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Longitudinal gut microbiome dynamics in relation to age and senescence in a wild animal population

Sarah F. Worsley¹  | Charli S. Davies¹ | Chuen Zhang Lee¹ | Maria-Elena Mannarelli¹ | Terry Burke²  | Jan Komdeur³ | Hannah L. Dugdale^{3,4}  | David S. Richardson^{1,5} 

¹School of Biological Sciences, University of East Anglia, Norfolk, UK

²NERC Biomolecular Analysis Facility, Department of Animal and Plant Sciences, University of Sheffield, Sheffield, UK

³Groningen Institute for Evolutionary Life Sciences (GELIFES), University of Groningen, Groningen, The Netherlands

⁴Faculty of Biological Sciences, School of Biology, University of Leeds, Leeds, UK

⁵Nature Seychelles, Mahé, Republic of Seychelles

Correspondence

Sarah F. Worsley and David S. Richardson, School of Biological Sciences, University of East Anglia, Norwich Research Park, Norfolk NR4 7TJ, UK.

Email: s.worsley@uea.ac.uk and david.richardson@uea.ac.uk

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Abstract

In humans, gut microbiome (GM) differences are often correlated with, and sometimes causally implicated in, ageing. However, it is unclear how these findings translate in wild animal populations. Studies that investigate how GM dynamics change within individuals, and with declines in physiological condition, are needed to fully understand links between chronological age, senescence and the GM, but have rarely been done. Here, we use longitudinal data collected from a closed population of Seychelles warblers (*Acrocephalus sechellensis*) to investigate how bacterial GM alpha diversity, composition and stability are associated with host senescence. We hypothesised that GM diversity and composition will differ, and become more variable, in older adults, particularly in the terminal year prior to death, as the GM becomes increasingly dysregulated due to senescence. However, GM alpha diversity and composition remained largely invariable with respect to adult age and did not differ in an individual's terminal year. Furthermore, there was no evidence that the GM became more heterogeneous in senescent age groups (individuals older than 6 years), or in the terminal year. Instead, environmental variables such as season, territory quality and time of day, were the strongest predictors of GM variation in adult Seychelles warblers. These results contrast with studies on humans, captive animal populations and some (but not all) studies on non-human primates, suggesting that GM deterioration may not be a universal hallmark of senescence in wild animal species. Further work is needed to disentangle the factors driving variation in GM-senescence relationships across different host taxa.

KEYWORDS

Acrocephalus sechellensis, ageing, gut microbiome, life history, senescence

1 | INTRODUCTION

Senescence—the decline in function with age—occurs in most organisms and results in substantial reductions to health and fitness (e.g. annual survival probability and reproductive success) (Nussey et al., 2013). Even within a single population of a species, considerable

variation may exist in the age at which individuals begin to senesce and the rate at which senescence occurs (Hammers et al., 2015; Nussey et al., 2013). Determining the biological processes that contribute to this individual variation may have implications for extending the health span of individuals living in ageing populations and could improve our understanding of the evolution of senescence.

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One factor that has received increasing interest for its possible role in senescence is the vertebrate gut microbiome (GM). The GM is a highly diverse microbial ecosystem that plays a significant role in many aspects of host physiology, including digestion, cognition and immunity (Davidson et al., 2020; Nicholson et al., 2012; Sommer & Bäckhed, 2013). However, it is also tightly regulated by the host, for example via gut epithelial cell function and the host immune system (Davies et al., 2022; Hooper et al., 2012; Zhou et al., 2022). As host systems deteriorate with age, due to the accumulation of molecular and cellular damage, the GM may become increasingly dysregulated; this could, in turn, have negative consequences for host health and exacerbate further functional declines in other host systems (Aleman & Valenzano, 2019; Bosco & Noti, 2021; Ghosh et al., 2022). Thus, the association between host senescence and the GM could involve complex interactions and feedback loops whereby GM shifts could be both a driver and consequence of ongoing senescence (Bosco & Noti, 2021; Ghosh et al., 2022).

Corresponding with this, a growing body of literature has identified age-related changes in the GM, particularly in human and captive animal populations. For example, several studies have reported a loss of bacterial diversity and a corresponding increase in the abundance of proinflammatory bacterial taxa in the GM of older individuals (e.g. Claesson et al., 2011; O'Toole & Jeffery, 2015; Smith et al., 2017; Xu et al., 2019). These changes have been correlated with chronic inflammation, impaired intestinal integrity and increased mortality risk (Bodogai et al., 2018; Clark et al., 2015; Mitchell et al., 2017). Furthermore, experiments on captive killifish (*Nothobranchius furzeri*) have shown that recolonising the GM of older individuals with bacteria from young donors can reverse functional declines and extend their lifespan, suggesting that the GM can play a causal role in host ageing (Smith et al., 2017). However, the extent to which these findings can be generalised to wild animals remains unclear given a range of confounding factors related to age and captivity (DeJong et al., 2020).

In humans, a variety of lifestyle factors can correlate with age (and thus apparently senescence) while also impacting the GM. For example, elderly individuals are more likely to take medication, experience malnutrition, and enter residential care, all of which can directly alter GM composition via processes that are independent of senescence (DeJong et al., 2020; Jeffery et al., 2016). Extrapolating from studies on laboratory animals is also difficult as they can exhibit differences in longevity and frequently harbour low diversity GM communities that differ radically in composition compared to their wild counterparts (Clayton et al., 2016; Kreisinger et al., 2014; Partridge & Gems, 2007; Rosshart et al., 2017). Given these discrepancies, an assessment of the extent to which GM imbalances are associated with age and senescence in wild hosts is warranted.

Recent studies on wild mammalian species have analysed the GM of older, post-prime individuals, however, no clear consensus has emerged from this research. While some studies have reported shifts in GM composition (Bennett et al., 2016; Trosvik et al., 2018)

and greater GM heterogeneity with increasing age (Sadoughi et al., 2022), others have found that GM dynamics and overall composition remain largely invariable throughout adulthood (Janiak et al., 2021; Reese et al., 2021; Risely et al., 2021).

One limitation of the studies done to date is that they have all focussed on changes associated with chronological age. To our knowledge, none have also included information about the biological condition of individuals. Individuals vary in the rate at which they undergo physiological deterioration in later life but die when this damage reaches a particular threshold (McNamara et al., 2009). This means that, although senescence can be correlated with chronological age, it may depend more strongly on the rate of damage accumulation. Thus, incorporating a measure of biological condition, for example 'time to death' (i.e. assessing time backwards from death instead of forwards from birth), could be more informative than chronological age when considering variation in senescent declines across individuals (Martin & Festa-Bianchet, 2011; McNamara et al., 2009).

Most wild studies also take a cross-sectional approach to examining GM changes with respect to age (but see Reese et al. [2021], Sadoughi et al. [2022]), by comparing samples from older adults to those from different, younger individuals (Bennett et al., 2016; Janiak et al., 2021; Trosvik et al., 2018). However, as senescence is a process that occurs within individuals, repeated measures from the same individual are needed, alongside accurate death dates, to properly ascertain the extent to which the GM becomes destabilised due to host senescence in the run up to death (Hammers et al., 2015; Nussey et al., 2008). Furthermore, since all studies on the late-life GM in wild animals have been conducted using mammalian systems (the majority on primates) investigations of other taxonomic groups are needed to assess whether patterns are consistent across host species.

Using the long-term study of the Seychelles warbler (*Acrocephalus sechellensis*), we expand on previous research by investigating the extent to which age and senescence predict changes in the GM of a wild, non-mammalian host. The Seychelles warbler population on Cousin Island is an excellent system to study senescence as the majority of individuals in this closed population are colour ringed (>96% since 1997), enabling longitudinal monitoring across their lives (Davies et al., 2021; Hammers et al., 2015). In many natural populations, most animals disperse or die before senescence can be measured, making it challenging to study this in wild animals (Hammers et al., 2015). However, there is virtually no migration into or out of the Cousin Island population (Komdeur et al., 2004) meaning that repeated sampling and accurate measures of survival can be achieved. Due to a lack of natural predators, a benign climate, and limited human disturbance, there is also very little extrinsic mortality within the Cousin Island population (Brouwer et al., 2006; Hammers et al., 2015). As such, Seychelles warblers can reach a remarkably old age for a passerine species (a maximum lifespan of 19 years), although substantial variation in longevity exists between individuals, with the median lifespan at fledging being 5.5 years (Hammers et al., 2019; Sparks et al., 2021). Previous research on this population

has identified senescent declines in fitness components, with individuals older than six years demonstrating a gradual decline in survival probability (Hammers et al., 2013) and reproductive success (Hammers et al., 2012, 2019). Reproductive success is also lower in the last year of life (a “terminal year effect”), particularly in older individuals, indicating that physiological condition declines prior to death due to senescence (Hammers et al., 2012).

Here, we use faecal samples collected across six consecutive years to investigate whether the Seychelles warbler GM shows signatures of change with age and senescent declines. The composition of the Seychelles warbler GM varies between juvenile and adult individuals (Davies et al., 2022; Worsley et al., 2021) and according to seasonal mortality in adults (Worsley et al., 2021). However, in these previous studies, all adults (ages 1–17 years) were grouped together into one age class as there were not enough longitudinal samples to investigate within individual dynamics in later life. We now have more samples (including within individual repeat samples) enabling a thorough investigation of how GM dynamics change with age, and in the time leading up to death.

We first tested whether GM alpha diversity and overall GM composition (beta diversity) change with increasing chronological age. We hypothesised that GM alpha diversity—the number and evenness of bacterial taxa within the GM—will be lower in older adults, consistent with previous studies on humans and captive animals (Claesson et al., 2011; O’Toole & Jeffery, 2015; Smith et al., 2017). We also hypothesised that GM composition will differ between senescent individuals and younger adults, with a decrease in core bacterial taxa and an increase in the abundance of potentially proinflammatory groups identified in other systems (Ghosh et al., 2022). Furthermore, we predicted that, similar to other traits (Hammers et al., 2012), these differences will be particularly pronounced in an individual’s terminal year (the year before death) as host condition deteriorates more rapidly due to senescence (McNamara et al., 2009). However, convergence on a typical “old” GM composition may be unlikely if senescence results in dysregulation and GM instability; instead, individual GMs may follow divergent trajectories. Thus, we also tested for greater intra- and inter-individual variation amongst samples taken in older age groups. Such heterogeneity has previously been identified in humans (Claesson et al., 2011; Ghosh et al., 2020) but is less well-studied in wild systems (but see Sadoughi et al. [2022]). We also extend this research by testing whether GM variation is greatest during the terminal year due to greater instability.

2 | MATERIALS AND METHODS

2.1 | Study species and sample collection

Samples were collected from a population of Seychelles warblers on Cousin Island (29 ha; 04° 20’ S, 55° 40’ E), which consists of ca 320 adult individuals distributed across ca 115 year-round territories (Hammers et al., 2019; Komdeur & Pels, 2005). Virtually all individuals are marked with a unique combination of a British Trust

for Ornithology (BTO) metal ring and three plastic colour rings enabling longitudinal monitoring throughout their lives (Richardson et al., 2001). Population monitoring is carried out in the minor (January–March) and major (June–September) breeding seasons of each year (Komdeur & Daan, 2005; Sparks et al., 2021). Most breeding activity (94% of territories) occurs in the major breeding season and corresponds with an increase in island-wide food abundance (Komdeur & Daan, 2005). The prevailing wind direction also differs between the two seasons which can have profound effects on coastal territories as trees become defoliated by salt spray (Komdeur & Daan, 2005). The annual resighting probability for adult individuals is very high (0.98 ± 0.01) (Brouwer et al., 2010) and dispersal from the island is virtually absent (Komdeur et al., 2004). Thus, if an individual is not seen in a breeding season it is assumed to be dead rather than having dispersed from the island, providing accurate survival data. Average annual survival probability is exceptionally high in adults (0.84 ± 0.04 SE) and juveniles (0.61 ± 0.09) compared to other passerine species (Brouwer et al., 2006). Survival was assessed each breeding season (until the end of the major season of 2023) and, for individuals that died during the study, death date was allocated as the final day of the breeding season in which the bird was last seen.

Faecal sampling took place over 10 breeding seasons (six major, and four minor seasons) from 2017 to 2022. Individuals were caught in mist nets and placed into a disposable, flat-bottomed paper bag containing a sterilised weigh boat protected by a metal grate. This established protocol (Davies et al., 2022; Knutie & Gotanda, 2018) allows faecal matter to fall into the tray and reduces chances of contact with the bird’s surface. Birds were removed from the bag after defecation or after 30 min. Faecal samples were collected using a sterile flocked swab and placed into a microcentrifuge tube containing 1 mL of absolute ethanol. Control swabs from fieldworker hands and collection bags were also collected at time of sampling. All samples were stored at 4°C for the remainder of the field season before being transferred to –80°C for long-term storage.

Prior to release, a blood sample was taken from the bird via brachial venipuncture and stored in absolute ethanol at 4°C. DNA was extracted from blood samples using the DNeasy Blood and Tissue kit (Qiagen, Crawley, UK); this was used for molecular sexing via a PCR-based method (Griffiths et al., 1998; Sparks et al., 2021). Age was calculated based on a combination of lay, hatch, or fledge date at point of first capture for 37% of birds in the final analysed dataset. If these exact dates were unknown, age was estimated for any new birds appearing in a territory in a given year (63% of birds). Estimates are based on a combination of behavioural observations (birds are often found begging from parents in their natal territory following a known breeding attempt) and eye colour which changes from grey in fledglings (<5 months old, still displaying begging behaviour) to light brown in sub-adults (<1 year of age), and red-brown in adults (>1 year) (Komdeur, 1992). Thus, these estimates are only used to further refine the age of new birds hatched within a year. The number of samples from birds with estimated birth dates did not differ according to sample year, sex, season or age (Figure S1).

Every breeding season, an index of quality was calculated for each territory on the island. As Seychelles warblers are insectivorous, this is based on the number of insect prey available, the territory size and the foliage cover during that breeding season (see Komdeur, 1992). For territories with missing scores in a season, quality was calculated as the mean of scores for that territory in the preceding and following sampling period of the same season type.

2.2 | Microbiome extraction and sequencing

Total genomic DNA was extracted from all faecal and control samples using the DNeasy PowerSoil kit (Qiagen) according to a modified version of the manufacturer's instructions (see Davies et al., 2022). Samples were randomised across extractions. Extracted DNA was submitted for 16S rRNA gene amplicon sequencing at the NEOF Centre for Genomic Research (Liverpool, UK). In total, 1015 samples were submitted for sequencing of which 969 were derived from faecal samples. There were also 21 collection controls (from hands and sample bags), 15 negative extraction blanks (approximately two per extraction kit) and 10 positive controls (at least one per sequencing run). Positive controls consisted of DNA extracted from a ZymoBIOMICS Microbial Community Standard (D6300). Amplicon sequencing libraries were generated using the V4 primers 515F and 806R (see Davies et al. [2022] for further information regarding the sequencing protocol). Libraries underwent 2 × 250bp, paired-end sequencing on an Illumina MiSeq platform. Samples were sequenced across seven runs. To check for batch effects, 69 samples were sequenced twice either within the same, or across different, runs. Additionally, 10 samples were extracted (and sequenced) twice within the same run to check that the extraction protocol was repeatable (previously confirmed in Worsley et al. [2021]).

2.3 | Bioinformatic processing of sequencing data

All sequencing reads were processed using QIIME2 2019.10 (Bolyen et al., 2019). Forward and reverse reads were truncated at 240bp. Low quality base calls were trimmed from the 5' end using the DADA2 plugin (Callahan et al., 2016). Amplicon sequencing variants (ASVs) were then inferred for each sample, followed by dereplication and pair-end joining. Putative chimeras and singleton reads were also removed. Following processing in DADA2, files from the seven separate sequencing runs were merged. ASVs were taxonomically classified by training a naïve-Bayes classifier on the SILVA 132 reference database for 16S rRNA gene sequences. ASVs classified as chloroplast or mitochondria were removed. A mid-point rooted phylogeny was then constructed using MAFFT (Kato, 2002) and the Fast Tree (Price et al., 2009) approach. The final ASV, taxonomy, and tree files were exported from QIIME2 into R 4.2.2 (R Core Team, 2020) using *phyloseq* 1.42.0 (McMurdie & Holmes, 2013).

Once imported, ASVs were filtered to remove non-bacterial sequences and those unassigned at phylum level (1.3% of ASVs). Eight

bacterial taxa were present in each of the positive controls; their identity matched those listed in the commercial mock community. Potential contaminants introduced during sample collection or laboratory processing were identified and removed from faecal samples using the prevalence method in *decontam* 1.18.0 (Davis et al., 2018). This method identifies putative contaminants by testing for their increased prevalence across negative controls compared to true samples. We ran the prevalence method in two steps by identifying ASVs that had greater prevalence in laboratory extraction controls followed by collection controls, respectively. A total of 6015 ASVs were conservatively filtered as possible contaminants. Following filtering, 51,360 ASVs remained across the 969 faecal samples. Faecal samples with fewer than 8000 reads (27 samples) were subsequently removed following an assessment of sample completeness and rarefaction curves generated using *iNEXT* 3.0.0 (Hsieh et al., 2016). These samples all had very low DNA concentrations. As a final filtering step, ASVs with fewer than 50 reads in total across all samples were removed prior to downstream analysis as these may represent possible sequencing errors. A total of 23,151 ASVs remained across 942 faecal samples (mean ASVs per sample = 225.62 ± 5.29 SE). Despite the loss of many ASVs only 1% of sequencing reads were removed by the abundance filtering step.

2.4 | Statistical analyses

2.4.1 | GM alpha diversity analysis

Faecal samples were rarefied to a depth of 8000 reads prior to alpha diversity analysis to control for variation in library size across samples. The observed ASV richness (number of ASVs) and Shannon diversity index (accounting for the evenness of ASV abundances) were calculated for each sample using *phyloseq* 1.42.0 (McMurdie & Holmes, 2013). To check for batch effects, pairwise Euclidean distances were calculated between samples that had been sequenced/extracted twice, based on their Shannon diversity index. Shannon diversity was consistent for samples sequenced twice within and across sequencing runs and for duplicate extractions of the same sample included in the same run (Figure S2a). As such, sample duplicates were filtered to retain the one with the highest read count for downstream analyses. Where multiple samples had been taken from the same individual during the same catch, only a single sample was retained; samples were prioritised if they had been taken from the sterile sampling tray instead of from inside the paper bag or, if both samples were taken from the same location, the one with highest read count was retained (58 samples removed, 13 samples remained that were taken from the paper bag). Samples from "floaters" individuals were removed as these individuals have no assigned territory and territory quality was controlled for in downstream analyses (12 samples removed). Finally, as we were interested in the association between the GM and senescence, only samples from adults (individuals >1 year of age which is the minimum age of first reproduction on Cousin [Hammers et al., 2012]) were retained for this analysis. A total

of 16,578 ASVs (mean number of ASVs per sample = 161.58 ± 5.14 SE) remained across 462 samples from 273 adult individuals in the final rarefied dataset.

To establish whether alpha diversity varied according to host age in adulthood, generalised additive mixed models (GAMMs) with a Gaussian (for Shannon diversity) or negative binomial (for observed ASV richness) distribution were constructed using *mgcv* 1.8.42 (Wood, 2017). This enabled evaluation of possible non-linear relationships between age and alpha diversity metrics which may be likely in the case of senescence. Age at sampling was included as a continuous smoothed term in the model. Time of sampling (minutes since sunrise at 06:00AM) and the number of days samples were stored at 4°C in the field were also included as smoothed terms in the model, since the GM can demonstrate circadian dynamics (Risely et al., 2021) and be influenced by sample storage methods (Blekhnman et al., 2016; Vargas-Pellicer et al., 2019). Storage time at 4°C ranged from 0 to 115 days in the major breeding season and 7–74 days in the minor breeding season. The median storage time was 41 days overall (46 and 35 days in major and minor seasons, respectively). Correlation coefficients between storage time and all other variables included in the model were $<.3$ suggesting collinearity was extremely unlikely to be an issue (Dormann et al., 2013). Smoothed terms were included by fitting cubic regression splines. Cubic regression splines divide the range of a predictor variable into smaller intervals (“knots”) using information on data density; cubic polynomials are then fitted to each interval and connected to form a smoothed curve (Wood, 2017). This method can be beneficial if data density is variable as it prevents periods of missing data, for example in the middle of the day, from generating unreliable, abrupt trends (Wood, 2017). Sex (male or female), season (major or minor) and two metrics of territory quality were included as linear parametric terms in the model. The two metrics of territory quality were (i) the mean territory quality (across all territories) for the sampling period and (ii) deviation of each territory score from the overall mean in that sampling period (mean-centred territory quality). This method enabled us to test whether alpha diversity varied according to differences in overall territory quality across years, and/or variation in territory quality across the island within a particular sampling period (van de Pol & Wright, 2009). To further test whether changes in alpha diversity were associated with host senescent declines, we included a term denoting whether a sample was taken in the bird's terminal year (yes or no) as an additional parametric fixed term. A total of 106 samples (out of 462) were taken in a bird's terminal year. Bird ID was included as a random effect in the model to control for repeated sampling of individuals. We tested for age-dependent effects of sex and terminal year by including interaction terms in the model, however, for all interactions described herein, these terms were removed sequentially if not significant (in order of least significance) to enable interpretation of the main effects. Variance inflation factors (VIF) were <3 for all fixed effects and terminal year samples were not restricted to the oldest individuals (Figure S3) suggesting collinearity was not an issue in the model.

2.4.2 | Compositional (beta diversity) analysis

Unrarefied reads were used, and ASVs were filtered to remove rare taxa that occurred in $<5\%$ of samples as these can disproportionately influence beta diversity metrics and may represent environmental transients (remaining ASVs represented 77% of sequencing reads). ASV abundances were then transformed using the centred log ratio (CLR) transform function in *microbiome* 1.20.0 (Lahti & Shetty, 2012) which controls for differences in library size and is appropriate for compositional datasets (Gloor et al., 2017). Batch effects were checked in the same way as for alpha diversity but using a matrix of pairwise sample Aitchison distances calculated using the CLR transformed ASV abundances in *vegan* 2.6.4 (Okansen et al., 2020). Beta diversity was consistent for samples sequenced twice within and across sequencing runs and for duplicate extractions of the same sample (Figure S2b). As such, repeat samples were filtered as described above. A total of 674 ASVs were retained across the remaining 462 samples from 273 adult individuals.

To quantify whether overall GM composition varied in association with host age, a marginal permutational analysis of variance (PERMANOVA) was performed on pairwise Aitchison distances using the *adonis2()* function within *vegan* 2.6.4 (Okansen et al., 2020), with 9999 permutations. As with alpha diversity analyses, host age, time of day, days stored at 4°C, sex, season, mean territory quality for a sampling period, mean-centred territory quality and a terminal year term were included as variables in the model. BirdID was included as a blocking factor to control for repeated sampling. We also tested age*sex and age*terminal year interactions as described above. To confirm that PERMANOVA results were robust to filtering ASVs to $>5\%$ prevalence the analysis was repeated on the full, unfiltered dataset. The results agreed with the findings of the filtered dataset (Table S1) and, as such, the more conservative analysis is presented in the main text. Differences in GM composition were visualised using principal components analysis (PCA).

2.4.3 | Changes in the abundance of core taxa with age

To understand whether abundances of individual bacterial taxa changed during adulthood, regardless of changes at the whole GM community level, we modelled the abundances of 54 core bacterial genera (defined as those found in at least 50% of adult samples and at a minimum relative abundance of 0.01%) which represented 30% of all identified genera and 63% of adult sequencing reads. A generalised linear latent variable model (GLLVM) was applied to CLR-transformed taxon abundances using *gllvm* 1.4.1 (Niku et al., 2019). The model was fitted with a Gaussian distribution and two latent variables (default). GLLVMs are a form of joint species distribution model which model the response of species to explanatory variables while accounting for correlations between the different species' abundances (Niku et al., 2019). The same predictor variables

were included as for beta diversity analysis. Bird ID was included as a random effect. As GLLVMs only model linear effects we also ran GAMM analyses with the same parameters to visualise any non-linear trends for taxon abundances in association with host age.

2.4.4 | GM personalisation and stability

Pairwise Aitchison distances between samples were scaled to similarity values ranging between zero and one using the following formula: $\text{similarity} = 1 - (\text{distance}/\text{maximum distance})$. A value of one indicates that samples are identical in terms of GM composition. We then modelled these pairwise GM similarities using dyadic Bayesian regression models in *brms* 2.19.0 following methods described in Raulo et al., (2021). Models were run on a High-Performance Computing Cluster at the University of East Anglia using a different version of R (R 3.6.2) because of memory constraints.

To assess if inter-individual differences in GM composition increase in older age groups (i.e. the GM becomes more personalised), we assigned samples taken from adults to different age classes: young adult (Y, 1–3 years), middle-aged adult (M, 3–6 years) or old adult (O, >6 years). We then modelled pairwise Aitchison similarities calculated between each individual and other members of its own age group by including a dyadic age comparison term in the model (YY, MM or OO). Lower GM similarity in the OO comparison group (pairwise comparisons between old adults) would indicate increased GM personalisation with age. To reduce the complexity of the model, we only included one sample taken at random per individual and, between individuals, samples that were taken no more than one year apart. This left 4867 pairwise comparisons (2349=YY; 1883=MM; 635=OO). The number of samples taken in the same (major-major or minor-minor) or different (major-minor) seasons were approximately equal within the same age group comparison, but the number of days between sampling points (temporal distance) was controlled for in the model. An interaction between the age group comparison and temporal distance terms was also initially included to test whether the rate of GM turnover differed amongst age groups but was removed (as not significant) to enable the interpretation of main effects. A three-level factor was included in the model indicating whether each pairwise comparison was made between two terminal year samples (TT, $N=385$ pairwise comparisons), between two non-terminal samples (NN, $N=2570$), or between a terminal year and non-terminal year sample (NT, $N=1912$). We expect GM similarity to be lowest in the NT and TT comparisons if the GM becomes more unstable in the last year of life due to senescence or pathology. Finally, a sex comparison term (0=different sex, 1=same sex) was also included as a covariate in the model. To control for the non-independence of datapoints, we fitted a multi-membership random intercept (following Raulo et al., 2021) which captures the samples included in each dyad (SampleID_A+SampleID_B). Models were run using a beta error distribution and a logit link function. To penalise extreme estimates, regularising priors were assigned as follows: $\beta \sim \text{normal}(0, 1)$ for fixed effects; $\Phi \sim \text{gamma}(1, 0.1)$ for the dispersion

parameter; student- $t(3, 0, 2.5)$ for intercept terms. Prior choice did not impact results but ensured model convergence. We ran 8000 iterations, with 2000 warmup iterations, on 4 chains. The thinning parameter was set to two. Convergence was assessed by inspection of caterpillar plots and Rhat values ≤ 1.01 .

To investigate whether GM composition becomes less stable within individuals with increasing age we ran the same analysis as above but only included pairwise comparisons of samples collected from the same individual within each age group. There were 155 pairwise comparisons in this analysis (56=YY; 58=MM group; 41=OO). The model included the dyadic age comparison term and temporal distance as fixed effects and the sample level multi-membership random effect. A terminal comparison term was not included as individuals were not sampled densely enough for there to be enough terminal comparisons (there were only 15 TT comparisons, and most were in young adults). Priors and model conditions were the same as above except that only 4000 iterations (with 2000 warmup iterations) were run with no thinning due to fewer samples.

3 | RESULTS

3.1 | GM alpha diversity does not vary with age or in the terminal year

A total of 462 GM samples were collected from 273 adult individuals over the sampling period (Figure S3); 211 of these samples were collected from females ($N=128$ individuals), 251 from males ($N=145$ individuals). Each adult individual had between one and six sequenced samples (mean 1.69 ± 0.05 SE); 129 individuals (47%) had >1 sample, and 43 individuals had >2 samples (16%) (Figure S3). In total, 345 samples were from dominant breeding individuals (159 females, 186 males), 88 from subordinate birds and 29 from individuals of unknown status. We did not include status in downstream analyses because it is significantly confounded with age (median age of subordinate versus breeding adults is 1.4 versus 4.1 years, respectively). Age at sampling ranged between 1 and 15.6 years for females (mean age = 4.2 ± 0.2 SE) and 1–17.2 years for males (mean = 4.2 ± 0.2 SE), respectively. A total of 98 samples ($N=43$ female, $N=55$ male) were collected from putatively senescent individuals >6 years of age. Furthermore, there were 106 samples (44 from females, 62 from males) taken in an individual's terminal year prior to death (Figure S3). Terminal samples were from individuals of different ages, ranging from 1 to 15.6 years in females (mean age = 5.1 ± 0.6 SE) and 1–17.2 (mean 4.3 ± 0.4 SE) in males, respectively. As such, there was substantial variation in the dataset with which to conduct a powerful analysis of the effects of chronological age and senescence on the GM.

There was no relationship between chronological age and observed ASV richness (Table 1) or Shannon diversity (Table S2) in adult Seychelles warblers. There was also no evidence that alpha diversity differed between samples taken in the terminal year of life and those taken in a non-terminal year during adulthood (Tables 1

TABLE 1 A generalised additive mixed model (GAMM) investigating the relationship between age, terminal year and observed ASV richness in the gut microbiome of adult Seychelles warblers ($N=462$ sample, 273 individuals).

| Predictor | Estimate | SE | z | p |
|--------------------------------|------------------|--------------|---------------|-------------|
| Intercept | 5.193 | 0.044 | 118.255 | <.001 |
| Sex (male) | -0.107 | 0.046 | -2.313 | .021 |
| Season (minor) | 0.040 | 0.070 | 0.570 | .569 |
| Mean territory quality | -0.122 | 0.064 | -1.904 | .057 |
| Mean-centred territory quality | -0.079 | 0.064 | -1.226 | .220 |
| Terminal year (no) | 0.031 | 0.054 | 0.574 | .566 |
| Smoothed terms | edf | χ^2 | p | |
| Age | 1.000 | 1.566 | .211 | |
| Time of day | 1.659 | 2.140 | .355 | |
| Time at 4°C | 1.002 | 2.380 | .123 | |
| Random | 462 Observations | | | Variance |
| Bird ID | 273 Individuals | | | 0.120 |

Note: Significant ($p < .05$) predictors are shown in bold. Reference categories for categorical variables were as follows: Female (sex), major (season) and yes (terminal year). Adjusted $R^2 = .04$.

and S2). The interaction term between chronological age and terminal year was not significant in either model ($p > .05$) and thus was removed to enable interpretation of the main effects. This suggests that GM alpha diversity did not vary according to age, or senescence, in adult Seychelles warblers.

Observed ASV richness was significantly lower in males than females (Table 1). A similar trend was identified between sex and Shannon diversity, but this was not statistically significant (Table S2). Since only Shannon diversity is weighted by ASV abundances, this suggests that the lower ASV richness observed in males compared to females was largely driven by the loss of rare taxa. There was a negative association between Shannon diversity and time stored at 4°C in the field (Table S2). None of the other predictors were associated with changes in GM alpha diversity (Tables 1 and S2).

3.2 | GM composition does not vary strongly with age or in the terminal year

We next explored whether overall GM composition differed with age, and between terminal year and non-terminal year samples collected during adulthood. A PERMANOVA analysis of CLR-transformed ASV abundances showed that there was a very weak, marginally significant association between age and GM composition (Table 2), however, age only explained an extremely low proportion of the overall variance in GM composition ($R^2 = .002$, Table 2). Consistent with this, sample points showed very little clustering according to age on a PCA ordination plot (Figure 1a). Furthermore, samples collected from adults in their terminal year versus those from a non-terminal year did not differ in terms of their GM composition (Table 2) indicating no association between senescence and changes in GM structure. An interaction term between adult age and

terminal year was also not significant ($p > .05$) and so was removed to interpret the main effects.

Host sex was not significantly associated with differences in GM composition (Table 2) despite an association between sex and GM richness (Table 1). Instead, environmental variables were the strongest predictors of GM composition in adults; GM composition varied significantly according to differences in territory quality between field periods (Table 2 and Figure 1b), season (Table 2 and Figure 1c), and the time of day at which an individual was sampled (Table 2 and Figure 1d). Clustering along the PC1 and PC2 axes of a PCA ordination plot was primarily associated with seasonal differences (Figure 1c) and variation in territory quality (Figure 1b), respectively, while clustering along the PC3 axis was associated with the time of day at which samples were collected (Figure 1d). The number of days each sample was stored at 4°C in the field was also a significant predictor of GM composition (Table 2); points clustered along the PC1 and PC2 axis of a PCA ordination according to whether samples were stored for less, or more than, 30 days at 4°C (Figure S4). Despite significant associations between environmental factors and the GM, all predictors explained a very low percentage of overall GM variance (maximum $R^2 = .009$, Table 2) suggesting drivers of GM variation remain poorly characterised in this system.

3.3 | Changes in taxon abundance

To identify whether the abundance of specific, prevalent microbial genera (rather than overall GM composition) changed with age in adult Seychelles warblers, we applied a GLLVM model to 54 core genera (those present in at least 50% of adults). The mean relative abundance of core genera ranged from 0.2% to 16.5% across samples (mean $1.85\% \pm 0.3\%$ SE). Only six core genera showed a significant

TABLE 2 A PERMANOVA analysis of associations between age, terminal year, and gut microbiome composition in adult Seychelles warblers.

| Predictor | Df | R ² | F | p |
|--------------------------------|----------|----------------|--------------|-----------------|
| Age | 1 | .002 | 1.134 | .046 |
| Sex | 1 | .002 | 1.106 | .619 |
| Mean-centred territory quality | 1 | .003 | 1.409 | .574 |
| Mean territory quality | 1 | .006 | 2.722 | <.001 |
| Season | 1 | .009 | 4.069 | <.001 |
| Time of day | 1 | .008 | 3.685 | <.001 |
| Time at 4°C | 1 | .007 | 3.158 | .005 |
| Terminal year | 1 | .002 | 0.906 | .988 |

Note: The analysis was performed using Aitchison distances calculated using centred log ratio (CLR)-transformed amplicon sequencing variant (ASV) abundances. Significant predictors ($p < .05$) are shown in bold. $N = 462$ samples from 273 individuals. Bird ID was included as blocking factor to control for repeated measures.

association with host age; the genera *Kineococcus*, *Pseudonocardia*, *Quadrisphaera* and one genus in the family *Micromonosporaceae* showed an increase in abundance with age, whereas the genus *Gordonia* and one genus in the family *Ruminococcaceae* decreased with age in adult Seychelles warblers (Figure S5). These findings were consistent with the output of GAMM models which identified the same linear trends for each of these taxa, apart from *Ruminococcaceae*, which showed a significant, weakly non-linear, decrease in abundance with age (Figure 2 and Table S3). Significant taxa were present at relatively low abundances within samples: *Kineococcus* ($0.24\% \pm 1.05\%$, mean relative abundance across samples \pm SD); *Pseudonocardia* ($0.89\% \pm 1.94\%$); *Quadrisphaera* ($0.30\% \pm 0.62\%$), *Gordonia* ($0.54\% \pm 1.05\%$); *Micromonosporaceae* genus ($0.30\% \pm 1.02\%$); and the *Ruminococcaceae* genus ($1.07\% \pm 1.89\%$).

Only two genera were differentially abundant in the terminal year of life in adults in the GLLVM model (Figure S5); *Friedmanniella* and *Microbacterium* were both present at greater abundance in terminal year samples. Many more genera were associated with environmental variables (Figure S6). For example, 20 core genera demonstrated a significant change in abundance according to season, while 11 core genera changed in association with the time of day and mean territory quality terms, respectively (Figure S6).

3.4 | GM personalisation and stability

The GM may not converge on a typical "old" composition if it becomes increasingly dysregulated with age. Instead, inter-individual variation may increase with age as the GM follows different, more unstable, trajectories. We found no evidence that GM samples taken from different individuals were compositionally less similar when pairwise comparisons were made between two old adults, versus two middle aged or two young adult individuals

(Figure 3a and Table S4). This indicates that the Seychelles warbler GM does not become more personalised in older individuals. Furthermore, there was no difference in GM similarity between comparisons involving samples taken from birds in their terminal year, versus those taken in a non-terminal year (Figure 3a and Table S4). Thus, GM personalisation did not increase the year before death. Only the time interval (in days) between samples was significantly negatively associated with GM similarity (Figure 3a and Table S4).

Relationships observed between individuals may not reflect patterns of change within individuals (van de Pol & Wright, 2009) and can be confounded, for example by the selective disappearance of individuals with extreme GM communities. However, an analysis of within individual GM similarities revealed very similar patterns to the between individual analysis. GM similarity in middle-aged sample comparisons (MM posterior mean -0.025 , CI $= -0.228$ to 0.173) and young adult sample pairs (YY posterior mean -0.080 , CI $= -0.286$ to 0.124), did not differ statistically from that of sample pairs taken when an individual was in the old adult age group (Figure 3b and Table S5). This indicates that, within individuals, the warbler GM does not become more unstable with increasing host age. As with between individual comparisons, the time interval between samples was negatively associated with GM similarity within the same individual (Figure 3b and Table S5).

4 | DISCUSSION

We used longitudinal data collected from Seychelles warblers to investigate the association between host age, senescence and GM characteristics. We found no evidence of senescent declines in the GM; both bacterial alpha diversity and composition remained largely invariable with respect to age in adults and did not differ in an individual's terminal year. Instead, environmental factors, including season and variation in mean territory quality across the study period, appeared to have the greatest impact on the GM during adulthood. Within individuals, we also found no evidence of increased GM personalisation or instability in older age groups, even in the terminal year. This is despite including some relatively very old individuals in the dataset (the oldest individual was c.a. 17 years of age, Figure S3).

Seychelles warblers have a median lifespan at fledging of 5.5 years (Hammers et al., 2019; Sparks et al., 2021) and, at the population level, there is evidence of reproductive and survival senescence from approximately six years of age (Hammers et al., 2012, 2013). In our analysis, we included samples from 66 individuals older than six years of age. Of these, 37 individuals had at least two samples taken longitudinally, including 19 individuals with samples taken before and after 6 years of age. There was a maximum of 4.8 years between longitudinal samples for putatively senescent individuals (mean 1.4 years ± 0.2 SE, Figure S3). These sample sizes are either comparable to, or greater than, the few studies that have identified statistically significant effects of age

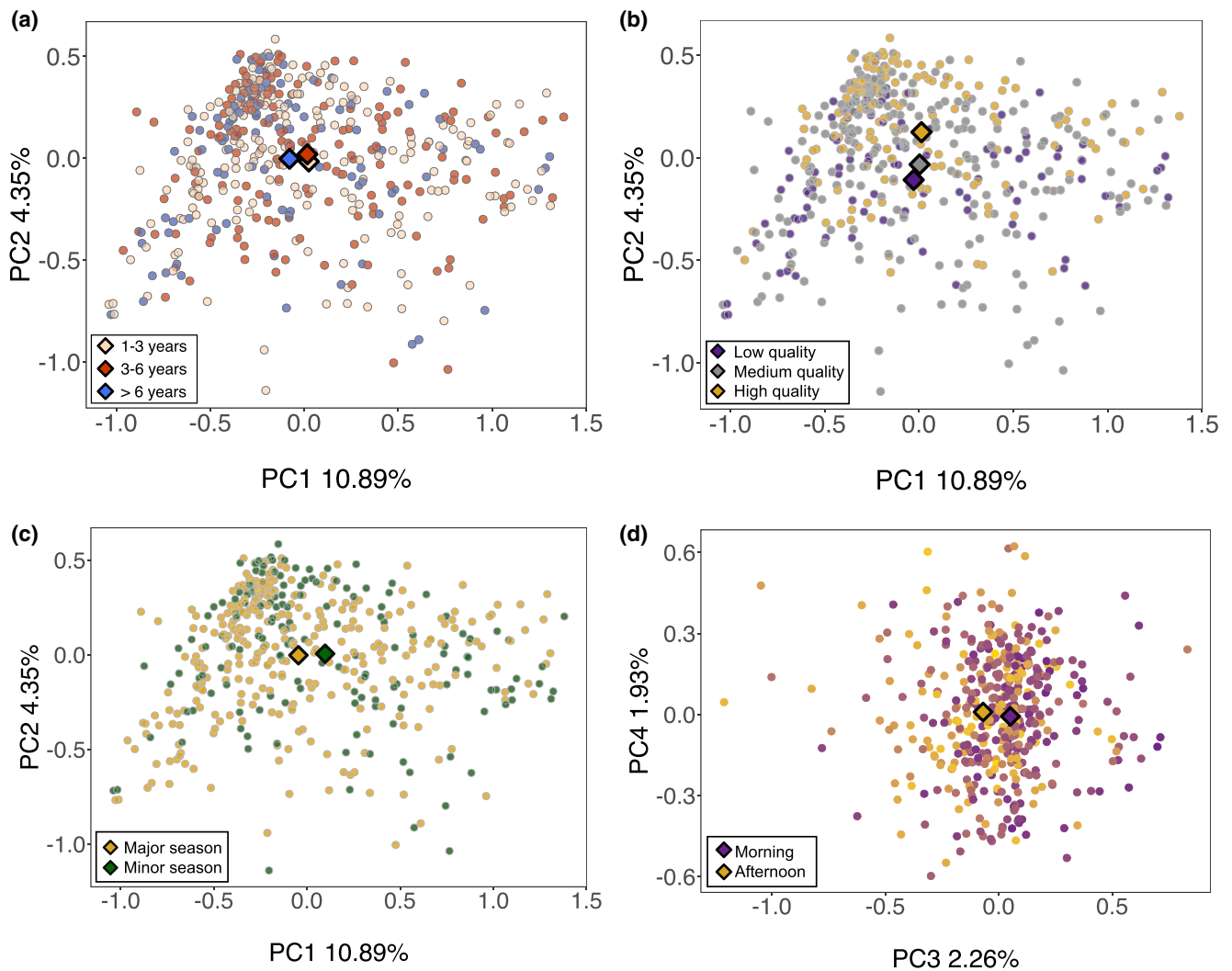


FIGURE 1 Shifts in gut microbiome composition according to (a) host age (b) mean territory quality (c) season and (d) time of day in adult Seychelles warblers. PCA ordination was carried out using Aitchison distances calculated on Centred Log Ratio (CLR)-transformed amplicon sequencing variant (ASV) abundances. Each point represents a unique gut microbiome sample ($N=462$ samples from 273 individuals). Large diamonds represent the group centroids. For clarity, samples were grouped into discrete categories for plotting: (a) Age: 1–3 years, 3–6 years, or >6 years; (b) Territory quality: Low (lower quartile $<17,136$), medium (interquartile range), or high (upper quartile $>36,602$); (c) Season: Major or minor; (d) Time of day: Samples collected in the morning (<6 h after sunrise at 06:00AM) or afternoon (>6 h after sunrise). Principal components 1, 2, 3 and 4 explained 10.9%, 4.4%, 2.3% and 1.9% of the variation in gut microbiome structure, respectively.

on GM structure in other wild systems (e.g. Bennett et al., 2016), $N=14$ post-prime individuals sampled cross-sectionally; (Trosvik et al., 2018), $N=70$ post-prime individuals sampled cross-sectionally; (Sadoughi et al., 2022), $N=11$ senescent individuals sampled longitudinally over 1.5 years). Furthermore, our study also included 97 individuals (out of a total of 273 adults) that had a sample taken in their terminal year of life allowing us to additionally test for changes in the GM close to death when other traits are showing effects of senescence; for example, Seychelles warblers demonstrate an age-dependent reduction in reproductive success in the terminal year of life, indicating a decline in condition prior to death (Hammers et al., 2012). Thus, this was a robust dataset with which to investigate the relationships between host age, senescence and the GM. As such, the absence of statistical significance

is unlikely to be due to a lack of power to detect effects that are large enough to be biologically meaningful to the host.

A wealth of studies on humans have identified a decline in bacterial diversity and shifts in GM composition in older age groups (e.g. Claesson et al., 2011; Jeffery et al., 2016; Xu et al., 2019). Only a few studies have been undertaken in the wild, however, cross-sectional studies on lemurs (*Lemur catta*) (Bennett et al., 2016) and geladas (*Theropithecus gelada*) (Trosvik et al., 2018, but see Baniel et al. [2021]) have also demonstrated shifts in GM composition, although not alpha diversity, between reproductively mature and post-prime adult individuals. We found no statistical evidence of an association between age and GM alpha diversity, and only identified extremely limited shifts in GM composition in adult Seychelles warblers. This was the case even after controlling for

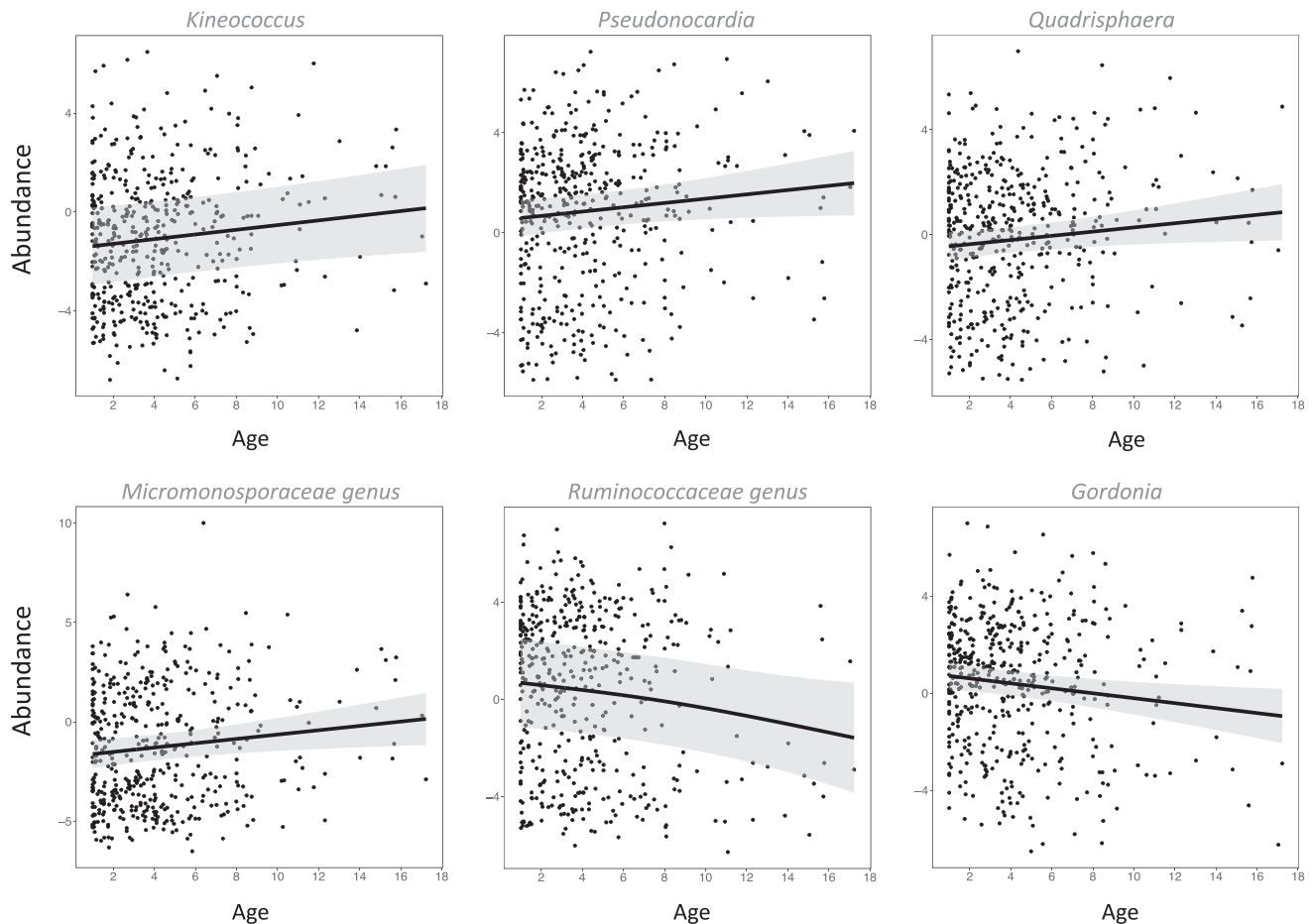


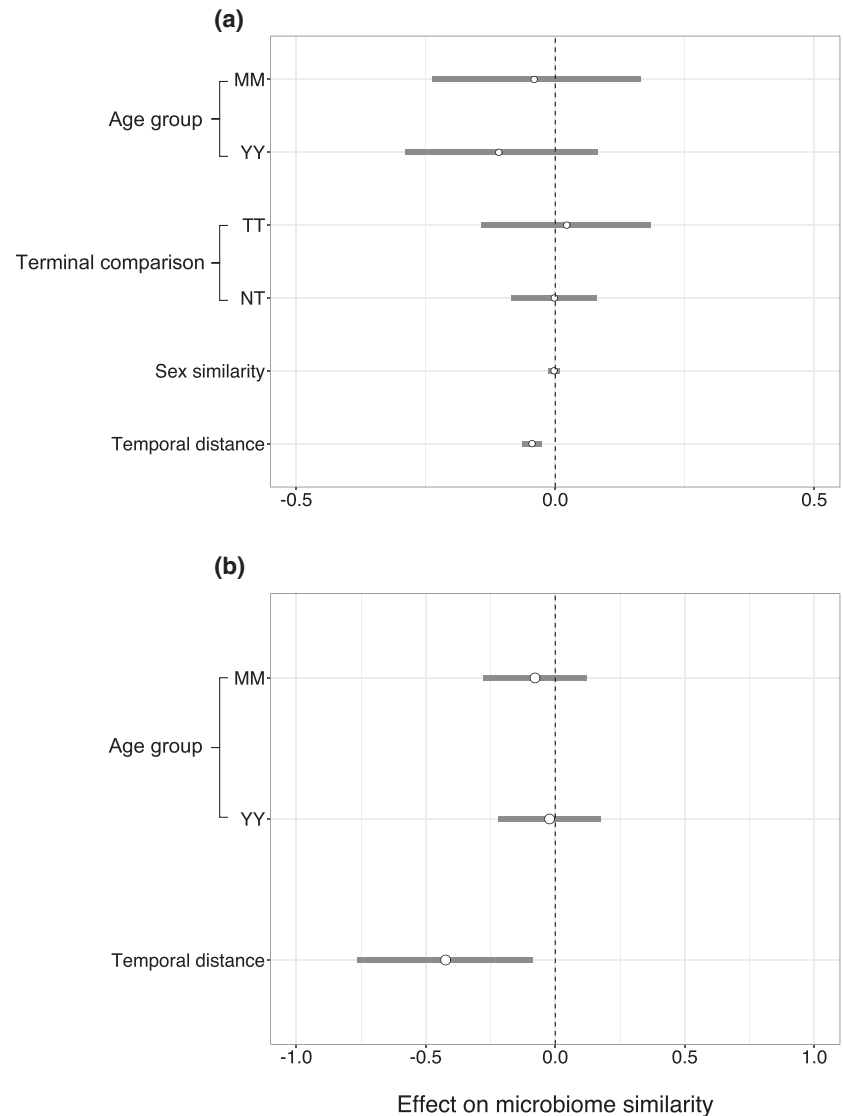
FIGURE 2 Changes in the abundance of six core bacterial genera with host age. Abundances are Centred Log Ratio (CLR) transformed to control for the compositionality of the dataset. Fitted lines are model predictions with 95% confidence intervals calculated from GAMM models ($p < .05$ in GAMMs and a GLLVM model). $N = 462$ samples from 273 adult individuals. The results of GAMMs are presented in full in [Table S3](#).

the possibility of differential rates of damage accumulation by investigating GM differences in the terminal year of life (McNamara et al., 2009). These results are consistent with the findings of several other studies on wild non-human primate populations in which GM diversity and composition remained largely invariable with respect to chronological age during adulthood (Janiak et al., 2021; Reese et al., 2021). However, these analyses were either fully cross-sectional with small sample sizes for old individuals ($N = 12$ samples from old individuals in McNamara et al. [2009]) or did not control for the time interval between sampling points when comparing the GM similarity of longitudinal samples (Reese et al., 2021). A recent study on wild meerkats also demonstrated that GM diurnal cycling remained consistent with chronological age, suggesting that functionality is largely maintained even in old individuals (Risely et al., 2021). However, although repeat samples were taken from individuals, this study did not investigate within individual dynamics (such as differences in GM stability) per se. Furthermore, none of the aforementioned studies controlled for differential damage accumulation by including a measure of biological condition such as time to death. Thus, we provide a more

robust test of the association between age, senescence and the GM and, by doing so, corroborate the results of these previous studies.

The discrepancy between studies on wild animals and those on humans might be explained, in part, by lifestyle and behavioural factors that change with age in human populations but that don't exist in wild systems. For example, in humans, medication intake and the probability of living in residential care increase with age, while physical activity and dietary quality decrease, all of which can directly impact the GM (Claesson et al., 2012; Ticinesi et al., 2017). It is possible that certain factors that impact the GM also vary with age in some wild mammalian species. For example, dental wear and tooth loss increase with age in some non-human primates (Cuozzo et al., 2010; King et al., 2005) which can, in turn, influence an individual's food choices and their ability to extract nutrients from dietary components (Venkataraman et al., 2014). However, Seychelles warblers ingest their food whole, feed almost exclusively on insects throughout their lives and show no decline in foraging efficiency with age (Komdeur, 1996). Thus, such effects are unlikely to be universal across wild species. This could potentially explain some of the

FIGURE 3 Compositional similarity of adult Seychelles warbler gut microbiome samples taken from (a) different individuals or (b) from the same individual. Effect sizes (points) are plotted with their 95% credible intervals and were calculated using Bayesian dyadic regression models. Pairwise GM Aitchison similarities among samples were used as the response in these models. Comparisons were made between samples taken in the same age group: YY, young adult comparisons (individuals 1–3 yrs); MM, middle aged comparisons (3–6 years). Old adult comparisons (OO, >6 years) were used as the reference category in the model. In (a), the terminal comparison term indicates whether a pairwise comparison was made between a non-terminal sample and a sample taken in a bird's terminal year (NT), or between two terminal year samples (TT). NN (two non-terminal year samples) was used as a reference. Sex similarity (same or different) was also included in (a). Temporal distance indicates the time interval (in days) between samples in each pairwise comparison. Significant predictors are those where the credible intervals do not overlap zero.



observed variation in GM-ageing patterns identified amongst different wild taxa.

Our findings are also in contrast to studies on captive animals demonstrating shifts in overall GM composition with increasing age (e.g. Clark et al., 2015; Langille et al., 2014; Smith et al., 2017). However, captive animals are often housed in highly controlled conditions and frequently harbour unrealistically low levels of GM diversity (Clayton et al., 2016; San Juan et al., 2021). In wild systems, environmental variation can strongly impact the GM (Baniel et al., 2021; Grieneisen et al., 2021; Risely et al., 2021) and may override host intrinsic effects observed in the laboratory. Indeed, time of day, seasonal differences, and changes in mean territory quality were strong determinants of GM composition in Seychelles warblers, consistent with previous studies on this system (Davies et al., 2022; Worsley et al., 2021, 2022). GM similarity also declined with an increasing number of days between samples suggesting a high level of turn-over within the GM. Corresponding with this, there were high levels of heterogeneity amongst GM samples; many ASVs were only found in a small proportion of

samples and only 30% of genera were shared across all individuals. Such heterogeneity is not unusual for a wild system (e.g. Baniel et al., 2021; Risely et al., 2022; Somers et al., 2023), particularly in passerines whose short intestinal tracts (an adaptation to flight) may make them more susceptible to transient environmental microbes (Bodawatta et al., 2021; Song et al., 2020). However, high levels of heterogeneity may eliminate, or make it difficult to detect, consistent longitudinal changes in GM diversity and stability. Thus, although Seychelles warblers demonstrate both reproductive and survival senescence (Hammers et al., 2012, 2013), constant environmental uptake of microbes may override any large-scale effects of senescence on the GM.

In addition to the lack of change in overall GM composition, only six (out of 54) individual core genera were associated with age in adult Seychelles warblers. One genus that decreased in abundance with increasing host age was in the family *Ruminococcaceae*. This is one of the most abundant families in the human GM (Tap et al., 2009). Members of the *Ruminococcaceae* are obligate anaerobes, interact directly with the mucosal layer of the gut epithelium and produce

important short chain fatty acids such as butyrate (Nava et al., 2011). The abundance of this family has also been shown to decrease with age in several long-term human studies (Biagi et al., 2016; Ghosh et al., 2022; Wang et al., 2015) and reduced abundances have been associated with increased gut inflammation and chronic intestinal disorders such as Crohn's disease (Sokol et al., 2008; Willing et al., 2009). Thus, it is possible that certain members of the GM are linked to senescent declines in the Seychelles warbler. However, further tests of the functionality of this genus, for example through metagenomic sequencing, would be needed to understand its role within the Seychelles warbler GM and whether a decline in its abundance in older age groups is biologically significant to the host. Furthermore, it is currently unclear if changes in the abundance of this taxon (and others) are driven by within-individual senescence effects or by the selective mortality of hosts colonised by high levels of this genus. Inclusion of an "age at death" (longevity) term in models could help to distinguish between these different drivers (van de Pol & Verhulst, 2006) but was unfeasible with the current dataset as >50% of individuals were still alive at the end of the study.

Aside from *Ruminococcaceae*, the core genus *Gordonia* also decreased in abundance with increasing host age, while four other genera (*Kineococcus*, *Pseudonocardia*, *Quadrisphaera* and a genus in the family *Micromonosporaceae*) increased with age. These genera are all in the aerobic phylum *Actinobacteria*, which is widely distributed in the environment (Arenskötter et al., 2004; Maldonado et al., 2005; Maszenan et al., 2005; Riahi et al., 2022; Yokota et al., 1993). It is possible that environmental bacteria may increase in abundance within the GM as host immune function declines with age and/or as other key bacterial genera, such as the *Ruminococcaceae*, are lost. However, due to their widespread distribution, it is also plausible that these bacteria are constantly acquired from the host's environment (e.g. via their diet) and may gradually accumulate and persist within the GM by outcompeting other microbes. Each actinobacterial genus that was associated with age was present at <1% mean relative abundance in adults suggesting that they may only play a very minor role within the gut ecosystem, although further functional analyses would be needed to confirm if this is the case. Furthermore, despite these few patterns, the vast majority of core genera showed no association with age suggesting senescence was not associated with a large-scale restructuring of the GM. This is consistent with results of the PERMANOVA analysis of overall GM composition.

Many more taxa were associated with variation in environmental factors, including time of sampling, season, and mean territory quality across sampling periods. This is consistent with other studies showing that environmental dynamics can play a significant role in structuring the GM of wild animals (Baniel et al., 2021; Grieneisen et al., 2021; Risely et al., 2021). For example, a study on wild meerkats showed that diurnal shifts in GM composition outweighed an association between the GM and host chronological age (Risely et al., 2021). Circadian GM dynamics have been identified in human and wild animal studies, and may be driven by differences in foraging regimes and dietary intake throughout the day (Risely et al., 2021; Schmid et al., 2023). In the Seychelles warbler, the genera *Lactococcus*

and *Enterococcus* showed the largest increases in abundance between samples collected in the morning and afternoon. Both genera are lactic acid producing bacteria and play an important role in dietary carbohydrate fermentation (George et al., 2018); as such, they may gradually increase in response to an influx of nutrients as individuals start to forage (Schmid et al., 2023). However, both genera are also found in insect microbiomes (Choi et al., 2023; Cox & Gilmore, 2007; Tang et al., 2012) and so it is possible that these taxa accumulate passively as feeding increases throughout the day.

Aside from time of day, differences in mean territory quality between sampling periods and season were also associated with GM differences. As territory quality (a measure of insect abundance) is likely to be linked to climatic factors, such as rainfall and temperature, which may also influence microbial abundances, it is possible that these GM differences were driven by differential exposure to microbes in the external environment. Seasonal GM differences could be driven by similar processes but may also be linked to host stress under low quality conditions (Stothart et al., 2019). Furthermore, since Seychelles warbler predominantly reproduce in the major breeding season (June–September) some of the seasonal differences in adult GM composition could be linked to physiological changes associated with host reproduction (Comizzoli et al., 2021; Hernandez et al., 2021). Further work, including dense sampling of the same individual pre- and post-reproductive attempts would be needed to understand if this is the case.

We found no evidence that changes in the GM were more extreme in an individual's terminal year of life when molecular and cellular damage accumulation is expected to be at its greatest due to senescence, and only two genera were more abundant in terminal year samples (*Friedmanniella* and *Microbacterium*). These are both environmental microbes that are frequently isolated from insects (Iwai et al., 2010; Kageyama et al., 2007) and neither of them were associated with host age. The Seychelles warbler benefits from very low levels of extrinsic mortality during adulthood (Brouwer et al., 2006). Indeed, an absence of natural predators, lack of human disturbance, and a relatively constant and benign climate, enables many individuals to reach an old age, providing the opportunity to detect and study senescence in this species (Hammers et al., 2015). Thus, although it is possible that a very small proportion of mortality in our study was stochastic, it is very unlikely that this would be at a level high enough to override any significant association between senescence and the GM. Therefore, the fact that we found little change in GM alpha diversity, composition or stability in the terminal year when other traits (e.g. reproductive success) decline (Hammers et al., 2012), and that this relationship did not depend on age, suggests that the GM is largely unaffected by host senescence in the Seychelles warbler. Our previous work has shown that differential survival is associated with differences in both bacterial and fungal GM composition in the Seychelles warbler, however, in both cases, survival was assessed over much shorter periods post sampling (in most cases less than 3 months post sampling, but in some cases less than 5 months) (Worsley et al., 2021, 2022). In this study we chose to look at changes over a longer period (the terminal year)

to try to identify factors linked to senescence rather than the more immediate changes in the GM just prior to death which could be a consequence of a rapid decline in health. We expected senescent changes in the GM to accumulate gradually over longer periods, but we found no evidence that this was the case.

Aside from overall GM alpha diversity and composition, we found no evidence of increased GM personalisation, or reduced within-individual GM stability, with increasing age or in the year leading up to death in adult Seychelles warblers. This also contrasts with long-term studies on humans (Claesson et al., 2011; Ghosh et al., 2020; Wilmanski et al., 2021), and a recent longitudinal study on wild macaques (*Macaca assamensis*) (Sadoughi et al., 2022), which identified increasing GM heterogeneity amongst individuals in older age groups. These studies were unable to determine the cause of these differences; whilst greater heterogeneity could be driven by reduced GM stability, it may also be driven by other factors such as reduced social contact amongst elderly individuals (Ghosh et al., 2022; Sadoughi et al., 2022). Social interaction strength predicts GM similarity in wild wood mice, *Apodemus sylvaticus* (Raulo et al., 2021), and reduced social network size has also been associated with lower GM diversity (Johnson, 2020; Raulo et al., 2021). However, social isolation may not be a hallmark of ageing in all species. Seychelles warblers are cooperative breeders that often live in groups consisting of a breeding pair and subordinate individuals, some of which may help with reproductive attempts (Kingma et al., 2016; Richardson et al., 2002, 2003). The presence of subordinates does not decline as dominant breeding individuals age and, indeed, the recruitment of helpers increases in elderly females (Hammers et al., 2019, 2021). Thus, there is currently no evidence to suggest that warblers become less social with age. Consequently, there may be ample opportunity for microbes to be shared amongst individuals in this population regardless of age.

One potential limitation of our study is that we cannot entirely rule out an effect of selective mortality. If individuals that have extreme GM compositions are rapidly lost from the population or are too sickly to be sampled using mist nets, then it is possible that changes occurring very close to death in old age would go undetected. However, terminal year samples were available for 35% of birds in our dataset suggesting we were still able to successfully sample birds when other traits, such as reproductive success, demonstrate age-dependent senescent declines (Hammers et al., 2012). Extremely dense repeat sampling would be needed to test whether the GM shifts much closer to death, particularly in older adults, which was unfeasible in our study. However, the lack of a terminal year effect for the GM, particularly in older birds, suggests that the GM does not follow the same senescent trajectory as other host traits in this species.

In conclusion, our study finds little evidence of senescent changes in the GM of the Seychelles warbler. Although the abundance of a very small number of individual genera were associated age, overall GM alpha diversity and composition remained stable throughout adulthood in this species. Instead, environmental factors were the major driver of GM differences amongst adult

warblers. While this contrasts with studies on humans and captive animals, our findings add to the growing body of literature reporting mixed effects of age in wild populations. Further work is needed to better understand whether variation in lifestyle and behavioural factors drive the observed variation in senescent changes in the GM of different taxa.

AUTHOR CONTRIBUTIONS

The study was conceived by SFW and DSR. SFW, CSD, CZL and DSR performed the fieldwork. SFW, CSD and MEM conducted the microbiome laboratory work. SFW performed the bioinformatics and statistical analyses and drafted the manuscript with input from DSR. DSR, HLD, JK and TB managed the Seychelles Warbler Project. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

All 16S rRNA gene amplicon sequences have been submitted to the European Nucleotide Archive (ENA) database under the study accession numbers PRJEB45408 (samples taken in 2017 and 2018) and PRJEB47095 (samples taken in 2019 and 2020) and PRJEB67634 (samples taken in 2021 and 2022). The scripts and metadata to reproduce all analyses and figures can be accessed via the GitHub repository, <https://github.com/Seychelle-Warbler-Project> and have been archived in the Dryad repository, <https://doi.org/10.5061/dryad.44j0zpcp7> (Worsley et al., 2024).

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).

ORCID

Sarah F. Worsley  <https://orcid.org/0000-0003-4736-0938>

Terry Burke  <https://orcid.org/0000-0003-3848-1244>

Hannah L. Dugdale  <https://orcid.org/0000-0001-8769-0099>

David S. Richardson  <https://orcid.org/0000-0001-7226-9074>

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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