Gut microbiome pattern reflects healthy ageing and predicts survival in humans.

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The gut microbiome has important effects on human health, yet its importance in human ageing remains unclear. In the present study, we demonstrate that, starting in mid-to-late adulthood, gut microbiomes become increasingly unique to individuals with age. We leverage three independent cohorts comprising over 9,000 individuals and find that compositional uniqueness is strongly associated with microbially produced amino acid derivatives circulating in the bloodstream. In older age (over ~80 years), healthy individuals show continued microbial drift towards a unique compositional state, whereas this drift is absent in less healthy individuals. The identified microbiome pattern of healthy ageing is characterized by a depletion of core taxa in those aged >104 years compared with younger individuals. The loss of core taxa, the exact identities of which may vary across different human populations (Bacteroides versus Prevotella spp.), and the increase in α-diversity reported in long-lived individuals, suggest that gut microbiomes may become increasingly divergent, or unique, to each individual as they age. This phenomenon of community compositional divergence seen in centenarians may be key to understanding how the gut microbiome contributes to the changing physiological landscape accompanying human ageing.

Gut microbial associations reported in centenarians are often inconsistent with studies of elderly populations. In particular, studies on the ELDERMET cohort (that is, the most extensively studied cohort of older people with gut microbiome data to date) have reported an increased dominance of the core genera Bacteroides, Alistipes and Parabacteroides in those aged 65+ years compared with healthy, younger controls. Measures of α-diversity have also been shown to negatively correlate with frailty, indicating that changes in the gut microbiome correspond to age-associated decline. Studies on older long-term-care residents further characterized a gradual shift in gut microbiome composition associated with the duration of stay in the care facility, whereas a more recent study demonstrated that older individuals (aged 60+ years) exhibited higher variability in gut microbiome composition relative to younger controls. Some studies have also reported higher levels of gut α-diversity in centenarians compared with younger individuals, indicating that gut microbiomes continue to develop within their hosts, even in the later decades of human life. The loss of core taxa, the exact identities of which may vary across different human populations (Bacteroides versus Prevotella spp.), and the increase in α-diversity reported in long-lived individuals, suggest that gut microbiomes may become increasingly divergent, or unique, to each individual as they age. This phenomenon of community compositional divergence seen in centenarians may be key to understanding how the gut microbiome contributes to the changing physiological landscape accompanying human ageing.

The ecological dynamics of the human gut microbiome have been characterized by rapid change in early life (0–3 years), followed by a long period of relative stability, ending with gradual changes associated with advanced age. Particularly in older populations (65+ years), studies over the past few years have revealed a number of associations between gut microbiome composition and measures of physical fitness, frailty and diet, highlighting the importance of proper gut microbiome function into the later decades of human life. Despite substantial progress in our understanding of the human gut microbiome, still very little is known about when age-associated changes in the gut microbiome begin, how these changes influence host physiology, and whether ageing patterns within the gut microbiome simply reflect, or contribute to, long-term health and survival outcomes. Importantly, identifying ageing patterns within the gut microbiome could have major clinical implications for both monitoring and modifying gut microbiome health throughout the human lifespan.

Several studies conducted on centenarian populations provided potential insight into gut microbial trajectories associated with ageing. Biagi et al. demonstrated that gut microbiomes of centenarians (aged ≤104 years) and supercentenarians (104+ years) show a depletion in core abundant taxa (Bacteroides, Roseburia and Faecalibacterium spp., among others), complemented by an increase in the prevalence of rare taxa. Similar findings have since been reported in other centenarian populations across the world, such as in Sardinian, Chinese and Korean centenarians, relative to healthy, younger controls. Some studies have also reported higher levels of gut α-diversity in centenarians compared with younger individuals, indicating that gut microbiomes continue to develop within their hosts, even in the later decades of human life. The loss of core taxa, the exact identities of which may vary across different human populations (Bacteroides versus Prevotella spp.), and the increase in α-diversity reported in long-lived individuals, suggest that gut microbiomes may become increasingly divergent, or unique, to each individual as they age. This phenomenon of community compositional divergence seen in centenarians may be key to understanding how the gut microbiome contributes to the changing physiological landscape accompanying human ageing.

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individuals (aged 20–60 years), which has been attributed to a putative increase in abundance of pathobionts at the expense of beneficial gut bacteria [12]. Collectively, these and other studies provide a view of the human gut microbiome across the adult lifespan as relatively stable up until old age, at which point gradual compositional shifts occur that reflect, and potentially contribute to, declining health.

The heterogeneity of findings in ageing studies indicates that there may be multiple gut microbiome patterns of ageing, some of which reflect better health and life expectancy outcomes than others. Although recent analyses have demonstrated a link between gut microbiome composition and long-term health outcomes [13,14], the scarcity of cohorts with longitudinal follow-up data, the lack of detailed molecular phenotyping and health metrics, and the relatively small sample sizes of existing studies on ageing limit our understanding of gut microbial changes seen across the human lifespan. In the present study, we overcome these limitations and present an analysis of the gut microbiome and phenotypic data from over 9,000 individuals from 3 independent cohorts spanning 18–101 years of age, with longitudinal follow-up data in an older cohort of predominantly community-dwelling individuals, which allowed us to track survival outcomes.

Results
Study design and cohort descriptions. We primarily studied two distinct cohorts: a deeply phenotyped population of individuals who were enrolled in a scientific wellness company (the ‘Arivale cohort’, ages 18–87 years; Extended Data Fig. 1) and the Osteoporotic Fractures in Men (MrOS) cohort (ages 78–98 years) [17–19] (Extended Data Fig. 2 and Fig. 1). These cohorts further subdivide into two groups each. The Arivale cohort separates into group A (n = 2,539) and group B (n = 1,114), where the distinguishing factor was the use of different vendors for the collection and processing of stool samples (Methods). The MrOS cohort separates into a discovery cohort (n = 599) and a validation cohort (n = 308), because, although stool samples from this population were all collected at the same time point, they were processed in two separate batches approximately 3 years apart. We further confirmed the identified ageing pattern in an additional external dataset from the American Gut Project (AGP) (n = 4,575) [20]. We began by analysing baseline data from the Arivale cohort to identify gut microbiome ageing patterns across most of the adult human lifespan, and investigate how these patterns correspond to host physiology. We then extended our analysis into the MrOS cohort, where we had detailed health metrics and follow-up data on mortality, to evaluate how the patterns identified within the Arivale cohort correspond to health and survival in the later decades of human life.

A gut microbiome ageing pattern spans much of the adult lifespan. To characterize gut microbial patterns associated with ageing, we initially performed a β-diversity analysis comparing all available baseline microbiome samples from a heterogeneous, and relatively healthy, Arivale population (Fig. 1 and Extended Data Fig. 1). To capture the compositional divergence indicative of healthy ageing observed in centenarians, our analysis involved extracting the minimum value for each individual from a calculated Bray–Curtis dissimilarity matrix. This value reflects how dissimilar an individual is from their nearest neighbour, given all other gut microbiome samples in the cohort. We refer to this as a measure of ‘uniqueness’: the higher the value, the more distinct the gut microbiome from everyone else’s in the studied population. Arivale participants showed initial drift towards an increasingly unique gut microbiome composition, starting between 40 and 50 years of age at the genus level, and 50 and 60 years at the amplicon sequence variant (ASV) level, and this continued to increase with every passing decade (Fig. 2a). The correlation between uniqueness and age was consistent across two different microbiome vendors used for gut microbiome processing, at both the ASV and the genus level, independent of sex, body mass index (BMI) and Shannon diversity (Fig. 2b and Extended Data Fig. 3). We replicated our analysis using additional β-diversity metrics. Uniqueness, based on weighted UniFrac, demonstrated a similar positive association with age across both vendors at both the ASV and the genus levels, whereas the unweighted Jaccard metric resulted in comparable associations with age at the ASV level, but considerably weaker associations at the genus level (Extended Data Fig. 3). Similar results were obtained when using unweighted UniFrac, although the association was weaker in vendor A compared with vendor B. Genus-level analysis once again showed weak to no association with age when using unweighted UniFrac (Extended Data Fig. 3). We expected unweighted (that is,
Fig. 2 | Associations between gut microbiome uniqueness and age across the Arivale cohort. a. Boxplots showing gut microbiome uniqueness scores calculated using the ASV-level (grey) and genus-level (blue) Bray–Curtis dissimilarity metric across the adult lifespan in the Arivale cohort, adjusted for vendor. Asterisks (*) indicate significant differences relative to the youngest group (<30 years), from a linear regression model adjusted for vendor, sex, BMI and Shannon diversity (ASV level: (50–59 years) $P = 3.52 \times 10^{-2}$, (60–69 years) $P = 1.88 \times 10^{-5}$, (70–79 years) $P = 1.47 \times 10^{-5}$, (80+ years) $P = 1.12 \times 10^{-2}$; genus level: (40–49 years) $P = 7.15 \times 10^{-2}$, (50–59 years) $P = 3.57 \times 10^{-1}$, (60–69 years) $P = 4.33 \times 10^{-1}$, (70–79 years) $P = 8.16 \times 10^{-1}$, (80+ years) $P = 7.90 \times 10^{-1}$, two sided). Also shown is the distribution of uniqueness calculated using the Bray–Curtis metric on both the ASV and the genus level. b. Spearman correlation coefficients for measures of Bray–Curtis uniqueness with age in individuals whose stool samples were processed by vendor A or B, as well as an additional external dataset (AGP). c. Boxplots showing gut microbiome uniqueness scores calculated using the ASV-level Bray–Curtis metric across early, mid- and late adulthood in the AGP dataset. Asterisks (*) indicate significant differences relative to the youngest group (aged <30 years), from a linear regression model adjusted for sex and Shannon diversity ((50–59 years) $P = 2.77 \times 10^{-5}$, (80+ years) $P = 2.95 \times 10^{-1}$). In both a and c, boxplots represent the IQR (25th to 75th percentiles), with the middle line denoting the median; the whiskers span 1.5x IQR, and the points beyond this range are shown individually. d. Percentage of variance explained in genus-level Bray–Curtis uniqueness by a diverse number of demographic and lifestyle factors, as well as a subset of clinical laboratory tests. ALAT, alanine aminotransferase; ALP, alkaline phosphatase; CRP, C-reactive protein; GGT, $\gamma$-glutamyltransferase; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; LDL, low-density lipoprotein; med., medication.

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presence–absence) $\beta$-diversity metrics to show a weaker association with age, given our initial hypothesis that the shifting relative dominance between core taxa and accessory taxa would drive rising uniqueness (that is, this pattern should be apparent only when using weighted metrics that incorporated taxon abundances). From this point forward, we focus primarily on the Bray–Curtis uniqueness measure, because it is a weighted measure not influenced by phylogeny and well suited to capture changes in gut bacterial dominance previously observed in smaller cohorts of extremely long-lived individuals (that is, a decline in core taxa) $9$. We next validated the identified uniqueness ageing signal in the AGP dataset $20$, a self-selected citizen–scientist cohort of thousands of individuals spanning a wide age range (18–101 years), which demonstrated a consistent pattern (Fig. 2b,c and Extended Data...
Fig. 3). Although the association was significant across the ASV- and genus-level analyses of all three cohorts, we conservatively focused on the genus-level Bray–Curtis measure in our downstream analysis. As we have described previously, genus-level data are less sensitive to batch effects and more amenable to cross-cohort comparisons15.

To further characterize the observed gut microbiome ageing pattern, and understand how it is reflected in host physiology and health, we tested the correspondence between genus-level Bray–Curtis uniqueness and a wide variety of clinical laboratory tests, demographic information and self-reported lifestyle/health measures in the Arivale cohort, adjusting for microbiome vendor (Fig. 2d and Extended Data Fig. 4). Of all the factors tested, age demonstrated the strongest association with gut microbiome uniqueness. Several other factors were significantly associated with uniqueness, including prescription medication use and alcohol consumption (Extended Data Fig. 4). However, after adjusting for age, mainly lipid markers remained significantly associated with gut microbiome uniqueness, with the direction of association indicating healthier metabolic and lipid profiles in individuals with more unique gut microbiomes: for example, lower low-density lipoprotein-cholesterol, higher vitamin D and lower triglycerides in individuals with more unique microbiomes (Fig. 2d). It is interesting that self-reported dietary measures showed little to no association with our gut microbiome uniqueness metric. However, the depth of information on dietary habits captured by our questionnaires was limited, so these results need to be interpreted with caution. More detailed dietary questionnaires are needed to capture the broad impact of diet on gut microbial composition reported previously16. The same age-adjusted analysis was replicated using the ASV-level Bray–Curtis uniqueness measure, showing similar results in terms of lipid profiles (high-density lipoprotein, vitamin D) and sex, as well as additional significant associations with antibiotic use and creatinine levels (Extended Data Fig. 4).

Reflection of gut microbiome uniqueness in the host metabolome. Our research group has previously demonstrated a strong reflection of gut microbiome community structure in the human plasma metabolome17. For a better understanding of how host physiology reflects the increasingly unique composition of the gut microbiome seen with ageing, and to gain potential mechanistic insight into the functional changes that take place in the microbiota, we regressed our uniqueness measure against each of the 653 plasma metabolites identified six analytes significantly associated with gut microbiome uniqueness. Several other factors were significantly associated with uniqueness, both beneficial (Christensenellaceae) and potentially pathogenic (Methanobrevibacter and Desulfbif). They have been previously implicated in human longevity, enriched in long-lived individuals18. We further replicated the same analysis in the MrOS validation cohort, the Arivale cohort across vendors and stratified by sex, as well as the AGP dataset. In each case, we observed a high level of congruence in the major taxa that correlate with compositional uniqueness, indicating that rising uniqueness with age is characterized by the rise and decline of the same sets of taxa across considerably diverse populations (Extended Data Fig. 6).

Consistent with our initial analysis, age showed a positive association with our uniqueness score in the MrOS cohort, although to a weaker extent than in the larger Arivale population (genus-level Bray–Curtis Spearman’s ρ = 0.11, P = 0.0097; ASV-level Bray–Curtis Spearman’s ρ = 0.07, P = 0.072). Unlike the Arivale cohort, MrOS participants were considerably more health heterogeneous at the time of sampling, with a large proportion of participants reporting chronic conditions (Extended Data Fig. 2). The health heterogeneity of MrOS participants provided an opportunity to better understand whether the observed increase in microbiome dissimilarity with age depends on host health, and thus may be indicative of healthy ageing. Hence, we re-ran the above analysis under four different stratifications based on: medication use, self-perceived health, lifestyle–space score (LSC) and walking speed. We chose these four health metrics because collectively they encompass a diverse repertoire of health in older populations (Table 1).

Under all stratifications considered, we observed a stronger positive association between age and genus-level microbiome uniqueness in healthier individuals, whereas the association was lower or absent altogether in individuals demonstrating worse health (Fig. 4e and Extended Data Fig. 7). We further generated a composite stratification (composite healthy), where MrOS participants had to meet all of the above criteria. The association between age and genus-level microbiome uniqueness remained significant across all stratifications.
in the Arivale cohort, from OLS regression models predicting genus-level Bray–Curtis uniqueness adjusted for microbiome vendor, sex, age, age², a sex x age interaction term, BMI and Shannon diversity. Metabolites are colour coded by their superfamily. Metabolites are colour coded by their superfamily. All metabolites above the light-red line are significant after multiple-hypothesis correction (Bonferroni’s $P < 0.05$, two sided), whereas the blue line indicates the unadjusted $P$-value threshold. Asterisks (*) indicate metabolites that were confidently identified on the basis of MS data, but for which no reference standards are available to verify the identity. b, Spearman correlation coefficients for each of the metabolites significantly associated with genus-level Bray–Curtis uniqueness after adjusting for covariates and multiple-hypothesis correction (Bonferroni’s $P < 0.05$, two sided). c, Spearman correlation coefficients for each of the metabolites significantly associated with the ASV-level Bray–Curtis uniqueness measure after adjusting for covariates and multiple-hypothesis correction (Bonferroni’s $P < 0.05$, two sided). For both b and c, the bars are colour coded as in a. d, Scatter plot of genus-level Bray–Curtis uniqueness and the strongest metabolite predictor, phenylacetylglutamine, adjusted for vendor. e, Scatter plot of ASV-level Bray–Curtis uniqueness and the strongest metabolite predictor, phenylacetylglutamine, adjusted for vendor. The lines shown are the $y$–$x$ regression lines, and the shaded regions are 95% CIs for the slope of the line. The $P$ values reported in d and e are a result of two-sided statistical tests.

In this limited group of 133 individuals, we observed an even stronger association between gut microbiome uniqueness and age than under any individual stratification. We replicated the analysis on the second batch of MrOS gut microbiome samples (validation cohort, $n = 308$), demonstrating very similar results (Fig. 4c and Extended Data Fig. 7). We also ran the same analysis using genus-level weighted UniFrac dissimilarity, and observed a high level of congruence between results (Extended Data Figs. 7 and 8). We further replicated the analysis on the ASV level. Although the strength of association between healthy ageing and uniqueness at the ASV level was weaker, it still showed the same directional trends, with healthier individuals showing a stronger positive correlation, particularly in the validation cohort (Extended Data Fig. 8). In contrast, measures of $\alpha$-diversity at the genus and ASV levels were not significantly associated with age under any stratification considered (Extended Data Fig. 8).

Drugs are known to impact the composition and function of the gut microbiome\(^\text{35}\). Given the high number of medications taken by MrOS participants, we explored whether the identified healthy ageing pattern is confounded by drug use. To this end, we performed additional analysis using the genus-level Bray–Curtis uniqueness measure in the MrOS discovery cohort. First, we focused specifically...
on the highly medicated individuals (more than eight reported meds). In this high-med group, we identified a subset of individuals on multiple medications who are nevertheless healthy using the criteria for walking speed described in Table 1. The correlation between uniqueness and age was still significant in healthy, highly medicated individuals (Pearson’s r = 0.26, P = 0.047; Spearman’s ρ = 0.27, P = 0.01, n = 81). Once again, less healthy individuals did not show the same pattern (Pearson’s r = −0.05, P = 0.50; Spearman’s ρ = 0.02, P = 0.76, n = 223).

Next we explored the impact of adjusting age-uniqueness linear regression models for individual medication use. The five most prevalent supplements and drugs reported by MrOS participants, as well as antibiotic use in the last month, did not impact the association between age and Bray–Curtis uniqueness in healthy composite individuals, and did not change the lack of the same relationship in the remainder of the cohort (Extended Data Fig. 7). Although drug–microbiome interactions are more complex than what can be captured by our approach, we conclude that medication use is not the main driver behind the observed variability in gut microbiome ageing patterns across health states.

**Gut microbiome predicts mortality in extreme ageing.** Next, we focused exclusively on community-dwelling individuals (that is, excluding participants in assisted living and nursing homes, and/or who have been hospitalized in the past 12 months) from the two MrOS datasets, combined together for increased power (n = 706) (Fig. 1). We performed genus-level differential abundance analysis to identify genera associated with age in healthy composite individuals (n = 172) and the remainder of the cohort (n = 534), separately, adjusting for batch (discovery/validation) and BMI. In healthy...
The major findings of our analysis were: (1) individual gut microbiome patterns associated with ageing across over 9,000 individuals from 3 distinct study populations spanning the ages 18–101 years. The major findings of our analysis were: (1) individual gut microbiomes became increasingly more unique to each individual with age, starting in mid-to-late adulthood, and this uniqueness was positively associated with known microbial metabolic markers previously implicated in immune regulation, inflammation, ageing and longevity; (2) in the later decades of human lifespan, healthy individuals continued to show an increasingly unique gut microbial compositional state (associated with a decline in core taxa) with age, whereas this pattern was absent in those in worse health; (3) in individuals approaching extreme age (85+ years), retention of high relative Bacteroides abundance and a low gut microbiome uniqueness measure were both associated with significantly decreased survival in the course of a 4-year follow-up. These observations are strengthened by the presence of similar age-related trends across two independent microbiome vendors and three demographically distinct cohorts (Arivale, MrOS and AGP).

Although the ecological composition of the gut microbiome becomes increasingly divergent with ageing, we found a corresponding convergence in plasma concentrations of microbial metabolites, indicating that ageing is characterized by gradual shifts in gut metabolic capacity. A number of the identified microbial metabolites in our analysis have been previously characterized as mildly toxic.

Our findings from both β-diversity and differential abundance analysis in healthy elderly MrOS participants indicate that the identified gradual ageing pattern may, in its end stages, resemble gut microbiome changes previously observed in extremely long-lived individuals (that is, a loss of core taxa)\(^9\). Therefore, we utilized longitudinal data from the MrOS cohort to investigate whether the characterized gut microbiome pattern is not only reflective of healthy ageing, but also predictive of survival. We performed the analysis in two steps: (1) on all community-dwelling participants (n = 706) and (2) on community-dwelling participants in the top age tertile (aged 85+, n = 256) at the time of gut microbiome sampling, because these participants were the closest to achieving extreme age in the course of the study’s follow-up period (~4 years). When focusing on all individuals in the cohort, we identified a significant positive association between relative Bacteroides abundance and increased risk of all-cause mortality, independent of age, BMI, clinical site, self-perceived health, diagnosis of congestive heart failure and batch in which stool samples were processed. Replicating the analysis in the oldest individuals (aged 85+) revealed a stronger association and higher hazard ratios (HRs) compared with the whole cohort (Table 2 and Fig. 5b). Using the participants’ calculated Bray–Curtis and weighted UniFrac uniqueness scores yielded comparable results in those aged 85+, where mortality risk decreased in individuals with more unique gut microbiomes independent of the same covariates. In contrast, the same associations between Bray–Curtis and weighted UniFrac uniqueness measures and mortality were not present when younger participants were included in the analysis (Table 2). Once again, we replicated the same analysis on the ASV level. Across both dissimilarity metrics, the trends were consistent with the genus-level measures; however, only the weighted UniFrac metric passed the significance threshold across all multivariable models (Table 2 and Fig. 5c).

### Discussion

There is limited understanding of how the human gut microbiome changes throughout adulthood and how these changes influence host physiology. In the present study, we evaluated gut microbiome patterns associated with ageing across over 9,000 individuals from 3 distinct study populations spanning the ages 18–101 years. The major findings of our analysis were: (1) individual gut microbiomes became increasingly more unique to each individual with age, starting in mid-to-late adulthood, and this uniqueness was positively associated with known microbial metabolic markers previously implicated in immune regulation, inflammation, ageing and longevity; (2) in the later decades of human lifespan, healthy individuals continued to show an increasingly unique gut microbial compositional state (associated with a decline in core taxa) with age, whereas this pattern was absent in those in worse health; (3) in individuals approaching extreme age (85+ years), retention of high relative Bacteroides abundance and a low gut microbiome uniqueness measure were both associated with significantly decreased survival in the course of a 4-year follow-up. These observations are strengthened by the presence of similar age-related trends across two independent microbiome vendors and three demographically distinct cohorts (Arivale, MrOS and AGP).

### Table 1 | Description of health metrics used for stratification in the MrOS cohort

| Health metric     | Description                                                      | Stratification                                                                 |
|-------------------|------------------------------------------------------------------|--------------------------------------------------------------------------------|
| Medication use    | Medication use is associated with chronic diseases and comorbidities, and is an important modulator of the gut microbiome\(^9\). High medication use is particularly prevalent in older populations, with nearly 40% of individuals aged 65+ years reporting taking five or more medications\(^4\). | High: >8, low: ≤8 medications. This allowed us to generate two groups of participants with similar age distribution but very different pharmacological profiles. |
| Self-perceived health | Self-perceived health has been previously shown to be an independent predictor of earlier mortality in older populations\(^5\)\(^6\). | In the MrOS cohort, individuals chose one out of five possible responses (excellent, good, fair, poor, very poor). We stratified the cohort into individuals who reported excellent health and those who reported anything less than excellent. |
| LSC               | LSC is an indicator of mobility, that is, how often an individual leaves their room, house or neighbourhood, and has been previously associated with risk of mortality in MrOS participants\(^5\). Its strength as a measure lies in that it provides insight into not only whether an individual is physically capable of performing activities, but also whether that individual actually performs these activities\(^6\). | For both the LSC and walking speed, we stratified the cohort into tertiles and defined the top tertile as the healthy group (high), whereas the bottom two tertiles were combined into the less healthy group (low). |
| Walking speed     | Walking speed is a validated measure used to assess functional status and overall health\(^9\), and had previously been shown to be associated with executive function and predictive of cognitive decline\(^10\). | Healthy: individuals who met ≥3+ of the above criteria (Extended Data Fig. 2). |
| Composite         | A composite of all four of the above measures.                   | Healthy group (high), whereas the bottom two tertiles were combined into the less healthy group (low). |

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| Composite         | A composite of all four of the above measures.                   | Healthy group (high), whereas the bottom two tertiles were combined into the less healthy group (low). |
indicates that phenylacetylglutamine and p-cresol concentrations of certain plasma indole metabolites (indoxyl sulfate and indolelactate) were positively associated with improved physical function in older adults. Furthermore, a number of microbial indole metabolites (indoleacetate, indolepropionate, indoxyl sulfate and indolelactate) have been recently shown to be depleted in the plasma of obese individuals relative to normal weight controls. Although most indole metabolites identified in our study were positively associated with gut microbiome uniqueness, ASV-level analysis revealed a negative association with indolepropionate, indicating that there is probably a more complex

Additional metabolites associated with our observed gut microbial pattern were dominated by indoles (3-indoxyl sulfate, 6-hydroxyindole sulfate, indoleacetate and indolepropionate), which are gut bacterial degradation products of tryptophan. Bacterial indole metabolism is emerging as an important mediator of the gut microbiome–host immune homoeostasis. Indole (the precursor to the human microbial co-metabolite indoxyl sulfate identified in our analysis) has been shown to increase health span and extend survival in a number of animal models. One of the most recognized mechanisms of action of indole metabolites is the mediation of inflammation through binding of the aryl hydrocarbon receptor. Studies in mice demonstrated that indole metabolites can alleviate lipopolysaccharide-induced liver inflammation and protect from chemically induced colitis, highlighting their role in mediating host inflammatory responses. Consistent with their positive effect on health span and inflammation in animal models, concentrations of certain plasma indole metabolites (indoxyl sulfate and indolelactate) were positively associated with improved physical function in older adults. Furthermore, a number of microbial indole metabolites (indoleacetate, indolepropionate, indoxyl sulfate and indolelactate) have been recently shown to be depleted in the plasma of obese individuals relative to normal weight controls. Although most indole metabolites identified in our study were positively associated with gut microbiome uniqueness, ASV-level analysis revealed a negative association with indolepropionate, indicating that there is probably a more complex

Fig. 5 | Associations between identified gut microbial ageing patterns and survival in older adults. a, Boxplots demonstrating the relative abundance of the genus Bacteroides across tertiles of age in community-dwelling individuals identified as healthy on 3+ criteria specified (composite healthy) and the remainder of the cohort. b, Kaplan–Meier curve demonstrating the association between overall survival and relative Bacteroides abundance, grouped into tertiles in community-dwelling MrOS participants aged 85+ years at the time of sampling (n = 256). c, Kaplan–Meier curve demonstrating the association between overall survival and ASV-level weighted (W.) UniFrac uniqueness grouped into tertiles in community-dwelling MrOS participants who were aged 85+ years at the time of sampling (n = 256). P values shown in b and c are a result of log-rank tests (two sided) comparing the two survival curves, and have not been corrected for multiple-hypothesis testing.

phenylalanine/tyrosine microbial fermentation products (p-cresol sulfate, phenylacetylglutamine and p-cresol glucurononide), indicating a rising burden of gut xenobiotic metabolites in an ageing host. This is consistent with a recent study in the ELDERMET cohort demonstrating that faecal concentrations of p-cresol correlated with increased frailty, particularly in long-term-care residents. The reported association between p-cresol and frailty indicates that p-cresol may reflect, or potentially contribute to, age-associated decline in this population. However, the association between microbial protein fermentation products and ageing in community-dwelling individuals, as well as certain animal models of longevity, show conflicting results. For example, urine levels of phenylacetylglutamine and p-cresol sulfate have both been previously shown to be enriched in centenarians relative to elderly and young controls. Similarly, metabolomic profiles of naked mole rats, a model organism characterized by extreme lifespan that exhibits negligible age-associated decline, demonstrated increased levels of phenylalanine and tryptophan degradation products (phenylacetylglutamine and indoleacetate) in the blood, relative to a group of control mice. Therefore, it is likely that protein microbial metabolites reflect different ageing gut ecosystem dynamics in community-dwelling individuals versus severely frail long-term-care residents. Alternatively, resilient individuals may be able to effectively neutralize and excrete these microbial compounds, whereas others succumb to the negative effects of these same metabolites on host physiology throughout the course of ageing.
A striking finding in our analysis was that increasing compositional uniqueness both reflected healthy ageing and predicted survival in older adults. This suggests that the gut microbiome may not only reflect, but also potentially contribute to, a longer host lifespan, which is consistent with some recent non-human animal studies. Concomitant with increasing uniqueness in healthy older individuals, a decline in the core genus *Bacteroides* emerged as a major characteristic of healthy ageing. *Bacteroides* and other major taxa showed consistent associations with uniqueness across both men and women, and across the demographically distinct MrOS, Arivale and AGP cohorts. This indicates that the identified uniqueness trajectory can be traced back to consistent taxonomic changes, providing better interpretability to the identified pattern. The role of *Bacteroides* in ageing has been inconsistent in the literature. Early investigations of the gut microbiome and ageing reported an increased dominance of *Bacteroides* in older people (65+ years) relative to healthy younger controls. Conversely, several studies have shown a decline in core taxa, such as *Bacteroides*, in extremely long-lived individuals. These latter studies often focused on highly co-abundant groups of taxa (CAGs) and their association with age. *Bacteroides* was grouped into the CAG of core microbes that decline with age, along with the primary taxa that drove this signal, such as *Faecalibacterium* and *Coprococcus* spp.7,8. There are several possible explanations for these conflicting results throughout the literature. For example, there may be multiple patterns associated with ageing in healthy and less healthy individuals. In the MrOS cohort, we were able to define healthy ageing through a relatively strict cut-off. Thus, we were able to show that a gradual decline in *Bacteroides* with age was observed only in the healthiest individuals, which was not the case in less healthy, yet still functionally independent, older adults of similar age. The importance of declining *Bacteroides* and other core taxa in ageing requires further investigation. However, we propose that the optimal trajectory for the adaptation of the gut microbiome to an ageing human host depends on an increase of rare taxa capable of synthesizing bioactive microbial metabolites (that is, indoles) at the expense of *Bacteroides* and other core taxa. This makes particular sense in the context of the changing immunological landscape with age, and the potential anti-inflammatory and immune-modulatory effects of several of the microbial metabolites associated with the rising uniqueness described above.

Previously, studies on the gut microbiome in older cohorts have highlighted patterns separating community-dwelling individuals from frailer older people residing in long-term-care facilities. These studies revealed significant associations between gut microbiome composition and health markers, inflammation and diet43, and characterized gut microbial patterns indicative of age-associated decline. Coupling these findings with results from studies investigating gut microbiome development in children and adults has led some to hypothesize that the gut microbiome remains relatively stable throughout adulthood and into old age, at which point gradual compositional changes reflect, and potentially contribute to, declining health. In the current analyses, we have expanded the investigation of the gut microbiome in ageing to cover most of the adult lifespan in multiple, large, deeply phenotyped cohorts. Although it is evident from previous research that gut microbiomes

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**Table 2 | Associations between gut microbiome measures and mortality in the MrOS cohort**

| Gut microbiome measure | Unadjusted HR (95% CI) | Age, clinical site and batch adjusted HR (95% CI) | Multivariable adjusted HR (95% CI) |
|------------------------|------------------------|--------------------------------------------------|----------------------------------|
| Community-dwelling participants, all ages (n = 706) | | | |
| Genus-level analysis | | | |
| Relative *Bacteroides* abundance | 1.21 (0.95–1.54) | 1.31 (1.02–1.68) | 1.28 (1.00–1.65) |
| Uniqueness (Bray–Curtis) | 1.15 (0.91–1.46) | 1.08 (0.85–1.38) | 1.09 (0.85–1.39) |
| Uniqueness (weighted UniFrac) | 1.03 (0.81–1.31) | 0.94 (0.74–1.19) | 0.94 (0.75–1.19) |
| ASV-level analysis | | | |
| Uniqueness (Bray–Curtis) | 1.26 (0.98–1.62) | 1.19 (0.92–1.54) | 1.13 (0.87–1.47) |
| Uniqueness (weighted UniFrac) | 0.88 (0.64–1.21) | 0.82 (0.59–1.14) | 0.82 (0.58–1.16) |
| Community-dwelling participants, 85+ years only (n = 256) | | | |
| Genus-level analysis | | | |
| Relative *Bacteroides* abundance | 1.74 (1.27–2.37) | 1.89 (1.36–2.63) | 1.91 (1.38–2.66) |
| Uniqueness (Bray–Curtis) | 0.76 (0.54–1.07) | 0.70 (0.49–0.99) | 0.70 (0.50–0.99) |
| Uniqueness (weighted UniFrac) | 0.65 (0.43–0.98) | 0.60 (0.40–0.90) | 0.59 (0.40–0.87) |
| ASV-level analysis | | | |
| Uniqueness (Bray–Curtis) | 0.89 (0.65–1.23) | 0.86 (0.62–1.21) | 0.77 (0.54–1.11) |
| Uniqueness (weighted UniFrac) | 0.40 (0.19–0.86) | 0.32 (0.14–0.71) | 0.26 (0.12–0.59) |

Unadjusted, age, clinical site, and batch-adjusted and multivariable adjusted HRs and the 95% CIs of relative *Bacteroides* abundance, genus-level Bray–Curtis and weighted UniFrac uniqueness scores, as well as the same metrics calculated at the ASV level, from Cox’s proportional hazard regression models evaluating mortality risk in all community-dwelling MrOS participants (n = 706) and exclusively community-dwelling MrOS participants aged 85+ years (n = 256). Multivariable models were adjusted for age, clinical site, BMI, self-perceived health, diagnosis of congestive heart failure and batch in which stool samples were processed. Both relative *Bacteroides* abundance and the Bray–Curtis uniqueness measures were scaled and centred before mortality analysis. Significant HRs are emboldened (P ≤ 0.05, two sided). The HRs and 95% CI values reported have not been corrected for multiple-hypothesis testing.
of older individuals (65+ years) change with deteriorating health, we propose that gut microbiomes of healthy individuals continue to develop along a distinct trajectory. This trajectory originates in adulthood, is accompanied by a rise in specific plasma microbial metabolites, reflects a healthy ageing phenotype and is predictive of extended survival in the latest decades of human life. As our understanding of the ageing microbiome increases, monitoring and identifying modifiable features that may promote healthy ageing and longevity will have important clinical implications for the world’s growing older population.

**Methods**

**IRB approval.** Arivale cohort. Procedures for the current study were run under the Western Institutional Review Board with Institutional Review Board (IRB) study no. 20170658 at the Institute for Systems Biology and 1178906 at Arivale (both in Seattle, WA).

MrOS cohort. The IRB at each of the six MrOS study sites approved the study protocol, and written informed consent was obtained from all participants.

**Cohorts.** The Arivale cohort consists of individuals aged ≥18 years who between 2015 and 2019 self-enrolled in a now closed scientific wellness company. Briefly, most of the Arivale participants (~80%) were residents of Washington or California when in the programme. Although the cohort tends to be healthier than the general US population, it is more reflective of populations in these two states4. The cohort is also predominantly female (~40%), which may be due to women being more likely to join these types of programmes. For the present study, only baseline measurements were considered for each participant, and individuals who provided a stool sample were included in the analysis. Demographic information on the cohort is provided in Extended Data Fig. 1.

The MrOS study is an ongoing prospective study of close to 6,000 men recruited across 6 clinical US sites. The cohort, recruitment criteria and stool sample collections have been described previously in detail40. Briefly, the recruitment for the MrOS cohort was designed to obtain a population sample representative of older (65+) US men in the community. As this was a volunteer programme, it is probably enriched for healthier individuals. However, comparison between MrOS participants and National Health and Nutrition Examination Survey (NHANES) data (representative of the US population) showed the two cohorts to be quite similar41. The cohort was not chosen to enrich for individuals with frailty, fractures or osteoporosis. During the fourth follow-up visit of the original study, a subset of participants across all six clinics was asked if they would consent to have their stool sampled for microbiome analysis. Participants who agreed were given the OMNIgene-GUT stool/faeces collection kit (OMR-200, DNA Genotek) and collected the faecal sample at their homes. Demographic information on MrOS participants is provided in Extended Data Fig. 2. In the initial uniqueness analysis, all participants with available high-quality microbiome data were included (n = 907). Subsequent differential abundance analysis focused exclusively on community-dwelling individuals (n = 706) (excluding individuals in assisted living and nursing homes, and those who have been hospitalized in the past 12 months). Finally, survival analysis was conducted on all community-dwelling individuals as well as specifically on community-dwelling individuals in the latest stages of ageing (85+ years, n = 256). The number of deaths in the whole community-dwelling group and in 85+-year community-dwelling group was 66 and 41, respectively.

**Microbiome analysis.** Arivale cohort. Independent of the vendor used, stool samples were collected at the participants’ homes using DNA collection kits, with a proprietary chemical DNA stabilizer provided by the microbiome vendor to maintain DNA integrity at ambient temperatures after collection. Gut microbiome sequencing data in the form of FASTQ files were provided on the basis of either 300-bp paired-end MiSeq profiling of the 16S V3 region (vendor A) or 250-bp paired-end MiSeq profiling of the 16S V4 region (second genome, vendor B). Further analysis was performed using the demose workflow from mbotls (https://github.com/gibbons-lab/mbotls) that wraps DADA2.

In summary, we first trained DADA2 (ref. 42) error models separately for each sequencing run and used those to obtain sequence variants for each sample. This was followed by de novo chimera removal, which removed ~17% of all reads as chimeric and resulted in about 89,000 final sequence variants across all samples. Taxonomy was assigned using the RDP classifier with the SILVA database (v.132). In the present study, 99% of the reads could be classified at the family level, 89% at the genus level and 32% at the species level. Species-level taxonomy was identified by exact alignment to the SILVA reference sequences. Sequence variants were aligned to each other using DECIPHER43 and the multiple sequence alignment was converted to a phylogenetic tree using FastTree44. Downstream gut microbiome data analysis was conducted using the Phyloseq package45. In two separate analyses, gut microbiome samples were rarefied to 21,123 (vendor A, DNA Genotek) and 25,596 (vendor B, second genome) reads, the minimum number of reads per sample for each vendor. For uniqueness analysis, the Bray–Curtis46, unweighted and weighted UniFrac47, and lacciard matrices were calculated for all samples for each vendor, using the rarefied genus and ASV tables. The minimum value for each row, corresponding to the dissimilarity of each sample to their nearest neighbour, was then extracted from the matrix and used for downstream analysis.

MrOS cohort. Stool samples were processed at the Alkek Center for Metagenomics and Microbiome Research (CMMR) at Baylor College of Medicine; using their customized analytical pipeline in two separate batches (discovery and validation, n = 320). Samples for both batches were collected during the same follow-up visit (visit 4), at which point all health-related data analysed in the present study were also collected. Sequencing data in the form of FASTQ files were then processed using the same pipeline as described above for the Arivale cohort. middle metabolomics analysis was conducted using the Phyloseq package. For α-diversity and uniqueness analysis, reads were rarefied to an even depth of 10,000 reads. A total of 12 samples in the validation cohort had fewer reads than the specified cut-off, and hence were excluded from the analysis (validation n = 308). α-Diversity measures were calculated at both the ASV and the genus levels using the Phyloseq package. Both the rarefied ASV and genus tables were used for β-diversity analysis comparing samples across the whole cohort. Uniqueness was calculated as described for the Arivale cohort. The calculated uniqueness measure for each participant was then used for downstream analysis.

As part of our analytical pipeline, we also performed differential abundance analysis assessing the relationship of individual genera with age in individuals diagnosed with healthy and unhealthy individuals. Analysis was conducted in R (v.3.4.4) using β-binomial regression through the Corncob package (v.1.0)48. Models were adjusted for BMI and batch (discovery/validation). The type I error was controlled using Bonferroni’s method (P < 0.01).

AVS abundances from the AGP were obtained from the 125-bp RIOM table deposited in fileshare49 provided in the original manuscript by McDonald et al.50. Metadata for study participants were obtained from the table provided in the same publication and also deposited in fileshare51. Taxonomy was again assigned using the ‘assignTaxonomy’ function contained in DADA2 on the raw ASVs’s returned by deblur and using the SILVA database (v.138). The combined data were then converted to the Phyloseq object for further downstream analysis. In summary, only samples with corresponding age in the metadata and a minimum of 10,000 reads was used for analysis. Individuals aged <18 years were further removed from the dataset. This resulted in a sample size of 4,575. Additional information on the cohorts, data and software can be found in the Nature Research Reporting Summary.

**Plasma metabolomics and clinical laboratory tests.** Blood draws for all assays were performed by trained phlebotomist at LabCorp or Quest service centres. For the 24 h period leading up to the blood draw, Arivale participants were required to avoid alcohol, vigorous exercise, aspartame and monosodium glutamate, and to begin fasting 12 h in advance. Metabolomics analysis was conducted on baseline plasma samples from the Arivale cohort by Metabolon Inc. Each ethylendiaminetetraacetic acid (EDTA)-drawn blood sample was thawed on ice, after which a recovery standard was added to each sample for quality control purposes. As part of the sample preparation, aqueous methanol extraction was performed to remove the protein fraction while retaining the maximum amount of small-molecular-mass compounds in the sample. The sample extract was then aliquoted into five separate fractions, one for each of the four methods used for subsequent metabolite quantification and one as a potential backup. Excess organic solvent was removed from the aliquoted samples by placing the samples on a TurboVap (Zymark). Aliquoted sample extracts were stored overnight under nitrogen before analysis. Regardless of which method for metabolite detection was used, all samples were run on the Waters ACQUITY ultra-performance liquid chromatography and a Thermo Fisher Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyser operated at 35,000 mass resolution. The four aliquoted sample extracts were dried, then reconstituted in solvents compatible with each of the four methods used for downstream metabolite quantification. To ensure injection and chromatographic consistency, each solvent further contained a series of standards at fixed concentrations. Two of the four aliquots were analysed using acid-positive ion conditions chromatographically optimized for either more hydrophobic (solvent consisting of water, methanol, acetonitrile, 0.05% perfluoropentanoic acid (PFPA) and 0.01% formic acid (FA)) or hydrophilic compounds (water and methanol, containing 0.5% PFPA and 0.1% FA). Both of these aliquots were diluted using a C18 column (Waters UPLC BEH C18-2.1 x 100 mm, 1.7 μm). Aliquot 3 was analysed under basic negative ion optimized conditions with elution performed using a dedicated C18 column in solvent containing methanol and water, with 6.5 mM ammonium bicarbonate, pH 8. The fourth and final aliquot was analysed via negative ionization after elution from a HILIC column (Waters UPLC BEH Amide 2.1 x 150 mm, 1.7 μm) using a gradient consisting of water and acetonitrile with 10 mM ammonium formate, pH 10.8. Mass spectrometry (MS) analysis was performed using dynamic exclusion and alternating between MS
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has been described in detail previously and is summarized below. The LSC captured each individual’s rating of their own health compared with other self-perceived health and the LSC were all self-reported. Self-perceived health that were collected from MrOS participants during their fourth follow-up visit, Health measures in the MrOS cohort.

for different questionnaires vary. The number of missing values for each response times per day’, Sleep was reported as the average amount +

once per month’ , (1) ‘1–3 times per month’ , (2) ‘Once per week’ , (3) ‘2–4 times per

(2) ‘3–4’ , (3) ‘5–6’ and (4) ‘7 or more’ ; (fruits, vegetables): (0) ‘Zero/less than 1 per

corresponding ordinal variables: (grains): (0) ‘Zero/less than 1 per day’ , (1) ‘1–2’ ,

‘1–2 drinks’ , (2) ‘3–4 drinks’ , (3) ‘5–6 drinks’ and (4) ‘More than 6 drinks’ . Current

which was recoded into corresponding ordinal variables: (0) ‘I do not drink’ , (1)

(1) ‘1–2 drinks’ , (2) ‘3–4 drinks’ , (3) ‘5–6 drinks’ and (4) ‘More than 6 drinks’ . Current

the walking speed low group in our analysis. More than

in your neighbourhood, other than your own yard or apartment building’ ; (4) ‘places outside your neighbourhood, but within your town’ ; or (5) ‘places outside your town’ . Within each of these levels, participants answered how often they travelled to that area and whether assistance in the form of equipment or

the findings in this study for research purposes. Requests should be sent to Andrew Magis (andrew.magis@ibsrs.org). The MrOS dataset is available to researchers through the following website: https://mrosonline.ucsf.edu/. The data are available to qualified researchers on submission and approval of a research plan. The AGP biom table and the accompanying metadata are publicly available through figshare, reference nos. 6137192 (ref. 4) and 6137315 (ref. 4), respectively.

Quality researchers can access the full Arivale deidentified dataset supporting the findings in this study for research purposes. Requests should be sent to Andrew Magis (andrew.magis@ibsrs.org). The MrOS dataset is available to researchers through the Arivale cohort. Lifestyle/health questionnaires in the Arivale cohort.

Clinical laboratory tests were conducted by either Quest or LabCorp. A 10% missing value threshold was set, which was passed by 653 metabolites. Missing values for metabolites were imputed to be the median observed value for that metabolite. A total of 1,459 Arivale participants had paired gut microbiome–plasma metabolome data and met the inclusion criteria. Values for each metabolite were log-transformed before analysis. Clinical laboratory tests were conducted by either Quest or LabCorp. A 10% missing value threshold was set for each clinical laboratory test used in the analysis. All but 104 participants (n = 3,549) had paired clinical laboratory gut microbiome data. Both metabolomics and clinical laboratory tests were scaled and centred before analysis and only baseline measures for each individual were used.

Lifestyle/health questionnaires in the Arivale cohort. Data on lifestyle, diet and health were obtained through self-administered questionnaires completed by Arivale participants during their initial assessment. For reporting antibiotic use, participants chose from three possible responses (‘not in the past year’, ‘in the past year’ and ‘never’), which were recoded into two ordinal variables: (7) ‘never’, (6) ‘1–3 times per week’ and (5) ‘1–2 times per week’ . Similarly, alcohol use (no. of drinks per day) was reported on the following scale, which was recoded into corresponding ordinal variables: (0) ‘I do not drink’ , (1) ‘1–2 drinks’ , (2) ‘3–4 drinks’ , (3) ‘5–6 drinks’ and (4) ‘More than 6 drinks’ . Current

interquartile range (IQR) = 2–4). Individuals with missing values for each ordinal variable were excluded before statistical analysis. A 10% missing value threshold was set, which was passed by 653 metabolites. Missing values for metabolites were imputed to be the median observed value for that metabolite. A total of 1,459 Arivale participants had paired gut microbiome–plasma metabolome data and met the inclusion criteria. Values for each metabolite were log-transformed before analysis. Clinical laboratory tests were conducted by either Quest or LabCorp. A 10% missing value threshold was set for each clinical laboratory test used in the analysis. All but 104 participants (n = 3,549) had paired clinical laboratory gut microbiome data. Both metabolomics and clinical laboratory tests were scaled and centred before analysis and only baseline measures for each individual were used.

Health measures in the MrOS cohort. We utilized four different health measures that were collected from MrOS participants during their fourth follow-up visit, when stool samples were collected for microbiome analysis. Medication use, self-perceived health and the LSC were all self-reported. Self-perceived health captured each individual’s rating of their own health compared with other individuals their own age. The implementation of the LSC in the MrOS cohort has been described in detail previously and is summarized below. The LSC consisted of five levels pertaining to the question: ‘During the past 4 weeks, have you been to: (1) other rooms of your home besides the room where you sleep?; (2) an area outside your home such as your porch, deck, or patio, hallway (of an apartment building), or garage, in your own yard or driveway?; (3) ‘places in your neighbours’ area other than your own or your apartment building’; (4) ‘places outside your neighbourhood, but within your town’ ; or (5) ‘places outside your town’ . Within each of these levels, participants answered how often they travelled to that area and whether assistance in the form of equipment or another person was required. The LSC was then calculated by assigning a score to each of the five levels outlined above and summing them. Level scores were obtained by multiplying the level number (1–5) by an independence factor (2 = no assistance; 1.5 = use of equipment only; and 1 = use of another person with/without equipment) and a frequency factor (1 = less than once per week; 2: 1–3 times per week; 3: 4–6 times per week; and 4 = daily). The final LSC measure could range from 0 (restricted to one’s bedroom) to 120 (travelled outside one’s town daily without assistance). We identified healthy individuals as those in the top tertile of the LSC cohort distribution. This corresponded to an LSC value of ≥296. Walking speed was calculated based on the time it took each participant to walk 6 m (m−1). As with the LSC, we defined healthy individuals based on walking speed if their speed was in the top tertile (≥1.17). A total of seven MrOS participants did not have available walking speed data. This is due to the participants either not coming to the clinic or not being able to attempt the test. These individuals were classified in the walking speed low group in our analysis.

Statistical analysis. Depending on the statistical approach, analysis was conducted using either R (v.3.6.0) or Python (v.3.7). The relationship between the calculated uniqueness measure and age in the Arivale cohort was modelled using ordinary least square (OLS) linear regression (Python) where square root-transformed Bray–Curtis uniqueness was modelled as the dependent variable and each age decade was considered within the youngest reference group (<30 years), adjusting for sex, BMI, and either genus- or ASV-level Shannon diversity, depending on what level the uniqueness measure was calculated. We chose to adjust for Shannon diversity because, in the Arivale cohort, it was associated with both age and microbiome uniqueness (higher α-diversity makes you more likely to be unique). We wanted to assess the significance of our dissimilarity pattern independently of the sex in the age groups and previously reported in literature. The same adjustment was not made for MrOS participants, because α-diversity measures showed no association with age in that cohort. When assessing the interrelationship of clinical, lifestyle and demographic variables with gut microbial uniqueness, Bray–Curtis uniqueness values ≥3 or <3.s.d. from the mean were removed. OLS linear regression was then used to assess the individual relationship between each factor and square root-transformed Bray–Curtis gut microbial uniqueness, with microbiome vendor included as a covariate. Percentage variance explained by each factor was calculated by taking the percentage variance explained by the complete OLS model (variable of interest and microbiome vendor) and subtracting the percentage variance explained by the microbial signature alone. The same analysis was then repeated with age included as a covariate (age-adjusted models). When investigating the relationship between plasma metabolite concentrations and gut microbial uniqueness, each metabolite was log-transformed and subsequently scaled and centred. The square root-transformed Bray–Curtis uniqueness measure was then regressed against each metabolite individually, adjusting for microbiome vendor, sex, age, sex, a sex × age interaction term, BMI and Shannon diversity using OLS regression.

In each instance, where multiple hypotheses were tested, type I error was controlled for using Bonferroni’s method (P < 0.05). In the MrOS cohort, correlation between Bray–Curtis uniqueness and age was calculated using the Python statistical functions package (SciPy.stats) and the square root-transformed uniqueness measure. Mortality analysis was conducted in R using the package survival (v.2.44–1.1). Relative Bacteroides abundance (after rarefaction) and uniqueness scores were scaled and centred before survival analysis. Cox proportional hazard regression models were generated assessing the relationship between survival and relative Bacteroides abundance. Bray–Curtis uniqueness weighted UniFrac uniqueness independently, adjusting for clinical site, batch (discovery/validation) and age, and adjusting for clinical site, age, BMI, self-perceived health (excellent, good, <good (fair, poor, very poor)), diagnosis of congestive heart failure and batch in which stool samples were processed (discovery/validation).

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Qualified researchers can access the full Arivale deidentified dataset supporting the findings in this study for research purposes. Requests should be sent to Andrew Magis (andrew.magis@ibsrs.org). The MrOS dataset is available to researchers through the Arivale cohort. Lifestyle/health questionnaires in the Arivale cohort.

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Code availability

Code used to process gut microbiome samples is available on the Gibbons lab GitHub page (https://github.com/gibbons-lab/microtools) and code used for statistical analysis is available through the Hosé-Price lab GitHub (https://github.com/PriceLab/AgingMicrobiome).

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Author contributions
T.W., S.M.G., L.H., E.S.O. and N.D.P. conceptualized the study. T.W., J.W., J.L., J.A.C., S.M.G., E.S.O and N.D.P. participated in the study design. T.W., C.D., N.R., S.P., J.W., J.L., J.C.E., A.Z. and J.T.Y. performed data analysis and figure generation. G.G. and M.R. aided in dissimilarity analysis. G.G., M.R., N.E.L., J.Z., J.A.C. and D.M.K. assisted in results interpretation. A.T.M. and J.C.L. managed the logistics of data collection and integration. T.W., S.M.G., E.S.O. and N.D.P. were the primary authors of the paper, with contributions from all others. All authors read and approved the final manuscript.

Competing interests
The authors declare no competing interests.

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### Extended Data Fig. 1 | Arivale cohort Demographics table

For comparisons between males and females, χ² tests were run for categorical variables and two-sided t-tests for continuous variables. Obese was defined as BMI ≥ 30. Abbreviations: BMI—body mass index; LDL—low-density lipoprotein cholesterol; HDL—high-density lipoprotein cholesterol; s.d.—standard deviation. P-values <0.05 (two-sided) are colored in red.

|                              | Males (N=1475) | Females (N=2178) | P-Value |
|------------------------------|----------------|------------------|---------|
| **Mean Age (s.d.)**          | 48.1 (12.4)    | 48.0 (12.1)      | NS      |
| Race (% non-white)           | 23.90%         | 19.00%           | <0.001  |
| **Mean BMI (s.d.)**          | 27.1 (4.8)     | 27.3 (6.8)       | NS      |
| **Mean LDL (mg dl⁻¹) (s.d.)**| 118.2 (34.4)   | 111.3 (33.2)     | <0.001  |
| **Mean HDL (mg dl⁻¹) (s.d.)**| 53.6 (14.6)    | 67.4 (19.1)      | <0.001  |
| **Mean blood triglycerides (mg dl⁻¹) (s.d.)** | 113.8 (62.6) | 99.1 (56.0) | <0.001 |
| **Mean Shannon Diversity (s.d.)** | 4.3 (0.5)     | 4.3 (0.5)        | NS      |
| % Obese                      | 19.20%         | 25.50%           | <0.001  |
| % Microbiome vendor A        | 64.50%         | 72.90%           | <0.001  |
### Extended Data Fig. 2 | MrOS discovery cohort characteristics table stratified into composite healthy and remainder of cohort

Statistical tests used to compare groups are as follows: independent samples t-tests were used for comparing age, body mass index (BMI), Shannon diversity and Observed Species; χ² or Fisher’s exact (if assumptions of χ² were not met) tests were used to compare ethnicity (percentage Hispanic), and prevalence of each of the specified diseases. P-values <0.05, two-sided are colored in red.

|                             | Composite Healthy (n=133) | Rest of Cohort (n=466) | Whole Cohort (N=599) | P-Value |
|-----------------------------|---------------------------|-------------------------|----------------------|---------|
| Median age (s.d.)           | 83.5 (3.6)                | 84.4 (4.2)              | 84.2 (4.1)           | 0.013   |
| Mean BMI (s.d.)             | 26.6 (3.6)                | 27.1 (3.8)              | 27.0 (3.8)           | NS      |
| Hispanic (%)                | 3.8                       | 1.5                     | 2                    | NS      |
| Mean Shannon diversity (s.d.) | 3.6 (0.6)                | 3.5 (0.6)               | 3.5 (0.6)            | NS      |
| Mean Observed Species (s.d) | 167.6 (53.5)              | 162.5 (55.7)            | 163.6 (55.2)         | NS      |
| Diabetes (%)                | 6                         | 17.8                    | 15.2                 | 0.001   |
| Congestive heart failure (%) | 0                         | 10.5                    | 8.2                  | <0.001  |
| Hypertension/high blood pressure (%) | 41.4                | 56.7                    | 53.3                 | 0.003   |
| COPD (%)                    | 6                         | 12                      | 10.7                 | NS      |
| Depression (%)              | 8.3                       | 9.9                     | 9.5                  | NS      |
Extended Data Fig. 3 | Associations between age and gut microbiome uniqueness across cohorts using different distance metrics. a, Age β-coefficients and corresponding P-values from OLS models predicting Bray-Curtis uniqueness at the ASV- and genus-level in the American Gut Project (AGP) and two vendors in the Arivale cohort. In the AGP dataset, the analysis was performed on all samples, and then repeated on the subset of samples who had available sex and BMI data for covariate adjustment. P-values reported are derived from OLS linear regression models and result from a two-sided hypothesis. b, Spearman correlations of different β-diversity metrics with age on both the ASV- and genus-level independently in each vendor used for gut microbiome processing in the Arivale cohort.
Extended Data Fig. 4 | Table with associations between Bray-Curtis gut microbiome uniqueness and clinical, demographic, and diet/lifestyle/health measures in the Arivale Cohort. *pvalue* corresponds to the unadjusted P-Value of the β-coefficient (B-coef column) for each analyte from an OLS model adjusted for gut microbiome vendor. *r_squared* reflects the percent of variance explained beyond microbiome vendor for each analyte independently for the Genus-level Bray-Curtis measure. *age_adjusted_coeff* and *age_adjusted_corr_pvalue* correspond to the β-coefficient and the Bonferroni corrected P-Value (two-sided) for each analyte predicting Genus-level Bray-Curtis Uniqueness, adjusting for gut microbiome vendor and age. The *age_adj_coeff (ASV-level)* and the *age_adj_corr_pvalue (ASV-level)* correspond to analysis done on the ASV-level Bray-Curtis Uniqueness measure, where models were adjusted for vendor and age. *Missing* shows the number of missing observations for each analyte. Values highlighted in red are statistically significant after multiple-hypothesis correction (Bonferroni P-Value < 0.05, two-sided).

| analyte      | pvalue   | r_squared | B-coef  | corr_pval | age_adjusted_coeff | age_adj_coeff (ASV-level) | age_adj_corr_pvalue (ASV-level) | missing |
|--------------|----------|-----------|---------|-----------|--------------------|---------------------------|-------------------------------|---------|
| age          | 0.0000   | 3.0465    | 0.0009  | 0.0000    | 1                  | 0.0028                    | 0.856600877                   | 2732    |
| Prescription Med | 0.0004  | 1.4045    | 0.0148  | 0.0126    | 0.0041             | 1                         | 0.00028                      | 2732    |
| Vitamin D    | 0.0000   | 0.8027    | 0.0054  | 0.0000    | 0.0037             | 0.0049436                 | 0.0034                        | 104     |
| Alcohol      | 0.0000   | 0.6581    | -0.0079 | 0.0000    | -0.0036            | 0.007137                  | -0.0012                       | 1       |
| HDL          | 0.0000   | 0.4724    | 0.0042  | 0.0006    | 0.0027             | 0.1457227                 | 0.0029                        | 104     |
| sex          | 0.0001   | 0.3703    | 0.0076  | 0.0041    | 0.0037             | 0.0032924                 | 0.0046                        | 9.02E-11| 0       |
| Triglycerides| 0.0008   | 0.2934    | -0.0033 | 0.0245    | -0.0041            | 0.0005793                 | -0.0021                       | 104     |
| Diarrhea     | 0.0017   | 0.2634    | -0.0041 | 0.0539    | -0.0020            | 1                         | 0.0014                        | 1       |
| Antibiotics  | 0.0203   | 0.1956    | 0.0076  | 0.6296    | 0.0023             | 1                         | 0.0033                        | 0.001505089 | 1092 |
| HbA1c        | 0.0085   | 0.1807    | 0.0026  | 0.2621    | 0.0001             | 1                         | 0.0004                        | 1       |
| Grains       | 0.0123   | 0.1697    | -0.0031 | 0.3826    | -0.0018            | 1                         | -0.0021                       | 0.07261846 | 237  |
| nGnRd        | 0.0157   | 0.1522    | -0.0024 | 0.4858    | -0.0003            | 1                         | -0.0014                       | 1       |
| BMI          | 0.0175   | 0.1501    | -0.0004 | 0.5430    | -0.0028            | 0.1270441                 | -0.0017                       | 0.424955136 | 170  |
| LDL          | 0.0253   | 0.1303    | -0.0022 | 0.7856    | -0.0034            | 0.0154571                 | -0.0015                       | 0.672038106 | 104  |
| Tobacco      | 0.0313   | 0.1300    | -0.0106 | 0.9703    | -0.0017            | 1                         | -0.0014                       | 1       |
| GGT          | 0.0298   | 0.1230    | -0.0021 | 0.9238    | -0.0033            | 0.0205866                 | -0.0012                       | 1       |
| Albumin      | 0.0301   | 0.1226    | -0.0021 | 0.9328    | -0.0011            | 1                         | -0.0017                       | 0.378553173 | 104  |
| Homocysteine | 0.0536   | 0.0971    | 0.0019  | 1         | -0.0001            | 1                         | -0.0019                       | 0.159030702 | 104  |
| ALT          | 0.0619   | 0.0909    | -0.0018 | 1         | -0.0020            | 1                         | -0.0007                       | 1       |
| SWEETS       | 0.4699   | 0.0599    | -0.0009 | 1         | 0.0004             | 1                         | 0.0011                        | 1       |
| ALP          | 0.1817   | 0.0465    | 0.0013  | 1         | -0.0004            | 1                         | -0.0006                       | 1       |
| Insulin      | 0.2019   | 0.0425    | -0.0012 | 1         | -0.0016            | 1                         | -0.0008                       | 1       |
| CRP          | 0.2250   | 0.0384    | -0.0012 | 1         | -0.0013            | 1                         | -0.0012                       | 1       |
| Glucose      | 0.2873   | 0.0295    | 0.0010  | 1         | -0.0009            | 1                         | 0.0002                        | 1       |
| Fruits       | 0.3120   | 0.0274    | 0.0012  | 1         | 0.0000             | 1                         | 0.0003                        | 1       |
| Sleep        | 0.4018   | 0.0273    | 0.0020  | 1         | 0.0013             | 1                         | 0.0013                        | 1       |
| Race(ref.white) | 0.4697   | 0.0136    | 0.0017  | 1         | -0.0007            | 1                         | -0.0008                       | 1       |
| Sodium       | 0.4710   | 0.0136    | 0.0007  | 1         | -0.0003            | 1                         | -0.0016                       | 0.620191505 | 104  |
| HOMA-IR      | 0.5392   | 0.0098    | -0.0006 | 1         | -0.0012            | 1                         | -0.0003                       | 1       |
| Creatinine   | 0.8226   | 0.0013    | -0.0002 | 1         | -0.0007            | 1                         | -0.0024                       | 0.01264996 | 104  |
| Vegetables   | 0.8626   | 0.0008    | -0.0002 | 1         | -0.0006            | 1                         | 0.0002                        | 1       |
Extended Data Fig. 5 | Table of associations between Bray-Curtis gut microbiome uniqueness and plasma metabolites in the Arivale cohort. ‘pvalue’ corresponds to the unadjusted P-Value of the β-coefficient (covariate_adj. Beta_coeff column) for each analyte from an OLS model adjusted for age, age², sex, a sex*age interaction term, BMI, Shannon diversity, and vendor with Genus-level Bray-Curtis uniqueness as the dependent variable. ‘corr_pvalue’ corresponds to the Bonferroni corrected P-value. ‘SUPER_PATHWAY’ indicates what pathway the metabolite belongs to. The last three columns are the same as the first three, but for Bray-Curtis uniqueness calculated on the ASV level. All metabolites with an unadjusted P-Value < 0.01 are shown. Values highlighted in red are statistically significant after multiple-hypothesis correction (Bonferroni P-Value < 0.05, two-sided).
Extended Data Fig. 6 | Associations between taxa and gut microbiome uniqueness across cohorts and sex.  

(a–d) Plots demonstrating the correlation coefficients between genus-level Bray-Curtis gut microbiome uniqueness and individual taxa in the (a) Discovery MrOS cohort, (b) Vendor A in the Arivale Cohort, (c) Validation MrOS cohort, (d) and vendor B of the Arivale cohort. Only correlations $>|0.20|$ are shown. (e) Plots demonstrating the strength of correlation between genus-level Bray-Curtis microbiome uniqueness and individual taxa in the AGP dataset. The strongest 20 associations are shown. (f–e) Plots demonstrating the strength of correlation between gut microbiome uniqueness and individual taxa in Vendor A of the Arivale cohort in (f) females and (g) males. (h) Scatter plot of correlation coefficients for each genus tested between males and females. The correlation of the coefficients for each genus between sexes is shown. Only genera that had less than 5% zero values and a mean greater than five counts were tested.
Extended Data Fig. 7 | Table of Spearman correlation coefficients and Beta-coefficients testing associations between age and uniqueness in the MrOS cohort. Uniqueness measures reported in this table were calculated at the genus level. ‘Health Stratification’ corresponds to the metric used to define healthy individuals. ‘Spearman Rho’ reports the Spearman correlation coefficient between age and microbiome uniqueness for the specified group of participants, while the ‘pvalue’ column provides the corresponding p-value. ‘Beta_coeff’ is the BMI adjusted age beta-coefficient predicting uniqueness across the same stratifications as the ‘Spearman Rho’ column. ‘Coef_pvalue’ provides the p-value corresponding to the age Beta-coefficient from linear regression models. ‘Sample_size’ is the number of participants in each stratification while the last column ‘Healthy (yes=1/no=0)’ specifies whether the group of participants is the healthy subgroup (yes(1)), or the remainder of the cohort (No(0)). Significant p-values (P < 0.05, two-sided) are highlighted in red. No multiple hypothesis correction was performed.
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | Associations between age and gut microbiome measures across health stratifications in the MrOS cohort. a–e. Plots demonstrating the strength of Spearman correlation between age and gut microbiome measures at different taxonomic resolutions. a, The blue/red panel corresponds to the calculated Weighted UniFrac (β-diversity) uniqueness score at the genus level, while b the grey/green and c grey/yellow panels correspond to Shannon diversity and Observed species (α-diversity measures) at the ASV level, respectively. Significant correlations (two-sided) are indicated with asterisks. Exact correlation coefficients and corresponding p-values for (a) are provided in Extended Data Fig. 7. d–e, The same plots as in (b–c), with α-diversity calculated at the genus level. f, Comparison of ASV level and genus-level analysis in healthy ageing in the MrOS cohort. Barplots represent correlation coefficients comparing age and uniqueness at the ASV level across composite healthy MrOS individuals, and the remainder of the cohort in both the discovery and validation groups. g, β-coefficients for age from OLS regression models predicting genus-level Bray-Curtis uniqueness in healthy composite individuals and remainder of the cohort, adjusted individually for the most commonly reported supplements and medications in the MrOS cohort.
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Software and code

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Data collection

Arivale cohort: Blood samples were collected by trained phlebotomists at LabCorp of America and Quest Diagnostics centers. Stool samples were collected by participants at their homes and processed by DNAGenetek or Second Genome.

The Osteoporotic Fractures in Men Study (MrOS) cohort: Stool samples were collected at participants’ homes. Samples were processed at the Alkek Center for Metagenomics and Microbiome Research (CMMR) at Baylor College of Medicine using their custom analytic pipeline. Upon delivery, samples were reconstituted with the provided manifest and stored at −80°C until further processing. For bacterial genomic DNA extraction, samples were thawed at room temperature to re-liquefy the samples and 200μl of stool suspension were transferred to the extraction deep-well plate. DNA extraction was carried out in the Hamilton STARlet platform following the standard MoBio PowerMag Soil DNA extraction protocol. Extracted DNA was subjected to 16S (v4) rDNA amplification using primers 515F and 806R containing Illumina adapters and a single-end barcode allowing pooling and direct sequencing of PCR product. Further details on gut microbiome data collection can be found in the methods section of the manuscript.

Data analysis

Gut Microbiome analysis was performed on FASTQ files using our in-house pipeline available at https://github.com/gibbons-lab/mbtools.

Additional details on our analytical pipeline are provided in the methods section of the manuscript.

Depending on the statistical approach, analysis was conducted using either R (v 3.6) or Python (v 3.7). Specific packages can be found in the methods section of the manuscript, where each analytical pipeline is described. The code used in our analysis is available at https://github.com/gibbons-lab/mbtools and https://github.com/PriceLab/AgingMicrobiome. Software versions used for the analysis are as follows: Phylseq (v 1.30.0), survival (v 2.44-1.1), Corncomb (v 1.00), DADA2 (v 1.12.1), DECIPHER (v 2.12.0), FastTree2 (v 2.1.10), gibbons-lab/mbtools (v 0.37.1).

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The Arivale data is made freely available for academic use. Qualified researchers interested in analyzing the data should contact Nathan Price at nathan.price@ibdsscience.org.
The MoS dataset is managed by the San Francisco Coordinating Center, University of California San Francisco and California Pacific Medical Center Research Institute. The data used in this study is freely available to qualified researchers. Further information on getting access to the data is available at: https://mrosdata.sfcc.cpmc.net/home.
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Life sciences study design

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| Sample size | No sample-size calculation was performed prior to analysis. Our analytical pipeline utilized two independent cohorts spanning the majority of the adult lifespan. We utilized the Arivale cohort because of its dense phenotyping and large sample size. The MoS cohort is one of the larger cohorts of older persons with gut microbiome data available to date. Because our pipeline involved beta-diversity analysis where samples are compared among each other, we aimed at utilizing the greatest number of high quality samples to test our hypothesis that gut microbiomes become increasingly unique with age. We were able to identify significant associations with sample sizes ranging from N=308 [MoS validation] to N=2539 [Arivale vendor A], indicating we were sufficiently powered for the proposed analysis. |
| Data exclusions | The inclusion criteria for this study are described in detail in the methods section of the manuscript. Briefly, for the Arivale cohort, if a participant provided a stool sample and research consent, they were included in our analytical pipeline. In the MoS cohort, a total of 12 samples were excluded from the validation cohort during the course of analysis because of low read counts in their microbiome sample. This exclusion was performed as a quality control, where samples with too low of a read count can introduce artifacts in the analysis. This exclusion is described in the methods section. Finally, certain exploratory analysis involved focusing on specific subsets of the studied population. Analysis was performed for men and women separately in the Arivale cohort to assess sex-dependent differences in gut microbiome aging patterns. In the MoS cohort, a sub-analysis focused on community dwelling individuals and also individuals closest to achieving extreme age. All of these specific analyses, and the rationale behind them, are described in detail in the results and methods section of the manuscript. For the American Gut Project validation cohort, only individuals with at least 10,000 reads were included in the analysis. Each participant had to further have provided their age. All individuals <18 years of age were excluded from the analysis. These exclusion criteria are described in the methods section of the manuscript. |
| Replication | Experimental findings in this manuscript were verified through validation in separate cohorts described in detail in the manuscript. Briefly, the aging patterns within the gut microbiome were confirmed in two separate vendors (DNAgenotek and Second Genome) in the Arivale cohort, and two separate groups of samples analyzed several years apart in the MoS cohort. We further replicated our main finding the American Gut Project dataset. Majority of findings were reproduced across all cohorts. In the MoS validation cohort, some associations did not reach significance, though they demonstrated a consistent trend. These associations, significant or not, are fully reported in the manuscript. The associations between clinical chemistries and lifestyle factors reported in the Arivale cohort could not be reproduced in the MoS cohort due to lack of the same data available for those participants. Finally, certain secondary findings, such as a positive association between alpha diversity and age in the Arivale cohort, were assessed in the MoS cohort, but did not replicate. These findings are once again described in detail in the manuscript and complemented by appropriate figures. |
| Randomization | Randomization was not performed in this analysis as this was not an intervention trial. |
| Blinding | Blinding was not performed in this analysis as there was no intervention in this study. |

Reporting for specific materials, systems and methods
We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.
Human research participants

Policy information about studies involving human research participants

Population characteristics
A detailed description of population characteristics is available in the submitted text in Figure 1, Supplementary Tables S1, S2.

Recruitment
Arivale Cohort: Participants were self-enrolled in the Arivale program, since it is a subscription service. Researchers do not play a role in recruiting participants for the program. Upon entering the program, participants are provided with the option to permit the use of their de-identified data for scientific discovery. These participants then join the research cohort and are used for downstream analysis.

MrOS Cohort: Recruitment criteria for the MrOS cohort have been described extensively in the literature. Briefly, The Osteoporotic Fractures in Men Study (MrOS) is a multi-center observational study designed to determine risk factors for osteoporosis, fractures and prostate cancer in older men. It recruited 5995 participants over a 25-month period across six clinical U.S. sites. The present manuscript utilized data on a subset of the original cohort who provided stool samples on their fourth follow-up visit (2014). Recruitment details of the original study can be found at: https://www.sciencedirect.com/science/article/pii/S1551144050010607 via 3D hub as well as the MrOS website: https://mrosdata.sfcc-cpnc.net

Ethics oversight
Arivale Cohort: Procedures for the current study were run under the Western Institutional Review Board (WIRB) with Institutional Review Board (IRB) Study Number 20170658 at the Institute for Systems Biology and 11/8906 at Arivale (both in Seattle, WA).

MrOS Cohort: The institutional review board at each of the 6 MrOS study sites approved the study protocol, and written informed consent was obtained from all participants.

Note that full information on the approval of the study protocol must also be provided in the manuscript.