Understanding the gut microbiome; the role of host senescence, sociality, and genetics.

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Abstract

Gut microbiomes (GMs) – microbes living in the intestine – play a central role in host health, survival and evolution, yet what affects their dynamics, and how that impacts host individuals in wild populations remains poorly understood. This thesis investigates host–GM interactions in a natural population of Seychelles warblers, integrating longitudinal sampling, shotgun metagenomics, and host genomic analyses to identify ecological, genetic, and social drivers of GM variation and their consequences for host survival.

I show that both taxonomic and functional GM diversity decline progressively with age, with compositional shifts and an age-related increase in transposase abundance. Host immunogenetics, measured through major histocompatibility complex (MHC) variation, shaped GM structure, revealing trade-offs between microbial defence and microbial metabolic function. Social interactions also influenced the GM: individuals sharing space harboured more similar GMs, and individuals that interact closely (e.g. breeding pairs and helpers) shared more similar anaerobic, but not aerotolerant, taxa. Host inbreeding effects were detectable at both individual and parental levels, correlating with GM taxonomic and functional composition. Genome-wide association analysis further identified nine loci linked to GM composition, the genes these loci encompass implicate host immune and gut physiological pathways shaping the GM. All nine loci were associated with microbial taxa that are related to survival in the warbler, and two loci were directly linked to host survival, demonstrating genomic pathways through which host–GM interactions influence fitness.

Overall, this thesis demonstrates that age, host genetics, and social environment all shape the GM through distinct but interacting mechanisms. By integrating ecological and genomic perspectives, this thesis advances understanding of how GMs are structured in the wild and their potential fitness consequences. More broadly, it emphasises the importance of viewing the hosts and their microbiomes as an interconnected system, with implications for both evolutionary biology and conservation.

Access Condition and Agreement

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Animal ethics

All procedures involving animals during fieldwork was carried out in accordance with local ethical regulations and agreements (UEA ethics approval ID ETH2223-0665). The Seychelles Department of Environment and the Seychelles Bureau of Standards approved the fieldwork (permit number A0157).

Contents

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Chapter contribution

This thesis has resulted in the following manuscripts presented in Box 1.1, Chapters 2, 3, 4, 5, and 6. At the time of submission, Box 1.1 and Chapter 2 in this thesis have been published in peer-reviewed journals. Chapter 3 is being revised for resubmission, following the reviewer's comments. Chapter 4 is submitted and is under review. Below, I detail the specific contributions I have made to each chapter. I am the lead author and responsible for the largest contribution to all chapters.

Box 1.1 | **Lee, C.Z.** Preserving microbial functional biodiversity, Nature Reviews Biodiversity (2025). https://doi.org/10.1038/s44358-025-00037-w

I wrote the manuscript (100%)

Chapter 2 | **Lee, C.Z.**, Worsley, S.F., Davies, C.S., Silan, E., Burke, T., Komdeur, J., Dugdale, H.L., Richardson, D.S. Metagenomic analyses of gut microbiome composition and function with age in a wild bird; little change, except increased transposase gene abundance, ISME Communications (2025). https://doi.org/10.1093/ismeco/ycaf008

I compiled and analysed the data, and drafted the manuscript (75%)

Chapter 3 | **Lee, C.Z.**, Worsley, S.F., Komdeur, J., Dugdale, H.L., Richardson, D.S. Host immunogenetic variation and gut microbiome functionality in a wild vertebrate population, In review at Microbiome

I conceived and designed the study, compiled and analysed the data and drafted the manuscript (80%)

Chapter 4 | **Lee, C.Z.**, Worsley, S.F., Burke, T., Komdeur, J., Dugdale, H.L., Richardson, D.S. Social interactions shape anaerobic, but not aerotolerant, gut microbiome composition in a cooperative breeding species, In review at Molecular Ecology

I conceived and designed the study, compiled and analysed the data and drafted the manuscript (80%)

Chapter 5 | | **Lee, C.Z.**, Worsley, S.F., Pinto, A.V., Lee, K.G.L., Burke, T., Komdeur, J., Dugdale, H.L., Richardson, D.S. Inbreeding, intergenerational inbreeding and the gut microbiome

I conceived and designed the study, compiled and analysed the data and drafted the manuscript (80%)

Chapter 6 | | **Lee, C.Z.**, Worsley, S.F., Burke, T., Lee, K.G.L., Dong, S., Komdeur, J., Dugdale, H.L., Richardson, D.S.The holobiont and survival in a wild vertebrate population

I conceived and designed the study, compiled and analysed the data and drafted the manuscript (80%)

Chapter 1 |

General Introduction

Perched quietly among the branches, this Seychelles warbler appears deep in thought—perhaps contemplating its life-history, genetic makeup, social bonds, and the hidden world of its gut microbiome!

“How far along the gut have we gone?”

1.1 History of microbiome research

The microbiome refers to all microbes living in or on a body that can influence host health and disease (Round and Mazmanian, 2009). It encompasses bacteria, viruses, fungi, and protozoa (Weinstock, 2012) and is defined (Berg et al., 2020) as:

> "… a characteristic microbial community occupying a reasonably well-defined habitat that has distinct physio-chemical properties. The term thus not only refers to the microorganisms involved but also encompasses their theatre of activity" (Whipps et al., 1988).

This definition captures not only the organisms present but also their ecological and functional roles.

Microbiome research began with Antonie van Leeuwenhoek's observations of bacteria under a handmade microscope, describing them as animalcules (a microscopic animal) (Leeuwenhoek, 1677). Subsequent advances included microbial culturing techniques, which revealed discrepancies between the number of observed bacterial cells (under the microscope) and those successfully grown in the lab (Stewart, 2012). The sequencing of 16S ribosomal RNA genes (16S rRNA) by Carl Woese and George Fox (1977) enabled taxonomic identification of bacteria. The human gut microbiome (GM) was then studied with the 16S rRNA, uncovering substantial inter-individual variation (Eckburg, 2005). More recently, shotgun metagenomics, metatranscriptomics, and metabolomics have expanded GM research beyond taxonomy, providing insights into microbial function, host-microbe interactions, and links to health and disease (Worsley et al., 2024c).

Research has subsequently shifted toward host-centric perspectives, examining how microbiomes influence host physiology, behaviour, health and fitness (Claesson et al., 2012; Langille et al., 2014). In vertebrates, work has largely focused on the microbiome of the gastrointestinal tract (gut microbiome, GM), a dense community (10^7 – 10^{14}) normally dominated by mutualistic taxa (Ferranti et al., 2014; A. R. Wang et al., 2018). Oxygen availability declines along the gut, allowing both aerotolerant (oxygen-resistant) and anaerobic (oxygen-sensitive) microbes to thrive (Chikina and Matic Vignjevic, 2021). Some microbes anchor in the gut and form mutualistic relationships with the host's mucosal layer, where the mucus protects them. The microbes then provide nutrients and metabolites to the host and prevent pathogen colonisation (Rathore et al., 2025). Over time, gut

microbial taxa form stable populations in individuals and become personalised gut microbes (Claesson et al., 2011; Ghosh et al., 2020).

To date, most GM studies have been performed on humans or captive and model organisms (Sharma, 2022). However, studies on humans are often confounded by lifestyle factors such as antibiotics, malnutrition, and residential care (DeJong et al., 2020). With captive animals, captivity influences the GM through differences in environmental and social conditions (Oliveira et al., 2020; San Juan et al., 2021), and is associated with often extensive differences in GM diversity and composition. Effects of captivity have been repeatedly shown in birds (Oliveira et al., 2020; San Juan et al., 2021; Wang et al., 2016), fish (Dhanasiri et al., 2011), reptiles (Keenan et al., 2013), amphibians (Bataille et al., 2016), and mammals (Clayton et al., 2016; Delport et al., 2016; Gibson et al., 2019). Thus, captive animal studies cannot accurately portray the GM in wild populations (Hird, 2017). Research on wild animal populations is necessary to fully understand natural GM changes associated with age and senescence, host genetics, and sociality (Hird, 2017).

While work on humans and captive animals has provided a wealth of important information, it is important to recognise these limitations. Accordingly, I deliberately diversify the examples to assess whether findings from humans and captive animals are confirmed in wild systems. Despite substantial progress, we still know relatively little about how these factors interact in natural populations, or the extent to which patterns observed in controlled settings translate to the wild. This review synthesises current GM research, focusing on how senescence, sociality, and host genetics shape the GM. It also explores the consequences of GM changes, the importance of selecting an appropriate study population, and the methodologies used to investigate the GM effectively.

1.2 Environmental factors affecting the GM

1.2.1 Diet

Diet is one of the most influential factors shaping the GM (Albenberg and Wu, 2014; Cotillard et al., 2013; Wolters et al., 2019), due to both external dietary inputs and intrinsic host factors that determine what the host can eat (Trevelline and Kohl, 2022). This influence is evident at multiple levels, including animal dietary classification, differences among species, among populations, and even among individuals within a population (Baniel et al., 2021; Bodawatta et al., 2021; Griffin et al., 2017; Zoelzer et al., 2021). Numerous studies have shown that shifts in diet can

rapidly and predictably alter gut microbial composition, diversity, and function, highlighting the strong and dynamic link between what hosts consume and their microbiome across ecological and evolutionary contexts (Bodawatta et al., 2021; Cotillard et al., 2013; Suriano et al., 2022; van Leeuwen et al., 2020).

In wild animals, changes in diet also impact GM composition (Amato et al., 2015; Ren et al., 2016; Springer et al., 2017). For instance, the GM structure of lowland gorillas (*Gorilla gorilla gorilla*), mice (*Mus musculus*), and myrmecophagous mammals (*Orycteropus afer*, *Vermilingua* and *Proteles cristata*) is highly dependent on their diet (Carmody et al., 2015; Delsuc et al., 2014; Hicks et al., 2018). However, in some species, the GM does not reflect dietary input (Youngblut et al., 2019), for example, Giant Pandas (*Ailuropoda melanoleuca*) subsist almost entirely on a herbivorous diet, yet their digestive system and GM resemble those of carnivores (Guo et al., 2020).

Additionally, the GM plays a critical functional role in host digestion and nutrition. For example, the mammal GM aids in the degradation of xyloglucans, which are found in vegetables (Larsbrink et al., 2014) and the degradation of cellulose, which is found in grass and most plants (Cholewińska et al., 2020). Thus, allowing herbivorous mammals to access otherwise inaccessible sources of nutrients (Froidurot and Julliand, 2022). Short-chain fatty acids, produced via fermentation of indigestible dietary fibres by gut microbes, serve as the primary source of energy for colonocytes- absorptive epithelial cells lining the large intestine (Ahmad, 2000). Ultimately, the interplay between diet, host physiology, and microbial function not only shapes the composition of the GM but also influences the host's overall health and metabolic efficiency.

1.2.2 Temporal environmental factors

Seasonal changes are associated with GM composition in wild animals (Amato et al., 2016; Ren et al., 2017; Wang et al., 2015), largely attributed to fluctuations in diet, i.e. resource abundance and diversity (David et al., 2014; Góngora et al., 2021; Schmiedová et al., 2022). However, these seasonal shifts also encompass broader environmental changes, including shifts in temperature, humidity, rainfall, and the start or end of breeding seasons, all of which may also shape the GM (Baniel et al., 2021; Góngora et al., 2021; Marsh et al., 2022).

In addition to these broader seasonal influences, finer-scale temporal factors such as host circadian rhythm can also modulate GM communities (Voigt et al., 2016). Multiple studies have shown that the GM taxonomic composition changes throughout the day (Schmid et al., 2023; Voigt et al., 2016; Zhang et al., 2023), with up to 60% of GM composition oscillating over 24 hours in mice (Thaiss et al., 2014). Moreover, microbial gene expression in mice varies by time of day (light-dark cycle): microbial gene functions such as energy metabolism, DNA repair and cell growth are upregulated in the dark phase (lights off), whereas genes such as detoxification, motility, and environmental sensing are upregulated in the light phase (lights on) of mice (Thaiss et al., 2014). Similarly, in wild meerkats (*Suricata suricatta*), GM *Clostridium* abundance peaked at dawn, aligning with the species' foraging schedule (Risely et al., 2021).

1.2.3 Pathogens

Viral outbreaks, such as the avian flu H5N1 in seabirds and chickens or the COVID-19 pandemic in humans, are factors that can alter the GM by activating host immune response and inducing inflammation (Chakraborty et al., 2022; Huang et al., 2023). Bacterial outbreaks, such as a pathogenic strain of *Escherichia coli,* could also directly lead to changes in the GM, especially if commensals are outcompeted by the pathogenic strain (Doranga et al., 2024).

Climate change has the potential to impact the GM through changes in abundance and quality of food, altering host physiology, and potentially increasing pathogenic microbes (Litchman, 2025). Global warming may increase enteric pathogens as microbes that previously could not survive in vertebrate systems (e.g., many fungi) adapt to higher viable temperatures. Such shifts could enable colonisation of hosts, while existing microbiota may fail to tolerate these changes, weakening colonisation resistance and increasing disease outbreaks (Konkel Neabore, 2024). Furthermore, climate change could impact host physiology, such as reduced hormonal expression due to increasing temperature, which could be associated with changes in GM composition (Maeng and Beumer, 2023; Santos-Marcos et al., 2023). Therefore, GM dynamics are not only a reflection of host biology, but are also deeply intertwined with broader environmental, ecological, and anthropogenic influences.

1.3 Intrinsic host factors and the GM

1.3.1 Age

Numerous studies link the GM with host age (Claesson et al., 2012; Xu and Zhang, 2021). At birth, gut microbial loads are low and gradually increase with age in mammals (Du et al., 2023; Shi et al., 2018; Wampach et al., 2017; J. Wang et al., 2018). Aerotolerant taxa (e.g. *Staphylococcus*, *Streptococcus*, *Escherichia coli* and *Enterobacteria*) are first to colonise the gut, with the role of consuming oxygen, producing a suitable environment for obligate anaerobic bacteria (e.g. *Bacteroides*, *Clostridium*, *Actinomyces* and *Fusobacterium*) to grow (Clemente et al., 2012a; Du et al., 2023; Minato et al., 1992). Therefore, the GM of human infants is typically of low diversity, dominated by *Bifidobacteria* and *Bacteroidetes* (Mitsuoka, 1996). At one to three years, the human GM shifts towards an adult-like state (Clemente et al., 2012b; Tamburini et al., 2016). Similarly, in other vertebrates – Koala (*Phascolarctos cinereus*), dog (*Canis lupus familiaris*), Great tit (*Parus major*), and ostrich (*Struthio camelus*) - the developmental GM gradually increases in diversity and converges compositionally to resemble an adult GM (Blyton et al., 2022; Dong et al., 2022; Teyssier et al., 2018; Videvall et al., 2019).

1.3.2 Senescence

Senescence is an age-related decline in host function (Monaghan et al., 2008; Nussey et al., 2008), which varies in onset and rate across and within species and across traits (Jones et al., 2014; Nussey et al., 2013). The GM is thought to be one such trait. In mammals, the GM remains relatively stable over long periods of the adult life (Becker et al., 2015; Martínez et al., 2013), but changes in later life, often becoming dysbiotic – characterised by an imbalanced or disrupted GM community (Biagi et al., 2016; Luan et al., 2020). These shifts are associated with age-related declines in beneficial bacterial taxa like *Bifidobacteria* and increases in potentially harmful groups like *Gammaproteobacteria* (Claesson et al., 2011). Experimental studies support the idea that age-related changes in the GM impact health. Faecal microbiota transplants from older donors shorten lifespan, while those from younger donors extend it (Bárcena et al., 2019; Fransen et al., 2017; Smith et al., 2017). This may be due to reduced microbial production of short-chain fatty acids, vital microbial metabolites (Lee et al., 2020a, 2020b; Nagpal et al., 2018; Spychala et al., 2018).

In wild vertebrates, age-related GM studies have been mostly cross-sectional, comparing juveniles and adults (Funosas et al., 2021; Reese et al., 2021). Few studies include elderly individuals, and often yield mixed results (Reese et al., 2021). Such cross-sectional studies are limited by inter-individual variation and selective disappearance effects (Benson et al., 2010; Dzierozynski et al., 2023; Nussey et al., 2013). Therefore, a longitudinal approach is needed to better understand age-related GM changes in wild vertebrates. Several studies on wild mammalian species using longitudinal sampling have reported that ageing is associated with small shifts in GM composition (Reese et al., 2021; Risely et al., 2021; Sadoughi et al., 2022).

1.3.3 Sex

Sex-related GM differences are found in most vertebrates but vary across host species (Valeri and Endres, 2021; Xu and Zhang, 2021). Hormonal differences likely explain many of these patterns, as hormones can directly modulate bacterial metabolism via steroid receptors (Menon et al., 2013). However, sex hormones could also influence behavioural differences in animals, leading to differences in dietary choices, consequently influencing GM characteristics (Ma et al., 2020; Zucker et al., 1972). Sex differences in the GM may also reflect energy investment strategies, such as in yellow-bellied marmots (*Marmota flaviventer*), where males invest in mass gain for hibernation, while females are focused on nursing (Pfau et al., 2024).

1.3.4 Host genetics and the GM

1.3.4.1 Host and GM phylogenies

Phylosymbiosis describes evolutionary alignment between host species and their GM, arising from the close coevolution of the hosts and microbes, whereby microbial communities adapt alongside host physiology, immunity, and diet (Lim and Bordenstein, 2020). This has been observed in several mammals (Brooks et al., 2016a; Kohl et al., 2018b, 2018a) and birds (Hird et al., 2015; Laviad-Shitrit et al., 2019) and may be driven by coevolution between host and microbes (see below Vertical Transmission). Alternatively, it could be caused by environmental filtering, i.e. the host may filter for specific microbes that can adapt to their gastrointestinal system (Moran and Sloan, 2015). Similarly, host phylogeny at the vertebrate classes and between species has repeatedly been shown to be associated with the GM,

though this doesn't necessarily indicate phylosymbiosis – as they may arise from correlated factors such as shared diet, geographic distribution or amount of social interactions (Gomez et al., 2019; Knowles et al., 2019; Sherrill-Mix et al., 2018; Song et al., 2020; Trevelline et al., 2020; Youngblut et al., 2019). While host-GM phylogenetic congruence captures long-term evolutionary influences on the GM across species, genetic variation within a species may also shape the GM in important ways.

1.3.4.2 Host immune system

The GM and the host immune system influence each other bidirectionally (Russler-Germain et al., 2017; Zheng et al., 2020). Host immune genes may shape GM composition (Dzierozynski et al., 2023; Marietta et al., 2015; Zheng et al., 2020), as immune activation must balance defending against pathogens while tolerating commensals (Fuess et al., 2021; Tanoue et al., 2010). In particular, immune receptor genes (pathogen detection) could preferentially affect certain microbes, shaping the GM (Kurilshikov et al., 2017).

Further, in humans, growing evidence links the GM to diseases such as colorectal cancer (Wong and Yu, 2019), inflammatory bowel disease (Khan et al., 2019), obesity (Ley et al., 2005), diabetes (Gurung et al., 2020; J. Wang et al., 2018), and *Clostridium difficile* infection (Kelly et al., 2014). It has been suggested that ulcerative colitis may result from a disrupted host-GM interaction (Bullard et al., 2022). Germ-free animals show underdeveloped immune systems and reduced immune activity, likely due to the absence of a healthy GM (Sommer and Bäckhed, 2013). A major reason for this is that the GM plays a central role in shaping and maintaining host immune defences (Fuess et al., 2021; Tanoue et al., 2010). This can occur directly, through microbial stimulation of immune cells and the production of metabolites such as short-chain fatty acids that modulate inflammation, or indirectly by maintaining gut barrier integrity (Mann et al., 2024; Takiishi et al., 2017; Tanoue et al., 2010; Wu and Wu, 2012; Zhao et al., 2023). In addition, commensal microbes enhance colonisation resistance by preventing pathogens from establishing in the gut by competing with pathogens for nutrients and niches (Caballero-Flores et al., 2023; Tanoue et al., 2010).

1.3.4.3 Host genetics

Beyond the host immune system, specific host genes such as those involved in gut physiology, nutrient production, and antimicrobial properties could also shape the GM (Cornick et al., 2015; Ridlon et al., 2014; Rowland et al., 2018). For example,

genes regulating mucus production (e.g. MUC2) and epithelial barrier integrity can influence microbial colonisation (Birchenough et al., 2023; Cornick et al., 2015; Song et al., 2023). Similarly, genes linked to nutrient availability (e.g. ABO and FUT2 in humans) determine the abundance of microbes such as *Faecalibacterium prausnitzii* due to the utilisation of N-acetylgalactosamine (GalNAc), a sugar molecule (Zhang et al., 2024; Zhernakova et al., 2024). Genes linked to bile acid synthesis and detoxification (e.g. FXR, UGT family genes) also affect GM community structure, since bile acids have strong antimicrobial properties (Collins et al., 2023; Ridlon et al., 2014). Even taste receptors and olfactory genes can shape diet choice, indirectly modifying the GM. Together, these pathways illustrate that host control of the microbiome extends beyond the immune function, supporting the view that the GM is a polygenic trait (Benson et al., 2010; Liu et al., 2024).

Conversely, the GM can also mediate host genetic expression via microbial short-chain fatty acids, immune signalling and epigenetic mechanisms (Nichols and Davenport, 2021). These interactions highlight the importance of within-species or within-population studies for disentangling the nuanced host genetic and GM drivers of host health and fitness.

1.3.4.4 Host inbreeding

Within a species, inbreeding can expose deleterious recessive alleles and reduce heterosis, leading to inbreeding depression, causing a shorter lifespan and reduced fertility (Bertorelle et al., 2022; Charlesworth and Willis, 2009). Inbreeding may also affect the GM directly, by altering the expression of genes that regulate them (Bonder et al., 2016; Melis et al., 2023) or indirectly, through loss of heterosis at key GM-associated loci, such as immune genes, which are frequently under balancing selection (Spurgin and Richardson, 2010). Related individuals tend to have more similar GMs due to shared environment, thus making it difficult to disentangle relatedness from environmental effects (Yuan et al., 2015). Consequently, inbred offspring may inherit less diverse sets of genes and microbes (Grieneisen et al., 2021; Roche et al., 2023; Turnbaugh et al., 2009).

1.4 Transmission of the GM

1.4.1 Vertical transmission of the GM

Vertical transmission refers to the direct transfer of microbes from parent to offspring, shaping the early GM (Sarkar et al., 2024, 2020). In asexually reproducing organisms, microbes are transferred as tissues are separated during the vegetative reproduction, such as producing runners and budding (Rosenberg and Zilber-Rosenberg, 2021). In egg-laying animals, transfer occurs from the egg white before hatching and from the eggshell (Bunker and Weiss, 2024; Ding et al., 2017; Perlmutter and Bordenstein, 2020; Wilkinson et al., 2003). Eggs of birds showed a proportion of microbiota originating at the yolk sac and embryonic gut, suggesting vertical transmission before egg shelling (Bunker and Weiss, 2024; Gao et al., 2025; Trevelline et al., 2018). In mammals, vaginal birth plays an important role in GM transfer (Dominguez-Bello et al., 2010) and the maturation of the GM (Stewart et al., 2018), as shown by the depauperate GM of caesarean-born individuals (Inchingolo et al., 2024). Postnatal behaviours such as coprophagy, regurgitation, and nursing also contribute to vertical transmission of microbes (Daft et al., 2015; Rosenberg and Zilber-Rosenberg, 2021; Soave and Brand, 1991; van Dongen et al., 2013). Close physical contact, such as hugging and kissing, has also been linked to the transmission of microbes between parents and offspring (Reyman et al., 2019; Sakwinska et al., 2017; van den Elsen et al., 2019).

Vertical transmission will lead to greater congruence between host and the GM, because microbial lineages are passed along host lineages (Funkhouser and Bordenstein, 2013; Moran et al., 2008). Hence, closely related hosts often harbour more similar GM, a pattern that has been observed across species and higher taxonomic levels (Lim and Bordenstein, 2020; Mallott and Amato, 2020; Yuan et al., 2015). This phylogenetic concordance facilitates tighter coevolution between hosts and their GM, potentially reinforcing host-specific adaptations and contributing to the stability and functional integration of the holobiont (Brooks et al., 2016b; Lim and Bordenstein, 2020).

1.4.2 Horizontal transmission of the GM

Horizontal transmission refers to the transfer of microbes from the environment and may be mediated by direct (close physical contact) with other con- and hetero-

specifics and indirect (shared environment) mechanisms (Sarkar et al., 2024). Indeed, research has shown that conspecific social interactions are a driving force for GM convergence in animals (Archie and Tung, 2015; Raulo et al., 2018; Sarkar et al., 2020). For instance, cohabitation leads to more similar GMs than living apart (Dill-McFarland et al., 2019; Griffin et al., 2017; Hildebrand et al., 2013; Seedorf et al., 2014). Several studies in wild mammals have also found that the GM is correlated with the host's social networks, with individuals who interact frequently having more similar GM compositions (Archie and Tung, 2015; Degnan et al., 2012; Raulo et al., 2018; Tung et al., 2015). However, social species that have close interactions often share physical space/environment, making it challenging to disentangle direct and indirect social transmission components of the GM (Raulo et al., 2024). To address this, studies in baboons (*Papio*) and wild mice (*Apodemus sylvaticus*) used behavioural scores and GPS tracking, respectively, to quantify social intimacy (Raulo et al., 2024; Tung et al., 2015). These studies found that individuals who socially interact more intimately tend to share more anaerobic (oxygen-sensitive) bacteria (Raulo et al., 2024; Tung et al., 2015), highlighting the role of close interaction in microbial sharing.

The interaction between hetero-specific has also been shown to increase GM similarities, which suggests that spatial proximity between host species promotes convergence in the GM (Moeller et al., 2017, 2013; Song et al., 2013). Although, much of the work on hetero-specific has focused on the transfer of pathogens between species, such as the spread of tuberculosis or SARS-CoV-2 between mammals (Gryseels et al., 2021; Torgerson et al., 2024). However, hetero-specific interactions may be beneficial; in one case, mice exposed to dog-associated house dust had reduced inflammation and were protected against respiratory infection and pathology (Fujimura et al., 2014).

1.5 Techniques used to characterise the GM

1.5.1 Amplicon Sequencing

The introduction of next-generation sequencing (NGS) enabled high-throughput amplicon sequencing of the 16S rRNA gene, allowing culture-independent profiling of bacterial communities (Clarridge, 2004). This technique revolutionised microbiome research (MacLeod et al., 2022; Wu et al., 2022; Zhu et al., 2022), but it has several limitations. Quantitative bias arises from DNA extraction efficiency,

PCR amplification, and primer specificity (Sinha et al., 2017). For instance, some bacteria (e.g. Gram-negative) are easier to lyse than others (e.g. Gram-positive), therefore, the DNA yield of some bacteria may be higher than others (Costea et al., 2017; Morgan et al., 2010). The inability to accurately determine microbial function is another limitation of amplicon sequencing (Aßhauer et al., 2015). This is because microbial function can differ between species and strains (Chang et al., 2022; Worsley et al., 2024c), but 16S rRNA (V3-V4 region) sequencing is only accurate to the genus level (Frioux et al., 2020; Srinivas et al., 2025). For example, most *Escherichia coli* strains are harmless, but some cause disease (Nataro and Kaper, 1998). Additionally, microbial functions are inferred from reference genomes, so poorly characterised genera often result in unreliable or incomplete functional annotations (Aßhauer et al., 2015). To access accurate functional data of the microbiome, other -omics options are preferred (Sharpton, 2014; Worsley et al., 2024c).

1.5.2 Metagenomic Sequencing

Shotgun metagenomic sequencing includes all DNA in the GM, which enables detection of all microbes, including fungi, bacteria, archaea and viruses (Yang et al., 2018). However, analyses typically focus on bacteria, as bacterial DNA dominates the GM metagenomic datasets (Xie et al., 2023). Shotgun sequencing also enables lower-level taxonomic assignment – often to species and strain level and is important for accurate functional assignment (Ferrer et al., 2012; Frioux et al., 2020). Additionally, any genes identified can be traced back to the microbes that carry them (Ferrer et al., 2012). The importance of understanding and preserving microbial function is discussed in Box 1.1. Finally, shotgun sequencing also reduces PCR-related bias, although sample handling and DNA extraction still affect results (McLaren et al., 2019). However, despite its strengths, shotgun sequencing is costly, technically challenging, and lacks standardised workflows. Therefore, most studies of microbiome research utilise 16S sequencing to determine the bacterial community before proceeding with metagenomics.

1.5.3 Other -omic options

Several other -omic approaches have been used to study the GM, including metatranscriptomics, metaproteomics, metabolomics, and culturomics. Metatranscriptomics is the shotgun sequencing of all expressed genes (RNA to cDNA) in a microbiome sample, which provides excellent data on gene activity in a

sample but is expensive (relative to amplicon and shotgun metagenomics). Furthermore, the bioinformatics steps are not well developed to filter out host transcripts, especially in non-model organisms (Worsley et al., 2024c). Similarly, metaproteomics and metabolomics provide even stronger evidence of functional profiles by directly measuring proteins and metabolites, respectively, but are also expensive and have much lower throughput than other methods (Worsley et al., 2024c). Additionally, there is a high number of unknown metabolites, further reducing metabolomics' ability to accurately measure GM function. Culturomics is a technique that isolates the microbe from the sample, followed by whole-genome or amplicon sequencing (Worsley et al., 2024c). However, culturomics underestimates the true GM composition because only culturable microbes will be characterised (Worsley et al., 2024c).

Box 1.1. Preserving microbial functional biodiversity (Lee, 2025)

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When thinking of conservation, people often picture elephants roaming the savannah, sea turtles on beaches, or lush rainforests. Although these species and ecosystems are undeniably important, people often overlook another vital, smaller world — the realm of microorganisms. In 2021, a paper by Dodd and Grueber highlighted the importance of conserving the microbial ecosystems that exist in, or on, animals in natural populations (Dodd and Grueber, 2021). This exciting paper inspired me to pursue a PhD in wildlife functional microbiomes.

In addition to providing a useful summary of emerging techniques in functional microbiome research, Dodd and Grueber offer a compelling description of the key functions of host microbial communities and their importance to species' conservation. Human activity can disrupt microbial ecosystems, which affects species' health and survival. For example, rhinoceroses are endangered primarily owing to poaching, but deforestation and pollution has led to dietary changes and exposure to pathogens, which alters their microbiomes and contributes to the declining population. Therefore, the authors emphasize that understanding the association between functional microbiomes and host health would help to identify host species that might suffer most from microbiome change. With this knowledge, informed conservation actions — such as introducing beneficial microorganisms — can be taken to help species to maintain a healthy wild microbiome.

The authors also made an unexpected point that well-intentioned conservation efforts can inadvertently harm host microbiomes and, therefore, the host species itself. For example, the process of translocating individuals — a common tool in animal conservation — could damage the vertebrate gut microbiome if it involves a period of captivity, supplementary feeding or antibiotic treatment. By highlighting the complexity of host–microorganism interactions and their implications for host health (and, thus, conservation), the authors illustrate the importance of understanding of the host microbiome's role in guiding effective conservation strategies.

To date, microbial function in wild animal hosts remains poorly understood, primarily owing to the costs associated with sampling, sequencing and analysis (Worsley et al., 2024c). Dodd and Grueber point out that research on wild endangered species is constrained by the difficulty of obtaining sufficient sample sizes. Analysing these samples presents further challenges, as the field is still relatively new — particularly in the context of wild systems. Moreover, changes in the wild animal microbiome are often influenced by many interacting variables, including biotic and abiotic environmental factors and intrinsic host factors, which must be accounted for in analyses to ensure accurate interpretations.

Dodd and Grueber recommend thoughtful study designs to reduce costs and optimize insights when studying wild animal microbiomes. They suggest reusing genomic and microbiome data from well-studied species to aid decision-making in small, isolated populations and speed up research. Given the complexity of host–microorganism interactions in wild populations, it is essential to develop effective strategies to protect life across all scales — from taxonomy to functional diversity.

1.6 The Seychelles warbler as a model system

The Seychelles warbler (*Acrocephalus sechellensis*) is a wild-living passerine endemic to the Seychelles archipelago (Figure 1.1AB; Spurgin et al., 2014). In 1968, only a single population of less than 30 warblers remained on Cousin Island, following anthropogenic destruction of their natural habitat (Crook, 1960; Loustau-Lalanne, 1968; Vesey-Fitzgerald, 1940). Subsequently, total population size has been increased by conservation efforts, including habitat restoration and translocations of Seychelles warblers to other islands: Cousine Island in 1988, Aride Island in 1990, Denis Island in 2004 and Fregate Island in 2011 (Komdeur, 1994; Richardson et al., 2006; Wright et al., 2014). Translocation is necessary to establish new populations of this species as there is virtually no inter-island dispersal (Komdeur et al., 2004). These successful conservation efforts have moved the Seychelles warblers from a critically endangered species to a near-threatened species, which now consists of over 3000 individuals spread across five islands (BirdLife International, 2022).

The Seychelles warbler population on Cousin Island (Figure 1.1C; 29 ha; 04° 20′ S, 55° 40′ E) has been monitored continuously since 1985. From 1997, even more extensive monitoring has been undertaken covering both the minor (January-March) and major (June-October) breeding seasons (Barrett et al., 2013; Brown et al., 2022; Komdeur, 1992). Each season, as many birds as possible are caught with mist nets or at the nest (chicks) and ringed with a British Trust of Ornithology metal ring and a unique combination of three colour rings (Figure 1.1B). Individuals are normally ringed as a nestling or as a still dependent fledgling on their natal territory (Komdeur, 1992). As Seychelles warblers do not disperse to other islands (Komdeur et al., 2004), each individual within this small population can be closely monitored throughout its life.

Figure 1.1. The Seychelles warbler study system. (A) A begging fledgling. https://youtu.be/zlTC9InGhPM , (B) An adult with a BTO metal ring and colour rings (Black-white (M)/orange(O) orange(O)/metal(X)). (C) Cousin Island, Republic of Seychelles. 29 ha in size and 69 m elevation.

The extensive long-term monitoring of the Cousin Island Seychelles warbler population has created a valuable extensive dataset, including accurate survival, reproductive success and social status data, alongside biological samples (blood and faeces) (Davies et al., 2022; Hammers et al., 2015a; D S Richardson et al., 2003). Birth and death dates of individual birds can be accurately estimated (Hammers et al., 2015), as individuals are first identified at their nest, and 98 ± 1% of adult Seychelles warblers are resighted each season (Brouwer et al., 2010a). Therefore, if an individual is not sighted for more than one year, they are presumed dead (Brouwer et al., 2010b). There are no natural adult predators; hence, extrinsic mortality is low (Komdeur, 1999). Seychelles warblers have an average lifespan of 5.5 years, with one individual previously documented living to 19 years (Barrett et al., 2013). On Cousin Island, there have been up to 115 territories, varying in quality

based on foliage cover, insect availability, and territory size (Komdeur and Pels, 2005).

Seychelles warblers are facultative cooperative breeders, with social groups comprising a monogamous dominant pair and subordinate individuals (Komdeur, 1991; Komdeur and Pels, 2005). The dominant pair usually pair for life and defends their territory year-round (Komdeur, 1992; Richardson et al., 2007). Some subordinates act as helpers, helping to raise the offspring of the dominant pair, while others (non-helpers) share the territory but do not contribute to breeding (Komdeur, 1991). Subordinates likely occur due to the population reaching carrying capacity (Komdeur and Pels, 2005). Helpers significantly improve the reproductive success of the breeding pair (Richardson et al., 2002, 2001). This system enables the disentangling of genetics from social interactions because subordinates vary extensively in how related they are to the dominant pair (Richardson et al., 2003b), partly due to the frequent dispersal of offspring into non-natal groups to become helpers (Groenewoud et al., 2018). Even subordinates originating from within their natal group could be the result of extra-pair paternity (EPP) and/or cobreeding (Hadfield et al., 2006; Raj Pant et al., 2019; Richardson et al., 2003b, 2002; Sparks et al., 2022).

The intensive monitoring of the Seychelles warblers enables long-term studies and represents a valuable model system for studies of ageing in a wild population. In this population, actuarial senescence begins at approximately seven years of age in both sexes (Hammers et al., 2013). For females, reproductive senescence begins at ca. six years, while in males it begins ca. eight years (Hammers et al., 2012; Raj Pant et al., 2020). In line with previous studies on other species (McCleery et al., 1996; Orell and Belda, 2002; Reed et al., 2008; Reid et al., 2003), early-life reproductive investment affects senescence in the Seychelles warbler (Hammers et al., 2013).

The Seychelles warbler system is well-suited for studying the GM because of the long-term individual-level data, including age, survival, social groups, genetic and social parents, territory quality, immunogenetics, and whole genome variation (Raj Pant et al., 2019; Wright et al., 2016). Faecal specimens for GM assessment have also been collected since 2017, so a relatively large, partially longitudinal sample is now available (Davies et al., 2022). This longitudinal dataset allows me to identify within individual changes in the GM, which is especially crucial for studies on senescence and sociality. In addition, as warblers are tree-foraging insectivores,

they are rarely exposed to conspecific faeces, thus limiting non-contact horizontal transfer post-fledging. Additionally, low extrinsic mortality allows warblers to reach remarkably old ages, increasing the likelihood of detecting GM-mediated fitness consequences.

A limitation of the Seychelles warbler system is the inability to manipulate the population, such as creating germ-free individuals or performing microbiome transplantation experiments to determine functional mechanisms. Nonetheless, the Seychelles warbler is a very tractable population to study the GM due to the availability of longitudinal sampling and extensive ecological, demographic, and environmental data.

Prior to my work in this system, studies on the Seychelles warbler GM had shown that the GM composition is correlated with adult host survival (Worsley et al., 2021) and Major Histocompatibility Complex (MHC) gene variation (Davies et al., 2022). In addition, adult birds that died the next breeding season carried a higher number of opportunistic bacteria compared to adult birds that survived the next breeding season (Worsley et al., 2021). Since I have begun working on the system, a study on the gut mycobiome (fungal component of the GM) has shown that the MHC is associated with alpha diversity and composition of the gut mycobiome (Worsley et al., 2022). Additionally, 16S rRNA has been used to examine the gut microbiome in relation to ageing (Worsley et al., 2024b), fine-scale geographic variation (Worsley et al., 2025), and the effects of translocation (Worsley et al., 2024a). The GM was not associated with GM diversity and had small effects on composition (Worsley et al., 2024b). Despite the small size of Cousin Island, the GM beta diversity was associated in a quadratic manner with geographic distance, suggesting more similar GMs between coastal territories than coastal-inland territories (Worsley et al., 2025). Additionally, the GM had lower alpha diversity in all translocated populations and varied in GM composition compared to the source Cousin Island population (Worsley et al., 2024a).

1.6.1 Conclusions and Perspectives

The past two decades of GM research have made it clear that animals live in a symbiotic relationship with microorganisms (Lim and Bordenstein, 2020). Gut microbe variation has been linked with environmental factors, host physiology, genetics, senescence, and social interactions (Biagi et al., 2010; Bonder et al., 2016; Hicks et al., 2018; Raulo et al., 2024; Sharma, 2022). The complexity of these

associations highlights the need for continued, in-depth research. Given that the GM can vary significantly between individuals, repeated longitudinal sampling is necessary to track changes within individuals over time, and to understand why they occur (Levy et al., 2020). Furthermore, captivity drastically changes the GM (Hird, 2017), highlighting the need to study GMs in wild populations to reveal true fitness effects and the evolutionary significance of host-microbe interactions. Such studies will allow a better understanding of host-microbe interactions, deciphering factors that shape the GM and uncovering the consequences of these changes on host senescence, reproductive success and mortality – and thus host evolution. Integration of metagenomic approaches will deepen functional GM insights, and long-term research in wild systems could ultimately identify the microbial factors critical for maintaining host health.

1.6.2: Thesis aims

Overall aim:

I use the Seychelles warbler as a model system to investigate the causes and consequences of GM variation within a wild vertebrate population. My research uses both amplicon and shotgun sequencing to explore what may generate differences among and within individual hosts in terms of GM taxonomy and function. The following chapters each aim to address a key aspect:

Chapter 2 will test how the GM taxonomy and functionality changes within individuals in relation to age and senescence using a longitudinal sampling approach.

Chapter 3 aims to determine the role of host immunogenetics in shaping the GM. Specifically, how Major Histocompatibility Complex (MHC) variation influences the GM taxonomy and function.

Chapter 4 endeavours to disentangle how close social contact and shared environment affect horizontal transmission of the GM in the cooperatively breeding Seychelles warblers. Specifically, I will also investigate how the transmission dynamics of both aerotolerant and anaerobic microbes differ with social contact to understand how social structures influence microbial exchange.

Chapter 5 aims to test the association between host inbreeding, including intergenerational inbreeding, and GM variation. By integrating host genomic

sequencing, accurate pedigree data, and amplicon and shotgun metagenomics GM sequencing, I will determine if and how levels of inbreeding are linked to variations in GM taxonomy and function.

Chapter 6 will identify host genomic loci that are associated with GM composition, and determine if these loci mediate host survival through the GM.

Discussion synthesises the findings from Chapters 2-6, highlighting the key contributions, identifying recurring themes, and proposing avenues for future research.

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Chapter 2 |

Metagenomic analyses of gut microbiome composition and function with age in a wild bird; little change, except increased transposase gene abundance

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A version of this chapter (Appendix 2) is published as:

Credit: Eugenio Carlon

2.1 Abstract

Studies on wild animals, mostly undertaken using 16S metabarcoding, have yielded ambiguous evidence regarding changes in the gut microbiome (GM) with age and senescence. Furthermore, variation in GM function has rarely been studied in such wild populations, despite GM metabolic characteristics potentially being associated with host senescent declines. Here, we used seven years of repeated sampling of individuals and shotgun metagenomic sequencing to investigate taxonomic and functional changes in the GM of Seychelles warblers (*Acrocephalus sechellensis*) with age. Our results suggest that taxonomic GM species richness declines with age and in the terminal year, with this terminal decline occurring consistently across all ages. Taxonomic and functional GM composition also shifted with host age. However, the changes we identified occurred linearly with age (or even mainly during early years prior to the onset of senescence in this species) with little evidence of accelerated change in later life or during their terminal year. Therefore, the results suggest that changes in the GM with age are not linked to senescence. Interestingly, we found a significant increase in the abundance of a group of transposase genes with age, which may accumulate passively or due to increased transposition induced as a result of stressors that arise with age. These findings reveal taxonomic and functional GM changes with age, but not senescence, in a wild vertebrate and provide a blueprint for future wild functional GM studies linked to age and senescence.

Keywords: gut microbiome, age, senescence, metagenomics, transposase, *Acrocephalus sechellensis*

2.2 Introduction

Senescence - a decline in physiological function in later life- occurs in most organisms (Jones et al., 2014; Nussey et al., 2013). However, its onset and rate often differ greatly among individuals within populations (Hammers et al., 2015; Nussey et al., 2013). One factor that may contribute to individual differences in senescence is variation in host-associated microbial communities. The intestinal tract of animals contains a diverse collection of microbes and their genomes (the gut microbiome; GM), which play an important role in host adaptation and fitness (Hildebrand et al., 2021; Petersen et al., 2023). The GM influences the regulation of essential processes, such as digestion, reproduction, and immune function (Caviedes-Vidal et al., 2007; Cholewińska et al., 2020). However, shifts in GM composition can be detrimental to the host; certain microbes may be pathogenic, while overall dysbiosis may impair host function (Davenport et al., 2017; Thevaranjan et al., 2017).

Studies in humans and laboratory animals have shown that GM composition generally changes rapidly in early life (Blyton et al., 2022; Guard et al., 2017) before stabilising during adulthood (Dong et al., 2022). This is often followed by greater GM instability in advanced age including a loss of diversity and changes to composition (Biagi et al., 2016; Dillin et al., 2017; Maynard & Weinkove, 2018). These late-life compositional shifts are generally characterised by a loss of commensal or probiotic bacteria and an increase in pathogenic microbes (Ghosh et al., 2022). GM functional changes with age have also been identified. For example, healthy ageing has been associated with microbes that enable increased biodegradation and metabolism of xenobiotics (Ghosh et al., 2022; Rampelli et al., 2020), whereas unhealthy ageing has been linked to increased production of detrimental microbial metabolites (Ghosh et al., 2022).

Studies have demonstrated links between the GM and senescence in humans and laboratory animals, however, their GM composition varies markedly from their counterparts living in natural environments because of the artificial environments they are exposed to (Gibson et al., 2019; Reese et al., 2021). It remains unclear if

these effects can be generalised to wild animals (Gibson et al., 2019; Oliveira et al., 2020; Reese et al., 2021).

Recent studies on wild organisms have not reached a consensus on what characterises the ageing microbiome. Some have documented altered GM composition (Fenn et al., 2023; Pannoni et al., 2022; Ren et al., 2017), increased GM diversity (Fenn et al., 2023; Hernandez et al., 2021), and reduced GM stability (Sadoughi et al., 2022) with increasing age. Other studies have indicated that GM characteristics remain relatively stable throughout adulthood (Baniel et al., 2021; Sadoughi et al., 2022; Worsley, Davies, et al., 2024). However, these studies have been based on 16S rRNA gene metabarcoding, which is limited in resolution (Durazzi et al., 2021; Scholz et al., 2012; Worsley, Mazel, et al., 2024). Shotgun metagenomic sequencing enables higher taxonomic resolution (species or strain level), as well as informing on the functional potential of microbial communities based on gene content (Cerk et al., 2024; Frioux et al., 2020; Hugenholtz & Tyson, 2008). In humans and captive primates, metagenomics has revealed an increase in pathogenic microbial genes, and a decrease in beneficial genes, with age (Duan et al., 2019; Rampelli et al., 2013, 2020). To our knowledge, no previous studies have investigated GM functional changes with age and senescence using shotgun metagenomics in a wild population.

Also, most GM studies on wild animals have relied on a cross-sectional sampling of differently aged individuals (Bennett et al., 2016; Janiak et al., 2021; Pereira et al., 2020) and, therefore, may be confounded by the selective appearance/disappearance of individuals with particular GM characteristics. A lack of longitudinal samples also makes it difficult to infer changes in GM stability with age (Chen et al., 2021). Understanding what drives this GM variation is important, as it may lead to a deeper comprehension of the evolution of senescence and life-history trade-offs (Hammers et al., 2015), and enhance our ability to prolong healthy lifespans. As senescence occurs at different rates across individuals, a longitudinal approach is crucial for accurately evaluating age-associated effects (Nussey et al., 2008). Given this rate variation, and because declines are expected to be greatest at the end of life, GM changes may be more closely associated with proximity to death than chronological age. Including such information in analyses requires accurate estimates of the point of death that are not confounded by dispersal.

The long-term study of the Seychelles warbler population on Cousin Island provides a powerful natural system to study GM variation and host senescence (Hammers et al., 2015). Its isolated nature allows for the longitudinal sampling of uniquely marked, known-age individuals across their entire lifespan and the collection of accurate survival and reproductive success data (Raj Pant et al., 2020; Richardson et al., 2001). Previous studies using 16S metabarcoding have demonstrated that Seychelles warbler GM composition is linked to subsequent survival (Worsley et al., 2021) but identified no overall patterns of GM senescence (Worsley, Davies, et al., 2024). Additionally, host age was not associated with GM diversity, but a very marginal effect of host age on GM composition was reported (Worsley, Davies, et al., 2024).

Here, we use shotgun metagenomics to assess fine-scale changes in the GM with age and senescence in the Seychelles warbler. First, we determine how GM taxonomic diversity and composition change with host age, particularly in a bird's terminal year when GM dysregulation is expected to be at its greatest. Then we test the hypothesis that GM functional characteristics (assessed via microbiome gene content) will change with age, senescence, and in the terminal year.

2.3 Materials and Methods

2.3.1 Study system and sample collection

Seychelles warblers are insectivorous passerines endemic to the Seychelles archipelago. The population on Cousin Island (29 ha; 04° 20′ S, 55° 40′ E) has been extensively monitored since 1985 in the winter (January – March) and summer (June – October) breeding seasons (Brown et al., 2022; Hammers et al., 2015; Komdeur, 1992). Each season nearly all new birds (offspring) are caught, in the nest or as dependent fledglings in the natal territory (Komdeur, 1992). As many adult birds as possible are re-caught each season using mist nets. Bird age is determined using either lay/fledgling date (Komdeur, 1992) for the majority of individuals, if birds are first caught without a fledging date being recorded, eye colour is used to estimate age instead (see (Komdeur, 1992)).

The population on Cousin Island consists of ca. 320 individuals grouped into ca. 115 territories, defended year-round by a dominant breeding pair (Hammers et al., 2019; Komdeur & Pels, 2005). Territory quality is calculated each season using arthropod counts, vegetation density, and territory size information (Brouwer et al., 2009; Komdeur, 1992).

Nearly every bird in the population (> 96% since 1997 (Raj Pant et al., 2019)) has been caught and marked with a unique combination of a British Trust for Ornithology (BTO) metal ring and three plastic colour rings, which enables them to be monitored throughout their lives (Davies et al., 2021; Hammers et al., 2015). Individuals almost never disperse between islands and the annual resighting probability is around 98% ± 1% (Komdeur et al., 2004; Raj Pant et al., 2020; Richardson et al., 2001). If an individual is not seen for two consecutive seasons it is assumed to have died (an error rate of 0.04%) (Raj Pant et al., 2020; Richardson et al., 2001). Death dates for individuals were set as the final day of the season in which the bird was last seen. Benign climatic conditions and a lack of predators result in relatively long-lived individuals (median lifespan 5.5 years, max lifespan 19 years) (Hammers et al., 2019; Sparks et al., 2022). Previous studies have found that male and female Seychelles warblers are sexually mature at one-year-old, and senescence (survival

and reproductive) begins at ca. 6 years of age (Hammers et al., 2015, 2019, 2021; Raj Pant et al., 2020). The annual survival of adults does not differ between sexes, remaining around 80% up to six years of age and then decreasing (Brouwer et al., 2006; Hammers et al., 2015). Thus, there were no differences in survival senescence between the sexes (Hammers et al., 2015, 2019, 2021). In addition, elderly females in their last year of life (terminal year) had reduced reproductive success (Hammers et al., 2012).

Faecal samples were collected from caught birds and stored as described previously (see (Worsley, Davies, et al., 2024)). Between 2017 and 2023 all caught birds were placed in a disposable flat-bottom waxed paper bag containing a sterilised plastic weighing tray underneath a sterilised metal grate (Davies et al., 2022). This allows the bird to stand on the grate and faeces to fall into the sterile tray, minimising contact with the bird's surface. After ca 15 minutes (after defaecation) the bird was removed. The sample was collected, using a sterile flocked swab, and placed into a microcentrifuge tube containing 1 mL of absolute ethanol. Samples were stored at 4°C in the field before being transferred to -80°C for long-term storage. Contamination (hand) controls were collected from fieldworkers each season. The time-of-day that samples were collected and the number of days for which samples were stored at 4°C, were recorded. A ca 25 µl blood sample was also taken via brachial venepuncture and stored in 1 mL of absolute ethanol at 4°C.

2.3.2 DNA extraction and sequencing

Blood samples were processed with a salt extraction method (Richardson et al., 2001) or Qiagen DNeasy Blood and Tissue Kit and the resulting DNA was used for molecular sexing (Griffiths et al., 1998; Sparks et al., 2022).

DNA from faecal samples was extracted using the Qiagen DNeasy PowerSoil Kit with a modified protocol (see (Davies et al., 2022)). Samples were lysed using both mechanical agitation and enzymic processes (Davies et al., 2022). Individuals for which multiple longitudinal samples were available were prioritised for metagenomic sequencing to capture within-individual changes. In total, 155 faecal samples from 92 individuals across 7 years were sequenced, as well as three positive controls (two extractions from a ZymoBIOMICS Microbial Community Standard (D6300), and

one extraction from a ZymoBIOMICS Fecal Reference with TruMatrix™ Technology (D6323)), and six hand controls. Library preparation was performed in two lanes per run using the LITE protocol (Perez-Sepulveda et al., 2021) and sequencing undertaken in two runs of 2 x 150 bp NovaSeq X platform. The D6300 extraction control was sequenced on both runs to compare extraction and batch effects.

2.3.3 Bioinformatics

Shotgun metagenomic sequence analysis was carried out using the MATAFILER pipeline (see (Hildebrand et al., 2021) and supplementary materials). Briefly, MATAFILER removes host reads, assembles reads, predicts and annotates genes, builds metagenome-assembled genomes (MAGs) and metagenomic species (MGSs), and taxonomically assigned MGSs. Due to the high individuality of the Seychelles warbler GM and the high sequencing coverage required to assign MGS, Metaphlan4 was also used to taxonomically classify reads (see supplementary materials for details).

2.3.4 **Gut microbiome analyses**

A total of 162 samples were successfully processed bioinformatically (153 faecal samples, 4 controls). Positive controls were successfully recovered, and hand controls did not contribute to substantial contamination in samples (Figure S2.1).

The 153 faecal samples (Figure S2.2) included 71 from 40 females and 82 from 51 males. In total, 41 individuals had one sample, 41 had two, eight individuals had three, and one individual had four samples. Age at sampling ranged from 0.6-17.0 years (mean 5.7 ± 0.3 SE). Of these, 48 were from 22 individuals in their terminal year (the year in which they died); with ages in terminal year ranging from 1.4–17.0 years. From all these samples, 1025 unique metaphlan4 species-genome-bins assignments were used for the subsequent taxonomic analysis (mean 29.3 ± 2.0 SE per sample).

All statistical analysis was performed using R version 4.33 (Posit team, 2024; R Core Team, 2024). Variance Inflation Factor (VIF) scores (*car* version 3.1.2) were

used to test for collinearity between variables in all models; all had a score <3 indicating no issues with collinearity (Fox John & Weisberg Sanford, 2019).

2.3.5 **Taxonomic GM changes with age**

2.3.5.1 Taxonomic GM alpha diversity

A rarefaction curve of Metaphlan4 species was constructed with *iNEXT* version 3.0.1 to determine the read depth required to recover 95% of theoretically present species (Figure S2.3) (Chao et al., 2014). Taxonomic classifications were rarefied to a depth of 5,500 reads before alpha diversity analysis; two samples were removed due to insufficient read depth. Species richness and Shannon diversity metrics were calculated per sample using R packages *phyloseq* version 1.46.0 and *microbiome* 1.24.0 (Leo Lahti & Sudarshan Shetty, 2019; McMurdie & Holmes, 2013). Wilcoxon rank sum tests were used to examine whether different sequencing plates affected species diversity (Shannon index, $p = 0.353$) and species richness (Observed index, $p = 0.124$), both were not significantly different.

A linear mixed effect model with a Gaussian distribution (lmer), and a generalised linear mixed effect model with a negative binomial distribution (glmer.nb), were used to model changes in species diversity (Shannon index) and richness (observed taxa), respectively, using *lme4* version 1.1-35.5 (Bates et al., 2015). Fixed effect variables included in models were: host age (years); terminal year (yes/no); sex (male/female); breeding season (winter/summer); sample year (as a factor: 2017-2023); territory quality; storage days at 4°C (days); time of day collected (minutes since sunrise at 6:00 am). Bird ID was included as a random effect.

Storage at 4°C in the field ranged from 4 days to 104 days (mean 36.3 ± 1.8 SE). A quadratic age term, and an interaction between terminal year and host age, were tested to assess whether GM changes became more extreme with age or if GM changes in the terminal year differ depending on age. These terms were dropped if not significant to allow interpretation of the main effects. Age was measured in years, but all samples taken when birds were >12 years of age were designated as 12 years because these samples were rare (n = 9, max age = 17 years). Previous analysis shows that body condition is not associated with Seychelles warbler gut

microbiome diversity and composition, thus, it was not included in analysis (Worsley et al., 2021). Model diagnostics were run using *DHARMa* version 0.4.6, with no significant issues in each chosen model (Florian Hartig, 2022). Herein, all models were tested with the same variables unless stated otherwise.

A within-subject centering approach was used to separate between-individual (cross-sectional) GM differences with age (which could be driven by the selective appearance/disappearance of individuals with particular GM characteristics), from within-individual (longitudinal) change (which could indicate senescence) (van de Pol & Verhulst, 2006). This involves calculating the mean age of each individual across all its sampling events (mean age) and the within-individual deviation from that mean age at each separate sampling event (delta age). These terms replace host age in the model. The fixed effect of terminal year was also replaced by a "terminal year bird" term (yes/no) which indicates whether individuals have at least one sample collected in the terminal year or not. An interaction between the terminal year bird and delta age, as well as quadratic delta age, were tested to assess whether within-individual GM changes were more extreme in birds with a sample taken in the terminal year of life and/or in older individuals, respectively (which would be indicative of senescence). In addition, an interaction between delta age and mean age was included in the models to test if within-individual changes with time occur differently depending on host age. The analysis was repeated with non-rarefied reads to determine if rarefaction influenced the results. These terms were dropped if not significant to allow interpretation of the main effects.

2.3.5.2 Taxonomic GM composition

A permutational multivariate analysis of variances (PERMANOVA) was carried out on a Euclidean distance matrix calculated using centered log ratio (CLR)-transformed reads, using the adonis2() function in *vegan* version 2.6.6 (Oksanen Jari et al., 2024). A blocking effect of Bird ID was used to account for repeated measures. The same predictors were included as for the main model in the Alpha diversity analysis above. Differences in composition were visualised with a principal component analysis (PCA) in *phyloseq* version 1.46.0 (McMurdie & Holmes, 2013).

2.3.5.3 Taxonomic GM differential abundance analysis (DAA)

Two different DAA methods were used to identify differentially abundant GM species with host age (as recommended by (Cappellato et al., 2022; Nearing et al., 2022); *ANCOMBC2* version 2.4.0 and *GLLVM* version 1.4.3 (Lin & Peddada, 2024; Niku et al., 2019). ANCOMBC2 calculates log fold change of species one at a time before adjusting p-values, whereas GLLVM calculates log fold change of all species all at the same time, accounting for correlation between species (Lin & Peddada, 2024; Niku et al., 2019). A total of 22 common species, defined as species found in 20% of the population at more than 0.01% abundance, were retained. Species that were significantly differentially abundant in the same direction using both DAA methods were considered robustly significant. Variables included in each model were the same as in models above.

2.3.6 **Functional GM changes with age**

2.3.6.1 Functional GM alpha diversity

Initially, 4727 different eggNOG orthologues (mean = 3616.6 ± 64.4 SE per sthe ample) were identified in our gene catalogues. A rarefaction curve of eggNOG orthologues was constructed using *iNEXT* to determine sample completeness (Chao et al., 2014). Samples were then rarefied to 100,000 reads based on >95% completeness. One sample was removed due to insufficient reads. Following rarefication, 4685 eggNOG orthologues were retained (mean = 3054.3 ± 47.1 SE per sample). Due to the (negative) skewness of the observed richness and Shannon diversity of eggNOG annotations, a scaled exponential transformation and an exponential transformation were used for analyses, respectively, to improve residual fit. Both these alpha diversity indices were then analysed with linear mixed models containing the same predictors as for taxonomic alpha diversity above.

2.3.6.2 Functional GM composition

To test for changes in functional microbiome beta diversity, a PERMANOVA of Euclidean distances calculated from CLR-transformed read abundances per

orthologue was used, using the same model structure as for taxonomic compositional analysis (described above). Differences in composition were visualized with a PCA plot as above.

2.3.6.3 Functional GM differential abundance analysis (DAA)

Differential abundance analysis was performed on eggNOG annotations using their assigned categories from the database of clusters of orthologous genes (COG) (Supplementary Table S2.1) (Tatusov et al., 2000) using *ANCOMBC2* and *GLLVM* as described above (Lin & Peddada, 2024; Niku et al., 2019). Post-hoc DAA were performed on individual eggNOG members found within differentially abundant COG categories to establish the drivers of any significant differences (see Supplementary material for details).

2.4 Results

2.4.1 **Taxonomic GM changes with age**

2.4.1.1 Taxonomic GM alpha diversity

GM species richness declines with host age, and individuals in their terminal year had significantly lower species richness than those in a non-terminal year (Table S2.2 & Figure S2.4). However, Shannon diversity was not significantly associated with host age, and did not differ between samples taken in a terminal or non-terminal year (Table S2.3). A quadratic age term, and an interaction between host age and terminal year were not significantly associated with species richness or Shannon diversity ($p > 0.05$) and were dropped from the final model.

The within-individual centering approach revealed that a decline in GM species richness with host age occurred longitudinally within individuals (Table 2.1, Figure 2.1). However, the slope of declining species richness within an individual (delta age) decreases with increasing mean age, i.e. a decline in GM species richness with time occurs more at earlier host ages than in later life (Table 2.1, Figure 2.1). Indeed, after the age of 6 there doesn't appear to be any significant decline in GM species richness with increasing age (Figure 2.1). This shows that contrary to our prediction that GM may show senescent effects, within-individual changes were less extreme in older individuals (in the ages we know senescence is occurring). There was also no evidence of between-individual selective disappearance effects (Table 2.1). Shannon diversity did not change significantly with mean or delta age (Table S2.4). There was also no evidence of a quadratic relationship between within-individual delta age and species richness or Shannon diversity, hence the quadratic age term was dropped from the final model. We also tested for an interaction between within-individual age and whether an individual's final sample was in their terminal year, but this was not significant ($p > 0.05$) and was dropped. Additionally, the results were consistent with Table 2.1 when non-rarefied reads were used (Table S2.5). This result indicates that within-individual changes in species richness with age had a similar slope whether the bird was sampled in its terminal year or not.

Table 2.1 A generalised linear mixed effect model with a negative binomial distribution (glmer.nb) investigating gut microbiome species richness in relation to within- (delta) and between- (mean) individual variation in age amongst Seychelles warblers (n = 151 samples, 91 individuals). Conditional R^2 = 53.1%. Reference categories for categorical variables are shown in brackets.

Predictor	Estimate	SE	*z*	*P*
(Intercept)	**2.705**	**0.317**	**8.536**	**< 0.001**
Delta Age	**-0.308**	**0.095**	**-3.233**	**0.001**
Mean Age	-0.036	0.023	-1.534	0.125
Terminal Year Bird (yes)	-0.189	0.142	-1.329	0.184
Season (winter)	0.020	0.157	0.126	0.900
Sex (female)	-0.020	0.144	-0.139	0.889
Days at 4℃	-0.238	0.137	-1.734	0.083
Time of day	0.237	0.122	1.938	0.053
Territory quality	-0.081	0.125	-0.645	0.519
Sample Year (2017)				
2018	0.439	0.280	1.568	0.117
2019	0.399	0.323	1.233	0.217
2020	**0.701**	**0.351**	**1.997**	**0.046**
2021	**0.755**	**0.338**	**2.231**	**0.026**
2022	**0.725**	**0.346**	**2.099**	**0.036**
2023	**0.879**	**0.400**	**2.197**	**0.028**
Delta Age * Mean Age	**0.034**	**0.014**	**2.440**	**0.015**
Random				
Individual ID	151 observations	91 individuals	Variance	0.2321

Note: Significant ($p < 0.05$) predictors are shown in bold.

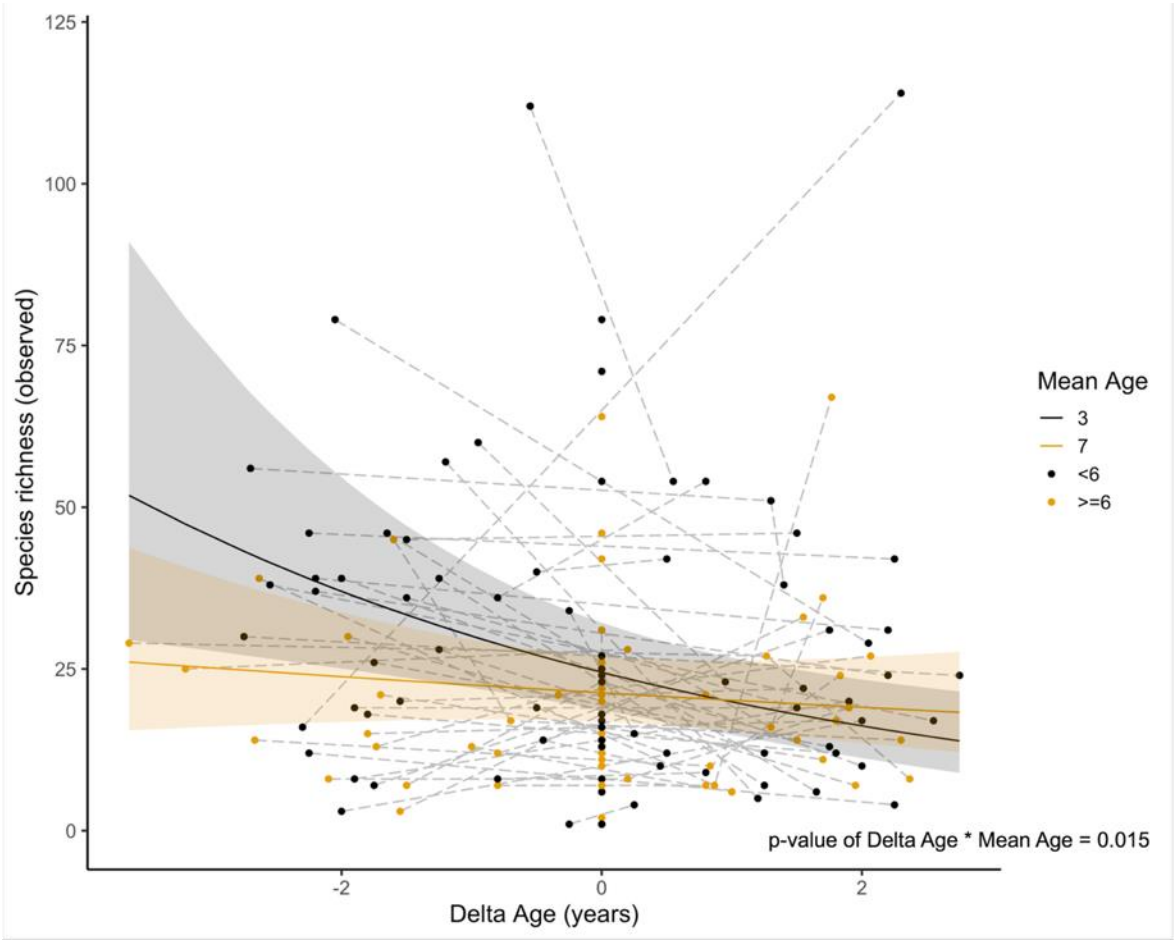

Figure 2.1. Gut microbiome species richness in relation to within-individual, longitudinal differences in age (delta age in years) in Seychelles warblers. The solid lines represent model predictions with 95% confidence intervals calculated from the generalised linear mixed effect model (Table 2.1). Coloured lines are model predictions at mean age of 3 (black) and 7 (gold) points before and after the start of senescence in this species (Hammers et al., 2015). Each point represents an individual gut microbiome sample, coloured by mean age of less than 6 (black) and greater or equal to 6 (gold), and the dashed grey lines connect samples from the same individual (n = 151 samples, 91 individuals).

2.4.1.2 Taxonomic GM composition

A PERMANOVA analysis found that cross-sectional host age was a marginally significant predictor of GM taxonomic composition (Table 2.2), but terminal year was not (Table 2.2). Sample year, season, and catch time were significant and explain the largest proportion of GM compositional variance (Table 2.2) followed by days sample stored at 4°C and sex. An interaction between age and terminal year was not significant ($p > 0.05$). A PCA showed limited sample clustering according to age, which is consistent with the small amount of variance explained in the PERMANOVA (Figure S2.5).

Table 2.2. A PERMANOVA analysis of gut microbiome taxonomic composition in relation to age and terminal year in the Seychelles warbler. The PERMANOVA was performed using a Euclidean distance matrix of CLR-transformed taxon abundances. N = 153 samples from 91 individuals. Bird ID was included as a blocking factor.

Predictor	*df*	R^2	F	*P*
Age	**1**	**0.009**	**1.368**	**0.043**
Terminal Year	1	0.007	1.051	0.569
Season	**1**	**0.013**	**2.021**	**0.001**
Sample Year	**6**	**0.056**	**1.479**	**< 0.001**
Sex	**1**	**0.007**	**1.096**	**0.064**
Days at 4℃	**1**	**0.008**	**1.193**	**0.034**
Time of day	**1**	**0.010**	**1.583**	**< 0.001**
Territory Quality	1	0.005	0.813	0.982

Note: Significant ($p < 0.05$) predictors are shown in bold.

2.4.1.3 Taxonomic GM differential abundance analysis (DAA)

Five of the 22 common GM species found in the Seychelles warbler population (i.e. in >20% individuals) differed significantly in relative abundance with age in the GLLVM analysis (*Escherichia coli*, *Lactococcus lactis*, *Brucella pseudogrignonensis*, *Lactococcus garvieae*, *Microbacterium enclense*), but none were differentially abundant with age in the ANCOMBC2 analysis (Figure S2.6A & S2.6B). Similarly, six species were differentially abundant in the terminal year in the GLLVM analysis (*Lactococcus garvieae*, *Pantoea anthophila*, *Escherichia coli*, *Rothia* sp AR01, *Microbacterium enclense*, *Brucella pseudogrignonensis*), but none were differentially abundant with terminal year in the ANCOMBC2 analysis (Figure S2.6C & S2.6D). Thus, there is no clear consensus of significant variation in the abundance of specific GM species with age or in the terminal year.

2.4.2 Functional GM changes with age

2.4.2.1 Functional GM alpha diversity

Alpha diversity of eggNOG gene orthologues declined significantly with host age for both observed richness and Shannon diversity metrics (Table S2.6, Figure S2.7). Alpha diversity of eggNOG orthologues did not differ between terminal year and non-terminal year samples (Table S2.6). Additionally, the interaction between host age (or quadratic age) and terminal year was not significant ($p > 0.05$).

The decrease in functional alpha diversity with host age is best explained by within-individual longitudinal changes with age for both tested indices (Table 2.3, Figure 2.2). Cross-sectional, between-individual age was a marginally significant predictor of Shannon diversity but not observed richness (Table 2.3). Alpha diversity did not differ between individuals that had at least one sample taken in their terminal year and those that did not. The interaction of terminal year bird and within-individual age, quadratic within-individual age, and the interaction between within-individual age and mean age were also not significant ($p > 0.05$) predictors of either index. Sample year was a significant variable of both eggNOG observed richness and Shannon diversity.

Table 2.3. A linear mixed effect model investigating variation in gut microbiome functional diversity (observed richness and Shannon diversity) in relation to within- (delta) and between- (mean) individual age in Seychelles warblers (n = 152 samples, 90 individuals). Functional diversity is based on eggNOG annotations. Observed richness and Shannon diversity were transformed using a scaled exponential and exponential function, respectively. Conditional R^2 = 35.6% and 13.7% respectively. Reference categories for categorical variables are shown in brackets.

Observed Richness					
Predictor	Estimate	SE	*df*	*t*	*P*
(Intercept)	**0.99**	**0.17**	**124.77**	**5.68**	**< 0.001**
Delta Age	**-0.12**	**0.04**	**137.00**	**-3.31**	**0.001**
Mean Age	-0.03	0.01	89.42	-1.97	0.052
Terminal Year Bird (yes)	0.01	0.08	83.34	0.17	0.870
Season (winter)	-0.06	0.10	136.94	-0.64	0.525
Sex (female)	-0.06	0.08	81.33	-0.79	0.430
Days at 4℃	**-0.19**	**0.09**	**127.35**	**-2.23**	**0.028**

Time of day	-0.07	0.08	137.00	-0.88	0.381
Territory quality	-0.07	0.08	129.62	-0.88	0.381
Sample Year (2017)					
2018	0.13	0.15	135.76	0.82	0.416
2019	0.08	0.18	135.88	0.46	0.647
2020	0.36	0.20	136.54	1.82	0.071
2021	**0.39**	**0.19**	**136.94**	**2.04**	**0.044**
2022	**0.56**	**0.19**	**128.48**	**2.90**	**0.004**
2023	**0.57**	**0.23**	**122.81**	**2.50**	**0.014**
Random					
Individual ID	152 observations	90 individuals	Variance		0.050
Shannon Diversity					
Predictor	Estimate	SE	*df*	t	*P*
(Intercept)	**757.59**	**182.06**	**119.47**	**4.16**	**< 0.001**
Delta Age	**-117.01**	**41.06**	**135.71**	**-2.85**	**0.005**
Mean Age	**-27.30**	**13.54**	**83.56**	**-2.02**	**0.047**
Terminal Year Bird (yes)	17.93	79.75	76.74	0.23	0.823
Season (winter)	173.07	104.67	127.74	1.65	0.101
Sex (female)	-4.98	80.46	69.67	-0.06	0.951
Days at 4℃	-48.55	95.70	133.26	-0.51	0.613
Time of day	-21.18	81.57	132.14	-0.26	0.796
Territory quality	-0.74	85.97	136.99	-0.01	0.993
Sample Year (2017)					
2018	88.02	168.08	136.67	0.52	0.601
2019	32.22	200.48	136.71	0.16	0.873
2020	169.50	210.62	131.73	0.81	0.422
2021	**464.12**	**206.85**	**136.39**	**2.24**	**0.026**
2022	**484.95**	**202.78**	**124.82**	**2.39**	**0.018**
2023	453.37	238.55	116.14	1.90	0.060
Random					
Individual ID	152 observations	90 individuals	Variance		5046

Note: Significant ($p < 0.05$) predictors are shown in bold.

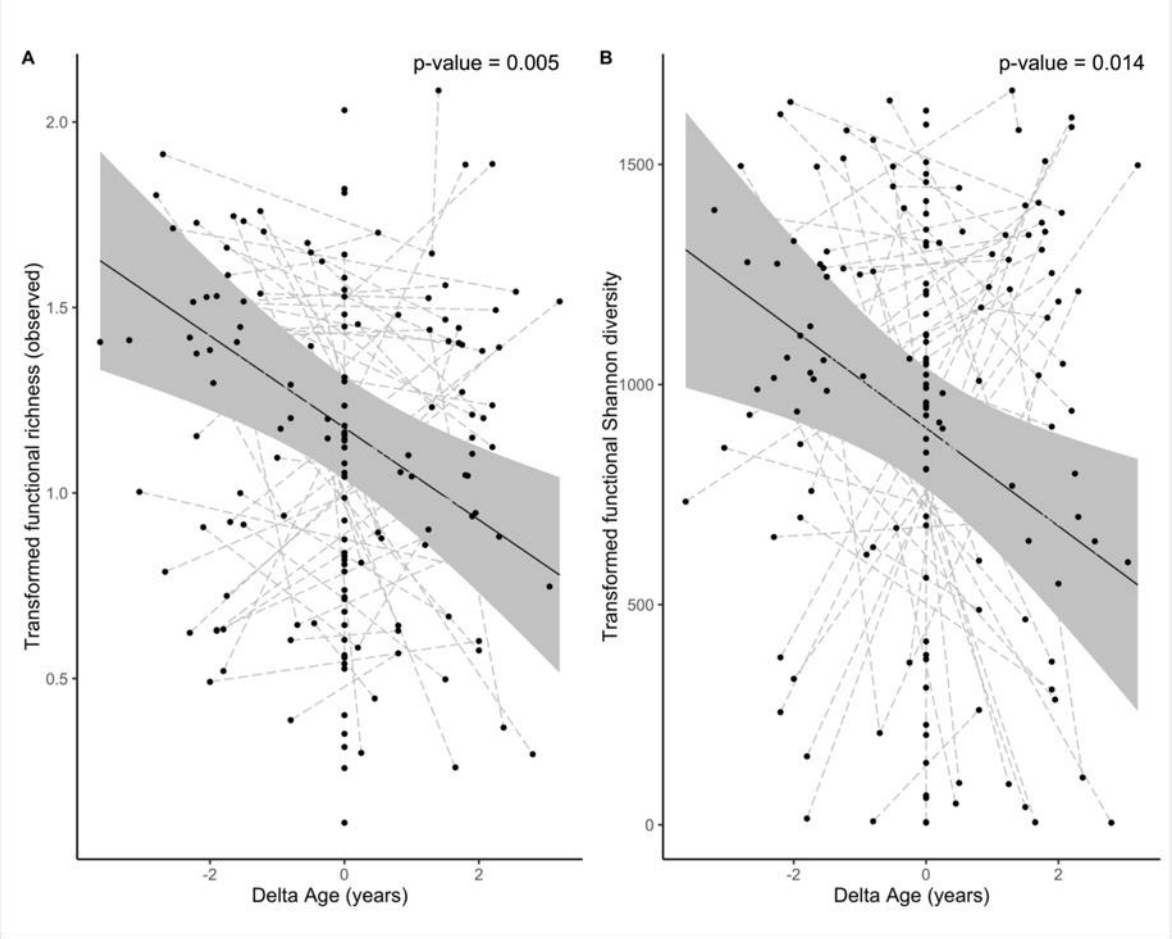

Figure 2.2. Gut microbiome functional diversity measured as (A) observed richness and (B) Shannon diversity in relation to within-individual host age (years). Functional diversity calculations are based on eggNOG orthologue groups. Solid lines represent model predictions (± 95% confidence interval) from linear mixed effects models (Table 2.3). Each point represents a unique gut microbiome sample, and the dashed grey lines connect samples collected from the same individual (n = 152 samples, 90 individuals).

2.4.2.2 Functional GM beta diversity

A PERMANOVA analysis identified factors that were significantly related to GM functional composition (Table 2.4). Host age, but not terminal year, was a marginally significant predictor of functional composition (Table 2.4). An interaction between age and terminal year was not significant ($p > 0.05$). The largest effect sizes were found in relation to season, sample year, sex, and days stored at 4℃ (Table 2.4). Time of day was not significantly related to GM functional composition (in contrast to GM taxonomic composition). A PCA plot showed limited clustering of GM samples

according to age, consistent with the small amount of variance explained by this variable (Figure S2.8).

Table 2.4. A PERMANOVA analysis of gut microbiome functional composition in relation to age (and other factors) in the Seychelles warbler. The PERMANOVA was performed using a Euclidean distance matrix calculated using CLR-transformed (eggNOG) abundances. N = 153 samples. 91 individuals. Bird ID was included as a blocking factor.

Predictor	df	R^2	F	*P*
Age	**1**	**0.007**	**1.096**	**0.044**
Terminal Year	1	0.006	0.890	0.292
Season	**1**	**0.011**	**1.823**	**0.042**
Sample Year	**6**	**0.052**	**1.374**	**0.020**
Sex	**1**	**0.008**	**1.250**	**0.001**
Days at 4℃	**1**	**0.010**	**1.569**	**0.007**
Time of day	1	0.008	1.200	0.139
Territory quality	1	0.007	1.094	0.413

Note: Significant ($p < 0.05$) predictors are shown in bold.

2.4.2.3 Functional GM differential abundance analysis (DAA)

Only one cluster of orthologous genes (COG) category was differentially abundant in relation to age. The COG category “X”, which represents mobilome COGs such as prophages and transposons, significantly increased in abundance with age in both the ANCOMBC2 and the GLLVM analyses (Figure 2.3). Several COG categories were significantly differentially abundant with environmental variables including Cat A (RNA processing and modification) with season and Cat C (Energy production and conversion) with sample year (Figure S2.9, Figure S2.10).

Within category X (mobilome), only COG2801 (transposase genes) was found to significantly increase in abundance with age in both GLLVM and ANCOMBC2 analyses (Figure S2.11, Table S2.1). A within-subject centering approach within a linear mixed model showed an increase in COG2801 was associated with both within-individual (longitudinal) age and between-individual (cross-sectional) age (Table S2.7, Figure 2.4). However, the interaction between within-individual age and terminal year, as well as the interaction between within-individual age and mean age, was not significant ($p > 0.05$).

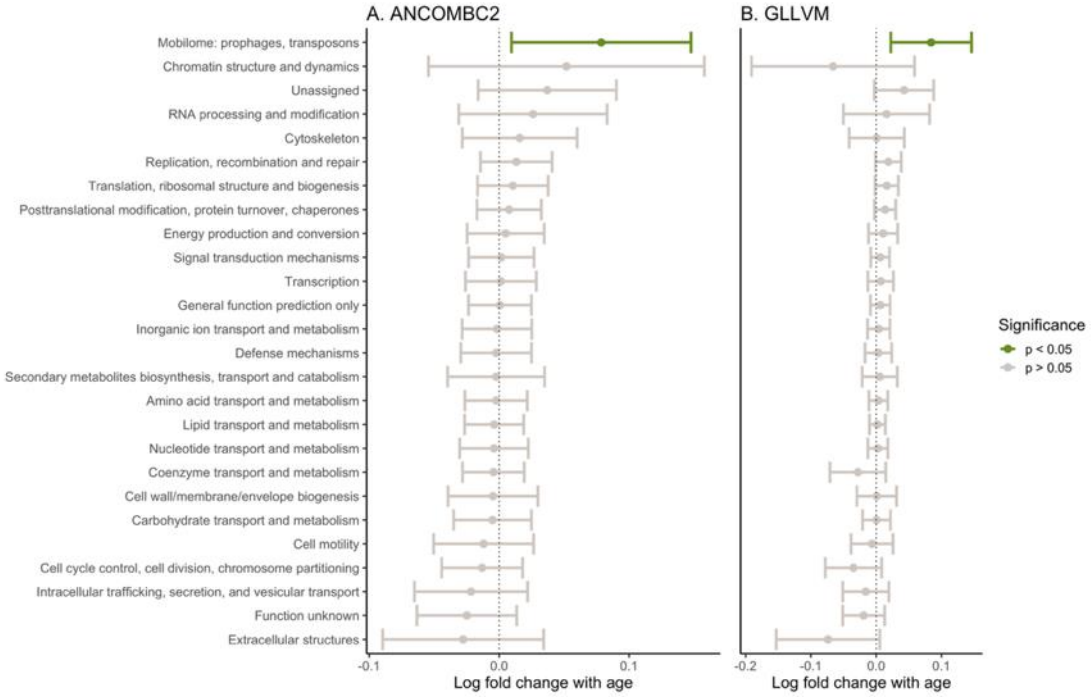

Figure 2.3. Differential abundance analysis of functional gut microbiome cluster of orthologous genes (COG) categories in Seychelles warblers using (A) ANCOMBC2 and (B) GLLVM. Each COG category is represented on the y-axis. Points and error bars are coloured according to significance (green: $p < 0.05$; grey: $p > 0.05$).

COG2801 located within MGSs (509 COG2801 copies from 160 MGS) were most closely related to the group insertion sequences (IS) 3 family of transposases (30%), other IS family transposases (12%), partial or putative transposases (33%) or other/unknown function (25%; Table S2.8). An increased abundance of COG2801 in the GM may be due to either an increase in the abundance of COG2801-carrying microbes or increased replication of the transposase gene itself. However, contrary to the first hypothesis, we found no relationship between the total abundance of COG2801-carrying MGSs (n = 160) and host age (Table S2.9). To further test this, COG2801-MGSs were matched with metaphlan4 annotations at the genus level; the abundance of COG2801-metaphlan4 genera was not significantly associated with host age (Table S2.10). Hence, the increase in COG2801 abundance with host age could not be attributed to an increased abundance of COG2801-carrying bacteria. Additionally, within COG2801, ten gene catalogues were commonly shared across 50% of samples. Each of these ten COG2801 gene catalogues was

not significantly ($p > 0.05$) differentially abundant with age individually when tested using both ANCOMBC2 or GLLVM analysis (Figure S2.12). Thus, the increase in abundance of COG2801 with age was not being driven by the abundance of a single prevalent, gene catalogue but rather the cumulative abundance of many.

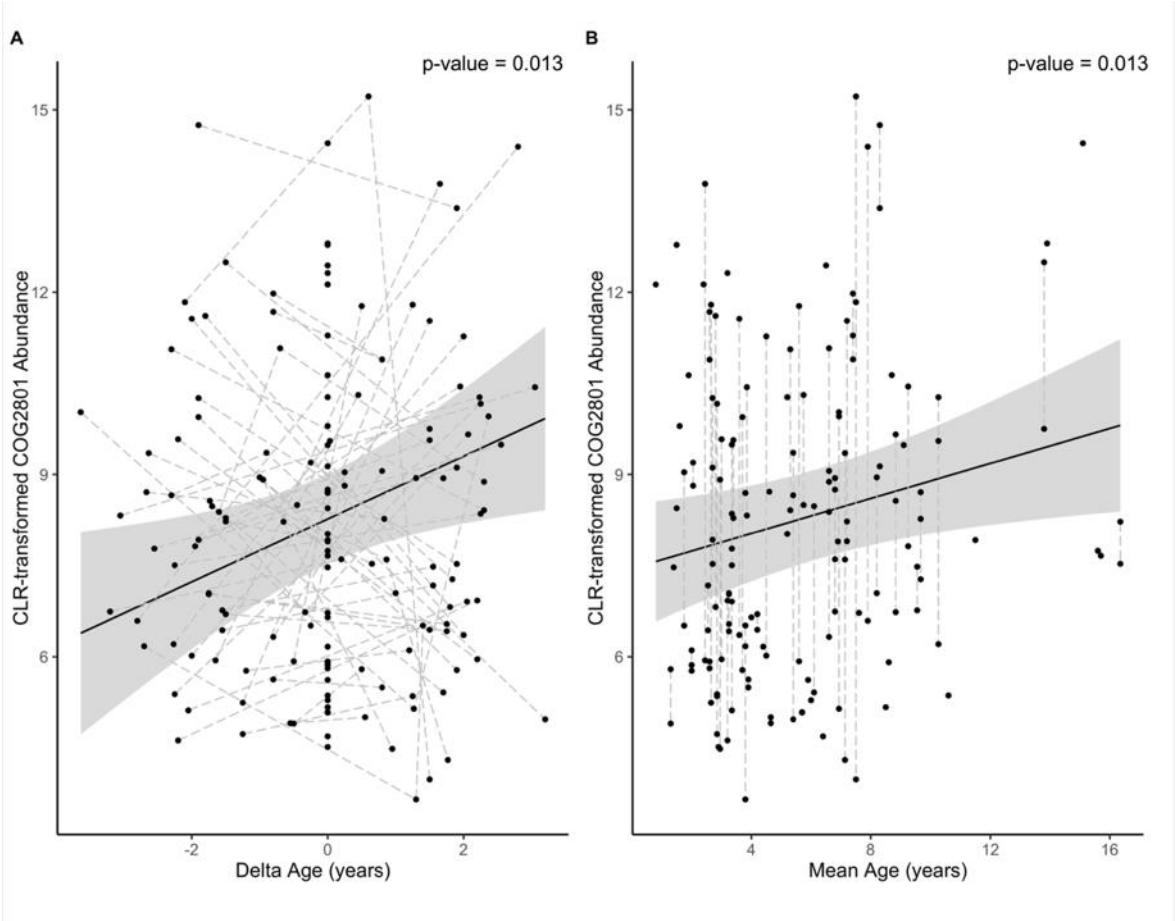

Figure 2.4. CLR-transformed COG2801 abundance in relation to (A) within-individual (delta) host age and (B) between-individual (mean) host age in the gut microbiome of Seychelles warblers. The solid line represents model predictions (± 95% confidence intervals) from a linear mixed effect model (Table S2.7). Each point represents a gut microbiome sample with dashed grey lines connecting samples from the same individual (n = 153 samples, 91 individuals).

2.5 Discussion

We used a repeated metagenomic dataset from individuals in a Seychelles warbler population to investigate how GM taxonomic and functional characteristics varied with host age. We identified a linear decrease in species richness, and small shifts in GM taxonomic composition, with host age. Additionally, species richness was lower in samples taken during an individual's terminal year, but taxonomic composition did not differ between terminal and non-terminal samples. We also identified a linear decrease in the GM's functional richness and diversity, and differences in functional GM composition, with host age. Finally, COG categories representing the mobilome increased in prevalence with bird age, driven by an increase in the abundance of COG2801, a group of transposases.

The small reduction in GM richness, but not Shannon diversity, with age suggests a loss of rare taxa that is not linked with a major restructuring of the evenness of the GM. The reduction in species richness was also age-dependent, with younger individuals experiencing greater reduction in species richness over time compared to older individuals, indicating that changes in GM species richness is not associated with senescence. This also concurs with the small changes in GM composition with age we identified; i.e showing a limited number of differentially abundant taxa with increasing host age. This result is consistent with a previous 16S metabarcoding analysis of senescence of the Seychelles warbler GM despite the increased taxonomic resolution afforded by a metagenomics approach (Worsley, Davies, et al., 2024). Additionally, the three dominant phyla identified in the metagenomics analysis (accounts for 95.6% of all taxonomic assignments) were the same three dominant phyla identified through the 16S analysis (Proteobacteria, Actinobacteria, and Firmicutes) (Worsley, Davies, et al., 2024). Overall, the results support the conclusion that, taxonomically, most of the GM stays the same with increasing age, apart from the loss of a few rare taxa.

Taxonomic changes in GM species diversity and composition with age have been repeatedly demonstrated in humans and captive animals (Ghosh et al., 2022). However, in these species, late-life changes in the GM may be due to external factors such as antibiotic use, lifestyle, and dietary changes (Gibson et al., 2019;

Oliveira et al., 2020). An increasing number of wild animal studies are finding little evidence of a late-life shift in GM taxonomic diversity without such external factors (see (Risely et al., 2022; Worsley, Davies, et al., 2024)). Our study supports this conclusion despite the repeated sampling and increased resolution yielded by shotgun metagenomics, which can potentially reveal more nuanced changes at lower taxonomic levels.

Few studies have directly investigated functional changes in the GM with age in wild animals (Levin et al., 2021). Some studies have been undertaken using functional inferences from metabarcoding sequence homology. However, this can be misleading due to being limited to variation within the same genus thus providing potentially inaccurate functional profiles. (Chang et al., 2022; Wilson & Nicholson, 2017). In our study using a higher resolution metagenomic approach, we found evidence of small, linear, changes in GM functional diversity and composition with age in the Seychelles warbler. Functional observed richness and Shannon diversity declined with age, which suggests not only that rare functions are lost, but that the evenness of these GM functions also changes linearly with adult age. Age-related decreases in functional richness and shifts in functional composition have previously been identified in elderly humans (Armour et al., 2019; Mosca et al., 2016). Such changes have been linked to the onset of specific disease states, such as inflammation and pathogenesis and changes to diet degradation and digestion, in humans and laboratory mice (Singh et al., 2021). However, other studies have either found no change in functional alpha diversity, or even an increase in microbial functional richness and diversity with age (Rampelli et al., 2013; Ruiz-Ruiz et al., 2020). Whether the loss of functional diversity, and minor changes in functional composition, with host age in Seychelles warbler is linked to declines in health and condition remains unclear and requires further study. The decline in taxonomic richness (but not taxonomic diversity) along with declines of functional richness and diversity with host age suggests that as the host age, less rare taxa contribute to the number and evenness of functional genes in the GM.

Despite the small changes in functional diversity and composition with age in the Seychelles warbler, we only identified one specific functional category whose abundance was significantly associated with host age. An increase in the abundance of COG2801 transposases occurred with age. However, this was not

due to an increase in COG2801-carrying microbes. COG2801 are a group of transposases that are primarily found in bacteria (89.5%) and have been shown to be the most widely transferred genes among prokaryotes (Powell et al., 2014). Most COG2801 genes found within MGSs were group insertion sequences 3 (IS3), which use a copy-out-paste-in mechanism to replicate (Ohtsubo et al., 2004). This could lead to an increased number of transposon copies in the same individual bacterial genome over time, or to horizontally transfer to other bacterial genomes. (Siguier et al., 2015; Wells & Feschotte, 2020). Thus, the increased abundance of COG2801 with age in Seychelles warbler GM's may be the result of self-replication, independent of microbial host cell DNA replication. An increase in transposition has been observed when bacteria are stressed and COG2801 is one of the most horizontally transferable eggNOG genes (Lysnyansky et al., 2009; Nakamura, 2018). Therefore, as vertebrate hosts get older, the GM may be exposed to a greater number or intensity of stressors, such as mucus barrier thinning or inflammation, which may induce activation of COG2801 (Elderman et al., 2017). However, there was not an accelerated increase (i.e. a quadratic relationship) of COG2801 abundance with host age, which would be expected if the cumulative effects of host senescence were driving these changes. Therefore, stressors to the host that occur linearly in adulthood, such as cell death in the gastrointestinal autonomic nervous system (Phillips et al., 2007; Phillips & Powley, 2001), may better explain the increased abundance of COG2801 with host age.

We also focused on assessing terminal year effects in the Seychelles warbler GM. Only species richness was found to be significantly lower in the final year of a bird's life. Moreover, the effect of terminal year was uniform across age, i.e. it was not more extreme in older individuals. Previous research has identified age-dependent terminal-declines in fitness components (reproductive success and survival probability) in the Seychelles warbler (Hammers et al., 2012). However, the lack of age-dependent terminal changes in GM characteristics identified in our study suggests that the GM does not undergo senescence in association with these other traits. As such, the declines in microbial species richness in terminal year samples (and linearly with age) may rather reflect the stabilisation of the GM with age rather than a senescence effect. These results concur with the previous 16S metabarcoding analysis of the Seychelles warbler GM which found little evidence of GM senescence (Worsley, Davies, et al., 2024).

Across analyses, environmental factors explained most of the variance in the Seychelles warbler GM. This concurs with previous research on this species (Davies et al., 2022; Worsley, Davies, et al., 2024; Worsley et al., 2021) as well as studies of other taxa (Gacesa et al., 2022; Ren et al., 2017; Wang et al., 2023). Temporal variation -specifically year and season- explained the most variance in both taxonomic and functional GM composition. This may be explained by many factors including climate variability, differences in insect prey availability, or host population density (Foster et al., 2012; Li et al., 2016; Sepulveda & Moeller, 2020). Most Seychelles warbler individuals breed in the summer rather than the winter season, and GM shifts may therefore reflect reproductive activity and related hormonal changes (Hernandez et al., 2021). Time of day was also associated with GM composition. Differences in insect activity might drive this pattern due to light availability and/or temperature (Totland & Totland, 1994; Welti et al., 2022). However, such patterns could also be due to host intrinsic circadian rhythms (Schmid et al., 2023). In addition, differences in the amount of time samples were stored at 4℃ resulted in differences in the GM characteristics and it is very important that these are controlled for. Given that samples are stored directly in absolute ethanol, the changes related to the time in storage at 4℃ are likely to do with DNA degradation affecting the assignment of reads rather than an actual biological change in storage.

These factors lead to a substantial amount of noise in GM studies that can confound studies on ageing, reproduction, and disease outcomes in wild populations. Therefore, accounting for these factors is important when investigating the GM in natural systems.

Our findings highlight the need for more studies investigating the functional characteristics of wild microbiomes as taxonomic relationships might not capture functional GM changes that occur (e.g. the increased prevalence of COG2801). However, researchers should not totally discount the utility of 16S metabarcoding for investigating general GM questions, as it may, in many cases, provide sufficient taxonomic resolution to answer specific questions (Durazzi et al., 2021). Indeed, we identified similar taxonomic patterns using shotgun metagenomics to those revealed by a previous metabarcoding study on the Seychelles warbler (Worsley, Davies, et al., 2024). The cost-effectiveness of 16S rRNA allows greater sample sizes, and

thus power, to resolve certain questions. A combination approach that harmonises both 16S metabarcoding and shotgun metagenomics has been proposed to maximise sample size, although such analyses are limited to genus-level comparisons (Usyk et al., 2023). On the other hand, shotgun metagenomics not only allows higher taxonomic resolution and functional analysis of the GM, but also an assessment of the interaction between taxa and their functions. As described with transposable elements, our functional analysis uncovered changes in GM function that were not detectable using 16S metabarcoding analysis.

In conclusion, while we found that the Seychelles warbler GM changes in terms of diversity, composition and even function with age, this happens gradually over the adult lifespan and there is little evidence of late-life GM senescence. Whilst species richness is lower in the terminal year, this occurs at all ages and is not more extreme in the oldest individuals. Interestingly, we found that the abundance of a group of transposase gene increases considerably with age in the GM, probably because of more frequent transposition within the GM community over time. Future work is required to determine exactly why these transposable element changes occur and what impact they may have. Additionally, work should investigate the generality of these conclusions by assessing whether functional changes occur in the GM of other wild vertebrates.

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2.7 Supplementary material

2.7.1 Supplementary methods

2.7.1.1 Bioinformatics

Briefly, host reads were removed by mapping sequences to the Seychelles warbler genome (unpublished; complete BUSCO = 96.0% with a total length = 1,081,018,985 bp), using Kraken 2 (version 2.1.3). Remaining reads underwent quality filtering using sdm software version 2.14 beta [101,102]. After trimming, two samples and five hand controls were removed because they did not return enough reads for subsequent analysis (< 300,000 reads). An average of 20,481,040 (±1,109,059 SE) paired-end reads per sample were retained across the remaining samples.

The same trimmed reads were also used for *de novo* metagenome assembly, as implemented in MATAFILER: MEGAHIT version 1.2.9 [103] was used for metagenomic assemblies, on these genes were predicted using Prodigal version 2.6.3 [104] and clustered into a gene catalogue (95 % identity) of 19,527,109 gene

clusters, and a gene abundance matrix created using rtk2 [105]. Functional annotations of clustered genes were done using eggNOGmapper version 2.1.12 and the evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG) database version 4 [82,106]. Subsequently, genome binning was done with SemiBin which created 4,176 bins (mean completeness = 34.95%, mean contamination = 1.41%) [107]. The bins were then filtered based on >80% completeness and <5% contamination using CheckM2 [108]; this retained 824 metagenome-assembled genomes (MAGs). MAGs were dereplicated across samples to generate 323 non-redundant metagenomic species (MGS) level bins, using clusterMAGs (https://github.com/hildebra/clusterMAGs). For MGSs, taxonomic assignment was performed using a marker-based approach with GTDB database version 214 [109]. Due to the high individuality of the warbler GM and the high sequencing coverage required to assign MGS, only one MGS was present in more than 50% of sequenced samples and relatively fewer MGSs were identified per sample (average 17 ± 1.3 SE per sample) which is likely to be an underestimate of the true diversity of the GM.

Therefore, Metaphlan4 version 4.1.0 (which is assembly-free and therefore requires lower coverage) was used to taxonomically classify reads using the default parameters [110]. Metaphlan4 assignments identified an average of 29.3 ± 2.0 species genome bins per sample and were used for the subsequent taxonomic analysis and MGS was only used for tracking functional annotations back to their taxonomy.

2.7.1.2 Post-hoc functional differential abundance analysis

Posthoc investigations were performed on individual eggNOG members found within the COG categories that were significantly differentially abundant with age. Firstly, a linear model was performed for each significant eggNOG member to test whether age-related changes were driven by between- or within-individual processes. Second, we tested if changes in the abundance of significant eggNOG members could be driven by changes in the abundance of the taxa from which these genes originate. To test this, the total abundance of MGSs carrying the eggNOG gene orthologs of interest was used as the response variable and age was included as a predictor in a lmer model. Furthermore, genera of eggNOG-carrying MGSs were matched with metaphlan4 genera to test whether the total abundance of known eggNOG-carrying genera was significantly associated with host age. Lastly, a protein-protein Basic Local Alignment Search Tool (BLASTp) analysis of each eggNOG gene ortholog of interest embedded within each MGS was performed to determine the identity of genes [111,112]. To test if the differential abundance of eggNOG members was driven by changes in the abundance of a specific gene (versus the cumulative abundance of many genes), gene catalogues assigned to the eggNOG cluster of interest (filtered to those with > 20% prevalence and 0.1% detection) were tested for differential abundance.

2.7.2 Supplementary Figures and Tables

Components of positive controls were successfully recovered as high-quality MGSs in acceptable relative abundances (Figure S2.2). Only 2 out of the 18 MGS from controls were found in faecal samples, both were widespread species *Enterococcus faecalis* and *Klebsiella pneumoniae* [113,114]. *E. faecalis* was part of the positive control but not found in the hand controls. *K. pneumoniae* was found in hand controls as well as samples but due to the low abundance in hand controls, we decided to retain all species for taxonomic analysis.

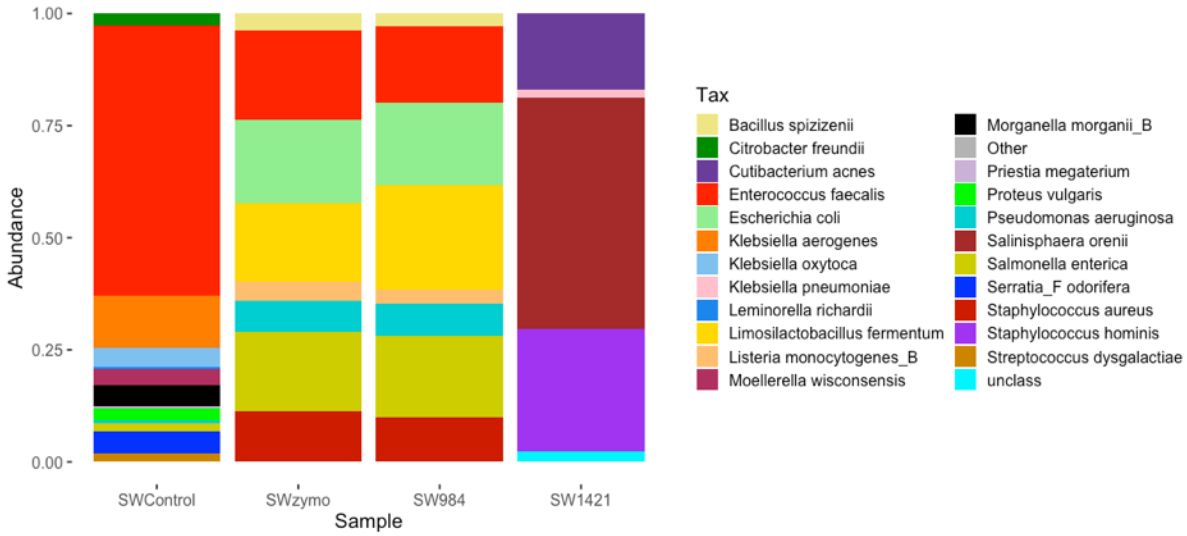

Figure S2.1. Controls and relative abundance of MGS at the species level. SWControl is positive control (ZymoBIOMICS Fecal Reference with TruMatrix™ Technology), SW984 and SWzymo are positive controls (ZymoBIOMICS Microbial Community Standard) sequenced separately, and SW1421 is a contamination (hand) control from 2023. We identified subspecies of *Bacillus subtilis* - *Bacillus spizizenii* and *Lactobacillus fermentum* – *Limosilactobacillus fermentum* . In SW1421 hand control, *Cutibacterium acnes* is linked to acne, *Klebsiella pneumoniae* is commonly found in the gut, *Salinisphaera orenii* are bacteria commonly isolated in high salinity environments, *Staphylococcus hominis* is commonly found to be harmless on human and animal skin.

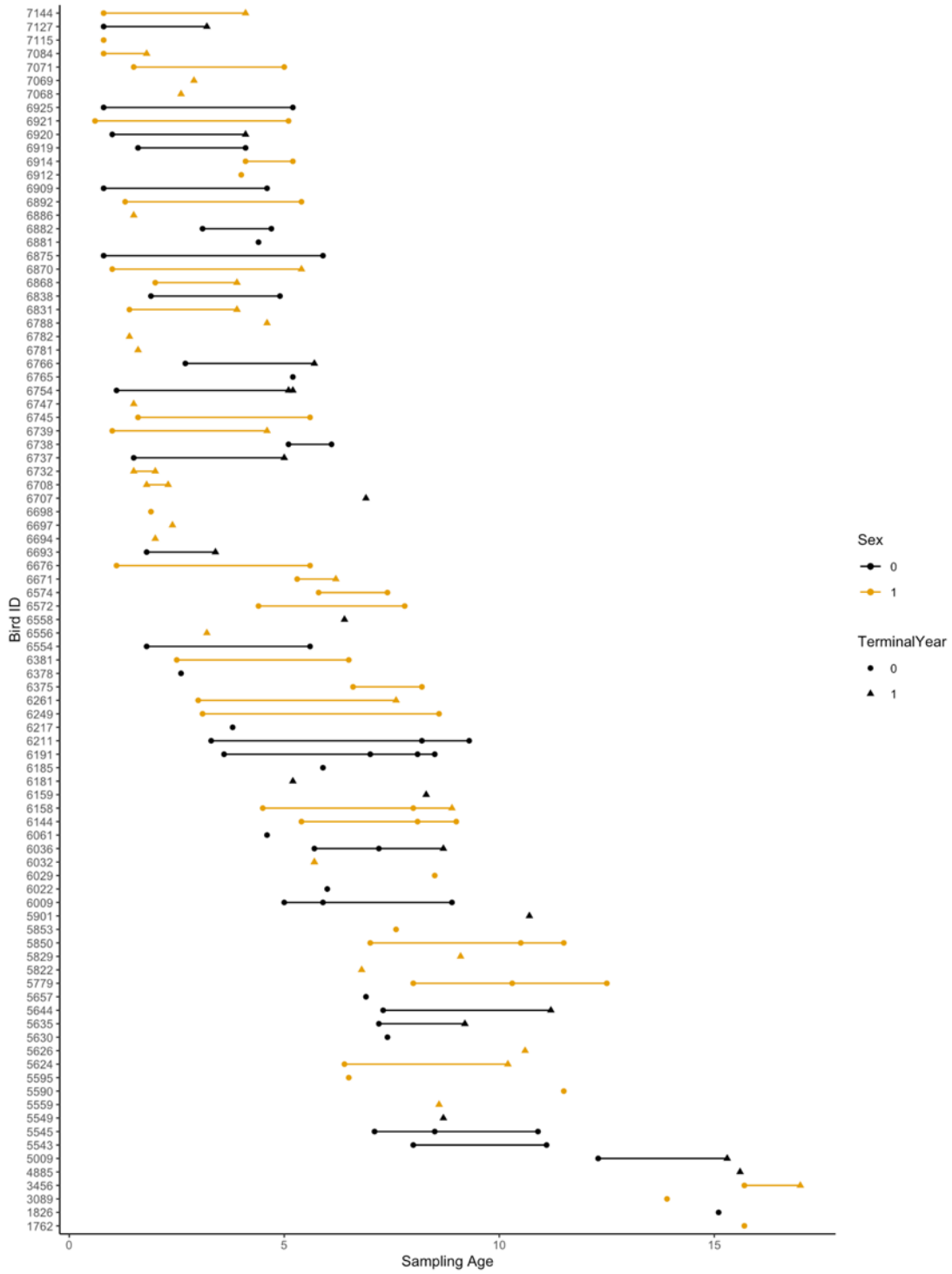

Figure S2.2. Seychelles warbler gut microbiome samples that were retained for analysis after sequencing and bioinformatics (n = 153 from 91 individuals). Points represent each sample, the x-axis represents individual's age at sampling, whilst the y-axis represents individuals. Solid lines connect samples that were collected from the same individual. Colours represent the different sex (black = female, gold = male). Shape represents whether the sample was collected in the individual's terminal year (circle = no, triangle = yes).

Taxonomy

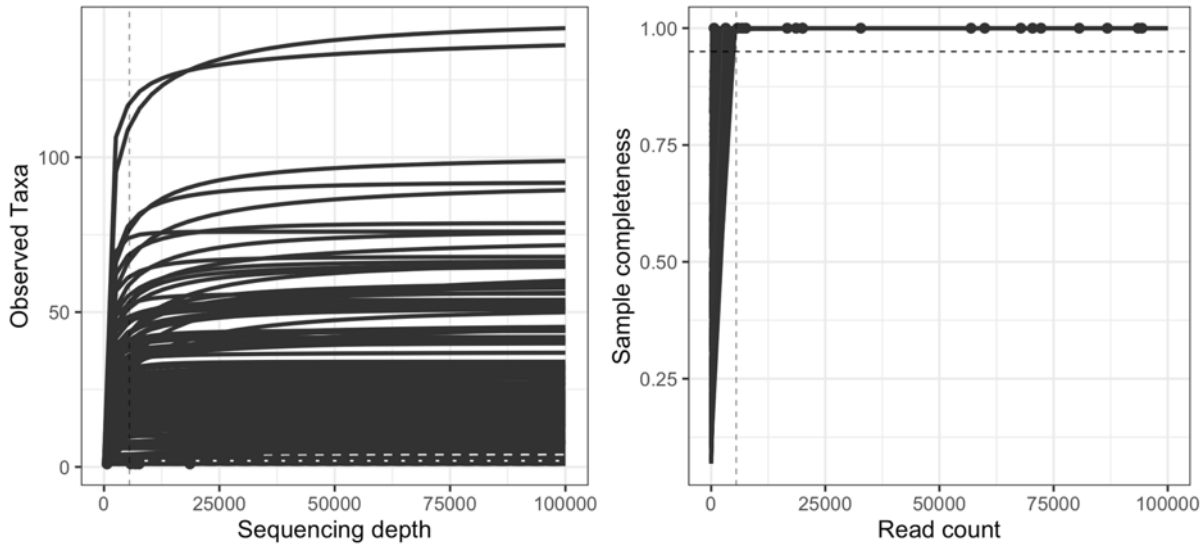

Figure S2.3. Sequencing depth against number of observed (metaphlan4) assembly-free taxonomic assignments (left) and read count against sample completeness (right) of each gut microbiome sample from Seychelles warblers (n = 153). 5500 reads at 95% completeness.

Table S2.1. COG functional categories [71]

Abbreviation	COG Functional Categories
A	RNA processing and modification
K	Transcription
L	Replication, recombination and repair
B	Chromatin structure and dynamics
D	Cell cycle control, cell division, chromosome partitioning
V	Defense mechanisms
Y	Nuclear structure
T	Signal transduction mechanisms
M	Cell wall/membrane/envelope biogenesis
N	Cell motility
Z	Cytoskeleton
W	Extracellular structures
U	Intracellular trafficking, secretion, and vesicular transport
O	Posttranslational modification, protein turnover, chaperones
X	Mobilome: prophages, transposons

C	Energy production and conversion
G	Carbohydrate transport and metabolism
E	Amino acid transport and metabolism
F	Nucleotide transport and metabolism
H	Coenzyme transport and metabolism
I	Lipid transport and metabolism
P	Inorganic ion transport and metabolism
R	General function prediction only
Q	Secondary metabolites biosynthesis, transport and catabolism
S	Function unknown
`	Unassigned

Table S2.2. A generalised linear mixed effect model with a negative binomial distribution investigating the relationship between age, terminal year, and species richness in the gut microbiome of Seychelles warblers (n = 151 samples, 91 individuals). Significant ($p < 0.05$) predictors are shown in bold. Conditional R^2 = 38.9%.

Predictor	Estimate	SE	*z*	*P*
(Intercept)	-125.20	71.62	-1.75	0.081
Age	**-0.04**	**0.02**	**-2.10**	**0.036**
Terminal Year (yes)	**-0.26**	**0.13**	**-2.06**	**0.039**
Season (winter)	0.01	0.13	0.09	0.932
Sex (female)	0.01	0.13	0.05	0.959
Time at 4℃	-0.18	0.14	-1.33	0.183
Time of day	0.22	0.12	1.82	0.069
Territory quality	-0.08	0.12	-0.67	0.506
Sample Year	0.06	0.04	1.79	0.073
Random				
Individual ID	151 observations	91 individuals	Variance	0.14

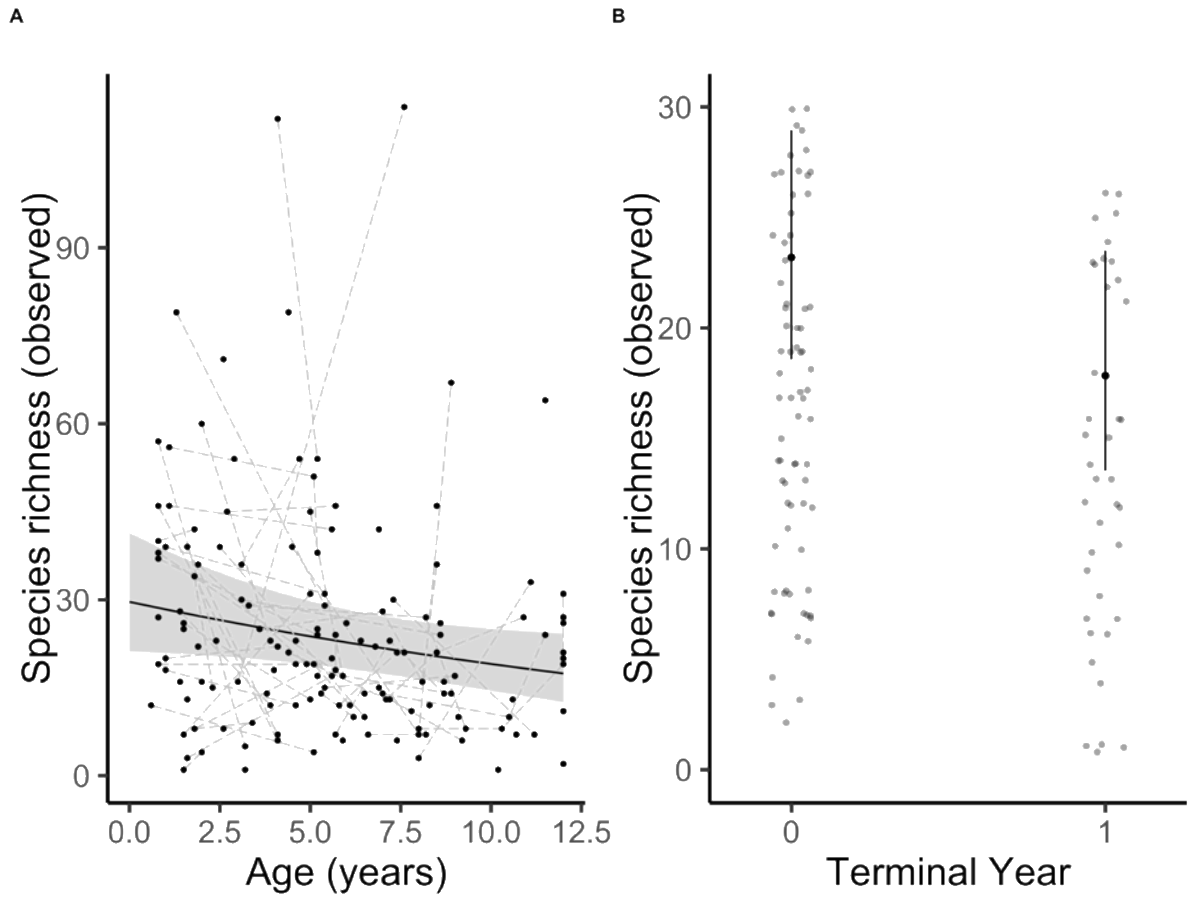

Figure S2.4. Species richness prediction from glmer.nb of the gut microbiome in the Seychelles warblers (n = 151 samples from 91 individuals). (A) Species richness against host age in years, solid black line and grey shaded area represent model predictions and confidence intervals respectively (Table S2.1, p = 0.036), points represent raw data. (B) Species richness against terminal year (0: No, 1: Yes), black dot and lines represent model predictions and error bars respectively, grey dots represent raw data points (Table S2.1, p = 0.039).

Table S2.3. A linear mixed effect model of Shannon diversity with chronological age and terminal year in the gut microbiome of Seychelles warblers (n = 151 samples, 91 individuals). Significant (p < 0.05) predictors are shown in bold. Conditional R^2 = 46.4%.

Predictor	Estimate	SE	*df*	*t*	*P*
(Intercept)	**-152.40**	**76.85**	**142.00**	**-1.98**	**0.049**
Age	-0.01	0.02	86.36	-0.46	0.644
Terminal Year (yes)	-0.16	0.14	133.79	-1.17	0.244
Season (winter)	-0.12	0.17	130.60	-0.69	0.491
Sex (female)	0.10	0.16	74.64	0.63	0.529
Time at 4℃	**-0.32**	**0.15**	**113.36**	**-2.15**	**0.034**
Time of day	-0.01	0.13	133.62	-0.10	0.920

Territory quality	-0.14	0.14	124.33	-1.02	0.311
Sample Year	0.08	0.04	142.00	2.00	0.047
Random					
Individual ID	151 observations	91 individuals	Variance	0.27	

Table S2.4. A linear mixed effect model of Shannon diversity within- and between-individual age analysis, accounting for subsequent close-to-death samples in the gut microbiome of Seychelles warblers (n = 151 samples, 91 individuals). Significant ($p < 0.05$) predictors are shown in bold. Conditional R^2 = 49.7%.

Predictor	Estimate	SE	*df*	*z*	*P*
(Intercept)	**0.95**	**0.35**	**129.65**	**2.75**	**0.007**
Delta Age	-0.07	0.07	135.41	-1.12	0.265
Mean Age	-0.18	0.16	77.16	-1.14	0.257
Terminal Year Bird (yes)	-0.01	0.03	81.30	-0.24	0.809
Sample Year	0.09	0.06	105.90	1.60	0.11
Season (winter)	-0.12	0.17	128.97	-0.72	0.470
Sex (female)	0.10	0.16	75.58	0.62	0.535
Time at 4℃	**-0.33**	**0.15**	**112.75**	**-2.24**	**0.027**
Time of day	-0.02	0.13	131.47	-0.12	0.908
Territory quality	-0.15	0.14	122.92	-1.08	0.281
Random					
Individual ID	151 observations	91 individuals	Variance	0.3003	

Table S2.5. A linear mixed effect model of non-rarefied reads species richness within- and between- individual age analysis, accounting for subsequent close-to-death samples in the gut microbiome of Seychelles warblers (n = 151 samples, 91 individuals). Significant ($p < 0.05$) predictors are shown in bold. R2 = 0.4587057

Predictor	Estimate	SE	*z*	*P*
(Intercept)	**2.809**	**0.336**	**8.362**	**< 0.001**
Delta Age	**-0.320**	**0.105**	**-3.043**	**0.002**
Mean Age	-0.035	0.024	-1.432	0.152
Terminal Year	-0.170	0.147	-1.161	0.246

Bird (yes)				
Season (winter)	0.008	0.170	0.046	0.963
Sex (female)	-0.063	0.149	-0.424	0.672
Days at 4℃	-0.188	0.149	-1.259	0.208
Time of day	0.240	0.132	1.819	0.069
Territory quality	-0.099	0.136	-0.724	0.469
Sample Year (2017)				
2018	0.461	0.297	1.552	0.121
2019	0.556	0.345	1.611	0.107
2020	**0.851**	**0.373**	**2.283**	**0.022**
2021	**0.917**	**0.358**	**2.561**	**0.010**
2022	**0.834**	**0.362**	**2.302**	**0.021**
2023	**0.953**	**0.418**	**2.280**	**0.023**
Delta Age * Mean Age	**0.033**	**0.016**	**2.118**	**0.034**
Random				
Individual ID	153 observations	91 individuals	Variance	0.2119

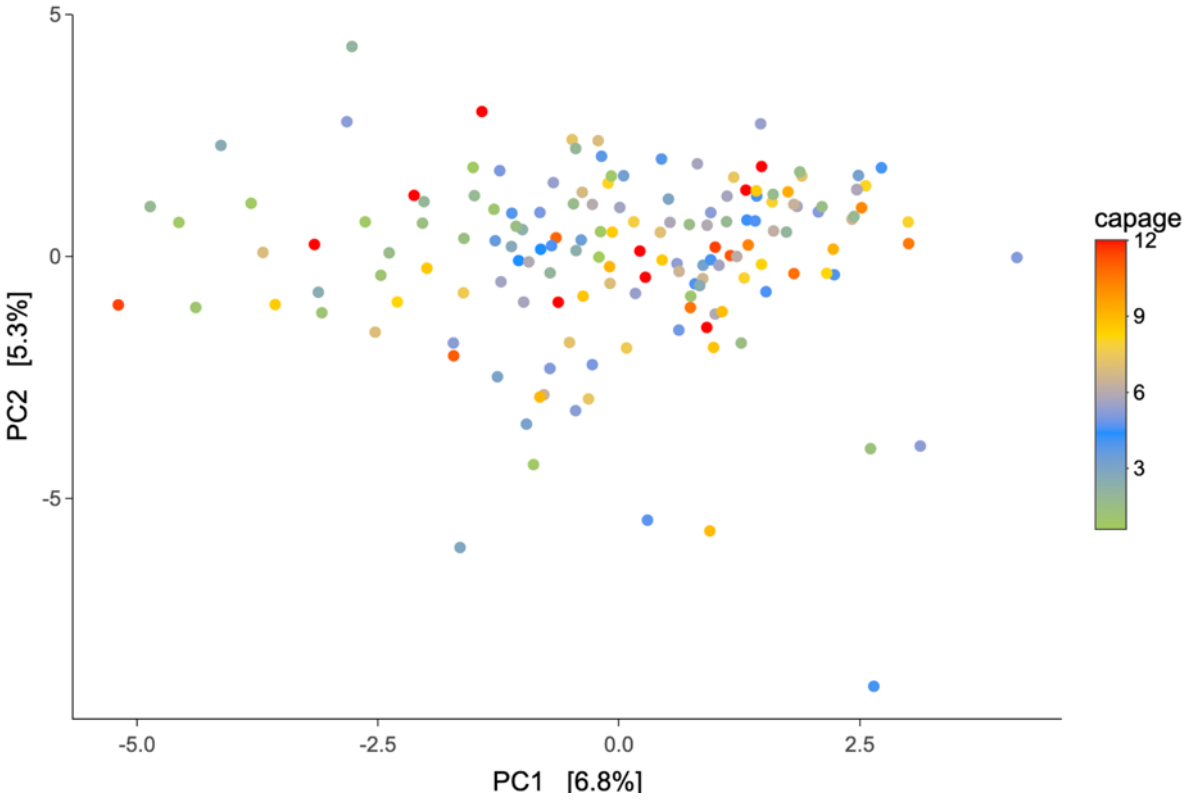

Figure S2.5. PCA plot of CLR-transformed reads in Euclidean distance, coloured by age.

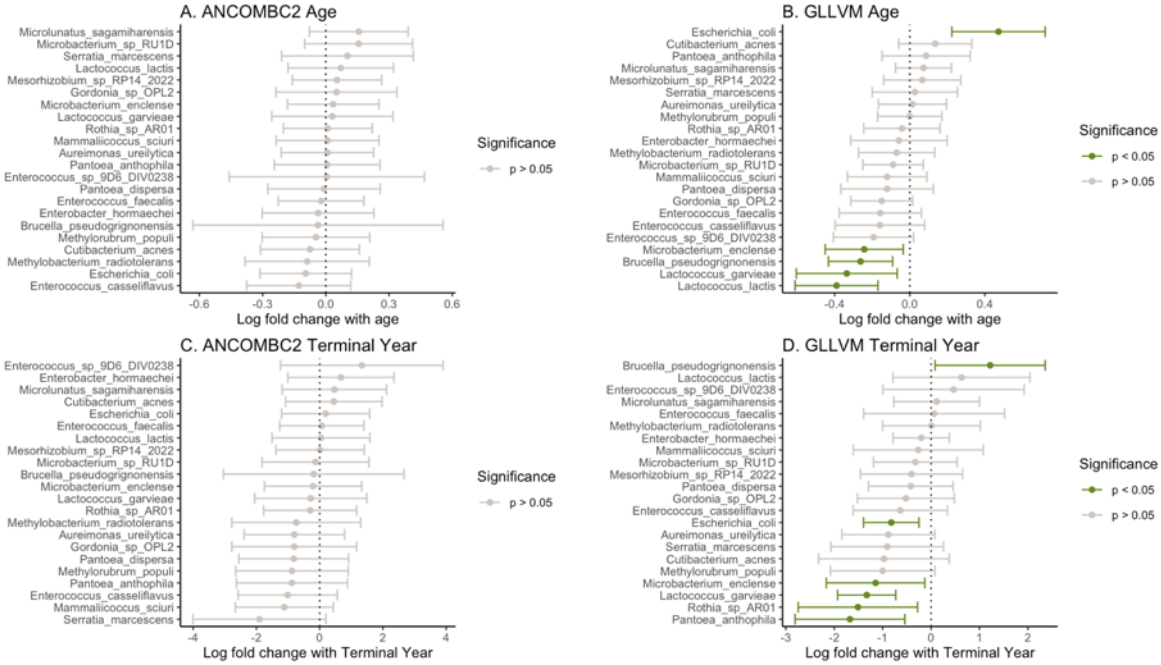

Figure S2.6. Taxonomic differential abundance analysis for common species (> 20% prevalence in the population). (A) ANCOMBC2 with age, (B) GLLVM with age, (C) ANCOMBC2 with terminal year, (D) GLLVM with terminal year. Significant ($p < 0.05$). Green = significant ($p < 0.05$) log fold change, grey = insignificant log fold change.

Table S2.6. A linear mixed effect model testing for age-related changes in functional scaled exponentially transformed observed richness and exponentially transformed Shannon diversity of eggNOG annotations in the gut microbiome of Seychelles warblers (n = 152 samples, 90 individuals). Conditional R^2 = 33.7% and 9.2% respectively.

Observed Richness					
Predictor	Estimate	SE	*df*	*t*	*P*
(Intercept)	-109.417	42.293	142.715	-2.587	0.011
Age (years)	**-0.036**	**0.013**	**92.620**	**-2.877**	**0.005**
Terminal Year (yes)	-0.124	0.077	142.784	-1.605	0.111
Season (winter)	-0.080	0.078	141.089	-1.024	0.307
Sex (female)	-0.080	0.080	78.890	-1.008	0.317
Days at 4℃	**-0.198**	**0.082**	**130.818**	**-2.422**	**0.017**
Time of day	-0.027	0.071	142.930	-0.373	0.710
Territory quality	-0.074	0.072	134.361	-1.030	0.305
Sample Year	**0.055**	**0.021**	**142.686**	**2.618**	**0.010**
Random					
Individual ID	152 observations	90 individuals	Variance		0.047

Shannon Diversity					
Predictor	Estimate	SE	*df*	*t*	*P*
(Intercept)	-92473.06	46119.45	143.00	-2.01	0.047
Age (years)	**-31.31**	**12.59**	**143.00**	**-2.49**	**0.014**
Terminal Year (yes)	-20.41	83.74	143.00	-0.24	0.808
Season (winter)	105.32	85.76	143.00	1.23	0.221
Sex (female)	-21.32	78.14	143.00	-0.27	0.785
Time at 4℃	-36.85	92.11	143.00	-0.40	0.690
Time of day	27.32	76.97	143.00	0.36	0.723
Territory quality	-1.21	79.70	143.00	-0.02	0.988
Sample Year	46.31	22.85	143.00	2.03	0.045
Random					
Individual ID	152 observations	90 individuals	Variance		108.9

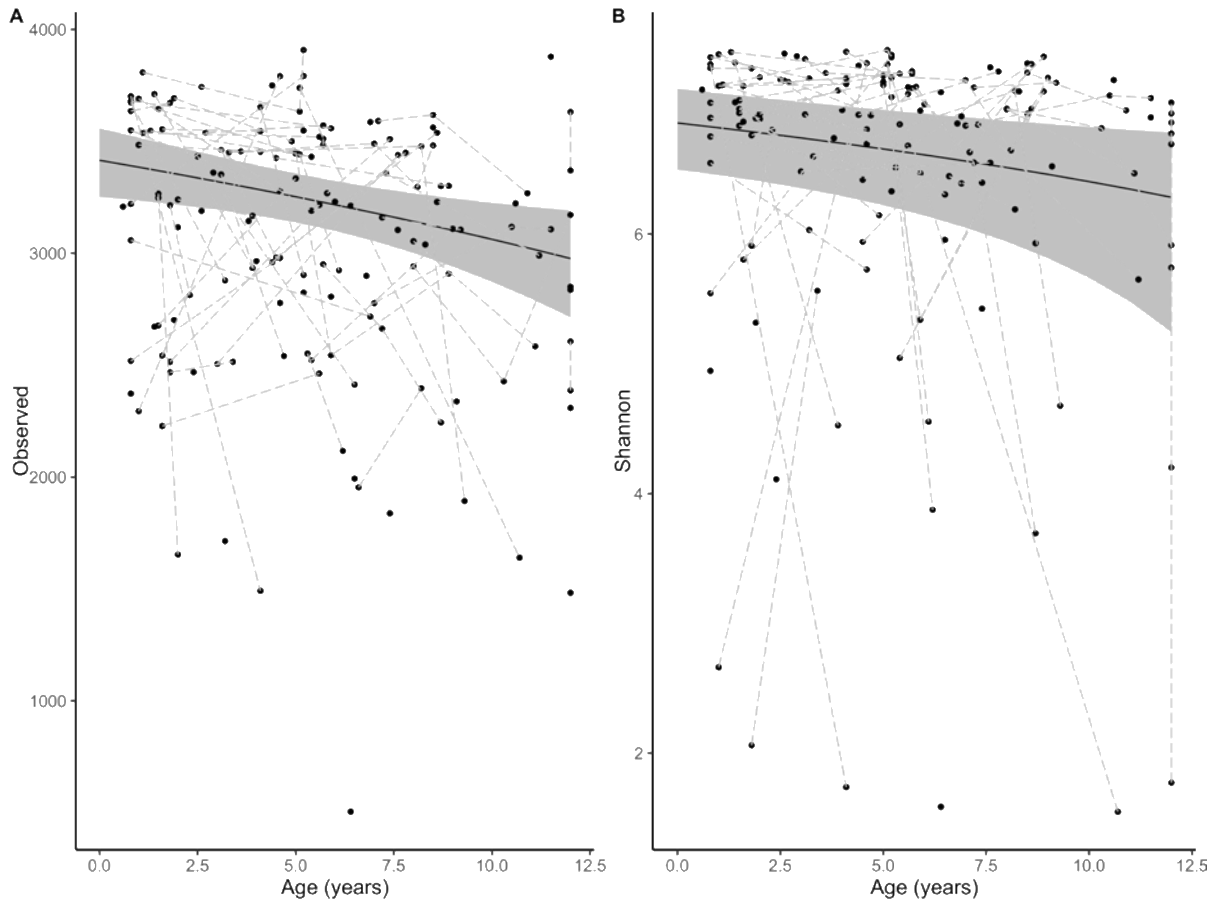

Figure S2.7. Evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG) (A) observed richness and (B) Shannon diversity against host age (years) model prediction from linear mixed effect model in the gut microbiome of Seychelles warblers (Table S2.4, p = 0.005 in A and p = 0.014 in B). The solid line represents model predictions and ribbon-shadding represent confidence intervals from model predictions. Each point represents a sample, and the dashed grey lines connect samples collected from the same individual (n = 152 samples from 90 individuals).

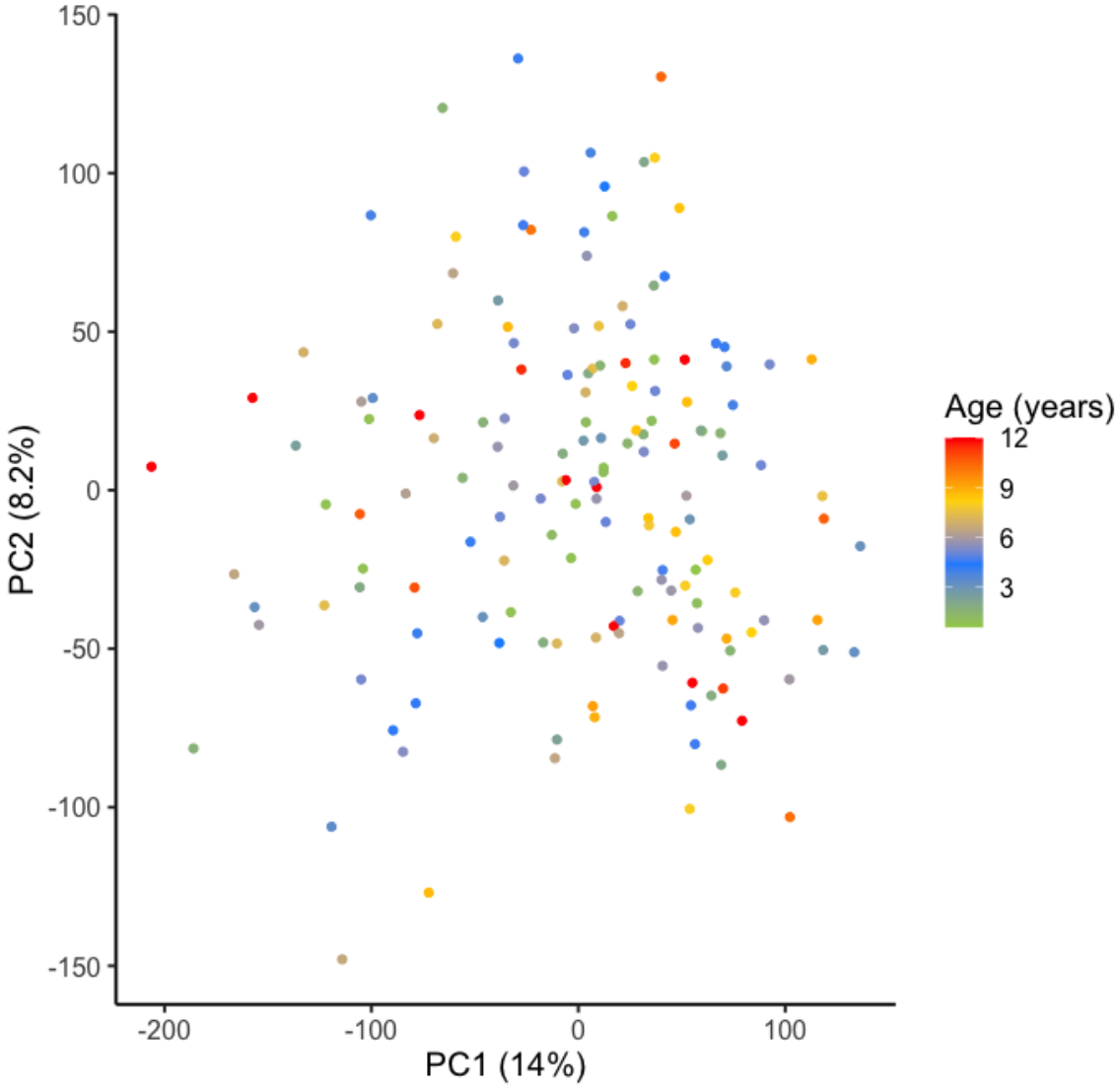

Figure S2.8. Functional PCA plot of CLR-count, euclidean distances of eggNOG annotations

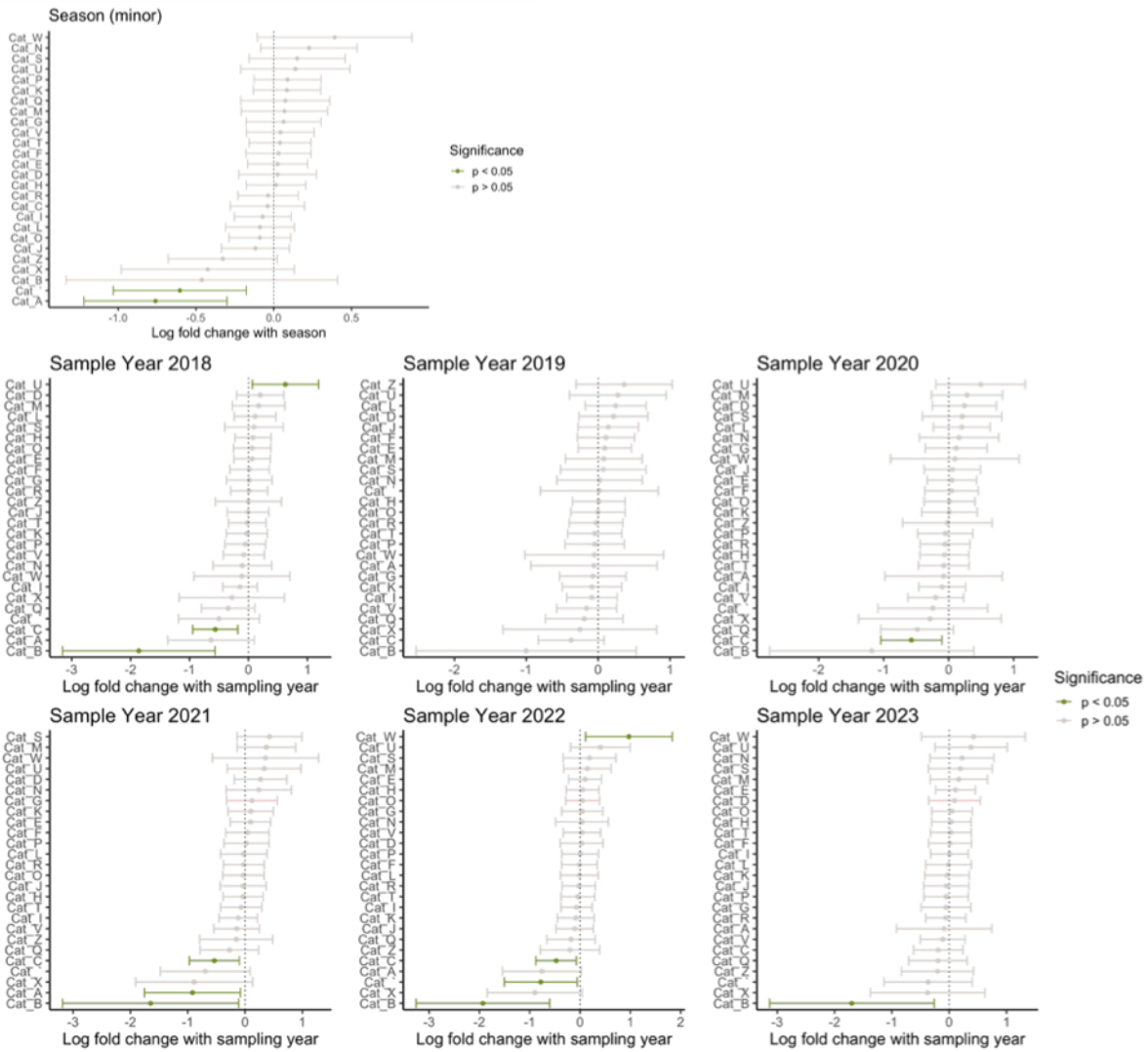

Figure S2.9. Differential abundance analysis of functional gut microbiome cluster of orthologous genes (COG) categories in Seychelles warblers using ANCOMBC2 with season and sample year. Each COG category is represented by a letter on the y-axis. Details of all COG categories are given in Table S2.5 [71]. “Cat_`” represents eggNOG annotations that were not assigned a COG category. Points and error bars are coloured according to significance (green: $p < 0.05$; grey: $p > 0.05$).

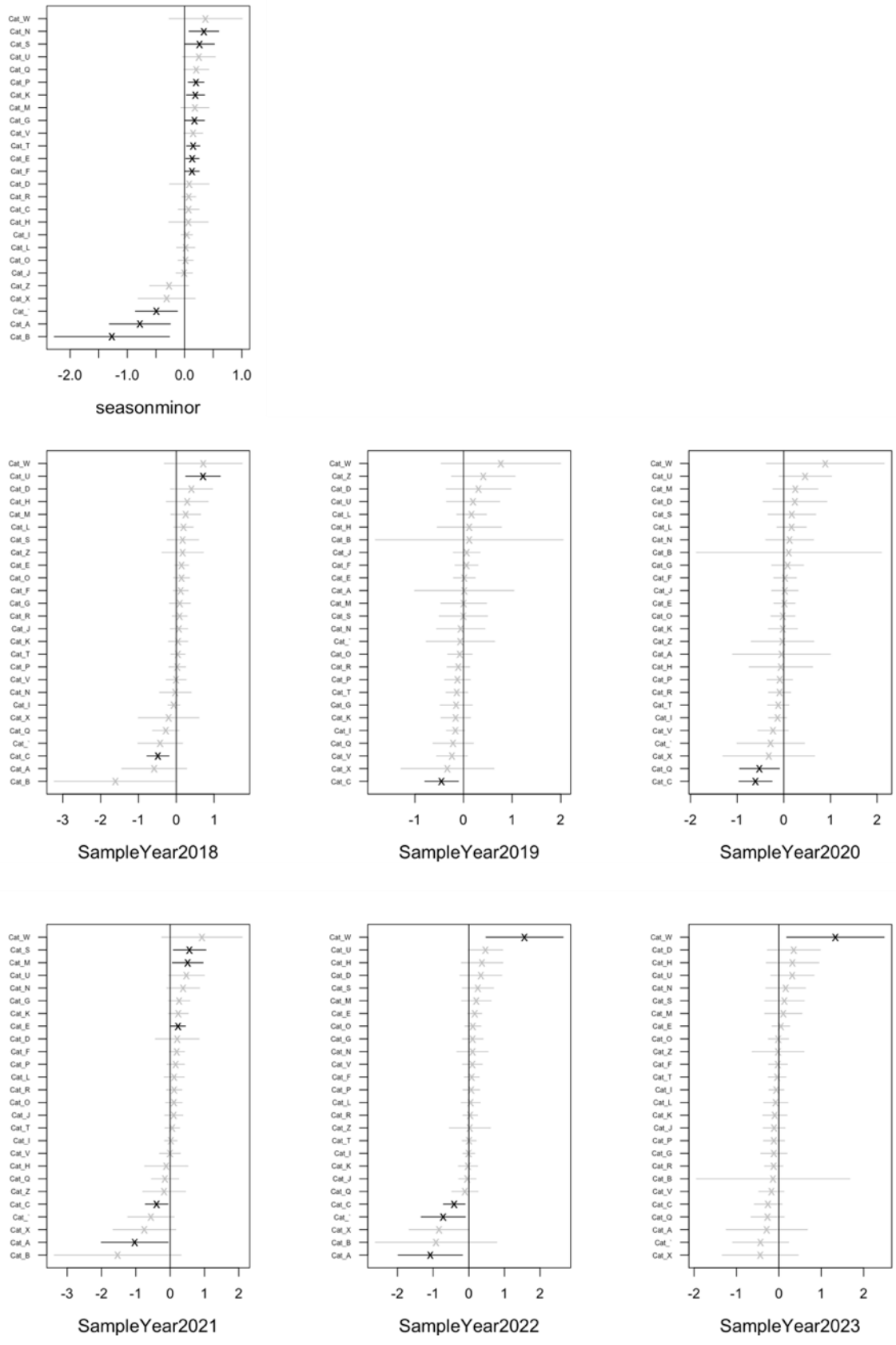

Figure S2.10. Differential abundance analysis of functional gut microbiome cluster of orthologous genes (COG) categories in Seychelles warblers using GLLVM with season and sample year. Each COG category is represented by a letter on the y-axis. Details of all COG categories are given in Table S2.5 [71]. “Cat_`” represents eggNOG annotations that were not assigned a COG category. Points and error bars are coloured according to significance (black: $p < 0.05$; grey: $p > 0.05$).

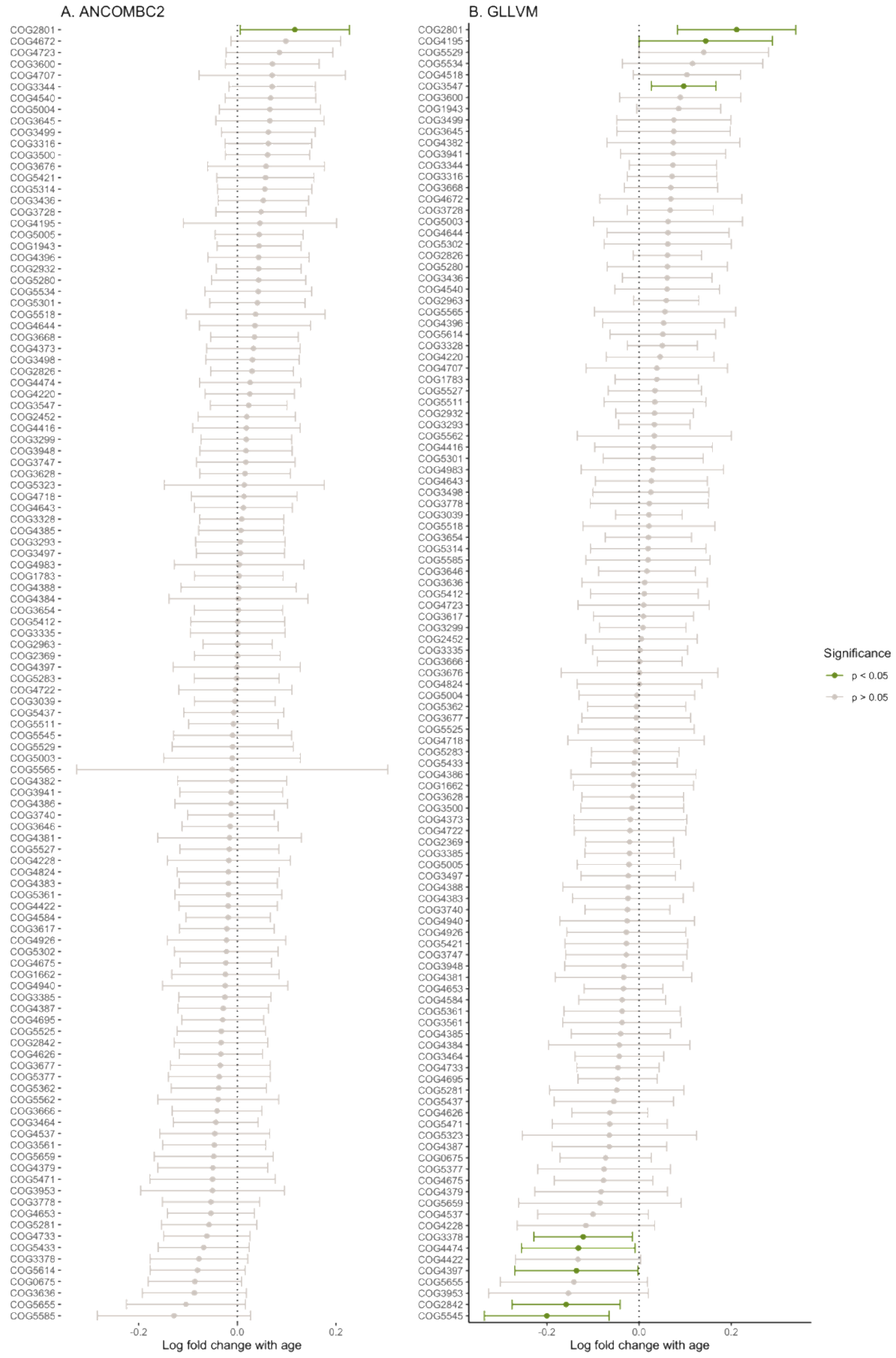

Figure S2.11. Differential abundance of COG X eggNOG members (A) ANCOMBC2 and (B) GLLVM.

Table S2.7. A linear mixed effect model of COG2801 abundance in the gut microbiome of Seychelles warblers in relation to within- (delta) and between-individual (mean) age. n = 153 samples, 91 individuals. Significant ($p < 0.05$) predictors in bold. Conditional R^2 = 14.7%. Reference categories for categorical variables are shown in brackets

Predictor	Estimate	SE	df	t	*P*
(Intercept)	9.700	0.971	115.370	9.989	**< 0.001**
Delta Age	**0.549**	**0.218**	**141.991**	**2.516**	**0.013**
Mean Age	**0.157**	**0.062**	**85.606**	**2.534**	**0.013**
Terminal Year Bird (yes)	0.028	0.420	69.803	0.067	0.947
Season (winter)	-0.502	0.553	132.368	-0.908	0.365
Sex (female)	0.219	0.422	63.434	0.520	0.605
Days at 4℃	-0.196	0.495	136.509	-0.396	0.693
Time of day	-0.313	0.428	136.421	-0.730	0.466
Territory quality	-0.315	0.452	141.901	-0.697	0.487
Sample Year (2017)					
2018	-1.662	0.902	140.921	-1.844	0.067
2019	-1.457	1.068	141.645	-1.363	0.175
2020	-2.200	1.129	134.384	-1.949	0.053
2021	**-2.911**	**1.119**	**140.585**	**-2.601**	**0.010**
2022	**-3.341**	**1.098**	**118.243**	**-3.042**	**0.003**

2023	**-3.215**	**1.289**	**111.442**	**-2.495**	**0.014**
Random					
Individual ID	153 observations	91 individuals		Variance	0.1776

Table S2.8. BLASTp top hits for each COG2801 found in the genomes of all constructed metagenomics species (MGS) from the gut microbiome of Seychelles warblers (n = 153 from 91 individuals).

Top hit (contains keyword)	Count	Percentage
IS3 transposase	154	30%
otherIS transposase	64	13%
transposase	170	33%
integrase	30	6%
Mobile element protein	4	1%
Helix-turn-helix	19	4%
Hypothetical protein	45	9%
Unknown	23	5%

Table S2.9. Linear mixed model on the CLR-transformed abundance of metagenomic species in the gut microbiome of Seychelles warblers (n = 2589 from 89 individuals). To test if COG2801-carrying MGS significantly differed in abundance with host age. Significant ($p < 0.05$) predictors are shown in bold. Conditional R^2 = 46.9%.

Predictor	Estimate	SE	*df*	t	*P*
(Intercept)	5.44	0.41	233.52	13.34	< 0.001
Age	0.03	0.04	69.32	0.79	0.432
Terminal Year (yes)	0.24	0.19	339.71	1.26	0.210
Season (winter)	-0.09	0.22	394.36	-0.43	0.671
Sex (female)	0.01	0.26	69.10	0.02	0.982
Time at 4℃	**-0.44**	**0.18**	**434.74**	**-2.40**	**0.017**
Time of day	**-0.35**	**0.18**	**395.12**	**-2.01**	**0.045**
Territory quality	**-0.47**	**0.17**	**379.46**	**-2.85**	**0.005**

Sample Year (2017)					
2018	**-0.77**	**0.41**	**402.12**	**-1.90**	**0.059**
2019	**-1.69**	**0.46**	**416.15**	**-3.71**	**0.000**
2020	**-1.19**	**0.48**	**360.43**	**-2.50**	**0.013**
2021	-0.70	0.46	334.48	-1.53	0.127
2022	-0.56	0.45	266.74	-1.25	0.213
2023	-0.65	0.49	239.09	-1.33	0.186
Random					
Individual ID	874 observations	85 individuals		Variance	1.042

Table S2.10. Linear mixed model on the CLR-transformed abundance of metaphlan4 genera in the gut microbiome of Seychelles warblers (n = 4477 from 91 individuals). To test if known COG2801-carrying genera significantly differed in abundance with host age. Significant ($p < 0.05$) predictors are shown in bold. Conditional R^2 = 16.8%.

Predictor	Estimate	SE	df	t	*P*
(Intercept)	9.08	0.45	316.13	20.37	< 0.001
Age	0.04	0.04	77.18	0.91	0.363
Terminal Year (yes)	0.30	0.22	272.48	1.37	0.173
Season (winter)	-0.30	0.27	271.10	-1.09	0.276
Sex (female)	0.15	0.27	70.01	0.54	0.589
Time at 4℃	**-0.52**	**0.22**	**373.62**	**-2.34**	**0.020**
Time of day	**-0.60**	**0.21**	**224.79**	**-2.82**	**0.005**
Territory quality	0.03	0.21	486.10	0.13	0.898
Sample Year (2017)					
2018	-0.15	0.47	519.08	-0.33	0.745
2019	-0.85	0.54	423.70	-1.57	0.116
2020	-0.80	0.55	380.92	-1.46	0.145
2021	**-1.13**	**0.52**	**377.58**	**-2.20**	**0.029**
2022	-0.56	0.49	363.36	-1.14	0.254
2023	-0.06	0.55	281.62	-0.11	0.916
Random					
Individual ID	1794 observations	89 individuals		Variance	0.995

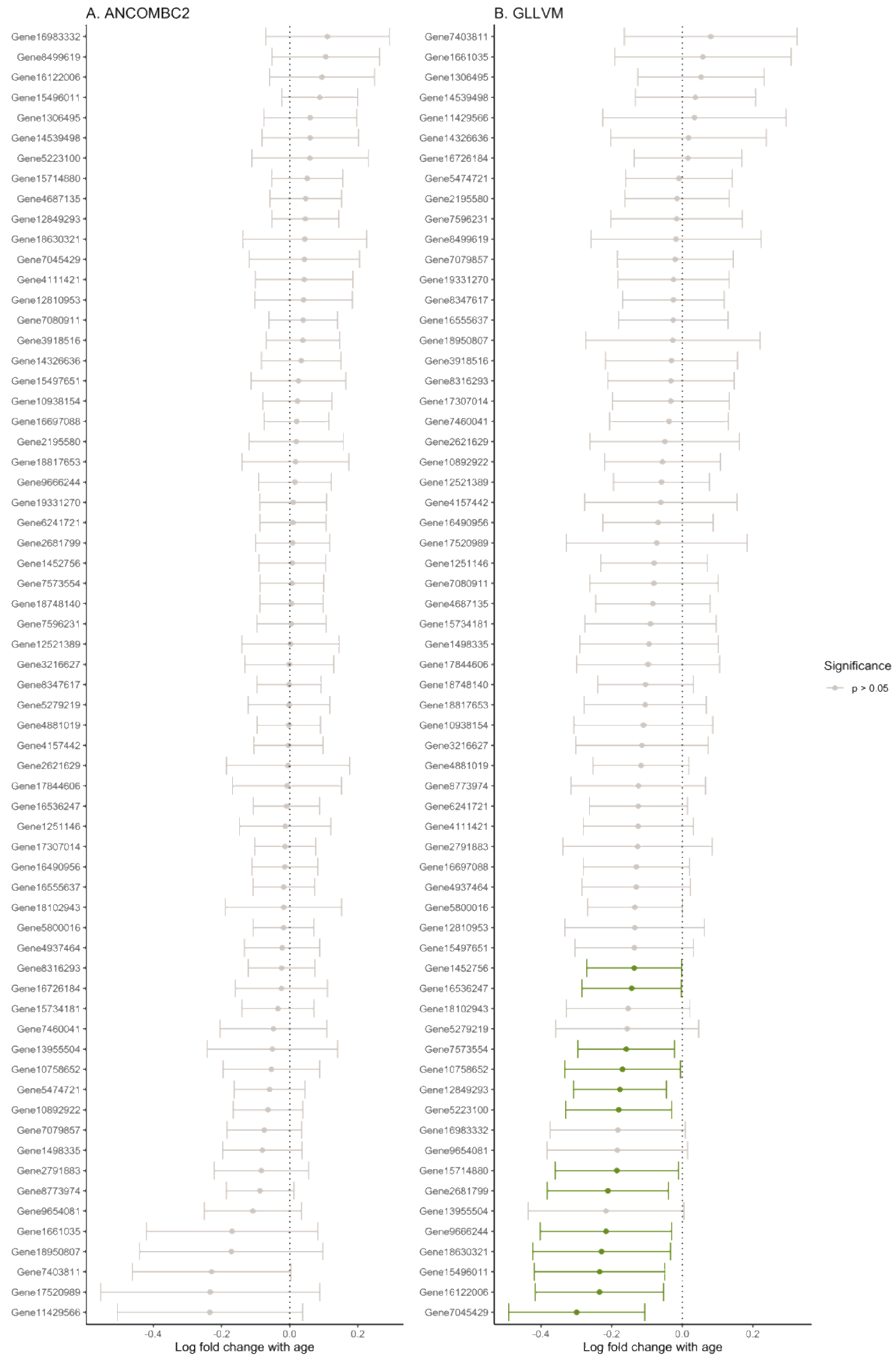

Figure S2.12. Differential abundance analysis of functional gut microbiome COG2801 gene catalogue that were commonly (20% prevalence) found in Seychelles warblers using (A) ANCOMBC2 and (B) GLLVM. Each gene catalogue (95% average nucleotide identity) are represented on the y-axis by their gene

catalogue number. Points and error bars are coloured according to significance (black: $p < 0.05$; grey: $p > 0.05$).

Chapter 3 |

Host immunogenetic variation and gut microbiome functionality in a wild vertebrate population

Promoting diversity and safeguarding communities for enhanced interconnectedness.

3.1 Abstract

Background: The gut microbiome (GM) –important for host health and survival– is partially shaped by host immunogenetics. However, to date, no study has investigated the influence of host Major Histocompatibility Complex (MHC) genes on gut microbiome functionality in a wild population. Here we use a natural population of the Seychelles warbler (*Acrocephalus sechellensis*) to assess the effects of MHC genes on GM taxonomy and functionality using shotgun metagenomics.

Results: Our results show that taxonomic GM composition was associated with MHC-II diversity and the presence of one specific MHC-I allele (*Ase-ua 7*). Specifically, MHC-II diversity was associated with decreased *Lactococcus lactis* and increased *Staphylococcus lloydii* abundance, while *Ase-ua 7* was linked to reduced *Enterococcus casselifavus* and *Gordonia sp* OPL2 but increased *Escherichia coli* and *Vulcaniibacterium thermophilum*. These taxonomic changes may reflect differences in MHC-mediated microbial recognition. In contrast, functional GM composition was significantly associated with increasing individual MHC-I diversity but not MHC-II diversity. Potentially importantly, MHC-I diversity was associated with an increased prevalence of microbial defence genes but a reduced prevalence of microbial metabolism genes. Analysis also revealed that taxonomic and functional GM networks were more fragmented but had stronger connections in high compared to low MHC-I diversity hosts, suggesting higher GM resilience in high MHC-I diversity individuals.

Conclusion: These results suggest that MHC-I variation (surprisingly more than MHC-II variation) is important in shaping the GM in this wild vertebrate population. MHC-I diversity induces microbial defence and metabolism trade-offs and increases GM resilience, which may, in turn, result in individual variation in health and survival in the Seychelles warbler. Consequently, this study highlights the importance of host immunogenetics in shaping the gut microbiome, both taxonomically and functionally.

Keywords: Acrocephalus sechellensis; Metagenomics; Gut microbiome; Major Histocompatibility Complex; wild population.

3.2 Introduction

The vertebrate gut microbiome (GM), a complex ecosystem of microorganisms inhabiting the gastrointestinal tract, is increasingly recognised as a critical determinant of host health and fitness (Zhu et al., 2021). However, the composition and function of the GM exhibit extensive variability across individuals, particularly in natural populations (Fenn et al., 2023; Ren et al., 2016; Worsley et al., 2021). This variation has been attributed to a range of factors, such as diet, age, sex, location and host genetic variation (Davies et al., 2022; Worsley et al., 2025; Zoelzer et al., 2021).

A growing body of evidence links GM characteristics to host immunogenetic variation (Dzierozynski et al., 2023; Zheng et al., 2020). Immune genes influence the immune system's ability to recognise, tolerate, or eliminate microbial populations (Criscitiello & de Figueiredo, 2013; McConnell et al., 2023). Therefore, the immune system must maintain a balance – tolerating beneficial microbes while combating pathogens- to optimise host health (Fuess et al., 2021; Tanoue et al., 2010). Furthermore, the GM also appears to play a role in the immune defences of the host, with GM dysbiosis (an imbalance in the composition of microbes) resulting in a reduction of host immune function, emphasising the interconnected nature of immune health and GM stability (Kuhn & Stappenbeck, 2013; Reikvam et al., 2011).

The major histocompatibility complex (MHC) is a family of immune genes, forming part of the vertebrate acquired immune system (Piertney & Oliver, 2006). These genes encode cell-surface glycoprotein receptor molecules that bind to antigens before presenting them to T lymphocytes and B cell receptors, which trigger an immune or tolerogenic response (Blum et al., 2013; Roland et al., 2020). The MHC has two main classes, MHC-I and MHC-II, based on the encoded receptors presenting intracellular or extracellular antigens, respectively (Rock et al., 2016; Roland et al., 2020). The role of the MHC in combating pathogens has been well-studied (Janeway et al., 2001; Özer & Lenz, 2021), with the extraordinarily high polymorphism of MHC genes observed in natural populations thought to be driven by pathogen-mediated selection mechanisms and sexual selection (Spurgin & Richardson, 2010). Individual MHC variation determines the range of microbial

antigens recognised by the immune system (Bolnick et al., 2014), and is associated with variation in commensal gut microbial communities (Davies et al., 2022; Kubinak et al., 2015; Silverman et al., 2017; Toivanen et al., 2001). Thus, different MHC genotypes could shape individual GM variation by initiating immune responses to potentially pathogenic microbes while maintaining beneficial species (Russell et al., 2019; Silverman et al., 2017).

Previous studies examining the impact of MHC variation on the GM in wild animals using 16s metabacoding, have reported mixed findings. Several have found that increased MHC diversity is associated with decreased microbiome diversity (Bolnick et al., 2014; Leclaire et al., 2019; Uren Webster et al., 2018) but others associated it with increased (Hernández-Gómez et al., 2018) or unchanged GM diversity (Davies et al., 2022; Montero et al., 2021). Similarly, some studies have observed shifts in taxonomic composition with MHC diversity (Bolnick et al., 2014; Hernández-Gómez et al., 2018; Montero et al., 2021), while others have not (Davies et al., 2022; Fleischer et al., 2020, 2022; Uren Webster et al., 2018). Additionally, the presence/absence of specific MHC alleles (rather than the overall diversity of alleles) has been found to be correlated with GM taxonomic composition (Bolnick et al., 2014; Davies et al., 2022).

The functional composition of the GM – represented through microbial genes – could provide a more direct representation of host-microbe interactions (Worsley, Mazel, et al., 2024). However, the consequences of MHC variation for GM functionality have remained underexplored so far (Fuess et al., 2021). Many microbes share genes and, consequently, have similar functional roles (Louca et al., 2018). Therefore, changes in microbial taxa do not always result in altered GM function – i.e. there is functional redundancy (Louca et al., 2018; Worsley, Mazel, et al., 2024). Functional redundancy refers to the ecological concept that multiple species within an ecosystem can perform similar roles, encoding the same gene and/or different genes with the same function, buffering against species loss (Louca et al., 2018; Worsley, Mazel, et al., 2024). Studying functionality is important for understanding if and how host genetic variation interacts with the GM to influence host fitness and evolutionary trajectories (Worsley, Mazel, et al., 2024). Most studies on MHC and microbial functionality rely on 16S metabarcoding markers and infer function based on known microbial taxa-function association (Gill et al., 2018, 2019;

Sun et al., 2020; Wadud Khan et al., 2019). However, in less studied systems, such as wild animals, functional inferences from 16S metabarcoding markers may lead to misassignments due to the lack of representation of the specific microbes observed in existing databases (Sun et al., 2020; Toole et al., 2021).

In humans and transgenic captive mice (*Mus musculus*), MHC haplotype is associated with GM functional composition (Berryman et al., 2024; Bonder et al., 2016). However, captive/domesticated populations often harbour greatly reduced genetic variation and microbial diversity compared to natural populations (Williams et al., 2024); thus, these results may not be transferable to wild systems. To our knowledge, the only pioneering study of host MHC and GM function in a wild animal so far used 16S functional inferences (Montero et al., 2021), which are likely to lead to inaccuracies (Sun et al., 2020; Toole et al., 2021). Shotgun metagenomics or transcriptomic approaches are needed to accurately determine gut microbiome function in response to host MHC variation in wild animal populations.

Here, we investigate the relationship between MHC and GM variation in a population of Seychelles warblers (*Acrocephalus sechellensis*). Despite reduced neutral genetic variation due to past population bottlenecks (Spurgin et al., 2014), the Seychelles warbler has maintained variation (albeit reduced) at MHC-I and MHC-II loci (Davies et al., 2022; Hansson & Richardson, 2005; Richardson & Westerdahl, 2003). Furthermore, one specific MHC allele (*Ase-ua 4*) and MHC-I diversity overall have been positively correlated with survival and reproductive success (Brouwer et al., 2010; Richardson et al., 2005). A previous 16S-based analysis of this population has demonstrated that MHC alleles are associated with changes in bacterial GM taxonomic diversity and composition (Davies et al., 2022). An analysis of the fungal mycobiome also reported changes in species diversity and composition associated with MHC alleles and MHC-I diversity, respectively (Worsley et al., 2022). Since these studies, we have greatly expanded our sample size, identified key environmental control variables, and conducted shotgun sequencing for metagenomic analysis (Lee et al., 2024).

We leverage a powerful combination of both 16S rRNA metabarcoding (larger sample size) and shotgun metagenomics to provide a comprehensive and high-

resolution assessment of the association between host MHC variation and both taxonomic and, importantly, functional components of the bacterial GM in adult Seychelles warblers. First, we test if GM taxonomic diversity and composition correlate with MHC-I and MHC-II diversity or alleles. Next, we test if GM functional diversity and functional composition are associated with this MHC variation. Finally, we assess the role of functional redundancy in preserving the functionality of the GM despite changes in host MHC variation.

3.3 Methods

3.3.1 Study system

The population of insectivorous Seychelles warblers on Cousin Island (29 ha; 04° 20′ S, 55° 40′ E) has been extensively monitored since 1985 (Brown et al., 2022; Komdeur, 1992) Two field seasons are undertaken annually from ca. January to March (minor) and June to September (major). Each season, as many individuals as possible are caught in the nest (chicks) or using mist nets and sampled (see below). New individuals are marked with a British Trust for Ornithology (BTO) metal ring and a unique combination of three colour rings, allowing them to be monitored throughout their lives. Almost every bird (>96%) on Cousin has been marked this way since 1997 (Raj Pant et al., 2019; Richardson et al., 2007). Age is calculated based on fledge or hatch dates, or eye colour at first catch (Komdeur, 1991). This population includes ca. 320 individuals in approximately 115 territories (Hammers et al., 2019; Komdeur & Pels, 2005).

3.3.2 Sample collection

Faecal sample collection, storage, DNA extraction, library preparation and sequencing were conducted between 2017 and 2023, as part of (and described in full in) previous studies using 16S rRNA metabarcoding (Davies et al., 2022; Worsley, Davies, et al., 2024) and shotgun metagenomics (Lee et al., 2024). In brief, caught birds were placed in a flat-bottom paper bag with a sterilised weigh boat under a metal grate, allowing faeces to drop to the weigh boat, while minimising contact with the birds' surface. Faecal matter was transferred into a sterile microcentrifuge tube containing 1 mL of absolute ethanol, stored at 4°C during fieldwork and then at -80°C for long-term storage at the University of East Anglia (UEA). The time-of-day (minutes after sunrise; 06:00 AM) of sampling was recorded. Each season, control samples were also taken from the hands of fieldworkers using a sterile swab and stored in the same manner. A small (ca 25 µL) blood sample was also collected from each bird via brachial venepuncture and stored in 0.7 ml of absolute ethanol at 4°C. Samples may be collected from the same individual in different field seasons; thus, the identify of each individual sampled (Bird ID) is recorded and used to control for these repeated measures in statistical analyses.

3.3.3 Molecular genotyping

Total genomic DNA was extracted from blood samples using the DNeasy Blood and Tissue kit (Qiagen, Crawley, UK) according to the manufacturer's protocol. All Individuals were genotyped using up to 30 polymorphic microsatellite loci and 3 sexing markers following (Hadfield et al., 2006; Richardson et al., 2001; Sparks et al., 2022) as part of the ongoing determination of parentage and pedigree within this population (Raj Pant et al., 2022). Individual genome-wide heterozygosity (Hs) at these neutral loci was calculated using *genhet* 3.1 in R 4.33 (COULON, 2010; R Core Team, 2024) as per (Wright et al., 2016).

Sequencing of amplified MHC-I exon 3 and MHC-II exon 2 variants using Illumina MiSeq technology had already been undertaken for 314 warblers (Davies et al., 2022). All confirmed variants (20 MHC-I and 14 MHC-II; hereafter termed alleles) at each of these MHC regions (which contain 4 replicated loci) (Hutchings, 2009; Richardson & Westerdahl, 2003) were used to calculate individual MHC diversity. However, due to statistical power limitations, only alleles present in >5% and <95% of individuals were included in the presence/absence analysis. Two MHC-I alleles (*Ase-ua1* and *Ase-ua10*) were co-occurring; thus, only one of them, Ase-ua1, was retained for downstream analyses. Therefore, nine MHC-I alleles and three MHC-II alleles were used in the presence/absence statistical analyses. Each allele in the presence/absence analysis each translates to unique amino acid sequences with different antigen-binding properties (Davies et al., 2022).

3.3.4 Gut microbiome screening

Microbial DNA from faecal samples was extracted using the DNeasy PowerSoil Kit (Qiagen, Crawley, UK) and a modified version of the manufacturer's protocol (described in detail (Davies et al., 2022)). Samples were randomised across extractions to minimise batch effects.

Faecal DNA samples were submitted for 16S rRNA amplicon sequencing. Amplicon sequencing libraries were generated using the V4 primers 515F (5'TGCCAGCMGCCGCGGTAA3') and 806R (5'GGACTACHVGGGTWTCTAAT3'). Libraries were sequenced across seven batches using 2 x 250bp, paired-end

sequencing on an Illumina MiSeq Platform (see (Davies et al., 2022; Worsley, Davies, et al., 2024)). Control samples were also extracted, library prepped and sequenced the same way (n = 21 hand controls, 15 negative controls, and 10 positive ZymoBIOMICS Microbial Community Standard (D6300) controls).

Faecal DNA samples underwent library preparation using the LITE protocol (Perez-Sepulveda et al., 2021) and were sequenced using 2 x 150 bp, paired-end shotgun metagenomic sequencing in two runs on an Illumina NovaSeq X platform (see (Lee et al., 2024)). Hand controls (n =6) and positive controls (n =3, two ZymoBIOMICS Microbial Community Standard (D6300), and one ZymoBIOMICS Fecal Reference with TruMatrix™ Technology (D6323)) were also prepped and sequenced as part of the metagenomic samples sequencing.

3.3.5 Bioinformatics

Read processing for 16S metabarcoding was performed as previously described (Worsley, Davies, et al., 2024). Briefly, 16S rRNA reads were processed using QIIME2 2019.10; reads were truncated, filtered, and classified into amplicon sequencing variants (ASV) using DADA2 (Callahan et al., 2016). ASVs were then taxonomically assigned using the naïve-Bayes classifier on the SILVA 132 reference database for 16S rRNA gene sequences (Bolyen et al., 2019). These ASVs were then imported into R 4.3.3 using *phyloseq* 1.46.0 (Callahan et al., 2016; McMurdie & Holmes, 2013), then filtered to remove non-bacterial sequences, reads unassigned to phylum level, and potential contaminants (based on hand controls). Rarefaction curves were constructed with *iNEXT* version 3.0.1 with the default 50 bootstrap replications (Chao et al., 2014), reaching an asymptote at 8000 reads, indicating sample completeness (Figure S3.1A). In addition, 27 faecal samples with <8000 reads were removed and ASVs with <50 reads across all samples were also removed.

Shotgun metagenomic sequence processing was performed using *MATAFILER* (Hildebrand et al., 2021) as previously described (Lee et al., 2024). Host reads were removed by mapping reads with *Kraken2* 2.1.3 to the Seychelles warbler genome (unpublished; complete BUSCO = 96.0% with a total length = 1,081,018,985 bp), followed by read quality filtering using *sdm* 2.14 beta; minimum sequence length of

50, minimum average quality of 27 (Hildebrand et al., 2014; Wood et al., 2019), an average of 21% (±0.07SE) reads were removed. After trimming, two samples and five hand controls were removed because they did not have enough reads for metagenome assembly. An average of 20,481,040 (SD = 13,718,305) paired-end reads per sample were retained for de novo metagenome assembly using *MEGAHIT* 1.2.9 with default parameters and kmer-list of 25,43,67,87,111,131 (Li et al., 2015). Using the resulting assemblies, genes were predicted using Prodigal 2.6.3 (Hyatt et al., 2010) and clustered into gene catalogues (95% identity). Genes were functionally annotated using *eggNOGmapper* 2.1.12 with default parameters and the eggNOG database 4 (Cantalapiedra et al., 2021; Powell et al., 2014). Functional categories were also assigned to each functional annotation based on the cluster of gene orthologs (COG) database (Tatusov et al., 2000). Metaphlan4 assignments were used to taxonomically assign shotgun sequencing reads. Rarefaction curves were constructed for metagenomics taxonomy and functional reads with *iNEXT* version 3.0.1 with the default 50 bootstrap replications (Chao et al., 2014) and showed an asymptote and sample completeness at 5,500 and 100,000 reads, respectively (Figure S3.1B-C).

3.3.6 Statistical analysis

Adult warblers with both microbiome and MHC data were analysed. For 16S analysis, 253 samples from 149 individuals were included in this study. Of these, 99 samples from 57 adult individuals also had GM shotgun metagenomic data. Individuals carried a mean of 5.13 (SE: 0.088, range 2-7) MHC-I alleles and 2.88 (SE: 0.060, range 1-5) MHC-II alleles. Due to the low number of samples for which we had shotgun metagenomic data, we had to limit the number of predictor variables (i.e. <9) in each model to avoid overfitting and unreliable estimates. Thus, for MHC diversity models, all control variables (see below) were included, but we first used the 16S metabarcoding dataset to shortlist which genetic metrics (including specific MHC alleles) should be included in the shotgun metagenomic models. Unless stated otherwise, all statistical analyses were conducted in R 4.3.3 in R Studio 2024.12.0+467 (Posit team, 2024; R Core Team, 2024) and linear mixed effect (LMMs) and generalised linear mixed effect models (GLMMs) were constructed using *lme4* 1.1-35.5 (Bates et al., 2015).

3.3.6.1 GM diversity

3.3.6.1.1 16S rRNA metabarcoding diversity

Reads were rarefied to 8,000 reads with the *rarefy_even_depth* function in *vegan* 2.6.6 (Oksanen Jari et al., 2024) – the point at which the number of ASVs identified reached an asymptote in rarefaction curves (Figure S3.1A) - before calculation of alpha diversity metrics. Both ASV richness and Shannon diversity were calculated for each sample using *phyloseq* 1.46.0 (McMurdie & Holmes, 2013).

A GLMM with a negative binomial distribution was constructed with ASV richness as a response variable. An LMM with a Gaussian distribution was used to model Shannon diversity. Hereafter, all 16S models were tested with the same set of variables (described below) with either MHC alleles or MHC diversity as the response unless stated otherwise. MHC-I and MHC-II diversity (i.e. the number of alleles per individual) were included as predictors, along with genome-wide heterozygosity, age, season, sample year, sex, sample days at 4°C, and time of day sampled, as fixed-term control variables and bird ID as a random effect. Quadratic effects of MHC-I diversity and MHC-II diversity were included to test if an intermediate number of alleles influenced GM characteristics, but were dropped if not significant, least significant first, to allow interpretation of the main terms. Standardised effect sizes of each fixed effect were determined using partial R^2.

To determine if specific MHC alleles were shaping the GM, a second model was constructed using the presence/absence of MHC-I (*Ase-ua 1, Ase-ua 3, Ase-ua 4, Ase-ua 5, Ase-ua 6, Ase-ua 7, Ase-ua 8, Ase-ua 9, Ase-ua 11*) and MHC-II alleles (*Ase-dab 3*, *Ase-dab 4*, *Ase-dab 5*) as predictors in place of MHC diversity.

3.3.6.1.2 Metagenomic taxonomic diversity

Metaphlan4 assignments were rarefied to 5,500 reads (Figure S3.1B) – prior to alpha diversity analysis. A GLMM with a negative binomial distribution was then used to model species richness, and an LMM was used to model Shannon diversity. All metagenomics analyses were performed with the same structure (described below, i.e. MHC diversity models included all terms, while MHC presence/absence models only included genetic variables that were identified as significant in the corresponding 16S analysis).

A second model with the presence/absence of specific MHC alleles (identified as significant in the 16S metabarcoding model above) was constructed.

3.3.6.1.3 Metagenomic functional diversity

Functional gene annotations (determined using eggNOG mapper described above) were rarefied to 100,000 reads (Figure S3.1C) before functional alpha diversity analysis. Scaled exponentially transformed functional gene richness and exponentially transformed functional Shannon diversity were modelled separately with LMMs, with either the MHC diversity or the presence/absence of MHC alleles, along with genome-wide heterozygosity and environmental control variables (as described for 16S analyses above).

3.3.6.2 GM composition

3.3.6.2.1 16S rRNA metabarcoding composition

Unrarefied reads were used. Rare ASVs (<5% prevalence) were removed prior to analysis, and a centred log ratio (CLR) transformation was applied to the remaining ASV abundances using *microbiome* 1.24.0 (Leo Lahti & Sudarshan Shetty, 2019). Pairwise Aitchison distances (i.e. composition differences) among GM samples were then modelled via a PERMANOVA using the *adonis2*() function in *vegan* 2.6.6 with 9999 permutations. A blocking effect of bird ID was included to account for repeated sampling (Oksanen Jari et al., 2024). The first PERMANOVA model included MHC diversity, genome-wide heterozygosity, age, season, sample year, sex, days at 4°C and time of day as predictors. The second PERMANOVA model had the presence/absence of individual MHC alleles instead of MHC diversity. Both these and all subsequent GM composition models were set up in the same way and visualised with a PCA generated in *phyloseq* 1.46.0 (McMurdie & Holmes, 2013) unless stated otherwise.

Metagenomic taxonomic composition

Rare species were removed (<5% prevalence), the remaining unrarefied reads were CLR transformed and used in a PERMANOVA to identify differences in taxonomic composition associated with MHC variation (as described for 16S analysis above). For the MHC alleles model, only genetic predictors identified as significant in the

16S rRNA metabarcoding composition analysis were included, along with all control variables (as described for 16S analyses above).

3.3.6.2.3 Metagenomic functional composition

Rare functional genes (<5% prevalence) were removed and the remaining unrarefied reads were CLR transformed and used in a PERMANOVA to test for differences in functional composition linked to MHC variation (as described for metagenomic taxonomic composition analyses above).

3.3.6.3 Differential abundance analyses

3.3.6.3.1 Differential abundance of metagenomic taxonomic species

Differential abundance tests were carried out using *ALDEx2* 1.34.0 (Fernandes et al., 2013). Only common species (>10% prevalence and >0.001% abundance resulting in 49 metagenomic identified species) were included. Abundances were CLR transformed as part of the *ALDEx2* method (Fernandes et al., 2013). Genome-wide heterozygosity, MHC-I and MHC-II diversity as well as significant variables identified in the metagenomic taxonomic composition analysis were included as predictors.

3.3.6.3.2 Differential abundance of metagenomic functional genes

Abundances of common functional genes (>50% prevalence, >0.1% abundance resulting in 94 eggNOG members) were CLR-transformed using *ALDEx2* 1.34.0 (Fernandes et al., 2013) and included in this analysis. Predictors were included as above but based on significant variables in metagenomic functional composition analysis.

3.3.6.4 Network analysis

3.3.6.4.1 Network analysis of metagenomic taxonomic species

Networks of metagenomic taxonomic species were constructed with SParse InversE Covariance Estimation for Ecological Association Inference (SPIEC-EASI) version 1.0.7 (Kurtz et al., 2015). The samples were split into two categories based on average MHC diversity (see above): low (<6) and high (≥6) MHC-I diversity, or low (<3) and high (≥3) MHC-II diversity. The raw counts of common bacterial species

were used as inputs, with SPIEC-EASI applying a CLR transformation. Common species were used to capture relevant, stable GM species and minimise the influence of rare taxa (Fabbrini et al., 2023). The number of nodes (species), the number of edges (interaction between species), the average number of connections per node, modularity and negative-to-positive ratios were calculated. The networks were then plotted with the *ggnet2* function in ggnet 0.1.0 (Briatte, 2025). Nodes were coloured by Phylum, and size was based on mean abundance per species.

3.3.6.4.2 Network analysis of metagenomic functional genes

Networks were constructed exactly as described above but using Metagenomic functional genes (eggNOG genes) instead of metagenomic species.

3.4 Results

3.4.1 GM diversity

3.4.1.1 16S rRNA metabarcoding diversity

GM alpha diversity (Shannon diversity or richness) was not significantly associated with MHC-I or MHC-II diversity (Table 3.1A, Table S3.1A & S3.2A & S3.3A). The presence of the MHC-I allele *Ase-ua 11* - but no other MHC allele - was significantly positively associated with 16S richness (Table 3.1B, Table S3.1B & S3.2B & S3.3B). No alleles were associated with Shannon diversity.

Table 3.1. The relationship between gut microbiome alpha diversity (richness) and variation in host (A) Major histocompatibility complex (MHC) diversity and (B) the presence/absence of specific MHC alleles in adult Seychelles warblers. Generalised linear mixed models with a negative binomial distribution were used for 16S ASV diversity (N = 253 samples from 149 individuals) and metagenomics taxonomy diversity (N = 99 samples, 57 individuals), and linear mixed models were used for metagenomics functional diversity (N = 99 samples, 57 individuals). Reference categories for categorical variables were as follows: Female (sex), winter (season), 2017 (Sample year), and absent (in all MHC alleles). Significant (P < 0.05) variables are shown in bold. Shannon diversity results are similar and shown in Supplementary Table S3.1.

Predictors	16S ASV diversity				Metagenomics taxonomic diversity				Metagenomics functional diversity			
	Est	SE	z	P	Est	SE	z	P	Est	SE	t	P
A) MHC Diversity												
(Intercept)	**5.37**	**0.32**	**16.82**	**< 0.001**	**3.23**	**0.70**	**4.62**	**< 0.001**	**1.06**	**0.45**	**2.39**	**0.02**
Heterozygosity	-0.19	0.21	-0.93	0.35	0.43	0.43	1.02	0.31	-0.13	0.28	-0.46	0.65
MHC-I Diversity	0.04	0.03	1.26	0.21	-0.01	0.06	-0.18	0.85	0.07	0.04	1.60	0.12
MHC-II Diversity	-0.01	0.04	-0.23	0.82	-0.14	0.08	-1.83	0.07	-0.02	0.06	-0.26	0.79
Age	-0.02	0.02	-1.28	0.20	-0.04	0.03	-1.12	0.26	**-0.04**	**0.02**	**-2.07**	**0.04**
Season (summer)	0.04	0.12	0.30	0.76	0.19	0.22	0.90	0.37	-0.04	0.14	-0.26	0.79
Sex (male)	**-0.22**	**0.09**	**-2.51**	**0.01**	0.15	0.17	0.90	0.37	-0.04	0.12	-0.31	0.76
Days at 4°C	-0.01	0.09	-0.05	0.96	0.02	0.19	0.13	0.90	-0.07	0.10	-0.63	0.53
Time of day	0.02	0.08	0.19	0.85	0.26	0.18	1.46	0.15	-0.06	0.10	-0.60	0.55
Sample Year (2018)	-0.03	0.13	-0.27	0.79	0.01	0.27	0.05	0.96	0.13	0.16	0.84	0.40
Sample Year (2019)	0.14	0.16	0.87	0.39	-0.29	0.37	-0.79	0.43	-0.03	0.21	-0.13	0.90
Sample Year (2020)	**0.48**	**0.21**	**2.29**	**0.02**	-0.02	0.45	-0.05	0.96	0.08	0.26	0.31	0.76
Sample Year (2021)	0.21	0.16	1.32	0.19	0.00	0.37	-0.01	0.99	0.07	0.20	0.33	0.75
Sample Year (2022)	0.14	0.16	0.88	0.38	0.45	0.31	1.44	0.15	0.31	0.18	1.70	0.09
Sample Year (2023)					0.08	0.36	0.22	0.83	-0.05	0.21	-0.25	0.81
B) Presence/absence of MHC alleles												
(Intercept)	**5.66**	**0.33**	**17.37**	**< 0.001**	**3.24**	**0.29**	**11.15**	**< 0.001**	**1.24**	**0.17**	**7.41**	**< 0.001**
Heterozygosity	-0.09	0.21	-0.42	0.68								

Ase-dab3	0.29	0.15	1.95	0.05								
Ase-dab4	-0.27	0.15	-1.76	0.08								
Ase-dab5	0.18	0.16	1.15	0.25								
Ase-ua1	0.12	0.18	0.64	0.52								
Ase-ua3	-0.11	0.19	-0.60	0.55								
Ase-ua4	-0.19	0.14	-1.37	0.17								
Ase-ua5	-0.09	0.18	-0.52	0.60								
Ase-ua6	-0.18	0.17	-1.04	0.30								
Ase-ua7	-0.16	0.20	-0.80	0.43								
Ase-ua8	-0.04	0.14	-0.29	0.78								
Ase-ua9	-0.07	0.17	-0.39	0.70								
Ase-ua11	**0.38**	**0.18**	**2.07**	**0.04**	0.00	0.16	0.01	0.99	0.05	0.11	0.44	0.66
Age	-0.03	0.02	-1.63	0.10	-0.06	0.03	-1.67	0.10	**-0.05**	**0.02**	**-2.38**	**0.02**
Season (summer)	0.04	0.12	0.31	0.75	0.09	0.22	0.42	0.68	-0.01	0.13	-0.10	0.92
Sex (male)	**-0.25**	**0.09**	**-2.88**	**< 0.001**	0.12	0.17	0.75	0.45	-0.08	0.11	-0.73	0.47
Days at 4°C	-0.03	0.09	-0.33	0.75	-0.03	0.19	-0.16	0.88	-0.07	0.10	-0.68	0.50
Time of day	0.08	0.08	0.92	0.36	0.27	0.18	1.48	0.14	-0.05	0.10	-0.52	0.61
Sample Year (2018)	-0.06	0.12	-0.45	0.65	0.08	0.28	0.30	0.76	0.13	0.16	0.85	0.40
Sample Year (2019)	0.09	0.16	0.56	0.58	-0.13	0.37	-0.35	0.73	-0.05	0.21	-0.22	0.83
Sample Year (2020)	**0.42**	**0.21**	**2.02**	**0.04**	-0.08	0.46	-0.18	0.86	0.08	0.26	0.31	0.76
Sample Year (2021)	0.21	0.16	1.30	0.19	0.14	0.37	0.38	0.71	0.10	0.20	0.50	0.62
Sample Year (2022)	0.12	0.15	0.79	0.43	0.57	0.32	1.79	0.07	0.34	0.18	1.86	0.07
Sample Year (2023)					0.29	0.37	0.78	0.43	-0.05	0.21	-0.23	0.82

3.4.1.2 Metagenomic taxonomic diversity

Taxonomic alpha diversity (Shannon diversity or richness) calculated using shotgun metagenomics data was not associated with MHC-I or MHC-II diversity (Table 3.1A, Table S3.1A & S3.2A & S3.3A), nor with the presence/absence of *Ase-ua 11* (the MHC variant identified in the 16S analysis above (Table 3.1B, Table S3.1B & S3.2B & S3.3B)).

3.4.1.3 Metagenomic functional diversity

Functional alpha diversity (Shannon diversity or richness) of gene annotations derived from shotgun metagenomics data was not associated with MHC-I or MHC-II diversity (Table 3.1A, Table S3.1A & S3.2A & S3.3A) nor with *Ase-ua 11* (Table 3.1B, Table S3.1B & S3.2B & S3.3B).

3.4.2 GM composition

3.4.2.1 16S rRNA metabarcoding composition

16S GM composition was associated with both a quadratic function of MHC-I diversity and of MHC-II diversity (Table 3.2A, Figure 3.1A-B). It was also associated with season, sample year, days at 4°C, and time of day (Table 3.2A) but not with genome-wide heterozygosity, age, and sex.

Table 3.2. PERMANOVA analyses of gut microbiome composition in relation to individual major histocompatibility complex (MHC) characteristics in adult Seychelles warblers. Performed using Euclidean distance matrices of CLR-transformed abundances of (I) 16S amplicon sequencing variants (ASV) composition, (II) metagenomic taxonomic composition, (III) metagenomic functional gene composition categories. Separate models included (A) MHC diversity and (B) the presence/absence of MHC alleles. Significant predictors ($p<0.05$) are in bold. N=253 samples from 149 individuals were included in the 16S metabarcoding analyses. N=99 samples from 57 individuals were used for analyses of metagenomic taxonomic and functional composition. Bird ID was included as a blocking factor.

Predictor	(I) 16S ASV composition				(II) Metagenomics taxonomic composition				(III) Metagenomics functional gene composition			
	df	R^2	F	*p*	*df*	R^2	F	*p*	*df*	R^2	F	*p*
A) MHC Diversity												
Heterozygosity	**1**	0.003	0.719	0.067	**1**	**0.008**	**0.797**	**0.030**	1	0.015	1.485	0.052
MHC-I Diversity	**1**	**0.003**	**0.919**	**0.007**	1	0.010	1.034	0.626	**1**	**0.014**	**1.423**	**0.045**
MHC-I Diversity^2	**1**	**0.004**	**0.951**	**0.006**		-				-		
MHC-II Diversity	**1**	**0.003**	**0.839**	**0.036**	**1**	**0.009**	**0.925**	**0.034**	1	0.011	1.069	0.642
MHC-II Diversity^2	**1**	**0.003**	**0.913**	**0.028**		-				-		
Age	1	0.003	0.918	0.930	1	0.015	1.625	0.528		0.010	0.976	0.893
Season	**1**	**0.007**	**1.955**	**<0.001**	1	0.019	1.995	0.001	1	0.014	1.368	0.155
Sample Year	**5**	**0.038**	**2.023**	**<0.001**	**6**	**0.085**	**1.492**	**<0.001**	6	0.063	1.044	0.202
Sex	1	0.003	0.914	0.870	**1**	**0.012**	**1.228**	**<0.001**	1	0.012	1.206	0.185
Days at 4°C	**1**	**0.010**	**2.618**	**0.008**	1	0.011	1.116	0.494	**1**	**0.014**	**1.394**	**0.015**
Time of day	**1**	**0.010**	**2.525**	**0.001**	**1**	**0.017**	**1.773**	**<0.001**	1	0.013	1.325	0.168
B) Presence/absence of MHC alleles												
Heterozygosity	1	0.003	0.679	0.246		**-**				-		
Ase-dab3	1	0.004	1.004	0.999		-				-		
Ase-dab4	1	0.003	0.894	0.371		-				-		
Ase-dab5	1	0.004	0.991	0.740		-				-		
Ase-ua1	1	0.004	1.107	0.084		-				-		
Ase-ua3	1	0.004	0.934	1.000		-				-		

Ase-ua4	1	0.005	1.273	0.876			-			-		
Ase-ua5	**1**	**0.004**	**0.986**	**0.048**	1	0.006	0.584	0.584	1	0.008	0.799	0.604
Ase-ua6	1	0.003	0.873	0.185			-			-		
Ase-ua7	**1**	**0.004**	**1.150**	**0.010**	**1**	**0.009**	**0.995**	**<0.001**	1	0.006	0.611	0.502
Ase-ua8	1	0.006	1.514	0.605			-			-		
Ase-ua9	**1**	**0.003**	**0.899**	**0.011**	1	0.008	0.834	0.118	1	0.007	0.644	0.824
Ase-ua11	1	0.007	1.743	0.976			-			-		
Age	**1**	0.004	0.937	0.971	1	0.015	1.532	0.518	1	0.011	1.080	0.796
Season	**1**	**0.007**	**1.966**	**0.001**	**1**	**0.020**	**2.145**	**<0.001**	1	0.015	1.421	0.278
Sample Year	**5**	**0.038**	**1.986**	**<0.001**	**6**	**0.083**	**1.455**	**<0.001**	6	0.062	1.011	0.354
Sex	1	0.003	0.889	0.862	**1**	**0.014**	**1.481**	**<0.001**	1	0.009	0.900	0.198
Days at 4°C	**1**	**0.010**	**2.543**	**0.009**	1	0.010	1.083	0.485	**1**	**0.013**	**1.297**	**0.014**
Time of day	**1**	**0.009**	**2.401**	**<0.001**	**1**	**0.017**	**1.794**	**<0.001**	1	0.014	1.345	0.224

16S GM composition was associated with the MHC-I variants, *Ase-ua 5*, *Ase-ua 7*, and *Ase-ua 9* (Table 3.2B, Figure 3.1C-E), and also season, sample year, days at 4°C, and time of day (Table 3.2B), but not genome-wide heterozygosity, age, and sex. Despite these significant associations between GM composition and MHC variation, the overall effect sizes were small across all MHC variables (R^2<0.4%, Table 3.2, Figure 3.1).

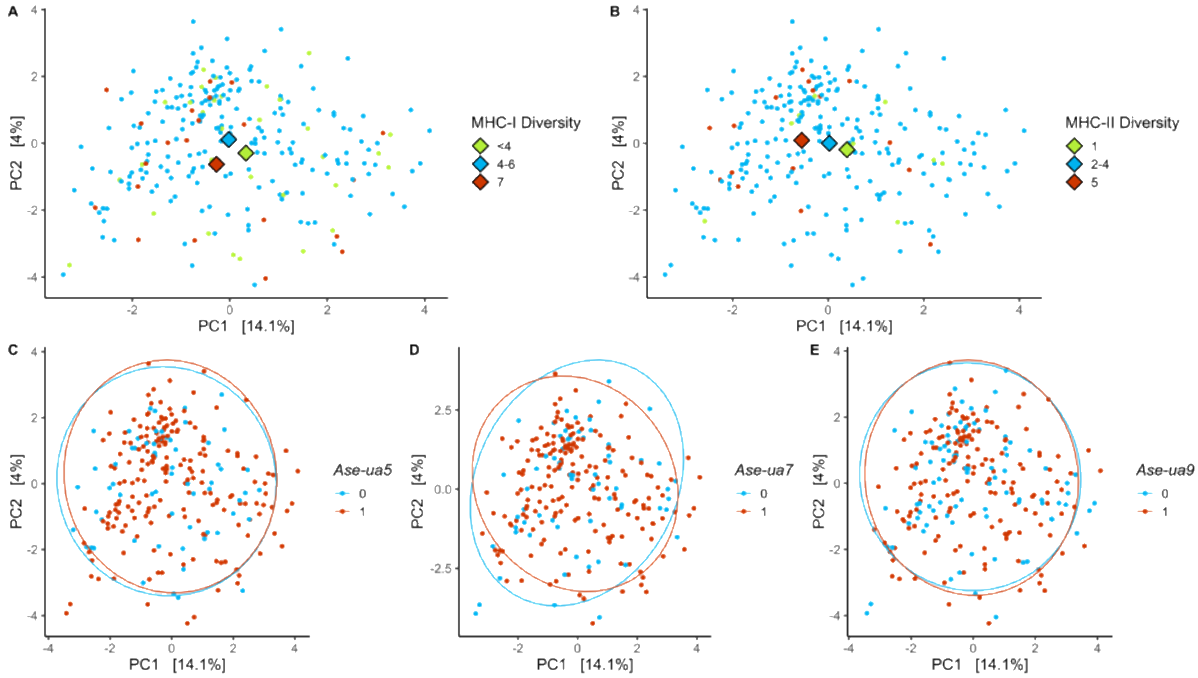

Figure 3.1. Principal Component Analyses (PCA) of gut microbiome compositional variation determined using 16S rRNA metabarcoding of adult Seychelles warbler faecal samples in relation to (A) MHC-I diversity, (B) MHC-II diversity, and the presence/absence (1/0) of (C) MHC-I allele *Ase-ua 5*, (D) MHC-I allele *Ase-ua 7*, (E) MHC-I allele *Ase-ua 9*. N=253 from 149 birds. Large diamonds represent the group centroids. For clarity, samples were grouped into discrete categories for plotting. In plots A-B, the coloured points represent low (green), medium (blue), and high (red) MHC diversity. In plots C-E, blue = absence, red = presence of the allele. Ellipses of 95% confidence intervals of each group are drawn around the points. Principal components 1 and 2 explained 14.1% and 4% of the variation in gut microbiome structure, respectively.

3.4.2.2 Metagenomic taxonomic composition

Variation in GM metagenomic taxonomic composition was associated with genome-wide heterozygosity and MHC-II diversity, but not MHC-I diversity (Table 3.2A, Figure 3.2A-B). Of the control variables, sex, sample year, and time of day were

associated with metagenomics taxonomic composition (Table 3.2A), but age, season, and days at 4°C were not.

When assessing MHC variants identified in the 16S analysis, GM metagenomic taxonomic composition was associated with the presence of MHC-I *Ase-ua 7* (Table 3.2B, Figure 3.2C) but not *Ase-ua 5* and *Ase-ua 9*. Sex, season, sample year, and time of day were also associated with metagenomic taxonomic composition (Table 3.2B), but age and days at 4°C were not. Despite significant differences in GM composition, the overall effect sizes were small across all MHC variables (R^2<0.9%, Table 3.2, Figure 3.2).

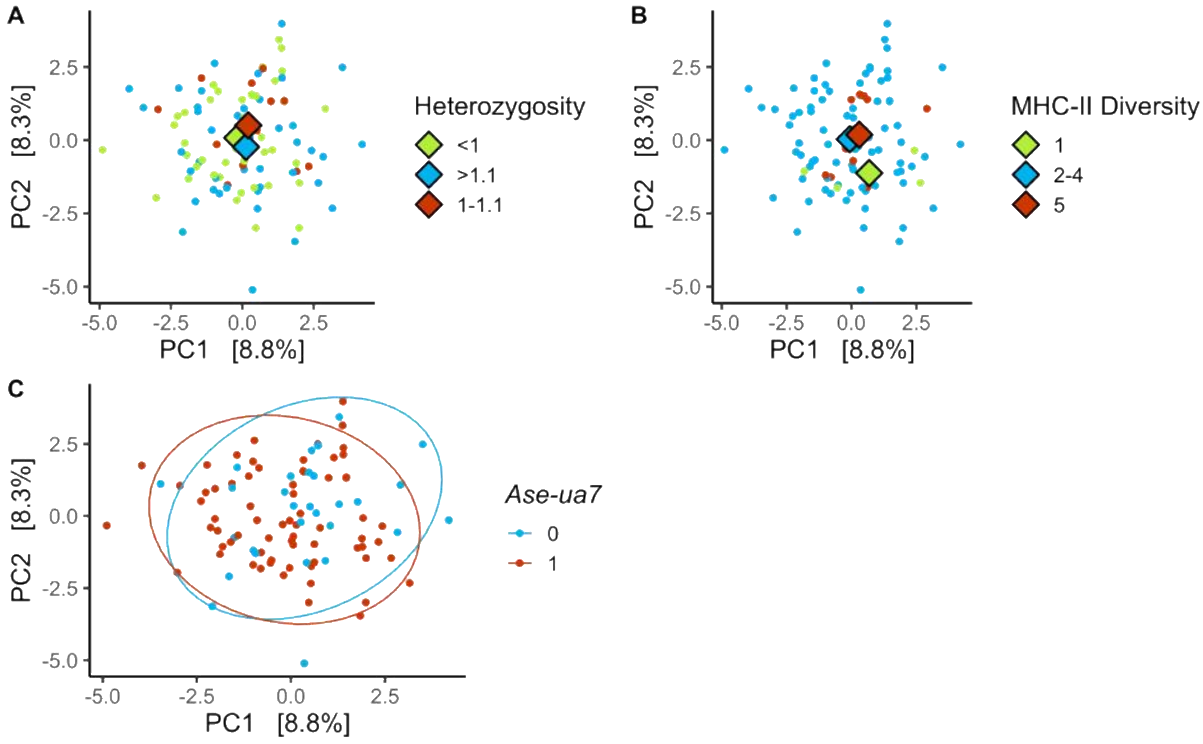

Figure 3.2. Principal Component Analyses (PCA) of gut microbiome metagenomic taxonomic compositional variation of Seychelles warbler faecal samples in relation to (A) genome-wide heterozygosity, (B) MHC-II diversity, and (C) MHC-I allele *Ase-ua 7.* N=99 from 57 birds. Large diamonds represent the group centroids. For clarity, samples were grouped into discrete categories for plotting. In plots A and B, the coloured points indicate low (green), middle (blue), and high (red) genome-wide heterozygosity (Heterozygosity) and MHC-II diversity, respectively. In plot C, blue = absence (0) and red = presence (1) of *Ase-ua 7*. Ellipses of each group are drawn around the points.

3.4.2.3 Metagenomics functional composition

Functional GM composition was significantly associated with increasing individual MHC-I diversity (Table 3.2A, Figure 3.3) and with days at 4°C (Table 3.2A). However, genome-wide heterozygosity, MHC-II diversity and all other control variables (age, sex, season, sample year, and time of day) were not (Table 3.2A).

Functional GM composition was not significantly associated with the MHC alleles *Ase-ua 5*, *Ase-ua 7*, and *Ase-ua 9* identified in the 16S analysis above (Table 3.2B). Functional GM composition was associated with days stored at 4°C (Table 3.2B), but not with any other control variables (age, sex, season, sample year, time of day) (Table 3.2B). Despite significant differences in GM composition, the overall effect sizes were small across all MHC variables (R^2<1.4%, Table 3.2, Figure 3.3), but the MHC-I diversity effect size is the largest among GM compositional analyses.

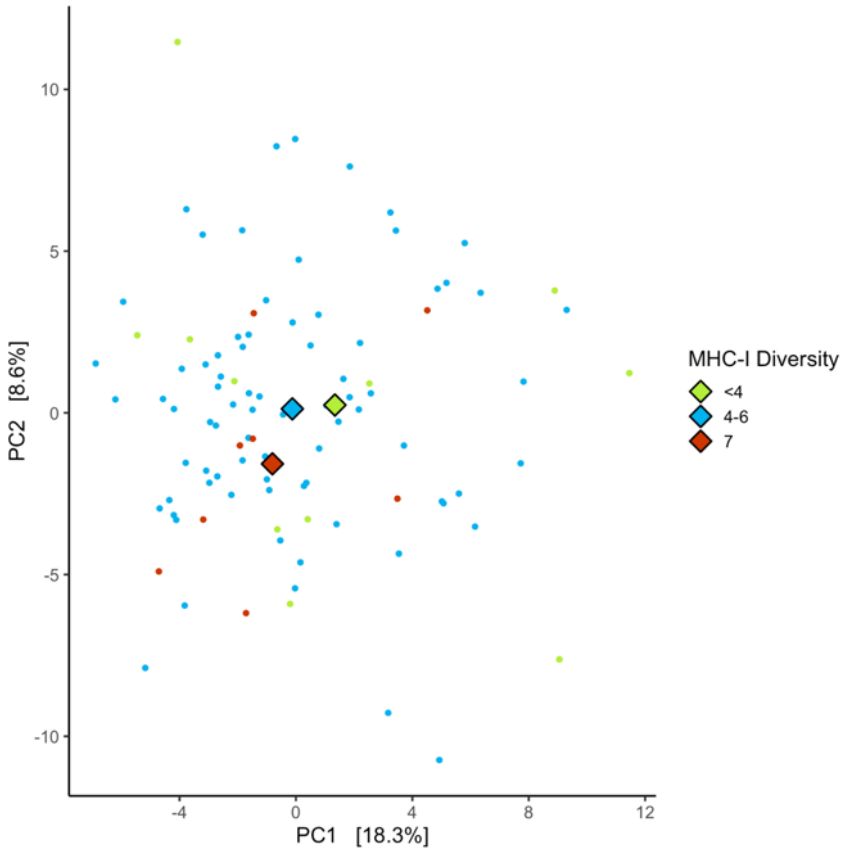

Figure 3.3. Principal Component Analyses (PCA) of gut microbiome compositional variation determined using metagenomics function with MHC-I diversity in the gut microbiome of Seychelles warblers (n = 99 from 57 birds). Large diamonds represent the group centroids. For clarity, samples were grouped into discrete categories for plotting. The coloured points represent the count <4 (green), 4-6 (blue), and 7 (red) of MHC-I diversity. Principal components 1 and 2 explained 18.3% and 8.6% of gut microbiome structure, respectively.

3.4.3 Differential abundance analysis

3.4.3.1 Differential abundance of metagenomic taxonomic species

The abundance of some individual bacterial species (identified using metagenomics) varied in relation to MHC characteristics (Figure 3.4AB); the abundance of *Enterococcus casselifavus* decreased, and *Microbacterium enclense* increased with increasing MHC-I diversity (Figure 3.4A). The abundance of *Lactococcus lactis* decreased, and the abundance of *Staphylococcus lloydii* increased with increasing MHC-II diversity (Figure 3.4B).

The abundances of four bacterial species were significantly associated with the presence/absence of the MHC-I allele *Ase-ua 7* (identified as associated with GM taxonomy composition), i.e. there was decreased prevalence of *Enterococcus casselifavus* and *Gordonia* sp OPL2, and an increased prevalence of *Escherichia coli* and *Vulcaniibacterium thermophilum,* when *Ase-ua 7* was present (Figure 3.4C). The abundance of bacterial species was not significantly related to host genome-wide heterozygosity (Figure 3.4D).

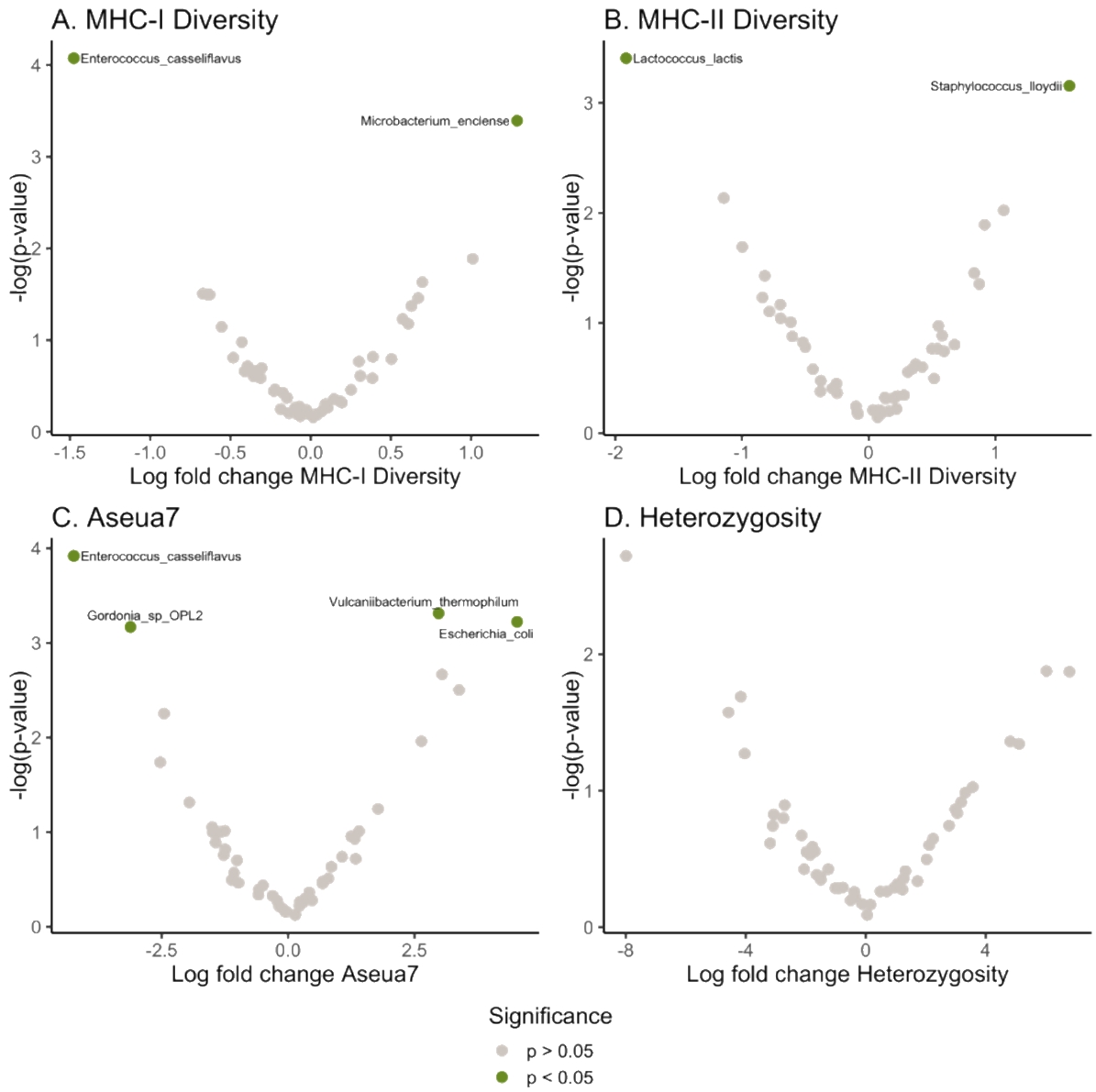

Figure 3.4. Differential abundance of metagenomically identified bacterial species in adult Seychelles warblers according to host (A) genome-wide heterozygosity, (B) MHC-I diversity, (C) MHC-II diversity, and (D) presence of MHC-I *Ase-ua 7* (n=99 from 57 birds). Points represent bacterial species and are coloured according to significance; green points (with species-level taxonomic annotations) are significantly differentially abundant (p<0.05), and grey points are not.

3.4.3.2 Differential abundance of metagenomic functional genes

Abundances of 24 GM functional gene annotations differed significantly in relation to individual host MHC-I diversity (Table S3.4, Figure 3.5A). In total, 9 GM genes increased in abundance and 15 genes decreased in abundance with increasing MHC-I diversity.

The 24 gene annotations were derived from 13 functional gene categories (defined by Cluster of Gene Orthologs (COG)). With increasing MHC-I diversity, two COG categories only increased in prevalence, four COG categories increased and decreased in prevalence, and seven COG categories only decreased in prevalence (Figure 3.5B). Five of the seven COG categories that only decreased in prevalence are involved in bacterial metabolism. In addition, one GM functional gene annotation (COG1216) increased in prevalence with increasing genome-wide heterozygosity.

The KEGG pathways of MHC-I diversity associated genes (Table S3.4) further support the findings, as core microbial function pathways decreased in prevalence; Carbohydrate metabolism (K01885, K01886, K01652, K06131, K01115), lipid metabolism (K00666), amino acid metabolism (K00852, K00847, K00874), transcription (K03043, K02335, K04799), translation (K08832, K15409), and replication and repair (K03581, K04043, K03283) (Table S3.4). In addition, MHC-I diversity was positively associated with KEGG pathways involved in environmental defence or stress adaptation (K02004, K18138, K09800, K04763; Table S3.4).

Three GM functional gene annotations were differentially abundant with increasing MHC-II diversity: decreased in COG0318 (K00666 – fatty-acyl-CoA synthase), and increased in COG1609 (K02529 – LacI family transcriptional regulator, galactose operon repressor) and COG1653 (K02027 – multiple sugar transport system substrate-binding protein).

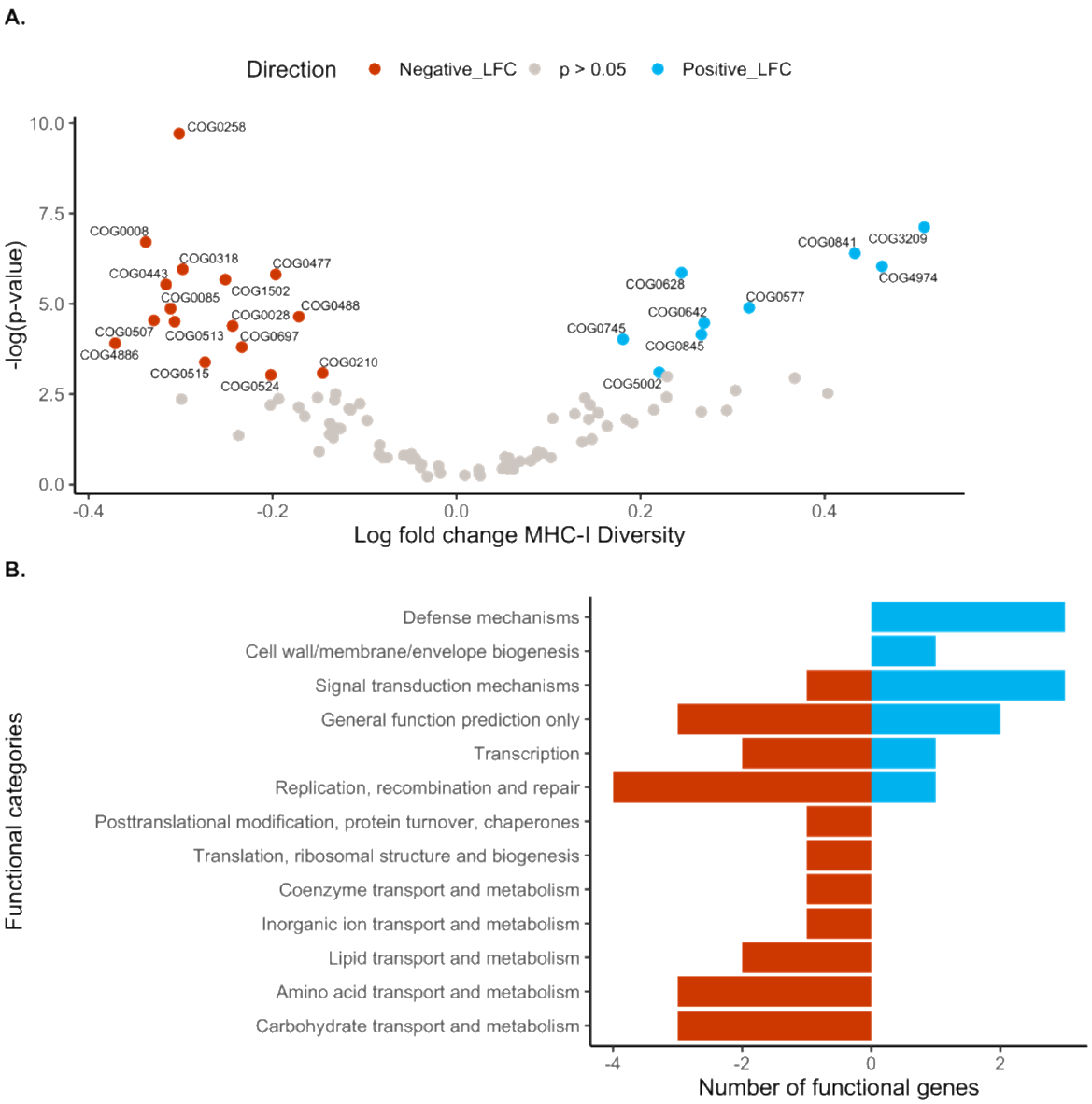

Figure 3.5. Variation in the abundance of bacterial functional genes (determined using eggNOG) in the gut microbiome of adult Seychelles warblers in relation to individual MHC-I diversity (n=99 from 57 birds). (A) The results of an ALDEx2 differential abundance test showing the log fold change in abundance of each eggNOG gene annotation with increasing MHC-I diversity. Blue points are significantly ($p<0.05$) more abundant, red points are significantly ($p<0.05$) less abundant, and grey points do not differ significantly with MHC-I diversity. Labels on significant points represent eggNOG members. (B) Counts of functional genes per eggNOG category that demonstrated a significant positive (blue) or negative (red) log fold change with increasing MHC-I diversity, respectively.

3.4.4 Network analysis

3.4.4.1 Network analysis of metagenomic taxonomic species

The metagenomic taxonomic species network had a higher number of connected nodes and edges in low MHC-I diversity (19 nodes, 13 edges) compared to high (2 nodes, 1 edge) MHC-I diversity individuals (Figure 3.6AB). The average number of

edges per connected node was also higher in low (mean = 1.4 edges per node) than in high MHC-I diversity (mean = 1.0 edges per node). Modularity was 0.82 in low MHC-I diversity, but zero in high MHC-I diversity due to only having one edge. The ratio of negative to positive edges in low MHC-I diversity was 0.3; there was only a single positive edge and no negative edges in MHC-I diversity. This shows that the metagenomic taxonomic species network is more fragmented in high MHC-I diversity individuals than in low MHC-I diversity individuals.

In relation to MHC-II diversity, the metagenomic taxonomic species network had a higher number of connected nodes in low (14 nodes) than in high (10 nodes) MHC-II diversity individuals (Figure 3.6CD). However, the number of edges did not differ between low (8 edges) and high (8 edges) MHC-II diversity (Figure 3.6CD). The average number of edges per connected node was lower in low (mean = 1.1 edges per node) than in high (mean = 1.6 edges per node) MHC-II diversity. Modularity was also higher in low (0.81) than in high (0.59) MHC-II diversity. The ratio of negative to positive edges was 0.33 in low MHC-II diversity and was 0.14 in high MHC-II diversity. This shows that the metagenomic taxonomic species network of individuals with high MHC-II diversity is slightly more fragmented and has weaker connections than in individuals with low MHC-II diversity.

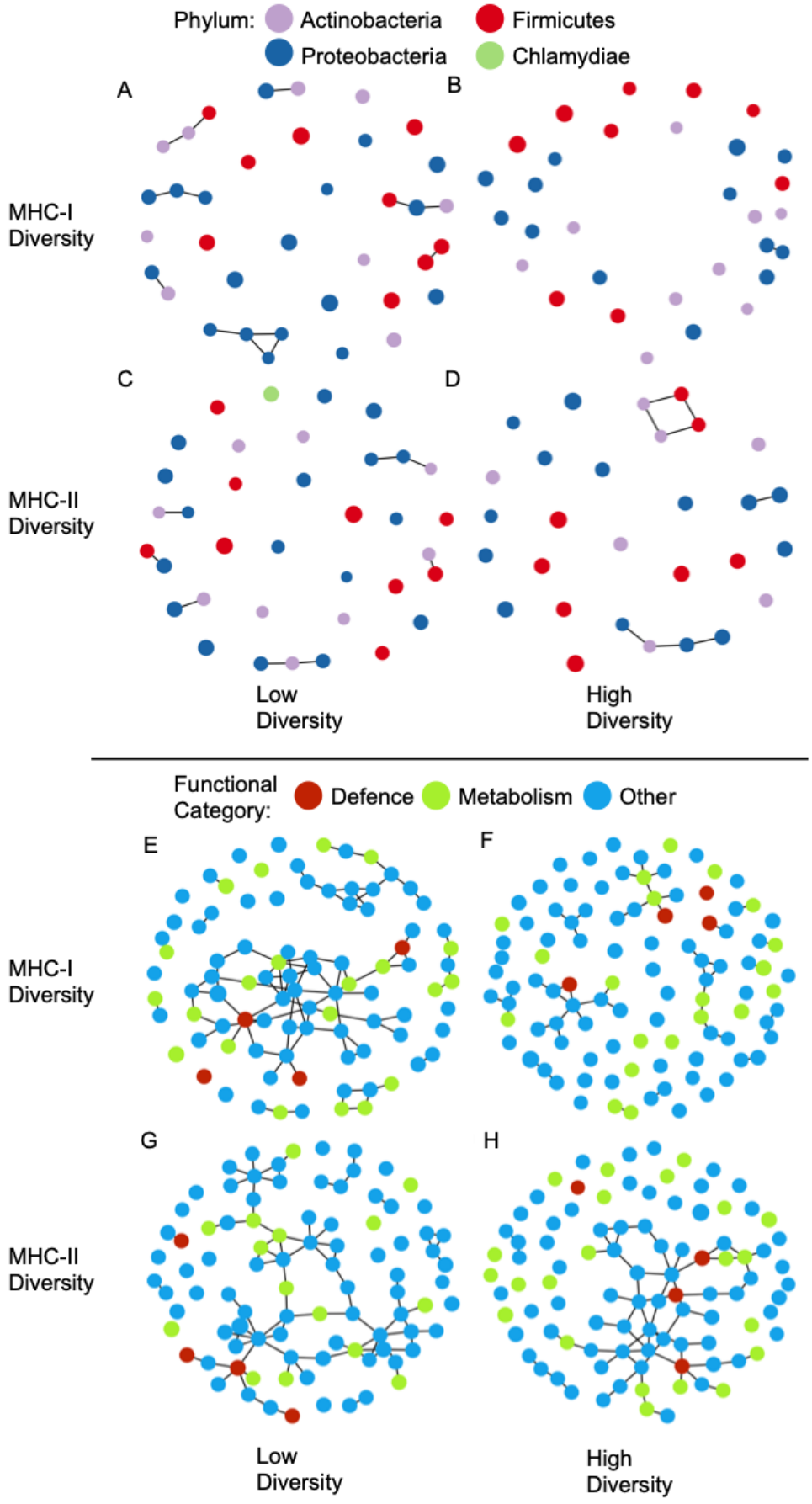

Figure 3.6. Network analysis between MHC diversity and the gut microbiome in adult Seychelles warblers (n=99 from 57 birds). Each node represents A-D) metagenomic

taxonomic species or E-H) metagenomic functional genes. ABEF) MHC-I diversity, CDGH) MHC-II diversity, ACEG) low MHC diversity, BDFH) high MHC diversity. Nodes (species/genes) are coloured by A-D) Phylum and E-H) functional category. The size of the nodes is proportional to the mean abundance. Lines are edges (interaction between species/genes), connecting nodes that are linked.

3.4.4.2 Network analysis of metagenomic functional genes

The metagenomic functional genes network had a higher number of connected nodes and edges in individuals with low MHC-I (76 nodes, 87 edges) than high MHC-I (56 nodes, 44 edges) diversity (Figure 3.6EF). The average number of edges per connected node was also higher in low MHC-I (mean = 2.3 edges per node) than in high MHC-I diversity (mean = 1.6 edges per node). Modularity was lower in low MHC-I (0.72) than in high (0.85) MHC-I diversity. The ratio of negative to positive edges was 0.45 in low MHC-I diversity and 0.19 in high MHC-I diversity. This shows that the metagenomically derived functional genes network is more fragmented in high MHC-I diversity individuals, but has stronger connections than in low MHC-I diversity individuals.

In relation to MHC-II diversity, the metagenomic functional genes network had a higher number of connected nodes and edges in low (74 nodes, 83 edges) than in high MHC-II diversity individuals (55 nodes, 62 edges) (Figure 3.6GH). The average number of edges per connected node was very similar between low (mean = 2.2 edges per node) and high (mean = 2.3 edges per node) MHC-II diversity individuals. Modularity was also higher in low (0.76) than in high (0.65) MHC-II diversity individuals. The ratio of negative to positive edges was 0.26 in low MHC-II diversity and was 0.44 in high MHC-II diversity. This suggests that the metagenomic functional genes network of high MHC-II diversity individuals is more fragmented and has weaker connections than in low MHC-II diversity individuals.

3.5 Discussion

Our study shows that in adult Seychelles warblers, there was no association between GM diversity (characterised by either 16S ASV or metagenomics) and MHC diversity, though one specific MHC variant (MHC-I allele *Ase-ua 11*) was associated with 16S GM richness but not metagenomic GM diversity. However, GM composition (16S and metagenomic-derived) was associated with MHC diversity. The 16S GM composition was associated with MHC-I and MHC-II diversity in a non-linear (quadratic function) manner, and with the MHC-I alleles *Ase-ua 5*, *Ase-ua 7*, and *Ase-ua 9*. The metagenomic taxonomic composition was associated with MHC-II diversity and the MHC-I allele *Ase-ua 7*. Furthermore, the functional composition of the GM (metagenomically derived) was associated with MHC-I diversity. Additionally, two metagenomic bacterial species were differentially abundant with increasing MHC-I and MHC-II diversity, and four bacterial species differed in relation to the presence/absence of MHC-I allele *Ase-ua 7*. Furthermore, 24 were differentially abundant with increasing MHC-I diversity (driving increases in microbial defence mechanisms and decreases in microbial metabolism) and three with increasing MHC-II diversity. Network analysis showed that high (compared to low) MHC-I diversity was associated with greater fragmentation in both taxonomic and functional GM structure, higher modularity, and a lower negative-to-positive interaction ratio, indicating fewer but strongly interconnected nodes in high MHC-I diversity individuals. In contrast, individuals with high MHC-II diversity were associated with slightly more fragmentation, lower modularity, and a higher negative-to-positive interaction ratio, suggesting fewer and weaker connections.

Consistent with previous findings on the Seychelles warbler, we found that MHC diversity (either class-I or class-II) was not significantly associated with GM diversity (Davies et al., 2022; Worsley et al., 2022). This contradicts results found in other wild populations (Bolnick et al., 2014; Hernández-Gómez et al., 2018; Leclaire et al., 2019; Uren Webster et al., 2018). For example, in giant salamanders (*Cryptobranchus alleganiensis bishopi* and *C. a. alleganiensis*) and sticklebacks (*Gasterosteus aculeatus*), increasing MHC allelic diversity is correlated with increases and decreases in overall skin microbiome diversity, respectively (Bolnick et al., 2014; Hernández-Gómez et al., 2018). Given that the Seychelles warblers had a recent population bottleneck (<50 individuals in the 1960s, Spurgin et al., 2014), the MHC is less diverse than similar species (Richardson & Westerdahl,

2003), and may therefore have a reduced impact on the GM diversity. However, the MHC region in the Seychelles warblers maintains considerable variation despite the population bottleneck (Richardson & Westerdahl, 2003). Thus, the MHC and GM diversity association may be host-species dependent, regardless of overall MHC variation levels.

In contrast to the GM diversity results, shifts in GM composition (both 16S and metagenomic) were associated with MHC characteristics. An association between MHC-II diversity and GM taxonomic composition, as seen in our study, has been shown multiple times in captive and wild systems (see review (Roland et al., 2020)). In our study, only two bacterial species were differentially abundant (increased *Staphylococcus loydii* and decreased *Lactococcus lactis*). There is evidence of *Lactococcus lactis* causing detrimental infections in avian species (Goyache et al., 2001), but it has also been used as a probiotic for broiler chickens (*Gallus gallus domesticus*) (Boodhoo et al., 2023; Navale et al., 2024). *Staphylococcus loydii* hasn't been observed to cause infections in any species. These results may suggest that in the Seychelles warblers, increasing MHC-II diversity may suppress pathogenic species. In addition, the MHC-I allele *Ase-ua 7* was significantly correlated with GM composition, consistent with previous work on this population (Davies et al., 2022). *Ase-ua 7* was associated with decreases in *Enterococcus casselifavus* and *Gordonia* sp OPL2, (gram-positive bacteria), but increased prevalence of the gram-negative bacteria, *Escherichia coli* and *Vulcaniibacterium thermophilum*, (Larke-Mejía et al., 2020; Lim et al., 2010; Niu et al., 2020; Yoshino, 2023). The World Health Organization (WHO) priority list of pathogens primarily consists of Gram-negative bacteria (Breijyeh et al., 2020). Therefore, we speculate that the effect of *Ase-ua 7* on GM composition could be based on microbial cell wall structure and could be important in controlling pathogens. Indeed, during inflammation, MHC expression was upregulated in the small intestinal stem cells of humans and mice (*Mus musculus*) (Heuberger et al., 2021).

In our study, the effects of MHC-II diversity and the MHC-I allele *Ase-ua 7* on taxonomic GM are relatively small (two and four, out of 49, core species were differentially abundant, respectively). Such limited effects have also been found in the few previous similar studies, with the MHC variations influencing only a very small number of bacterial species rather than the overall composition (Bolnick et al.,

2014; Montero et al., 2021; Worsley et al., 2022). This small taxonomic effect could explain why both MHC-II diversity and MHC-I allele *Ase-ua 7* were not significantly associated with metagenomic function (see below). As only a few species are influenced, other microbes may replace their function through functional redundancy (Louca et al., 2018; Worsley, Mazel, et al., 2024). Although network analyses of both taxonomic and functional GM revealed that high MHC-II diversity was associated with greater fragmentation, lower modularity, and a higher negative-to-positive interaction ratio, these differences were less pronounced than those observed for MHC-I diversity. This further supports the conclusion that MHC-II diversity has a relatively small effect on the gut microbiome.

In contrast, the diversity of MHC-I alleles (but not class-II) did appear to influence GM functional differences (determined metagenomically), with higher MHC-I diversity leading to the presence of an increased number of microbial defence genes, whilst decreasing the number of metabolism-related genes. This is further shown in network analysis, where taxonomic and functional microbial networks appear more fragmented in individuals with high MHC-I diversity, indicating reduced microbial interactions. These patterns may reflect increased microbial competition or immune-mediated disruption under high MHC-I diversity. In addition, high MHC-diversity also had higher modularity, suggesting higher resilience to environmental perturbations (Coyte et al., 2022; Fabbrini et al., 2023), which supports the findings of increased genes involved in defence in our study. Trade-offs between defence and growth are common (although not ubiquitous) in microbial species (Ferenci, 2016; Liu et al., 2024). Our results suggest that these microbial trade-offs can also amount to costs for the host, whereby control of the microbiome (via the immune system) can result in a reduction in the GM's metabolic potential. Presumably, these costs may be outweighed by the benefits of eliminating pathogens and maintaining a healthy microbiome (Gillingham et al., 2025; Metcalf & Koskella, 2019). Quantifying the relative costs and benefits of maintaining a dynamic microbiome remains largely unexplored but is essential for understanding how host-microbiome interactions, and host control mechanisms (including the immune system) evolve (Gillingham et al., 2025; Metcalf & Koskella, 2019; Wilde et al., 2024).

Overall, our study indicates that MHC-I, not the MHC-II, plays a greater role in shaping the GM, both taxonomically and functionally. This is consistent with

previous findings in the Seychelles warbler (Davies et al., 2022; Worsley et al., 2022), as well as in the reddish-gray mouse lemur (*Microcebus griseorufus*) (Montero et al., 2021). MHC-I molecules encode receptors that typically act intracellularly, while MHC-II receptors interact extracellularly, therefore, one would think that the class-II receptors should have greater interaction with and influence over gut microbes (Rock et al., 2016). However, the mechanisms by which MHC-I alleles and diversity influence the GM functions remain unclear. MHC-I receptors can be triggered by bacteriophages (Bazan et al., 2012), which play an important role in shaping bacterial composition (Hughes & Yeager, 1998). Future work on host MHC and the gut virome in the Seychelles warbler could help understand the mechanisms behind how MHC-I affect GM composition. It is still surprising to have detected less of an effect of the extracellular-acting MHC-II on the GM (Rock et al., 2016; Roland et al., 2020). However, spatial segregation of microbes and epithelial cells, suppression of the host immune system, and peripheral tolerance (elimination of self-reactive immune cells) could be potential mechanisms in maintaining a mutualistic relationship between the host and the GM (Roland et al., 2020).

In the Seychelles warblers, MHC-I diversity has been positively correlated with both survival and reproductive success (Brouwer et al., 2010; Richardson et al., 2005; Richardson & Westerdahl, 2003). In the current study, we also found that MHC-I diversity was associated with GM metagenomics function. It is plausible that MHC-I diversity affects the GM functionality by controlling mutualistic bacteria, indirectly influencing host survival. However, MHC-I variation could affect other components of the host's overall health, indirectly leading to differences in the GM function. Consequently, multiple mechanisms may be involved in the relationship between MHC-I diversity and individual differences in the GM.

An individual's sex and genome-wide heterozygosity, independent of MHC variation, were also associated with shifts in GM metagenomic species. However, these changes were not detectable in terms of 16S ASVs and metagenomics function, which indicates that the changes are species/strain specific and do not influence microbial function. This result is reinforced by the fact that no common species were differentially abundant with increasing levels of genome-wide heterozygosity. Various other variables including sample year, season, and time of day, were also predictors of 16S ASVs and metagenomic species, but not

metagenomic function. The fact that so many factors affect the species present in the GM, but not the overall GM functionality is likely attributed to functional redundancy, as the types of environmental microbes change with time, the overall GM function is replaced by other microbes (potentially due to changes in diet), thereby preserving the overall GM function (Louca et al., 2018; Worsley, Mazel, et al., 2024).

Interestingly, the number of days stored at 4°C was associated with shifts in metagenomics function but not 16S ASVs and metagenomic species. Larger DNA fragments degrade quicker in storage, and these larger DNA fragments are required for accurate gene recognition and gene annotation, as these steps require a start codon followed by an open reading frame (Hyatt et al., 2010). Whereas smaller DNA fragments may still be sufficient for accurate taxonomic assignments in both 16S ASV and metagenomic species, as specific marker genes are typically used for taxonomic annotations (Blanco-Míguez et al., 2023; Parks et al., 2022).

One limitation of our study on this wild population of Seychelles warbler is that it is purely correlative, and we are unable to validate our findings experimentally. Nonetheless, our findings in both datasets (16S and metagenomic methods) are comparable despite the smaller sample size in the metagenomic work. Future experimental work in more amenable study systems could investigate potential pathways by which host MHC-I diversity and alleles may affect the GM, for example by introducing immune-triggering bacteriophages and measuring their impact on the gut bacteriophage and bacteria community. (González-Mora et al., 2020; Wan et al., 2001). However, such experiments are likely lab-based and the GM would be radically altered (van Leeuwen et al., 2020), hence the generalisation to natural populations would be limited. A further limitation of our study is that it lacks gene expression data for the MHC and the GM. Therefore, the functional relevance is only on the DNA level, reflecting potential rather than actual function of the MHC and GM. However, in the bottlenecked Seychelles warbler population, the few remaining MHC alleles are highly divergent, preventing further reduction into functional supertypes (Davies et al., 2022; Richardson & Westerdahl, 2003; Wright et al., 2016). Future research could use transcriptomics and proteomics to quantify both MHC and GM function. The lack of expression data could plausibly explain the modest effect sizes in this study as a locus could be present but not expressing. In

addition, although multiple studies have found that MHC is associated with the microbiome (Bolnick et al., 2014; Hernández-Gómez et al., 2018; Leclaire et al., 2019; Uren Webster et al., 2018), the host regulation of the GM may be more directly shaped by innate immunity, such as by toll-like receptors, NOD-like receptors or defensins (Wilde et al., 2024). However, a previous study in the Seychelles warbler found no associations between toll-like receptor 3 (variation at which influences host survival in this species) and the GM alpha diversity or composition (Davies et al., 2022). Therefore, while the MHC may only explain a limited amount of variation in the GM (as shown in this study and (Davies et al., 2022; Worsley et al., 2022)), this variation may still be important.

We found an association between three MHC-I alleles and GM 16S taxonomy, but only one MHC-I allele *Ase-ua 7* was associated with GM metagenomic taxonomy, none with GM function. The lack of associations in metagenomic dataset may be due to the limited sample size, constraining our ability to assess all other MHC alleles. To robustly assess all 12 MHC alleles and to include seven essential control variables in the same model, we would need at least 190 samples from different individuals (10 samples per variable, (Kelly et al., 2015)).

Despite reporting several associations between MHC characteristics and the GM, our study is limited to only assessing genome-wide heterozygosity and the specific MHC candidate alleles we had already screened for. A genome-wide association study on the host and its GM could reveal more loci, and potentially more nuanced or polygenic effects by accounting for multiple genes concurrently (Xiang et al., 2024). However, this requires whole-genome data on a large number of individual birds, in combination with metagenomic sequencing of their GM, which would be very costly and labour-intensive (La Reau et al., 2023; Yoshida et al., 2019). Leveraging a sequencing approach that targets specific individuals and utilises recent technological advances, such as the imputation of low-coverage samples, could be a feasible way to conduct such studies (Yoshida et al., 2019). In the same vein, while heterozygosity measured using 30 neutral microsatellite loci does reflect genome-wide heterozygosity/inbreeding in this species (Spurgin et al., 2014), whole-genome sequencing of individuals would be a more accurate measure. It would enable us to determine runs of homozygosity, and thus provide the resolution

needed for a powerful investigation of the effect of host inbreeding on the GM (Ceballos et al., 2018).

In conclusion, our study suggests that both MHC class I and II influence an individual's GM in the Seychelles warbler, but that despite being an intracellular receptor, MHC-I has a greater influence on GM composition, than MHC-II. We also found that MHC-I allele *Ase-ua 7* changes the GM taxonomic composition, while MHC-I diversity alters GM function. These results may explain previous findings that MHC-I diversity is positively correlated with fitness in this population (Brouwer et al., 2010; Richardson et al., 2005), potentially as a result of inducing changes in the GM functionality. GM functional network analyses further support the increased GM resilience with high MHC-I diversity, which could be important for host health (Fassarella et al., 2021). However, this could be an indirect effect, rather than the GM actively contributing to increased fitness. Various pathways are involved in regulating the immune system, underscoring the need for host and gut transcriptomics and metabolomic data to enable mechanistic investigation of immunogenetics and the GM (Eshleman et al., 2023; Roland et al., 2020).

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3.7 Supplementary material

Table S3.1. The relationship between gut microbiome alpha diversity (Shannon) and variation in host (A) MHC diversity and (B) the presence/absence of specific MHC alleles in Seychelles warblers. Linear mixed models were used for all models. N = 253 samples from 149 individuals in 16S ASV diversity and N = 99 samples from 57 individuals in metagenomic taxonomy diversity and functional diversity. Reference categories for categorical variables were as follows: Female (sex), winter (season), 2017 (Sample year), and absent (0) in all MHC alleles. Significant (P < 0.05) variables are shown in bold.

Model	Predictor	16S ASV Shannon					Metagenomics Taxonomy Shannon					Metagenomics Functional Shannon				
		Est	SE	df	t	P	Est	SE	df	t	P	Est	SE	df	t	P
A) MHC Diversity	(Intercept)	**3.256**	**0.609**	**151.658**	**5.344**	**< 0.001**	0.707	0.843	51.987	0.838	0.406	810.308	458.228	63.471	1.768	0.082
	Heterozygosity	0.035	0.400	127.858	0.087	0.931	0.549	0.529	43.259	1.039	0.304	84.219	284.505	56.648	0.296	0.768
	MHC-I Diversity	0.043	0.059	126.354	0.726	0.470	0.056	0.079	39.364	0.705	0.485	18.886	41.001	47.343	0.461	0.647
	MHC-II Diversity	-0.038	0.085	107.453	-0.444	0.658	-0.106	0.101	36.388	-1.043	0.304	-8.708	54.004	49.176	-0.161	0.873
	Age	-0.026	0.034	167.775	-0.742	0.459	-0.015	0.040	54.211	-0.383	0.703	-41.418	22.222	64.476	-1.864	0.067
	Season	-0.152	0.218	232.175	-0.698	0.486	0.002	0.246	81.629	0.007	0.994	147.236	147.488	69.650	0.998	0.322
	Sex	-0.276	0.164	116.709	-1.677	0.096	0.204	0.217	37.363	0.941	0.353	4.794	113.824	47.563	0.042	0.967
	Days in fridge	-0.084	0.177	237.601	-0.475	0.635	-0.235	0.191	65.090	-1.232	0.222	30.756	124.123	75.900	0.248	0.805
	Catch Time	-0.023	0.159	238.584	-0.145	0.885	0.112	0.188	74.829	0.594	0.554	79.915	117.418	78.638	0.681	0.498
	SampleYear2018	0.168	0.237	232.771	0.710	0.478	0.216	0.299	81.766	0.721	0.473	95.562	172.637	76.217	0.554	0.582
	SampleYear2019	-0.092	0.306	238.742	-0.301	0.764	-0.188	0.397	79.019	-0.473	0.637	90.988	238.156	78.404	0.382	0.704
	SampleYear2020	0.488	0.393	232.913	1.243	0.215	0.353	0.482	75.198	0.733	0.466	-75.215	292.792	78.329	-0.257	0.798
	SampleYear2021	-0.179	0.300	238.987	-0.597	0.551	0.074	0.375	67.298	0.198	0.844	84.213	237.966	75.655	0.354	0.724
	SampleYear2022	0.114	0.292	232.157	0.391	0.696	0.270	0.347	81.205	0.778	0.439	285.653	202.309	76.433	1.412	0.162
	SampleYear2023						0.425	0.391	81.858	1.087	0.280	133.373	243.410	78.918	0.548	0.585
B) Presence/absence of MHC alleles	(Intercept)	**3.770**	**0.643**	**133.977**	**5.864**	**< 0.001**	**1.262**	**0.314**	**82.917**	**4.018**	**< 0.001**	**1024.450**	**184.100**	**80.340**	**5.565**	**< 0.001**
	Heterozygosity	**0.119**	**0.411**	**111.652**	**0.291**	**0.772**										
	Ase-dab3	0.306	0.289	142.300	1.061	0.290										
	Ase-dab4	-0.288	0.301	126.639	-0.958	0.340										
	Ase-dab5	-0.031	0.304	146.426	-0.103	0.918										
	Ase-ua1	0.153	0.360	123.331	0.424	0.672										
	Ase-ua3	-0.332	0.370	122.950	-0.898	0.371										
	Ase-ua4	-0.277	0.276	107.669	-1.005	0.317										
	Ase-ua5	-0.240	0.348	133.132	-0.690	0.491										

	Ase-ua6	-0.004	0.342	140.968	-0.012	0.991										
	Ase-ua7	-0.272	0.401	118.988	-0.679	0.499										
	Ase-ua8	0.004	0.284	127.433	0.013	0.990										
	Ase-ua9	-0.188	0.337	115.587	-0.557	0.579										
	Ase-ua11	0.494	0.363	115.143	1.359	0.177	0.199	0.203	41.077	0.977	0.334	-60.530	103.310	48.260	-0.586	0.561
	Age	-0.027	0.036	170.485	-0.753	0.453	-0.034	0.039	59.801	-0.857	0.395	-41.700	21.230	64.560	-1.964	0.054
	Season	-0.208	0.222	223.919	-0.939	0.349	-0.010	0.243	83.916	-0.040	0.968	125.180	141.490	70.070	0.885	0.379
	Sex	-0.268	0.171	114.037	-1.565	0.120	0.102	0.210	42.108	0.487	0.628	-8.360	107.100	48.910	-0.078	0.938
	Days in fridge	-0.155	0.179	228.097	-0.866	0.387	-0.264	0.187	67.914	-1.406	0.164	12.680	121.900	78.130	0.104	0.917
	Catch Time	0.045	0.161	228.854	0.279	0.780	0.123	0.187	75.298	0.658	0.513	88.540	115.770	80.730	0.765	0.447
	SampleYear2018	0.147	0.238	226.562	0.615	0.539	0.242	0.299	83.252	0.810	0.420	79.380	171.340	78.680	0.463	0.644
	SampleYear2019	-0.099	0.306	228.897	-0.325	0.746	-0.173	0.392	80.974	-0.440	0.661	94.310	232.050	79.830	0.406	0.686
	SampleYear2020	0.479	0.398	226.558	1.203	0.230	0.331	0.478	76.291	0.693	0.491	-99.300	288.020	80.150	-0.345	0.731
	SampleYear2021	-0.164	0.304	228.678	-0.538	0.591	0.205	0.370	69.594	0.553	0.582	99.280	232.440	78.440	0.427	0.671
	SampleYear2022	0.087	0.300	218.106	0.291	0.771	0.333	0.346	83.825	0.964	0.338	283.560	198.730	78.970	1.427	0.158
	SampleYear2023						0.514	0.388	83.636	1.325	0.189	141.400	234.950	80.630	0.602	0.549

Table S3.2. The standardised effect sizes (partial R^2) of the relationship between gut microbiome alpha diversity (richness) and variation in host (A) Major histocompatibility complex (MHC) diversity and (B) the presence/absence of specific MHC alleles in Seychelles warblers (Table 3.1 in main text).

Model	Predictor	16S ASV diversity			Metagenomics taxonomic diversity			Metagenomics functional diversity		
		Rsq	upper.CL	lower.CL	Rsq	upper.CL	lower.CL	Rsq	upper.CL	lower.CL
A) MHC Diversity	Model	0.088	0.202	0.067	0.128	0.357	0.114	0.169	0.397	0.140
	Heterozygosity	0.003	0.032	<0.001	0.011	0.089	<0.001	0.003	0.066	<0.001
	MHC-I Diversity	0.006	0.039	<0.001	<0.001	0.052	<0.001	0.034	0.139	<0.001
	MHC-II Diversity	<0.001	0.021	<0.001	0.028	0.125	<0.001	0.001	0.058	<0.001

	Age	0.007	0.043	<0.001	0.013	0.094	<0.001	0.049	0.165	0.001
	Season (summer)	<0.001	0.021	<0.001	0.008	0.081	<0.001	0.001	0.057	<0.001
	Sex (male)	0.024	0.075	0.001	0.006	0.074	<0.001	0.001	0.060	<0.001
	Days at 4°C	<0.001	0.020	<0.001	<0.001	0.052	<0.001	0.004	0.069	<0.001
	Time of day	<0.001	0.020	<0.001	0.024	0.119	<0.001	0.003	0.068	<0.001
	Sample Year (2018)	0.036	0.110	0.015	0.043	0.207	0.024	0.064	0.236	0.032
B) Presence/absence of MHC alleles	Model	0.130	0.273	0.122	0.090	0.309	0.082	0.139	0.359	0.109
	Heterozygosity	<0.001	0.022	<0.001						
	Ase-dab3	0.013	0.055	<0.001						
	Ase-dab4	0.011	0.050	<0.001						
	Ase-dab5	0.004	0.036	<0.001						
	Ase-ua1	0.002	0.027	<0.001						
	Ase-ua3	0.001	0.025	<0.001						
	Ase-ua4	0.006	0.041	<0.001						
	Ase-ua5	0.001	0.024	<0.001						
	Ase-ua6	0.004	0.034	<0.001						
	Ase-ua7	0.002	0.029	<0.001						
	Ase-ua8	<0.001	0.021	<0.001						
	Ase-ua9	0.001	0.023	<0.001						
	Ase-ua11	0.015	0.059	<0.001	0.002	0.060	<0.001	0.003	0.065	<0.001
	Age	0.010	0.049	<0.001	0.026	0.121	<0.001	0.065	0.188	0.004
	Season	<0.001	0.021	<0.001	0.004	0.068	<0.001	<0.001	0.054	<0.001
	Sex	0.028	0.081	0.002	0.001	0.056	<0.001	0.007	0.081	<0.001
	Days at 4°C	<0.001	0.022	<0.001	0.001	0.057	<0.001	0.004	0.071	<0.001
	Time of day	0.003	0.030	<0.001	0.028	0.126	<0.001	0.003	0.065	<0.001
	Sample Year	0.031	0.103	0.012	0.037	0.198	0.022	0.074	0.248	0.036

Table S3.3. Differentially abundant eggNOG members with increasing MHC-I Diversity in the gut microbiome of the Seychelles warblers (n = 99 from 57 birds). Categories are COG functional categories: E - Amino acid transport and metabolism, G - Carbohydrate transport and metabolism,

I - Lipid transport and metabolism, J – Translation, ribosomal structure and biogenesis, K - Transcription, L - Replication, recombination and repair, M - Cell wall/membrane/envelope biogenesis, O - Posttranslational modification, protein turnover, chaperones, P - Inorganic ion transport and metabolism, R - General function prediction only, T - Signal transduction mechanisms, V - Defense mechanisms.

eggNOG members	Direction of log fold change	Annotation	Category	KEGG pathways	KEGG pathway names
COG0008	Negative	Glutamyl- or glutaminyl-tRNA synthetase	J	K01885, K01886	glutamyl-tRNA synthetase [EC:6.1.1.17] & glutaminyl-tRNA synthetase [EC:6.1.1.18]
COG0028	Negative	Acetolactate synthase large subunit or other thiamine pyrophosphate-requiring enzyme	E H	K01652	acetolactate synthase I/II/III large subunit [EC:2.2.1.6]
COG0085	Negative	DNA-directed RNA polymerase, beta subunit/140 kD subunit	K	K03043	DNA-directed RNA polymerase subunit beta [EC:2.7.7.6]
COG0258	Negative	5'-3' exonuclease Xni/ExoIX (flap endonuclease)	L	K02335, K04799	DNA polymerase I [EC:2.7.7.7] & RAD2; flap endonuclease-1 [EC:3.1.-.-]
COG0318	Negative	O-succinylbenzoic acid-CoA ligase MenE or related acyl-CoA synthetase (AMP-forming)	I	K00666	fatty-acyl-CoA synthase [EC:6.2.1.-]

COG0443	Negative	Molecular chaperone DnaK (HSP70)	O	K04043, K03283	molecular chaperone DnaK & heat shock 70kDa protein 1/6/8
COG0477	Negative	MFS family permease, includes anhydromuropeptide permease AmpG	G E P R	None	
COG0488	Negative	ATPase components of ABC transporters with duplicated ATPase domains	R	K06158, K15738	ATP-binding cassette, subfamily F, member 3 & ABC transport system ATP-binding/permease protein
COG0507	Negative	ATPase/5'-3' helicase helicase subunit RecD of the DNA repair enzyme RecBCD (exonuclease V)	L	K03581	exodeoxyribonuclease V alpha subunit [EC:3.1.11.5]
COG0513	Negative	Superfamily II DNA and RNA helicase	L	None	
COG0515	Negative	Serine/threonine protein kinase	T	K08832, K15409	serine/threonine-protein kinase SRPK3 [EC:2.7.11.1] & SRPK1 [EC:2.7.11.1]
COG0524	Negative	Sugar or nucleoside kinase, ribokinase family	G	K00852, K00847, K00874	ribokinase [EC:2.7.1.15] & fructokinase [EC:2.7.1.4] & 2-dehydro-3-deoxygluconokinase [EC:2.7.1.45]
COG0577	Positive	ABC-type antimicrobial peptide transport system, permease component	V	K02004	putative ABC transport system permease protein
COG0628	Positive	Predicted PurR-regulated permease PerM	R	None	

COG0642	Positive	Signal transduction histidine kinase	T	None	
COG0697	Negative	Permease of the drug/metabolite transporter (DMT) superfamily	G E R	None	
COG0745	Positive	DNA-binding response regulator, OmpR family, contains REC and winged-helix (wHTH) domain	K T	None	
COG0841	Positive	Multidrug efflux pump subunit AcrB	V	K18138	multidrug efflux pump
COG0845	Positive	Multidrug efflux pump subunit AcrA (membrane-fusion protein)	V M	None	
COG1502	Negative	Phosphatidylserine/phosphatidylglycerophosphate/cardiolipin synthase	I	K06131, K01115	cardiolipin synthase A/B [EC:2.7.8.-] & phospholipase D1/2 [EC:3.1.4.4]
COG2911	Positive	Phospholipid transport to the outer membrane protein TamB	M	K09800	translocation and assembly module TamB
COG3209	Positive	Uncharacterized conserved protein RhaS, contains 28 RHS repeats	R	None	
COG4886	Negative	Leucine-rich repeat (LRR) protein	K	None	
COG4974	Positive	Site-specific recombinase XerD	L	K04763	integrase/recombinase XerD

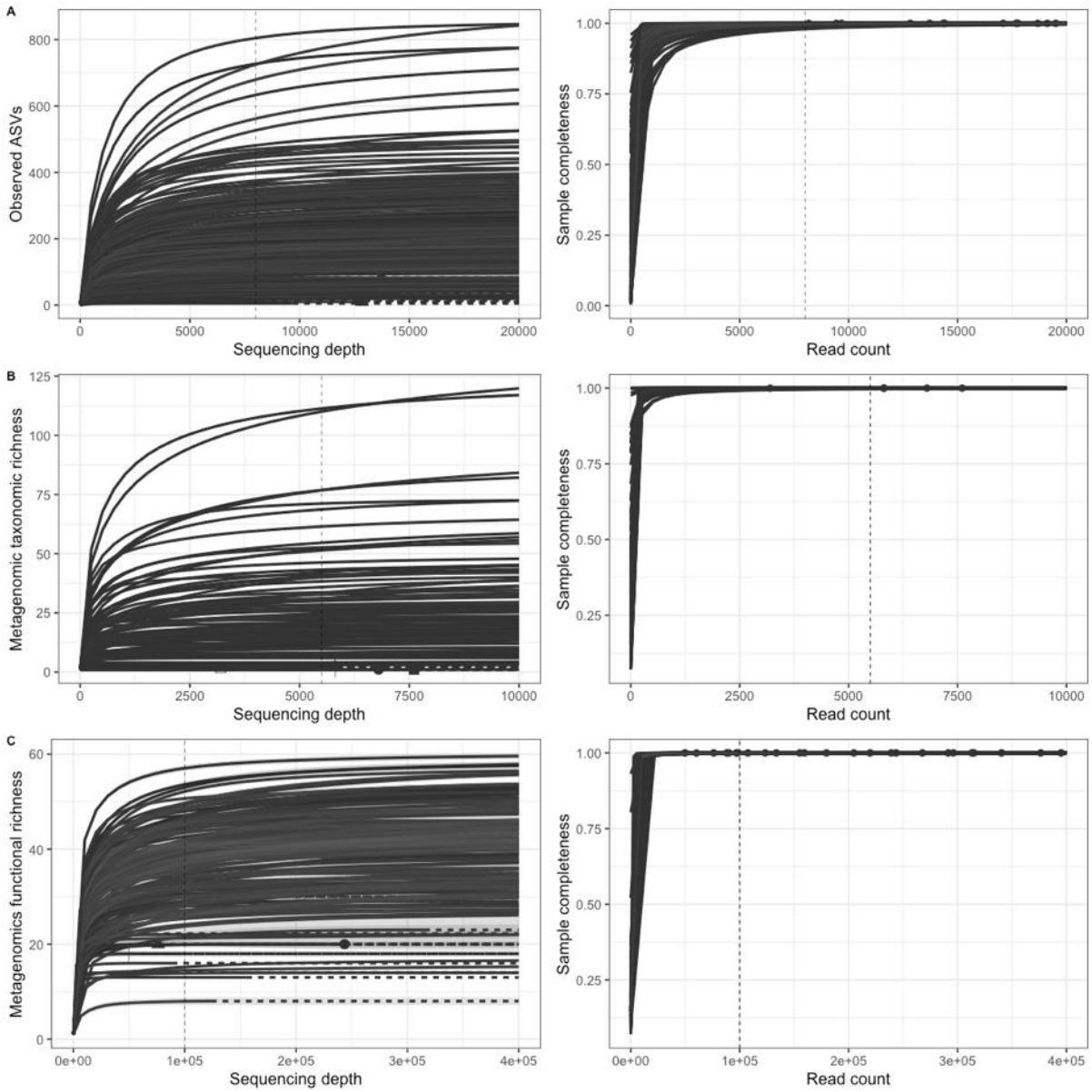


Figure S3.1. Rarefaction curve of 16S gut microbiome sequencing of the Seychelles warbler.

Chapter 4 |

Social interactions shape anaerobic, but not aerotolerant, gut microbiome composition in a cooperative breeding species

Image edited by Google Gemini.

4.1 Abstract

Background

Social transmission of microbes has profound impacts on disease epidemiology and host health. However, how social factors influence gut microbiome (GM) transmission in wild populations is not well understood. Here, we use a wild population of the Seychelles warbler, a facultative cooperatively breeding passerine, to determine whether cooperative breeding behaviour influences the GM. Specifically we hypothesis that close social interactions as part of cooperative breeding should encourage the sharing of anaerobic microbes, that may be less likely to transmit indirectly through the environment.

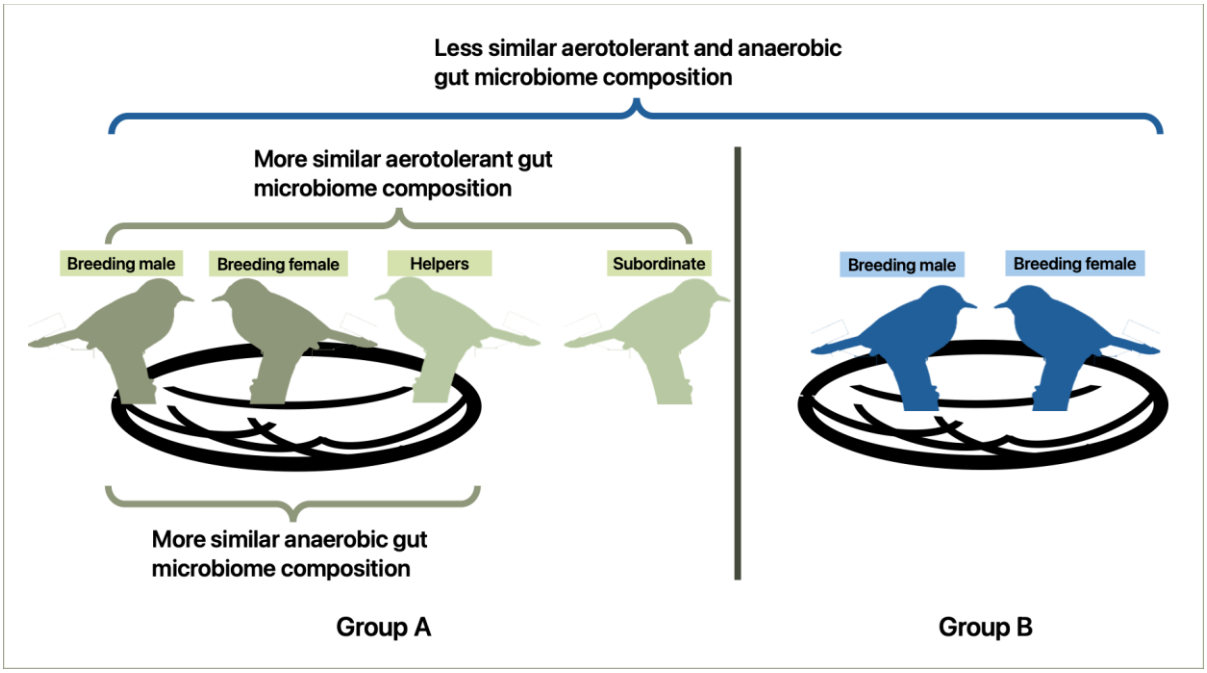
Results

We found that GM composition was more similar within versus between social groups, and this effect was driven by sharing both aerotolerant and anaerobic bacterial genera. GM diversity was also more similar between dominant individuals and helpers than between the dominant male and female mates within a breeding group. As predicted, the similarity of anaerobic, but not aerotolerant, GM communities between pairs of individuals within a group was positively correlated with the strength of their social interactions (defined by their cooperative breeding status). Specifically, anaerobic GM composition was more similar between pairs of individuals that cooperate at the nest (dominant breeders and dominant-helper pairs) than for non-cooperative pairs (involving non-helping subordinate individuals). This is likely because breeders and helpers directly interact while caring for offspring at a nest.

Conclusions

This work reveals how cooperative social interactions lead to microbial transmission and thus contribute to shaping specific components of a host's gut microbiome.

Keywords: *Acrocephalus sechellensis*; Cooperative breeding; Gut microbiome; social transmission; wild population.



Graphical abstract. Individuals within groups were more similar in aerotolerant and anaerobic gut microbiome composition than between groups. Breeders and helpers share more similar anaerobic gut microbiome composition than with subordinates.

4.2 Introduction

The vertebrate gut microbiota (GM) –the ecosystem of microbes that live within the gastrointestinal tract– plays a role in many important processes within the host, including metabolism, immune defences, and cognition (Corbin et al., 2023; Davies et al., 2022; Foster & McVey Neufeld, 2013; Zheng et al., 2020). In turn, many factors, such as host genetics, environment, and diet, are important in shaping the GM (Bonder et al., 2016; Davies et al., 2022; Grieneisen et al., 2021; Hicks et al., 2018). Consequently, the GM can vary significantly not just across species and populations but also across individuals within populations (Hicks et al., 2018). Individual variation in GM composition has been associated with host health, being linked to, for example, nutrient extraction and immune function in vertebrates and, therefore, survival and reproductive success in wild animals (Cholewińska et al., 2020; Worsley et al., 2021; Zheng et al., 2020).

Despite evidence of the GM's significant role in host health and fitness (de Vos et al., 2022; Gould et al., 2018), there are still substantial gaps in our understanding of the factors that shape individual variation in GM composition. Among the least understood, yet potentially most important, factors is host sociality. The microbial metacommunity within social networks of hosts (the social microbiome) needs to be investigated to understand how social microbial transmission impacts host health and disease (Sarkar et al., 2024). To date, most research on microbial transmission across social networks has focused on pathogens, neglecting commensal microbes (Sarkar et al., 2020). In most vertebrates, the GM is initially acquired through parental transmission and then quickly becomes shaped by a combination of direct (via physical contact) and indirect (via the environment) transmission (see (Sarkar et al., 2024)). However, it is often difficult to distinguish between these mechanisms as socially interacting individuals also normally share the same environment (Raulo et al., 2024).

In captivity, conspecifics that socially interact share a more similar GM composition than those that do not (Bensch et al., 2023; Hildebrand et al., 2013; Hufeldt et al., 2010). However, captive animals are exposed to much less microbial diversity than their wild counterparts, which likely contributes to greater microbial sharing.

Consequently, the GM of captive animals may be simpler (lower diversity and variation) than in nature and show many artefacts (Bensch et al., 2023). In contrast, wild animals encounter a much broader range of microbes due to factors such as exposure to other species, diverse and variable food sources, habitat and climatic variation and anthropogenic influences (Bensch et al., 2023; White et al., 2023). Very few studies have investigated the role of sociality in shaping the GM of wild animals, but see (Archie & Tung, 2015; Raulo et al., 2018, 2024). Most work has focused on differences in GM between social groups (Antwis et al., 2018; Bennett et al., 2016; Raulo et al., 2018; Theis et al., 2012; Tung et al., 2015), but now we need to understand the links between GM and the degree of sociality within highly social animals.

Social organisation has also been associated with the microbiome communities of social insects (Gamboa et al., 2025; Jones et al., 2018; Shimoji et al., 2021) and non-group-living mice (Raulo et al., 2021, 2024), with individuals that interact more frequently having more similar microbial communities. Socially acquired GM similarity is likely driven by having a shared environment (indirect) and repeated social interactions (direct), such as grooming, food sharing and close contact (including copulations), which facilitate microbial transmission (Dill-McFarland et al., 2019; Raulo et al., 2018, 2024). Related individuals that are from the same social group also have a more similar GM composition than unrelated individuals, highlighting the importance of host genetics in shaping the microbiome in groups (Grieneisen et al., 2021; Roche et al., 2023; Turnbaugh et al., 2009).

Aerotolerance may play a significant role in determining the likelihood of environmental versus direct transfer of microbial species (Raulo et al., 2024). Aerotolerant (aerobic and facultatively anaerobic) bacteria may grow outside the host and are therefore more likely to survive long enough to undergo indirect environmental social transmission (Mazel et al., 2024). By contrast, anaerobic bacteria survive less well outside the body and are likely limited to vertical and close-contact transmission (Mazel et al., 2024; Moeller et al., 2018). Consistent with this, a couple of studies have suggested that social proximity facilitates the transfer of anaerobic bacteria (Dill-McFarland et al., 2019; Raulo et al., 2024).

Some group-living vertebrates practice cooperative breeding, whereby additional adult group members provide care to offspring produced by a limited number of breeders (often just a dominant pair) (Cockburn, 1998; García-Ruiz et al., 2022; Koenig & Dickinson, 2016). Such subordinate 'helpers' enable dominant breeders to increase their reproductive success, while potentially providing the helpers with inclusive fitness benefits (including indirect (kin-selected) and direct benefits (e.g (Cockburn, 1998; Koenig & Dickinson, 2016; Richardson et al., 2002)). These 'helpers' interact closely with the breeders, potentially facilitating the direct transmission of microbes (Sarkar et al., 2024). However, given that helpers normally share the same space/territory and may be genetically related to the dominants (Cockburn, 1998), separating the role of direct and indirect transmission in shaping the GM can be difficult. Research using suitable cooperative systems which allow these routes of transmission to be untangled and better understood is now needed.

Here, we use the facultatively cooperative breeding Seychelles warbler (*Acrocephalus sechellensis*) to assess how cooperative interactions shape individual GM variation. This system enables us to disentangle the effects of genetic relatedness from social interactions, as subordinates vary extensively in how related they are to the dominant breeders due to the frequent dispersal of offspring into non-natal groups to become subordinates (Groenewoud et al., 2018), and even subordinates within their natal group being the result of extra-pair paternity (Hadfield et al., 2006) and/or cobreeding (Raj Pant et al., 2019). In addition, as warblers are tree-foraging insectivores, they are rarely exposed to other conspecifics' faeces, thus limiting non-contact horizontal transfer post-fledging. The insects they eat typically contain a high proportion of aerotolerant bacteria (Engel & Moran, 2013; Yun et al., 2014). Therefore, we hypothesise that warblers will share aerotolerant bacteria through a shared environment, whereas close physical contact is needed to transfer anaerobic bacteria. We test the following predictions: (1) Individuals sharing a territory have more similar GM than those who do not. (2) Individual GM similarity is correlated with the closeness of the social relationship within the cooperative breeding system. (3) The cooperative relationship between individuals will more strongly affect the anaerobic, rather than the aerotolerant, GM components.

4.3 Materials and Methods

4.3.1 Study systems

The Seychelles warbler population on Cousin Island (29 ha; 04° 20′ S, 55° 40′ E) has consisted of ca. 320 individuals from ca. 115 territories since 1985 (Brouwer et al., 2009; Kingma et al., 2016). This population has been extensively monitored during the minor (January–March) and major (June–October) breeding season each year, with the major season accounting for 94% of breeding (Brown et al., 2022; Hammers et al., 2015; Komdeur, 1992). Since 1997, nearly all individuals (>96%) have been uniquely marked with a combination of three colour rings and a British Trust for Ornithology metal ring (Davies et al., 2021; Hammers et al., 2015). The age of individuals is determined during their first catch, either directly when accessing them in the nest, or as begging fledglings, or using their eye colour (Komdeur, 1992). Individuals almost never disperse between islands (Komdeur, Piersma, et al., 2004) and the annual resighting rate is high (98% ± 1% SE) (Raj Pant et al., 2020; Richardson et al., 2001).

Seychelles warblers often breed successfully in socially monogamous pairs (Komdeur, 1996). Individuals who attain a breeding position typically remain in the same territory, defending it with the same partner until their death (Richardson et al., 2007). However, due to a shortage of suitable breeding opportunities, some individuals delay independent breeding and become subordinates, often, but not always, in their natal territory (Groenewoud et al., 2018; Komdeur, 1992). In any given breeding event, some subordinates (20% males and 42% females (Hammers et al., 2019)) contribute to alloparental care (defined as ‘helpers’), assisting with incubation (only females) and provisioning (both sexes), while others do not (non-helper subordinates) (Komdeur, 1992). Helpers benefit by gaining breeding experience, through indirect fitness benefits (kin-selected). Each season, every group member is given a breeding status: dominant male, dominant female, helper, non-helper subordinate. Breeding attempts normally produce single egg clutches (80%) (Richardson et al., 2001). Extra-group paternity occurs frequently (~44%) (Hadfield et al., 2006; Richardson et al., 2001). Fledglings leave the nest after 18-20 days but are provided with extended post-fledgling care for up to three months (Komdeur, 1996; Komdeur et al., 2016; Richardson et al., 2001).

Genetic relatedness of individuals within a group varies considerably (mean 0.26 ± 0.23 SD, range 0.00-0.77) because, (a) not all subordinates are from the natal territory (Komdeur, 1992), (b) subordinates hatched in the territory may be the result of extra-pair paternity (Hadfield et al., 2006; Richardson et al., 2001) or subordinate maternity (Raj Pant et al., 2019; Richardson et al., 2001, 2002) and (c) dominant breeders are replaced over time when individuals die or are deposed (Richardson et al., 2007).

4.3.2 Sample collection

Faecal samples were collected from 2017-2022 across ten breeding seasons (Worsley, Davies, et al., 2024). Birds were captured in mist nets and placed in a clean disposable flat-bottom paper bag containing a sterile metal grate covering a sterile plastic tray. This established protocol (Davies et al., 2022; Knutie & Gotanda, 2018) allows and any faecal sample that is produced by the bird to fall onto the plastic tray, minimising contact with the outside of the bird and the bag. After defaecation (ca. 15 min), the bird was released and the sample collected using a sterile flocked swab and placed in 1ml of absolute ethanol in a sterile screw-cap microcentrifuge tube. Control microbiome samples were taken from each fieldworker's hands by swabbing with a sterile flocked swab. Samples were stored at 4°C during the field season and transferred to -80°C for long-term storage on reaching UEA. The time-of-day of each sample was recorded (minutes after sunrise – 06.00 h GMT+4), and the number of days between sampling and -80°C storage was recorded. A blood sample (ca. 25µl) was collected through brachial venipuncture and stored in 1ml of absolute ethanol at 4°C.

4.3.3 Molecular methods

Total genomic DNA was extracted from faecal samples using the Qiagen DNeasy PowerSoil Kit with a modified version of the manufacturer's protocol (see (Davies et al., 2022)). To minimise batch effects of extraction, samples were randomised. DNA was submitted for 16S rRNA amplicon sequencing using the amplicon libraries of

V4 primers 515F (5'TGCCAGCMGCCGCGGTAA3') and 806R (5'GGACTACHVGGGTWTCTAAT3') and sequenced across seven batches using 2x250bp, paired-end sequencing on an Illumina MiSeq Platform (see detailed methodology in (Davies et al., 2022; Worsley, Davies, et al., 2024)). Control samples were also extracted and sequenced this way (n=21 hand controls, 15 negative controls, and 10 positive, ZymoBIOMICS Microbial Community Standard (D6300), controls).

DNA had previously been extracted from blood with the DNeasy blood and tissue kit (Qiagen) and used in molecular sexing (Griffiths et al., 1998; Sparks et al., 2022) and microsatellite genotyping for parentage analyses (Richardson et al., 2001; Sparks et al., 2022). All offspring hatched between 1991 and 2022 (2282 offspring, 1935 (85%) mothers, 2016 (88%) fathers had been assigned parentage at >80% confidence using *MasterBayes* 2.52 as part of previous studies (detailed in (Edwards et al., 2018; Hadfield et al., 2006; Sparks et al., 2022)). Relatedness between individuals was calculated from the *MasterBayes* pedigree using *sequoia* 2.11.4 in R Studio 2024.12.0+467 (Huisman, 2017; Posit team, 2024; R Core Team, 2024).

4.3.4 Bioinformatics

The processing of DNA reads followed previously described steps using QIIME2 2019.10 (Bolyen et al., 2019; Worsley, Davies, et al., 2024). In brief, read truncation, filtering and classification into amplicon sequencing variants (ASV) was undertaken using DADA2 (Callahan et al., 2016). Taxonomic assignment of ASVs was performed using the naïve-Bayes classifier on the SILVA 132 reference database (Quast et al., 2012). The resulting ASVs were imported to R using *phyloseq* 1.46.0 (Leo Lahti & Sudarshan Shetty, 2019; McMurdie & Holmes, 2013). Samples were filtered to remove non-bacterial sequences, reads not assigned to phylum level, and potential contaminants (based on hand and lab controls). Based on evidence from rarefaction curves showing sample completeness of 95% at 8000 reads (Worsley, Davies, et al., 2024), 27 faecal samples with less than 8000 reads were removed. ASVs that had fewer than 50 reads across all samples were also removed, as these represented possible sequencing errors.

The aerotolerance status of each bacterial genus (1111 genera) was assigned using both Google Gemini 2.0 and ChatGPT 3.5 on 21st January 2025. The text used was "Assign aerotolerance status for the following genera", followed by the list of genera. Google Gemini returned a table of genera and aerotolerance statuses, while ChatGPT responded with text. 'Facultative anaerobic' and 'Aerobic' were categorised as 'Aerotolerant', 'Anaerobic' was categorised as 'Anaerobic', and everything else was categorised as 'Unknowns'. After excluding unknown or unassigned genera (n = 891 genera assigned), the accuracy of these assignments was checked by comparing the assignments obtained with the manually assigned genera in Raulo et al. (2024) using Bergey's Manual of Systematics of Archaea and Bacteria (Trujillo et al., 2015). The correspondence to the previous manual assignment in Raulo et al. (2024) using Google Gemini was 92.5% and ChatGPT was 74.2% (n =160 or n = 98 genera, respectively). However, the assignments in (Raulo et al., 2024) could also have been incorrect or out of date. So, in addition, 80 random genera were manually checked using Bergey's Manual of Systematics of Archaea and Bacteria (Trujillo et al., 2015) by CL, and the correspondence was 96.3% for Google Gemini and 73.4% for ChatGPT. The assignments from Google Gemini were therefore used for subsequent analysis.

4.3.5 Statistics

4.3.5.1 GM similarity within and between breeding groups

4.3.5.1.1 Alpha diversity

Both ASV richness and Shannon diversity were calculated for each sample (after rarefication) using *phyloseq* 1.46.0 (McMurdie & Holmes, 2013). A pairwise alpha diversity difference was calculated for ASV richness and Shannon diversity, which were made negative to reflect alpha diversity similarity. Importantly, samples were then filtered to include only sample pairs from individuals from the same field period (n = 27,821 pairwise comparisons across 648 samples from 345 birds) to control for temporal variation. A linear mixed effect multi-membership model (*lmer* with *lmerMultiMember*) using *lme4* 1.1-35.5 (Bates et al., 2015) was used to test whether the difference in alpha diversity was smaller when pairs were from the same breeding group than between breeding groups. Breeding group status (within a group, between groups), the age difference of individuals (0-16.7 years), sex

difference (no/yes), the number of days apart samples were collected (0-97 days), the difference in the time of day samples were collected (0-634 minutes), season (minor/major), present in nest at hatch (whether one individual was present in the other's nest at hatch e.g. as a sibling, helper or parent), and relatedness were included as explanatory variables. Sample year and a multi-membership ID (calculated using *lmerMultiMember* to account for the repeated occurrences of individual ID in both columns, and suitable for dyadic models (van Paridon et al., 2023)), were used as random variables. Hereafter, all models included the same explanatory and random variables unless stated otherwise. Variance inflation factor (VIF) scores were computed to test for collinearity among the terms (all VIF scores were <3).

4.3.5.1.2 GM composition

Differences in GM composition were modelled using the same pairwise approach as for Alpha diversity. Unrarefied raw reads were filtered to remove rare taxa (<5% occurrence), and then centred log ratio (CLR) transformed using *microbiome* 1.20.0, which controls for differences in library size and is suitable for compositional datasets (Gloor et al., 2017). A pairwise Aitchinson distance matrix was then calculated using *phyloseq* 1.46.0 (Callahan et al., 2016; McMurdie & Holmes, 2013), which was made negative to reflect GM composition similarity. A multi-membership lmer was used to test if samples from individuals within a group had more similar GM composition compared to those outside of the group, where GM Aitchison distance was used as a response variable and the explanatory and random variables were as described for alpha diversity above.

4.3.5.1.3 Aerotolerance

Bacterial taxa were split into an anaerobic dataset (205 anaerobic genera), and an aerotolerant dataset (686 aerotolerant genera). The same model structure (between/within breeding group GM composition model) was used to determine if within-group changes in GM composition were dependent on aerotolerance capability.

4.3.5.2 The GM and social status categories

4.3.5.2.1 Alpha diversity

A second alpha diversity model was constructed as above but replacing breeding group status with individual status. Pairs of samples were filtered from distance matrices to only include comparisons made within the same breeding group (n = 279 pairwise comparisons across 322 samples from 204 individual birds). There were five groupings for individual status pairs: (1) dominant breeding pair (Dom-Dom), (2) breeders—helpers (Dom-Help), (3) dominant breeders— other subordinates (Dom-Sub), (4) helpers—other subordinates (Help-Sub), (5) subordinates—subordinates (Sub-Sub). If the overall individual status pair predictor term was significant, a post-hoc pairwise comparison was performed using a Tukey test.

4.3.5.2.2 Overall GM composition

A social status category model was constructed (as above) to assess the impact of individual status on GM composition by replacing breeding group status with individual status comparisons and restricting comparisons to within-breeding group.

4.3.5.2.3 Aerotolerance vs. Anaerobic GM composition

The same model structure as directly above was used to test whether patterns of GM variation associated with within-group social status categories differed according to bacterial aerotolerance capability. Finally, the same model was run but lumping the within group social status categories to compare all categories that involved the pair of individuals interacting at a shared nest (Dom-Dom and Dom-Help combined) with all pairs that did not (Dom-Sub, Help-Sub, Sub-Sub combined), using the same model structure as above.

4.4 Results

4.4.1 GM similarity within versus between breeding groups

4.4.1.1 Alpha diversity

The observed ASV richness and Shannon diversity similarity did not significantly differ between pairs of individuals from within the same breeding group versus pairs from different breeding groups (Table S4.1, Table 4.1). ASV richness and Shannon diversity similarity did decline as the number of days between sampling points increased (Table S4.1, Table 4.1). Shannon diversity similarity was also marginally associated with season (positively) and time in season (negatively) (Table 4.1).

Table 4.1. A linear mixed effect model (lmer) investigating the relationship between breeding group membership and gut microbiome ASV Shannon diversity similarity in pairs of Seychelles warblers (N = 27,821 pairwise comparisons across 648 samples from 345 individual birds). Significant terms (P <0.05) are in bold, marginal terms (P<0.10) in italics. Reference categories for categorical variables were the first term in brackets. Time of day was measured as minutes apart, and time in season was measured as days apart.

Characteristic	**Beta**	**SE**[1]	**Statistic**	**df**	**p-value**
(Intercept)	**-1.279**	**0.072**	**-17.7**	**12.5**	**<0.001**
Breeding group (Between/Within)	-0.012	0.058	-0.206	27,548	0.837
Age difference	0.001	0.003	0.496	24,508	0.620
Sex (same/different)	-0.006	0.011	-0.567	27,560	0.571
Season (major/minor)	*-0.065*	*0.033*	*-1.94*	*1,654*	*0.053*
Time of day	*<0.001*	*<0.001*	*-1.96*	*27,712*	*0.050*
Time in season	**-0.001**	**<0.001**	**-3.98**	**27,775**	**<0.001**
Relatedness	-0.029	0.087	-0.333	27,582	0.739
Shared nest at hatch (no/yes)	-0.010	0.025	-0.381	26,525	0.703
Random	**27,821 observations**			**Variance**	
Multi membership ID (Intercept)		345 groups			0.374
Sample Year (Intercept)		6 years			0.137

Residual	0.880

4.4.1.2 GM composition

Pairs within breeding groups had a more similar GM composition than pairs in different breeding groups (Table 4.2, Figure 4.1). Additionally, pairs sampled in the minor season had a more similar GM composition compared to pairs sampled in the major season (Table 4.2). GM composition became increasingly different between individuals as the number of days between sampling of each of the pair increased. Moreover, individuals that shared a nest at hatch (including from different seasons; as either siblings, parents or helpers) had a significantly more similar GM composition.

Table 4.2. A linear mixed effect model investigating gut microbiome composition similarity in Seychelles warbler pairs from the same versus pairs from different breeding groups (N = 27821 pairwise comparisons across 648 samples from 345 individual birds). Significant terms (P <0.05) are in bold. Reference categories for categorical variables were the first term in the brackets. Time of day was measured as minutes apart, and time in season was measured as days apart.

Characteristic	**Beta**	**SE**[1]	**Statistic**	**df**	**p-value**
(Intercept)	**-83.21**	**2.38**	**-35.0**	**6.17**	**<0.001**
Breeding group Pair (Between/Within)	**3.683**	**0.581**	**6.34**	**27,490**	**<0.001**
Age difference	0.016	0.028	0.556	27,767	0.578
Sex (same/different)	-0.123	0.109	-1.13	27,493	0.259
Season (major/minor)	**2.062**	**0.353**	**5.84**	**25,345**	**<0.001**
Time of day	<0.001	<0.001	-0.304	27,572	0.761
Time in season	**-0.007**	**0.003**	**-2.08**	**27,590**	**0.038**
Relatedness	0.494	0.870	0.568	27,502	0.570
Shared nest at hatch (no/yes)	**0.538**	**0.257**	**2.09**	**27,806**	**0.036**
Random	**27,821 observations**				**Variance**

Multi membership ID	(Intercept)	345 groups	6.898
Sample Year	(Intercept)	6 years	5.514
Residual			8.808

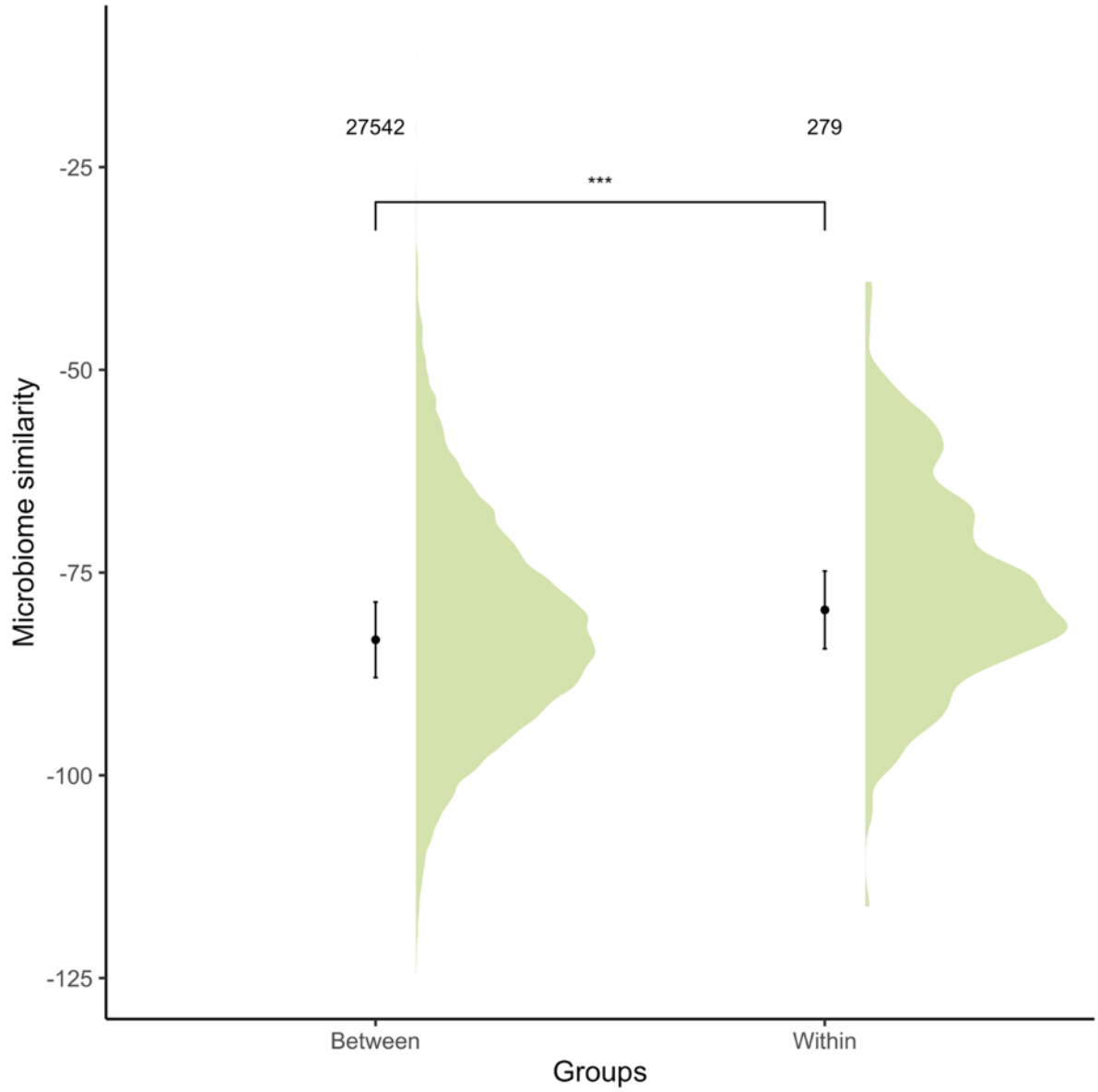


Figure 4.1. Gut microbiome composition similarity of pairs of individuals from the same versus pairs of individuals taken from different breeding groups in the Seychelles warbler (N = 27821 pairwise comparisons across 683 samples from 345 individual birds). Dots and lines represent model predictions with 95% confidence intervals calculated from lmer models. The density plot represents the distribution of raw data. *** represent p<0.001.

4.4.1.3 Aerotolerant vs. Anaerobic bacteria

Considering aerotolerant bacterial genera, GM compositional similarity was significantly higher in pairs from the same breeding group compared to pairs from different breeding groups (Table 4.3). Aerotolerant GM composition was also significantly less similar with increasing age differences, time of day difference, and time in season difference, but more similar if the pair shared a nest at hatch (Table 4.3).

Table 4.3. A linear mixed effect model (lmer) investigating the relationship between **aerotolerant** gut microbiome composition similarity in pairs of Seychelles warblers from the same breeding group versus pairs generated from individuals sampled from different breeding groups (N = 27821 pairwise comparisons across 648 samples from 345 individual birds). Significant terms (P <0.05) are in bold. Reference categories for categorical variables were the first term in the bracket. Time of day was measured as minutes apart, and time in season was measured as days apart.

	Estimate	SE	df	t	P
(Intercept)	**-46.49**	**1.10**	**7.04**	**-42.4**	**<0.001**
Breeding group Pair (Between/Within)	**1.957**	**0.325**	**27,489**	**6.02**	**<0.001**
Age difference	**-0.098**	**0.007**	**27,603**	**-13.3**	**<0.001**
Sex (same/different)	-0.019	0.061	27,492	-0.317	0.752
Season (major/minor)	0.273	0.197	22,916	1.38	0.167
Time of day	**<0.001**	**<0.001**	**27,566**	**-2.13**	**0.033**
Time in season	**-0.006**	**0.002**	**27,583**	**-3.41**	**0.001**
Relatedness	0.756	0.486	27,498	1.55	0.120
Shared nest at hatch (no/yes)	**0.312**	**0.145**	**27,803**	**2.16**	**0.031**
Random	**27821 observations**			**Variance**	
Multi membership ID	(Intercept)	345 groups			16.018
Sample Year	(Intercept)	6 years			6.029

Residual	24.243

Considering only anaerobic bacterial genera, pairs within the same breeding group had more similar GM compositions compared to pairs from separate breeding groups (Table 4.4). The anaerobic GM composition was significantly negatively associated with increasing time of day difference, and time in season difference but more similar if the pair shared a nest at hatch (Table 4.4).

Table 4.4. A linear mixed effect model (lmer) investigating the relationship between **anaerobic** gut microbiome composition similarity in pairs of Seychelles warblers from the same breeding group versus pairs generated from individuals sampled in different breeding groups (N = 27821 pairwise comparisons across 648 samples from 345 individual birds). Significant terms (P <0.05) are indicated in bold. Reference categories for categorical variables were the first term in brackets. Time of day was measured as minutes apart, and time in season was measured as days apart.

	Estimate	SE	df	t	P
(Intercept)	**-24.53**	**0.807**	**6.45**	**-30.4**	**<0.001**
Breeding group Pair (Between/Within)	**0.844**	**0.285**	**27,179**	**2.96**	**0.003**
Age difference	-0.002	0.006	27,370	-0.366	0.714
Sex (same/different)	0.061	0.053	27,185	1.14	0.255
Season (major/minor)	-0.247	0.170	19,017	-1.45	0.147
Time of day	**-0.001**	**0.000**	**27,310**	**-3.38**	**0.001**
Time in season	**-0.007**	**0.002**	**27,337**	**-4.34**	**<0.001**
Relatedness	-0.431	0.425	27,196	-1.01	0.310
Shared nest at hatch (no/yes)	**0.266**	**0.126**	**27,326**	**2.11**	**0.035**

Random	**27,821 observations**		**Variance**
Multi membership ID	(Intercept)	345 groups	6.342
Sample Year	(Intercept)	6 years	3.408
Residual			18.298

4.4.2 The GM and within-group social status categories

4.4.2.1 Alpha diversity

We assessed similarity in ASV richness (Table S4.2) and Shannon diversity (Table S4.3) between pairs of birds with different statuses within the same breeding group. Only Shannon diversity was significantly more similar for dominant-helper status pairs than for dominant pairs (Table S4.3, Figure 4.2). All other pairwise comparisons were not significantly different from each other (Tables S4.2, S4.3 & S4.4) and lower than for dominant-helper status pairs.

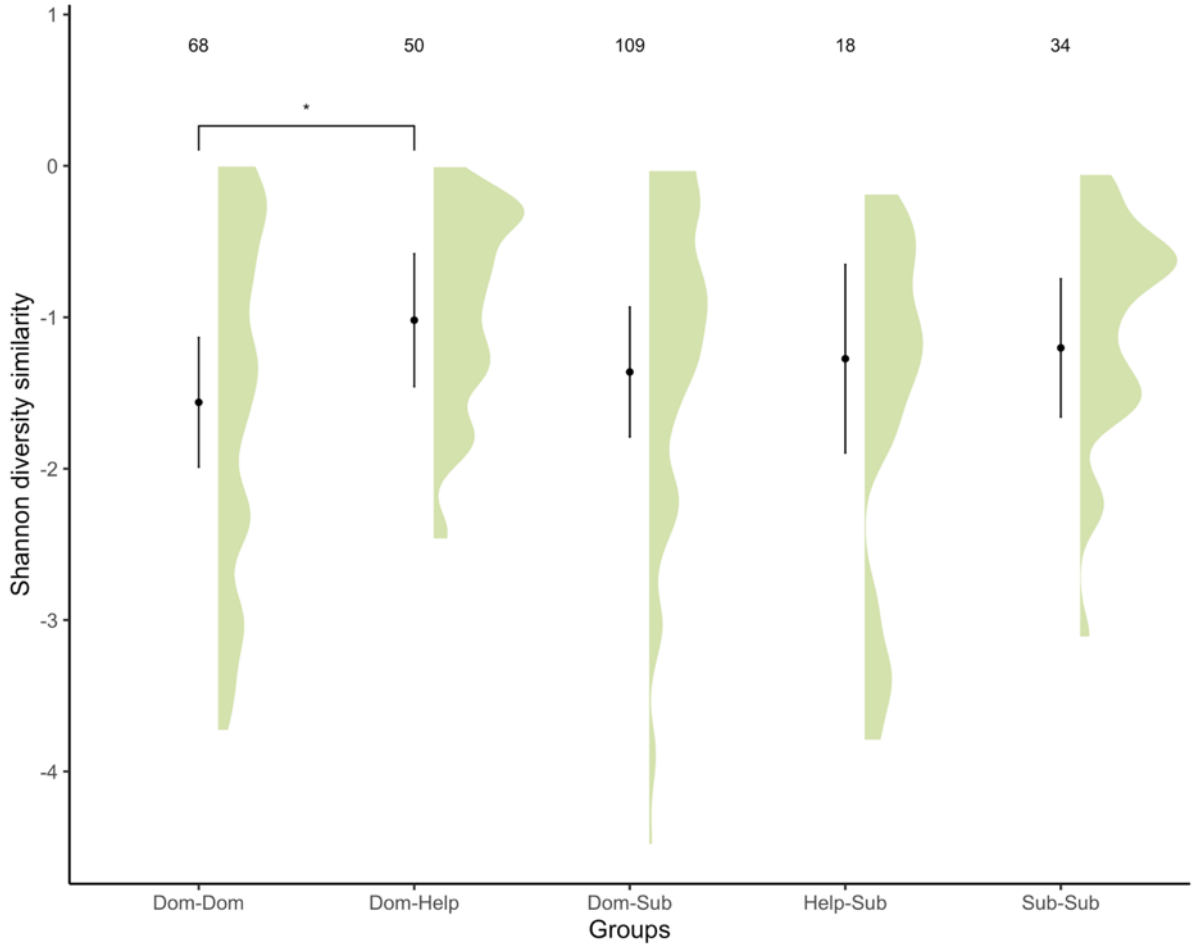


Figure 4.2. Gut microbiome Shannon diversity similarity of different breeding group status pairs of Seychelles warblers. Dots and lines represent model predictions with 95% confidence intervals calculated from lmer models. The density plot represents the distribution of raw data. N = 279 pairwise comparisons across 322 samples from 204 individual birds.

4.4.2.2 Overall GM composition

None of the social status pair categories significantly differed in overall GM composition similarity (Table S4.5).

4.4.2.3 Aerotolerant vs. Anaerobic GM composition

Pairwise similarities in aerotolerant GM composition did not differ between social status pair categories (Table S4.6). The only significant effect in this model was a negative association between aerotolerant GM composition similarity and increasing differences in host age (Table S4.6).

In contrast, anaerobic GM composition similarity did significantly differ between social status pair categories (Table 4.5, Figure 4.3). Specifically, the anaerobic GM compositional similarity of dominant-dominant and dominant-helper categories did not differ (Table 4.5, Figure 4.3). However, anaerobic GM composition was significantly more similar in dominant-dominant pairs than for pairs in the other three categories (dominant-subordinate (marginal), helper-subordinate, and subordinate-subordinate pairs) (Table 4.5, Figure 4.3). The anaerobic GM composition was not significantly different in all other pairwise comparisons (Table S4.7).

Finally, when combining the nest-sharing pairs and the non-nest-sharing pairs into two overall categories, anaerobic GM composition similarity was higher for nest-sharing pairs (Dom-Dom and Dom-Help) than for non-nest-sharing pairs (Dom-Sub, Help-Sub, Sub-Sub) (Estimate=-2.317, p=0.003, Table S4.8, Figure 4.3).

Table 4.5. A linear mixed effect model (lmer) investigating the relationship between individual breeding group status pairs and **anaerobic** GM composition similarity of Seychelles warblers (N = 279 pairwise comparisons across 320 samples from 204 individual birds). Significant terms (P <0.05) are indicated in bold, marginal terms (P <0.1 are indicated in italics. Reference categories for categorical variables were the first term in brackets. Time of day was measured as minutes apart, and time in season was measured as days apart.

Characteristic	**Beta**	**SE**[1]	**Statistic**	**df**	**p-value**
(Intercept)	**-22.44**	**1.30**	**-17.3**	**39.0**	**<0.001**
Individual Status Pair					
Dom - Dom	—	—	—		
Dom - Help	-0.661	1.23	-0.539	209	0.590
Dom - Sub	*-2.231*	*1.14*	*-1.96*	*194*	*0.051*
Help - Sub	**-3.483**	**1.63**	**-2.13**	**160**	**0.034**
Sub - Sub	**-3.319**	**1.34**	**-2.47**	**189**	**0.014**
Age difference	0.009	0.067	0.135	258	0.893
Sex (same/different)	0.335	0.735	0.456	239	0.649
Season (major/minor)	0.049	1.05	0.046	91.8	0.963
Time of day	-0.002	0.003	-0.591	250	0.555

Time in season	0.001	0.018	0.083	260	0.934
Relatedness	1.622	1.82	0.893	194	0.373
Shared nest at hatch (no/yes)	-0.283	0.863	-0.328	233	0.743
Random		**274 observations**			**Variance**
Multi membership ID	(Intercept)	204 groups			1.836
Sample Year	(Intercept)	6 years			1.576
Residual					4.341

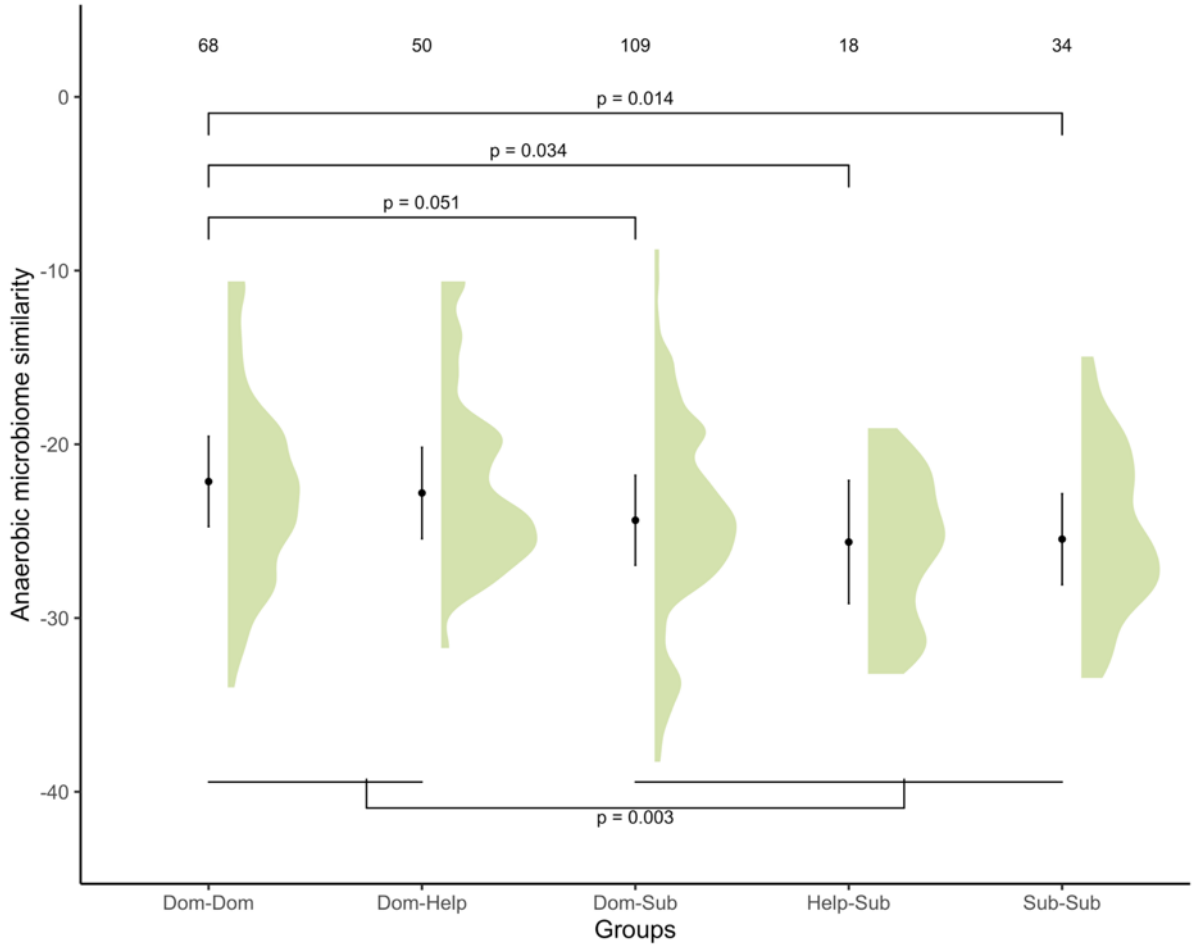


Figure 4.3. Anaerobic gut microbiome composition similarity of different social status pair categories of Seychelles warblers (comparison within groups). Dots and lines represent model predictions with 95% confidence intervals calculated from lmer models. The density plot represents the distribution of raw data. N = 279 pairwise comparisons across 322 samples from 204 individual birds. P-values between categories shown above the plots (Table 4.5) and nest-sharing groups of categories shown below the plots (Table S4.7) are shown with brackets.

4.5 Discussion

We investigated how sociality GM shapes the GM in the cooperative breeding Seychelles warbler. GM alpha diversity did not differ between individuals from the same breeding group or individuals from different breeding groups. However, individuals within a group had a more similar GM composition compared to individuals from different groups. When separating aerotolerant from anaerobic bacteria, individuals within a breeding group shared more of both categories than did individuals from different groups. When we focus on cooperative breeding status differences within breeding groups, dominants and helpers shared a more similar GM Alpha diversity than the dominant pair, but no other pairs were significantly more similar in terms of GM diversity. When looking at all GM genera we found no differences in GM compositional similarity between any of the within group social status categories. However, when separating aerotolerant and anaerobic bacterial genera we find that, as predicted, anaerobic GM composition was more similar between birds that directly cooperate during breeding and thus interact closely at the nest than between categories of pairs that interact less.

Seychelles warbler groups have defined territory boundaries that they defend year-round to secure resources (Hammers et al., 2019). Individuals from the same group do not differ in how similar their GM alpha diversity is compared to pairs of individuals from different groups, which suggests that social transmission does not influence the overall diversity of the GM. This is not surprising, as GM alpha diversity is highly variable and may not reflect GM composition; individuals that live in different territories can have differing GM composition but still retain the same alpha diversity (Johnson & Burnet, 2016; Worsley, Lee, et al., 2024). However, as predicted, GM composition was more similar for individuals from the same groups than individuals from different groups, even when controlling for relatedness. Recent research on the social transmission of microbes in other group-living animals has yielded similar results (Raulo et al., 2018; Tung et al., 2015). This increase in GM composition similarity within groups likely arises from such individuals sharing the same resources, but also because of increased physical interaction among individuals. Indeed, non-group living wild mice (*Apodemus sylvaticus*) that interact more frequently tend to share a more similar microbiome composition (Raulo et al.,

2021, 2024). In our results, that both aerotolerant and anaerobic bacterial communities were more similar within than between breeding groups further supports the idea that shared microbes occur because of a combination of shared environment/diet (e.g. aerobes from insects) and close physical contact (e.g. the transmission of anaerobes). However, it would be challenging to distinguish between resource sharing and social contact modes of transmission when only comparing between and within social groups, as the two modes would overlap (but see below).

Associations between GM characteristics and social interactions have been previously reported in social insects, the harvester ants (*Veromessor andrei*) and honey bees (*Apis mellifera*) (Gamboa et al., 2025; Jones et al., 2018), wild baboons (*Papio cynocephalus*) (Tung et al., 2015) and wild mice (Raulo et al., 2024), but researchers have not directly investigated social interactions within cooperative breeders. In social systems where cooperative breeding occurs, a hierarchy of closeness of interactions between individuals exists, with the dominant breeding pair interacting most frequently, followed by breeders-helpers, breeders-non-helping subordinates, helpers-non-helping subordinates, and subordinates-subordinates (Cant & Field, 2005; Komdeur, 1994). Interestingly, in Seychelles warblers, breeders-helpers have a more similar GM diversity than do the dominant breeding pair. This may be because the helpers (who are normally female) also share in incubating with the dominant female (Richardson et al., 2001) while male dominants do not. Importantly, when comparing all bacterial genera, GM compositional similarity was not associated with the closeness of cooperative breeding relationships within a group. This may be because individuals from the same environment tend to have a similar diet, which leads to homogenisation of the GM irrespective of social interactions. However, as predicted, if we only focus on anaerobic genera we do find that the closeness of cooperative breeding relationships influences GM composition similarity. This was not the case for the aerotolerant GM. These results support the hypothesis that aerotolerant microbes are likely transmitted through a shared general environment (i.e. the territory), while anaerobic microbes require closer social interactions, such as direct interactions at the nest, for transmission. The logic being that oxygen-sensitive anaerobic bacteria do not survive long outside of a host and therefore require close direct contact for transmission (Raulo et al., 2024). Our findings concur with previous work that

investigated anaerobic versus aerotolerant GM similarity in relation to social intimacy using GPS data tracking or grooming behaviour (Raulo et al., 2024; Tung et al., 2015).

Is there likely to be any benefit of GM transmission through close social interactions in cooperatively breeding species? One benefit may be gaining beneficial anaerobic microbes (as observed in the Seychelles warbler). Anaerobic gut microbes are more likely to form close symbiotic relationships with their host as they cannot survive in the aerotolerant conditions outside of the intestinal tract. Indeed, most probiotics – living microbes that provide health benefits - are anaerobic bacteria (El Enshasy et al., 2015). Benefits include aiding gut homeostasis and aid digestion (Kelsey & Colpoys, 2018; Nalla et al., 2022; Zhang et al., 2016) and supporting the host's immune system by preventing pathogens from colonising the GM (Murata et al., 2025; Wells et al., 1988). However, there are also potential downsides to increased transmission, such as pathogen transmission. Although many life-threatening pathogens are aerotolerant (André et al., 2021), previous studies tracking pathogen transmission have suggested that there is an increased risk of spread in animals due to social proximity and shared resources (Duncan et al., 2021; Lebarbenchon et al., 2015).

The Seychelles warbler is an excellent system for studying the social transmission of the GM. However, several limitations exist, such as samples not always being collected from all individuals within a breeding group within the same field period. All tests were restricted to samples within the same field seasons to ensure that individuals had the opportunity to interact recently, and in a similar environment, as temporal effects are known to influence GM communities in the Seychelles warbler, as well as other wild animals (Hicks et al., 2018; Marsh et al., 2022; Worsley, Davies, et al., 2024). Furthermore, although the finding that social closeness makes anaerobic GM composition more similar is clear and important, incorporating shotgun metagenomic data would help determine whether differences in taxonomy alter GM function and the possible contribution of these microbes to host health (Worsley, Mazel, et al., 2024). Additionally, metagenomics would enable the analysis of the GM at the species or strain-level (Anyansi et al., 2020), which would provide higher resolution when asking how GM components are correlated with social closeness rather than environmental transmission. Strain-tracking between

family members and how long strains persist in the GM during an individual life would also improve our understanding of how social closeness shapes the GM (Hildebrand et al., 2021). However, the overall patterns as detected in our study are still valid and shotgun metagenomics for the number of samples required would be very costly. In addition, the use of GPS logger data would allow us to generate more nuanced social networks and determine the strength of social relationships (Kingma et al., 2016). Unfortunately, GPS monitoring of Seychelles warblers within territories is not yet effective, as the accuracy of current tracking technology (that is sufficiently light weight to use on the birds) relative to the size of the Seychelles warbler's extremely small territories (0.18-0.46 ha per territory)(Komdeur & Pels, 2005), limits our ability to track individual interactions. Given the quality of the data on the Seychelles warblers gained through intense fieldwork observations, we are confident of the reliability of our estimates used here regarding the closeness of relationships between individuals (Brouwer et al., 2009; Hammers et al., 2019; Komdeur, 1994).

Overall GM composition was also more similar when one individual (parent/helper) attended the other when they were a nestling,suggesting that the developmental GM tends to persist into later life and remains more similar due to a shared natal environment. This finding is consistent with that found in humans, where an individual shares gut microbial strains with their mothers, and these are maintained throughout life (Eikenaar et al., 2007; Valles-Colomer et al., 2023).

In the present study on the Seychelles warbler when assessing the GM both within and across groups relatedness was not a predictor of GM composition similarity. This may be because highly related individuals, such as siblings, may not share the same territory later in life when we sample them (all samples were post-fledgling), especially since most individuals disperse from their natal territory as soon as a breeding opportunity elsewhere becomes available (Eikenaar et al., 2007). In wild mice and Verreaux's sifaka (*Propithecus verreauxi*), kinship and relatedness did not predict GM similarity (Perofsky et al., 2017; Raulo et al., 2021). However, in humans and wild baboons, related individuals share more similar GMs (Grieneisen et al., 2021; Roche et al., 2023; Turnbaugh et al., 2009).

The Seychelles warbler GM was also influenced by environmental variables, especially the number of days apart that samples were collected, which is consistent with previous studies on this species (Lee et al., 2025; Worsley, Davies, et al., 2024; Worsley, Lee, et al., 2024). The effect of this variable on GM diversity and composition could be explained by changes in weather and food availability throughout the season or the storage time of our samples (Cunningham et al., 2020). However, we cannot separate these two possibilities as they are strongly correlated. Additionally, GM composition was more similar between pairs sampled within the minor breeding season than in the major breeding season. The more relaxed territory boundaries in the minor breeding season and possibly fewer seasonal changes due to a shorter minor season, as well as less breeding attempts, could explain this, as groups are likely to share more of their geographic range and diet and, hence, a more similar GM (Komdeur, 1992, 2001).

In conclusion, our study has been able to separate the effect of sharing habitat from the effect of close social interactions (within cooperative breeding) in shaping the GM of a wild vertebrate. Importantly we show that different components of the GM are differentially affected by such social interactions: anaerobic microbes are more likely to be transmitted through the cooperative breeding behaviours. Further research is needed to determine whether this elevated sharing of specific microbes due to cooperative breeding is beneficial or detrimental to host fitness.

4.6 References

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4.7 Supplementary material

Table S4.1. A linear mixed effect model investigating the relationship between breeding group membership and gut microbiome ASV richness similarity in pairs of Seychelles warblers (N = 27,821 pairwise comparisons across 648 samples from 345 individual birds). Significant terms (P <0.05) are indicated in bold. Reference categories for categorical variables were the first term in brackets. Time of day was measured as minutes apart, and time in season was measured as days apart.

Characteristic	**Beta**	**SE**[1]	**Statistic**	**df**	**p-value**
(Intercept)	**-125.7**	**9.14**	**-13.8**	**13.4**	**<0.001**
Breeding group (Between/Within)	-0.995	6.43	-0.155	27,528	0.877
Age difference	-0.067	0.308	-0.217	26,377	0.828
Sex (same/different)	-0.636	1.21	-0.527	27,537	0.598
Season (major/minor)	-7.117	3.76	-1.89	2,446	0.059
Time of day	-0.003	0.004	-0.675	27,669	0.500
Time in season	**-0.157**	**0.035**	**-4.50**	**27,719**	**<0.001**
Relatedness	6.394	9.63	0.664	27,553	0.507
Shared nest at hatch (no/yes)	2.631	2.83	0.931	27,372	0.352
Random	**27,821 observations**			**Variance**	
Multi membership ID (Intercept)		345 groups			49.49
Sample Year (Intercept)		6 years			17.19
Residual					97.56

Table S4.2. A linear mixed effect model (lmer) investigating the relationship between the social status categories of pairs of Seychelles warblers within breeding groups and the gut microbiome ASV richness similarity between them (N = 279 pairwise comparisons across 322 samples from 204 individual birds). Significant terms (P <0.05) are indicated in bold. Reference categories for categorical variables were the first term in brackets. Time of day was measured as minutes apart, and time in season was measured as days apart.

Characteristic	Beta	SE[1]	Statistic	df	p-value
(Intercept)	**-128.9**	**31.2**	**-4.13**	**107**	**<0.001**
Individual Status Pair					
Dom - Dom	—	—	—		
Dom - Help	13.04	31.0	0.420	217	0.675
Dom - Sub	-29.20	28.5	-1.02	200	0.308
Help - Sub	-4.601	41.2	-0.112	163	0.911
Sub - Sub	46.08	34.6	1.33	193	0.185
Age difference	-2.380	3.20	-0.743	136	0.459
Sex (same/different)	-2.730	18.6	-0.147	243	0.884
Season (major/minor)	-11.05	23.1	-0.479	43.8	0.635
Time of day	0.099	0.077	1.29	262	0.199
Time in season	-0.176	0.441	-0.398	267	0.691
Relatedness	-41.71	44.4	-0.940	187	0.348
Shared nest at hatch (no/yes)	42.05	21.7	1.94	243	0.053
Random		**279 observations**			**Variance**
Multi membership ID	(Intercept)	204 groups			47.05
Sample Year	(Intercept)	6 years			18.09
Residual					109.5

Table S4.3. A linear mixed effect model (lmer) investigating the relationship between the social status categories of pairs of Seychelles warblers within breeding groups and the gut microbiome ASV Shannon diversity similarity between them (N = 279 pairwise comparisons across 322 samples from 204 individual birds). Significant terms (P <0.05) are in bold. Reference categories for categorical variables were the first term in brackets. Time of day was measured as minutes apart, and time in season was measured as days apart.

Characteristic	Beta	SE[1]	Statistic	df	p-value
(Intercept)	**-1.425**	**0.230**	**-6.18**	**78.3**	**<0.001**

Individual Status Pair					
Dom - Dom	—	—	—		
Dom - Help	**0.542**	**0.226**	**2.40**	**219**	**0.017**
Dom - Sub	0.200	0.208	0.963	204	0.337
Help - Sub	0.288	0.303	0.950	169	0.344
Sub - Sub	0.359	0.254	1.42	194	0.159
Age difference	-0.012	0.024	-0.510	142	0.611
Sex (same/different)	-0.077	0.135	-0.570	243	0.569
Season (major/minor)	-0.075	0.173	-0.436	46.3	0.665
Time of day	0.001	0.001	1.37	265	0.170
Time in season	<0.001	0.003	0.059	264	0.953
Relatedness	-0.613	0.324	-1.89	191	0.060
Shared nest at hatch (no/yes)	0.129	0.157	0.820	247	0.413
Random		**279 observations**			**Variance**
Multi membership ID	(Intercept)	204 groups			0.376
Sample Year	(Intercept)	6 years			0.166
Residual					0.762

Table S4.4. Pairwise comparison of social status categories of pairs of Seychelles warblers within breeding groups and the gut microbiome ASV Shannon diversity similarity between them using Tukey method p-values (from Table S4.2; N = 279 pairwise comparisons across 322 samples from 204 individual birds). Significant terms (P <0.05) are indicated in bold.

Contrast	Estimate	SE	df	t.ratio	p.value
Dom-Dom vs Dom-Help	0.542	0.229	217	2.364	0.129
Dom-Dom vs Dom-Sub	0.200	0.210	202	0.954	0.875
Dom-Dom vs Help-Sub	0.288	0.307	166	0.938	0.882

Dom-Dom vs Sub-Sub	0.359	0.260	192	1.384	0.639
Dom-Help vs Dom-Sub	-0.342	0.190	212	-1.805	0.374
Dom-Help vs Help-Sub	-0.255	0.291	192	-0.876	0.906
Dom-Help vs Sub-Sub	-0.183	0.267	214	-0.687	0.959
Dom-Sub vs Help-Sub	0.087	0.282	177	0.310	0.998
Dom-Sub vs Sub-Sub	0.159	0.247	206	0.642	0.968
Help-Sub vs Sub-Sub	0.072	0.333	196	0.215	1.000

Table S4.5. A linear mixed effect model (lmer) investigating the relationship between social status pair categories within a breeding group and GM composition similarity in Seychelles warblers (N = 279 pairwise comparisons across 322 samples from 204 individual birds). Significant terms (P <0.05) are indicated in bold. Reference categories for categorical variables were the first term in brackets. Time of day was measured as minutes apart, and time in season was measured as days apart.

Characteristic	**Beta**	**SE**	**Statistic**	**df**	**p-value**
(Intercept)	**-75.42**	**3.30**	**-22.8**	**24.2**	**<0.001**
Individual Status Pair					
Dom - Dom	—	—	—		
Dom - Help	-0.841	2.91	-0.289	237	0.773
Dom - Sub	-2.150	2.69	-0.799	228	0.425
Help - Sub	-2.380	4.02	-0.592	198	0.555
Sub - Sub	-2.352	3.35	-0.702	209	0.483
Age difference	-0.451	0.322	-1.40	174	0.163
Sex (same/different)	-0.199	1.69	-0.117	249	0.907
Season (major/minor)	3.977	2.39	1.66	92.6	0.100
Time of day	-0.001	0.007	-0.222	254	0.824
Time in season	0.002	0.037	0.065	222	0.948
Relatedness	-3.252	4.23	-0.768	220	0.443
Shared nest at hatch (no/yes)	1.368	1.95	0.701	262	0.484

Random		**279 observations**	**Variance**
Multi membership ID	(Intercept)	204 groups	6.449
Sample Year	(Intercept)	6 years	4.151
Residual			8.037

Table S4.6. A linear mixed effect model (lmer) investigating the relationship between individual status pairs and **aerotolerant** GM composition similarity of Seychelles warblers (N = 279 pairwise comparisons across 322 samples from 204 individual birds). Significant terms (P <0.05) are indicated in bold. Reference categories for categorical variables were the first term in brackets. Time of day was measured as minutes apart, and time in season was measured as days apart.

Characteristic	**Beta**	**SE**[1]	**Statistic**	df	**p-value**
(Intercept)	**-41.42**	**1.86**	**-22.2**	**18.7**	**<0.001**
Individual Status Pair					
Dom - Dom	—	—	—		
Dom - Help	-0.831	1.68	-0.495	232	0.621
Dom - Sub	-1.804	1.56	-1.16	227	0.248
Help - Sub	-3.017	2.33	-1.29	198	0.197
Sub - Sub	-1.902	1.88	-1.01	207	0.313
Age difference	**-0.245**	**0.074**	**-3.29**	**212**	**<0.001**
Sex (same/different)	-1.102	0.973	-1.13	245	0.258
Season (major/minor)	1.833	1.40	1.31	116	0.192
Time of day	<0.001	0.004	-0.040	254	0.968
Time in season	-0.001	0.021	-0.055	224	0.956
Relatedness	-2.428	2.45	-0.991	221	0.323
Shared nest at hatch (no/yes)	-0.389	1.12	-0.346	261	0.729
Random		**279 observations**			**Variance**
Multi membership ID	(Intercept)	204 groups			3.785
Sample Year	(Intercept)	6 years			2.638

Residual	4.588

Table S4.7. A pairwise comparison with Tukey method p-values of the relationship between individual status pairs and **anaerobic** GM composition dissimilarity of Seychelles warblers (from Table 4.5) (N = 279 pairwise comparisons across 322 samples from 204 individual birds). Significant terms (P <0.05) are indicated in bold.

Contrast	**Estimate**	**SE**	**df**	**t.ratio**	**p.value**
Dom-Dom vs Dom-Help	-0.661	1.240	202	-0.534	0.984
Dom-Dom vs Dom-Sub	-2.231	1.150	186	-1.945	0.297
Dom-Dom vs Help-Sub	-3.483	1.650	150	-2.110	0.221
Dom-Dom vs Sub-Sub	-3.319	1.360	179	-2.434	0.111
Dom-Help vs Dom-Sub	-1.570	1.030	205	-1.521	0.550
Dom-Help vs Help-Sub	-2.822	1.580	182	-1.790	0.383
Dom-Help vs Sub-Sub	-2.658	1.440	204	-1.841	0.353
Dom-Sub vs Help-Sub	-1.252	1.530	169	-0.819	0.925
Dom-Sub vs Sub-Sub	-1.088	1.310	200	-0.829	0.921
Help-Sub vs Sub-Sub	0.164	1.790	190	0.092	1.000

Table S4.8. A linear mixed effect model (lmer) investigating the relationship between the **anaerobic** GM composition similarity of nest-sharing pairs of Seychelles warblers compared to non-nest-sharing pairs (N = 279 pairwise comparisons across 320 samples from 204 individual birds). Significant terms (P <0.05) are indicated in bold. Reference categories for categorical variables were the first term in brackets. Time of day was measured as minutes apart, and time in season was measured as days apart.

Characteristic	**Beta**	**SE**[1]	**Statistic**	**df**	**p-value**
(Intercept)	**-22.970**	**1.141**	**-20.135**	**30**	**<0.001**
Nest sharing group (yes/no)	**-2.317**	**0.778**	**-2.977**	**197**	**0.003**
Age difference	0.015	0.066	0.223	261	0.824
Sex (same/different)	0.490	0.694	0.706	240	0.481
Season (major/minor)	0.157	1.040	0.151	84	0.880

Time of day	-0.001	0.003	-0.463	254	0.644
Time in season	<0.001	0.018	-0.024	263	0.981
Relatedness	1.622	1.754	0.925	206	0.356
Shared nest at hatch (no/yes)	-0.096	0.730	-0.131	242	0.896
Random		**274 observations**		**Variance**	
Multi membership ID	(Intercept)		204 groups	3.432	
Sample Year	(Intercept)		6 years	2.180	
Residual					

Chapter 5 |

Inbreeding, intergenerational inbreeding and the gut microbiome

Photo of a demographic rescue event in the Cousin Island population of Seychelles warbler fieldworkers (n = 3 ± 1)

5.1 Abstract

Background

Inbreeding can detrimentally impact the fitness of individuals and their offspring (inbreeding depression). However, whether being inbred impacts the gut microbiome (GM) of individuals in natural populations remains largely unexplored, despite this being a potentially important cause of reduced host health and fitness. Moreover, the intergenerational effects of having inbred parents on the GM have never been explored.

Methods

We used a natural, closed population of Seychelles warblers (*Acrocephalus sechellensis*) to investigate how host inbreeding impacts GM communities. Inbreeding avoidance does not occur in this population, leading to high variance in individual inbreeding coefficients (FRoH). Furthermore, extra-pair paternity is high (~44%) in this socially monogamous species, which helps us separate genetic and social effects influencing the GM. Using faecal samples, we undertook both 16S rRNA amplicon sequencing variants (ASV) metabarcoding (n = 439 from 235 individuals) and metagenomic sequencing (n = 143 from 80 individuals) to investigate how being inbred, or having inbred parents, affects an individual's taxonomic and functional GM variation.

Results

Individuals with higher FRoH had lower metagenomic taxonomic alpha diversity and distinct shifts in ASV and functional composition, although no specific taxa or functions differed in abundance, and GM stability was unaffected.

Intergenerational effects were also evident: genetic father FRoH was positively associated with offspring functional richness, while maternal and social father FRoH influenced offspring species composition. However, no specific taxa or functions and GM stability were associated with parental inbreeding.

Conclusions

Individuals with a higher inbreeding coefficient showed a small but detectable effect on the GM, particularly in relation to alpha diversity and composition. Likewise, intergenerational inbreeding had some limited effects on microbiome characteristics. Together, these findings provide evidence that inbreeding can influence (albeit with small effect sizes) not only an individual's microbiome but also that of their offspring.

5.2 Introduction

The gut microbiome (GM) plays a key role in many host processes, including metabolic functions, immune defence and cognition (Ross et al., 2024; Zhu et al., 2021). Studies of wild animals have shown that the GM varies amongst individuals in association with environmental differences (Hicks et al., 2018; Marsh et al., 2022; Schmid et al., 2023) as well as host factors such as age, sex, and sociality (C. Lee et al., 2025; Raulo et al., 2021; Risely et al., 2022; Tung et al., 2015; Xu and Zhang, 2021). Fine-scale within-population studies, where environmental factors (such as diet) remain relatively constant, and where individual variation in these other host factors is known, provide the best opportunity to reveal the effects of among-individual host genetic variation on the GM (Dzierozynski et al., 2023).

A few studies in natural populations have shown that individual differences in host genetics are associated with variation in the GM (Davies et al., 2022; Flynn et al., 2023; Grieneisen et al., 2021). For example, a study on baboons (*Papio cynocephalus*) revealed that the GM is significantly heritable (Grieneisen et al., 2021). Furthermore, host immunogenetic diversity has been associated with differences in GM diversity and composition in several species (Bolnick et al., 2014; Davies et al., 2022; Fuess et al., 2021; Tanoue et al., 2010), and specific host genes that shape gut physiology and nutrient metabolism may also shape the GM (Bonder et al., 2016; Kurilshikov et al., 2017; Schroeder, 2019). However, powerful studies that address host genetic variation across the entire genome are now needed to resolve if, to what extent, and why, host genetic characteristics impact the GM.

Inbreeding results in higher homozygosity across the genome in offspring, which, through greater expression of recessive deleterious alleles and a loss of heterosis, can lead to reduced health and fitness, i.e. inbreeding depression (Charlesworth and Willis, 2009). Despite these detrimental effects, inbreeding may become inevitable in small, isolated populations due to increasing relatedness among individuals (Ralls et al., 2007), especially where effective inbreeding avoidance mechanisms do not exist (Dorsey and Rosenthal, 2023; Eikenaar et al., 2008).

The GM may be affected by inbreeding through both direct and indirect effects.

Increased homozygosity can lead to the expression of deleterious alleles at loci that are directly involved in regulating the GM (Bonder et al., 2016; Melis et al., 2023) or lead to a loss of heterosis/heterozygote advantage at key genes that interact with the GM, e.g. immune genes under balancing selection (Spurgin and Richardson, 2010). Alternatively, the expression of deleterious alleles and loss of heterosis across loci may detrimentally impact the general physiological health of an individual (Fareed and Afzal, 2014). That reduced health may subsequently lead to differences in GM composition and stability (Keller, 2002), potentially leading to dysbiosis and more negative host health effects (Hooks and O'Malley, 2017; Martinez et al., 2021; Videvall et al., 2020).

Given that being inbred reduces individual health and condition, it is also possible that the offspring of inbred individuals may also suffer fitness loss independent of their own genetics (Ford et al., 2018). For example, such intergenerational inbreeding depression can occur due to reduced parental care quality, a key contributor to offspring fitness (Vedder et al., 2021). Another possible route is that inbred parents have differences in epigenetic regulation of genetic expression (Achrem et al., 2023; Vergeer et al., 2012), which may influence offspring development and health (Xu et al., 2021). Furthermore, given that the GM is to some degree vertically transmitted in many species (Sarkar et al., 2024, 2020), inbred parents with poorer GM characteristics may pass on a weaker initial GM to their offspring (Choo et al., 2017; Sarkar et al., 2024).

Links between inbreeding and GM variation are still not well characterised or understood. Studies on inbred captive animals suggest that inbreeding is associated with reduced GM alpha diversity and changes in GM composition, with decreases in probiotic, and increases in potentially pathogenic microbes (Hufeldt et al., 2010; Melis et al., 2023; Ørsted et al., 2022; Wei et al., 2020). However, captive animals harbour a simpler and different (artificial) GM compared to their wild counterparts (Gibson et al., 2019; Ning et al., 2020; Oliveira et al., 2020). Thus, findings from captive animals may not reflect what is occurring in natural populations. Furthermore, long-term captive host lines are often purged of recessive deleterious alleles and hence capable of surviving incredibly high inbreeding coefficients

(Festing and Lutz, 2010; Wei et al., 2020). Therefore, studies on these lines are unlikely to reflect how inbreeding affects GM characteristics in the wild.

In wild populations, some studies have shown that genome-wide heterozygosity is associated with increased GM alpha diversity (Davies et al., 2022; Yuan et al., 2015), while others have not (Guimaraes Sales et al., 2024). Additionally, genome-wide heterozygosity has been associated with differences in GM composition (Guimaraes Sales et al., 2024; Steury et al., 2019). However, overall, the effects of inbreeding on the GM in wild animals. has received little, or in the case of intergenerational effects, no attention.

The power of modern sequencing methodologies enables us to accurately and efficiently assess both host inbreeding and GM characteristics. Whole-genome sequencing allows for accurate quantification of recent inbreeding via inbreeding coefficients (e.g. the fraction of genome in runs of homozygosity; FRoH (Ceballos et al., 2018)). GM composition can be determined in a cost-effective manner using 16S rRNA metabarcoding, which allows for low-resolution taxonomic analysis of the bacterial GM across many samples due to its low sequencing cost (Worsley et al., 2024d). Additionally, shotgun metagenomic sequencing, though much more expensive, can be used to analyse the GM at a higher taxonomic resolution (species or strain level) and can shed light on GM functional characteristics via information on gene content (Worsley et al., 2024d). An assessment of GM function could extend our insights into the mechanisms by which GM variation influences the host or vice versa (C. Z. Lee et al., 2025b, 2025a; Worsley et al., 2024d, 2021).

Here, we use the Seychelles warblers (*Acrocephalus sechellensis*) on Cousin Island to investigate how inbreeding affects the GM in a wild vertebrate population. This isolated population of ca. 300 individuals has been intensively monitored since 1997 (Hammers et al., 2015; Richardson et al., 2001; Speelman et al., 2025). Over 2500 individuals have been followed throughout their lives and an extensive genetically verified pedigree generated (Hadfield et al., 2006; Sparks et al., 2022). In this socially monogamous, territorial species, extra-pair paternity (EPP) is common (~44% of offspring) (Raj Pant et al., 2019; Richardson et al., 2001), and nearly all genetic fathers are assigned (Hadfield et al., 2006; Sparks et al., 2022), thus

enabling us to separate genetic and environmental paternal effects. The small size and lack of inbreeding avoidance in this population (Eikenaar et al., 2008) have resulted in a high variance of inbreeding (Richardson et al., 2004) and inbreeding depression (Bebbington et al., 2016; Richardson et al., 2004). Prior GM studies on this population have shown that genome-wide heterozygosity is positively associated with 16S GM diversity (Davies et al., 2022), and major histocompatibility complex (MHC) diversity with GM composition (Davies et al., 2022; C. Z. Lee et al., 2025b; Worsley et al., 2022). Additionally, GM differences have been associated with several host factors, including relatedness and sociality(C. Lee et al., 2025; Worsley et al., 2024c), as well as environmental factors including season, sampling year, and time of day (Davies et al., 2022; C. Z. Lee et al., 2025a, 2025b; Worsley et al., 2024c, 2024b, 2021). Importantly, another study also identified 28 ASVs whose abundances were associated with differential survival (22 negatively and 6 positively), suggesting pathogenic or beneficial microbes (Worsley et al., 2021).

Here, we utilise individual inbreeding coefficients (FRoH) derived from whole genome sequencing of 1900 Seychelles warblers in combination with previously generated 16S metabarcoding (Worsley et al., 2024b) and metagenomic data (C. Z. Lee et al., 2025a) to investigate the effects of host inbreeding, and intergenerational inbreeding, on the GM. We hypothesise that inbreeding will lead to lower quality hosts and GMs. Specifically, we predict that i) individual FRoH will be associated with lower GM diversity and ii) negative changes in GM taxonomical and functional composition, and reduced GM stability. Specifically, inbred individuals will show an increased prevalence of negative/pathogenic microbes, reduced beneficial functional genes, and greater inter-individual variation. We also predict that iii) offspring of inbred social parents will have lower GM diversity and differences in GM taxonomical and functional compositions, and reduced GM stability.

5.3 Methods

5.3.1 Study system

The Seychelles warblers on Cousin Island, Republic of Seychelles (29 ha; 04° 20′ S, 55° 40′ E) are a population of small, insectivorous passerines that has been monitored since 1985 (Hammers et al., 2015; Komdeur, 1992; Speelman et al., 2025). The population consists of ca. 300 individuals inhabiting ca 100 territories (Komdeur, 1992) and is closed, with virtually no dispersal to other islands (Komdeur et al., 2004). Two field seasons are conducted per year: January to March (minor breeding season) and June to September (major breeding season). During these as many individuals as possible are caught using mist nets or in the nest (chicks), and new individuals are marked using a British Trust for Ornithology (BTO) metal ring and a unique combination of three colour rings. Since 1997, almost every individual (>98%) has been marked and monitored throughout their life (Brown et al., 2022). Age is calculated from an individual's hatch or fledge date and eye colour (Komdeur, 1991).

A 12-generation, genetically verified pedigree has been constructed from this population (Hadfield et al., 2006; Sparks et al., 2022), allowing accurate assignment of parentage and detection of inbreeding events (Figure 5.1A). The pedigree also informs intergenerational inbreeding events (Figure 5.1B) and EPP (Figure 5.1C).

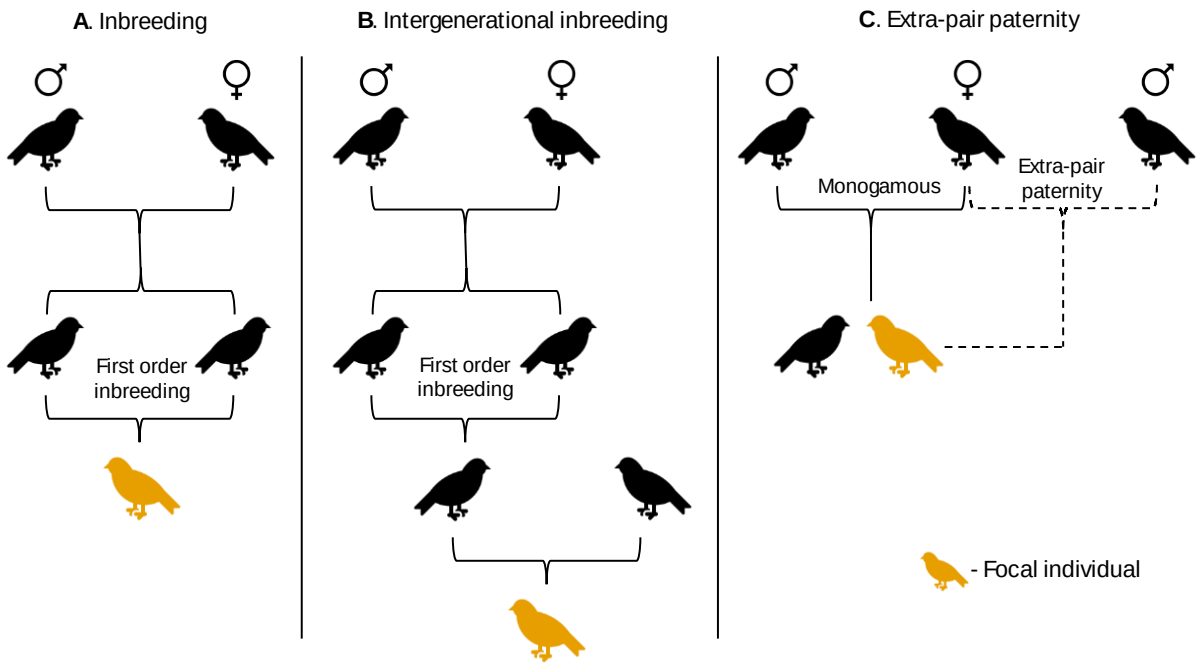

Figure 5.1. Representation of a focal individual resulting from (A) inbreeding, (B) intergenerational inbreeding and (C) extra-pair paternity. Focal individuals are in gold.

5.3.2 Sample collection

Faecal and blood samples were collected from caught individuals. Briefly, birds were placed in a flat-bottom paper bag with a sterilised plastic tray under a metal grate allowing faeces to drop onto the plastic, while minimising contact with the birds' surface (Davies et al., 2022; Knutie and Gotanda, 2018). Faecal samples are placed into a sterile microcentrifuge tube containing 1 mL of absolute ethanol, stored at 4°C during fieldwork and then at -80°C for long-term storage. Each season, control samples were collected by swabbing collection bags and fieldworker hands. The time of day (minutes after sunrise; 06:00 AM) of sampling was recorded, as was faecal storage time (days) at 4°C. A small (ca. 25 µl) blood sample was also collected from each bird via brachial venepuncture and stored in 0.7 ml of absolute ethanol at 4°C.

5.3.3 Gut (bacterial) microbiome molecular methods

Microbial DNA were extracted from faecal samples using the DNeasy PowerSoil Kit (Qiagen, Crawley, UK) with a modified version of the manufacturer's protocol (described in (C. S. Davies et al., 2022)). Microbial DNA were sequenced as part of

previous studies using 16S rRNA amplicon (Worsley et al., 2024b) and shotgun sequencing for metagenomics (C. Z. Lee et al., 2025a).

Briefly, microbial DNA samples were submitted for 16S rRNA amplicon sequencing across seven batches from libraries generated with V4 primers 515F (5'TGCCAGCMGCCGCGGTAA3') and 806R (5'GGACTACHVGGGTWTCTAAT3') (Worsley et al., 2024b). Libraries were sequenced with 2 x 250 bp paired end reads on an Illumina MiSeq Platform. Control samples were extracted, and amplicon sequenced in the same way (n = 21 hand controls, 15 negative controls, and 10 positive ZymoBIOMICS Microbial Community Standard (D6300) controls).

A subset of DNA samples was submitted for shotgun sequencing in two batches from libraries generated with the LITE protocol (Perez-Sepulveda et al., 2021). Libraries were sequenced using 2 x 150 bp paired end sequencing on an Illumina NovaSeq X platform. Controls samples were library prepped and sequenced in the same way (n = 6 hand controls, n = 2 ZymoBIOMICS Microbial Community Standard (D6300), and n = 1 ZymoBIOMICS Fecal Reference with TruMatrix™ Technology (D6323)).

5.3.4 Host genome molecular methods

Genomic DNA was extracted from blood samples using the DNeasy Blood and Tissue kit (Qiagen, Crawley, UK) or a salt extraction protocol (Richardson et al., 2001) according to the manufacturer's protocol. Genomic DNA was used for molecular sexing following (Griffiths et al., 1998) and microsatellite genotyping for parentage analyses (Richardson et al., 2001; Sparks et al., 2022).. Genomic DNA was submitted for whole genome sequencing in 20 batches (detailed in Kiran Lee et al., 2025 – in prep) from randomly selected libraries generated with NEBNext Ultra II FS DNA Library Prep (New England Biolabs). Libraries were sequenced using 2 x 150 bp, paired-end sequencing on an Illumina NovaSeq 6000 platform.

5.3.5 Bioinformatics

Amplicon sequenced reads were processed as described in (Worsley et al., 2024b). Briefly, 16S rRNA reads were truncated, filtered, and classified in amplicon

sequencing variants (ASV) using DADA2 as part of QIIME2 (Bolyen et al., 2019). ASVs were taxonomically assigned with the naïve-Bayes classifier on the SILVA 132 reference database for 16S rRNA gene sequences (Quast et al., 2012). The resulting ASVs were imported to R using *phyloseq* 1.46.0 (Leo Lahti and Sudarshan Shetty, 2019; McMurdie and Holmes, 2013) and filtered to remove ASVs that were non-bacterial, unassigned to phylum level, or had less than 50 reads, as well as potential contaminants (based on controls). Amplicon sequencing reads were rarefied to 8,000 reads, based on samples reaching >95% completeness in rarefaction curves (Worsley et al., 2024b). ASV richness and Shannon diversity were calculated using *phyloseq* version 1.46.0 (McMurdie and Holmes, 2013).

Shotgun sequence processing was performed using MATAFILER (Hildebrand et al., 2021) as previously described in detail (C. Lee et al., 2024). Briefly, host reads were removed by mapping reads to the Seychelles warbler genome (see below) with Kraken 2 (version 2.1.3), followed by read quality filtering using sdm software version 2.14 beta (Hildebrand et al., 2014; Wood et al., 2019). After removing host reads and read trimming, two samples and five hand controls were removed because they did not have enough reads for metagenome assembly. An average of 20,481,040 (SD = 13,718,305) paired end reads per sample were retained for de novo metagenome assembly using MEGAHIT version 1.2.9 (Li et al., 2015). Genes were predicted from the resulting assembly using Prodigal version 2.6.3 (Hyatt et al., 2010) and clustered into gene catalogues (95% identity). Functional annotations of genes were performed using eggNOGmapper version 2.1.12 and the eggNOG database version 4 (Cantalapiedra et al., 2021; Powell et al., 2014). Metaphlan4 assignments were used to taxonomically assign shotgun sequencing reads. Metagenomic species reads were rarefied to 5500 reads, the point of asymptote of the metagenomic species rarefaction curve (C. Z. Lee et al., 2025a). Metagenomic functional annotation reads were rarefied to 100,000 reads, the point of asymptote of the metagenomic functional annotation rarefaction curve (C. Z. Lee et al., 2025a).

Whole host genome sequencing reads were processed as described previously (Kiran Lee, et al., 2025). Briefly, reads were filtered to keep only high-quality reads (Phred quality score of >33 and a minimum length of 80 bp) using *Trimmomatic* version 0.39 (Bolger et al., 2014). These reads were mapped to the Seychelles warbler reference genome (Kiran Lee, et al., 2025; BUSCO: 96.0%

with a total length = 1,081,018,985 bp) before imputation with *STITCH* version 1.7.0 (Davies et al., 2016), with the following parameters: diploid, eight founding haplotypes, and 25 generations since founding (Kiran Lee, et al., 2025). Runs of homozygosity (ROH) were then calculated for each sample using *PLINK* version 2.0 (Purcell et al., 2007) by including SNPs with genotyping rate >99% and minor allele frequency >99% as well as a maximum allowed density of heterozygous SNPs of 200 kb, maximum allowed gap of 300 kb, minimum length of 3750 kb, minimum number of SNPs of 50, maximum number of heterozygous SNPs in a sliding window of 2, maximum number of missing genotypes within the sliding window of 4, and minimum number of SNPs required in a sliding window of 50 (Kiran Lee, et al., 2025). The fraction of the genome in ROH (FROH) was calculated by dividing the ROH length by genome size.

5.3.6 Pedigree

Parentage assignment was generated with MasterBayes 2.5.2 (Hadfield et al., 2006) using microsatellites (Richardson et al., 2001; Sparks et al., 2022). All offspring hatched between 1991 and 2022 (2282 offspring, 1935 (85%) mothers, 2016 (88%) fathers were assigned parentage at >80% confidence using *MasterBayes* 2.52 as part of previous studies (Hadfield et al., 2006; C. Lee et al., 2025; Sparks et al., 2022)).

In addition, parentage assignment was also performed with *sequoia* version 2.11.4 (Huisman, 2017) using SNPs filtered in *PLINK* for genotyping rate of >99.9 %, minor allele frequency of >30% and linkage of 1000 SNP window, 2 step size, 0.1 pairwise r^2, sex chromosomes and chromosomes with <90% imputation accuracy were excluded. Both pedigrees showed excellent consistency (>95% concordance; Kiran Lee et al., 2025). Therefore, the genomic pedigree was used for all subsequent analyses.

5.3.7 Statistical analysis

Individuals >0.5 years age (when the mature GM stabilises (Worsley et al., 2021)).for which we had gut microbiome samples, genomic data, and known parents were included (16S, n = 439 samples from 235 individuals; Metagenomics, n = 143

samples from 80 individuals). All statistical analysis was conducted in R 4.3.3 (Posit team, 2024; R Core Team, 2024). Linear mixed effect (LMMs) and Generalised linear mixed effect models (GLMMs) were constructed using *lme4* version 1.1-35.5 (Bates et al., 2015), and PERMANOVAs were constructed using the *adonis2*() function in *vegan* version 2.6.6 with 9999 permutations and a blocking effect of bird ID to account for repeated measures (Oksanen Jari et al., 2024).

5.3.7.1 Inbreeding and GM alpha diversity

16S rRNA metabarcoding diversity: A GLMM with negative binomial distribution was constructed with ASV richness as the response variable and the individual's, mother's, genetic father's, social father's inbreeding coefficient and extra-pair paternity (no/yes) as predictor variables. Age (years), sex, season, sample year, days at 4°C, and time of day were also included as fixed-term control variables, and bird ID, mother ID, genetic father ID and social father ID were included as random variables. An interaction between inbreeding coefficient of genetic father and EPP is tested to determine if the effect was only present only during EPP, but the interaction was dropped if it was not significant to allow interpretation of the main effects. Shannon diversity was also then modelled with an LMM including the same variables.

All subsequent models of GM diversity (below) included the same control variables unless stated otherwise.

For all analyses, if any inbreeding coefficient had a significant ($P < 0.05$) effect on the dependent variable, then an additional model was constructed with only extra-pair offspring included to confirm results using only cases where the social and genetic fathers were different.

Metagenomic taxonomic diversity: A GLMM with negative binomial distribution was constructed with metagenomic species richness as the dependent variable, and an LMM was constructed with metagenomic species Shannon diversity.

Metagenomic functional diversity: LMMs were constructed for scaled exponentially transformed functional richness and exponentially transformed functional Shannon diversity.

5.3.7.2 Inbreeding and GM composition

16S rRNA metabarcoding composition: Unrarefied amplicon sequencing reads were filtered to remove rare ASVs (<5% prevalence), and a centred log ratio (CLR) transformation was applied to the ASVs abundance using *microbiome* 1.24.0 package (Leo Lahti & Sudarshan Shetty, 2019). A PERMANOVA was constructed using pairwise Aitchison distance with the inbreeding coefficient of the focal individual, and its mother, genetic father, social father and extra-pair paternity (EPP; no/yes) as predictors, along with control variables (age, sex (female/male), season (major/minor), sample year, days at 4°C, and catch time). An interaction between inbreeding coefficient of genetic father and EPP is tested to determine if the effect was only present only during EPP, but the interaction was dropped if it was not significant to allow interpretation of the main effects. PCA was generated using *phyloseq* version 1.46.0 (McMurdie & Holmes, 2013) to visualise compositional changes.

Metagenomic taxonomic composition: Unrarefied metagenomic species reads were filtered, and a CLR transformation was applied to species abundance as for ASV composition above. A PERMANOVA (also as described above), was constructed to test for inbreeding and intergenerational inbreeding coefficient effects on metagenomic species composition.

Metagenomic functional composition: Unrarefied metagenomic functional annotations were filtered and a CLR transformation was applied to functional annotation abundance as for ASV composition above. A PERMANOVA (also as described above) was constructed to test for intergenerational inbreeding coefficient effects on metagenomic functional annotation composition.

5.3.7.3 Inbreeding and GM differential abundance analysis

16S rRNA metabarcoding abundance: Differential abundance analysis was performed using *ALDEx2* 1.34.0 (Fernandes et al., 2013). CLR transformed ASVs from GM composition were used as the response variable with the inbreeding coefficient of the focal individual and its mother, genetic father and social father as predictors, along with control variables (age, sex, season, sample year, days at 4°C, and catch time). The differential abundant ASVs were then compared to previously identified survival-related ASVs (Worsley et al., 2021), to assess whether inbreeding is associated with an increase in potentially pathogenic microbes and/or a decrease in beneficial ones.

Metagenomic taxonomic abundance: Metagenomic species and metagenomic function were also analysed with *ALDEx2* as described for ASV abundance above.

5.3.7.4 Inbreeding and GM stability

16S rRNA metabarcoding GM stability: Pairwise Aitchison distances of CLR-transformed reads from between samples were scaled to similarity values (0-1) using the formula as previously described (Worsley et al., 2024b): similarity = 1-(distance/maximum distance), where a value closer to one indicates samples are identical in GM composition. We then modelled pairwise GM similarities using a LMM multi-membership model (*lmer* with *lmerMultiMember*) using *lme4* 1.1-35.5 (Bates et al., 2015). Inbreeding coefficients were categories by the average population FRoH (low $\leq$ 0.25 and high >0.25) to assess whether inbreeding of the focal individual or its parents had more or less stable GMs. A total of 96141 pairwise comparisons of individual inbreeding category (low-low, high-high, and mixed), mother inbreeding category, genetic father inbreeding category, social inbreeding category, age difference (in years) and temporal distance (days between sampling) were included as fixed effects in the model. A multi-membership ID variable (calculated using *lmerMultiMember* to account for the repeated occurrences of individual ID in both columns, and suitable for dyadic models (van Paridon et al., 2023)) was used as a random variable.

Metagenomic GM stability: Metagenomic species and metagenomic function were analysed as for ASV GM stability (as described above).

5.4 Results

5.4.1 Inbreeding and GM alpha diversity

16S rRNA metabarcoding diversity: An individual's GM ASV alpha diversity (richness or Shannon diversity) was not significantly associated with the individual's inbreeding coefficient, nor that of its mother, genetic or social father (Table 5.1AB, Table S5.1-5.2), but was (both richness and Shannon) negatively associated with age (Table S5.1). GM ASV richness was also associated with sample year, while Shannon diversity was also significantly negatively associated with days at 4°C (Tables S5.1-S5.2).

Table 5.1. Models investigating associations between gut microbiome (GM) alpha diversity and inbreeding (of individuals and their parents) in the Seychelles warbler. The inbreeding coefficient (FROH) predictors in all models are shown on the left side of the table: the inbreeding coefficient of 1) the focal individual, and its 2) mother, 3) genetic father, and 4) social father. The different metrics describing GM characteristics used as the dependent variables are given along the top. The estimate (Est), P-value (P), and sample size (N) of each predictor in each model are represented by the numbers in the boxes. An asterisk (*) denotes predictors that were also significant (P < 0.05) in an extra-pair only model (Table S5.5 & S5.8). Significant (P < 0.05) effects of the inbreeding coefficients with the GM characteristic in each model are shown by bold text and shading the box in blue (positive relationship) or orange (negative relationship).

	Model					
Inbreeding coefficient	A) Metabarcoding ASV Richness	B) Metabarcoding ASV Shannon	C) Metagenomics species richness	D) Metagenomics species Shannon	E) Metagenomics functional richness	F) Metagenomics functional Shannon
1) Individual	Est: -0.22 P: 0.744 N: 439	Est: -0.84 P: 0.489 N: 439	**Est: -3.09** **P: 0.046*** **N: 141**	Est: -2.46 P: 0.184 N: 141	Est: -1.75 P: 0.061 N: 139	Est: -1206 P: 0.205 N: 139
2) Mother	Est: 0.31 P: 0.606 N: 439	Est: 0.05 P: 0.962 N: 439	Est: 2.19 P: 0.057 N: 141	Est: 1.70 P: 0.238 N: 141	Est: 1.12 P: 0.103 N: 139	Est: 175 P: 0.798 N: 139
3) Genetic Father	Est: -0.06 P: 0.920 N: 439	Est: 0.52 P: 0.611 N: 439	Est: 0.20 P: 0.861 N: 141	Est: -0.53 P: 0.710 N: 141	**Est: 1.54** **P: 0.027** **N: 139**	Est: 904 P: 0.212 N: 139
4) Social Father	Est: 0.30 P: 0.628 N: 439	Est: 0.58 P: 0.605 N: 439	Est: -0.16 P: 0.894 N: 141	Est: 0.31 P: 0.840 N: 141	Est: -0.92 P: 0.208 N: 139	Est: -1167 P: 0.117 N: 139

Metagenomics taxonomic alpha diversity: Species richness (but not Shannon diversity) was significantly negatively associated with the focal individual's inbreeding coefficient (Table 5.1C, Table S5.3 & S5.4, Figure 5.2A). Using only extra-pair offspring, metagenomic species richness remained significantly associated with individual inbreeding coefficient, despite the smaller sample size (Table S5.5). Neither Richness or Shannon diversity were associated with the inbreeding coefficient of the focal individual's mother, genetic or social father (Table 5.1C, Table S5.3 & S5.4). In terms of the other control variables, metagenomic species richness was significantly negatively associated with age (Table S5.3), and metagenomic species Shannon diversity was significantly negatively associated with days at 4°C (Table S5.4), but no other variables.

Metagenomic functional diversity: Functional richness and Shannon diversity was not associated with inbreeding coefficient of the focal individual or its mother or social father, but functional richness was significantly positively associated with the inbreeding coefficient of the genetic father's (Table 5.1E, Table S5.6 & S5.7, Figure 5.2B). Using only extra-pair offspring, metagenomic functional richness remained related to the genetic father's inbreeding coefficient (with a similar positive effect size), though this was no longer significant in this reduced sample size model (Table S5.8), which suggests that, despite a lack of power, the results remain consistent. Metagenomic functional richness and Shannon diversity were both significantly negatively associated with age (Table S5.6 & S5.7) and metagenomic functional richness was also significantly associated with sample year (Table S5.6).

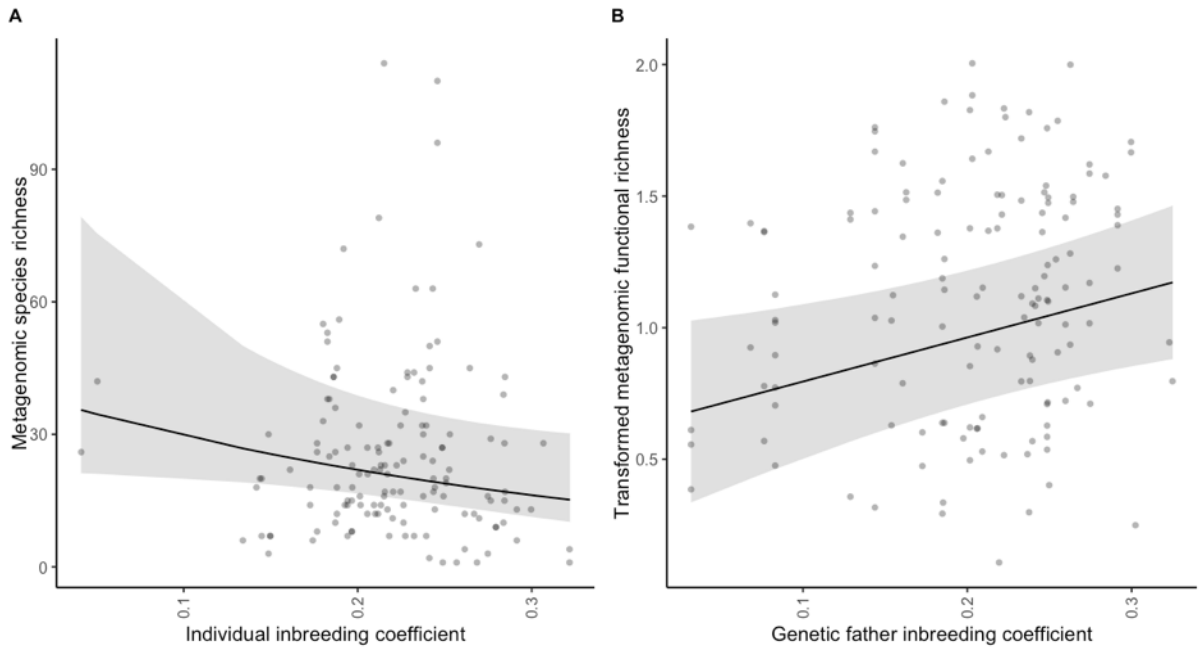

Figure 5.2. Seychelles warbler gut microbiome metagenomic-derived (A) species richness in relation to individual inbreeding coefficient (FROH), and (B) functional richness and genetic father's inbreeding coefficient. Solid lines represent model predictions (±95% confidence interval) from a generalised linear mixed effect model with negative binomial distribution (Table S5.3) and a linear mixed effects model (Table S5.6), respectively. Each point represents a unique gut microbiome sample (n = 141 samples from 80 individuals).

5.4.2 Inbreeding and GM composition

16S rRNA metabarcoding composition: GM ASV composition was significantly associated with the inbreeding coefficient of an individual, though it only explained a small amount of the overall GM variance (R^2 = 0.002, Tables 5.2 & 5.3A, Figure S5.3). Using only extra-pair offspring, GM ASV composition was still significantly associated with individual inbreeding coefficient (Table S5.9). GM ASV composition was also significantly associated with season, sample year, days at 4°C, and time of day (Table 5.2). The inbreeding coefficient of the focal individual's mother, genetic and social father was not associated with GM composition, nor was age and sex (Table 5.2 & 5.3A).

Table 5.2. A PERMANOVA of the relationship between gut microbiome (GM) ASV compositional differences and the inbreeding coefficients (FROH) of Seychelles warblers and its mother, genetic and social fathers. The analysis was performed using Aitchison distances calculated using centred log ratio (CLR) transformed amplicon sequencing variant (ASV) abundances. Significant predictors (P < 0.05)

are shown in bold. N = 439 samples from 235 individuals. Bird ID was included as a blocking factor to control for repeated measures.

	Df	R^2	F	P
Individual inbreeding coefficient	**1**	**0.002**	**0.855**	**0.005**
Mother inbreeding coefficient	1	0.003	1.147	0.872
Genetic Father Inbreeding coefficient	1	0.003	1.495	0.653
Social Father Inbreeding coefficient	1	0.002	1.058	0.648
Extra-pair paternity	1	0.002	0.995	0.448
Age	1	0.002	1.106	0.973
Sex	1	0.003	1.188	0.523
Season	**1**	**0.005**	**2.197**	**<0.001**
Sample Year	**5**	**0.024**	**2.137**	**<0.001**
Days at 4°C	**1**	**0.005**	**2.344**	**<0.001**
Time of day	**1**	**0.008**	**3.496**	**<0.001**
Residual	424	0.935		
Total	438	1		

Table 5.3. The relationship between GM composition and inbreeding in the Seychelles warbler. The inbreeding coefficient (FROH) predictors in all models are shown on the left side of the plot: the inbreeding coefficient of 1) the individual and that of its, 2) mother, 3) genetic father, and 4) social father. The different metrics describing GM characteristics used as the dependent variables are given along the top: A) ASV composition (Table 5.2), B) Metagenomic species composition (Table S5.10), C) Metagenomic functional composition (Table S5.12). The effect size (R^2), P-value, and sample size (N) of each predictor in each model are represented by the numbers in the boxes. An asterisk (*) denotes predictors that were also significant (P < 0.05) in an extra-pair only model (Table S5.9 & S5.11 & S5.13). Significant (P < 0.05) effects of the inbreeding coefficients with the GM composition in each model are shown by bold text and box shaded in light green.

Model

Inbreeding coefficient	A) Metabarcoding ASV composition	B) Metagenomics species composition	C) Metagenomics functional composition
Individual	**R^2: 0.002** **P: 0.005*** **N: 439**	R^2: 0.007 P: 0.314* N: 143	**R^2: 0.010** **P: 0.020** **N: 143**
Mother	R^2: 0.003 P: 0.872 N: 439	**R^2: 0.008** **P: 0.024** **N: 143**	R^2: 0.008 P: 0.553 N: 143
Genetic Father	R^2: 0.003 P: 0.653 N: 439	-	-
Social Father	R^2: 0.002 P: 0.648 N: 439	**R^2: 0.009** **P: <0.001*** **N: 143**	R^2: 0.007 P: 0.238 N: 143
Genetic Father * EPP	-	**R^2: 0.006** **P: <0.001** **N: 143**	**R^2: 0.006** **P: 0.028** **N: 143**

Metagenomic taxonomic composition: This was not significantly associated with inbreeding coefficient of individual's (Table S5.10) but was significantly associated with the inbreeding coefficient of the mother and social father as well as the

interaction between the inbreeding coefficient of genetic father and EPP (R^2 = 0.008, 0.009, and 0.006, respectively, Table 5.3B, Table S5.10, Figure S5.4). Metagenomic taxonomic composition was also significantly associated with sex, season, days at 4°C, and time of day (Table S5.7). The overall effect sizes were small for inbreeding coefficient of the mother and social father (R^2 = 0.007 and 0.008, respectively). Metagenomic species composition within an individual was not significantly associated with age and sample year. Using only extra-pair offspring (N=64), metagenomic species composition was still significantly associated with the social fathers' inbreeding coefficient (Table S5.11) but not with the inbreeding coefficient of mothers. In addition, using only extra-pair offspring, metagenomic species composition was also significantly associated with individual inbreeding coefficient (Table S5.11). Metagenomic species composition was also not related to age or sample year (Table 5.3B, Table S5.10).

Metagenomic functional composition: This was significantly associated with increases in the inbreeding coefficient of individuals, explaining a small amount of the overall variance (R^2 = 0.01, Table 5.3C, Table S5.12, Figure S5.5). Metagenomic functional composition was also significantly associated with the interaction between the inbreeding coefficient of genetic fathers and EPP but was not associated with the inbreeding coefficient of mothers or social fathers (Table 5.3C, Table S5.12). Using only extra-pair offspring (N=64), metagenomic functional composition was not significantly associated with the inbreeding coefficient of individuals or any other parent (Table S5.13). Metagenomic functional composition was significantly associated with age, sample year and days at 4°C (Table S5.12).

5.4.3 Inbreeding and differential abundance analysis

16S rRNA metabarcoding abundance: No differentially abundant ASVs, were identified as changing in abundance in association with the inbreeding coefficients of the individual, mother, genetic father or social father. Thus, the absence of differentially abundant ASVs suggests that inbreeding is not linked to survival-related ASVs.

Metagenomic abundance: No metagenomic species or functions were identified as changing in abundance in association with the inbreeding coefficients of the individual, mother, genetic father or social father.

5.4.4 Inbreeding and GM stability

16S rRNA metabarcoding GM stability: GM ASV stability was not associated with the inbreeding coefficients of the individual, mother, genetic father or social father (Table S5.14). GM ASV stability was significantly negatively associated with age difference and positively associated with temporal difference (Table S5.14).

Metagenomic GM stability:

GM metagenomic species and function stability was not associated with the inbreeding coefficients of the individual, mother, genetic father or social father (Table S5.15 & S5.16). GM Metagenomic species was significantly negatively associated with temporal difference (Table S5.15). GM metagenomic function was significantly positively associated with age difference and negatively associated with temporal difference (Table S5.16).

5.5 Discussion

In the Seychelles warbler, we found that the individual inbreeding coefficient was negatively associated with metagenomic species richness but was not associated with species Shannon diversity, ASV or functional GM alpha diversity. Individual inbreeding was also associated with changes in ASV and functional GM composition, but not metagenomic species composition. However, no specific taxa (ASV or metagenomics) or function varied significantly in abundance with increasing individual inbreeding. Additionally, individuals with low and high inbreeding coefficients did not differ in GM stability (metabarcoding ASV, metagenomic species or metagenomic function).

In terms of intergenerational effects, none of the parents' inbreeding coefficients were associated with ASV or metagenomic species alpha diversity. However, the genetic father's (but not the mother's or social father's) inbreeding coefficient was positively associated with metagenomic functional richness. Furthermore, the inbreeding coefficient of both the mother and social father was significantly associated with differences in metagenomic species composition. None of the parental inbreeding coefficients were significantly associated with changes in the abundance of any specific taxa or function. Finally, parental inbreeding coefficients were not associated with changes in GM stability (metabarcoding ASV, metagenomic species and metagenomic function).

The evidence from the few previous studies undertaken on inbreeding in wild animals is mixed on whether individual inbreeding is linked to lower GM diversity. We found increased individual inbreeding to be linked to decreased metagenomic species richness in the Seychelles warbler. However, individual inbreeding was not associated with ASV or functional GM alpha diversity. This contrasts with a previous study on the Seychelles warbler, which reported that lower microsatellite heterozygosity was associated with lower ASV GM bacterial richness (Davies et al., 2022). However, microsatellite variation offers low-resolution estimates of genome-wide heterozygosity and shows limited correlation with inbreeding in the Seychelles warbler, thus, it has been replaced by the more powerful whole-genome sequencing approach used in this study. The evidence is equally ambiguous in other species;

captive studies in house mice (*Mus musculus*), wild and inbred populations did not differ in GM alpha diversity (Kreisinger et al., 2014; Wang et al., 2015), but a different study in mice, and studies in banna minipig (*Sus scrofa domesticus*), and fruit flies (*Drosophila melanogaster*) identified significant differences (Hanski et al., 2025, 2024; Ørsted et al., 2022; Wei et al., 2020). In wild animals, a study on northern muriqui (*Brachyteles hypoxanthus*) found no link between heterozygosity and GM diversity (Guimaraes Sales et al., 2024), while a study on three-spined stickleback (*Gasterosteus aculeatus*) found a positive association (Steury et al., 2019). Further research is needed to fully understand how inbreeding affects GM alpha diversity in animals (especially wild animals), but this metric may be species-specific.

In the Seychelles warbler, we also found that the individual inbreeding was associated with variation in both ASV and functional GM composition. Consistent with our results, inbred individuals in previous captive studies also displayed changes in ASV (Hanski et al., 2025, 2024; Wang et al., 2015) and functional GM composition (Hanski et al., 2025; Wang et al., 2015). Similarly in wild animals, inbred individuals showed changes in ASV GM composition ((Guimaraes Sales et al., 2024; Yuan et al., 2015). However, in the Seychelles warbler, inbreeding was not associated with differences in the abundance of any specific ASVs; thus, we found no evidence that inbreeding alters any previously identified survival-related ASVs (potentially pathogenic or beneficial ASVs; (Worsley et al., 2021)). Although inbreeding was significantly associated with GM composition, it accounted for very little variance, which may explain the absence of differentially abundant taxa. This may also suggest that inbreeding likely influences a broad spectrum of microbes rather than impacting particular ones. The small effects across many taxa/function may arise from systemic host physiology changes that alter the gut environment, thereby reshaping GM composition without consistently affecting specific taxa or function (Nearing et al., 2022).

The effects of parental inbreeding varied by GM metric and by parent. The genetic father's inbreeding coefficient was positively correlated with functional richness but showed no association with GM composition. Since the GM can mediate genetic influences on social behaviour (Jin et al., 2021; Smith et al., 2023), the increase in functional richness may reflect a compensatory response to inherited genetic deficits. For example, juvenile Hihi's (*Notiomystis cincta*) sociability was associated

with father's inbreeding but not mother's inbreeding, suggesting that inbred fathers may shape the social behaviour of juveniles through genetics (Franks et al., 2023). However, in Seychelles warblers, it remains unclear whether fathers' inbreeding affects offspring social behaviour or fitness. Thus, whether the GM is compensating for genetic deficits or responding to another mechanism requires further investigation.

The inbreeding coefficient of mothers and social fathers was associated with metagenomic species composition. Social parents could be correlated with their offspring's GM as a result of physical contact or because they experience a shared environment (Sarkar et al., 2020; Tochitani et al., 2024). In other species, the GM composition of cross-fostered offspring quickly changes to reflect their foster siblings (Daft et al., 2015; Teyssier et al., 2018), indicating that parental transmission and natal environment play an important role in shaping the GM. Social parents that are inbred may have a dysbiotic GM, and this may be transferred to offspring (leading to compositional correlations with parental inbreeding), which may, in turn, impact the offspring's health and fitness (Argaw-Denboba et al., 2024). This highlights the importance of considering social parental effects on GM characteristics and how these may subsequently influence offspring health and fitness.

Inbred parents were not associated with the abundance of any specific taxa or function. This may be due to different sets of inbred parents passing on different sets of gut microbes. Thus, the changes would be individual-specific and would not be detected. Similarly, inbred parents were not associated with changes in GM stability, which is consistent with individual inbreeding coefficients, suggesting that GM stability is not linked to inbreeding.

One limitation of our study on the Seychelles warbler is that inbreeding coefficients can only be calculated from individuals that hatch and survive until being sampled. If inbreeding leads to higher mortality in our population (Pinto et al., 2026 – in prep, Kiran Lee et al., 2025 – in prep), then our results will be impacted by the selective disappearance of highly inbred individuals from our dataset. This may then reduce our statistical power to assess the effect of inbreeding on the GM, which could explain the small effect sizes. Despite that, we still detected individual inbreeding

effects on ASV and functional GM composition, as well as intergenerational inbreeding effects on metagenomic species GM composition. Future studies on the GM of wild animals where the individuals could be repeatedly sampled in early life (before mortality) may provide further resolution of this question.

Beyond genetic effects, environmental variables emerged as crucial factors shaping the GM. Specifically, temporal factors such as season and time of day explain a significant proportion of ASV and metagenomic species GM composition. In addition, sampling year was also a significant predictor of ASV GM composition. Similarly, the sampling year and host age were significant predictors of metagenomic functional GM composition. Temporal factors have previously been shown to affect GM composition in Seychelles warblers (C. Z. Lee et al., 2025a; Worsley et al., 2024b) as well as in other wild animals (Hicks et al., 2018; Marsh et al., 2022; Schmid et al., 2023; Xu and Zhang, 2021). Across GM compositional analyses, the number of days samples were stored at 4°C was also a significant predictor; hence, it was included as a control variable and has been found in previous studies (C. Z. Lee et al., 2025a; Worsley et al., 2024b). This underscores the importance of accounting for relevant confounding variables such as storage time to improve model reliability and reproducibility (Holzhausen et al., 2021).

5.6.1 Conclusion

Greater habitat fragmentation and escalating climate change will likely result in an increasing number of small, isolated populations, potentially leading to more inbreeding events in animals (Pinto et al., 2024; Surina et al., 2024). Population bottlenecks have been shown to contribute to lower GM diversity (Ørsted et al., 2022; Worsley et al., 2024a), which could compound the effects of inbreeding depression. Given the negative consequences of inbreeding, it is crucial to understand the mechanisms that lead to inbreeding depression. Our study highlights the importance of host genetics, specifically individual and parental inbreeding, in shaping the GM, which may have downstream consequences for influencing fitness.

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5.8 Supplementary material

16s richness

Table S5.1. A generalised linear mixed effect model with a negative binomial distribution (glmer.nb) investigating GM ASV richness in relation to intergenerational inbreeding coefficient among Seychelles warblers (*n* = 439 samples, 235 individuals). Conditional R^2 = .09. Significant (P<.05) terms are indicated in bold. Reference categories for categorical variables are shown as the first term in brackets and are as follows: no (EPP), major (Season), female (Sex), 2017 (Sample Year).

Characteristic	Beta	SE[1]	Statistic	p-value
(Intercept)	**5.16**	**0.26**	**20.10**	**<0.001**
Individual inbreeding coefficient	0.22	0.68	0.33	0.744
Mother inbreeding coefficient	0.31	0.60	0.52	0.606
Genetic Father Inbreeding coefficient	-0.06	0.57	-0.10	0.920
Social Father Inbreeding coefficient	0.30	0.63	0.49	0.628
EPP (no/yes)	0.09	0.07	-1.35	0.179
Age	**-0.04**	**0.01**	**-2.64**	**0.008**
Season (major/minor)	0.15	0.09	1.72	0.085
Sex (female/male)	-0.12	0.07	-1.79	0.073
Days at 4°C	-0.04	0.08	-0.55	0.585
Time of day	0.01	0.07	0.15	0.882
Sample Year				
2017	—	—	—	
2018	0.04	0.11	0.36	0.719
2019	0.17	0.13	1.38	0.169
2020	**0.46**	**0.17**	**2.78**	**0.006**
2021	**0.31**	**0.13**	**2.37**	**0.018**
2022	0.12	0.13	0.91	0.364

Random variables	Variance	SD	n
Bird ID	<0.001	<0.001	235
Mother ID	<0.001	<0.001	139
Genetic Father ID	<0.001	<0.001	131
Social Father ID	<0.001	<0.001	134

16s Shannon

Table S5.2. A linear mixed effect model investigating GM ASV Shannon diversity in relation to intergenerational inbreeding coefficient among Seychelles warblers (*n* = 439 samples, 235 individuals). Conditional R^2 = .06. Significant (P<.05) terms are indicated in bold. Reference categories for categorical variables are shown as the first term in brackets and are as follows: no (EPP), major (Season), female (Sex), 2017 (Sample Year).

Characteristic	**Beta**	**SE**[1]	**Statistic**	**p-value**
(Intercept)	3.36	0.46	7.32	**<0.001**
Individual inbreeding coefficient	-0.84	1.21	-0.69	0.489
Mother inbreeding coefficient	0.05	1.06	0.05	0.962
Genetic Father Inbreeding coefficient	0.52	1.01	0.51	0.611
Social Father Inbreeding coefficient	0.58	1.12	0.52	0.605
EPP (no/yes)	0.13	0.12	-1.10	0.271
Age	**-0.06**	**0.02**	**-2.41**	**0.017**
Season (major/minor)	0.10	0.16	0.65	0.517
Sex (female/male)	-0.20	0.12	-1.67	0.096
Days at 4°C	**-0.31**	**0.14**	**-2.23**	**0.027**
Time of day	-0.10	0.12	-0.82	0.413
Sample Year				
2017	—	—	—	
2018	0.13	0.19	0.66	0.508
2019	0.01	0.23	0.04	0.971
2020	0.31	0.30	1.05	0.296
2021	0.09	0.24	0.38	0.702
2022	0.20	0.24	0.82	0.414
Random variables	Variance	SD	n	
Bird ID	<0.001	<0.001	235	
Mother ID	<0.001	<0.001	139	
Genetic Father ID	<0.001	<0.001	131	
Social Father ID	<0.001	<0.001	134	

Mpa obs

Table S5.3. A generalised linear mixed effect model with a negative binomial distribution (glmer.nb) investigating GM species richness in relation to intergenerational inbreeding coefficient among Seychelles warblers (*n* = 141 samples, 80 individuals). Conditional R^2 = .35. Significant (P<.05) terms are indicated in bold. Reference categories for categorical variables are shown as the

first term in brackets and are as follows: no (EPP) major (Season), female (Sex), 2017 (Sample Year).

Characteristic	**Beta**	**SE**[1]	**Statistic**	**p-value**
(Intercept)	3.36	0.54	6.27	**<0.001**
Individual inbreeding coefficient	**-3.09**	**1.55**	**-2.00**	**0.046**
Mother inbreeding coefficient	*2.19*	*1.15*	*1.91*	*0.057*
Genetic Father Inbreeding coefficient	0.20	1.14	0.18	0.861
Social Father Inbreeding coefficient	-0.16	1.22	-0.13	0.894
EPP (no/yes)	-0.04	0.15	0.23	0.815
Age	-0.05	0.02	-2.35	0.019
Season (major/minor)	-0.02	0.16	-0.09	0.927
Sex (female/male)	0.02	0.14	0.14	0.893
Days at 4°C	-0.16	0.14	-1.19	0.235
Time of day	0.10	0.12	0.84	0.402
Sample Year				
2017	—	—	—	
2018	0.06	0.24	0.25	0.806
2019	0.07	0.29	0.23	0.821
2020	0.28	0.32	0.87	0.383
2021	0.31	0.28	1.11	0.269
2022	0.41	0.27	1.51	0.132
2023	0.20	0.28	0.71	0.477
Random variables	Variance	SD	N	
Bird ID	0.129	0.359	80	
Mother ID	<0.001	0.008	68	
Genetic Father ID	<0.001	<0.001	60	
Social Father ID	<0.001	0.003	67	

Mpa Shannon

Table S5.4. A linear mixed effect model (lmer) investigating GM species Shannon diversity in relation to intergenerational inbreeding coefficient among Seychelles warblers (n = 141 samples, 80 individuals). Conditional R^2 = .57. Significant (P<.05) terms are indicated in bold. Reference categories for categorical variables are

shown as the first term in brackets and are as follows: no (EPP), major (Season), female (Sex), 2017 (Sample Year).

Characteristic	**Beta**	**SE**[1]	**df**	Statistics	**p-value**
(Intercept)	1.60	0.64	69.41	2.49	0.015
Individual inbreeding coefficient	-2.46	1.84	72.72	-1.34	0.184
Mother inbreeding coefficient	1.70	1.42	51.43	1.19	0.238
Genetic Father Inbreeding coefficient	-0.53	1.42	57.37	-0.37	0.710
Social Father Inbreeding coefficient	0.31	1.53	49.04	0.20	0.840
EPP (no/yes)	0.17	0.18	66.28	-0.95	0.348
Age	-0.03	0.02	72.68	-1.06	0.293
Season (major/minor	-0.16	0.17	114.38	-0.92	0.357
Sex (female/male)	0.18	0.17	64.27	1.05	0.297
Days at 4°C	-0.41	0.14	105.16	-2.87	0.005
Time of day	-0.02	0.13	111.76	-0.14	0.892
Sample Year					
2017	—	—	—		
2018	0.16	0.25	112.81	0.65	0.516
2019	0.00	0.30	105.77	0.01	0.989
2020	0.24	0.33	109.79	0.73	0.470
2021	0.23	0.28	106.93	0.80	0.426
2022	0.36	0.28	114.30	1.28	0.204
2023	0.51	0.30	118.99	1.71	0.091
Random variables	Variance	SD	N		
Bird ID	0.264	0.514	80		
Mother ID	0.071	0.267	68		
Genetic Father ID	<0.001	<0.001	60		
Social Father ID	<0.001	<0.001	67		

Mpa obs EPP

Table S5.5. A generalised linear mixed effect model with a negative binomial distribution (glmer.nb) investigating GM species richness in relation to intergenerational inbreeding coefficient among Seychelles warblers that were born in **extra-pair paternity** ($n = 64$ samples, 38 individuals). Conditional $R^2 = .28$. Significant (P<.05) terms are indicated in bold. Reference categories for categorical variables are shown as the first term in brackets and are as follows: major (Season), female (Sex), 2017 (Sample Year).

Characteristic	Beta	SE[1]	Statistic	p-value
(Intercept)	**3.377**	**0.600**	**5.625**	**<0.001**
Individual inbreeding coefficient	**-3.876**	**1.608**	**-2.411**	**0.016**
Mother inbreeding coefficient	0.941	1.247	0.755	0.450
Genetic Father Inbreeding coefficient	1.484	1.028	1.443	0.149
Social Father Inbreeding coefficient	0.667	1.136	0.587	0.557
Age	-0.024	0.023	-1.037	0.300
Season (major/minor)	**0.520**	**0.224**	**2.323**	**0.020**
Sex (female/male)	0.136	0.169	0.801	0.423
Days at 4°C	-0.219	0.181	-1.211	0.226
Time of day	-0.063	0.159	-0.398	0.691
Sample Year	**-0.481**	**0.294**	**-1.640**	**0.101**
2017	-0.675	0.367	-1.837	0.066
2018	-0.768	0.380	-2.022	0.043
2019	0.276	0.345	0.798	0.425
2020	0.274	0.314	0.872	0.383
2021	-0.130	0.364	-0.357	0.721
2022	**3.377**	**0.600**	**5.625**	**<0.001**
2023	-3.876	1.608	-2.411	0.016
Random variables	Variance	SD	N	
Bird ID	0.129	0.359	80	
Mother ID	<0.001	0.008	68	
Genetic Father ID	<0.001	<0.001	60	
Social Father ID	<0.001	0.003	67	

NOG richness

Table S5.6. A linear mixed effect model (lmer) investigating GM functional richness in relation to intergenerational inbreeding coefficient among Seychelles warblers (*n* = 139 samples, 79 individuals). Conditional R^2 = .31. Significant (P<.05) terms are indicated in bold. Reference categories for categorical variables are shown as the first term in brackets and are as follows: no (EPP), major (Season), female (Sex), 2017 (Sample Year).

Characteristic	Beta	SE[1]	df	Statistics	p-value
(Intercept)	1.19	0.32	66.76	3.71	**<0.001**

Individual inbreeding coefficient	-1.75	0.92	70.73	-1.91	0.061
Mother inbreeding coefficient	1.12	0.68	48.14	1.66	0.103
Genetic Father Inbreeding coefficient	**1.54**	**0.67**	**44.44**	**2.29**	**0.027**
Social Father Inbreeding coefficient	-0.92	0.72	35.27	-1.28	0.208
EPP (no/yes)	-0.07	0.09	56.59	0.84	0.405
Age	-0.04	0.01	69.26	-3.06	0.003
Season (major/minor	-0.10	0.10	116.37	-1.02	0.309
Sex (female/male)	0.01	0.09	50.54	0.07	0.942
Days at 4°C	-0.13	0.09	117.20	-1.56	0.121
Time of day	-0.05	0.08	117.66	-0.62	0.535
Sample Year					
2017	—	—	—		
2018	0.04	0.14	120.44	0.25	0.804
2019	0.02	0.17	116.76	0.13	0.895
2020	0.22	0.19	121.86	1.16	0.248
2021	0.18	0.17	115.74	1.04	0.300
2022	0.38	0.16	120.36	2.35	0.021
2023	0.22	0.17	117.86	1.27	0.207
Random variables	Variance	SD	N		
Bird ID	0.023	0.173	79		
Mother ID	<0.001	<0.001	67		
Genetic Father ID	<0.001	<0.001	59		
Social Father ID	<0.001	0.080	66		

NOG Shannon

Table S5.7. A linear mixed effect model (lmer) investigating GM functional Shannon diversity in relation to intergenerational inbreeding coefficient among Seychelles warblers (*n* = 139 samples, 79 individuals). Conditional R^2 = .18. Significant (P<.05) terms are indicated in bold. Reference categories for categorical variables are shown as the first term in brackets and are as follows: no (EPP) major (Season), female (Sex), 2017 (Sample Year).

Characteristic	**Beta**	**SE**[1]	**df**	Statistics	**p-value**
(Intercept)	1276.67	334.95	69.41	3.81	**<0.001**
Individual inbreeding coefficient	-1206.09	945.01	100.55	-1.28	0.205

Mother inbreeding coefficient	175.47	684.02	83.04	0.26	0.798
Genetic Father Inbreeding coefficient	903.98	707.96	29.11	1.28	0.212
Social Father Inbreeding coefficient	-1167.24	730.92	48.63	-1.60	0.117
EPP (no/yes)	-45.37	90.11	75.46	0.50	0.616
Age	-36.33	12.58	72.85	-2.89	0.005
Season (major/minor	164.00	107.25	118.11	1.53	0.129
Sex (female/male)	58.06	87.07	99.35	0.67	0.506
Days at 4°C	28.16	93.46	120.10	0.30	0.764
Time of day	-26.54	80.38	121.30	-0.33	0.742
Sample Year					
2017	—	—	—		
2018	-45.50	154.14	118.67	-0.30	0.768
2019	-136.02	187.36	121.08	-0.73	0.469
2020	36.93	206.39	120.98	0.18	0.858
2021	223.96	182.90	120.46	1.22	0.223
2022	210.22	176.02	121.52	1.19	0.235
2023	121.93	185.43	121.31	0.66	0.512
Random variables	Variance	SD	N		
Bird ID	<0.001	<0.001	79		
Mother ID	<0.001	<0.001	67		
Genetic Father ID	13610	116.7	59		
Social Father ID	<0.001	<0.001	66		

Table S5.8. A linear mixed effect model (lmer) investigating GM functional richness in relation to intergenerational inbreeding coefficient among Seychelles warblers that were born in **extra-pair paternity** (*n* = 64 samples, 38 individuals). Conditional R^2 = .65. Significant (P<.05) terms are indicated in bold. Reference categories for categorical variables are shown as the first term in brackets and are as follows: major (Season), female (Sex), 2017 (Sample Year).

Characteristic	**Beta**	**SE**[1]	**df**	Statistics	**p-value**
(Intercept)	1.136	0.561	23.795	2.023	0.054
Individual inbreeding coefficient	-1.731	1.378	31.604	-1.255	0.219
Mother inbreeding coefficient	1.873	1.140	28.375	1.643	0.111
Genetic Father Inbreeding coefficient	1.312	1.099	15.906	1.194	0.250
Social Father Inbreeding coefficient	-1.031	1.172	19.449	-0.879	0.390

Age	-0.010	0.021	26.114	-0.497	0.623
Season (major/minor	**0.314**	**0.153**	**42.929**	**2.049**	**0.047**
Sex (female/male)	0.031	0.149	26.395	0.206	0.839
Days at 4°C	0.041	0.113	35.760	0.362	0.720
Time of day	-0.136	0.104	41.387	-1.308	0.198
Sample Year					
2017	—	—	—		
2018	-0.307	0.204	44.658	-1.506	0.139
2019	**-0.509**	**0.233**	**36.265**	**-2.185**	**0.036**
2020	-0.445	0.253	39.905	-1.763	0.086
2021	-0.170	0.223	40.254	-0.764	0.449
2022	-0.041	0.205	41.652	-0.199	0.843
2023	-0.335	0.239	45.020	-1.404	0.167
Random variables	Variance	SD	N		
Bird ID	0.032	0.180	38		
Mother ID	<0.001	<0.001	36		
Genetic Father ID	<0.001	<0.001	35		
Social Father ID	<0.001	0.080	31		

Intergenerational inbreeding on GM composition

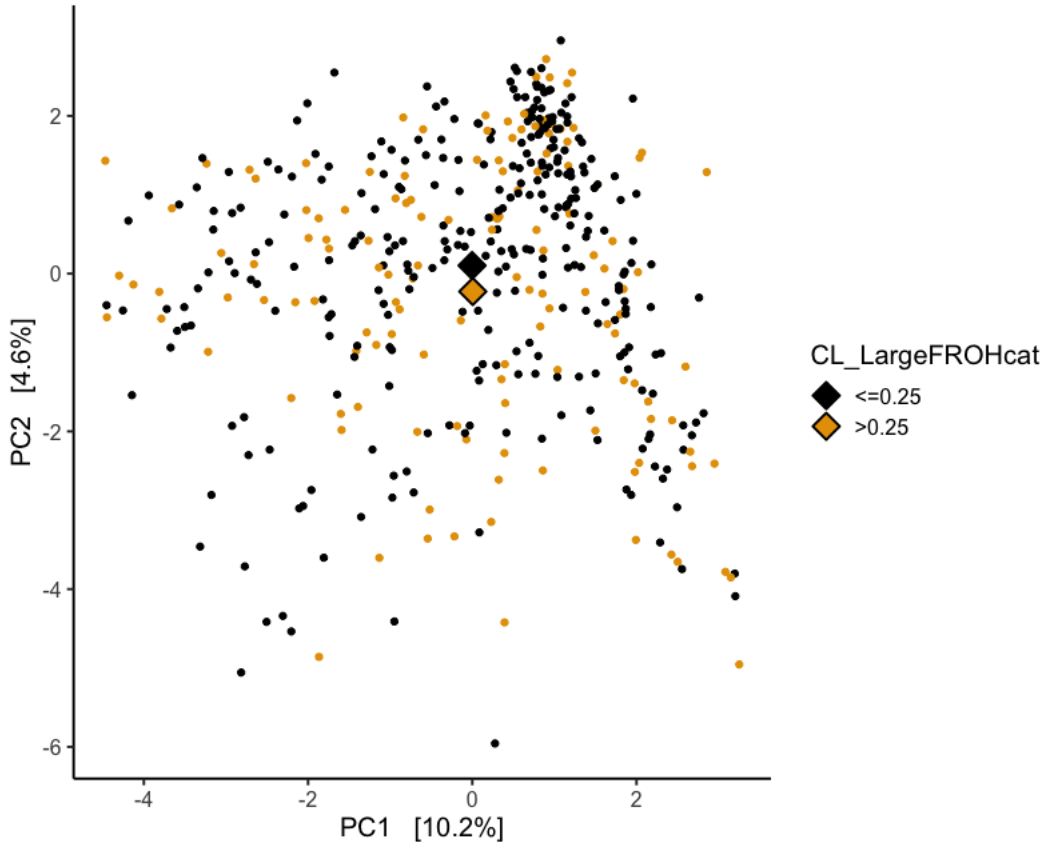


Figure S5.3. Principal Components Analyses (PCA) of gut microbiome 16S rRNA amplicon sequencing variants of Seychelles warbler faecal samples in relation to host inbreeding coefficient, N=439 from 235 birds. Large diamonds represent the group centroids. For clarity, samples were grouped into discrete categories for plotting. In plots, the coloured points indicate low (black) and high (orange) individual inbreeding coefficient.

ASV EPP

Table S5.9. A PERMANOVA of the relationship between gut microbiome (GM) ASV compositional differences and the inbreeding coefficients (FROH) of Seychelles warblers that were born in **extra-pair paternity**. The analysis was performed using Aitchison distances calculated using centred log ratio (CLR) transformed amplicon sequencing variant (ASV) abundances. Significant predictors (P < 0.05) are shown in bold. N = 183 samples from 101 individuals. Bird ID was included as a blocking factor to control for repeated measures.

	Df	R^2	F	Pr (>F)
Individual inbreeding coefficient	**1**	**0.005**	**0.871**	**0.004**
Mother inbreeding coefficient	1	0.005	1.034	0.974
Genetic Father Inbreeding coefficient	1	0.008	1.577	0.107
Social Father Inbreeding coefficient	1	0.005	0.981	0.053
Age	1	0.006	1.049	0.571
Sex	1	0.007	1.267	0.176
Season	1	0.007	1.239	0.079

Sample Year	**5**	**0.041**	**1.568**	**0.001**
Days at 4°C	1	0.009	1.660	0.104
Time of day	**1**	**0.012**	**2.309**	**<0.001**
Residual	168	0.887		
Total	182	1		

Mpa

Table S5.10. A permutational multivariate analysis of variance analysis of the relationship between metagenomics species gut microbiome compositional differences and individual, mother's, genetic father's and social father's inbreeding coefficients (fraction of the genome in runs of homozygosity) in Seychelles warblers. The analysis was performed using Aitchison distances calculated using centred log ratio (CLR) transformed amplicon sequencing variant (ASV) abundances. Significant predictors (P<0.05) are shown in bold. N=143 samples from 80 individuals. Bird ID was included as a blocking factor to control for repeated measures.

	Df	R2	F	Pr(>F)
Inbreeding coefficient	1	0.007	1.031	0.314
Mother inbreeding coefficient	**1**	**0.008**	**1.191**	**0.024**
Social Father Inbreeding coefficient	**1**	**0.009**	**1.437**	**<0.001**
Age	1	0.011	1.625	0.196
Sex	**1**	**0.017**	**2.541**	**<0.001**
Season	**6**	**0.065**	**1.671**	**<0.001**
Sample Year	1	0.005	0.772	0.455
Days at 4°C	**1**	**0.008**	**1.294**	**0.008**
Time of day	**1**	**0.012**	**1.866**	**0.001**
Genetic Father Inbreeding coefficient * EPP	**1**	**0.006**	**0.958**	**<0.001**
Residual	127	0.828		
Total	142	1		

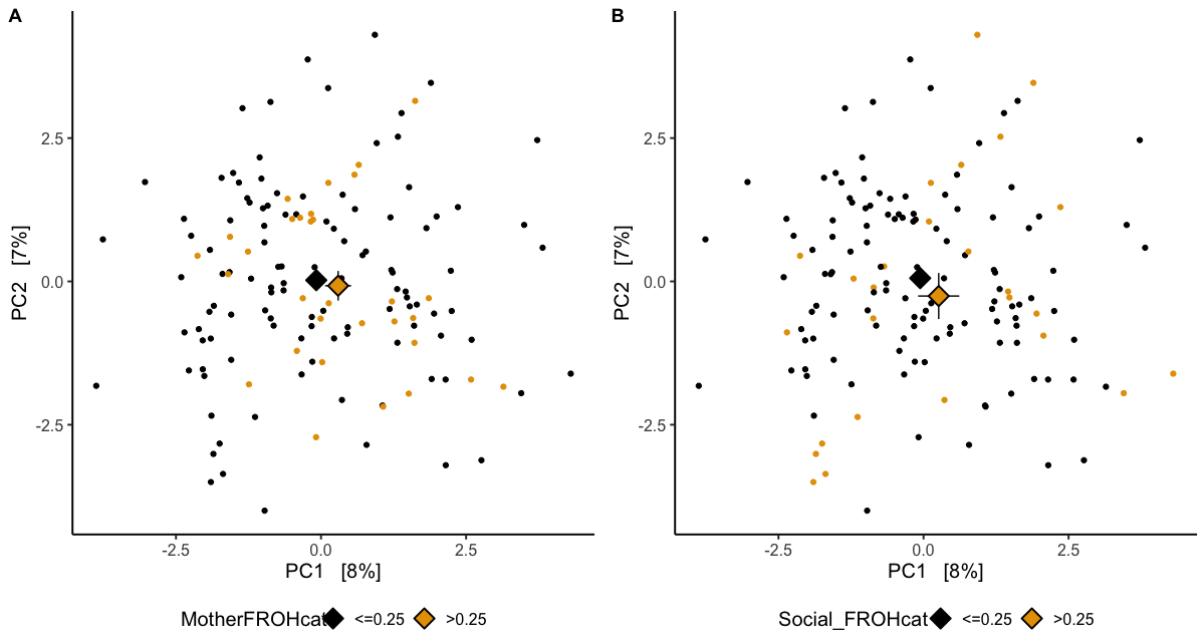


Figure S5.4. Principal Components Analyses (PCA) of gut microbiome metagenomic taxonomic composition of Seychelles warbler faecal samples in relation to (A) mother's and (B) social father's inbreeding coefficient, N=143 from 80 birds. Large diamonds represent the group centroids. For clarity, samples were grouped into discrete categories for plotting. In plots, the coloured points indicate low (black) and high (orange) inbreeding coefficient.

Table S5.11. A permutational multivariate analysis of variance analysis of the relationship between metagenomics species gut microbiome compositional differences and individual, mother's, genetic father's and social father's inbreeding coefficients (fraction of the genome in runs of homozygosity) in Seychelles warblers that were born in **extra-pair paternity.** The analysis was performed using Aitchison distances calculated using centred log ratio (CLR) transformed amplicon sequencing variant (ASV) abundances. Significant predictors (P<0.05) are shown in bold. N=64 samples from 38 individuals. Bird ID was included as a blocking factor to control for repeated measures.

	Df	R2	F	Pr(>F)
Inbreeding coefficient	**1**	**0.022**	**1.483**	**0.005**
Mother inbreeding coefficient	*1*	*0.017*	*1.201*	*0.073*
Genetic Father Inbreeding coefficient	1	0.022	1.505	0.187
Social Father Inbreeding coefficient	**1**	**0.020**	**1.372**	**0.006**
Age	1	0.025	1.706	0.222
Sex	**1**	**0.027**	**1.844**	**0.018**
Season	**6**	**0.123**	**1.414**	**0.010**
Sample Year	1	0.011	0.779	0.292
Days at 4°C	1	0.017	1.137	0.356

Time of day	1	0.020	1.344	0.114
Residual	48	0.698		
Total	63	1		

NOG

Table S5.12. A permutational multivariate analysis of variance analysis of the relationship between metagenomics functional gut microbiome compositional differences and individual, mother's, genetic father's and social father's inbreeding coefficients (fraction of the genome in runs of homozygosity) in Seychelles warblers. The analysis was performed using Aitchison distances calculated using centred log ratio (CLR) transformed amplicon sequencing variant (ASV) abundances. Significant predictors (P<0.05) are shown in bold. N=143 samples from 80 individuals. Bird ID was included as a blocking factor to control for repeated measures.

	Df	R2	F	Pr(>F)
Inbreeding coefficient	**1**	**0.010**	**1.465**	**0.020**
Mother inbreeding coefficient	1	0.008	1.128	0.553
Social Father Inbreeding coefficient	1	0.007	0.978	0.238
Age	**1**	**0.009**	**1.259**	**0.033**
Sex	1	0.013	1.868	0.050
Season	6	0.051	1.249	0.147
Sample Year	**1**	**0.007**	**0.988**	**0.001**
Days at 4°C	**1**	**0.013**	**1.891**	**0.001**
Time of day	1	0.009	1.290	0.072
Genetic Father Inbreeding coefficient * EPP	**1**	**0.006**	**0.924**	**0.028**
Residual	127	0.867		
Total	142	1		

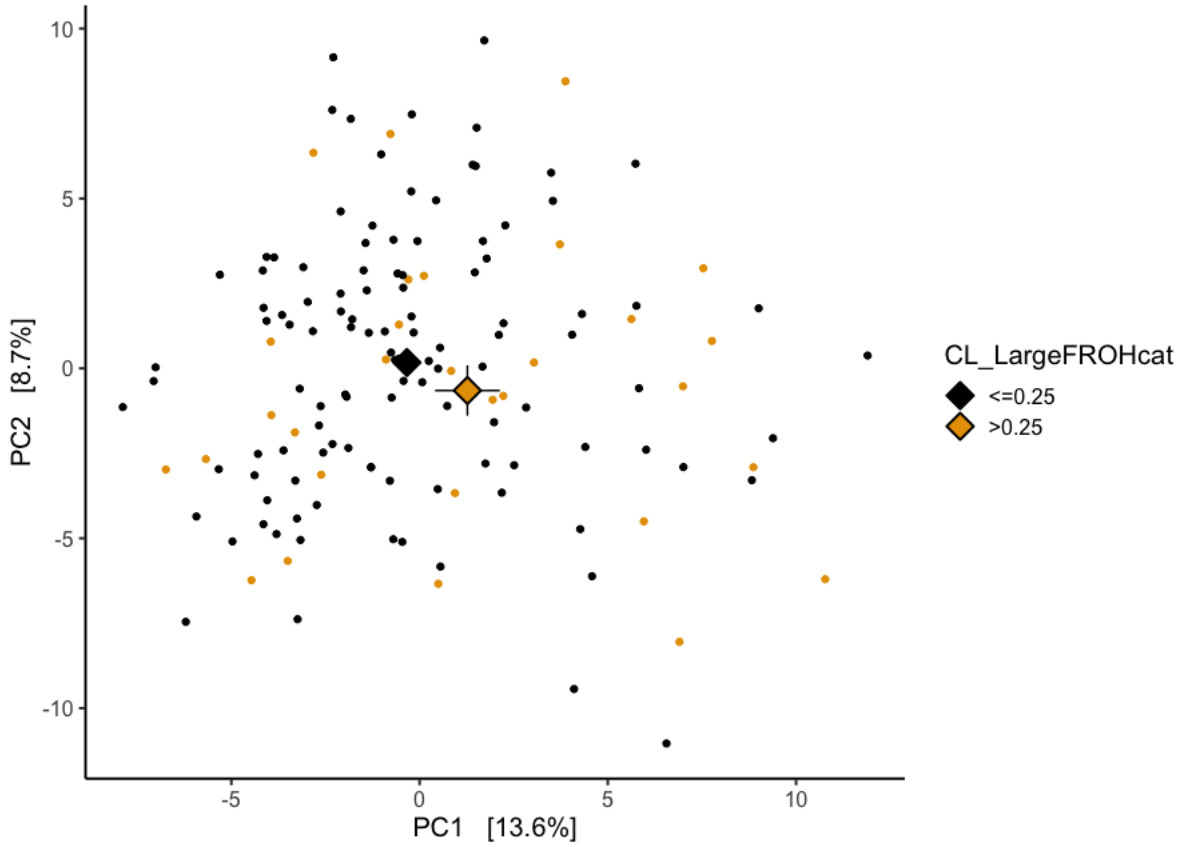


Figure S5.5. Principal Components Analyses (PCA) of gut microbiome metagenomic functional composition of Seychelles warbler faecal samples in relation to host inbreeding coefficient, N=143 from 80 birds. Large diamonds represent the group centroids. For clarity, samples were grouped into discrete categories for plotting. In plots, the coloured points indicate low (black) and high (orange) individual inbreeding coefficient.

NOG EPP

Table S5.13. A permutational multivariate analysis of variance analysis of the relationship between metagenomics functional gut microbiome compositional differences and individual, mother's, genetic father's and social father's inbreeding coefficients (fraction of the genome in runs of homozygosity) in Seychelles warblers that were born in **extra-pair paternity**. The analysis was performed using Aitchison distances calculated using centred log ratio (CLR) transformed amplicon sequencing variant (ASV) abundances. Significant predictors (P<0.05) are shown in bold. N=64 samples from 38 individuals. Bird ID was included as a blocking factor to control for repeated measures.

	Df	R2	F	Pr(>F)
Inbreeding coefficient	1	0.021	1.418	0.198
Mother inbreeding coefficient	1	0.014	0.930	0.752
Genetic Father Inbreeding coefficient	1	0.019	1.272	0.211
Social Father Inbreeding coefficient	1	0.015	0.987	0.699
Age	1	0.013	0.840	0.577

Sex	1	0.030	1.987	0.167
Season	6	0.102	1.123	0.408
Sample Year	1	0.011	0.720	0.266
Days at 4°C	**1**	**0.027**	**1.765**	**0.036**
Time of day	1	0.019	1.230	0.043
Residual	48	0.726		
Total	63	1.000		

Intergeneration inbreeding and Differential abundance analysis

All DAA not significant.

Inbreeding and GM stability

Table S5.14. A linear mixed effect model (lmer) investigating the relationship between **metabarcoding amplicon sequencing variant (ASV)** gut microbiome composition similarity in pairs of Seychelles warblers from individuals with low individual inbreeding coefficient versus high individual inbreeding coefficient (N = 96141 pairwise comparisons across 439 samples from 235 individual birds). Significant terms (P <0.05) are indicated in bold. Reference categories for inbreeding are low inbred individuals.

	Estimate	SE	df	t	P
(Intercept)	**0.354**	**0.013**	**231**	**28.002**	**<0.001**
Individual inbreeding (High)	-0.007	0.014	230	-0.487	0.627
Individual inbreeding (Mix)	-0.003	0.007	232	-0.460	0.646
Mother inbreeding (High)	-0.027	0.015	230	-1.755	0.081
Mother inbreeding (Mix)	-0.013	0.008	231	-1.755	0.081
Genetic father inbreeding (High)	0.001	0.018	230	0.056	0.955
Genetic father inbreeding (Mix)	0.000	0.009	231	0.049	0.961
Social father inbreeding (High)	-0.005	0.018	230	-0.276	0.783
Social father inbreeding (Mix)	-0.002	0.009	231	-0.265	0.791
Age difference	**-0.0003**	**0.000**	**96130**	**-2.630**	**0.009**
Temporal difference	**<0.001**	**0.000**	**95960**	**3.384**	**0.001**
Random		**54767 observations**		**Variance**	

Multi membership ID	(Intercept)	235 groups	0.003
Residual			0.005

Table S5.15. A linear mixed effect model (lmer) investigating the relationship between **metagenomic functional** gut microbiome composition similarity in pairs of Seychelles warblers from mothers and social fathers with low inbreeding coefficient versus high inbreeding coefficient (N = 10153 pairwise comparisons across 141 samples from 80 individual birds). Significant terms (P <0.05) are indicated in bold. Reference categories for categorical variables were the first term in brackets.

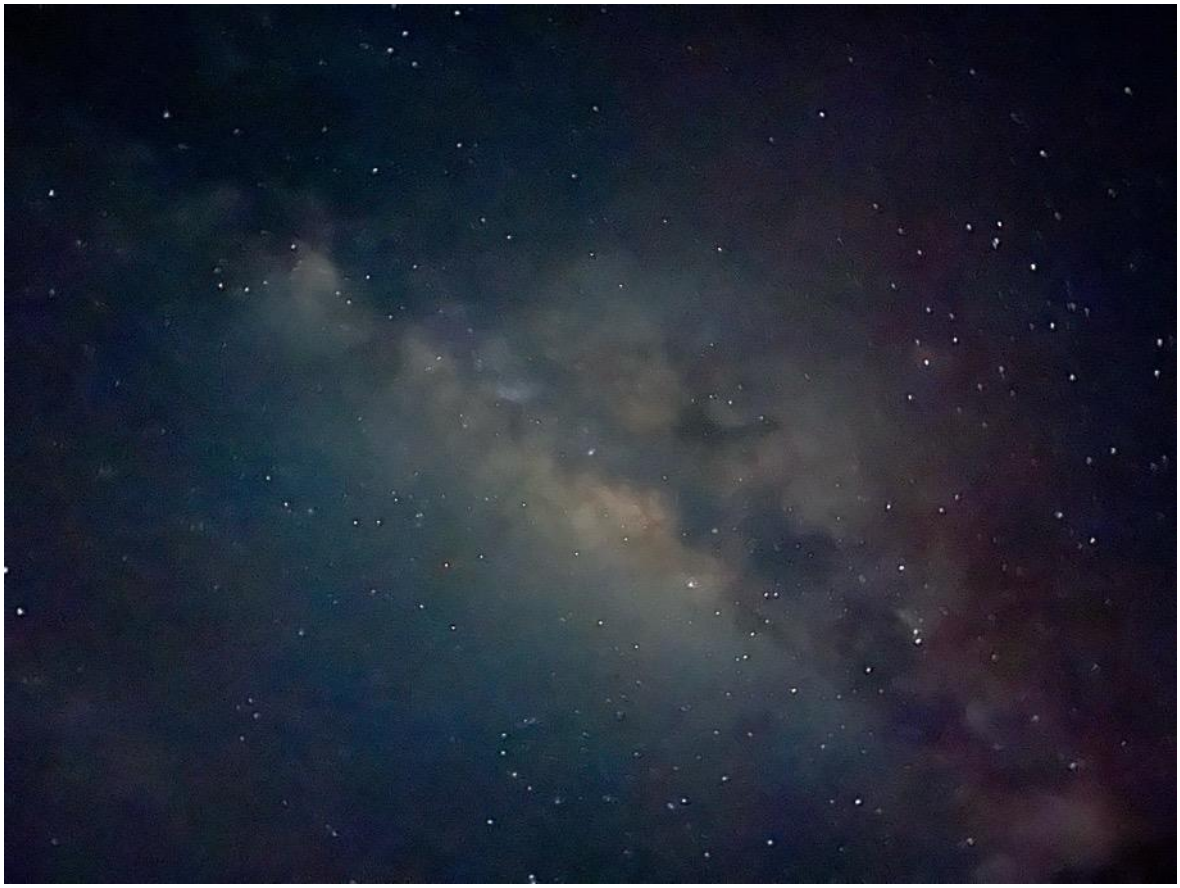
	Estimate	SE	df	t	P
(Intercept)	0.344	0.025	75	13.966	**<0.001**
Individual inbreeding (High)	0.028	0.028	75	0.969	0.336
Individual inbreeding (Mix)	0.014	0.014	76	0.960	0.340
Mother inbreeding (High)	-0.004	0.030	75	-0.126	0.900
Mother inbreeding (Mix)	-0.002	0.015	76	-0.104	0.917
Genetic father inbreeding (High)	0.006	0.033	75	0.175	0.861
Genetic father inbreeding (Mix)	-0.001	0.017	76	-0.054	0.957
Social father inbreeding (High)	0.004	0.033	75	0.121	0.904
Social father inbreeding (Mix)	0.002	0.017	76	0.104	0.918
Age difference	**<0.001**	0.000	10130	-1.554	0.120
Temporal difference	**<0.001**	0.000	10080	-7.406	**<0.001**
Random		**54767 observations**		**Variance**	
Multi membership ID	(Intercept)		235 groups	0.003	
Residual					0.005

Table S5.16. A linear mixed effect model (lmer) investigating the relationship between **metagenomic functional** gut microbiome composition similarity in pairs of Seychelles warblers from individuals with low individual inbreeding coefficient versus high individual inbreeding coefficient (N = 10153 pairwise comparisons across 141 samples from 80 individual birds). Significant terms (P <0.05) are indicated in bold. Reference categories for categorical variables were the first term in brackets.

	Estimate	SE	df	t	P
(Intercept)	0.443	0.029	75	15.213	**<0.001**
Individual inbreeding (High)	-0.041	0.034	75	-1.219	0.227
Individual inbreeding (Mix)	-0.023	0.017	76	-1.362	0.177
Mother inbreeding (High)	-0.005	0.036	75	-0.152	0.880
Mother inbreeding (Mix)	-0.003	0.018	76	-0.185	0.854
Genetic father inbreeding (High)	0.004	0.039	75	0.109	0.913
Genetic father inbreeding (Mix)	0.003	0.020	76	0.139	0.890
Social father inbreeding (High)	-0.014	0.039	75	-0.353	0.725
Social father inbreeding (Mix)	-0.009	0.020	76	-0.447	0.656
Age difference	0.001	0.000	10130	4.139	**<0.001**
Temporal difference	**<0.001**	0.000	10080	-6.764	**<0.001**
Random		**54767 observations**		**Variance**	
Multi membership ID	(Intercept)		235 groups	0.003	
Residual					0.005

Chapter 6 |

The holobiont and survival in a wild vertebrate population



“On Cousin Island, the night sky mirrors the challenge of microbiome–genome research: just as stars become constellations when connected, individual data points only reveal their true meaning when viewed as part of a larger pattern.”

6.1 Abstract

Background

The gut microbiome (GM) plays a key role in host health, influencing digestion, immunity, cognition, and survival. While environmental factors like diet and age affect GM composition, host genetics also play an important role. However, which loci across the host genome are key to shaping the GM, and the extent to which host genomic variation contributes to GM-mediated survival remains unclear—especially in natural populations.

Method

Here, we use a natural population of the Seychelles warbler (*Acrocephalus sechellensis*) on Cousin Island to investigate host genome-mediated GM compositional differences (including survival-related microbes) among individuals, and whether these differences are associated with host survival. This population provides exceptionally accurate survival data, as the small size and a lack of migration allows life-long monitoring of individuals. We analyse 205 individuals for which both whole-genome sequences and gut microbiome 16S rRNA amplicon sequencing profiles are available to address these questions.

Results

Our study provides some of the first evidence in a wild population that host genomic variation shapes gut microbiome composition. Nine host genome loci, spanning 14 genes, were strongly associated with GM composition. Variation at these loci was correlated with significant differences in the abundance of 107 unique GM ASVs. Ten out of the 107 ASVs were also differentially abundant in relation to host survival to the next season. Importantly, all nine host loci were linked to the differential abundance of at least one of these survival-related ASVs. In addition, two of the loci, *rs95_2409799* and *rs728642*, were linked to opposing effects on survival-related ASVs, and also directly on host survival.

Discussion

Our study demonstrates that the host genome influences GM composition via multiple pathways, including the immune system and gut physiology. Host loci were linked to survival-associated microbes, and the opposing effects of rs95_2409799 and rs728642 on both GM and host survival underscores the complex and potentially consequential role of host genomic variation in shaping microbiome–fitness relationships. These findings highlight the importance of exploring host genetic influences on the GM to better understand host health and survival.

6.2 Introduction

The gut microbiome (GM) has been linked with a multitude of important host processes such as digestion, immunity, behaviour and cognition (Archie and Tung, 2015; Cholewińska et al., 2020; Davidson et al., 2018; Davies et al., 2022). Therefore, it is important that we also understand what factors shape individual variation in the GM. Recent studies have identified various such factors, including diet, host age, sex, and social interactions (Bonder et al., 2016; Davies et al., 2022; Lee et al., 2025; Ruiz-Ruiz et al., 2020; Worsley et al., 2022; Xu and Zhang, 2021). Research has also highlighted the role of host genetics in shaping individual variation in the GM (Bonder et al., 2016; Goodrich et al., 2014; Melis et al., 2023). For example, host immunogenetic variation can alter the GM composition by favouring or eliminating specific microbes (Davies et al., 2022; Roland et al., 2020; Worsley et al., 2022). In turn, changes to the GM can alter the immunological function of the host, for example, by fostering beneficial microbes which prevent pathogenic microbe colonisation (Noh, 2021; Sommer and Bäckhed, 2013). Therefore, the GM may be an integral part of a host's defences. Indeed, the host genome and GM may interact to form a functional unit termed "the hologenome"; this is a potentially important concept that could improve our understanding of host traits, disease and fitness (Zilber-Rosenberg and Rosenberg, 2008). Growing evidence for this hologenome concept has significantly expanded our understanding of host–microbe coevolution (Lan et al., 2021; Rosenberg and Zilber-Rosenberg, 2018).

Given the GM is a determinant of host evolution, an important next step is to determine how host genetics influences the component of the microbiome that impacts host survival. Studies in humans and captive animals have shown that a reduction in GM diversity and a dysbiotic GM composition are correlated with poor health and the onset of diseases (Shreiner et al., 2015; Sommer et al., 2017; Videvall et al., 2020). Additionally, work on a wide range of human conditions has demonstrated that host genetic factors shape health outcomes partly through their interactions with the GM (Liu et al., 2024; Park et al., 2020). Microbiome genome wide association studies (GWAS) in humans have identified numerous genomic regions and GM characteristics that are associated with diseases (Liu et al., 2024; Markowitz et al., 2022; Priya et al., 2022; Xu et al., 2020). A common set of these loci collectively regulate the host immune response, gut physiology, and gut-microbe

interactions (Hao et al., 2025; Ntunzwenimana et al., 2021; Priya et al., 2022). Such findings highlight that neither host genetics nor the GM alone fully explain variation in health. and underscore the importance of adopting a hologenome perspective to understand health and fitness outcomes.

While valuable, humans and captive animals often have a very different GM community compared to their wild counterparts (Clayton et al., 2016; Malukiewicz et al., 2022; van Leeuwen et al., 2020). Captive animals, normally exhibit a reduced GM diversity, which may not provide a representative model for detecting natural (and/or subtle) GM shifts that precede disease in wild populations (Gibson et al., 2019; Ning et al., 2020; Oliveira et al., 2020). Wild animals tend to have a more diverse GM, and, unlike in captive animals, diversity alone is often not been a good indicator of health (Davidson et al., 2021; Williams et al., 2024; Worsley et al., 2021). In addition, captive animals often harbour limited genomic variation, further restricting the generalisation of findings to wild populations (Festing and Lutz, 2010; Wei et al., 2020). Finally, human/captive GM studies are limited by confounding variables such as medical intervention and lifestyle changes (Haran et al., 2021; Konstantinidis et al., 2020; Martinez et al., 2021; Thorburn et al., 2014). Direct studies on wild animals are needed to fully understand the complex co-evolutionary relationship between host genetics, the GM, and host survival.

Quantifying survival in wild animals is inherently challenging because it requires accurate, individual-level survival data, which is often confounded by dispersal and imperfect detection. Despite these challenges, a few studies have investigated how the GM is associated with host survival in wild animals, but the evidence remains mixed. One study found that higher alpha diversity is correlated with higher survival (Bestion et al., 2017), while three others did not (Davidson et al., 2021; Stothart et al., 2024; Worsley et al., 2021) In addition, two studies reported association between GM composition and host survival (Stothart et al., 2024; Worsley et al., 2021) but not one other one (Davidson et al., 2021). Furthermore, a study in feral horses (*Equus ferus caballus*) also reported functional GM differences related to host survival (Stothart et al., 2024). However, to our knowledge, no studies have investigated the influence of the host genome on the gut microbes that influence survival in a wild population.

Whether variation in the host genome contributes to the strength or direction of any GM-host survival associations is unexplored in the wild. This represents a major gap in our understanding, as linking host genetics with the microbiome and survival could reveal fundamental mechanisms of host-microbe coevolution. A microbiome GWAS approach offers a powerful way to pinpoint host loci that shape microbial community structure (Hua et al., 2022), enabling us to link specific genetic variants with survival-associated microbes. To our knowledge, ours is the first study to take such an approach in a wild species, providing a unique window into how natural genetic variation interacts with the GM to influence fitness. Such knowledge could inform targeted interventions, such as personalised probiotics to support survival, when the natural GM is disturbed (e.g. in captivity or during translocation events) (Chong et al., 2019).

Here, we use a population of the Seychelles warbler (*Acrocephalus sechellensis*) to investigate links between individual host genomic variation and GM composition, including specifically with survival-related gut microbes. The Seychelles warbler provides an excellent system for such a study as intensive long-term monitoring, provides accurate survival data, while extensive whole genome resequencing provides aligned genetic data (Kiran Lee et al., 2025, in prep). A previous analysis on this population has demonstrated that adult survival is associated with GM composition (Worsley et al., 2021). We hypothesise that host genomic variation is linked to GM composition, leading to differences in survival. Specifically, we aim to determine, 1) which host loci are correlated with GM composition, 2) more specifically, which host loci are associated with variation in the abundance of survival-related ASVs, and 3) to what extent that host genomic variation is associated directly with host survival.

6.3 Method

6.3.1 Study system

The Seychelles warblers on Cousin Island have been monitored since 1985 during both the January to March (minor) and June to October (major) breeding seasons that occur each year (Brown et al., 2022; Komdeur, 1992). Individuals are caught using mist nets or in the nest (age is determined based on hatch/lay dates and eye colour (Komdeur, 1992)), and all new individuals are marked using a British Trust for Ornithology (BTO) metal ring and a unique combination of three colour rings. Since 1997, nearly all individuals have been marked (>96%), allowing them to be closely monitored throughout their lives (Richardson et al., 2001). The annual resighting rate of individuals is high at 98% ± 1% SE (Brouwer et al., 2010) and there is virtually no dispersal between islands (Komdeur et al., 2004), therefore, if an individual has not been sighted during a breeding season, it is confidently presumed dead. The population normally consists of ca. 300 individuals from ca. 110 stable year-round territories (Kingma et al., 2016; Komdeur, 1992). Seychelles warblers are monogamous facultative cooperative breeders, whereby the dominant breeding pair in each territory may be joined by other subordinate adult individuals that may also help (Komdeur, 1991; Richardson et al., 2003, 2002). Cobreeding and extra-pair paternity occur frequently (~44%) (Hadfield et al., 2006; Richardson et al., 2001), thus, all parentage is genetically verified thus allowing a multi-generation pedigree to be generated (Sparks et al., 2022).

6.3.2 Sample collection

Faecal samples were collected by placing caught birds in a flat-bottom paper bag with a sterilised weigh boat under a metal grate, thus allowing faeces to drop to the plastic, while minimising contact with the birds' surface (Davies et al., 2022; Knutie and Gotanda, 2018). Faecal samples were stored in a microcentrifuge tube containing 1 mL of absolute ethanol, at 4°C during fieldwork and then at -80°C for long-term storage at UEA. Each season, control samples were collected by swabbing the insides of collection bags and fieldworker hands. A small (ca. 25 µl)

blood sample was collected from each bird via brachial venepuncture and stored in 0.7 ml of absolute ethanol at 4°C. The time of day (minutes after sunrise; 06:00 AM) of sampling and the number of days faecal samples were kept at 4°C during fieldwork were recorded.

6.3.3 DNA extraction and sequencing

Microbial DNA from faecal samples were extracted using the DNeasy PowerSoil Kit (Qiagen, Crawley, UK) with a modified version of the manufacturer's protocol (described in (Davies et al., 2022)). Samples were extracted in a random order to minimise batch effects. Microbial DNA samples were submitted for 16S rRNA gene amplicon sequencing in seven batches. Libraries were generated with V4 primers 515F (5'TGCCAGCMGCCGCGGTAA3') and 806R (5'GGACTACHVGGGTWTCTAAT3') and sequenced using 2 x 250 bp paired-end sequencing on an Illumina MiSeq platform. Control samples were also DNA extracted, amplified and sequenced in the same way (n = 21 hand controls, 15 negative controls, and 10 positive ZymoBIOMICS Microbial Community Standard (D6300) controls).

Total host genomic DNA from blood samples was extracted using the DNeasy Blood and Tissue kit (Qiagen, Crawley, UK) according to the manufacturer's protocol, or a salt extraction protocol (Richardson et al., 2001). Extracted DNA was used for molecular sexing following (Griffiths et al., 1998) and microsatellite genotyping for parentage analyses (Richardson et al., 2001; Sparks et al., 2022b). Genomic DNA was submitted for whole genome sequencing in 20 batches (detailed in Kiran Lee et al., 2025 – in prep) from randomly selected libraries generated with NEBNext Ultra II FS DNA Library Prep (New England Biolabs). Libraries were sequenced using 2 x 150 bp, paired-end sequencing on an Illumina NovaSeq 6000 platform.

6.3.4 Bioinformatics

Microbial 16S amplicon sequenced reads were processed as described in detail in (Worsley et al., 2024b). Briefly, 16S rRNA reads were truncated, filtered, and classified as amplicon sequencing variants (ASVs) using DADA2 as part of QIIME2 (Bolyen et al., 2019; S. F. Worsley et al., 2024a). ASVs were taxonomically assigned using a naïve-Bayes classifier on the SILVA 132 reference database for 16S rRNA gene sequences (Quast et al., 2012). The resulting ASVs were imported into R using *phyloseq* 1.46.0 (Leo Lahti and Sudarshan Shetty, 2019; McMurdie and Holmes, 2013). ASVs were filtered to remove non-bacterial sequences, those unassigned to the phylum level, or with fewer than 50 reads (which may represent sequencing errors). Potential contaminants were also filtered with *decontam* 1.18.0 (Davis et al., 2018) using negative lab and collection controls as a reference. Samples with fewer than 8000 reads were also removed based on samples reaching >95% completeness in rarefaction curves (Worsley et al., 2024b). In total, 205 adult samples/individuals remained with both gut microbiome and host genomic samples. Rare ASVs (<5% prevalence) were then removed, and pairwise UniFrac distances (beta diversity) were calculated using the distance() function in *phyloseq* (Leo Lahti and Sudarshan Shetty, 2019; McMurdie and Holmes, 2013).

Whole-genome sequencing reads were processed as described previously (Kiran Lee et al., 2025). Briefly, reads were filtered to remove low-quality reads (Phred quality score of 33 and a minimum length of 80 bp) using *Trimmomatic* version 0.39 (Bolger et al., 2014). The remaining reads were mapped to the Seychelles warbler reference genome (Kiran Lee et al., 2025; BUSCO: 96.0% with a total length = 1,081,018,985 bp) before imputation with *STITCH* version 1.7.0 (Davies et al., 2016), with the following parameters: diploid, eight founding haplotypes, and 25 generations since founding (Kiran Lee, et al., 2025). The accuracy of imputation was tested by down sampling high coverage samples (n = 57), imputing the reduced sample, and then comparing the imputed genotypes against the original high-coverage genotypes at each site (accuracy = 96%). Linkage disequilibrium (LD) was then calculated in *PLINK* version 2.0 (Purcell et al., 2007) with these high coverage samples using a genotyping rate of >99%, minor allele frequency of >30%, LD window of 5 Mb and LD of 0.

6.3.5 Statistical methods

All statistical analyses were conducted in R 4.3.3 on R Studio 2024.12.0+467 unless stated otherwise (Posit team, 2024; R Core Team, 2024).

6.3.5.1 Genome wide association study (GWAS) of GM composition

A GWAS was carried out using *MicrobiomeGWAS* (Hua et al., 2022) with GM beta diversity (UniFrac distance matrix) as the response variable. Firstly, the imputed host SNPs were filtered for >0.4 STITCH imputation INFO score, autosomes, >95% genotyping rate, and >5% minor allele frequency (no. of SNPs = 2,720,843). A minor allele frequency of 5% was chosen because this coincides with the most accurate p-values (lowest skewness and kurtosis) for small sample sizes in *MicrobiomeGWAS* (Hua et al., 2022). To account for relatedness, we calculated the top five principal components of host genome variation using --pca function in *PLINK* (Purcell et al., 2007). These and variables identified as important in previous Seychelles warbler GM studies (Lee et al., 2025; Worsley et al., 2024b, 2024c) (i.e. host age, sex, individual inbreeding coefficient, sample year, season (major/minor), time at 4°C, and time of day), were included as control variables in the GWAS. Significant SNPS were determined by applying a false discovery rate (FDR) correction with the *qvalue* package (Storey et al., 2024); giving $q < 0.05$. The highest SNP per peak in the GWAS (smallest q-value) that are also not co-occurring (i.e. VIF < 3) (Fox and Weisberg, 2019), were selected for downstream analysis. Genes within ±75 kb (half-LD, Figure S6.1) of all the significant SNPs were identified using the functionally annotated Seychelles warbler reference genome (Kiran Lee, et al., 2025).

6.3.5.2 Determining ASVs associated with GM-associated SNPs

To determine how the GM-associated SNPs (identified above) impacted the abundance of specific bacterial ASVs, an analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC2) (Lin and Peddada, 2024) was conducted. This included the presence/absence of all identified GM-associated SNPs as the primary independent variables, followed by host age, sex, individual inbreeding coefficient, sample year, season, storage time at 4°C, and the time-of-day samples were collected as control variables. The Holm method was used to correct *P*-values for multiple testing ($q<0.05$).

6.3.5.3 GM-associated SNPs and host survival to the next season

ASVs determined to be associated with any GM-associated SNPs were then tested to establish if they differed in abundance across hosts that survived or died by the following breeding season. Adult warblers with GM samples (including individuals without whole-genome sequencing data) and accurate survival records (excluding birds sampled in 2020 due to incomplete censusing during the covid pandemic) were selected. Only one sample per bird was used in this analysis as described previously (Worsley et al., 2021). In total, N = 266 samples/individuals were included in the analysis. An analysis of ANCOM-BC2 was conducted with survival to the next season (yes/no) as the primary variable, and the control variables, sample year and season as described previously (Worsley et al., 2021). Significance was determined following a Holm correction for multiple testing correction ($q<0.05$).

6.3.5.4 GM-associated SNPs and host survival (lifespan)

A Cox-regression mixed effect model was conducted to test if any of the GM-associated SNPs was directly associated with host survival using the long-term dataset of individuals with survival and genomic data. Annual survival from birth (lifespan) was the response variable, with individuals right censored if still alive (N = 1340 samples/individuals; 57 right censored), and the presence of the minor allele at all GM-associated SNPs was the predictor. Variables previous identified as important for Seychelles warblers' survival were included as control variables (Borger et al., 2023; Brouwer et al., 2006; Davies et al., 2021; Sparks et al., 2022a): sex (female/male), individual inbreeding coefficient, mother's age, group size, helper presence at birth (no/yes), sibling presence at birth (no/yes), and mean rainfall (the average annual rainfall experience by the bird in its lifetime) were included as control variables. Birth year was included as a random effect. A second model was constructed with the same structure, replacing presence of minor allele with allele genotypes.

6.4 Results

6.4.1 Genome wide association study (GWAS) of GM composition

In the Seychelles warbler's genome we identified 393 SNPs, spanning 9 peaks (labelled after and represented by the highest SNP (Table 6.1)), significantly ($q < 0.05$) associated with overall GM composition (Table S6.1, Figure 6.1). The peaks, -one in chromosome three, one in chromosome six, one in chromosome 12, and 11 in chromosome 17, -are in or close to 14 known genes (Table S6.1). Two peaks were not in or near any genes (Table 6.1). Three peaks were single SNPs, whereas six peaks consist of more than one SNP (Table 6.1). Gene functions are summarised in Table S6.2.

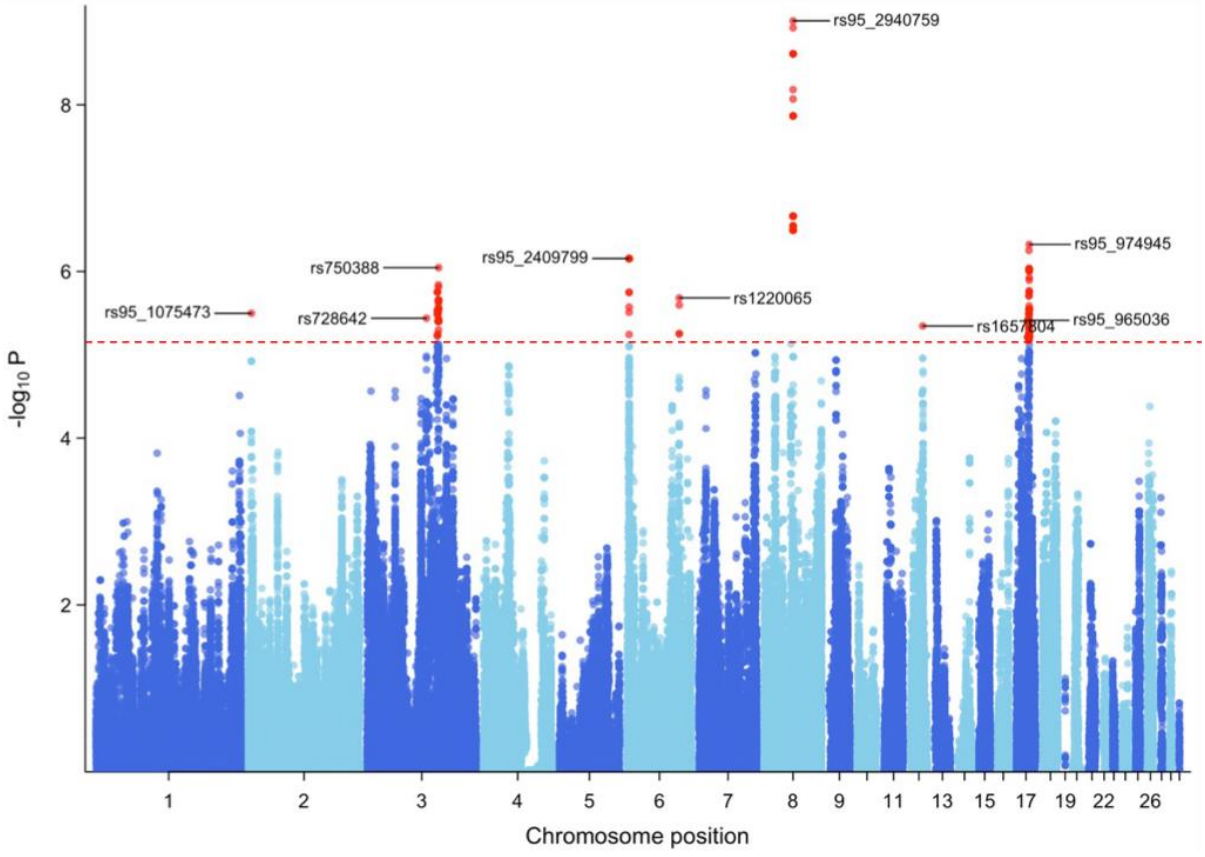


Figure 6.1. Genome-wide association analysis (GWAS) of host genetic variants and gut microbiome (GM) composition in Seychelles warblers (N = 205 individuals). Differences in GM composition were calculated using pairwise UniFrac distances. GWAS signals ($-\log_{10}P$) are reported for SNP markers across all chromosomes (displayed with alternating colours). SNPs that are significantly associated ($q < 0.05$) with differences in GM composition are coloured in red. Labelled SNPs represent the highest points (largest $-\log_{10}P$ value) that are not collinear (VIF < 3) with any other SNP.

Table 6.1. Host genetic variants associated with differences in gut microbiome (GM) composition among adult Seychelles warblers (N = 205). Associations were identified via a Genome-wide Association analysis (GWAS)- see Figure 6.1. The chromosome (Chr) number, position, number of SNPs, and minor allele freqeuncy are presented, as well as the genes encoded within that region. Gene functions are summarised in Table S6.2.

Region name	Chr	Position	No. of SNPs	Minor allele F	Genes
rs95_1075473	2	3,156,468	1	0.23	Unknown gene
rs728642	3	65,229,635	1	0.32	Unknown gene
rs750388	3	77,394,794 – 78,619,451	86	0.41	*GRIK2*
rs95_2409799	6	2,239,609 – 2,278,765	15	0.18	*CACHD1*
rs1220065	6	57,461,779 – 57,462,611	4	0.25	Not near any genes
rs95_2940759	8	31,789,764 – 31,799,923	68	0.31	Not near any genes
rs1657804	12	13,802,557	1	0.18	*MED7*
rs95_965036	17	12,982,837 – 12,986,427	12	0.43	*SEC16A*
rs95_974945	17	14,084,350 – 14,617,185	205	0.21	*SARDH*, *FAM163B*, *ADAMTSL2*, *TMEM8C*, *SLC2A6*, *SPACA9*, *AK8*, *DDX31*, *BARHL1*, *CFAP77*

6.4.2 Determining ASVs associated with GM-associated SNPs

The abundance of 107 unique ASVs differed significantly in association with the presence/absence of the minor allele at the nine identified loci (Figure 6.2). These ASVs are assigned to six phyla, 24 orders, and 37 families (differentially abundant ASV taxonomy is presented in full in Table S6.2). An average of 23.4 ± 3.67 SE ASVs (range = 7-44 ASVs) were associated with each GM-associated SNP. Each ASV was associated with 1-7 loci (average of 1.97 ± 0.12 SE); 49.5% of ASVs were associated with one locus.

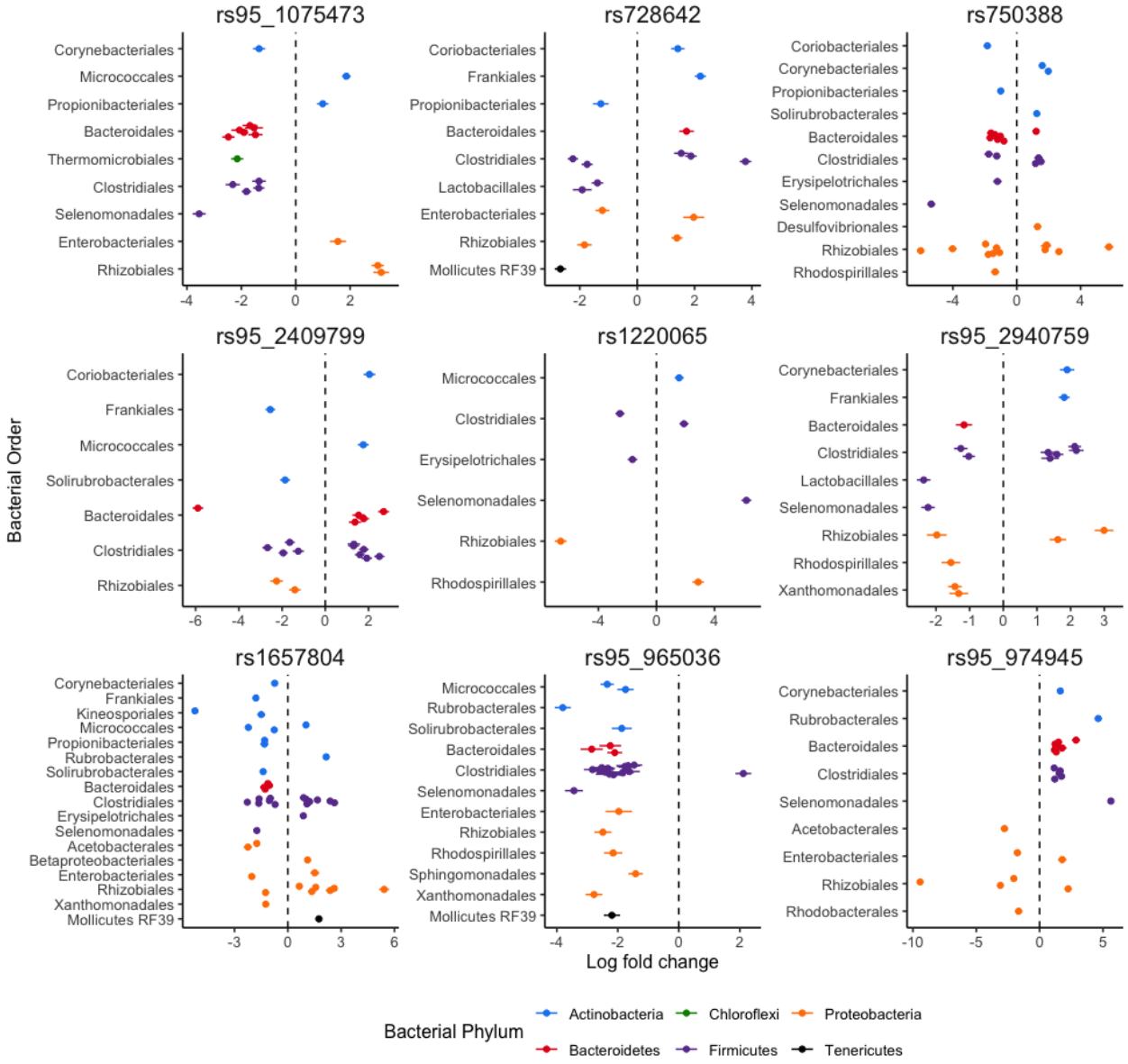


Figure 6.2. Differentially abundant amplicon sequencing variants (ASVs) in the gut microbiome (GM) of adult Seychelles warblers harbouring (or not) the minor allele at nine genomic loci. Host loci were selected based on a GWAS of GM composition (see Figure 6.1, Table 6.1). Points and error bars represent the log fold change in abundance of significant ASVs (P_{adj}<0.05) associated with each host genomic loci. A positive log fold change indicates that an ASV is more abundant in individuals containing the minor allele and a negative log fold change indicates a higher prevalence in individuals without the minor allele. ASVs are classified by bacterial order and coloured by bacterial phylum. Results of differential abundance tests and ASV taxonomies are presented in full in Table S6.3. N = 205 individuals were included in the analysis.

6.4.3 GM-associated SNPs and host survival to the next season

Analysis revealed 10 out of the 107 differentially abundant ASVs were also linked to host survival: five were more abundant in the GM of those that survived to the next breeding season, and five were more abundant in those that died (Figure 6.3,

Table S6.4). These ASVs were from seven bacterial orders (see Figure 6.3), nine bacterial families, and 10 bacterial genera (taxonomy of survival-related ASVs is presented in full in Table S6.4). All nine genomic loci identified in the GWAS were associated with at least one survival-related ASV; the presence of the minor allele in six loci was associated with both positive and negative survival-related ASVs (*r95_1075473*, *rs728642, rs95_2409799, rs95_2940759, rs95_965036, rs95_974945*), one locus was associated with only positive survival-related ASVs (*rs750388*), and two loci were associated with only negative survival-related ASVs (*rs1220065* and *rs1657804*) (Figure 6.3, Table S6.5).

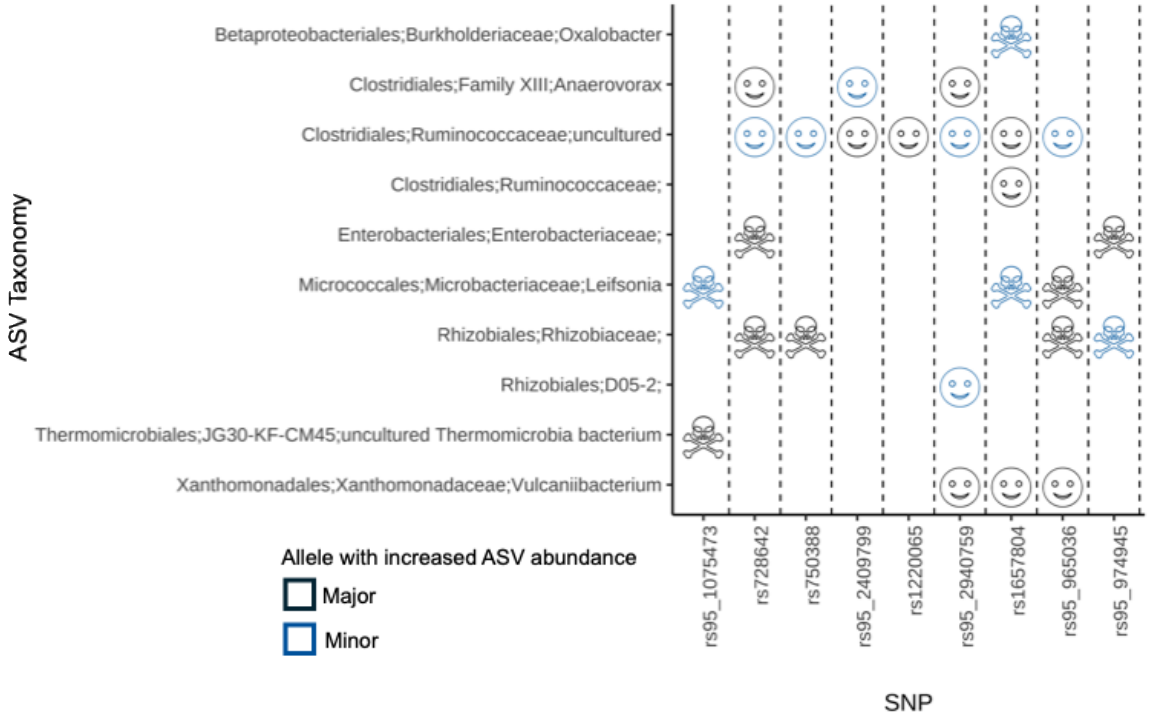


Figure 6.3. Gut microbiome (GM) amplicon sequencing variants (ASVs) associated with host genetic variants (Table S6.3,4.2) and with the differential survival of Seychelles warblers (N = 205 individuals). Survival was defined based on whether an individual survived to the breeding season following gut microbiome sampling. Skulls and smiley faces designate where ASV abundance was negatively or positively associated with survival, respectively. The allele with increased ASV abundance is coloured in black (without minor allele) and blue (presence of minor allele). ASV taxonomies are shown in bacterial order, family, genus (y-axis).

6.4.4 GM-associated SNPs and direct host survival

The presence of the minor allele at *rs95_2409799* was associated with significantly lower mortality risks, while the presence of the minor allele at *rs728642* was

significantly associated with higher mortality risks (indicated by a negative and positive coefficient, and a higher hazard ratio, respectively; Table 6.2A, Figure 6.4). The minor allele of both loci was inversely associated with survival-related *Clostridiales* ASVs- *rs95_2409799* was associated with increased abundance of Family XIII; *Anaerovorax* and a decrease in *Ruminococcaceae*; uncultured, whereas *rs728642* was associated with decreased abundance of Family XIII; *Anaerovorax* and an increase in *Ruminococcaceae*; uncultured (Figure 6.3, Table S6.5). No other GM-associated loci were significantly associated survival (Table 6.2A).

The heterozygous genotype of *rs95_2409799* was significantly associated with a lower mortality risk (Table 6.2B, Figure S6.2A). In addition, the heterozygous genotype of *rs728642* was marginally associated with a higher mortality risk (Table 6.2B, Figure S6.2B). However, the genotypes of other loci were not significantly associated with survival (Table 6.2B).

Table 6.2. Cox proportional hazard model to test the effects of (A) allelic variation and (B) the genotype of SNPs associated with gut microbiome composition, on survival in the Seychelles warbler (N = 1340). Individuals still alive at the end of the study are right censored (N = 57). Significant terms (P < 0.05) are indicated in bold, marginal and significant alleles (P < 0.1) are underlined. Reference categorical variables are as follows: (A) absence of minor allele, (B) major allele homozygous, sex (female), helper presence in natal territory (no), and sibling presence in natal territory (no). Abbreviations: Coef, hazard rate; HR, hazard ratio; SE(coef), standard error of the hazard rate.

Predictors	**Coef**	**HR**	**SE(coef)**	**z**	**p**
(A) Presence/absence of minor allele					
rs95_2940759	0.05	1.05	0.06	0.80	0.424
rs95_974945	0.09	1.09	0.06	1.39	0.163
rs95_2409799	**-0.14**	**0.87**	**0.06**	**-2.27**	**0.023**
rs750388	-0.10	0.90	0.06	-1.66	0.097
rs1220065	-0.06	0.94	0.06	-1.03	0.304
rs95_1075473	-0.01	0.99	0.06	-0.09	0.931
rs728642	**0.12**	**1.12**	**0.06**	**1.97**	**0.048**
rs95_965036	-0.02	0.98	0.06	-0.37	0.715
rs1657804	-0.01	0.99	0.06	-0.21	0.835
Sex (male)	0.09	1.10	0.06	1.59	0.112
Inbreeding coefficient	**2.20**	**9.07**	**0.49**	**4.50**	**<0.001**
Mother's age	0.01	1.01	0.01	1.13	0.260
Helper (yes)	0.13	1.14	0.09	1.42	0.154
Sibling (yes)	-0.08	0.93	0.06	-1.23	0.220
Group Size	0.02	1.02	0.05	0.51	0.612
Mean rainfall	**<0.01**	**1.00**	**<0.01**	**10.43**	**<0.001**
Random effects	Variance	SD			
Birth Year	0.078	0.278			
(B) SNP genotypes					
rs95_2940759					
Hz	0.07	1.07	0.07	0.96	0.335
Hm	0.02	1.02	0.07	0.28	0.782

rs95_974945						
	Hz	0.08	1.09	0.07	1.25	0.211
	Hm	0.20	1.22	0.13	1.50	0.133
rs95_2409799						
	Hz	**-0.14**	**0.87**	**0.07**	**-2.18**	**0.030**
	Hm	-0.17	0.85	0.21	-0.78	0.437
rs750388						
	Hz	-0.11	0.90	0.06	-1.66	0.097
	Hm	-0.07	0.93	0.09	-0.82	0.411
rs1220065						
	Hz	-0.07	0.94	0.06	-1.04	0.299
	Hm	-0.02	0.98	0.15	-0.11	0.910
rs95_1075473						
	Hz	-0.01	0.99	0.06	-0.14	0.887
	Hm	0.05	1.05	0.13	0.39	0.694
rs728642						
	Hz	0.12	1.13	0.06	1.90	0.057
	Hm	0.11	1.12	0.09	1.23	0.217
rs95_965036						
	Hz	0.01	1.01	0.07	0.22	0.829
	Hm	-0.14	0.87	0.09	-1.57	0.116
rs1657804						
	Hz	-0.01	0.99	0.06	-0.10	0.920
	Hm	0.01	1.01	0.14	0.05	0.962
Sex (male)		0.10	1.10	0.06	1.66	0.098
Inbreeding coefficient		2.21	9.07	0.50	4.44	**<0.001**
Mother's age		0.01	1.01	0.01	1.12	0.262
Helper		0.14	1.15	0.09	1.49	0.136
Sibling		-0.07	0.93	0.06	-1.09	0.275

Group size	0.03	1.03	0.05	0.58	0.562
Mean rainfall	**<0.01**	**1.00**	**<0.01**	**10.43**	**<0.001**
Random effects	Variance	SD			
Birth Year	0.079	0.28			

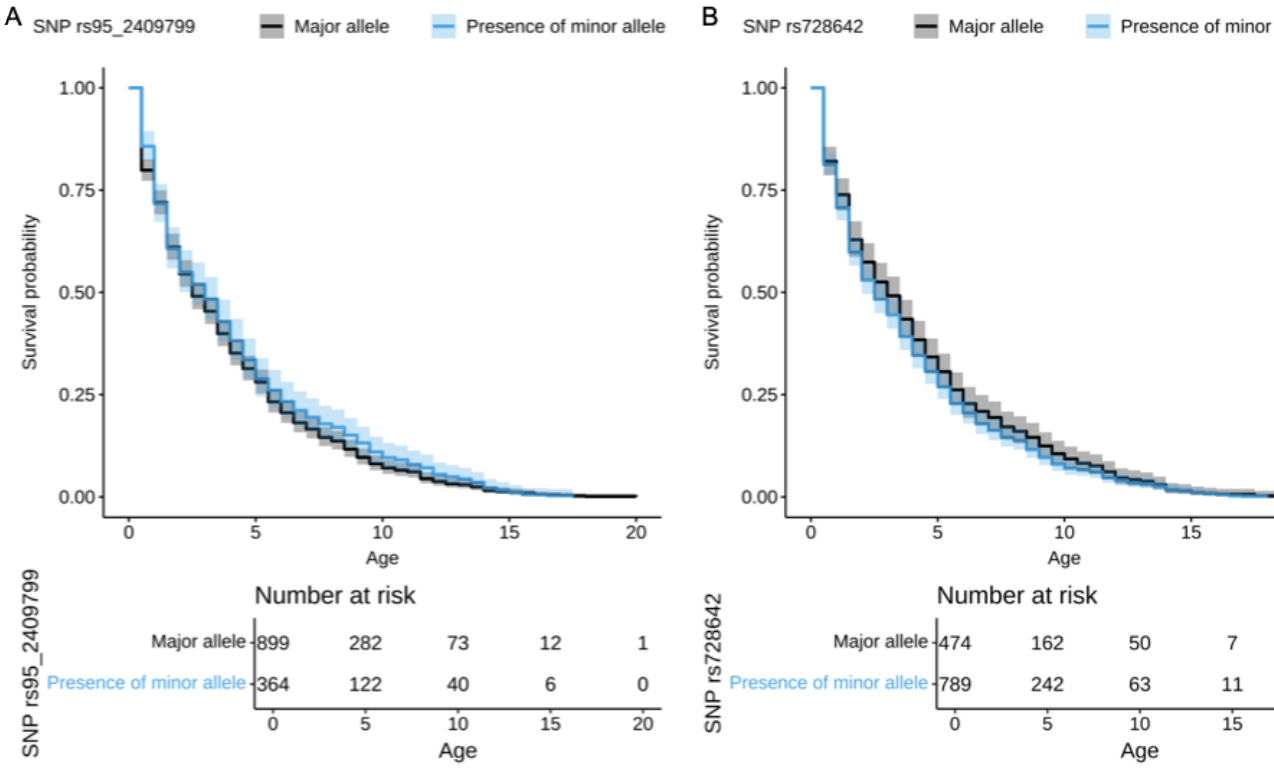


Figure 6.4. Survival probability in relation to variation at the genomic loci A) *rs95_2409799* and B) *rs728642* in Seychelles warblers (N=1340). Lifetime survival probabilities are denoted with different colours: absence of minor allele (black) and presence of minor allele (blue). The number of alive/at-risk individuals at each interval of 5 years is shown at the bottom of the plot. Individuals still alive at the end of the study are right censored (indicated with the symbol "+", N=57).

Individual inbreeding coefficient and mean rainfall (average yearly rainfall the bird experiences over its lifetime) were significantly associated with increased mortality in both the minor allele and genotype analyses (Table 6.2A&B).

6.5 Discussion

Nine host loci (encompassing 393 SNPs) were correlated with GM composition; these loci included 14 known genes. There were 107 unique differentially abundant ASVs associated with variation at these nine loci. Ten of these 107 ASVs were also linked to host survival to the next breeding season. Notably, the minor allele at two of these host loci *rs95_2409799* and *rs728642* was also directly associated with higher and lower host survival, respectively. These two loci were inversely associated with survival-related *Clostridiales* ASVs- *rs95_2409799* increases Family XIII; *Anaerovorax* and decreases *Ruminococcaceae*; uncultured, while *rs728642* decreases Family XIII; *Anaerovorax* and increases *Ruminococcaceae*; uncultured.

6.5.1 Host genomic regions and the GM

The finding that nine host genomic loci are associated with differences in GM composition in the Seychelles warbler and is in line with previous studies on humans, cows (*Bos taurus*), shrimp (*Litopenaeus vannamei*) and wild mice (*Mus musculus*) showing associations between host genome and the GM (Bonder et al., 2016; Brulin et al., 2025; Cornejo-Granados et al., 2025; Kurilshikov et al., 2021; Suzuki et al., 2019). However, such genome–GM comparisons remain rare in wild animal systems (but see (Suzuki et al., 2019)), making our study one of the first to demonstrate these links in a natural population. The nine loci identified in our study encompassed 14 known genes, each of which could be directly or indirectly linked to the GM.

The *GRIK2* (glutamate ionotropic receptor kainite type subunit 2) gene (*rs750388*; chromosome 3) plays a role in glutamatergic neurotransmission in which variation has been linked to changes in intestinal motility, secretions, and gut barrier function, all of which can shape microbial communities (Hamnett et al., 2025). Variation at GRIK2 has also been associated with the presence of *Faecalibacterium* in the human gut (Boulund et al., 2022).

The *CACHD1* (Cache Domain Containing 1) gene (*rs95_2409799*; Chromosome 6), which modulates voltage-gated calcium channel activity (Powell et al., 2024) is expressed in multiple tissues, including the gut (Dahimene et al., 2018; Powell et al., 2024). Given its role in calcium channel function, this locus could indirectly shape the GM through changes in mucus secretion, gut motility, and immune response (Diercks, 2024; Kirchhoff, 2006; Song et al., 2023).

The *MED7* (Mediator Complex Subunit 7) gene (*rs1657804*; Chromosome 12) encodes a component of the Mediator complex, important for DNA-bound transcription and RNA polymerase II (Kim et al., 1994; Kornberg, 2005). *MED7* has been associated with immune responses and may influence the GM via host transcriptional responses to microbial signals (Wu et al., 2023). Downregulation of *MED7* in humans has also been associated with increased gastrointestinal stromal tumour risk (Hur et al., 2010).

The *SEC16A* gene (*rs95_965036*; Chromosome 17) is involved in protein transport from the endoplasmic reticulum to the Golgi apparatus (Piao et al., 2017). Variants of this gene have been associated with inflammatory bowel disease (Hu et al., 2020). *SEC16A* is crucial in COPII vesicle formation, which is a target of some gastrointestinal pathogens such as *Escherichia coli* and Norovirus (Sharp and Estes, 2010; Wang et al., 2024).

The second locus identified in chromosome 17 (*rs95_974945*) encompasses 10 genes. Of these, the gene *SLC2A6*, involved in extracellular glucose uptake (DOEGE et al., 2000; Jiang et al., 2022), has been associated with the human intestinal-type alkaline phosphatase measurement (Loya et al., 2025) and thus, is the most plausibly linked to GM variation. The other genes do not appear to be directly linked to the GM but could also influence the GM pleiotropically. For instance, genomic regions in mice (Mus musculus) that were associated with body fat were also associated with GM composition (Leamy et al., 2014). Therefore, even in the absence of a direct association, the identified genes may still impact the GM indirectly through shared genetic architecture with other host traits.

Many of the genes we identify (*GRIK2*, *CACHD1*, *MED7*, *SEC16A*, and *SLC2A6*), appear to have been previously associated with the gastrointestinal tract, influencing the GM primarily through pathways related to gut physiology, immunity, and host-microbes interactions (see above). These findings suggest that host genetic effects on the GM are multifaceted, acting through diverse host systems but converging on pathways that alter the gut environment and immune system. This is consistent with previous findings, where immune-related genes and gut physiology are known to shape the GM of humans and captive animals (Bonder et al., 2016; Procházková et al., 2024; Tanoue et al., 2010). Similarly, studies on wild animals have shown that the host's immunogenetics also shape the GM (Davies et al., 2022; Marietta et al., 2015; Montero et al., 2021).

The loci we identified in chromosome two and one locus in chromosome three were in unknown genes, and hence, we are unable to speculate on potential pathways that could link the gene to the GM. In addition, there were two other loci (in chromosomes 6 and 8) that were not near any genes. These intergenic SNPs could be part of promoters, enhancers, transposable elements and tandem repeats, which can have significant influences on genome function (Pagni et al., 2022). However, since the reference genome of Seychelles warblers is only annotated with functional genes, we are unable to identify the gene regulatory impact of these regions.

6.5.2 Survival and GM-associated SNPs

Among the 107 ASVs that are associated with variation at GM-associated host loci, 10 were also linked with host survival to the next season. Five out of ten of these host survival-related ASVs were in the same bacterial order as identified in a previous study in the Seychelles warbler carried out using a smaller dataset (Worsley et al., 2021). Five ASVs were positively associated with survival, and five were negatively associated with survival, which suggests the GM could be associated with host survival through a variety of mechanisms (Shealy et al., 2021; Tanoue et al., 2010; Wang et al., 2015).

One ASV from an unknown genus (family: *Enterobacteriaceae*, order: *Enterobacteriales*), one from *Leifsonia* (family: *Microbacteriaceae*, order: *Micrococcales*), and one from *Oxalobacter* (family: *Burkholderiaceae*, order:

Betaproteobacteriales) were negatively linked to survival in the warbler. *Enterobacteriaceae* and *M. Leifsonia* are known opportunistic pathogens in humans (Al-Sardi et al., 2021; Shealy et al., 2021). However, *Oxalobacter* is not known to be pathogenic in any species and instead is important for preventing kidney stones in humans (Duncan et al., 2002). In the warbler, these ASVs identified from *Enterobacteriales* and *Micrococcales* could infect hosts that are already weakened by other infections. This is consistent with previous findings in feral horses, where opportunistic pathogens were associated with reduced survival (Stothart et al., 2024).

The three ASVs from the *Clostridiales* order (two *Ruminococcaceae* with unknown genus and one Family XIII, *Anaerovorax* genus) were identified as being positively linked to survival in the warbler. *Clostridiales* is a bacterial order found in other insectivorous passerines (Bodawatta et al., 2021, 2018), and is capable of fermenting carbohydrates and proteins and degrading toxic by-products (Yang et al., 2022). The *Ruminococcaceae* and *Anerovorax* are known producers of the short-chain fatty acids acetate and butyrate, which play key roles in maintaining gut health and homeostasis (González Hernández et al., 2019; Kim et al., 2024; Liu et al., 2018; Matthies et al., 2000). Both genera have also been positively associated with physical activity in humans, further supporting their potential beneficial roles in host physiology and fitness (Santarossa et al., 2021; Zhong et al., 2021). A reduction in *Clostridiales* was detected in the GM of juvenile ostriches (*Struthio camelus*) that subsequently died (Videvall et al., 2020), suggesting that it may be beneficial to the host. Collectively, these findings suggest that *Clostridiales* may represent an important microbial group mediating links between gut community composition, host condition, and survival in wild populations.

Finally, we also identified four Seychelles warbler survival-related ASVs that did not have a clear biological link to host health. The ASV from the bacterial order *Thermomicrobiales* and one ASV from *Rhizobiales* were negatively associated with host survival, and the one ASV in each of the orders *Rhizobiales* and *Xanthomonadales* (genus *Vulcaniibacterium)* was positively associated with survival. The *Vulcaniibacterium* and *Thermomicrobiales* are frequently found in high-temperature environments and are important for biofilm formation and nutrient cycling (Niu et al., 2020) but have no clear association with vertebrate GM or host

survival. Similarly, the ASVs *Rhizobiales* are typically environmental and have not been reported to be associated with host survival (Garrido-Oter et al., 2018). However, the ASVs identified in our study have not been functionally characterised in Seychelles warblers, and most inferred functions are derived from non-avian systems; therefore, these interpretations remain speculative.

All nine host genomic loci identified as being linked GM variation were associated with at least one, but no more than four, survival-related ASVs, suggesting that host genomic effects on survival may be mediated, at least in part, through the GM. In addition, five loci were associated with ASVs showing both positive and negative associations with survival. This pattern suggests that a given host locus may selectively suppress certain microbes while simultaneously tolerating others. Such contrasting effects could mask clear functional outcomes, as functional redundancy within the GM may buffer against the loss or gain of individual taxa (Louca et al., 2018; Worsley et al., 2024d). Therefore, while the host genome is associated with survival-related ASVs, whether the consequences of these associations are likely to depend on the functional roles of the specific ASVs involved, but the exact mechanisms remain to be determined.

Two GM-associated host loci (*rs95_2409799* and *rs728642*) were also directly associated with host survival, where the presence of the minor allele was associated with an increase and decrease in survival probability, respectively. The two loci involved showed opposite associations with the beneficial *Clostridiales* ASVs: the minor allele of *rs95_2409799* was linked to an increase in Family XIII (*Anaerovorax*) and a decrease in *Ruminococcaceae* (uncultured), whereas the minor allele of *rs728642* was associated with the reverse pattern, an increase in *Ruminococcaceae* (uncultured) and a decrease in Family XIII (*Anaerovorax*) (Table S6.5). Although both ASVs are positively associated with survival, the effect of Family XIII (*Anaerovorax*) on survival is stronger than *Ruminococcaceae* (uncultured), suggesting that survival not only depends on harbouring beneficial microbes, but on which beneficial microbe is favoured. These opposing effects underscore the role of host genomic variation in shaping specific GM components, which may ultimately influence host survival.

Our study highlights the intricate interplay between host genetics, the GM, and host survival. However, identifying causal mechanisms in wild populations remains challenging. While our analyses provide an important starting point, the analyses were based on independent associations, rather than an integrative framework, meaning that the effects of host genetic variants on survival could be dependent or independent of their effects on survival-related ASVs. It is also possible that changes in ASV abundance reflect declines in host health caused by genetic variants rather than the ASVs driving mortality themselves (i.e. they are a consequence, not a cause of imminent mortality). To establish this directionality would require future research that functionally characterises ASVs (e.g. metagenomics), employs experimental manipulation, for example, to test whether altering ASV abundance affects mortality, or with structural equation modelling in wild populations. Another limitation is that all analyses were conducted within a single population, which may restrict the generalisability of our findings to other populations and species. The influence of the host genome on GM composition could vary across populations due to differences in environmental conditions, ecological pressures, and local selection regimes (Degregori et al., 2025; Worsley et al., 2024a).

6.5.3 Conclusion

Our study provides evidence that the host genome is linked to GM variation through various pathways, including elements of the host immune system and gut physiology. In addition, host loci were associated with some host survival-related gut microbes, suggesting that the host genome interacts with the GM to influence host survival in this species. Two host loci, rs95_2409799 and rs728642, exhibited opposing associations with the survival-related ASVs *Anaerovorax* and *Ruminococcaceae*, mirroring their opposite effects on host survival. These findings highlight the complexity of host-GM-fitness relationships and underscore the importance of integrating genomics and microbial perspectives to gain deeper insights into the evolution of host traits in natural populations.

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6.7 Supplementary material

Table S6.1. Host single-nucleotide polymorphisms (SNP) that were significantly (q<0.05) associated with gut microbiome composition of Seychelles warblers (n=205). Abbreviations: SNP position (Pos) on the chromosome (Chr), major allele (A1), minor allele (A2), minor allele frequency (MAF), whether the SNP is in a gene or near (Inside Gene). The representative SNP of the locus is in bold.

SNP	Chr	Pos	q	MAF	Inside Gene	Gene ID	Gene start	Gene end
rs95_1075473	**2**	**3156468**	**0.039**	**0.227**	**near**	**Unknown**	**3156527**	**3170101**
rs728642	**3**	**65229635**	**0.039**	**0.317**	**near**	**Unknown**	**65221342**	**65226115**
rs95_1854197	3	77314535	0.039	0.418	None	None	-	-
rs95_1854198	3	77314702	0.039	0.418	None	None	-	-
rs746442	3	77358510	0.039	0.419	None	None	-	-
rs746475	3	77376834	0.039	0.419	None	None	-	-
rs746535	3	77390364	0.043	0.416	None	None	-	-
rs746540	3	77393927	0.043	0.416	None	None	-	-
rs746545	3	77394450	0.039	0.418	None	None	-	-
rs746547	3	77394794	0.039	0.419	None	None	-	-
rs746649	3	77496766	0.039	0.419	None	None	-	-
rs746696	3	77520320	0.039	0.419	None	None	-	-
rs746781	3	77565911	0.043	0.420	None	None	-	-
rs746782	3	77566060	0.039	0.419	None	None	-	-

rs746838	3	77584645	0.039	0.419	None	None	-	-
rs746839	3	77584784	0.039	0.419	None	None	-	-
rs95_1857837	3	78309141	0.039	0.418	near	*GRIK2*	78353417	78713749
rs95_1857850	3	78309763	0.039	0.415	near	*GRIK2*	78353417	78713749
rs749151	3	78310019	0.039	0.415	near	*GRIK2*	78353417	78713749
rs95_1857926	3	78329306	0.039	0.414	near	*GRIK2*	78353417	78713749
rs749246	3	78345567	0.039	0.414	near	*GRIK2*	78353417	78713749
rs749277	3	78357411	0.039	0.413	yes	*GRIK2*	78353417	78713749
rs749370	3	78373096	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749377	3	78374287	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749379	3	78374363	0.039	0.414	yes	*GRIK2*	78353417	78713749
rs749410	3	78378012	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749428	3	78381777	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749434	3	78382297	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749435	3	78382308	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749436	3	78382318	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749437	3	78382370	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749442	3	78382585	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749443	3	78382607	0.039	0.415	yes	*GRIK2*	78353417	78713749

rs749446	3	78382635	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749457	3	78382986	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749458	3	78382994	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749459	3	78383035	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749484	3	78386682	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749485	3	78386776	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749486	3	78387022	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749487	3	78387056	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749488	3	78387083	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749489	3	78387759	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749490	3	78390775	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749491	3	78390796	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs95_1858221	3	78390875	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749492	3	78390973	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749493	3	78391318	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749494	3	78391642	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749495	3	78391681	0.039	0.412	yes	*GRIK2*	78353417	78713749
rs749496	3	78392225	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749509	3	78393919	0.039	0.415	yes	*GRIK2*	78353417	78713749

rs749510	3	78394107	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749511	3	78394217	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs95_1858242	3	78394657	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs95_1858244	3	78396427	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749853	3	78476212	0.039	0.413	yes	*GRIK2*	78353417	78713749
rs749890	3	78483596	0.039	0.413	yes	*GRIK2*	78353417	78713749
rs749892	3	78483863	0.039	0.414	yes	*GRIK2*	78353417	78713749
rs749893	3	78483980	0.039	0.414	yes	*GRIK2*	78353417	78713749
rs749894	3	78485038	0.039	0.414	yes	*GRIK2*	78353417	78713749
rs749895	3	78485048	0.039	0.414	yes	*GRIK2*	78353417	78713749
rs749896	3	78485108	0.039	0.414	yes	*GRIK2*	78353417	78713749
rs749897	3	78485763	0.039	0.414	yes	*GRIK2*	78353417	78713749
rs749945	3	78492331	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749946	3	78492454	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749948	3	78492675	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749949	3	78492686	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749952	3	78493435	0.039	0.409	yes	*GRIK2*	78353417	78713749
rs749953	3	78493490	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749954	3	78493595	0.039	0.415	yes	*GRIK2*	78353417	78713749

rs749955	3	78493922	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749956	3	78493926	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749957	3	78494048	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749958	3	78494245	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs749960	3	78494388	0.039	0.414	yes	*GRIK2*	78353417	78713749
rs750119	3	78513425	0.039	0.414	yes	*GRIK2*	78353417	78713749
rs750124	3	78514312	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs750175	3	78521310	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs750177	3	78521552	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs750178	3	78521585	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs750183	3	78523284	0.039	0.415	yes	*GRIK2*	78353417	78713749
rs750184	3	78523505	0.043	0.416	yes	*GRIK2*	78353417	78713749
rs750252	3	78562154	0.039	0.414	yes	*GRIK2*	78367739	78713749
rs750253	3	78562255	0.039	0.414	yes	*GRIK2*	78367739	78713749
rs750256	3	78566935	0.039	0.414	yes	*GRIK2*	78367739	78713749
rs95_1859246	3	78616549	0.039	0.414	yes	*GRIK2*	78367739	78713749
rs750388	**3**	**78619451**	**0.029**	**0.412**	**yes**	***GRIK2***	**78367739**	**78713749**
rs95_2409590	6	2239609	0.043	0.171	yes	*CACHD1*	2219428	2312883
rs95_2409591	6	2239614	0.039	0.169	yes	*CACHD1*	2219428	2312883

rs95_2409592	6	2239615	0.039	0.169	yes	*CACHD1*	2219428	2312883
rs95_2409597	6	2240049	0.024	0.167	yes	*CACHD1*	2219428	2312883
rs95_2409598	6	2240058	0.024	0.167	yes	*CACHD1*	2219428	2312883
rs95_2409599	6	2240059	0.024	0.167	yes	*CACHD1*	2219428	2312883
rs95_2409642	6	2249271	0.039	0.174	yes	*CACHD1*	2219428	2312883
rs95_2409796	6	2277958	0.039	0.180	yes	*CACHD1*	2219428	2312883
rs95_2409799	**6**	**2278070**	**0.024**	**0.177**	**yes**	***CACHD1***	**2219428**	**2312883**
rs95_2409800	6	2278082	0.024	0.177	yes	*CACHD1*	2219428	2312883
rs95_2409801	6	2278163	0.024	0.177	yes	*CACHD1*	2219428	2312883
rs95_2409802	6	2278164	0.024	0.177	yes	*CACHD1*	2219428	2312883
rs95_2409803	6	2278479	0.024	0.177	yes	*CACHD1*	2219428	2312883
rs95_2409804	6	2278757	0.024	0.177	yes	*CACHD1*	2219428	2312883
rs95_2409805	6	2278765	0.024	0.177	yes	*CACHD1*	2219428	2312883
rs1220065	**6**	**57461779**	**0.039**	**0.251**	**None**	**None**	**-**	**-**
rs1220068	6	57462214	0.043	0.254	None	None	-	-
rs1220070	6	57462341	0.043	0.254	None	None	-	-
rs1220076	6	57462611	0.039	0.254	None	None	-	-
rs1441391	8	31789764	0.013	0.320	None	None	-	-
rs1441392	8	31789825	0.013	0.320	None	None	-	-

rs1441393	8	31789864	0.013	0.320	None	None	-	-
rs1441394	8	31790009	0.013	0.320	None	None	-	-
rs1441395	8	31790064	0.013	0.320	None	None	-	-
rs1441396	8	31790401	0.013	0.320	None	None	-	-
rs95_2940681	8	31790532	0.013	0.320	None	None	-	-
rs1441397	8	31790586	0.013	0.320	None	None	-	-
rs1441398	8	31790606	0.013	0.320	None	None	-	-
rs1441399	8	31790616	0.013	0.320	None	None	-	-
rs1441400	8	31791099	0.013	0.320	None	None	-	-
rs1441401	8	31791180	0.013	0.320	None	None	-	-
rs95_2940687	8	31791246	0.013	0.320	None	None	-	-
rs1441402	8	31791386	0.013	0.320	None	None	-	-
rs1441403	8	31791441	0.013	0.320	None	None	-	-
rs1441404	8	31791490	0.013	0.320	None	None	-	-
rs1441405	8	31791587	0.013	0.320	None	None	-	-
rs1441406	8	31791606	0.013	0.320	None	None	-	-
rs1441407	8	31791607	0.013	0.320	None	None	-	-
rs95_2940694	8	31791678	0.013	0.321	None	None	-	-
rs95_2940695	8	31791696	0.013	0.321	None	None	-	-

rs95_2940696	8	31791756	0.013	0.321	None	None	-	-
rs95_2940697	8	31791764	0.013	0.321	None	None	-	-
rs1441408	8	31791834	0.013	0.321	None	None	-	-
rs95_2940699	8	31791971	0.013	0.321	None	None	-	-
rs1441409	8	31792197	0.013	0.321	None	None	-	-
rs1441410	8	31792206	0.013	0.321	None	None	-	-
rs95_2940702	8	31792242	0.013	0.321	None	None	-	-
rs95_2940703	8	31792251	0.013	0.321	None	None	-	-
rs95_2940704	8	31792263	0.013	0.321	None	None	-	-
rs1441411	8	31792295	0.013	0.321	None	None	-	-
rs1441412	8	31792306	0.013	0.321	None	None	-	-
rs1441413	8	31792322	0.013	0.320	None	None	-	-
rs95_2940708	8	31792329	0.013	0.320	None	None	-	-
rs1441416	8	31793250	0.013	0.320	None	None	-	-
rs95_2940712	8	31793286	0.013	0.320	None	None	-	-
rs95_2940716	8	31793896	0.013	0.321	None	None	-	-
rs95_2940717	8	31793899	0.013	0.321	None	None	-	-
rs1441418	8	31794311	0.013	0.321	None	None	-	-
rs1441419	8	31794583	0.013	0.321	None	None	-	-

rs95_2940720	8	31795075	0.013	0.321	None	None	-	-
rs95_2940721	8	31795204	0.013	0.321	None	None	-	-
rs1441420	8	31795465	0.013	0.321	None	None	-	-
rs1441421	8	31795523	0.013	0.321	None	None	-	-
rs1441422	8	31795682	0.013	0.321	None	None	-	-
rs1441423	8	31795751	0.013	0.321	None	None	-	-
rs95_2940730	8	31797035	0.013	0.321	None	None	-	-
rs95_2940731	8	31797093	0.013	0.321	None	None	-	-
rs95_2940732	8	31797112	0.013	0.321	None	None	-	-
rs95_2940733	8	31797262	0.013	0.321	None	None	-	-
rs95_2940734	8	31797312	0.013	0.321	None	None	-	-
rs95_2940735	8	31797320	0.013	0.321	None	None	-	-
rs95_2940736	8	31797327	0.013	0.321	None	None	-	-
rs95_2940753	8	31798961	0.002	0.317	None	None	-	-
rs95_2940754	8	31798962	0.002	0.317	None	None	-	-
rs95_2940755	8	31799330	0.002	0.317	None	None	-	-
rs95_2940756	8	31799339	0.002	0.317	None	None	-	-
rs1441425	8	31799395	0.002	0.317	None	None	-	-
rs95_2940758	8	31799409	0.002	0.317	None	None	-	-

rs95_2940759	**8**	**31799410**	**0.001**	**0.314**	**None**	**None**	**-**	**-**
rs1441426	8	31799423	0.002	0.312	None	None	-	-
rs1441427	8	31799557	0.002	0.313	None	None	-	-
rs95_2940762	8	31799587	0.001	0.311	None	None	-	-
rs1441428	8	31799612	0.001	0.311	None	None	-	-
rs1441429	8	31799627	0.001	0.311	None	None	-	-
rs1441430	8	31799660	0.001	0.311	None	None	-	-
rs95_2940766	8	31799917	0.001	0.311	None	None	-	-
rs1441431	8	31799923	0.001	0.310	None	None	-	-
rs1657804	**12**	**13802557**	**0.043**	**0.177**	**near**	***MED7***	**13800481**	**13801173**
rs95_965023	17	12982837	0.043	0.428	yes	*SEC16A*	12972402	12994093
rs95_965026	17	12982958	0.043	0.428	yes	*SEC16A*	12972402	12994093
rs95_965028	17	12983044	0.043	0.428	yes	*SEC16A*	12972402	12994093
rs95_965029	17	12983134	0.043	0.428	yes	*SEC16A*	12972402	12994093
rs95_965030	17	12983137	0.043	0.428	yes	*SEC16A*	12972402	12994093
rs95_965031	17	12983143	0.043	0.428	yes	*SEC16A*	12972402	12994093
rs95_965033	17	12983214	0.043	0.428	yes	*SEC16A*	12972402	12994093
rs95_965034	17	12983220	0.043	0.428	yes	*SEC16A*	12972402	12994093
rs95_965035	17	12983244	0.043	0.428	yes	*SEC16A*	12972402	12994093

rs95_965036	**17**	**12983248**	**0.039**	**0.428**	**yes**	***SEC16A***	**12972402**	**12994093**
rs95_965037	17	12983250	0.039	0.428	yes	*SEC16A*	12972402	12994093
rs95_965056	17	12986427	0.039	0.428	yes	*SEC16A*	12972402	12994093
rs95_972005	17	14081628	0.045	0.267	yes	*SARDH*	14075427	14102674
rs95_972008	17	14081657	0.045	0.267	yes	*SARDH*	14075427	14102674
rs95_972010	17	14081694	0.045	0.267	yes	*SARDH*	14075427	14102674
rs95_972020	17	14081844	0.045	0.267	yes	*SARDH*	14075427	14102674
rs95_972024	17	14081886	0.045	0.267	yes	*SARDH*	14075427	14102674
rs95_972025	17	14081927	0.045	0.267	yes	*SARDH*	14075427	14102674
rs95_972027	17	14082509	0.045	0.267	yes	*SARDH*	14075427	14102674
rs95_972034	17	14083501	0.045	0.267	yes	*SARDH*	14075427	14102674
rs95_972042	17	14084350	0.039	0.268	yes	*SARDH*	14075427	14102674
rs95_972044	17	14085154	0.039	0.268	yes	*SARDH*	14075427	14102674
rs95_972058	17	14091248	0.039	0.268	yes	*SARDH*	14075427	14102674
rs95_972060	17	14091351	0.039	0.268	yes	*SARDH*	14075427	14102674
rs95_972062	17	14091383	0.039	0.268	yes	*SARDH*	14075427	14102674
rs95_972063	17	14091386	0.039	0.268	yes	*SARDH*	14075427	14102674
rs95_972069	17	14091513	0.039	0.268	yes	*SARDH*	14075427	14102674
rs1798058	17	14095417	0.039	0.268	yes	*SARDH*	14075427	14102674

rs95_972103	17	14096077	0.039	0.268	yes	*SARDH*	14075427	14102674
rs95_972104	17	14096086	0.039	0.268	yes	*SARDH*	14075427	14102674
rs1798061	17	14096292	0.039	0.268	yes	*SARDH*	14075427	14102674
rs1798064	17	14097611	0.039	0.268	yes	*SARDH*	14075427	14102674
rs95_972114	17	14097842	0.039	0.268	yes	*SARDH*	14075427	14102674
rs95_972116	17	14098231	0.039	0.268	yes	*SARDH*	14075427	14102674
rs95_972117	17	14098233	0.039	0.268	yes	*SARDH*	14075427	14102674
rs95_972123	17	14098783	0.039	0.268	yes	*SARDH*	14075427	14102674
rs95_972128	17	14099069	0.039	0.268	yes	*SARDH*	14075427	14102674
rs95_972133	17	14099631	0.039	0.268	yes	*SARDH*	14075427	14102674
rs1798081	17	14100140	0.039	0.268	yes	*SARDH*	14075427	14102674
rs95_972155	17	14100509	0.039	0.268	yes	*SARDH*	14075427	14102674
rs95_972157	17	14100579	0.039	0.268	yes	*SARDH*	14075427	14102674
rs95_972162	17	14100632	0.039	0.268	yes	*SARDH*	14075427	14102674
rs95_972213	17	14101729	0.039	0.268	yes	*SARDH*	14075427	14102674
rs95_972860	17	14151891	0.046	0.229	near	*FAM163B*	14155387	14156086
rs95_972864	17	14151910	0.044	0.228	near	*FAM163B*	14155387	14156086
rs95_972982	17	14171133	0.039	0.268	yes	*ADAMTSL2*	14159465	14187958
rs95_972993	17	14171308	0.039	0.268	yes	*ADAMTSL2*	14159465	14187958

rs95_972994	17	14171400	0.043	0.270	yes	*ADAMTSL2*	14159465	14187958
rs1798266	17	14172197	0.043	0.270	yes	*ADAMTSL2*	14159465	14187958
rs95_973009	17	14174018	0.043	0.270	yes	*ADAMTSL2*	14159465	14187958
rs95_973014	17	14176049	0.039	0.268	yes	*ADAMTSL2*	14159465	14187958
rs1798277	17	14177662	0.039	0.268	yes	*ADAMTSL2*	14159465	14187958
rs1798278	17	14177912	0.039	0.270	yes	*ADAMTSL2*	14159465	14187958
rs95_973024	17	14179230	0.039	0.268	yes	*ADAMTSL2*	14159465	14187958
rs1798282	17	14179790	0.039	0.268	yes	*ADAMTSL2*	14159465	14187958
rs95_973156	17	14191099	0.029	0.268	near	*TMEM8C*	14198859	14206190
rs95_973182	17	14194687	0.022	0.265	near	*TMEM8C*	14198859	14206190
rs95_973258	17	14205945	0.039	0.221	yes	*TMEM8C*	14198859	14206190
rs95_973259	17	14206027	0.039	0.219	yes	*TMEM8C*	14198859	14206190
rs1798330	17	14208180	0.039	0.223	near	*TMEM8C*	14198859	14206190
rs1798331	17	14208270	0.039	0.223	near	*TMEM8C*	14198859	14206190
rs1798332	17	14208282	0.039	0.223	near	*TMEM8C*	14198859	14206190
rs1798333	17	14208290	0.039	0.223	near	*TMEM8C*	14198859	14206190
rs95_973286	17	14208764	0.039	0.223	near	*TMEM8C*	14198859	14206190
rs1798334	17	14208857	0.039	0.223	near	*TMEM8C*	14198859	14206190
rs95_973294	17	14209272	0.039	0.223	near	*TMEM8C*	14198859	14206190

rs95_973295	17	14209295	0.039	0.223	near	*TMEM8C*	14198859	14206190
rs95_973298	17	14209312	0.039	0.223	near	*TMEM8C*	14198859	14206190
rs95_973299	17	14209388	0.039	0.223	near	*TMEM8C*	14198859	14206190
rs1798363	17	14219256	0.043	0.223	near	*SLC2A6*	14226604	14233714
rs95_974880	17	14468396	0.043	0.225	yes	*SPACA9*	14465731	14472331
rs95_974884	17	14468545	0.043	0.225	yes	*SPACA9*	14465731	14472331
rs95_974887	17	14468599	0.043	0.225	yes	*SPACA9*	14465731	14472331
rs95_974888	17	14468600	0.043	0.225	yes	*SPACA9*	14465731	14472331
rs95_974889	17	14468618	0.043	0.225	yes	*SPACA9*	14465731	14472331
rs95_974893	17	14469387	0.043	0.225	yes	*SPACA9*	14465731	14472331
rs95_974894	17	14469436	0.043	0.225	yes	*SPACA9*	14465731	14472331
rs95_974896	17	14469923	0.043	0.225	yes	*SPACA9*	14465731	14472331
rs95_974909	17	14473367	0.043	0.223	yes	*AK8*	14473227	14541222
rs1799056	17	14473444	0.043	0.223	yes	*AK8*	14473227	14541222
rs95_974913	17	14473708	0.043	0.223	yes	*AK8*	14473227	14541222
rs95_974916	17	14473984	0.043	0.223	yes	*AK8*	14473227	14541222
rs95_974920	17	14474951	0.043	0.223	yes	*AK8*	14473227	14541222
rs95_974921	17	14474983	0.043	0.223	yes	*AK8*	14473227	14541222
rs95_974922	17	14474985	0.043	0.223	yes	*AK8*	14473227	14541222

rs95_974923	17	14474995	0.043	0.223	yes	*AK8*	14473227	14541222
rs95_974924	17	14475025	0.043	0.223	yes	*AK8*	14473227	14541222
rs95_974925	17	14475193	0.043	0.223	yes	*AK8*	14473227	14541222
rs95_974926	17	14475817	0.039	0.222	yes	*AK8*	14473227	14541222
rs95_974936	17	14477367	0.043	0.221	yes	*AK8*	14473227	14541222
rs95_974937	17	14477368	0.043	0.221	yes	*AK8*	14473227	14541222
rs95_974941	17	14477653	0.037	0.219	yes	*AK8*	14473227	14541222
rs95_974945	**17**	**14478560**	**0.019**	**0.212**	**yes**	***AK8***	**14473227**	**14541222**
rs1799063	17	14479233	0.029	0.203	yes	*AK8*	14473227	14541222
rs95_974957	17	14479671	0.039	0.219	yes	*AK8*	14473227	14541222
rs1799065	17	14479840	0.029	0.219	yes	*AK8*	14473227	14541222
rs1799066	17	14479854	0.029	0.219	yes	*AK8*	14473227	14541222
rs1799070	17	14480129	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799071	17	14480240	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799072	17	14480272	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799073	17	14480426	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799074	17	14481005	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799075	17	14481137	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799076	17	14481138	0.039	0.221	yes	*AK8*	14473227	14541222

rs1799077	17	14481139	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799078	17	14481185	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799079	17	14481202	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799081	17	14481515	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799083	17	14481549	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799084	17	14481738	0.039	0.221	yes	*AK8*	14473227	14541222
rs95_974980	17	14482056	0.039	0.221	yes	*AK8*	14473227	14541222
rs95_974981	17	14482067	0.039	0.221	yes	*AK8*	14473227	14541222
rs95_974982	17	14482632	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799085	17	14482660	0.039	0.221	yes	*AK8*	14473227	14541222
rs95_974984	17	14482944	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799087	17	14483507	0.029	0.219	yes	*AK8*	14473227	14541222
rs1799088	17	14483550	0.029	0.219	yes	*AK8*	14473227	14541222
rs1799089	17	14484068	0.029	0.219	yes	*AK8*	14473227	14541222
rs1799090	17	14484420	0.029	0.219	yes	*AK8*	14473227	14541222
rs1799091	17	14484738	0.029	0.219	yes	*AK8*	14473227	14541222
rs1799092	17	14484874	0.029	0.219	yes	*AK8*	14473227	14541222
rs1799093	17	14484924	0.029	0.219	yes	*AK8*	14473227	14541222
rs1799095	17	14485290	0.039	0.221	yes	*AK8*	14473227	14541222

rs1799099	17	14486057	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799100	17	14486371	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799102	17	14487108	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799104	17	14488156	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799106	17	14488432	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799107	17	14488867	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799108	17	14489466	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799109	17	14489640	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799110	17	14489794	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799111	17	14489805	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799112	17	14490111	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799113	17	14490376	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799115	17	14491819	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799116	17	14492597	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799117	17	14495656	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799118	17	14496885	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799119	17	14497167	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799121	17	14497235	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799122	17	14497253	0.039	0.221	yes	*AK8*	14473227	14541222

rs1799123	17	14497973	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799124	17	14499286	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799125	17	14499369	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799126	17	14499603	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799128	17	14499963	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799129	17	14499997	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799133	17	14500768	0.039	0.221	yes	*AK8*	14473227	14541222
rs95_975042	17	14507911	0.039	0.221	yes	*AK8*	14473227	14541222
rs95_975046	17	14508098	0.039	0.221	yes	*AK8*	14473227	14541222
rs95_975048	17	14508758	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799142	17	14509273	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799172	17	14514614	0.039	0.221	yes	*AK8*	14473227	14541222
rs1799176	17	14514662	0.039	0.221	yes	*AK8*	14473227	14541222
rs95_975282	17	14559320	0.043	0.222	yes	*DDX31*	14554666	14597701
rs95_975287	17	14560260	0.043	0.222	yes	*DDX31*	14554666	14597701
rs95_975294	17	14560700	0.043	0.222	yes	*DDX31*	14554666	14597701
rs1799295	17	14562827	0.043	0.222	yes	*DDX31*	14554666	14597701
rs1799297	17	14563103	0.043	0.222	yes	*DDX31*	14554666	14597701
rs1799300	17	14563140	0.043	0.222	yes	*DDX31*	14554666	14597701

rs1799322	17	14564959	0.043	0.222	yes	*DDX31*	14554666	14597701
rs1799326	17	14565480	0.043	0.222	yes	*DDX31*	14554666	14597701
rs1799336	17	14566686	0.043	0.222	yes	*DDX31*	14554666	14597701
rs1799345	17	14567503	0.043	0.222	yes	*DDX31*	14554666	14597701
rs1799353	17	14568483	0.043	0.222	yes	*DDX31*	14554666	14597701
rs1799374	17	14570796	0.035	0.218	yes	*DDX31*	14554666	14597701
rs1799377	17	14571418	0.043	0.222	yes	*DDX31*	14554666	14597701
rs1799631	17	14603792	0.043	0.222	near	*BARHL1*	14603834	14609469
rs1799649	17	14605681	0.043	0.222	yes	*BARHL1*	14603834	14609469
rs1799686	17	14610211	0.043	0.222	near	*BARHL1*	14603834	14609469
rs1799688	17	14610768	0.043	0.222	near	*BARHL1*	14603834	14609469
rs1799693	17	14611471	0.043	0.222	near	*BARHL1*	14603834	14609469
rs1799694	17	14611504	0.043	0.222	near	*BARHL1*	14603834	14609469
rs1799695	17	14611571	0.043	0.222	near	*BARHL1*	14603834	14609469
rs1799696	17	14611582	0.043	0.222	near	*BARHL1*	14603834	14609469
rs1799698	17	14611723	0.043	0.222	near	*BARHL1*	14603834	14609469
rs1799699	17	14611811	0.043	0.222	near	*BARHL1*	14603834	14609469
rs1799700	17	14612045	0.043	0.222	near	*BARHL1*	14603834	14609469
rs1799701	17	14612202	0.043	0.222	near	*BARHL1*	14603834	14609469

rs1799702	17	14612383	0.043	0.222	near	*BARHL1*	14603834	14609469
rs1799703	17	14612741	0.043	0.222	near	*BARHL1*	14603834	14609469
rs1799704	17	14612820	0.043	0.222	near	*BARHL1*	14603834	14609469
rs1799705	17	14612880	0.043	0.222	near	*BARHL1*	14603834	14609469
rs1799706	17	14612899	0.043	0.222	near	*BARHL1*	14603834	14609469
rs1799708	17	14612953	0.043	0.222	near	*BARHL1*	14603834	14609469
rs95_975783	17	14617185	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs95_975787	17	14617524	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs95_975797	17	14619749	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs95_975798	17	14619765	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs1799732	17	14619829	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs1799738	17	14620366	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs95_975814	17	14622240	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs1799742	17	14622481	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs1799743	17	14622673	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs1799744	17	14623060	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs95_975819	17	14625251	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs95_975821	17	14625428	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs1799745	17	14625615	0.043	0.222	yes	*CFAP77*	14615352	14671769

rs95_975824	17	14626261	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs1799747	17	14627154	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs95_975827	17	14627368	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs95_975828	17	14627419	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs95_975829	17	14627532	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs1799748	17	14627600	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs95_975831	17	14627609	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs95_975832	17	14627788	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs1799749	17	14627990	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs95_975834	17	14628065	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs1799750	17	14628521	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs1799751	17	14629076	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs95_975837	17	14629894	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs95_975838	17	14630263	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs95_975846	17	14632389	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs1799758	17	14632570	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs1799763	17	14634026	0.043	0.222	yes	*CFAP77*	14615352	14671769
rs1799766	17	14634409	0.043	0.222	yes	*CFAP77*	14615352	14671769

Table S6.2. Host genes and gene functions that were associated with gut microbiome composition of Seychelles warblers (n=205).

Genes	Full Gene name	NCBI Refseq Gene Summary (https://www.ncbi.nlm.nih.gov/datasets/gene/)
GRIK2	Glutamate ionotropic receptor kainate type subunit 2	Glutamate receptors are the predominant excitatory neurotransmitter receptors in the mammalian brain and are activated in a variety of normal neurophysiologic processes. This gene product belongs to the kainate family of glutamate receptors, which are composed of four subunits and function as ligand-activated ion channels. The subunit encoded by this gene is subject to RNA editing at multiple sites within the first and second transmembrane domains, which is thought to alter the structure and function of the receptor complex. Alternatively spliced transcript variants encoding different isoforms have also been described for this gene. Mutations in this gene have been associated with autosomal recessive cognitive disability. [provided by RefSeq, Jul 2008]
CACHD1	Cache domain containing 1	Predicted to enable voltage-gated calcium channel activity. Predicted to be involved in calcium ion transmembrane transport. Predicted to be located in membrane. Predicted to be part of voltage-gated calcium channel complex. [provided by Alliance of Genome Resources, Jul 2025]
MED7	Mediator complex subunit 7	The activation of gene transcription is a multistep process that is triggered by factors that recognize transcriptional enhancer sites in DNA. These factors work with co-activators to direct transcriptional initiation by the RNA polymerase II apparatus. The protein encoded by this gene is a subunit of the CRSP (cofactor required for SP1 activation) complex, which, along with TFIID, is required for efficient activation by SP1. This protein is also a component of other multisubunit

		complexes e.g. thyroid hormone receptor-(TR-) associated proteins which interact with TR and facilitate TR function on DNA templates in conjunction with initiation factors and cofactors. Two transcript variants encoding the same protein have been found for this gene. [provided by RefSeq, Jul 2008]
SEC16A	SEC16 homolog A, endoplasmic reticulum export factor	This gene encodes a protein that forms part of the Sec16 complex. This protein has a role in protein transport from the endoplasmic reticulum (ER) to the Golgi and mediates COPII vesicle formation at the transitional ER. Alternative splicing results in multiple transcript variants that encode different protein isoforms. [provided by RefSeq, Feb 2013]
SARDH	Sarcosine dehydrogenase	This gene encodes an enzyme localized to the mitochondrial matrix which catalyzes the oxidative demethylation of sarcosine. This enzyme is distinct from another mitochondrial matrix enzyme, dimethylglycine dehydrogenase, which catalyzes a reaction resulting in the formation of sarcosine. Mutations in this gene are associated with sarcosinemia. Alternatively spliced transcript variants have been described. [provided by RefSeq, Oct 2008]
FAM163B	Family with sequence similarity 163 member B	Predicted to be located in membrane. [provided by Alliance of Genome Resources, Jul 2025]
ADAMTSL2	ADAMTS like 2	This gene encodes a member of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) and ADAMTS-like protein family. Members of the family share several distinct protein modules, including a propeptide region, a metalloproteinase domain, a disintegrin-like domain, and a thrombospondin type 1 (TS) motif. Individual members of this family differ in the

		number of C-terminal TS motifs, and some have unique C-terminal domains. The protein encoded by this gene lacks the protease domain, and is therefore of a member of the the ADAMTS-like protein subfamily. It is a secreted glycoprotein that binds the cell surface and extracellular matrix; it also interacts with latent transforming growth factor beta binding protein 1. Mutations in this gene have been associated with geleophysic dysplasia. [provided by RefSeq, Feb 2009]
TMEM8C	Transmembrane protein 8C	Provisional gene – unknown function
SLC2A6	Solute carrier family 2 member 6	Hexose transport into mammalian cells is catalyzed by a family of membrane proteins, including SLC2A6, that contain 12 transmembrane domains and a number of critical conserved residues.[supplied by OMIM, Jul 2002]
SPACA9	Sperm acrosome associated 9	Enables microtubule binding activity. Involved in axoneme assembly. Located in axonemal microtubule. [provided by Alliance of Genome Resources, Jul 2025]
AK8	Adenylate kinase 8	Enables AMP binding activity and nucleobase-containing compound kinase activity. Predicted to be involved in nucleoside monophosphate phosphorylation. Predicted to act upstream of or within ventricular system development. Located in 9+2 motile cilium. [provided by Alliance of Genome Resources, Apr 2025]
DDX31	DEAD-box helicase 31	DEAD box proteins, characterized by the conserved motif Asp-Glu-Ala-Asp (DEAD), are putative RNA helicases. They are implicated in a number of cellular processes involving alteration of RNA

		secondary structure such as translation initiation, nuclear and mitochondrial splicing, and ribosome and spliceosome assembly. Based on their distribution patterns, some members of this DEAD box protein family are believed to be involved in embryogenesis, spermatogenesis, and cellular growth and division. This gene encodes a member of this family. The function of this member has not been determined. Alternative splicing of this gene generates multiple transcript variants encoding different isoforms. [provided by RefSeq, Apr 2016]
BARHL1	BarH like homeobox 1	Enables sequence-specific double-stranded DNA binding activity. Predicted to be involved in regulation of transcription by RNA polymerase II. Predicted to act upstream of or within several processes, including negative regulation of outer hair cell apoptotic process; nervous system development; and sensory perception of sound. Predicted to be located in chromatin. Predicted to be active in nucleus. Biomarker of Alzheimer's disease; high grade glioma; and triple-receptor negative breast cancer. [provided by Alliance of Genome Resources, Jul 2025]
CFAP77	Cilia and flagella associated protein 77	Predicted to be involved in flagellated sperm motility. Located in axonemal microtubule. [provided by Alliance of Genome Resources, Jul 2025]

Table S6.3. Differentially abundant amplicon sequencing variants (ASVs) in the gut microbiome significantly ($P_{adj}<0.05$) associated with nine genomic loci in adult Seychelles warblers (N=204).

ASV	SNP	lfc	se	q	Kingdom	Phylum	Class	Order	Family
8f7c737007cfbed8b5ea17503e45422c	rs9510754773	-1.35	0.23	0.010	Bacteria	Actinobacteria	Actinobacteria	Corynebacteriales	Nocardiaceae
df98b3d20eafc1a8628a93e7b04a3325	rs9510754773	1.86	0.16	0.031	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae
e019a4db05822cabb7c9b4c2d1603056	rs9510754773	0.99	0.21	0.028	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardioidaceae
155fc453a9b2083b2246927750b3adb2	rs9510754773	-2.48	0.23	0.001	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Dysgonomonadaceae
d9072e1cebd91a61f8afca4f8963b5be	rs9510754773	-1.68	0.27	0.012	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Dysgonomonadaceae
d8b1a77dfc5fcc46c34936ac6d56c848	rs9510754773	-1.54	0.33	0.020	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Tannerellaceae
52e0efeb21d0e7d271464fbd25c19f2c	rs9510754773	-1.48	0.27	0.004	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae
bfb3000539dcc3f92f5596f1902aecc9	rs9510754773	-2.07	0.30	0.000	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae
68e632a89a7b3a65e318d15bd6a24af9	rs9510754773	-1.90	0.16	0.003	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Tannerellaceae
38f39d32b69c25e9c315a57df4cd6529	rs9510754773	-2.15	0.23	0.005	Bacteria	Chloroflexi	Chloroflexia	Thermomicrobiales	JG30-KF-CM45
44b093eb341528171b8bf175602a3eb3	rs9510754773	-1.81	0.18	0.007	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
a7bc7a114d44fc504e29440deaa177d4	rs9510754773	-2.31	0.26	0.000	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae

5073acc23b8393bff0d6b923c54d41cb	rs9510754 73	-1.36	0.21	0.004	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
e5102f241d2860ef10a22c7349943ea6	rs9510754 73	-1.35	0.25	0.003	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
64d51efb9e80efe006ba73ec4dc71a43	rs9510754 73	-3.55	0.24	0.010	Bacteria	Firmicutes	Negativicutes	Selenomonadales	Acidaminococcaceae
82dece6e35540738ba450a0c3a90b5a0	rs9510754 73	1.55	0.30	0.009	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae
0356b8bf9fd15d2b45124228f1e8a464	rs9510754 73	3.14	0.29	0.005	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Devosiaceae
240f1818162f44e37e45da79cf72aac7	rs9510754 73	3.02	0.23	0.016	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae
561cd8096aaf39bae17a35286191df07	rs728642	1.42	0.24	0.023	Bacteria	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriales Incertae Sedis
b8232a8b26d5f1bbb3e12aa2ce8c9ade	rs728642	2.20	0.20	0.037	Bacteria	Actinobacteria	Actinobacteria	Frankiales	Geodermatophilaceae
e019a4db05822cabb7c9b4c2d1603056	rs728642	-1.27	0.27	0.033	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardioidaceae
de4398f5aa45c3f4c65cea1276af9cde	rs728642	1.72	0.26	0.013	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Tannerellaceae
e3475214847c4c5ad349fa98e8d77b4b	rs728642	1.54	0.25	0.003	Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae
44b093eb341528171b8bf175602a3eb3	rs728642	-2.24	0.18	0.002	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
93fbf502ac0ca16e7746c15c7852884c	rs728642	1.87	0.20	0.006	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XIII

09666b1754e79c10181ea027c4e16595	rs728642	-1.75	0.19	0.000	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XIII
1f6a965b1db7f6b030e88767881ac6de	rs728642	3.78	0.20	0.000	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae
a443e786f67086749ef29ce0ab494c9e	rs728642	-1.39	0.20	0.017	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae
556864a5da3a811b67be9fc73488e926	rs728642	-1.92	0.33	0.001	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae
aee9f354c80ca7baa872c3da2fe462c2	rs728642	-1.22	0.24	0.026	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae
a0901407705992dc213ac509ede97d47	rs728642	1.97	0.36	0.021	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae
e04bebc06e6ea7571bca8406fc51a247	rs728642	1.39	0.19	0.010	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae
0414019fbf6990da9b14a5c07fa51e88	rs728642	-1.84	0.25	0.000	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae
c1049a7e04cbb23f9393be111a2fb40d	rs728642	-2.68	0.20	0.001	Bacteria	Tenericutes	Mollicutes	Mollicutes RF39	uncultured Firmicutes bacterium
561cd8096aaf39bae17a35286191df07	rs750388	-1.84	0.22	0.001	Bacteria	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriales Incertae Sedis
ad8664f21c744d16b06f829014b5511c	rs750388	1.97	0.15	0.000	Bacteria	Actinobacteria	Actinobacteria	Corynebacteriales	Mycobacteriaceae
b510a187af183f4f23d1b7548590e057	rs750388	1.59	0.20	0.032	Bacteria	Actinobacteria	Actinobacteria	Corynebacteriales	Tsukamurellaceae
e019a4db05822cabb7c9b4c2d1603056	rs750388	-1.01	0.19	0.005	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardioidaceae

7722655b922eeb9dc8b8f5540016684c	rs750388	1.25	0.19	0.032	Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales	67-14
d9072e1cebd91a61f8afca4f8963b5be	rs750388	-1.62	0.24	0.005	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Dysgonomonadaceae
de4398f5aa45c3f4c65cea1276af9cde	rs750388	1.21	0.21	0.041	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Tannerellaceae
1adcb4432c1748e25195afa084456f06	rs750388	-1.20	0.16	0.040	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Tannerellaceae
00bba75546dccb66972bcd3db2f21c70	rs750388	-0.81	0.16	0.048	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Marinifilaceae
52e0efeb21d0e7d271464fbd25c19f2c	rs750388	-1.67	0.24	0.000	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae
6c7697bc0a5b3fe71168932bca7a358b	rs750388	-1.03	0.20	0.008	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Tannerellaceae
bfb3000539dcc3f92f5596f1902aecc9	rs750388	-1.37	0.26	0.015	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae
7f00ef6dd3ed4b3ee63a718d37bccd25	rs750388	1.52	0.22	0.015	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae
1f6a965b1db7f6b030e88767881ac6de	rs750388	1.18	0.16	0.032	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae
d89ea01870634721df50d0484ceb5190	rs750388	1.36	0.27	0.007	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
e5102f241d2860ef10a22c7349943ea6	rs750388	-1.76	0.27	0.000	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
8cc1122edb65f25be0fe002b9215ef93	rs750388	1.40	0.21	0.004	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae

e3475214847c4c5ad349fa98e8d77b4b	rs750388	-1.25	0.23	0.014	Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae
04bb579482c97dd31aee2e295aa6b900	rs750388	-1.21	0.27	0.048	Bacteria	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae
64d51efb9e80efe006ba73ec4dc71a43	rs750388	-5.36	0.25	0.002	Bacteria	Firmicutes	Negativicutes	Selenomonadales	Acidaminococcaceae
0768028d5708206376cea53dc8216c61	rs750388	1.30	0.24	0.002	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae
36ecd054f5309a9658926a0926d7ee82	rs750388	-6.02	0.24	0.046	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae
4a97f30401e751b4ab95285d52081371	rs750388	1.78	0.23	0.044	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae
513d71b4885ea6a7ffb3b9bf79550ae1	rs750388	-4.02	0.26	0.039	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae
0356b8bf9fd15d2b45124228f1e8a464	rs750388	-1.78	0.19	0.013	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Devosiaceae
21045dfb2dc1f9ed56d360a3a292291c	rs750388	-1.08	0.18	0.044	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae
0414019fbf6990da9b14a5c07fa51e88	rs750388	-1.48	0.25	0.004	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae
5464b890bc8886e24432241fbd063406	rs750388	-1.27	0.24	0.025	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae
e04bebc06e6ea7571bca8406fc51a247	rs750388	-1.96	0.20	0.001	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae
240f1818162f44e37e45da79cf72aac7	rs750388	2.64	0.22	0.027	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae

89c35640e742b9d427778a4f35601c19	rs750388	5.77	0.28	0.012	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae
934a0a85cf44102603af0b354db60d22	rs750388	1.83	0.32	0.036	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae
b7f5af401f678ffbe9f7299cfa59e3bf	rs750388	1.87	0.26	0.000	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Devosiaceae
126dededfbef4551638cafb183ad8def	rs750388	-1.35	0.26	0.049	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae
561cd8096aaf39bae17a35286191df07	rs9524099	2.04	0.27	0.003	Bacteria	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriales Incertae Sedis
b8232a8b26d5f1bbb3e12aa2ce8c9ade	rs9524099	-2.55	0.22	0.032	Bacteria	Actinobacteria	Actinobacteria	Frankiales	Geodermatophilaceae
4b9243da4d6fa5e09c00a302851869a4	rs9524099	1.76	0.25	0.000	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Micrococcaceae
7722655b922eeb9dc8b8f5540016684c	rs9524099	-1.86	0.22	0.006	Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales	67-14
155fc453a9b2083b2246927750b3adb2	rs9524099	1.78	0.25	0.018	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Dysgonomonadaceae
06cdd022246e9427f51e85e2b84f9040	rs9524099	1.37	0.29	0.031	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Dysgonomonadaceae
de4398f5aa45c3f4c65cea1276af9cde	rs9524099	-5.89	0.25	0.000	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Tannerellaceae
52e0efeb21d0e7d271464fbd25c19f2c	rs9524099	1.54	0.28	0.005	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae
d9072e1cebd91a61f8afca4f8963b5be	rs9524099	2.70	0.25	0.000	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Dysgonomonadaceae

061062949bbf882e44007c5fba1bf43b	rs9524099	1.92	0.25	0.000	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
37c55ab92106d40a5b4eb8fc910c2e3a	rs9524099	-1.25	0.26	0.041	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae
09666b1754e79c10181ea027c4e16595	rs9524099	2.50	0.24	0.000	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XIII
917e5e80e61ae435d7483b3e826baffd	rs9524099	1.32	0.29	0.020	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
1f6a965b1db7f6b030e88767881ac6de	rs9524099	-1.95	0.21	0.006	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae
8d05637380a2682c88f955c19d9a1b4c	rs9524099	1.31	0.25	0.021	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
85a8ff4e8267be7ee22683a4853bb5e7	rs9524099	1.78	0.23	0.000	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae
0c1af63ebb72ddcb81901b7a55121c28	rs9524099	1.60	0.22	0.012	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae
93fbf502ac0ca16e7746c15c7852884c	rs9524099	-1.65	0.23	0.039	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XIII
8cc1122edb65f25be0fe002b9215ef93	rs9524099	-2.68	0.23	0.000	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
4a97f30401e751b4ab95285d52081371	rs9524099	-2.25	0.29	0.043	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae
2e4c00977817e0832a1188525ca6d7f8	rs9524099	-1.41	0.27	0.032	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae
4b9243da4d6fa5e09c00a302851869a4	rs1220065	1.56	0.31	0.026	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Micrococcaceae

1f6a965b1db7f6b030e88767881ac6de	rs1220065	-2.52	0.32	0.017	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae
061062949bbf882e44007c5fba1bf43b	rs1220065	1.89	0.31	0.001	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
04bb579482c97dd31aee2e295aa6b900	rs1220065	-1.65	0.33	0.015	Bacteria	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae
64d51efb9e80efe006ba73ec4dc71a43	rs1220065	6.20	0.33	0.003	Bacteria	Firmicutes	Negativicutes	Selenomonadales	Acidaminococcaceae
89c35640e742b9d427778a4f35601c19	rs1220065	-6.60	0.41	0.033	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae
126dededfbef4551638cafb183ad8def	rs1220065	2.87	0.39	0.001	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae
a35842bcf8fa5b819611065e58ad4038	rs9529407 59	1.90	0.21	0.002	Bacteria	Actinobacteria	Actinobacteria	Corynebacteriales	Nocardiaceae
b8232a8b26d5f1bbb3e12aa2ce8c9ade	rs9529407 59	1.81	0.16	0.038	Bacteria	Actinobacteria	Actinobacteria	Frankiales	Geodermatophilaceae
7b05524c9fc15b98949d166220fcd60f	rs9529407 59	-1.17	0.24	0.044	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae
06b6dc64ed1385fa4c5f3ee921267c37	rs9529407 59	1.39	0.26	0.002	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XIII
93fbf502ac0ca16e7746c15c7852884c	rs9529407 59	2.12	0.19	0.001	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XIII
8d05637380a2682c88f955c19d9a1b4c	rs9529407 59	-1.27	0.20	0.003	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
1f6a965b1db7f6b030e88767881ac6de	rs9529407 59	1.59	0.19	0.013	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae

09666b1754e79c10181ea027c4e16595	rs9540759	-1.03	0.18	0.032	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XIII
8cc1122edb65f25be0fe002b9215ef93	rs9540759	2.17	0.22	0.000	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
5073acc23b8393bff0d6b923c54d41cb	rs9540759	1.33	0.22	0.009	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
a443e786f67086749ef29ce0ab494c9e	rs9540759	-2.37	0.20	0.000	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae
64d51efb9e80efe006ba73ec4dc71a43	rs9540759	-2.24	0.20	0.038	Bacteria	Firmicutes	Negativicutes	Selenomonadales	Acidaminococcaceae
aa8290338f51566d44735c3ab76b67b0	rs9540759	2.99	0.28	0.044	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae
27d8f12f22ba9352acadc2f057b1895a	rs9540759	1.62	0.25	0.010	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	D05-2
3f73222e4a3ce7521ef471683fe61079	rs9540759	-1.98	0.29	0.001	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae
126dededfbef4551638cafb183ad8def	rs9540759	-1.56	0.28	0.025	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae
708e50f6b07a7fcee8183886a5f169b1	rs9540759	-1.44	0.20	0.021	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae
535486be478535576e961533b0f14f46	rs9540759	-1.33	0.28	0.032	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae
cff60bed359051dd65fe850fd0bd07e1	rs1657804	-0.74	0.15	0.046	Bacteria	Actinobacteria	Actinobacteria	Corynebacteriales	Nocardiaceae
b8232a8b26d5f1bbb3e12aa2ce8c9ade	rs1657804	-1.80	0.10	0.004	Bacteria	Actinobacteria	Actinobacteria	Frankiales	Geodermatophilaceae

144d2b8f94ec382e34460e8217d6a750	rs1657804	-1.49	0.22	0.007	Bacteria	Actinobacteria	Actinobacteria	Kineosporiales	Kineosporiaceae
b1dde0ed7ab1f036845b5643cfaa699e	rs1657804	-5.22	0.13	0.013	Bacteria	Actinobacteria	Actinobacteria	Kineosporiales	Kineosporiaceae
df98b3d20eafc1a8628a93e7b04a3325	rs1657804	1.02	0.09	0.039	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae
4b9243da4d6fa5e09c00a302851869a4	rs1657804	-0.76	0.13	0.004	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Micrococcaceae
a017c36e39ab836bcbcd6e1714cae758	rs1657804	-2.22	0.14	0.032	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Micrococcaceae
e019a4db05822cabb7c9b4c2d1603056	rs1657804	-1.31	0.16	0.000	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardioidaceae
cec2b53a478c65984fc5aaa6c8ad9f54	rs1657804	-1.32	0.23	0.034	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardioidaceae
b20c16d3ebe571ddb000e9072cfd0ddf	rs1657804	2.16	0.11	0.002	Bacteria	Actinobacteria	Rubrobacteria	Rubrobacterales	Rubrobacteriaceae
7722655b922eeb9dc8b8f5540016684c	rs1657804	-1.39	0.14	0.002	Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales	67-14
d9072e1cebd91a61f8afca4f8963b5be	rs1657804	-1.13	0.13	0.000	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Dysgonomonadaceae
47d567c28de6cd0c1148ba93ff4e3aa7	rs1657804	-1.34	0.20	0.000	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Tannerellaceae
1adcb4432c1748e25195afa084456f06	rs1657804	-1.27	0.10	0.002	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Tannerellaceae
bfb3000539dcc3f92f5596f1902aecc9	rs1657804	-1.04	0.20	0.023	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae

061062949bbf882e44007c5fba1bf43b	rs1657804	-0.72	0.13	0.006	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
44b093eb341528171b8bf175602a3eb3	rs1657804	-2.28	0.09	0.000	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
17327d6dc726d2503fb40b572df39de2	rs1657804	1.08	0.19	0.001	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae
7f00ef6dd3ed4b3ee63a718d37bccd25	rs1657804	2.38	0.17	0.000	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae
917e5e80e61ae435d7483b3e826baffd	rs1657804	1.04	0.24	0.044	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
93fbf502ac0ca16e7746c15c7852884c	rs1657804	-1.62	0.15	0.002	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XIII
1f6a965b1db7f6b030e88767881ac6de	rs1657804	-1.64	0.12	0.000	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae
465c3a41559c5655c0e1fa88362daf68	rs1657804	1.23	0.16	0.019	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
3230c86b61839502c9c1837e5c672a45	rs1657804	2.62	0.12	0.001	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae
d8f86f866900533770e5d5b27b0f042f	rs1657804	-0.98	0.19	0.014	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae
8cc1122edb65f25be0fe002b9215ef93	rs1657804	1.67	0.14	0.000	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
85a8ff4e8267be7ee22683a4853bb5e7	rs1657804	-1.04	0.16	0.001	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae
f32d7e288fa805b9816b49e08ea3eebe	rs1657804	0.89	0.19	0.011	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae

8b46fc7b8e646871ea666e40bf01f518	rs1657804	0.87	0.11	0.010	Bacteria	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae
64d51efb9e80efe006ba73ec4dc71a43	rs1657804	-1.74	0.17	0.046	Bacteria	Firmicutes	Negativicutes	Selenomonadales	Acidaminococcaceae
5fdfa9fc9810cce81578a1f6458bc64c	rs1657804	-1.75	0.15	0.000	Bacteria	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae
1e5094a00b54498a967ba1d8ffe79837	rs1657804	-2.25	0.23	0.004	Bacteria	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae
8029a92bd672e641733132c302a9108d	rs1657804	1.11	0.16	0.013	Bacteria	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae
c939ee112ba982665e012437dae05c1e	rs1657804	1.52	0.25	0.019	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae
13e95cfbeef5d976331e058e676dfdc6	rs1657804	-2.03	0.18	0.000	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae
664765dce1a593784085345685a67650	rs1657804	1.35	0.21	0.014	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae
21045dfb2dc1f9ed56d360a3a292291c	rs1657804	-1.25	0.16	0.005	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae
8cc8e6565ca94b2237fad1d81f5865c5	rs1657804	2.61	0.15	0.000	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae
78d965a900ff7e19d546b879c9b0bf12	rs1657804	2.38	0.25	0.010	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Labraceae
dd070e655d5c10f17ed045ab7bf6008b	rs1657804	0.65	0.11	0.003	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae
aa8290338f51566d44735c3ab76b67b0	rs1657804	1.57	0.13	0.020	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae

89c35640e742b9d427778a4f35601c19	rs1657804	5.42	0.28	0.014	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae
708e50f6b07a7fcee8183886a5f169b1	rs1657804	-1.24	0.17	0.014	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae
c1049a7e04cbb23f9393be111a2fb40d	rs1657804	1.75	0.11	0.000	Bacteria	Tenericutes	Mollicutes	Mollicutes RF39	uncultured Firmicutes bacterium
df98b3d20eafc1a8628a93e7b04a3325	rs95_965036	-2.35	0.22	0.042	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae
4b9243da4d6fa5e09c00a302851869a4	rs95_965036	-1.75	0.27	0.001	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Micrococcaceae
b20c16d3ebe571ddb000e9072cfd0ddf	rs95_965036	-3.80	0.26	0.010	Bacteria	Actinobacteria	Rubrobacteria	Rubrobacterales	Rubrobacteriaceae
d3db18b9320e3a4b9dfd3f7557dd0698	rs95_965036	-1.87	0.33	0.002	Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales	67-14
68e632a89a7b3a65e318d15bd6a24af9	rs95_965036	-2.10	0.24	0.021	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Tannerellaceae
d9072e1cebd91a61f8afca4f8963b5be	rs95_965036	-2.86	0.37	0.001	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Dysgonomonadaceae
e23e5f762f1db27f75a8e47367e69c64	rs95_965036	-2.25	0.36	0.000	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Tannerellaceae
06b6dc64ed1385fa4c5f3ee921267c37	rs95_965036	-2.14	0.33	0.000	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XIII
31ba992fb23ccb4ed50b819846c6f65d	rs95_965036	-1.83	0.33	0.012	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae
36af7a2ab3128912c105a2f5e15d135e	rs95_965036	-1.62	0.34	0.012	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae

a5065a7e5f0311dbdc4d65014189ee99	rs95_965036	-2.33	0.23	0.003	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
465c3a41559c5655c0e1fa88362daf68	rs95_965036	-2.43	0.28	0.010	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
f4f25b4d40944286bff7d571dde1ba20	rs95_965036	-1.47	0.27	0.025	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
1f6a965b1db7f6b030e88767881ac6de	rs95_965036	2.11	0.26	0.014	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae
e3475214847c4c5ad349fa98e8d77b4b	rs95_965036	-1.76	0.26	0.001	Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae
7f00ef6dd3ed4b3ee63a718d37bccd25	rs95_965036	-2.46	0.35	0.013	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae
dd45968f44cd0a920b784a516c7df1b2	rs95_965036	-1.71	0.26	0.001	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae
44b093eb341528171b8bf175602a3eb3	rs95_965036	-2.59	0.24	0.004	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
a7bc7a114d44fc504e29440deaa177d4	rs95_965036	-2.53	0.36	0.000	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
2560d4657d16cc98d87c20f8f064bda3	rs95_965036	-1.83	0.35	0.010	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae
f32d7e288fa805b9816b49e08ea3eebe	rs95_965036	-1.66	0.39	0.041	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae
09a261cd2ba6d5db1a38bfe0ef012286	rs95_965036	-2.27	0.45	0.004	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
5073acc23b8393bff0d6b923c54d41cb	rs95_965036	-2.83	0.29	0.000	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae

64d51efb9e80efe006ba73ec4dc71a43	rs95_96503 6	-3.44	0.30	0.029	Bacteria	Firmicutes	Negativicutes	Selenomonadales	Acidaminococcaceae
d46e2205f0c6ecf67b51f83d111c509c	rs95_96503 6	-1.97	0.43	0.014	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae
0414019fbf6990da9b14a5c07fa51e88	rs95_96503 6	-2.49	0.28	0.000	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae
126dededfbef4551638cafb183ad8def	rs95_96503 6	-2.15	0.30	0.002	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae
10afda2baef44de4c584a6641de399b1	rs95_96503 6	-1.42	0.24	0.004	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae
708e50f6b07a7fcee8183886a5f169b1	rs95_96503 6	-2.78	0.27	0.001	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae
c1049a7e04cbb23f9393be111a2fb40d	rs95_96503 6	-2.19	0.27	0.028	Bacteria	Tenericutes	Mollicutes	Mollicutes RF39	uncultured Firmicutes bacterium
b510a187af183f4f23d1b7548590e057	rs95_97494 5	1.63	0.17	0.011	Bacteria	Actinobacteria	Actinobacteria	Corynebacteriales	Tsukamurellaceae
b20c16d3ebe571ddb000e9072cfd0ddf	rs95_97494 5	4.63	0.27	0.004	Bacteria	Actinobacteria	Rubrobacteria	Rubrobacterales	Rubrobacteriaceae
00bba75546dccb66972bcd3db2f21c70	rs95_97494 5	1.32	0.16	0.000	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Marinifilaceae
52e0efeb21d0e7d271464fbd25c19f2c	rs95_97494 5	1.41	0.24	0.002	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae
47d567c28de6cd0c1148ba93ff4e3aa7	rs95_97494 5	1.79	0.34	0.002	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Tannerellaceae
155fc453a9b2083b2246927750b3adb2	rs95_97494 5	1.17	0.18	0.039	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Dysgonomonadaceae

b065b43b380ca0dcc8d17f2bd328f8c3	rs954975	1.49	0.26	0.002	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae
6c7697bc0a5b3fe71168932bca7a358b	rs954975	1.22	0.24	0.008	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Tannerellaceae
d9072e1cebd91a61f8afca4f8963b5be	rs954975	2.87	0.33	0.000	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Dysgonomonadaceae
31ba992fb23ccb4ed50b819846c6f65d	rs954975	1.73	0.26	0.001	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae
a7bc7a114d44fc504e29440deaa177d4	rs954975	1.62	0.30	0.010	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
f4f25b4d40944286bff7d571dde1ba20	rs954975	1.15	0.21	0.024	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
2e651aced8b895be03721d92af877963	rs954975	1.20	0.24	0.045	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae
5073acc23b8393bff0d6b923c54d41cb	rs954975	1.55	0.23	0.002	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae
64d51efb9e80efe006ba73ec4dc71a43	rs954975	5.63	0.20	0.000	Bacteria	Firmicutes	Negativicutes	Selenomonadales	Acidaminococcaceae
5fdfa9fc9810cce81578a1f6458bc64c	rs954975	-2.77	0.29	0.001	Bacteria	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae
11f0558dca37009cbf1a6083b0b658e5	rs954975	1.78	0.34	0.022	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae
aee9f354c80ca7baa872c3da2fe462c2	rs954975	-1.75	0.29	0.003	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae
0414019fbf6990da9b14a5c07fa51e88	rs954975	2.25	0.27	0.000	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae

36ecd054f5309a9658926a0926d7ee82	rs95_974945	-9.43	0.25	0.015	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae
240f1818162f44e37e45da79cf72aac7	rs95_974945	-3.10	0.22	0.013	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae
7265f8def29fe6c4baf366cbea6e9f52	rs95_974945	-2.04	0.26	0.020	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae
0ac723a8700b14f8613b2febd515d830	rs95_974945	-1.66	0.29	0.005	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae

Table S6.4. Differentially abundant amplicon sequencing variants were significantly (q<0.05) associated with the host's survival to the next season in the gut microbiome of adult Seychelles warblers (N=266).

ASV	lfc_Survive1	se_Survive1	q_Survive1	Phylum	Class	Order	Family	Genus
df98b3d20eafc1a8628a93e7b04a3325	-1.125	0.174	0.000	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	Leifsonia
38f39d32b69c25e9c315a57df4cd6529	-1.351	0.197	0.000	Chloroflexi	Chloroflexia	Thermomicrobiales	JG30-KF-CM45	uncultured Thermomicrobiabacterium

09666b1754e79c10181ea027c4e16595	0.911	0.190	0.002	Firmicutes	Clostridia	Clostridiales	Family XIII	Anaerovorax
1f6a965b1db7f6b030e88767881ac6de	0.677	0.177	0.047	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	uncultured
d8f86f866900533770e5d5b27b0f042f	0.915	0.186	0.001	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	NA
8029a92bd672e641733132c302a9108d	-1.157	0.187	0.000	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	Oxalobacter
aee9f354c80ca7baa872c3da2fe462c2	-2.076	0.243	0.000	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA
0414019fbf6990da9b14a5c07fa51e88	-0.916	0.221	0.014	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	NA
27d8f12f22ba9352acadc2f057b1895a	0.865	0.178	0.002	Proteobacteria	Alphaproteobacteria	Rhizobiales	D05-2	NA
708e50f6b07a7fcee8183886a5f169b1	1.432	0.203	0.000	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Vulcaniibacterium

Table S6.5. Differentially abundant amplicon sequencing variants (ASVs) significantly (q<0.05) associated with both the presence of the minor allele of genomic loci (Table S6.3) and host's survival to the next season (Table S6.4) in the gut microbiome of adult Seychelles warblers.

taxon	SNP	SNP_lfc	SNP_se	SNP_q	Survival_lfc	Survival_se	Survival_q	Phylum	Class	Order	Family	Genus

1f6a965b1db7f6b030e88767881ac6de	**rs728642**	**3.78**	**0.20**	**0.000**	**0.68**	**0.18**	**0.047**	**Firmicutes**	**Clostridia**	**Clostridiales**	**Ruminococcaceae**	**uncultured**
1f6a965b1db7f6b030e88767881ac6de	rs750388	1.18	0.16	0.001	0.68	0.18	0.047	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	uncultured
09666b1754e79c10181ea027c4e16595	**rs95_2409799**	**2.50**	**0.24**	**0.000**	**0.91**	**0.19**	**0.002**	**Firmicutes**	**Clostridia**	**Clostridiales**	**Family XIII**	**Anaerovorax**
1f6a965b1db7f6b030e88767881ac6de	rs95_2940759	1.59	0.19	0.001	0.68	0.18	0.047	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	uncultured
1f6a965b1db7f6b030e88767881ac6de	rs95_965036	2.11	0.26	0.001	0.68	0.18	0.047	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	uncultured
aee9f354c80ca7baa872c3da2fe462c2	rs728642	-1.22	0.24	0.002	-2.08	0.24	0.000	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	
aee9f354c80ca7baa872c3da2fe462c2	rs95_974945	-1.75	0.29	0.000	-2.08	0.24	0.000	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	
df98b3d20eafc1a8628a93e7b04a3325	rs95_965036	-2.35	0.22	0.002	-1.12	0.17	0.000	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	Leifsonia
0414019fbf6990da9b14a5c07fa51e88	rs728642	-1.84	0.25	0.000	-0.92	0.22	0.014	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	-
0414019fbf6990da9b14a5c07fa51e88	rs750388	-1.48	0.25	0.000	-0.92	0.22	0.014	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	-
27d8f12f22ba9352acadc2f057b1895a	rs95_2940759	1.62	0.25	0.001	0.86	0.18	0.002	Proteobacteria	Alphaproteobacteria	Rhizobiales	D05-2	-
0414019fbf6990da9b14a5c07fa51e88	rs95_965036	-2.49	0.28	0.000	-0.92	0.22	0.014	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	-

38f39d32b69c25e9c315a57df4cd6529	rs95_1075473	-2.15	0.23	0.001	-1.35	0.20	0.000	Chloroflexi	Chloroflexia	Thermomicrobiales	JG30-KF-CM45	uncultured Thermomicrobiabacterium
8029a92bd672e641733132c302a9108d	rs1657804	1.11	0.16	0.000	-1.16	0.19	0.000	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	Oxalobacter
09666b1754e79c10181ea027c4e16595	**rs728642**	**-1.75**	**0.19**	**0.000**	**0.91**	**0.19**	**0.002**	**Firmicutes**	**Clostridia**	**Clostridiales**	**Family XIII**	**Anaerovorax**
1f6a965b1db7f6b030e88767881ac6de	**rs95_2409799**	**-1.95**	**0.21**	**0.001**	**0.68**	**0.18**	**0.047**	**Firmicutes**	**Clostridia**	**Clostridiales**	**Ruminococcaceae**	**uncultured**
1f6a965b1db7f6b030e88767881ac6de	rs12200 65	-2.52	0.32	0.004	0.68	0.18	0.047	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	uncultured
09666b1754e79c10181ea027c4e16595	rs95_2940759	-1.03	0.18	0.002	0.91	0.19	0.002	Firmicutes	Clostridia	Clostridiales	Family XIII	Anaerovorax
1f6a965b1db7f6b030e88767881ac6de	rs1657804	-1.64	0.12	0.000	0.68	0.18	0.047	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	uncultured
d8f86f866900533770e5d5b27b0f042f	rs1657804	-0.98	0.19	0.000	0.91	0.19	0.001	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	-
df98b3d20eafc1a8628a93e7b04a3325	rs95_1075473	1.86	0.16	0.002	-1.12	0.17	0.000	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	Leifsonia
df98b3d20eafc1a8628a93e7b04a3325	rs1657804	1.02	0.09	0.001	-1.12	0.17	0.000	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	Leifsonia
0414019fbf6990da9b14a5c07fa51e88	rs95_974945	2.25	0.27	0.000	-0.92	0.22	0.014	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	-
708e50f6b07a7fcee8183886a5f169b1	rs95_2940759	-1.44	0.20	0.002	1.43	0.20	0.000	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Vulcaniibacterium

708e50f6b07a7fcee8183886a5f169b1	rs1657804	-1.24	0.17	0.000	1.43	0.20	0.000	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Vulcaniibacterium
708e50f6b07a7fcee8183886a5f169b1	rs95_965036	-2.78	0.27	0.000	1.43	0.20	0.000	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Vulcaniibacterium

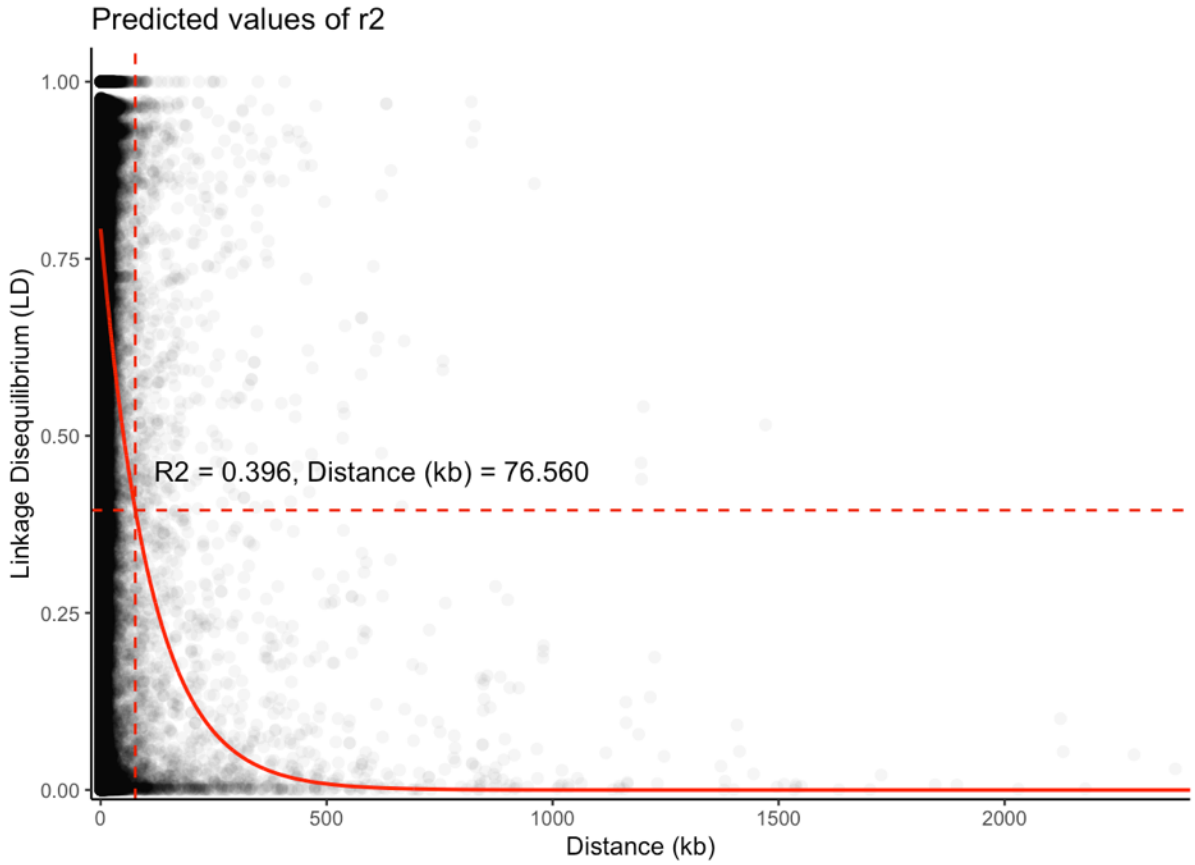

Figure S6.1. Linkage disequilibrium (LD) decay of Seychelles warblers. LD plotted using a random subsample consisting of approximately 1% of pairwise SNP comparisons from each chromosome. Maximum distance between SNPs for LD estimation: 5 Mbp (plotted to 2.5 Mbp). Red solid line represents model fit, red dotted line represent where LD decayed to 50%.

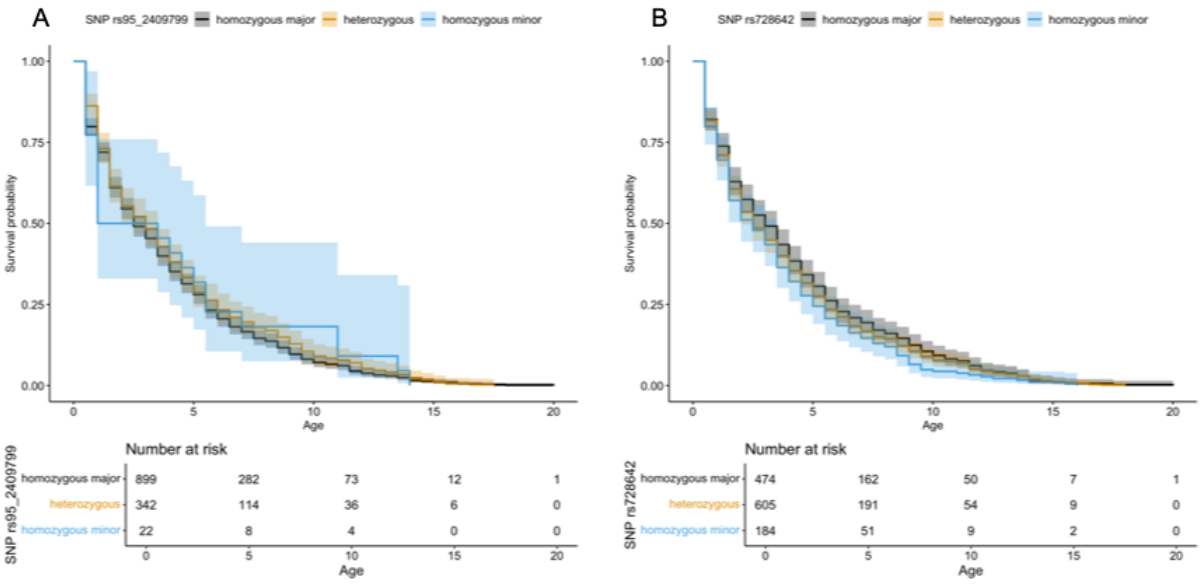

Figure S6.2. Survival probability in relation to variation at the genomic loci A) *rs95_2409799* and B) *rs728642* in Seychelles warblers (N=1340). Lifetime survival probabilities are denoted with different colours: homozygous major allele (black), heterozygous (orange), homozygous minor allele (blue). The number of alive/at-risk individuals at each interval of 5 years is shown at the bottom of the plot. Individuals still alive at the end of the study are right censored (indicated with the symbol "+", N=57).

Chapter 7 |

General Discussion

Photo Credit: Sen Dong – Seychelles warblers, having a pleasant viva discussion

“Time to digest – the journey thus far”

7.1 Overview

The aim of this thesis was to investigate the relationship between the host and its gut microbiome (GM) in a natural vertebrate population. Building upon existing research, incorporating shotgun metagenomics, and using modern computational methods, the aim was to extend our understanding of the factors that shape, and are shaped by, the GM using the Seychelles warbler. In this final chapter, I bring together results from earlier chapters to discuss what they may reveal about the GM in Seychelles warblers, their wider relevance to the field and possible directions for future research.

Chapter two aimed at identifying age and senescence-related changes in the GM using longitudinal sampling and shotgun metagenomics. I showed that the GM decreases linearly with age in both taxonomic and functional alpha diversity and change in composition within individuals. This provides support for a consistent change of the GM with age, rather than a sudden drop-off in alpha diversity or composition linked to senescence. I also showed that a group of microbial transposases (COG2801) increases linearly with age. This is despite functional alpha diversity decreasing with age, suggesting fewer functions are present.

Chapter three explored how different aspects of host immunogenetics could modulate the GM. I showed that variation on the host major histocompatibility complex (MHC) shapes the GM – both in terms of taxonomic and functional characteristics. Moreover, I identified a trade-off between microbial defence and metabolism in relation to increasing MHC-I diversity.

Chapter four assessed how social interactions influence the GM within a cooperative breeding population. I showed that individuals who shared space had a more similar GM composition, and that individuals who interact closely (i.e. breeding pair and helpers) shared more anaerobic GM composition.

Chapter five investigated the effects of inbreeding (including intergenerational inbreeding) on the GM. I found that the inbreeding coefficient of individuals was correlated with amplicon sequencing variants (ASVs) and functional GM composition. Additionally, I showed that the inbreeding coefficient of mothers and social fathers was correlated with the taxonomic GM composition.

Chapter six aimed to identify host loci that are associated with GM composition and determine if those loci contribute to GM-associated survival. I showed that nine genomic loci from 14 known genes were correlated with the GM, suggesting multiple

genetic pathways (immune-related genes and gut physiology), through which host genetics modulate the GM. All nine loci were also correlated with at least one survival-related ASV. Two of these loci were also directly associated with host survival.

Overall, this thesis presents a coherent research programme aimed at identifying the key drivers that shape the GM in a wild population. By integrating ecological, genetic, and social perspectives, it offers new insights into processes shaping GM taxonomy and function, within and between individuals.

7.2 Synthesis

Each chapter provides an in-depth discussion of its key findings. Thus, here, I focus more on connecting and synthesising the findings across chapters. I explain how my findings confirm and build upon previous work in the Seychelles warbler and in the broader field of wild animal GM research.

7.2.1 External environmental effects

Before my project began, temporal environmental effects on the GM in the Seychelles warbler had already been documented (Davies et al., 2022; Worsley et al., 2021, 2022). Across all of my chapters, I found consistent support for these effects in shaping the GM (including GM function newly revealed through my metagonomics approach). Sample year, season, and time of day were significant across multiple analyses, indicating that temporal variation at both broad (year and season) and fine (time of day) scales influences GM composition. These temporal factors affecting the GM have been seen in many other wild animal systems (Hicks et al., 2018; Marsh et al., 2022; Risely et al., 2022; Schmid et al., 2023; Voigt et al., 2016), revealing the importance of controlling for environmental variables in wild animal GM research. Unexpectedly, territory quality – a proxy for food abundance (Komdeur, 1992) – was not significantly associated with the GM, shown in two papers (Worsley, Davies, et al., 2024; Worsley et al., 2021) and Chapter 2. This contrasts with the frequent suggestion that diet is the major driver of GM variation in both wild and captive systems (Bodawatta, Freiberga, et al., 2021; Cotillard et al., 2013; Loo et al., 2019; Suriano et al., 2022; van Leeuwen et al., 2020). However, most of these studies examined dietary changes (e.g. seasonal shifts, supplementation, or replacement), rather than food abundance per se, so direct comparisons may be limited. Together, these results suggest that seasonal and

interannual differences are important contributors to GM variation in the Seychelles warbler, but that the underlying mechanisms may not involve food abundance.

7.2.2 Gut microbiome diversity

GM alpha diversity measures have been linked to host health and mortality in several captive species (Shreiner et al., 2015; Sommer et al., 2017; Videvall et al., 2020). In my chapters, I showed within-individual but limited between-individual effects. Age (Chapter 2) and environmental variables (across chapters) were significantly associated with GM alpha diversity, but host genetics and sociality had limited effects. This suggests that within-individual temporal changes had a larger influence on alpha diversity than between-individual host factors – revealing the importance of within-individual measures of GM alpha diversity. Additionally, previous studies support our findings as environmental variables are frequently associated with GM alpha diversity across diverse taxa, such as great tits (*Parus major*), giant panda (Ailuropoda melanoleuca) and meerkats (*Suricata suricatta*) (Liukkonen et al., 2024; Risely et al., 2021; Xue et al., 2015). However, studies into the role of between-individual host factors, such as host genetics, on GM alpha diversity have reported mixed findings (Bolnick et al., 2014; Hernández-Gómez et al., 2018; Leclaire et al., 2019; Montero et al., 2021; Uren Webster et al., 2018), with effects apparently species dependent (Williams et al., 2024). Thus, GM alpha diversity may be a valuable indicator for tracking within-individual changes in the GM but is less informative for between-individual comparisons in the Seychelles warbler. Additionally, GM alpha diversity may be limited in response in Seychelles warblers, where GM composition appears to be a better measure of individual differences (see below).

7.2.3 Gut microbiome composition

The GM composition of Seychelles warblers was associated with multiple variables: age (Chapter 2), immunogenetics (Chapter 3), sociality (Chapter 4), inbreeding (Chapter 5), and nine host genomic loci (Chapter 6), as well as environmental variables (discussed above). Indeed, all main variables tested impacted the GM composition in some way. However, as expected, each variable was associated with different sets of GM taxa and functions, indicating that their effects on the GM are distinct and act on different pathways. Although many previous separate studies across many species have suggested that the GM is shaped by many different factors (Bonder et al., 2016; Raulo et al., 2018; Xu & Zhang, 2021), here we show

that the GM of a single wild species/population was shaped by many of these same factors. This shows studying the GM of a single population in depth, as we did here, provides rare evidence that the GM is shaped by multiple interacting factors. It also points to the fact that these factors need to be considered together to avoid oversimplification and to more accurately interpret host–microbe dynamics.

Although multiple factors shape the GM, changes in GM composition do not necessarily indicate host deterioration, unless they have been directly linked to survival (Williams et al., 2024). Instead, these shifts in taxa and functional GM are often adaptive adjustments of the GM to differences in the host's physiological state, with certain GM taxa or functions selectively maintained to support host health and resilience (Williams et al., 2024). Therefore, in the Seychelles warbler, the GM shifts we observe may represent subtle compensatory mechanisms that buffer the effects of, for example, ageing, inbreeding, or environmental stress on host fitness.

The MHC was identified as a key factor shaping the GM in Chapter 3, but it was not identified as a significant locus in the GM genome-wide association study (GWAS) presented in Chapter 6. This is likely because the MHC is a highly repetitive region of the genome that is notoriously difficult to assemble and accurately map using standard short-read sequencing techniques (Vekemans et al., 2021). In the Seychelles warbler, the whole genome sequencing was performed with short-read sequencing. Thus, there are regions of the genome that are not assembled, and so it is impossible to include such regions in the GWAS. Therefore, while the nine genomic loci identified as being linked to GM variation in Chapter 6 highlight multiple genetic pathways by which the host genome may impact the GM, the true extent of host genetic influence on the GM may be underestimated. Future research using long-read whole-genome sequencing could assemble a better genome, improve mapping quality, and provide a more complete picture of these host-GM relationships.

7.2.4 Decoupling of gut microbiome taxonomy and function

While certain results showed agreement between differences in GM taxonomy and differences in GM function, other findings do not, highlighting discrepancies between the GM taxonomy and function. This is not surprising due to functional redundancy, where changes in taxa do not alter GM function because different taxa can carry the same genes/function or changes in function are not reflected in changes in taxa because many species can contribute to the functional change (Worsley, Mazel, et

al., 2024). For example, the increase in COG2801 (transposase) with age (see Chapter 2) was not linked to an increase in any one specific microbe. Therefore, the increase in COG2801 with age likely reflects shifts across multiple microbial species, with functional redundancy potentially masking these changes when examined solely from a taxonomic perspective.

In chapters three and five, taxonomic and functional GM composition were associated with different variables; taxonomic GM composition was associated with MHC-II diversity, whereas functional GM composition was associated with MHC-I diversity. Similarly, taxonomic GM composition was associated with the inbreeding coefficient of social fathers and mothers, whereas functional GM composition was associated with the inbreeding coefficient of the individual. These results indicate that taxonomic and functional GM composition can vary independently, suggesting that some taxa are able to functionally adapt to host requirements, while certain functions are maintained despite shifts in taxonomic composition. Whether the GM taxa or function changes likely depend on the selection pressures and the adaptability of the available bacterial species (Kohl et al., 2018; Petersen et al., 2023).

7.2.5 The benefits of metagenomics

Metagenomic data provides valuable insights into the functional aspects of the GM, but its high cost often limits sample size. In contrast, the more cost-effective 16S data, with its larger dataset, can be crucial for building large sample sizes and detecting small effect sizes, which are characteristic of host-GM relationships in wild birds (see below). Using both synergistically can be a good strategy. This is evident in Chapter 3, where I first used 16S data to identify specific MHC loci, which were then validated with the more detailed metagenomic dataset. Interestingly, there were some discrepancies between 16S data and metagenomic taxonomy (e.g. MHC-I allele *Ase-ua 11* was significant in 16S but not metagenomic taxonomy alpha diversity). This could be due to a few factors, such as primer bias in 16S sequencing, differing copy number variation of the 16S rRNA gene in bacteria, taxonomic resolution, and sample size. However, 16S data was also comparable to the metagenomic taxonomy data (e.g. ageing and MHC diversity), which is expected as both methods are comparing GM taxonomy. This combined approach allowed for deeper explorations that would have been missed with either dataset alone, demonstrating that both methods are essential but serve different, complementary purposes.

7.2.6 Peculiarities of the avian GM

The effect sizes of GM associations were small in my studies compared to those reported in mammalian, reptiles and amphibian studies (Marsh et al., 2022; Mazel et al., 2024; Song et al., 2020; Tung et al., 2015). This is consistent with other wild avian GM studies, often reporting modest effect sizes (Bodawatta, Koane, et al., 2021; Somers et al., 2023; Song et al., 2020). The effect sizes may also reflect the high variability of the GM both within and between individuals, which is likely driven by short gut retention times in passerines and a diverse insect-based diet in the Seychelles warbler (each type of insect carrying its unique microbiome) (Engel & Moran, 2013). Additionally, the GM may also be less important to a passerine compared to a ruminant that relies on the GM for digestion (Cholewińska et al., 2020). The detection of significant associations in my studies—despite relatively small effect sizes—emphasises the importance of a large dataset to robustly detect true associations, as smaller sample sizes typically fail to detect such effects (Kelly et al., 2015; Serdar et al., 2021). These results underscore the need, at least in avian studies, for large, well-powered datasets such as the Seychelles warbler to reliably detect subtle host-GM relationships.

7.2.7 Advances made

Across chapters, I have identified a suite of variables that have shaped the GM. This thesis advances the field by demonstrating within-host temporal changes (Chapter 2), host genetic influences (Chapters 3, 5, and 6) and social (Chapter 4) associations with the GM factors rarely integrated into a single wild vertebrate system. Synthesising these findings, I show that temporal variation consistently impacts the GM, and that composition offers a more powerful lens than alpha diversity for detecting host effects. Moreover, taxonomic and functional profiles often diverged due to redundancy, underscoring the need for integrative approaches. Together, these findings highlight the value of multi-faceted, longitudinal approaches and open new questions about the mechanisms driving GM stability, functional resilience, and their consequences for host fitness in natural populations.

7.3 Limitations

A central question I originally aimed to address—though ultimately decided I could not—was whether the GM contributes to reproductive success. The primary

limitation was sample size. The GM samples we collect linked to reproduction are largely restricted to the major breeding season (June to October), because the GM varies between the major and minor breeding seasons. Additionally, ideally, we would collect samples just before breeding begins to assess their predictive value. Post-breeding samples may reflect changes in the GM driven by behaviours like offspring care (Antwis et al., 2019; Sarkar et al., 2020, 2024). Compounding this, nest numbers were low during the years I participated in fieldwork, further limiting the availability of samples from successful breeders. In the larger 16S dataset, GM samples were available for 193 breeding birds, but only 16 of these successfully reproduced in the major season and had samples collected before reproduction. Hence, I decided to focus my energy elsewhere. However, the Seychelles warbler research project has continued to collect samples, thus, this idea may be possible once enough samples have been collected

In the same vein, the final chapter had a small sample size for GWAS (n=205), as although we have GM samples, we do not have the whole genome sequencing data from recent Seychelles warblers. Most human GWAS studies have sample sizes >1000 (Hong & Park, 2012). Future research could incorporate more samples into a GM composition GWAS, which should identify a greater number of loci that are correlated with the GM. Nonetheless, my sample size was greater than the recommended 100 samples for a GWAS study (Hong & Park, 2012), and, while I recognise that some loci may have been missed, it does not undermine the fact that nine genomic regions that are strongly associated with the GM were detected.

The correlative nature of this thesis is a key limitation, as the underlying causal mechanisms remain unknown. Future research should focus on uncovering the specific pathways through which each variable influences the GM. This is particularly relevant for Chapter 6, as each host genomic loci were associated with a different or multiple genes. Thus, pinpointing the causal gene(s) within these genomic regions could improve our understanding of how the host genome regulates the GM. Targeted resequencing of candidate regions in a larger sample panel would enhance resolution and validate the associations identified. Furthermore, experimental manipulations—such as transplanting GMs between hosts of different genotypes—could test whether specific taxa are selectively retained or excluded by the host genetics.

7.4 Future research

I see several other promising directions for future research on the Seychelles warbler GM, which I did not have time to investigate. One intriguing avenue involves the relationship between breeding pair divorce in this socially monogamous species and the duration the GM remains the same. In Chapter 4, I found that breeding pairs tend to share more similar GM, raising the question: Does pair separation also lead to GM divergence, and to what extent is the shared GM retained after divorce? However, given that divorce is rare (14%) in this species (Speelman et al., 2024), this question may be better suited to a system where divorce occurs more frequently, such as *Ciconiiformes* (Jeschke & Kokko, 2008). Similarly, future research could explore GM retention following dispersal from a territory (i.e in offspring)—investigating how much of an individual's GM remains the same when it moves to a new social and ecological environment.

Additionally, the potential link between the GM and personality traits in the Seychelles warbler remains unexplored. In Seychelles warblers, how individuals explore novel environments and objects is associated with their dispersal patterns: males with higher exploratory tendencies often delay natal territory dispersal, whereas highly exploratory females tend to disperse farther from their natal territory (Cox et al., 2023). While an exploratory personality is not associated with Seychelles warbler fitness (Edwards et al., 2018), the personality differences could be associated with the GM, perhaps through the microbiome-gut-brain axis (Davidson et al., 2018). This research idea may elucidate the associations between specific GM profiles and behavioural syndromes or competitive phenotypes, offering insights into physiological fitness, health outcomes, and social hierarchy dynamics.

An underexplored area of GM research is how microbial communities differ between growing and stable populations. A growing population may reflect an environment with higher resource availability and low competition, which may reduce conspecific social interactions, subsequently leading to reduced GM diversity (Archie & Tung, 2015; Raulo et al., 2024). Additionally, growing populations may have healthier individuals, hence GM stability and resilience may be higher. While direct experimental manipulation is not currently feasible in the Seychelles warbler, natural translocations provide a valuable pseudo-experimental framework to examine how a growing population influences GM composition. One hypothesis is that individuals in growing populations—facing less competition and greater food availability—can select preferred dietary items, leading to a more diverse or beneficial GM. If such a

"healthier" GM can be identified, it could potentially be used in future translocations via faecal microbiota transplants to provide a microbial “kickstart,” supporting the health and growth of new populations.

Epigenetics (such as telomere length and DNA methylation) has recently been bidirectionally associated with the GM (Pepke et al., 2024). GM metabolites may act as signalling molecules that can modify the host epigenome (Ha et al., 2025), and thus, may lead to differential host senescence patterns (Adams et al., 2025). Additionally, host epigenetics can influence the GM through a range of host genes and proteins (e.g. genes/proteins involved in gut barrier function, sirtuin proteins, and CHD1) (Pepke et al., 2024). In addition, recent tools such as Computel (Nersisyan & Arakelyan, 2015) and TelSeq (Ding et al., 2014) have enabled estimating telomere length from whole genome sequencing. In addition, the Seychelles warbler telomere length has also been estimated with quantitative polymerase chain reaction (qPCR) in past papers (Barrett et al., 2013; Bebbington et al., 2016; Sparks et al., 2022). Together, these approaches offer a promising research idea to explore how host biological age, mediated through epigenetic mechanisms, interacts with the GM to influence health, fitness, and ageing in the Seychelles warbler.

A key goal for future work is to integrate all the identified variables—age, host genetics, sociality, and environmental factors—into a single, powerful model. This approach would allow for a direct comparison of the relative influence of each driver on the GM, providing a more complete picture of host-GM interactions. This kind of multi-faceted analysis would require significantly more data, but would also be the most powerful. Integrating longitudinal microbiome data with host genomic, epigenetic, and ecological covariates would enable partitioning of variance attributable to each factor and testing for interactions, revealing the relative and combined effects of multiple drivers on GM structure and function. Ultimately, this integrative approach could provide predictive insights into how host biology and environment jointly shape GM communities and influence fitness in natural populations.

7.5 Final remarks

In conclusion, the exceptional long-term Seychelles warbler project has provided – and will continue to provide – a unique opportunity to investigate the factors shaping the GM in a wild vertebrate population. Beyond identifying individual drivers, it offers

the potential to integrate these factors to improve the predictive power of GM models. Collectively, my findings demonstrate that age, host genetics, sociality, and environmental variables all contribute to variation in the Seychelles warbler GM. This thesis underscores the value of a detailed, fine-scale approach to studying the GM within a single, well-characterised wild population. More broadly, it highlights the importance of embracing the complex, multi-faceted relationship between hosts and their GM in natural systems.

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Appendix 1 |

Published version of Box 1.1

Conservation biology

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Preserving microbial functional biodiversity

When thinking of conservation, people often picture elephants roaming the savannah, sea turtles on beaches, or lush rainforests. Although these species and ecosystems are undeniably important, people often overlook another vital, smaller world — the realm of microorganisms. In 2021, a paper by Dodd and Grueber highlighted the importance of conserving the microbial ecosystems that exist in, or on, animals in natural populations. This exciting paper inspired me to pursue a PhD in wildlife functional microbiomes.

In addition to providing a useful summary of emerging techniques in functional microbiome research, Dodd and Grueber offer a compelling description of the key functions of host microbial communities and their importance to species' conservation. Human activity can disrupt microbial ecosystems, which affects species' health and survival. For example, rhinoceroses are endangered primarily owing to poaching, but deforestation and pollution has led to dietary changes and exposure to pathogens, which alters their microbiomes and contributes to the declining population. Therefore, the authors emphasize that understanding the association between functional microbiomes and host health would help to identify host species that might suffer most from microbiome change. With this knowledge, informed conservation actions — such as introducing beneficial microorganisms — can be taken to help species to maintain a healthy wild microbiome.

The authors also made an unexpected point that well-intentioned conservation efforts can inadvertently harm host microbiomes and, therefore, the host species itself. For example, the process of translocating individuals — a common tool in animal conservation — could damage the vertebrate gut microbiome if it involves a period of captivity, supplementary feeding or antibiotic treatment. By highlighting the complexity of host–microorganism interactions and their implications for host health (and, thus, conservation), the authors illustrate the importance of understanding of the host microbiome's role in guiding effective conservation strategies.

To date, microbial function in wild animal hosts remains poorly understood, primarily owing to the costs associated with sampling, sequencing and analysis. Dodd and Grueber point out that research on wild endangered species is constrained by the difficulty of obtaining sufficient sample sizes. Analysing these samples presents further challenges, as the field is still relatively new — particularly in the context of wild systems. Moreover, changes in the wild animal microbiome are often influenced by many interacting variables, including biotic and abiotic environmental factors and intrinsic host factors, which must be accounted for in analyses to ensure accurate interpretations.

"the authors illustrate the importance of understanding of the host microbiome's role in guiding effective conservation strategies"

Dodd and Grueber recommend thoughtful study designs to reduce costs and optimize insights when studying wild animal microbiomes. They suggest reusing genomic and microbiome data from well-studied species to aid decision-making in small, isolated populations and speed up research. Given the complexity of host–microorganism interactions in wild populations, it is essential to develop effective strategies to protect life across all scales — from taxonomy to functional diversity.

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Competing interests
The author declares no competing interests.

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Appendix 2 |

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Original Article

Metagenomic analyses of gut microbiome composition and function with age in a wild bird; little change, except increased transposase gene abundance

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Abstract

Studies on wild animals, mostly undertaken using 16S metabarcoding, have yielded ambiguous evidence regarding changes in the gut microbiome (GM) with age and senescence. Furthermore, variation in GM function has rarely been studied in such wild populations, despite GM metabolic characteristics potentially being associated with host senescent declines. Here, we used 7 years of repeated sampling of individuals and shotgun metagenomic sequencing to investigate taxonomic and functional changes in the GM of Seychelles warblers (*Acrocephalus sechellensis*) with age. Our results suggest that taxonomic GM species richness declines with age and in the terminal year, with this terminal decline occurring consistently across all ages. Taxonomic and functional GM composition also shifted with host age. However, the changes we identified occurred linearly with age (or even mainly during early years prior to the onset of senescence in this species) with little evidence of accelerated change in later life or during their terminal year. Therefore, the results suggest that changes in the GM with age are not linked to senescence. Interestingly, we found a significant increase in the abundance of a group of transposase genes with age, which may accumulate passively or due to increased transposition induced as a result of stressors that arise with age. These findings reveal taxonomic and functional GM changes with age, but not senescence, in a wild vertebrate and provide a blueprint for future wild functional GM studies linked to age and senescence.

Keywords: gut microbiome, age, senescence, metagenomics, transposase, *Acrocephalus sechellensis*

Introduction

Senescence—a decline in physiological function in later life—occurs in most organisms [1, 2]. However, its onset and rate often differ greatly among individuals within populations [1, 3]. One factor that may contribute to individual differences in senescence is variation in host-associated microbial communities. The intestinal tract of animals contains a diverse collection of microbes and their genomes (the gut microbiome; GM), which play an important role in host adaptation and fitness [4, 5]. The GM influences the regulation of essential processes, such as digestion, reproduction, and immune function [6, 7]. However, shifts in GM composition can be detrimental to the host; certain microbes may be pathogenic, while overall dysbiosis may impair host function [8, 9].

Studies in humans and laboratory animals have shown that GM composition generally changes rapidly in early life [10, 11] before stabilizing during adulthood [12]. This is often followed by greater GM instability in advanced age including a loss of diversity and changes to composition [13–15]. These late-life compositional shifts are generally characterized by a loss of commensal or probiotic bacteria and an increase in pathogenic microbes [16]. GM functional changes with age have also been identified. For example, healthy aging has been associated with microbes that enable increased biodegradation and metabolism of xenobiotics [16, 17], whereas unhealthy aging has been linked to increased production of detrimental microbial metabolites [16].

Studies have demonstrated links between the GM and senescence in humans and laboratory animals, however, their GM composition varies markedly from their counterparts living in natural environments because of the artificial environments they are exposed to [18, 19]. It remains unclear if these effects can be generalized to wild animals [18–20].

Recent studies on wild organisms have not reached a consensus on what characterizes the aging microbiome. Some have documented altered GM composition [21–23], increased GM

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diversity [22, 24]„ and reduced GM stability [25] with increasing age. Other studies have indicated that GM characteristics remain relatively stable throughout adulthood [25–27]. However, these studies have been based on 16S ribosomal ribonucleic acid (rRNA) gene metabarcoding, which is limited in resolution [28–30]. Shotgun metagenomic sequencing enables higher taxonomic resolution (species or strain level), as well as informing on the functional potential of microbial communities based on gene content [31–33]. In humans and captive primates, metagenomics has revealed an increase in pathogenic microbial genes, and a decrease in beneficial genes, with age [17, 34, 35]. To our knowledge, no previous studies have investigated GM functional changes with age and senescence using shotgun metagenomics in a wild population.

Also, most GM studies on wild animals have relied on a cross-sectional sampling of differently aged individuals [36–38] and, therefore, may be confounded by the selective appearance/disappearance of individuals with particular GM characteristics. A lack of longitudinal samples also makes it difficult to infer changes in GM stability with age [39]. Understanding what drives this GM variation is important, as it may lead to a deeper comprehension of the evolution of senescence and life-history trade-offs [3], and enhance our ability to prolong healthy lifespans. As senescence occurs at different rates across individuals, a longitudinal approach is crucial for accurately evaluating age-associated effects [40]. Given this rate variation, and because declines are expected to be greatest at the end of life, GM changes may be more closely associated with proximity to death than chronological age. Including such information in analyses requires accurate estimates of the point of death that are not confounded by dispersal.

The long-term study of the Seychelles warbler population on Cousin Island provides a powerful natural system to study GM variation and host senescence [3]. Its isolated nature allows for the longitudinal sampling of uniquely marked, known-age individuals across their entire lifespan and the collection of accurate survival and reproductive success data [41, 42]. Previous studies using 16S metabarcoding have demonstrated that Seychelles warbler GM composition is linked to subsequent survival [43] but identified no overall patterns of GM senescence [26]. Additionally, host age was not associated with GM diversity, but a very marginal effect of host age on GM composition was reported [26].

Here, we use shotgun metagenomics to assess fine-scale changes in the GM with age and senescence in the Seychelles warbler. First, we determine how GM taxonomic diversity and composition change with host age, particularly in a bird's terminal year when GM dysregulation is expected to be at its greatest. Then we test the hypothesis that GM functional characteristics (assessed via microbiome gene content) will change with age, senescence, and in the terminal year.

Materials and methods

Study system and sample collection

Seychelles warblers are insectivorous passerines endemic to the Seychelles archipelago. The population on Cousin Island (29 ha; 04° 20′ S, 55° 40′ E) has been extensively monitored since 1985 in the winter (January–March) and summer (June–October) breeding seasons [3, 44, 45]. Each season nearly all new birds (offspring) are caught, in the nest or as dependent fledglings in the natal territory [45]. As many adult birds as possible are re-caught each season using mist nets. Bird age is determined using either lay/fledgling date [45] for the majority of individuals, if birds are first caught without a fledging date being recorded, eye color is used to estimate age instead (see [45]).

The population on Cousin Island consists of ca. 320 individuals grouped into ca. 115 territories, defended year-round by a dominant breeding pair [46, 47]. Territory quality is calculated each season using arthropod counts, vegetation density, and territory size information [45, 48].

Nearly every bird in the population (> 96% since 1997 [49]) has been caught and marked with a unique combination of a British Trust for Ornithology (BTO) metal ring and three plastic color rings, which enables them to be monitored throughout their lives [3, 50]. Individuals almost never disperse between islands and the annual resighting probability is ∼98% ± 1% [41, 42, 51]. If an individual is not seen for two consecutive seasons it is assumed to have died (an error rate of 0.04%) [41, 42]. Death dates for individuals were set as the final day of the season in which the bird was last seen. Benign climatic conditions and a lack of predators result in relatively long-lived individuals (median lifespan 5.5 years, max lifespan 19 years) [46, 52]. Previous studies have found that male and female Seychelles warblers are sexually mature at 1-year-old, and senescence (survival and reproductive) begins at ca. 6 years of age [3, 41, 46, 53]. The annual survival of adults does not differ between sexes, remaining ∼80% up to 6 years of age and then decreasing [3, 54]. Thus, there were no differences in survival senescence between the sexes [3, 46, 53]. In addition, elderly females in their last year of life (terminal year) had reduced reproductive success [55].

Fecal samples were collected from caught birds and stored as described previously (see [26]). Between 2017 and 2023 all caught birds were placed in a disposable flat-bottom waxed paper bag containing a sterilized plastic weighing tray underneath a sterilized metal grate [56]. This allows the bird to stand on the grate and feces to fall into the sterile tray, minimizing contact with the bird's surface. After ca 15 minutes (after defecation) the bird was removed. The sample was collected, using a sterile flocked swab, and placed into a microcentrifuge tube containing 1 ml of absolute ethanol. Samples were stored at 4°C in the field before being transferred to −80°C for long-term storage. Contamination (hand) controls were collected from fieldworkers each season. The time-of-day that samples were collected and the number of days for which samples were stored at 4°C, were recorded. A ca 25 μl blood sample was also taken via brachial venepuncture and stored in 1 ml of absolute ethanol at 4°C.

Deoxyribonucleic acid extraction and sequencing

Blood samples were processed with a salt extraction method [42] or Qiagen DNeasy Blood and Tissue Kit and the resulting deoxyribonucleic acid (DNA) was used for molecular sexing [52, 57].

DNA from fecal samples was extracted using the Qiagen DNeasy PowerSoil Kit with a modified protocol (see [56]). Samples were lysed using both mechanical agitation and enzymic processes [56]. Individuals for which multiple longitudinal samples were available were prioritized for metagenomic sequencing to capture within-individual changes. In total, 155 fecal samples from 92 individuals across 7 years were sequenced, as well as three positive controls (two extractions from a ZymoBIOMICS Microbial Community Standard [D6300], and one extraction from a ZymoBIOMICS Fecal Reference with TruMatrix™ Technology [D6323]), and six hand controls. Library preparation was performed in two lanes per run using the LITE protocol [58] and sequencing undertaken in two runs of 2 × 150 bp NovaSeq X

platform. The D6300 extraction control was sequenced on both runs to compare extraction and batch effects.

Bioinformatics

Shotgun metagenomic sequence analysis was carried out using the MATAFILER pipeline (see [5] and supplementary materials). Briefly, MATAFILER removes host reads, assembles reads, predicts, and annotates genes, builds metagenome-assembled genomes (MAGs) and metagenomic species (MGSs), and taxonomically assigned MGSs. Due to the high individuality of the Seychelles warbler GM and the high sequencing coverage required to assign MGS, Metaphlan4 was also used to taxonomically classify reads (see supplementary materials for details).

Gut microbiome analyses

A total of 162 samples were successfully processed bioinformatically (153 fecal samples, 4 controls). Positive controls were successfully recovered, and hand controls did not contribute to substantial contamination in samples (Fig. S1).

The 153 fecal samples (Fig. S2) included 71 from 40 females and 82 from 51 males. In total, 41 individuals had one sample, 41 had two, eight individuals had three, and one individual had four samples. Age at sampling ranged from 0.6–17.0 years (mean 5.7 ± 0.3 SE). Of these, 48 were from 22 individuals in their terminal year (the year in which they died); with ages in terminal year ranging from 1.4–17.0 years. From all these samples, 1025 unique metaphlan4 species-genome-bins assignments were used for the subsequent taxonomic analysis (mean 29.3 ± 2.0 SE per sample).

All statistical analysis was performed using R version 4.33 [59, 60]. variance inflation factor scores (*car* version 3.1.2) were used to test for collinearity between variables in all models; all had a score < 3 indicating no issues with collinearity [61].

Taxonomic gut microbiome changes with age

Taxonomic gut microbiome alpha diversity

A rarefaction curve of Metaphlan4 species was constructed with *iNEXT* version 3.0.1 to determine the read depth required to recover 95% of theoretically present species (Fig. S3) [62]. Taxonomic classifications were rarefied to a depth of 5500 reads before alpha diversity analysis; two samples were removed due to insufficient read depth. Species richness and Shannon diversity metrics were calculated per sample using R packages *phyloseq* version 1.46.0 and *microbiome* 1.24.0 [63, 64]. Wilcoxon rank sum tests were used to examine whether different sequencing plates affected species diversity (Shannon index, $P = .353$) and species richness (Observed index, $P = .124$), both were not significantly different.

A linear mixed effect model with a Gaussian distribution (lmer), and a generalized linear mixed effect model with a negative binomial distribution (glmer.nb), were used to model changes in species diversity (Shannon index) and richness (observed taxa), respectively, using *lme4* version 1.1–35.5 [65]. Fixed effect variables included in models were: host age (years); terminal year (yes/no); sex (male/female); breeding season (winter/summer); sample year (as a factor: 2017–2023); territory quality; storage days at 4°C (days); time of day collected (minutes since sunrise at 6:00 a.m.). Bird ID was included as a random effect.

Storage at 4°C in the field ranged from 4 days to 104 days (mean 36.3 ± 1.8 SE). A quadratic age term, and an interaction between terminal year and host age, were tested to assess whether GM changes became more extreme with age or if GM changes in the terminal year differ depending on age. These terms were dropped if not significant to allow interpretation of the main effects. Age was measured in years, but all samples taken when birds were > 12 years of age were designated as 12 years because these samples were rare (n = 9, max age = 17 years). Previous analysis shows that body condition is not associated with Seychelles warbler GM diversity and composition, thus, it was not included in analysis [43]. Model diagnostics were run using *DHARMa* version 0.4.6, with no significant issues in each chosen model [66]. Herein, all models were tested with the same variables unless stated otherwise.

A within-subject centering approach was used to separate between-individual (cross-sectional) GM differences with age (which could be driven by the selective appearance/disappearance of individuals with particular GM characteristics), from within-individual (longitudinal) change (which could indicate senescence) [67]. This involves calculating the mean age of each individual across all its sampling events (mean age) and the within-individual deviation from that mean age at each separate sampling event (delta age). These terms replace host age in the model. The fixed effect of terminal year was also replaced by a "terminal year bird" term (yes/no) which indicates whether individuals have at least one sample collected in the terminal year or not. An interaction between the terminal year bird and delta age, as well as quadratic delta age, were tested to assess whether within-individual GM changes were more extreme in birds with a sample taken in the terminal year of life and/or in older individuals, respectively (which would be indicative of senescence). In addition, an interaction between delta age and mean age was included in the models to test if within-individual changes with time occur differently depending on host age. The analysis was repeated with non-rarefied reads to determine if rarefaction influenced the results. These terms were dropped if not significant to allow interpretation of the main effects.

Taxonomic gut microbiome composition

A permutational multivariate analysis of variances (PERMANOVA) was carried out on a Euclidean distance matrix calculated using centered log ratio (CLR)-transformed reads, using the adonis2() function in *vegan* version 2.6.6 [68]. A blocking effect of Bird ID was used to account for repeated measures. The same predictors were included as for the main model in the Alpha diversity analysis above. Differences in composition were visualized with a principal component analysis (PCA) in *phyloseq* version 1.46.0 [64].

Taxonomic gut microbiome differential abundance analysis

Two different differential abundance analysis (DAA) methods were used to identify differentially abundant GM species with host age (as recommended by [69, 70]; *ANCOMBC2* version 2.4.0 and *GLLVM* version 1.4.3 [71, 72]. ANCOMBC2 calculates log fold change of species one at a time before adjusting p-values, whereas GLLVM calculates log fold change of all species all at the same time, accounting for correlation between species [71, 72]. A total of 22 common species, defined as species found in 20% of the population at >0.01% abundance, were retained. Species that were significantly differentially abundant in the same direction using both DAA methods were considered robustly significant. Variables included in each model were the same as in models above.

Functional gut microbiome changes with age

Functional gut microbiome alpha diversity

Initially, 4727 different eggNOG orthologues (mean = 3616.6 ± 64.4 SE per sthe ample) were identified in our gene catalogs. A rarefaction curve of eggNOG orthologues was constructed using *iNEXT* to

Table 1. A generalized linear mixed effect model with a negative binomial distribution (glmer.nb) investigating GM species richness in relation to within-(delta) and between-(mean) individual variation in age among Seychelles warblers ($n = 151$ samples, 91 individuals). Conditional $R^2 = 53.1\%$. Reference categories for categorical variables are shown in brackets.

Predictor	Estimate	SE	z	P
(Intercept)	**2.705**	**0.317**	**8.536**	**<.001**
Delta Age	**−0.308**	**0.095**	**−3.233**	**.001**
Mean age	−0.036	0.023	−1.534	.125
Terminal year bird (yes)	−0.189	0.142	−1.329	.184
Season (winter)	0.020	0.157	0.126	.900
Sex (female)	−0.020	0.144	−0.139	.889
Days at 4°C	−0.238	0.137	−1.734	.083
Time of day	0.237	0.122	1.938	.053
Territory quality	−0.081	0.125	−0.645	.519
Sample year (2017)				
2018	0.439	0.280	1.568	.117
2019	0.399	0.323	1.233	.217
2020	**0.701**	**0.351**	**1.997**	**.046**
2021	**0.755**	**0.338**	**2.231**	**.026**
2022	**0.725**	**0.346**	**2.099**	**.036**
2023	**0.879**	**0.400**	**2.197**	**.028**
Delta age * mean age	**0.034**	**0.014**	**2.440**	**.015**
Random				
Individual ID	151 observations	91 individuals	Variance	.2321

Note: Significant ($P < .05$) predictors are shown in bold.

determine sample completeness [62]. Samples were then rarefied to 100 000 reads based on >95% completeness. One sample was removed due to insufficient reads. Following rarefication, 4685 eggNOG orthologues were retained (mean = 3054.3 ± 47.1 SE per sample). Due to the (negative) skewness of the observed richness and Shannon diversity of eggNOG annotations, a scaled exponential transformation and an exponential transformation were used for analyses, respectively, to improve residual fit. Both these alpha diversity indices were then analysed with linear mixed models containing the same predictors as for taxonomic alpha diversity above.

Functional gut microbiome composition

To test for changes in functional microbiome beta diversity, a PERMANOVA of Euclidean distances calculated from CLR-transformed read abundances per orthologue was used, using the same model structure as for taxonomic compositional analysis (described above). Differences in composition were visualized with a PCA plot as above.

Functional gut microbiome differential abundance analysis

DAA was performed on eggNOG annotations using their assigned categories from the database of clusters of orthologous genes (COG; Supplementary Table S1) [73] using *ANCOMBC2* and *GLLVM* as described above [71, 72]. Post-hoc DAA were performed on individual eggNOG members found within differentially abundant COG categories to establish the drivers of any significant differences (see Supplementary material for details).

Results

Taxonomic gut microbiome changes with age

Taxonomic gut microbiome alpha diversity

GM species richness declines with host age, and individuals in their terminal year had significantly lower species richness than those in a non-terminal year (Table S2 and Fig. S4). However, Shannon diversity was not significantly associated with host age, and did not differ between samples taken in a terminal or non-terminal year (Table S3). A quadratic age term, and an interaction between host age and terminal year were not significantly associated with species richness or Shannon diversity ($P > .05$) and were dropped from the final model.

The within-individual centering approach revealed that a decline in GM species richness with host age occurred longitudinally within individuals (Table 1, Fig. 1). However, the slope of declining species richness within an individual (delta age) decreases with increasing mean age, i.e. a decline in GM species richness with time occurs more at earlier host ages than in later life (Table 1, Fig. 1). Indeed, after the age of 6 there doesn't appear to be any significant decline in GM species richness with increasing age (Fig. 1). This shows that contrary to our prediction that GM may show senescent effects, within-individual changes were less extreme in older individuals (in the ages we know senescence is occurring). There was also no evidence of between-individual selective disappearance effects (Table 1). Shannon diversity did not change significantly with mean or delta age (Table S4). There was also no evidence of a quadratic relationship between within-individual delta age and species richness or Shannon diversity, hence the quadratic age term was dropped from the final model. We also tested for an interaction between within-individual age and whether an individual's final sample was in their terminal year, but this was not significant ($P > .05$) and was dropped. Additionally, the results were consistent with Table 1 when non-rarefied reads were used (Table S5). This result indicates that within-individual changes in species richness with age had a similar slope whether the bird was sampled in its terminal year or not.

Taxonomic gut microbiome composition

A PERMANOVA analysis found that cross-sectional host age was a marginally significant predictor of GM taxonomic composition (Table 2), but terminal year was not (Table 2). Sample year, season, and catch time were significant and explain the largest proportion of GM compositional variance (Table 2) followed by days sample

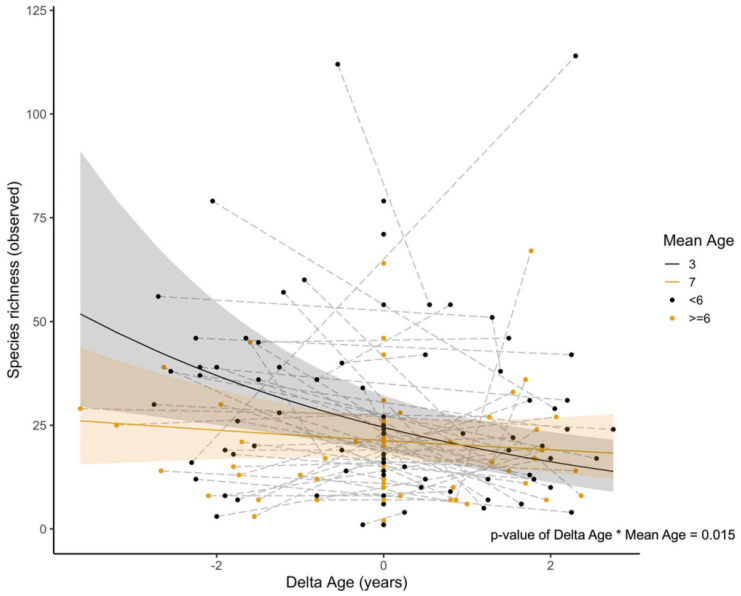

Figure 1. GM species richness in relation to within-individual, longitudinal differences in age (delta age in years) in Seychelles warblers. The solid lines represent model predictions with 95% confidence intervals calculated from the generalized linear mixed effect model (Table 1). Lines are model predictions at mean age of 3 and 7 before and after the start of senescence in this species [3]. Each point represents an individual GM sample, distinguished by mean age of <6 and greater or equal to 6, and the dashed lines connect samples from the same individual ($n = 151$ samples, 91 individuals).

stored at 4°C and sex. An interaction between age and terminal year was not significant ($P > 0.05$). A PCA showed limited sample clustering according to age, which is consistent with the small amount of variance explained in the PERMANOVA (Fig. S5).

Taxonomic gut microbiome differential abundance analysis

Five of the 22 common GM species found in the Seychelles warbler population (i.e. in >20% individuals) differed significantly in relative abundance with age in the GLLVM analysis (*Escherichia coli*, *Lactococcus lactis*, *Brucella pseudogrignonensis*, *Lactococcus garvieae*, *Microbacterium enclense*), but none were differentially abundant with age in the ANCOMBC2 analysis (Fig. S6A and B). Similarly, six species were differentially abundant in the terminal year in the GLLVM analysis (*L. garvieae*, *Pantoea anthophila*, *E. coli*, *Rothia* sp AR01, *M. enclense*, *B. pseudogrignonensis*), but none were differentially abundant with terminal year in the ANCOMBC2 analysis (Fig. S6C and D). Thus, there is no clear consensus of significant variation in the abundance of specific GM species with age or in the terminal year.

Functional gut microbiome changes with age

Functional gut microbiome alpha diversity

Alpha diversity of eggNOG gene orthologues declined significantly with host age for both observed richness and Shannon diversity metrics (Table S6, Fig. S7). Alpha diversity of eggNOG orthologues did not differ between terminal year and non-terminal year samples (Table S6). Additionally, the interaction between host age (or quadratic age) and terminal year was not significant ($P > .05$).

The decrease in functional alpha diversity with host age is best explained by within-individual longitudinal changes with age for both tested indices (Table 3, Fig. 2). Cross-sectional, between-individual age was a marginally significant predictor of Shannon diversity but not observed richness (Table 3). Alpha diversity did not differ between individuals that had at least one sample taken in their terminal year and those that did not. The interaction of terminal year bird and within-individual age, quadratic within-individual age, and the interaction between within-individual age and mean age were also not significant ($P > .05$) predictors of either index. Sample year was a significant variable of both eggNOG observed richness and Shannon diversity.

Functional gut microbiome beta diversity

A PERMANOVA analysis identified factors that were significantly related to GM functional composition (Table 4). Host age, but not terminal year, was a marginally significant predictor of functional composition (Table 4). An interaction between age and terminal year was not significant ($P > .05$). The largest effect sizes were found in relation to season, sample year, sex, and days stored at 4°C (Table 4). Time of day was not significant related to GM functional composition (in contrast to GM taxonomic composition). A PCA plot showed limited clustering of GM samples according to age, consistent with the small amount of variance explained by this variable (Fig. S8).

Functional gut microbiome differential abundance analysis

Only one cluster of orthologous genes (COG) category was differentially abundant in relation to age. The COG category "X", which represents mobilome COGs such as prophages and transposons, significantly increased in abundance with age in both the ANCOMBC2 and the GLLVM analyses (Fig. 3). Several COG

Table 2. A PERMANOVA analysis of GM taxonomic composition in relation to age and terminal year in the Seychelles warbler. The PERMANOVA was performed using a Euclidean distance matrix of CLR-transformed taxon abundances. $N = 153$ samples from 91 individuals. Bird ID was included as a blocking factor.

Predictor	*df*	R^2	F	P
Age	**1**	**0.009**	**1.368**	**.043**
Terminal year	1	0.007	1.051	.569
Season	**1**	**0.013**	**2.021**	**.001**
Sample year	**6**	**0.056**	**1.479**	**<.001**
Sex	**1**	**0.007**	**1.096**	**.064**
Days at 4°C	**1**	**0.008**	**1.193**	**.034**
Time of day	**1**	**0.010**	**1.583**	**<.001**
Territory quality	1	0.005	0.813	.982

Note: Significant ($P < .05$) predictors are shown in bold.

Table 3. A linear mixed effect model investigating variation in GM functional diversity (observed richness and Shannon diversity) in relation to within-(delta) and between-(mean) individual age in Seychelles warblers ($n = 152$ samples, 90 individuals). Functional diversity is based on eggNOG annotations. Observed richness and Shannon diversity were transformed using a scaled exponential and exponential function, respectively. Conditional $R^2 = 35.6\%$ and 13.7%, respectively. Reference categories for categorical variables are shown in brackets.

Observed richness

Predictor	Estimate	SE	*df*	t	P
(Intercept)	**0.99**	**0.17**	**124.77**	**5.68**	**<.001**
Delta age	**−0.12**	**0.04**	**137.00**	**−3.31**	**.001**
Mean age	−0.03	0.01	89.42	−1.97	.052
Terminal year bird (yes)	0.01	0.08	83.34	0.17	.870
Season (winter)	−0.06	0.10	136.94	−0.64	.525
Sex (female)	−0.06	0.08	81.33	−0.79	.430
Days at 4°C	**−0.19**	**0.09**	**127.35**	**−2.23**	**.028**
Time of day	−0.07	0.08	137.00	−0.88	.381
Territory quality	−0.07	0.08	129.62	−0.88	.381
Sample year (2017)					
2018	0.13	0.15	135.76	0.82	.416
2019	0.08	0.18	135.88	0.46	.647
2020	0.36	0.20	136.54	1.82	.071
2021	**0.39**	**0.19**	**136.94**	**2.04**	**.044**
2022	**0.56**	**0.19**	**128.48**	**2.90**	**.004**
2023	**0.57**	**0.23**	**122.81**	**2.50**	**.014**
Random					
Individual ID	152 observations	90 individuals	Variance	0.050	

Shannon diversity

Predictor	Estimate	SE	*df*	t	P
(Intercept)	**757.59**	**182.06**	**119.47**	**4.16**	**<.001**
Delta age	**−117.01**	**41.06**	**135.71**	**−2.85**	**.005**
Mean age	**−27.30**	**13.54**	**83.56**	**−2.02**	**.047**
Terminal year bird (yes)	17.93	79.75	76.74	0.23	.823
Season (winter)	173.07	104.67	127.74	1.65	.101
Sex (female)	−4.98	80.46	69.67	−0.06	.951
Days at 4°C	−48.55	95.70	133.26	−0.51	.613
Time of day	−21.18	81.57	132.14	−0.26	.796
Territory quality	−0.74	85.97	136.99	−0.01	.993
Sample year (2017)					
2018	88.02	168.08	136.67	0.52	.601
2019	32.22	200.48	136.71	0.16	.873
2020	169.50	210.62	131.73	0.81	.422
2021	**464.12**	**206.85**	**136.39**	**2.24**	**.026**
2022	**484.95**	**202.78**	**124.82**	**2.39**	**.018**
2023	453.37	238.55	116.14	1.90	.060
Random					
Individual ID	152 observations	90 individuals	Variance	5046	

Note: Significant ($P < .05$) predictors are shown in bold.

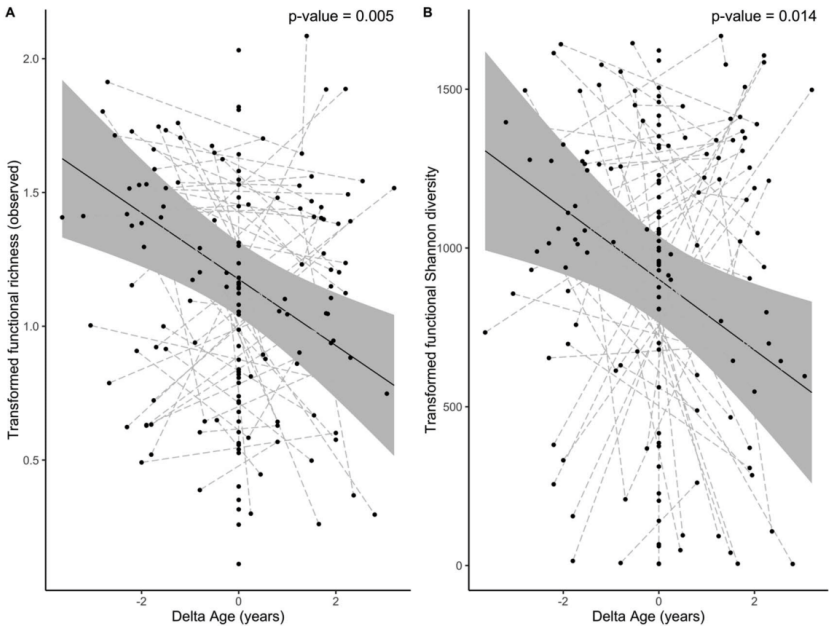

Figure 2. GM functional diversity measured as (A) observed richness and (B) Shannon diversity in relation to within-individual host age (years). Functional diversity calculations are based on eggNOG orthologue groups. Solid lines represent model predictions (± 95% confidence interval) from linear mixed effects models (Table 3). Each point represents a unique GM sample, and the dashed gray lines connect samples collected from the same individual ($n = 152$ samples, 90 individuals).

Table 4. A PERMANOVA analysis of GM functional composition in relation to age (and other factors) in the Seychelles warbler. The PERMANOVA was performed using a Euclidean distance matrix calculated using CLR-transformed (eggNOG) abundances. $N = 153$ samples; 91 individuals; bird ID was included as a blocking factor.

Predictor	df	R^2	F	P
Age	**1**	**0.007**	**1.096**	**0.044**
Terminal year	1	0.006	0.890	0.292
Season	**1**	**0.011**	**1.823**	**0.042**
Sample year	**6**	**0.052**	**1.374**	**0.020**
Sex	**1**	**0.008**	**1.250**	**0.001**
Days at 4°C	**1**	**0.010**	**1.569**	**0.007**
Time of day	1	0.008	1.200	0.139
Territory quality	1	0.007	1.094	0.413

Note: Significant ($P < .05$) predictors are shown in bold.

categories were significantly differentially abundant with environmental variables including Cat A (RNA processing and modification) with season and Cat C (Energy production and conversion) with sample year (Figs S9 and S10).

Within category X (mobilome), only COG2801 (transposase genes) was found to significantly increase in abundance with age in both GLLVM and ANCOMBC2 analyses (Fig. S11, Table S1). A within-subject centering approach within a linear mixed model showed an increase in COG2801 was associated with both within-individual (longitudinal) age and between-individual (cross-sectional) age (Table S7, Fig. 4). However, the interaction between within-individual age and terminal year, as well as the interaction between within-individual age and mean age, was not significant ($P > 0.05$).

COG2801 located within MGSs (509 COG2801 copies from 160 MGS) were most closely related to the group insertion sequences (IS) 3 family of transposases (30%), other IS family transposases (12%), partial or putative transposases (33%) or other/unknown function (25%; Table S8). An increased abundance of COG2801 in the GM may be due to either an increase in the abundance of COG2801-carrying microbes or increased replication of the transposase gene itself. However, contrary to the first hypothesis, we found no relationship between the total abundance of COG2801-carrying MGSs ($n = 160$) and host age (Table S9). To further test this, COG2801-MGSs were matched with metaphlan4 annotations at the genus level; the abundance of COG2801-metaphlan4 genera was not significantly associated with host age (Table S10). Hence, the increase in COG2801 abundance with host age could not be

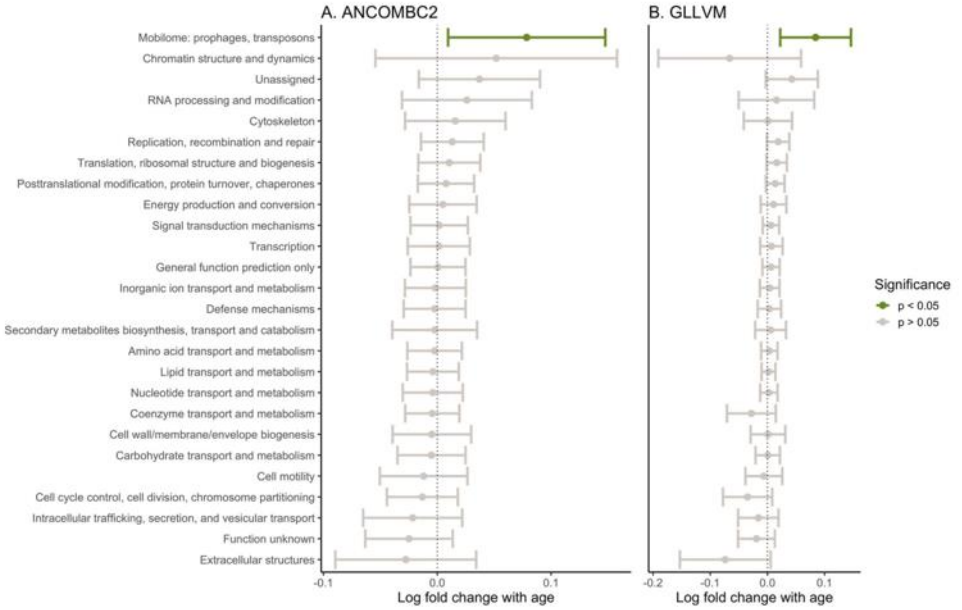

Figure 3. DAA of functional GM COG categories in Seychelles warblers using (A) ANCOMBC2 and (B) GLLVM. Each COG category is represented on the y-axis. Points and error bars are distinguished according to significance ($P < .05$).

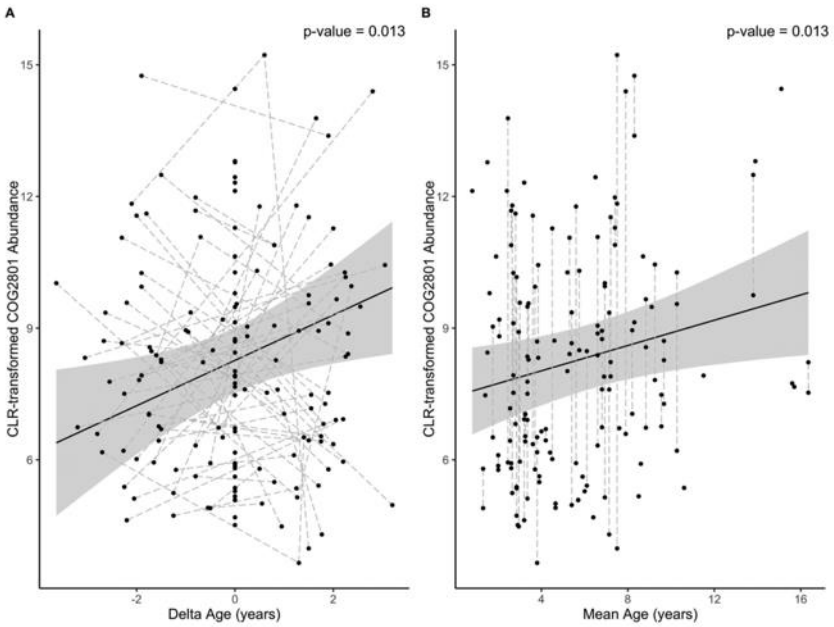

Figure 4. CLR-transformed COG2801 abundance in relation to (A) within-individual (delta) host age and (B) between-individual (mean) host age in the GM of Seychelles warblers. The solid line represents model predictions (± 95% confidence intervals) from a linear mixed effect model (Table S7). Each point represents a GM sample with dashed gray lines connecting samples from the same individual ($n = 153$ samples, 91 individuals).

attributed to an increased abundance of COG2801-carrying bacteria. Additionally, within COG2801, ten gene catalogs were commonly shared across 50% of samples. Each of these ten COG2801 gene catalogs was not significantly ($p > 0.05$) differentially abundant with age individually when tested using both ANCOMBC2 or GLLVM analysis (Fig. S12). Thus, the increase in abundance of COG2801 with age was not being driven by the abundance of a single prevalent, gene catalog but rather the cumulative abundance of many.

Discussion

We used a repeated metagenomic dataset from individuals in a Seychelles warbler population to investigate how GM taxonomic and functional characteristics varied with host age. We identified a linear decrease in species richness, and small shifts in GM taxonomic composition, with host age. Additionally, species richness was lower in samples taken during an individual's terminal year, but taxonomic composition did not differ between terminal and non-terminal samples. We also identified a linear decrease in the GM's functional richness and diversity, and differences in functional GM composition, with host age. Finally, COG categories representing the mobilome increased in prevalence with bird age, driven by an increase in the abundance of COG2801, a group of transposases.

The small reduction in GM richness, but not Shannon diversity, with age suggests a loss of rare taxa that is not linked with a major restructuring of the evenness of the GM. The reduction in species richness was also age-dependent, with younger individuals experiencing greater reduction in species richness over time compared to older individuals, indicating that changes in GM species richness is not associated with senescence. This also concurs with the small changes in GM composition with age we identified; i.e. showing a limited number of differentially abundant taxa with increasing host age. This result is consistent with a previous 16S metabarcoding analysis of senescence of the Seychelles warbler GM despite the increased taxonomic resolution afforded by a metagenomics approach [26]. Additionally, the three dominant phyla identified in the metagenomics analysis (accounts for 95.6% of all taxonomic assignments) were the same three dominant phyla identified through the 16S analysis (Proteobacteria, Actinobacteria, and Firmicutes) [26, 43]. Overall, the results support the conclusion that, taxonomically, most of the GM stays the same with increasing age, apart from the loss of a few rare taxa.

Taxonomic changes in GM species diversity and composition with age have been repeatedly demonstrated in humans and captive animals [16]. However, in these species, late-life changes in the GM may be due to external factors such as antibiotic use, lifestyle, and dietary changes [18, 20]. An increasing number of wild animal studies are finding little evidence of a late-life shift in GM taxonomic diversity without such external factors (see [26,74]). Our study supports this conclusion despite the repeated sampling and increased resolution yielded by shotgun metagenomics, which can potentially reveal more nuanced changes at lower taxonomic levels.

Few studies have directly investigated functional changes in the GM with age in wild animals [75]. Some studies have been undertaken using functional inferences from metabarcoding sequence homology. However, this can be misleading due to being limited to variation within the same genus thus providing potentially inaccurate functional profiles. [76,77]. In our study using a higher resolution metagenomic approach, we found evidence of small, linear, changes in GM functional diversity and composition with age in the Seychelles warbler. Functional observed richness and Shannon diversity declined with age, which suggests not only that rare functions are lost, but that the evenness of these GM functions also changes linearly with adult age. Age-related decreases in functional richness and shifts in functional composition have previously been identified in elderly humans [78,79]. Such changes have been linked to the onset of specific disease states, such as inflammation and pathogenesis and changes to diet degradation and digestion, in humans and laboratory mice [80]. However, other studies have either found no change in functional alpha diversity, or even an increase in microbial functional richness and diversity with age [35,81]. Whether the loss of functional diversity, and minor changes in functional composition, with host age in Seychelles warbler is linked to declines in health and condition remains unclear and requires further study. The decline in taxonomic richness (but not taxonomic diversity) along with declines of functional richness and diversity with host age suggests that as the host age, less rare taxa contribute to the number and evenness of functional genes in the GM.

Despite the small changes in functional diversity and composition with age in the Seychelles warbler, we only identified one specific functional category whose abundance was significantly associated with host age. An increase in the abundance of COG2801 transposases occurred with age. However, this was not due to an increase in COG2801-carrying microbes. COG2801 are a group of transposases that are primarily found in bacteria (89.5%) and have been shown to be the most widely transferred genes among prokaryotes [82]. Most COG2801 genes found within MGSs were group IS3, which use a copy-out-paste-in mechanism to replicate [83]. This could lead to an increased number of transposon copies in the same individual bacterial genome over time, or to horizontally transfer to other bacterial genomes. [84,85]. Thus, the increased abundance of COG2801 with age in Seychelles warbler GM's may be the result of self-replication, independent of microbial host cell DNA replication. An increase in transposition has been observed when bacteria are stressed and COG2801 is one of the most horizontally transferable eggNOG genes [86,87]. Therefore, as vertebrate hosts get older, the GM may be exposed to a greater number or intensity of stressors, such as mucus barrier thinning or inflammation, which may induce activation of COG2801 [88]. However, there was not an accelerated increase (i.e. a quadratic relationship) of COG2801 abundance with host age, which would be expected if the cumulative effects of host senescence were driving these changes. Therefore, stressors to the host that occur linearly in adulthood, such as cell death in the gastrointestinal autonomic nervous system [89,90], may better explain the increased abundance of COG2801 with host age.

We also focused on assessing terminal year effects in the Seychelles warbler GM. Only species richness was found to be significantly lower in the final year of a bird's life. Moreover, the effect of terminal year was uniform across age, i.e. it was not more extreme in older individuals. Previous research has identified age-dependent terminal-declines in fitness components (reproductive success and survival probability) in the Seychelles warbler [55]. However, the lack of age-dependent terminal changes in GM characteristics identified in our study suggests that the GM does not undergo senescence in association with these other traits. As such, the declines in microbial species richness in terminal year samples (and linearly with age) may rather reflect the stabilization of the GM with age rather than a senescence effect. These results concur with the previous 16S metabarcoding analysis of

the Seychelles warbler GM which found little evidence of GM senescence [26].

Across analyses, environmental factors explained most of the variance in the Seychelles warbler GM. This concurs with previous research on this species [26, 43, 56] as well as studies of other taxa [21,91,92]. Temporal variation -specifically year and season- explained the most variance in both taxonomic and functional GM composition. This may be explained by many factors including climate variability, differences in insect prey availability, or host population density [93–95]. Most Seychelles warbler individuals breed in the summer rather than the winter season, and GM shifts may therefore reflect reproductive activity and related hormonal changes [24]. Time of day was also associated with GM composition. Differences in insect activity might drive this pattern due to light availability and/or temperature [96,97]. However, such patterns could also be due to host intrinsic circadian rhythms [98]. In addition, differences in the amount of time samples were stored at 4°C resulted in differences in the GM characteristics and it is very important that these are controlled for. Given that samples are stored directly in absolute ethanol, the changes related to the time in storage at 4°C are likely to do with DNA degradation affecting the assignment of reads rather than an actual biological change in storage.

These factors lead to a substantial amount of noise in GM studies that can confound studies on aging, reproduction, and disease outcomes in wild populations. Therefore, accounting for these factors is important when investigating the GM in natural systems.

Our findings highlight the need for more studies investigating the functional characteristics of wild microbiomes as taxonomic relationships might not capture functional GM changes that occur (e.g. the increased prevalence of COG2801). However, researchers should not totally discount the utility of 16S metabarcoding for investigating general GM questions, as it may, in many cases, provide sufficient taxonomic resolution to answer specific questions [28]. Indeed, we identified similar taxonomic patterns using shotgun metagenomics to those revealed by a previous metabarcoding study on the Seychelles warbler [26]. The cost-effectiveness of 16S rRNA allows greater sample sizes, and thus power, to resolve certain questions. A combination approach that harmonizes both 16S metabarcoding and shotgun metagenomics has been proposed to maximize sample size, although such analyses are limited to genus-level comparisons [99]. On the other hand, shotgun metagenomics not only allows higher taxonomic resolution and functional analysis of the GM, but also an assessment of the interaction between taxa and their functions. As described with transposable elements, our functional analysis uncovered changes in GM function that were not detectable using 16S metabarcoding analysis.

In conclusion, while we found that the Seychelles warbler GM changes in terms of diversity, composition, and even function with age, this happens gradually over the adult lifespan and there is little evidence of late-life GM senescence. While species richness is lower in the terminal year, this occurs at all ages and is not more extreme in the oldest individuals. Interestingly, we found that the abundance of a group of transposase gene increases considerably with age in the GM, probably because of more frequent transposition within the GM community over time. Future work is required to determine exactly why these transposable element changes occur and what impact they may have. Additionally, work should investigate the generality of these conclusions by assessing whether functional changes occur in the GM of other wild vertebrates.

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Supplementary material

Supplementary material is available at *ISME Communications* online.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Data availability

All raw sequence data have been submitted to the European Nucleotide Archive database under the study accession numbers PRJEB81709.

Ethics statement

Fieldwork was carried out in accordance with local ethical regulations and agreements (UEA ethics approval ID ETH2223-0665). The Seychelles Department of Environment and the Seychelles Bureau of Standards approved the fieldwork (permit number A0157).

Code availability

The data files and script necessary to reproduce the statistical analysis and plots are provided at https://github.com/Chuen-Lee/SW_Senescence_GM

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