (A) and Bray Curtis distance of predicted functional groups (B) from 60 cane
580 toad individuals of the invasion-front (Kununurra, Old Theda, and Mary Pool) and the range-core
581 (Rossville, Croydon, and Lucinda).

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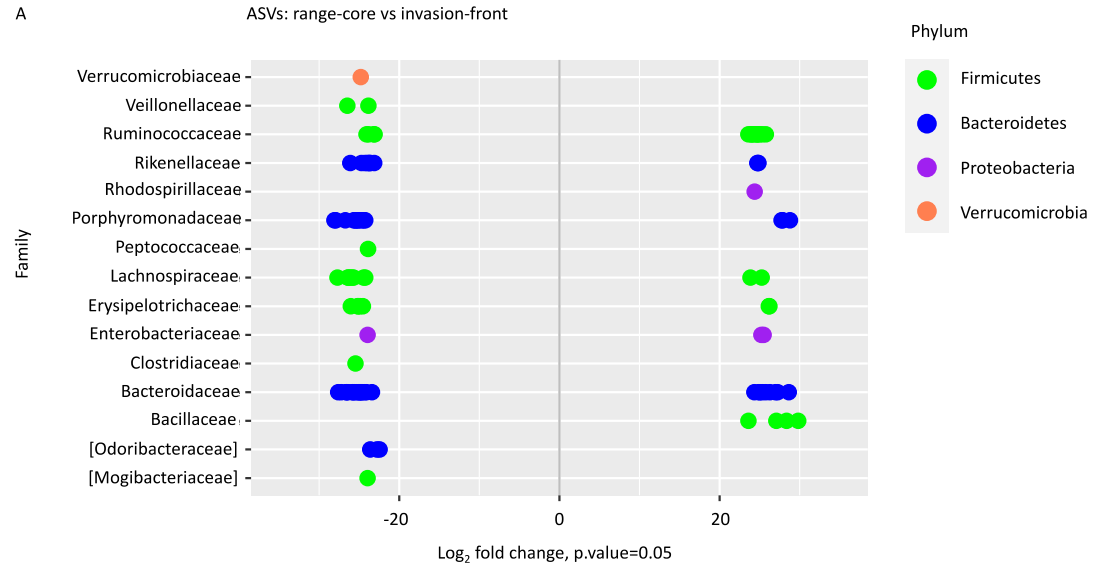

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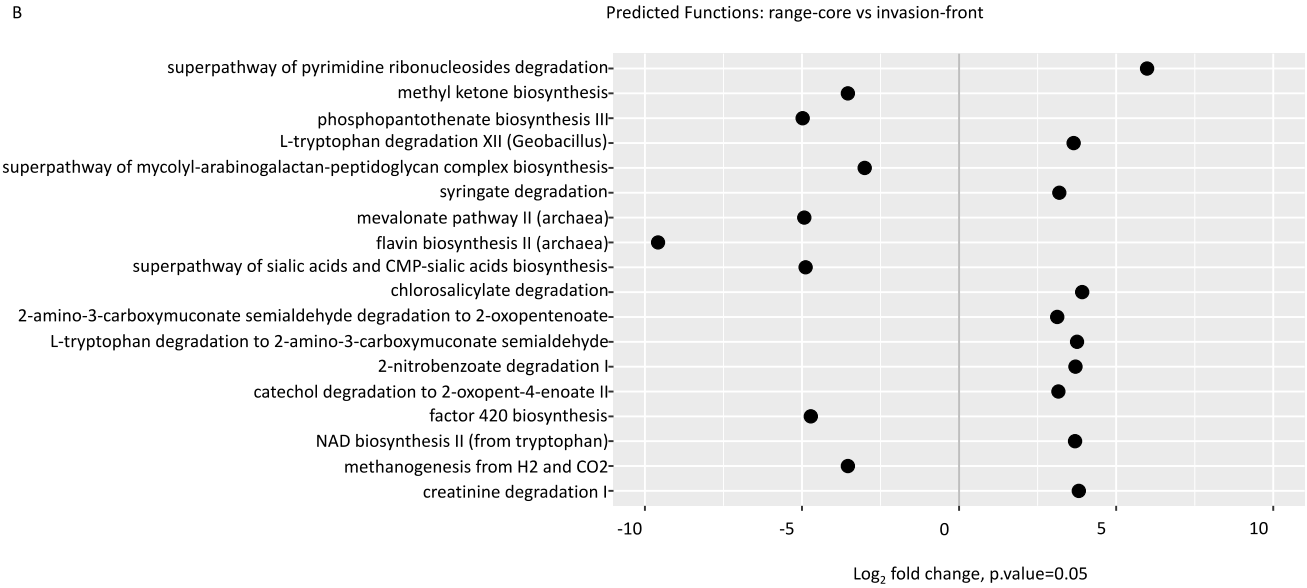

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588 **Figure 2. Significantly different bacterial taxa and predicted functions between range-core**
589 **(QLD) and invasion-front (WA) toads' colon.** Significant differences were identified between
590 locations via differential abundance testing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

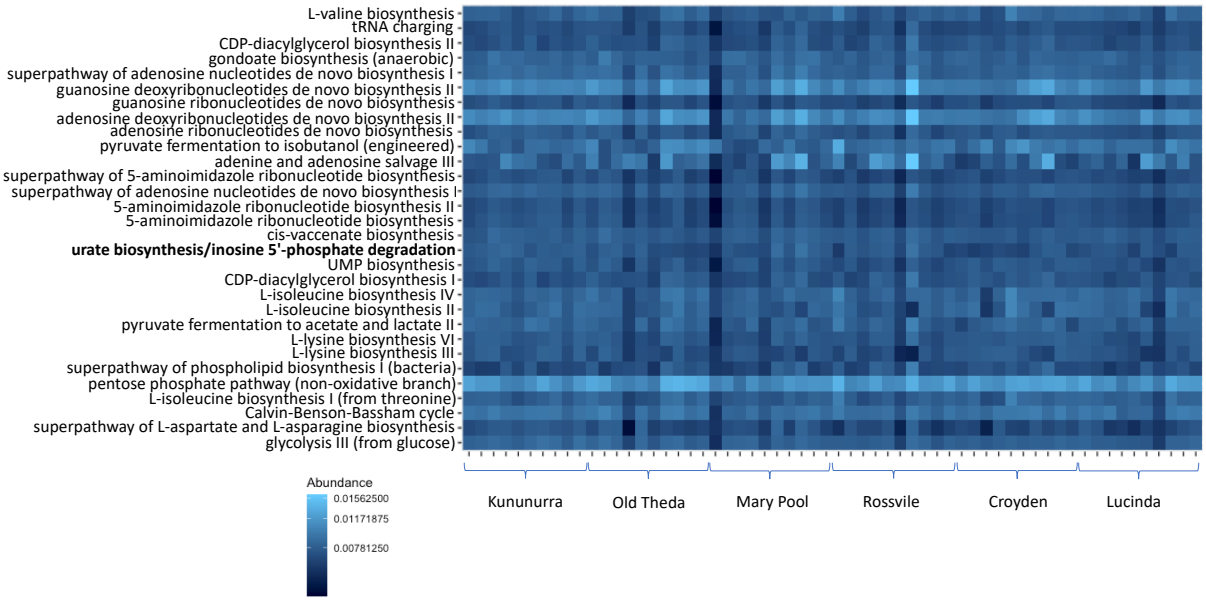
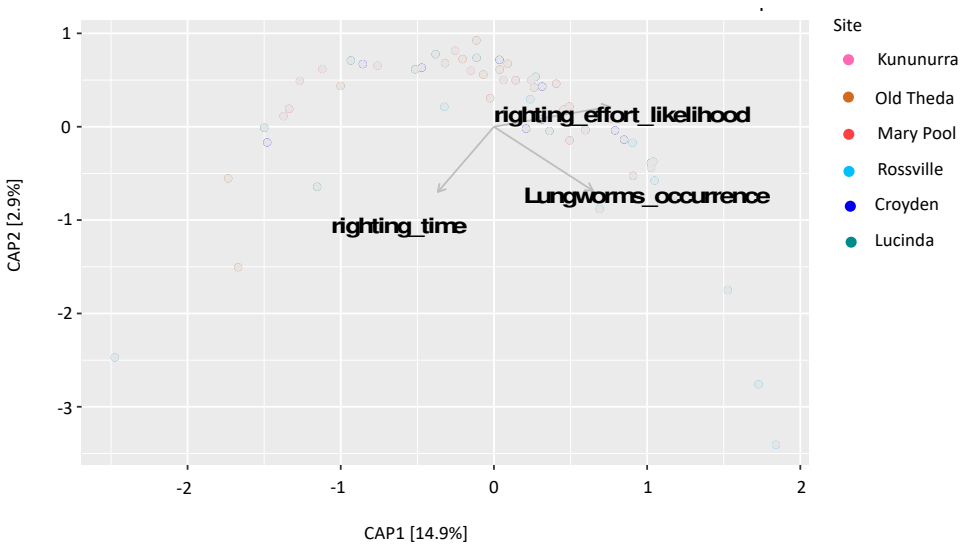


Figure 3. Heatmap for top 30 functional group abundance.

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. Range-core includes Rossville, Croyden, and Lucinda; invasion-front includes Kununurra, Old Theda, and Mary Pool.



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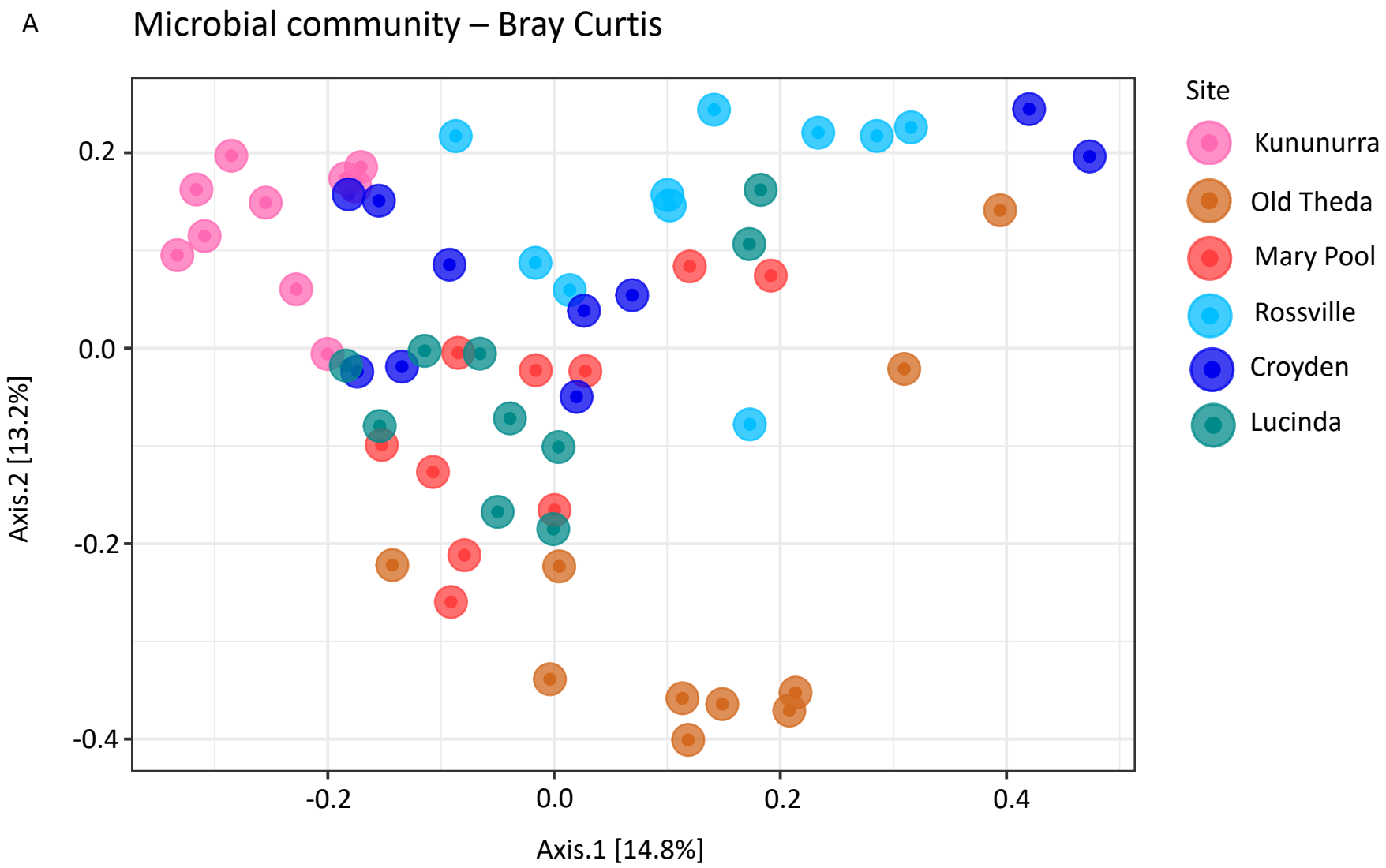
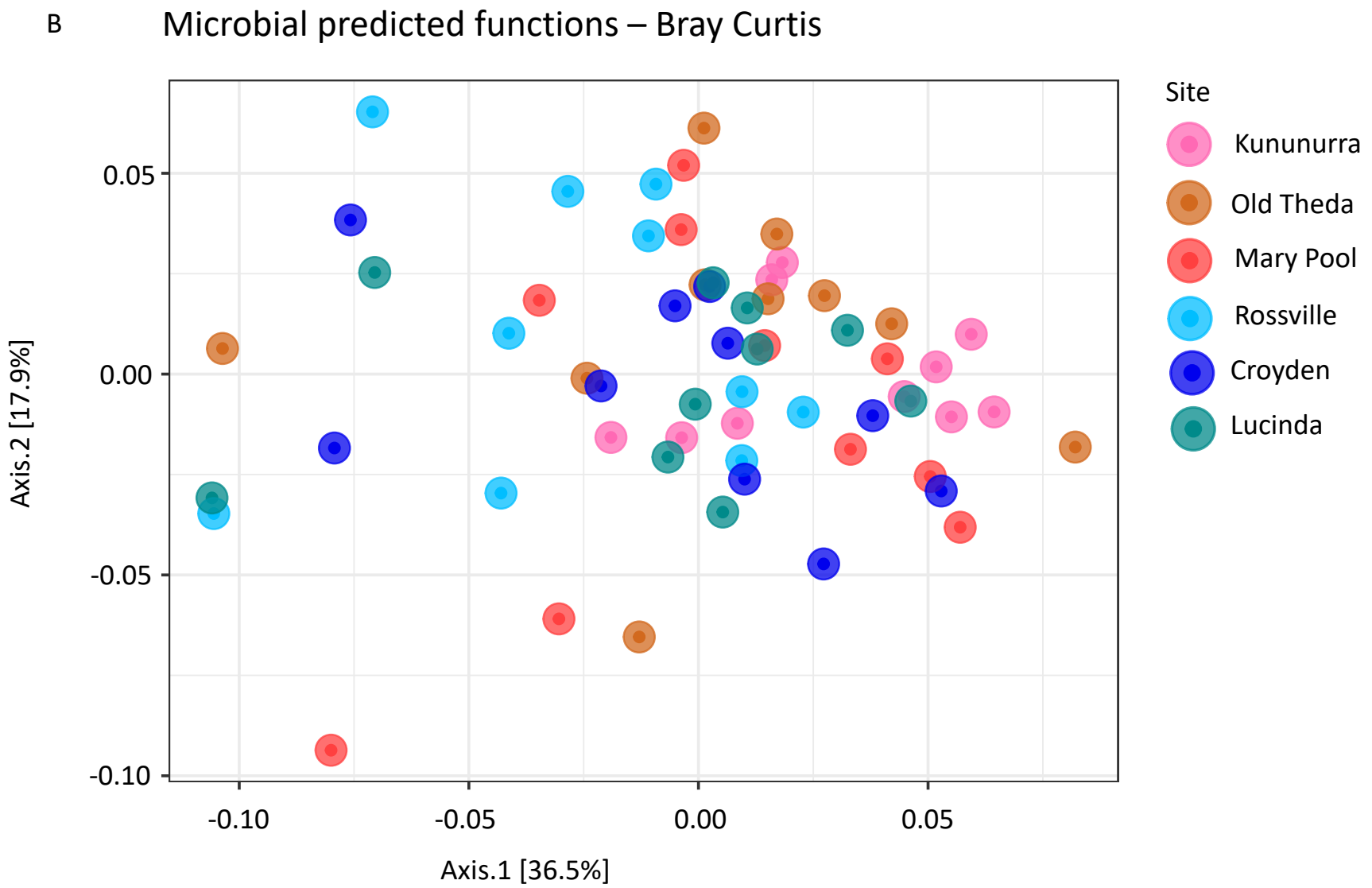
616 **Figure 4. Main variables that affect predicted function differentiation among individuals.** CAP
617 (*capscale*) plot displays the combination of variables that explained the greatest variation in the
618 predicted functions through model selection, using 60 cane toad individuals from the invasion-front
619 (Kununurra, Old Theda, and Mary Pool) and the range-core (Rossville, Croydon, and Lucinda). The
620 final model explained 17.8% of variation in the microbial predicted functions, which includes
621 righting effort likelihood (AIC = 53.613), occurrence of lung worms (AIC = 54.297) and righting
622 time (AIC = 56.912) explained the greatest variation.

623

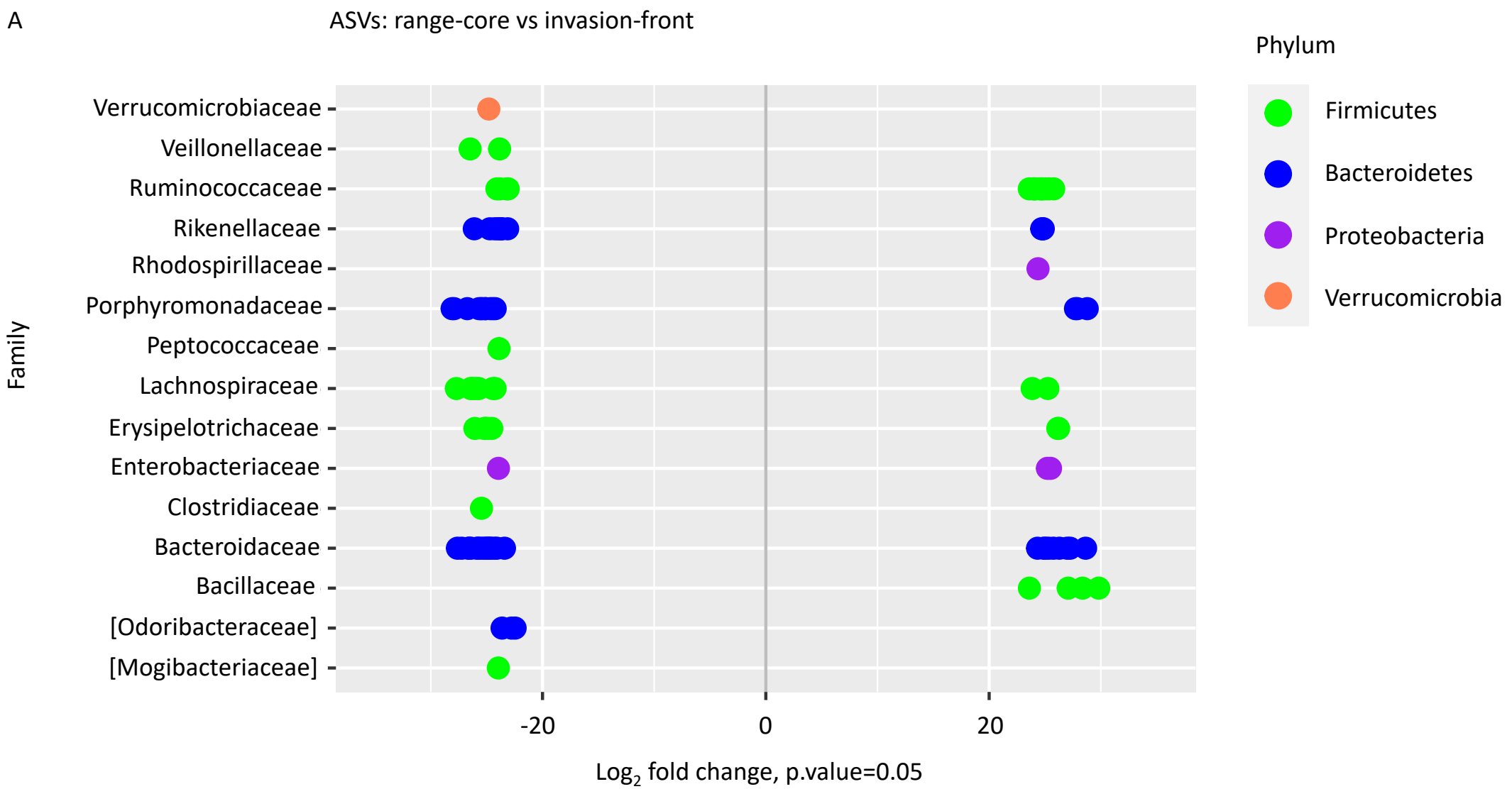
A


Microbial community – Bray Curtis
Axis.2 [13.2%]
0.2
0.0
-0.2
-0.4
-0.2
0.0
0.2
0.4
Axis.1 [14.8%]
Site
Kununurra
Old Theda
Mary Pool
Rossville
Croyden
Lucinda

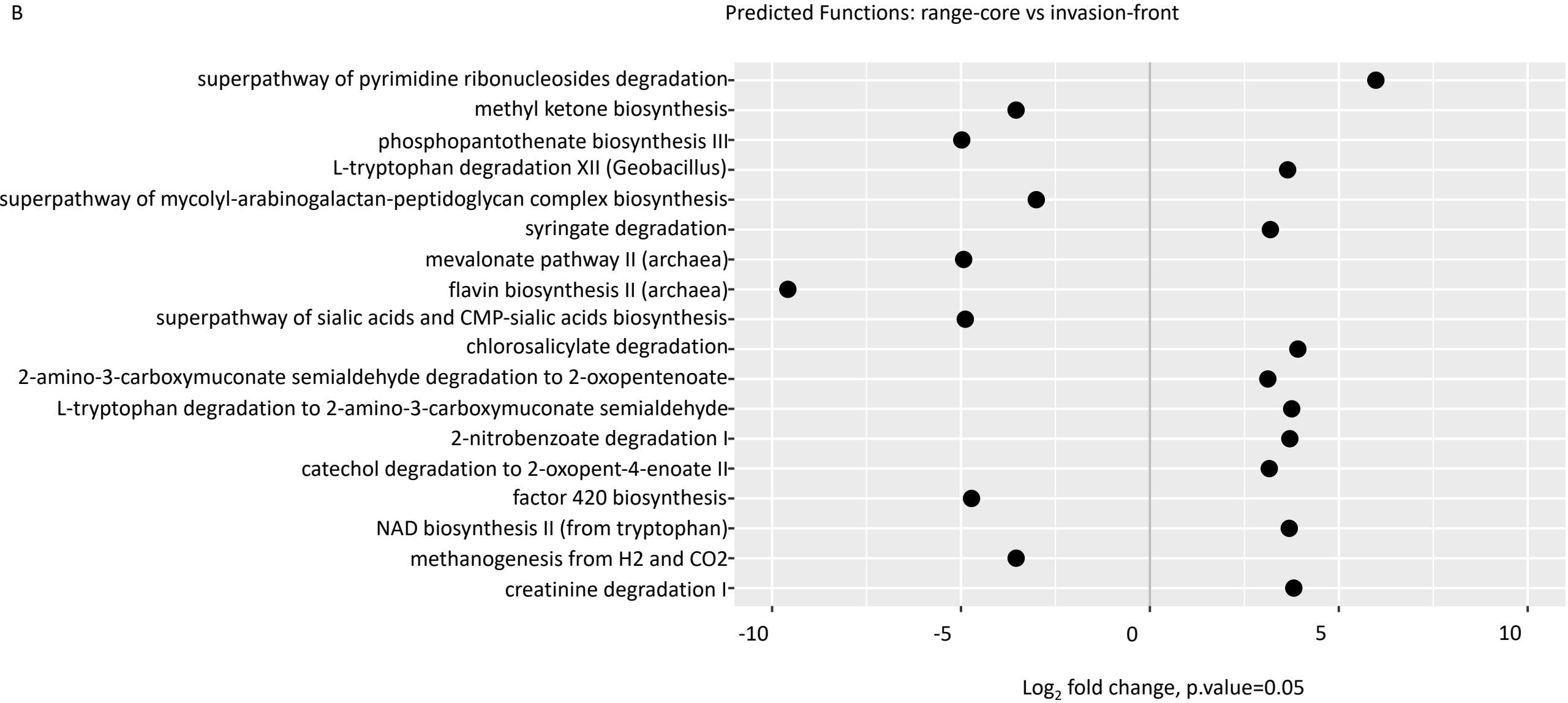
B

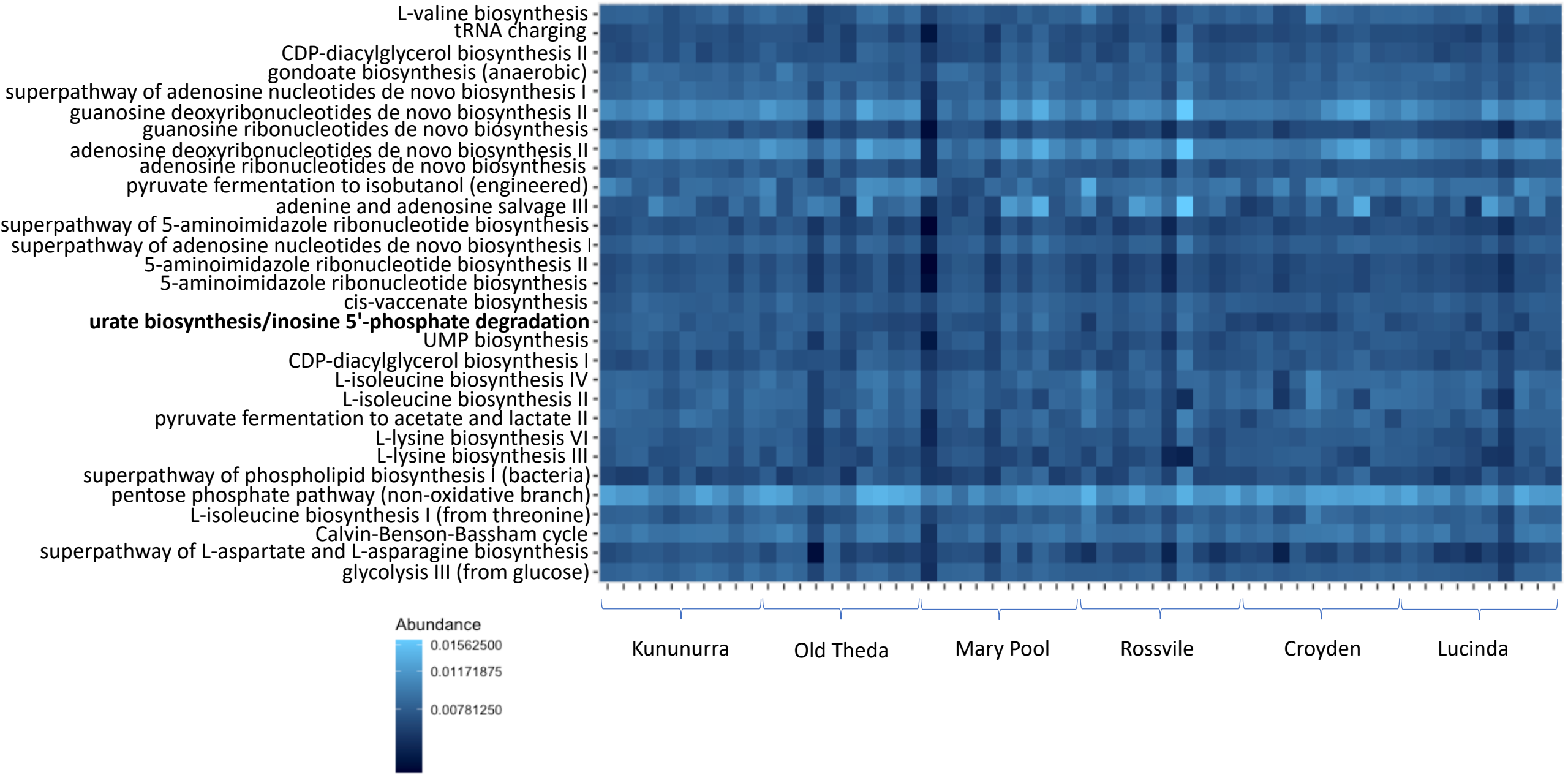

Microbial predicted functions – Bray Curtis
Axis.2 [17.9%]
0.05
0.00
-0.05
-0.10
-0.10
-0.05
0.00
0.05
Axis.1 [36.5%]
Site
Kununurra
Old Theda
Mary Pool
Rossville
Croyden
Lucinda

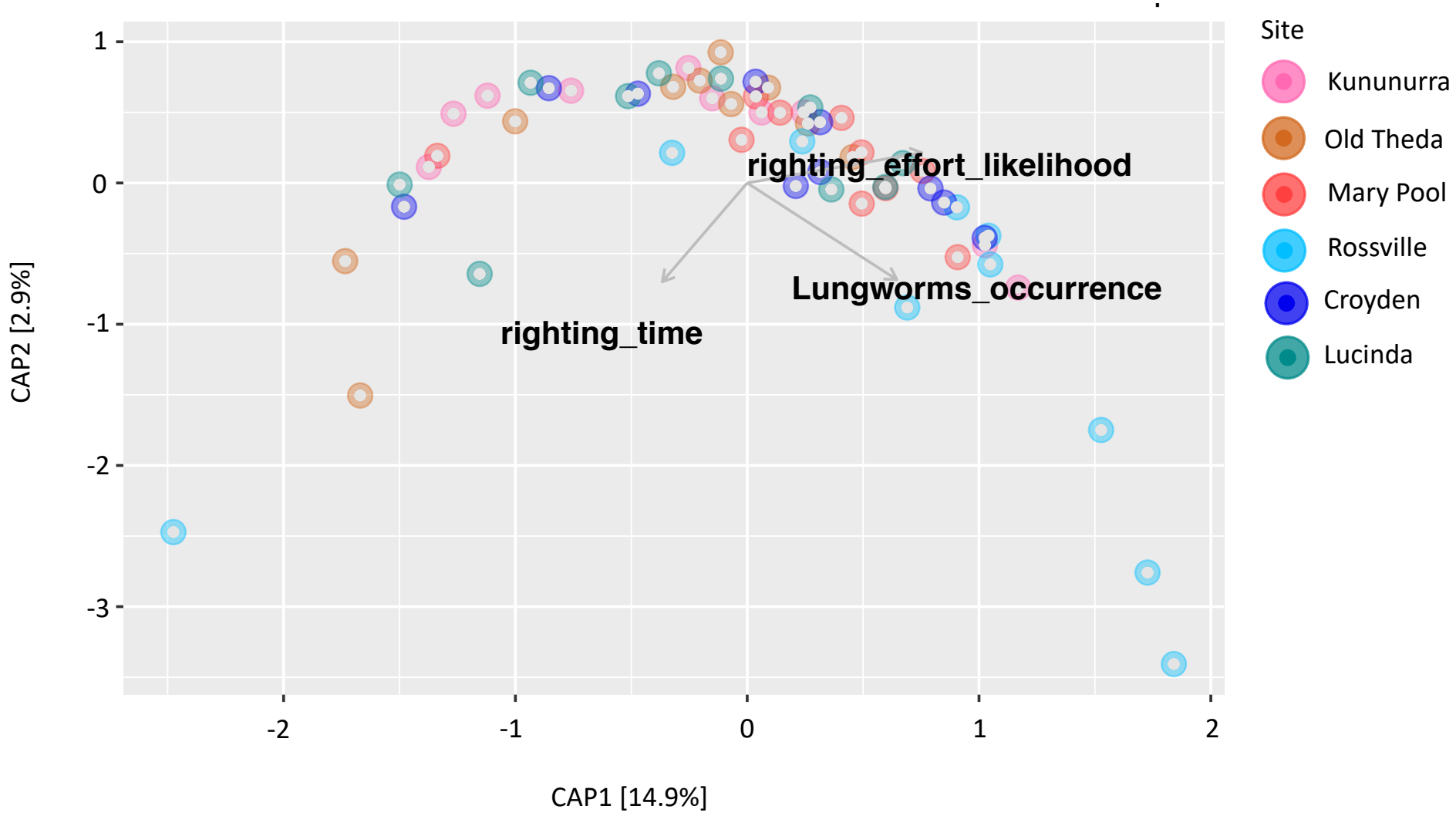
A


ASVs: range-core vs invasion-front
Phylum
Firmicutes
Bacteroidetes
Proteobacteria
Verrucomicrobia
Family
Verrucomicrobiaceae
Veillonellaceae
Ruminococcaceae
Rikenellaceae
Rhodospirillaceae
Porphyromonadaceae
Peptococcaceae
Lachnospiraceae
Erysipelotrichaceae
Enterobacteriaceae
Clostridiaceae
Bacteroidaceae
Bacillaceae
[Odoribacteraceae]
[Mogibacteriaceae]
-20
0
20
Log2 fold change, p.value=0.05

B


Predicted Functions: range-core vs invasion-front
superpathway of pyrimidine ribonucleosides degradation
methyl ketone biosynthesis
phosphopantothenate biosynthesis III
L-tryptophan degradation XII (Geobacillus)
superpathway of mycolyl-arabinogalactan-peptidoglycan complex biosynthesis
syringate degradation
mevalonate pathway II (archaea)
flavin biosynthesis II (archaea)
superpathway of sialic acids and CMP-sialic acids biosynthesis
chlorosalicylate degradation
2-amino-3-carboxymuconate semialdehyde degradation to 2-oxopentenoate
L-tryptophan degradation to 2-amino-3-carboxymuconate semialdehyde
2-nitrobenzoate degradation I
catechol degradation to 2-oxopent-4-enoate II
factor 420 biosynthesis
NAD biosynthesis II (from tryptophan)
methanogenesis from H2 and CO2
creatinine degradation I
-10
-5
0
5
10
Log2 fold change, p.value=0.05


L-valine biosynthesis
tRNA charging
CDP-diacylglycerol biosynthesis II
gondoate biosynthesis (anaerobic)
superpathway of adenosine nucleotides de novo biosynthesis I
guanosine deoxyribonucleotides de novo biosynthesis II
guanosine ribonucleotides de novo biosynthesis
adenosine deoxyribonucleotides de novo biosynthesis II
adenosine ribonucleotides de novo biosynthesis
pyruvate fermentation to isobutanol (engineered)
adenine and adenosine salvage III
superpathway of 5-aminoimidazole ribonucleotide biosynthesis
superpathway of adenosine nucleotides de novo biosynthesis I
5-aminoimidazole ribonucleotide biosynthesis II
5-aminoimidazole ribonucleotide biosynthesis
cis-vaccenate biosynthesis
urate biosynthesis/inosine 5'-phosphate degradation
UMP biosynthesis
CDP-diacylglycerol biosynthesis I
L-isoleucine biosynthesis IV
L-isoleucine biosynthesis II
pyruvate fermentation to acetate and lactate II
L-lysine biosynthesis VI
L-lysine biosynthesis III
superpathway of phospholipid biosynthesis I (bacteria)
pentose phosphate pathway (non-oxidative branch)
L-isoleucine biosynthesis I (from threonine)
Calvin-Benson-Bassham cycle
superpathway of L-aspartate and L-asparagine biosynthesis
glycolysis III (from glucose)
Abundance
0.01562500
0.01171875
0.00781250
Kununurra
Old Theda
Mary Pool
Rossvile
Croyden
Lucinda


righting_effort_likelihood
Lungworms_occurrence
righting_time
CAP1 [14.9%]
CAP2 [2.9%]
1
0
-1
-2
-3
-2
-1
0
1
2
Site
Kununurra
Old Theda
Mary Pool
Rossville
Croyden
Lucinda