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Oral Microbiome Metabarcoding in Two Invasive Small Mammals from New Zealand

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Abstract: All multicellular organisms host a wide diversity of microorganisms in and on their bodies, which are collectively known as their microbiome. Characterising microbial communities that inhabit different body niches in wild animals is critical to better understand the dynamics of microbiome diversityand its functional significance. The current study is the first to apply massively parallel sequencing of 16S rRNA to characterise the microbial diversity and functional content of oral microbiota in two of New Zealand's most important invasive mammals, the omnivorous common brushtail possum (*Trichosurus vulpecula*) and the carnivorous stoat (*Mustela erminea*). In total, strains of bacteria belonging to 19 different phyla, 27 classes, 52 orders, 103 families, 163 genera and 51 known species were identified from the oral cavities of the study species. Strains of the phyla Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria, and Actinobacteria dominated the core oral microbial diversity in both species, while other taxa were comparatively less abundant. Despite invasive populations typically demonstrating limited genetic variation, intraspecific variation of the core bacterial taxa in the oral microbiota was considerable. This suggests that a complex interaction between genetic, physiological, and environmental factors determines the diversity of the study species'oral microbiome.

Keywords: microbiome; oral cavity; microbiota; common brushtail possum; stoat; invasive species; microbiota diversity

1. Introduction

Microbes have dominated Earth's evolutionary landscape for billions of years and have developed a plethora of biochemical adaptations to exploit all conceivable ecological niches on the planet. Some of these microbial metabolic repertoires ultimately became integrated into multicellular organisms [1,2], and have been an essential component in organismal evolution and development ever since [3].

All eukaryotic organisms host a variety of bacteria, archaea, fungi, protists, and viruses in and on their bodies. The combined number of these microorganisms and their genome size, known collectively

as the microbiome, typically exceeds that of the host's somatic cells [4]. The spectrum of the relationships between eukaryote hosts and their microbial communities varies from obligate symbiosis to pathogenic dysbiosis [5,6]. Host physiology, immune systems, and genetic backgrounds influence microbiome dynamics and complexity. Similarly, microbial communities strongly affect various fitness-associated physiological functions, host development, innate and adaptive immune responses, and can even modulatehost behavior [6–14]. Evolutionary processes affect eukaryote hosts and their microbiomes concurrently [15,16]. Intricate and interdependent relationships between eukaryotic organisms and their microbiomes create a situation where the two evolve as a single entity, known as a holobiont [3]. This idea is the foundation of the hologenome theory of evolution [17], which maintains that eukaryotic organismal fitness is the product of the integrated activities of both the hosts and all their associated microorganisms at any given point in time. As such, holobionts and their associated hologenomes represent a previously underestimated hierarchical level of eco-evolutionary and genetic processes [18].

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Compared to the microbial diversity of mammalian distal digestive tracts [19–22], the microbial diversity of the oronasal cavity has received little attention [23–30]. The oral cavity contains multiple ecological niches, such as teeth, gingival sulcus, tongue, cheeks, hard and soft palates, as well as saliva and tonsils, each of which harbours a dynamic microbial community [31,32]. Food digestion commences in the oral cavity, which serves as the first line of exposure to, and interpretation of, physio-chemical stimuli that can modulate complex feeding and mating behaviours. Pathological imbalance in the oral microbiota may progress to cardiometabolic, respiratory, immunological, gastrointestinal and obstetric diseases, with negative consequences to the host's fitness [33–36].

New Zealand was colonised by humans approximately 800 years ago [37]. Two consecutive waves of human colonisation, habitat destruction, hunting, and the introduction of alien species have had catastrophic impacts on New Zealand's endemic ecosystems [38–40]. An omnivorous marsupial, the Australian common brushtail possum *(Trichosurus vulpecula)*, hereafter the brushtail possum, and a predatory placental mammal, the stoat (*Mustela erminea*), have had particularly destructive impacts. Soon after their introduction in the 1800s, these species spread rapidly in their new environments and preyed on native species that possessed no prior adaptations to evade mammalian land predators.

Moreover, brushtail possums, and to a lesser extent, stoats, form the largest reservoir of bovine tuberculosis in the wild, with potentially negative impacts on New Zealand's farming and dairy industries, as well as public health.

In 2017, New Zealand conservation and agriculture authorities started an ambitious project aimed at eradicating all invasive species from the archipelago by 2050. To reach the objectives of the"New Zealand Predator Free 2050" initiative, an in-depth understanding of the biology and natural history of the invasive species is required [41]. Characterisingthe microbial communities that live in and on the different host species is an indispensable step towards this objective.Studying the functional content of the microbiome in invasive species is of particular interest since behavioural, physiological,

and genomic adaptations to new environments take place on a comparatively short evolutionary time scale, implying that evolutionary mechanisms other than those originating from the slowly-evolved host genome may be involved [12,42].

In the current study, we used metabarcoding of 16S rRNA by means of massively parallel sequencing to characterise the taxonomic diversity and functional content of oral microbiota in the brushtail possum and the stoat in New Zealand and investigated the extent of intra- and inter-species variation in oral microbial diversity in the subject animals.

This study may serve as a starting point for more elaborate and robust analyses of oral microbiomes in the context of biological invasions. Such knowledge holds unprecedented therapeutic, environmental, and socio-economic promises that reach beyond the realm of wildlife genomics [43].

2. Materials and Methods

2.1. Animal Ethics Statements

The Lincoln University, Animal Ethics Committee approved oral sample collection and animal handling (SOP 29–14 "Protocol for sedation of possums"; SOP 33–14 "Protocol for anaesthesia of stoats"). All necessary steps were taken to minimise the pain and suffering of the test subjects.

2.2. Sample Collection and Genomic Library Preparation

Five adult brushtail possums (three females and two males) and five stoats (three females and two males) were live-captured from different populations on Bank Peninsula and West Coast, New Zealand. The animals were immediately transferred to the wild animal husbandry, Johnstone Memorial Laboratory at Lincoln University. After arrival at the facility, the health status of each animal was assessed by the Lincoln University wildlife veterinary team. To reduce the effects of diet on oral microbiome diversity, individuals of each species were fed the same diet prior to microbiome sample collection. The microbiome samples were collected between 9 a.m. and 12 p.m. from fasted subject animals within 36 h of arrival at the facility.

Stoats were anaesthetised using halothane and isoflurane inhalation protocols [44] for a maximum of 2 min [45], whereas the brushtail possums were lightly sedated by intravenous administration of an appropriate dosage of tiletamine-zolazepam (Zoleti®) [46]. After complete immobilisation was achieved, each animal's outer surfaces of the gingiva in the upper and lower dental arches, the inner cheek lining, the soft and hard palates, the surface of tongue, and the floor of the mouth were carefully swabbed using a sterile cotton swab. Special attention was paid to ensuring that sample collection was consistent between individuals. The cotton swabs were air-dried in a sterile cabinet for a maximum of 5 min, transferred to a sterile cylindrical plastic pouch, and stored at −80 °C until DNA was extracted, within a week.The health condition of each animal was closely monitored during and after the sampling procedure until full recovery was observed.

Metagenomic DNA of high molecular weight was extracted from the buccal swabs [47], and the hypervariable regions (V1–V2) of the 16S rRNA were amplified using the primer pair 8F (5′-TCG TCG GCAGCG TCA GAT GTG TAT AAG AGA CAG AGA GTT TGA TCC TGG CTC AG-3′) [48] and 338R (5′-GTC TCG TGG GCTCGG AGA TGT GTA TAA GAG ACA GGC TGC CTC CCG TAG GAG T-3′) [49]. Primer sequences contained overhang adapters appended to the 5′ end for compatibility with Illumina sequencing platforms. Each PCR reaction contained 12.5 ng of template DNA, 0.2 µM of each primer and 2 × KAPA HiFi HotStart Ready Mix (KAPA Biosystems, Wilmington, NC, USA). Each batch of PCR reactions included three positive and three negative control reactions. The thermal profile for PCR amplifications started with an initial denaturing step at 95 °C for 3 min, followed by 25 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s, and a 30 s extension at 72 °C, followed by a final 5 min extension at 72 °C. PCR amplicons were purified using the AMPure XP reagent (Beckman Coulter, Indianapolis, IN, USA).This was followed by eight cycles of barcoding PCRs, each with an initial denaturing step at 95 °C for 3 min, annealing at 55 °C for 30 s, 30 s extension

at 72 °C, and a final 5 min extension at 72 °C. The resulting libraries were purified using the AMPure XP reagent (Beckman Coulter, Pasadena, CA, USA) and pooled in equimolar concentrations before being sequenced on an Illumina MiSeq sequencing platform (Illumina, SanDiego, CA, USA) using a V2, 2 × 250 bp chemistry, at the Chapel Hill Microbiome Core Facility, University of North Carolina.

Demultiplexed sequences were processed using the Qiime2 v11.2017 package [50]. Illumina sequencing adapters, contaminants, and over-represented sequences were filtered using the Qiime2 Cutadapt v1.18 plugin [51]. The Qiime2 DADA2 plugin [52] was used to remove low-quality sequences (Phred score < 25), detect potential chimeric reads, and de-noise demultiplexed sequences into unique sequence features. To assign a taxonomy rank to the sequence features, the Qiime2 "blast-consensus feature classifier plugin" was used, with the number of best-accepted hits set to four, the minimum percentage identity to 70%, and the *p*-value to a maximum of 0.001. Quality-filtered sequences were searched against the Greengenes v13_8, 99% OTUs full-length database [53]. Sequences that were unassigned or lacked taxonomy IDs in the NCBI taxonomy database (https://www.ncbi.nlm.nih.gov/taxonomy) were discarded. The taxonomy ID of each consensus match was extracted using an in-house Python3 script, and a circular phylogenetic tree was reconstructed using the PhyloT online server [54] and visualised in ITol v.33 [55].

To estimate the phylogenetic distance necessary for computing some diversity indices, an approximate maximum-likelihood phylogenetic tree was constructed using the package FastTree2 [56]. Before running the core diversity script in Qiime2, the sequence counts across different individuals were randomly subsampled to an estimated equal rarefaction depth of 79,690 sequences. Rarefaction at this sequencing depth resulted in the maximum number of sequences (67.7% of the total sequences) being retained in nine individuals. To minimise the bias in diversity metrics estimation, one stoat with a lower number of sequences than the above threshold was discarded from the diversity analysis.

Four α-diversity metrics including Faith phylogenetic distance [57], group evenness [58], Shannon diversity [59], and Bray-Curtis dissimilarity [60] were estimated separately for each species. To calculate β-diversity variation between the two species, unweighted and [61] weighted UniFrac distance [62] were computed. Principal coordinates analysis [63] was performed on a subset of diversity metrics (Bray-Curtis, Jaccard, and UniFracs), and results were visually inspected using the Emperor Python package [64].

The statistical significance for differences in α-diversity (evenness and Faith phylogenetic indices) and β-diversity (unweighted UniFrac) between two species were tested by performing group-wise non-parametric Kruskal-Wallis [65] and permutational multivariate analysis of variance (PERMANOVA) tests [66].

To find modules of co-occurring bacterial taxa in each species, the SCNIC package (https://github.com/shafferm/SCNIC) was used to create a sparse positive co-occurrence correlation network of different bacterial taxa by setting the minimum pairwise correlation value to 0.30 (R = 30), and the network files were visualised in Gephi v0.9.2 [67].

Bacterial taxa whose abundance differed between two species were identified using the Gneiss balances method [68]. First, a correlation-clustering balance tree was constructed for each species. In the correlation-clustering algorithm, an unsupervised Ward's hierarchical clustering algorithm [69] groups different bacterial taxa interactively based on their co-occurrence in each species' oral microbiota. Then, a multivariate linear regression analysis [70] was performed independently on each node (balance) of the resulting tree and tested for significant changes in bacterial abundance between pairs of species. In the regression analysis, the description (animal ID) and taxonomic infraclass (marsupial versus placental) were selected as explanatory variables, and taxon abundance was set as the response variable. To evaluate the explanatory power of a single covariate, a leave-one-variable-out method was applied. The variable whose removal caused the most significant changes in the fitted model was reported as having the strongest effects on oral microbiome diversity. Overfitting of the regression model was

monitored by dividing the data into ten partitions and evaluating the prediction accuracy of the model built based on nine partitions on the remaining partition.

The PICRUSt2 [71] pipeline was executed to predict metabolic pathways for the different bacterial taxa present in each species' oral cavities. Alternatively, the biochemical functions of differentially abundant taxa between pairs of species were predicted using the BioCys online server [72]. Functional enrichments of the oral microbial communities for different biochemical functions were tested using Microbiome Analyst tools [73]. Raw sequences generated for this study are available on the NCBI SRA database under BioProject (accession number PRJNA61279).

3. Results

None of the animals showed any signs of major acute or systematic pathology during physical examination. Only minor gum inflammation, typical of wild populations, was observed. Negative control reactions in all PCR setups did not produce any products. Moreover, genomic DNA libraries prepared from negative control reactions failed quality checking prior to Illumina sequencing and were discarded.

The sequencing run yielded 1219,643 paired-end, quality-filtered sequences. Each buccal swab produced 121,964 sequences on average. Brushtail possum oral swabs yielded more raw sequences compared with stoat oral swabs. The DADA2 plugin collapsed the raw sequences into 1083 unique sequence features. The blast-consensus feature classifier assigned a taxonomy rank to 590 sequence features in brushtail possums and to 549 sequence features in the stoats, with a mean posterior confidence level of 92%.

In total, strains of bacteria belonging to 19 different phyla, 27 classes, 52 orders, 103 families, 163 genera and 51 known species were identified from the oral cavities of the study species. The oral cavity microbiome in both species was dominated by different strains of the phyla Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria, and Actinobacteria. Members of Tenericutes, Saccharibacteria (candidate phylumTM7), Absconditabacteria (candidate phylum SR1), Cyanobacteria, Gracilibacteria (candidate phylum GN02), Chlorobi, Spirochaetes, Acidobacteria, Thermi, Planctomycetes, Dependentiae (candidate phylum TM6), Armatimonadetes, Chloroflexi, and Thermotogae were also detected but were present at a comparatively lower abundance. Absconditabacteria(candidate phylum SR1), Gracilibacteria (candidate phylum GN02), Spirochaetes, and Thermotogae were only detected in the possum oral microbiota. Strains unique to stoat oral cavities were Chlorobi, Acidobacteria, Thermi, Planctomycetes, Armatimonadetes, and Chloroflexi. Strains of *Pasteurellaceae* (32.02%), *Neisseriaceae* (18.29%), *Streptococcaceae* (17.76%), and *Leptotrichiaceae* (10.04%) were the most abundant families in the oral cavities of brushtail possums. In stoats, members of *Neisseriaceae* (22.47%), *Mycoplasmataceae* (11.12%), *Pasteurellaceae* (10.97%), and *Flavobacteriaceae* (10.28%) dominated oral bacterial diversity (Table 1).

The variation in group-evenness and Faith phylogenetic α-diversity indices were not statistically significant between brushtail possums and stoats ($H = 3.84$, p-values = 0.05 and H = 0.24, p-values = 0.62, respectively). PERMANOVA test results on unweighted UniFrac metrics showed that β-diversity differed significantly between the two species (pseudo $F = 3.43$, p-values= 0.007, and 999 permutations).

Principal coordinates analysis of the oral microbiota based on Bray-Curtis, Jaccard's, and unweighted Unifrac metrics demonstrated that the oral microbiota of the brushtail possums clustered densely together, while stoat microbiota showed higher intra-individual variation and clustered more loosely. This pattern is less evident in weighted UniFrac metrics where the presence of low-abundance taxa most likely confounded the clustering pattern (Figure 1).

Table 1. Abundance of different bacterial families identified from the oral microbiota of brushtail possums and stoats.

Phylum	Family	Possum Specimens					Stoat Specimens				
		a	**b**	**c**	**d**	**e**	**a**	**b**	**c**	**d**	**e**
Actinobacteria	*Acidobacteriaceae*	2	0	0	9	6	0	0	0	0	0
	C111	0	0	0	0	4	0	0	0	0	0
	Actinomycetaceae	6671	4399	6709	0	0	890	324	1155	238	4265
	Brevibacteriaceae	5	7	0	11	7	0	0	0	0	0
	Cellulomonadaceae	0	0	0	0	3	0	0	0	0	4
	Corynebacteriaceae	226	949	397	50	23	150	604	392	26	79
	Dermabacteraceae	0	0	0	9	13	0	0	0	0	11
	Dermacoccaceae	0	0	0	3	0	0	0	0	0	0
	Dietziaceae	0	4	0	0	0	0	0	0	0	0
	Geodermatophilaceae	0	0	0	0	7	0	0	0	0	0
	Intrasporangiaceae	0	0	0	9	37	0	0	0	0	0
	Kineosporiaceae	0	0	0	0	15	0	0	0	0	0
	Microbacteriaceae	0	0	0	9	93	744	4	26	43	202
	Micrococcaceae	26	18	0	5	36	0	65	90	1855	12
	Nakamurellaceae	0	0	0	12	0	0	0	0	0	0
	Nocardioidaceae	0	0	0	16	36	0	0	0	0	0
	Propionibacteriaceae	6064	30	8846	123	42	113	6	257	120	115
	Pseudonocardiaceae	0	0	0	3	17	0	0	0	0	0
	Sporichthyaceae	0	0	0	0	5	0	0	0	0	0
	Streptomycetaceae	0	0	0	4	29	0	0	3	0	0
	Williamsiaceae	0	0	0	4	0	0	0	0	0	0
Bacteroidetes	*Coriobacteriaceae*	0	2	9	0	6	0	9	0	2	0
	Euzebyaceae	0	0	0	0	0	0	0	4	0	0
	Gaiellaceae	0	0	0	2	3	0	0	0	0	0
	Patulibacteraceae	0	0	0	0	14	0	0	0	0	0
	Solirubrobacteraceae	0	0	0	7	11	0	0	0	0	0
	Bacteroidaceae	31	9	5	18	4	6	0	3	165	0
	Porphyromonadaceae	73	1286	42	29	20	3092	2093	466	482	5361
	Prevotellaceae	38	63	53	35	91	156	572	62	157	494
	S24-7	0	0	0	0	0	0	0	4	7	4
	Barnesiellaceae	0	0	0	0	7	0	0	0	0	0
	Paraprevotellaceae	3	9	0	0	7	4	0	0	6	0
	Cyclobacteriaceae	2	0	0	0	0	0	0	0	0	0
	Cytophagaceae	4	0	0	0	15	0	0	0	0	0
	Flavobacteriaceae	4887	23,828	17,570	2099	17	2238	2809	937	1856	5121
	Weeksellaceae	11,660	7945	2085	4249	14,979	405	945	477	111	1068
	Sphingobacteriaceae	7	0	0	15	58	0	4	0	0	0
	Chitinophagaceae	0	0	0	9	21	0	0	0	0	4
Chloroflexi	*Dolo_23*	0	0	0	0	2	0	0	0	0	0
Firmicutes	*Bacillaceae*	13	4	6	5	12	5	0	0	0	0
	Paenibacillaceae	0	0	0	0	0	5575	1657	0	2645	61
	Planococcaceae	0	0	2	14	8	0	0	6	0	0
	Staphylococcaceae	86	7	0	246	300	0	3	3	0	3
	Exiguobacteraceae	0	4	0	0	0	3	0	0	0	0
	Thermicanaceae	5	0	0	4	9	0	0	0	0	0
	Gemellaceae	10,310	1821	1049	12,727	15,233	3438	829	84	2015	0
	Aerococcaceae	429	4295	218	1718	747	1226	4145	113	1258	1590
	Carnobacteriaceae	10	11	5	2	46	80	7	11	130	98
	Enterococcaceae	7	0	5	0	8	0	7	0	6	5
	Lactobacillaceae	4	4	0	0	28	0	2	14	12	0
	Leuconostocaceae	0	3	0	0	8	0	0	0	0	0
	Streptococcaceae	51	840	132	3196	12,656	26,169	19,442	14,569	32,630	11,017
	Turicibacteraceae	0	0	0	0	3	0	0	0	14	0
	Clostridiaceae	170	13	52	458	190	0	0	7	67	13
	Lachnospiraceae	24	31	22	2	41	511	1153	175	814	191
	Peptococcaceae	0	0	0	0	0	0	0	9	0	4
	Peptostreptococcaceae	37	6	0	166	53	0	28	26	0	2
	Ruminococcaceae	20	12	6	7	42	4	5	0	0	34
	Veillonellaceae	45	62	57	19	35	20	1666	97	454	57
	Acidaminobacteraceae	0	0	0	0	0	0	0	73	0	12
	Mogibacteriaceae	0	0	0	0	0	0	2	0	0	3
	Tissierellaceae	45	0	7	9	6	4	0	15	0	8
	Erysipelotrichaceae	0	1622	6	0	11	15	11	23	145	50

Table 1. *Cont.*

Phylum	Family	Possum Specimens					Stoat Specimens				
		a	**b**	**c**	**d**	**e**	**a**	**b**	**c**	**d**	**e**
Fusobacteria	*Fusobacteriaceae*	21	20	19	54	66	5151	17,078	1478	7176	15,051
	Leptotrichiaceae	784	26	7	0	55	3050	28,266	2224	7229	17,960
Proteobacteria	*Caulobacteraceae*	6	0	0	5	10	0	0	0	0	0
	Bradyrhizobiaceae	0	0	0	9	0	0	0	0	0	0
	Brucellaceae	0	0	0	0	6	0	0	0	0	0
	Hyphomicrobiaceae	0	0	0	0	9	0	0	0	0	0
	Methylobacteriaceae	0	0	0	14	72	0	0	0	0	0
	Methylocystaceae	0	0	0	0	6	0	0	0	0	0
	Rhizobiaceae	0	11	0	14	30	0	0	0	0	0
	Rhodobacteraceae	0	0	0	3	11	0	0	0	0	4
	Acetobacteraceae	5	0	2	0	29	0	0	0	0	0
	Rhodospirillaceae	0	0	0	0	3	0	0	0	0	0
	mitochondria	0	0	0	0	8	0	0	0	2	0
	Erythrobacteraceae	0	0	0	0	8	0	0	0	0	0
	Sphingomonadaceae	6	0	0	4	91	6	0	0	0	0
	Alcaligenaceae	4	0	0	0	0	3	0	2	23	4
	Burkholderiaceae	6992	29,794	1774	1606	66	1099	239	208	86	83
	Comamonadaceae	2032	39	1616	7	56	99	0	40	14	158
	Oxalobacteraceae	0	4	0	10	76	0	0	4	0	0
	Methylophilaceae	6	0	0	0	0	0	0	0	0	0
	Neisseriaceae	11,609	28,742	19,824	10,454	35,160	25,358	10,979	22,580	43,075	4938
	Rhodocyclaceae	9	0	0	7	6	8	0	0	6	0
	Bdellovibrionaceae	0	0	0	0	2	0	0	0	0	0
	Polyangiaceae	6	0	0	0	0	0	0	0	0	0
	Syntrophobacteraceae	0	0	0	0	4	0	0	0	0	0
	Campylobacteraceae	3	0	0	0	11	71	54	19	29	55
	Helicobacteraceae	71	4	95	128	3686	0	0	0	0	0
	Chromatiaceae	0	0	4	0	0	0	0	0	0	0
	Cardiobacteriaceae	3223	3521	6280	2	0	32	0	185	16	387
	Enterobacteriaceae	41	40	24	35	140	0	0	0	0	0
	Halomonadaceae	3	0	0	0	0	0	0	0	0	0
	Pasteurellaceae	6193	9601	9533	5981	20,350	56,748	27,617	48,242	43,199	11,382
	Moraxellaceae	8	0	0	66	281	1709	2623	588	298	59
	Pseudomonadaceae	5	13	6	13	26	0	0	0	0	0
	Vibrionaceae	0	0	0	0	4	0	0	2	0	0
	Sinobacteraceae	0	0	0	0	5	0	0	0	0	0
	Xanthomonadaceae	16	3	0	26	77	2	0	0	0	0
Spirochaetes	*Spirochaetaceae*	0	0	0	0	0	0	3	36	0	3
TM7	*F16*	0	0	0	0	0	0	0	0	7	0
Tenericutes	*Acholeplasmataceae*	0	0	0	0	0	0	0	51	0	0
	Anaeroplasmataceae	0	0	0	0	0	0	0	0	6	0
	Mycoplasmataceae	27,690	2951	13,142	4572	4017	0	4	14	0	0
Thermotogae	*Thermotogaceae*	0	0	0	0	0	0	0	0	2	0
Thermi	*Trueperaceae*	0	0	0	2	0	0	0	0	0	0
	Thermaceae	6	0	0	0	0	0	0	0	0	0

The linear regression summary in Gneiss indicated that approximately 45% of the variance in oral bacterial diversity were explained by our model. Taxonomic infraclass (brushtail possum versus stoat) had significantly more influence on bacterial diversity than intraspecies variation (Rdif of 0.188 versus 0.0796). The 10-fold cross-validation confirmed that within-model error was higher than prediction accuracy in all replicates, and that model overfitting was rejected.

Bacterial taxon abundance in one specific balance in the correlation tree differed significantly between possums and stoats (false discovery rate corrected coefficient p-values of 0.001 and 0.030 for intercept and a taxonomic rank, respectively). This balance, and its descending leaf nodes, mainly consisted of strains of *Streptococcus* spp., *Leptotrichia* spp., *Heamophilus* spp., and unidentified strains belonging to *Pasteurellaceae* and *Aerrococcaceae* families.

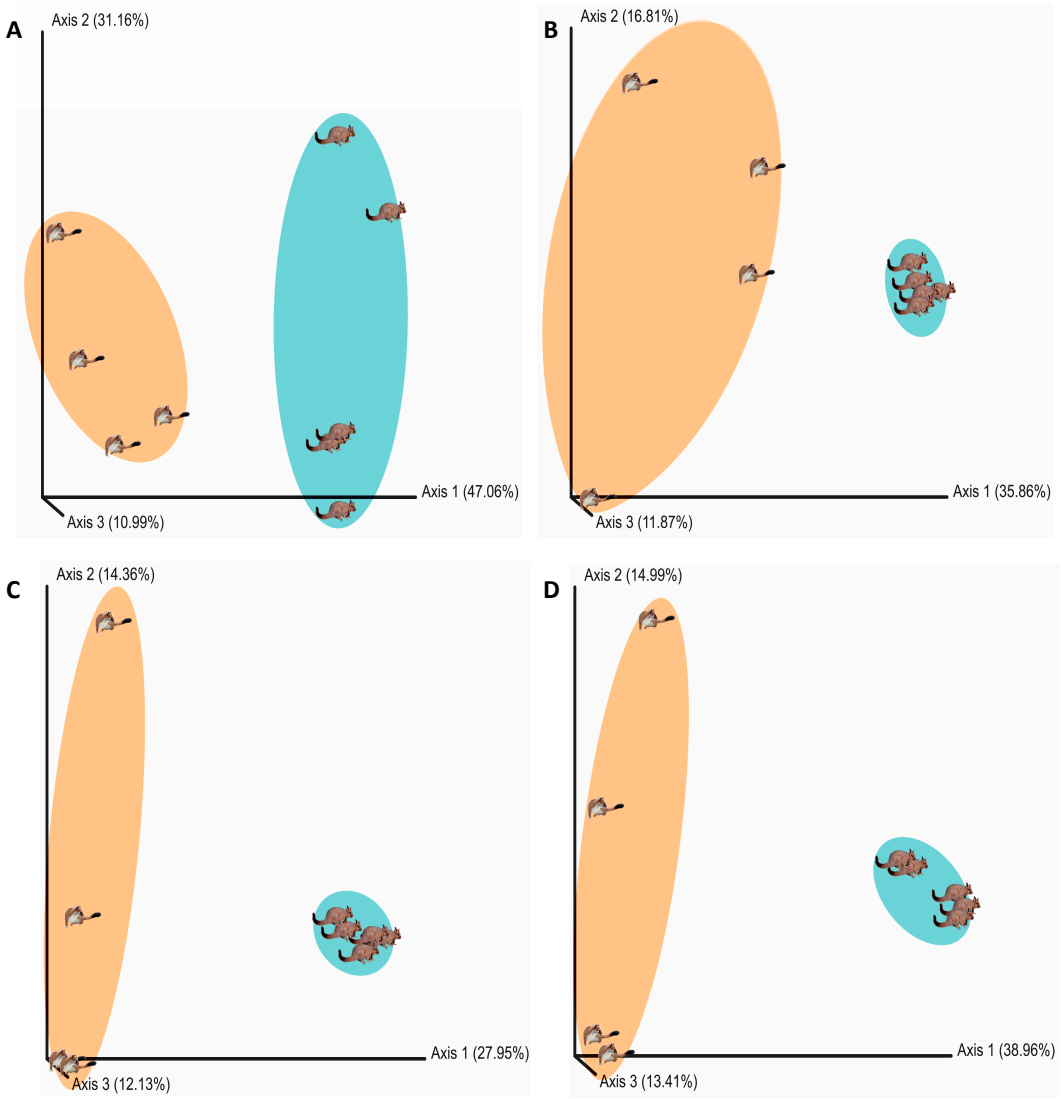

Figure 1. Component analyses of different α and β diversity indices in the oral microbiota of brushtail possums and stoats. (**A**) Weighted Unifrac, (**B**) unweighted Unifrac, (**C**) Jaccard's index, and (**D**) Bray Curtis. For each axis, the percentage of variance explained by that axis is indicated. brushtail possums (turquois) and stoats(orange).

In the modular network analysis, graph density was similar between brushtail possums and stoats (0.12). The average degree of node connectivity estimated for stoats was higher than that for brushtail possums (16.62 versus 11.01, respectively). The most connected nodes in the brushtail possum oral microbiome network consisted of *Campylobacter* sp. (n = 35), *Saccharibacteria* sp. (n = 34), and *Paenibacilus* sp. (n = 33). In stoats, *Patulibacter* sp. (n = 58), *Curtobacterium* sp. (n = 58), *Carnobacterium* sp. (n = 58), and *Streptomyces* sp. (n = 56) demonstrated the highest degree of node connectivity (Figure 2).

Metabolic pathway analysis of oral microbiome diversity revealed that the microbial taxa in the oral cavities of both species are functionally enriched for biochemical pathways involved in the biosynthesis of various metabolites (e.g., vitamins, aminoamides and organic carbon compounds), starch and sucrose metabolism, steroid hormone degradation, and antimicrobial activities (e.g., streptomycin biosynthesis) (Table 2). Functional content analysis of the bacterial taxa that differ in abundance between the two species illustrated that the majority of these bacteria are involved in the biosynthesis of different metabolites, biochemical degradation and assimilation, energy production, respiration, and detoxification.

Table 2. Functional enrichment of biochemical pathways identified from the oral microbiota of brushtail possums and stoats (names, total number of pathways and *p*-values).

Pathway	Possums		Stoats	
	Total	*p*-Values	Total	*p*-Values
Lipopolysaccharide biosynthesis	17	0	17	0
Biosynthesis of amino acids	222	<0.001	222	<0.001
Peptidoglycan biosynthesis	13	<0.001	13	<0.001
Terpenoid backbone biosynthesis	23	<0.001	23	<0.001
Streptomycin biosynthesis	12	<0.001	12	<0.001
Polyketide sugar unit biosynthesis	4	<0.05	4	<0.05
Valine, leucine and isoleucine biosynthesis	15	<0.05	-	-
Folate biosynthesis	29	<0.05	-	-
Porphyrin and chlorophyll metabolism	69	<0.001	69	<0.001
Alanine, aspartate and glutamate metabolism	62	<0.001	62	<0.001
Arginine and proline metabolism	115	<0.01	115	<0.01
Cysteine and methionine metabolism	71	<0.01	71	<0.01
Glycine, serine and threonine metabolism	78	<0.01	78	<0.01
D-Glutamine and D-glutamate metabolism	6	<0.01	6	<0.01
Thiamine metabolism	23	<0.01	23	<0.01
Starch and sucrose metabolism	65	<0.01	65	<0.05
Glyoxylate and dicarboxylate metabolism	51	<0.01	51	<0.05
Biotin metabolism	19	<0.05	19	<0.05
Riboflavin metabolism	22	<0.05	22	<0.05
Amino sugar and nucleotide sugar metabolism	64	<0.05	64	<0.05
Carbon metabolism	249	<0.05	249	<0.05
Butanoate metabolism	61	<0.05	-	-
Nicotinate and nicotinamide metabolism	36	<0.05	-	-
beta-Alanine metabolism	-	-	36	<0.05
Vitamin B6 metabolism	-	-	12	<0.05
Phenylalanine metabolism	41	<0.05	41	<0.01
Xylene degradation	20	<0.01	20	<0.01
Steroid degradation	9	<0.05	9	<0.05
Lysine degradation	-	-	30	<0.05
Synthesis and degradation of ketone bodies	5	<0.05	5	<0.05
Pentose and glucuronate interconversions	41	<0.05	41	<0,01
Carbon fixation in photosynthetic organisms	35	<0.05	35	<0.05
Pentose phosphate pathway	-	-	63	<0.01

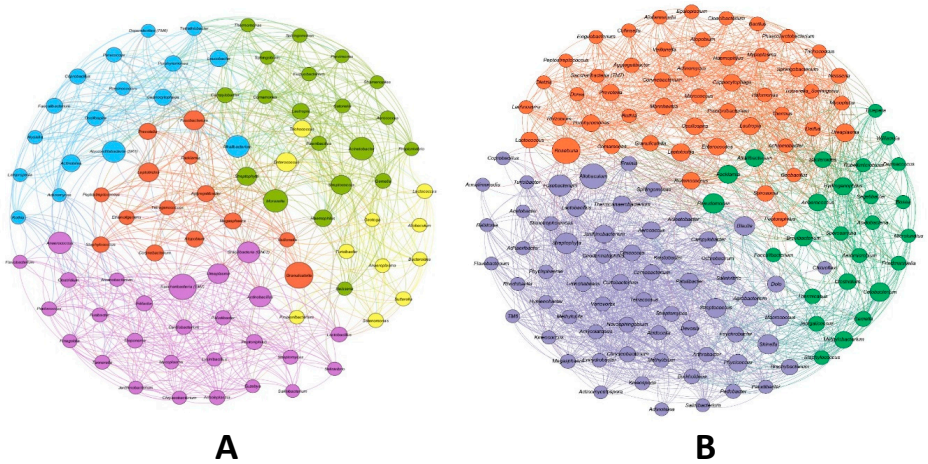

Figure 2. Modular network analysis of the bacterial taxa identified from (**A**) brushtail possums' and (**B**) stoats' oral microbiota. Node coloursrepresent the co-occurring bacterial taxa (modules). The node sizes are proportional to the degree of between centrality, and edges are coloured based on the node of origin.

4. Discussion

Recent advances in comparative genomics, biochemistry, and developmental biology have made it possible to demarcate species boundaries with an unprecedented level of accuracy. However, the interdependence between hosts and a wide diversity of microorganisms that reside in and on an individual has provided a new set of challenges in defining what constitutes an individual.

In the current study, we have described and discussed the taxonomic diversity and functional content of the oral microbiome of brushtail possums and stoats in New Zealand. The number of sequence features identified in the oral cavities of both species resembles that reported for humans [74]. However, the sequence features that were assigned the taxonomy rank of genus or species were limited, and potentially represented a high level of concealed microbial diversity that is only broadly known to science.

The oral microbiota in stoats and brushtail possums shared common features with those reported from other mammals. In both species, the oral microbiota were dominated by a small number of taxa, while other taxa were comparatively rare. The abundant bacterial taxa have been ubiquitously reported in different herbivorous, carnivorous, and omnivorous mammal species (Figure 3) [23–30].

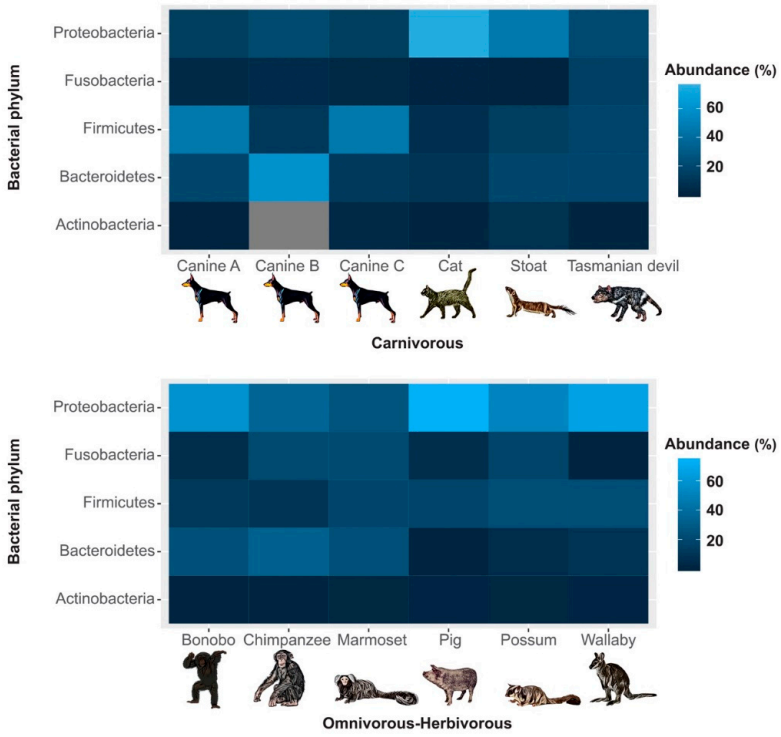

Figure 3. Major components of the oral bacterial communities of a selected number of carnivorous (top) andomnivorous-herbivorous (bottom) mammals. Colour intensities indicate the percentage abundance of five major bacterial phyla.

Diet has been suggested as one of the major determinants of oral microbial diversity [75–78]. However, consuming a similar diet is not necessarily a good predictor of oral microbiome diversity. For example, the microbiome diversity of pig (omnivore), cat (carnivore) and wallaby (herbivore) is quite similar, whereas the closely related chimpanzees and bonobos show considerable differences. In the present study, oral microbiome samples were obtained from several different niches within the oral cavity. Previous studies have shown that each niche may harbour a unique microbial community, and direct comparisons with such studies are thus limited by the fact that we pooled samples from multiple niches [79].

Our data suggest a potential link between diet and the presence of some bacterial taxa, and the biochemical functions they may perform in the oral cavity. For instance, a plant polysaccharide

metabolising taxon, *Treponema* sp., was completely absent from the oral microbiota of the carnivorous stoat. High diversity of Firmicutes in brushtail possum oral microbiota most likely reflects the presence of staple plant-based polysaccharides in their omnivorous diet (Figures 4 and 5). The distal digestive tract in this species consists of a well-developed caecum and an enlarged proximal colon that act as fermentation chambers [80]. Firmicutes bacteria in the oral cavity of brushtail possums can initiate an early digestion step that ameliorates overall energy uptake efficiency. The absence of enrichment for biochemical pathways involved in vitamin B6 (pyridoxine) biosynthesis in the brushtail possum's oral microbiome is also consistent with a diet rich in plants. Similarly, lack of metabolic pathways to biosynthesise essential aminoacids, such as valine, leucine, and isoleucine, in the stoats' oral microbiota may reflect a high intake of animal protein in the diet.

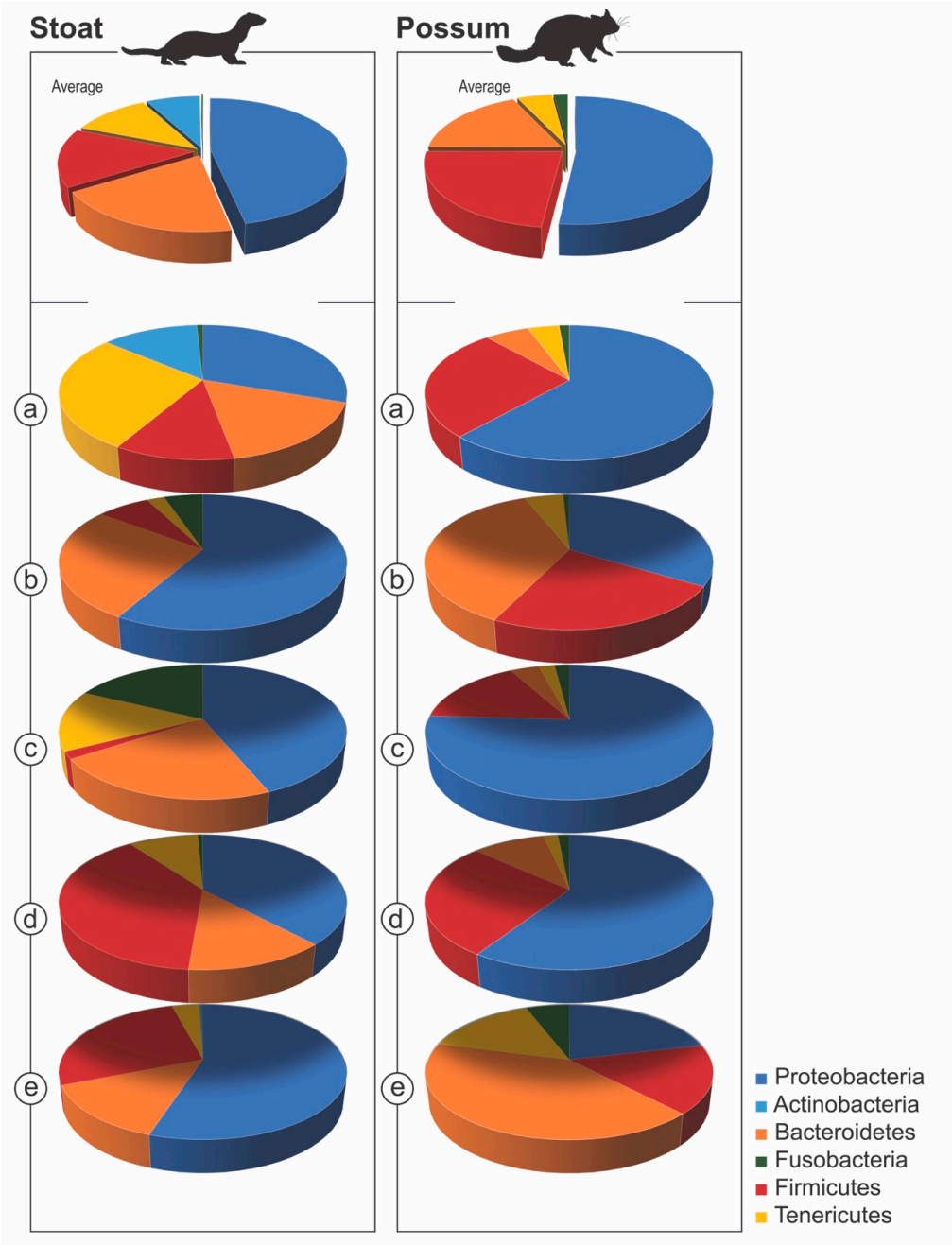

Figure 4. Inter- and intra-specific variations in the abundance of five core bacterial taxa from the oral cavities of brushtail possums and stoats. The top figure for each species depicts averages, and a–e show the variation between different individuals.

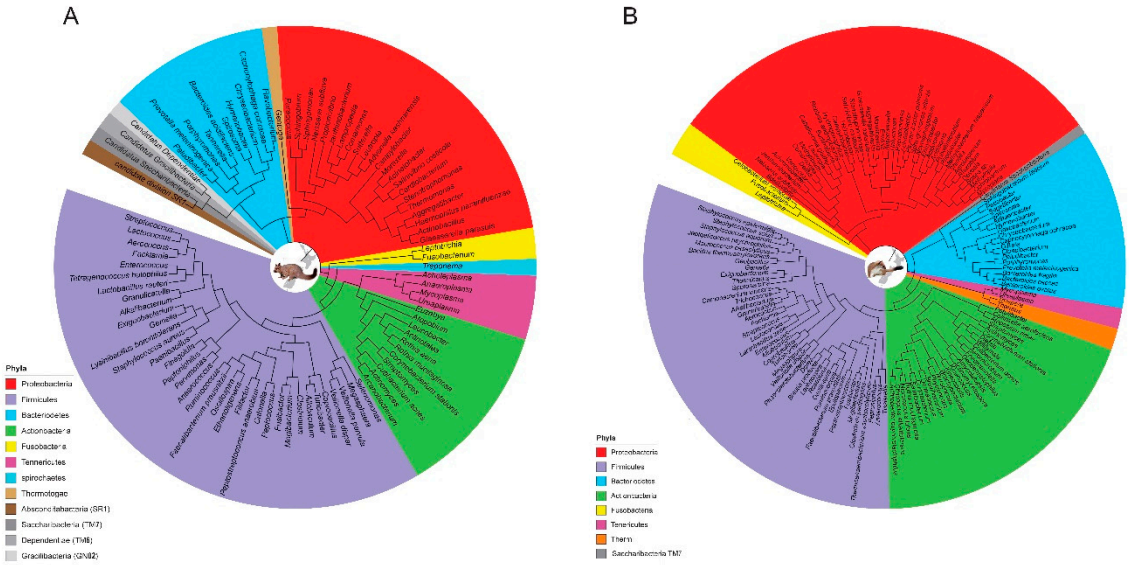

Figure 5. Phylogenetic trees showing bacterial taxa identified at the genus or species level identified from the oral cavities of (**A**) brushtail possums and (**B**) stoats. Colours represent different bacterial phyla.

Our results are amongst the first to report the presence of culture-independent candidate phylum TM6 (*Dependentiae*), thermophilic *Thermotogae*, Armatimonadetes (OP10) and Acidobacteria from the oral cavities of wild mammals. Reduced genome size and a limited biochemical repertoire in TM6 (*Dependentiae*) bacteria is indicative of an obligate endosymbiotic or parasitic lifestyle in the oral cavity [81]. Strains of Thermotogae, Armatimonadetes (OP10), and Actinobacteria have been reported from the microbiota in the gut of pregnant rats (*Rattus norvegicus*) [82], in the human oral cavity [83], and in the intestines of rice frogs (*Fejervaryalimnocharis*) [84]. The origin and functional significance of these taxa in the oral microbiota of the study species remain to be discovered.

The considerable intraspecific variation in the oral microbial diversity of each study species (Figures 4 and 5) suggests the influence of variables other than diet that were more difficult to control. These may include genetic and physiological factors, as well as differences in the environment prior to capture. These need to be taken into consideration to better understand the complexity of microbiome diversity [85–88].

Microorganisms in the alimentary tract represent a subset of the microbial diversity found in the environment. The exact mechanisms by which the host and its resident microbiome differentially select specific microbial diversity and functional content from the environment is unknown. However, earlier studies have shown that microbial diversity can be strongly predicted by the genetic make-up of the host species [15,89–97]. For examples, polymorphisms in the immunity-related gene interleukin-2 [90] and genes involved in core metabolic pathways, such as nucleotide-binding oligomerisation domain-containing protein 2 [91] and fucosyltransferase 2 [94], have been linked to a specific microbiome structure in the host species.

Despite considerable intra- and interspecific variations in oral microbiota, both species nonetheless display significant similarities in the metabolic functions of their oral bacterial communities. The observed pattern substantiates the idea that different hosts may assemble taxonomic diversity and functional content of their microbiome in different ways, but with a high level of taxonomic and functional redundancy that prevents a host's dependency on a limited number of bacterial taxa [98–100]. Sharpton [101] suggested that the functional content of the microbiome can be more heritable than its taxonomic diversity.

Species in the current study have been subjected to intensive population control measures using a combination of toxins and trapsfor more than 60 years. While numerous studies have underlined the

critical role that gut microbiota play in drug and xenobiotic substance metabolism [102], the role of microbial communities in the proximal alimentary tract is yet to be understood.

As conservation efforts to eradicate invasive species intensify around the globe, focusing on the microgenome rather than the slowly-evolving host genome in genetically deprived invasive species presents an under-appreciated means of studying evolutionary responses to altered environments over a short time-scale. Future comparative studies should focus on studying microbiome diversity in a larger sample of individuals from both native and invaded ranges, and investigate the significance of bacterial communities in modulating the host's physiological and behavioural responses. Such studies would benefit from a considerably more complex study design than the one described here, as they would require that the roles of biotic and abiotic factors be detangled, while controlling for the confounding effects of the host's diet, physiological condition, sex, age, and whether individuals were sampled in the wild or had spent some time in captivity.

5. Conclusion

Our study highlights the critical role that a combination of genetic, physiological, and environmental factors plays in shaping oral microbiome diversity. The integrated anatomical, metabolic and immunological fitness of the host and its associated microbiome depend on a combination of concurrent selective and random changes in the genomes of both the host and its microbiome in response to changing environmental conditions.

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