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Social and environmental predictors of gut microbiome age in wild baboons

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eLife Assessment

This study leverages an impressive and comprehensive longitudinal 16S rRNA gut microbiome dataset from baboons to provide **important** insight regarding the use of a microbiome-based clock to predict biological age. The evidence for age-associated microbiome features and environmental and social variables that impact microbiome aging is **convincing**. This study of microbiomes as markers of host age will fuel inquiries and studies and interest a broad range of researchers, especially those interested in alternatives to measuring biological aging.

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Abstract

Mammalian gut microbiomes are highly dynamic communities that shape and are shaped by host aging, including age-related changes to host immunity, metabolism, and behavior. As such, gut microbial composition may provide valuable information on host biological age. Here we test this idea by creating a microbiome-based age predictor using 13,563 gut microbial profiles from 479 wild baboons collected over 14 years. The resulting “microbiome clock” predicts host chronological age. Deviations from the clock’s predictions are linked to some demographic and socio-environmental factors that predict baboon health and survival: animals who appear old-for-age tend to be male, sampled in the dry season (for females), and have high social status (both sexes). However, an individual’s “microbiome age” does not

predict the attainment of developmental milestones or lifespan. Hence, in our host population, gut microbiome age largely reflects current, as opposed to past, social and environmental conditions, and does not predict the pace of host development or host mortality risk. We add to a growing understanding of how age is reflected in different host phenotypes and what forces modify biological age in primates.

Introduction

For most vertebrate species, physical declines with age are inevitable. These changes define the concept of biological aging and contribute to increased disease burden in older individuals (1, 2). While vertebrates have species-typical patterns of biological aging, those patterns can also differ between individuals within species. Hence, an animal's age in years—i.e., its chronological age— is not an exact reflection of age-related decline in physical functioning (3–6). Measuring individual differences in biological age is an important first step to understand how socio-environmental conditions influence aging processes and to identify strategies to improve health in old age.

In mammals, one valuable marker of biological aging may lie in the composition and dynamics of the gut microbiome (7–10). Age-related changes in gut microbiomes are well documented in humans and other animals, and the gut microbiome has the potential to reflect a wide variety of aging processes for individual hosts (11–24). Mammalian gut microbiomes interact with the immune, endocrine, nervous, and digestive systems, all of which change with age (25–28). Gut microbiomes are also sensitive to host environments and behaviors that change with age, including host diet, living conditions, and social integration (23, 29–32). Finally, gut microbiomes may play a causal role in age-related changes in host development and longevity and may, therefore, be directly involved in individual differences in biological age (16, 17, 22, 33, 34). For example, children who experience famine exhibit developmentally immature gut microbiomes that, when transplanted into mice, delay growth and alter bone morphology (35–38). Experiments in flies, mice, and killifish find that the gut microbiome can also influence longevity (16, 17, 22, 39).

One strategy for testing if gut microbiomes reflect host biological age is to apply supervised machine learning to microbiome compositional data to develop a model for predicting host chronological age, and then to test whether deviations from the resulting "microbiome clock" age predictions are explained by socio-environmental drivers of biological age and/or predict host development or mortality. A parallel approach is commonly applied to patterns of DNA methylation, and epigenetic clock age estimates have been shown to predict disease and mortality risk more accurately than chronological age alone (40–45). To date, at least five microbiome age-predicting clocks have been built for humans, which predict sample-specific age with median error of 6 to 11 years (46–49). However, to our knowledge, no clocks have tested whether microbiome age is sensitive to potential socio-environmental drivers of biological age or predicts host development or mortality.

Here we create a microbiome-based age-predicting clock using 13,476 16S rRNA gene sequencing-based gut microbiome compositional profiles from 479 known-age, wild baboons (*Papio sp.*) sampled over a 14-year period (**Figures 1A** and **1B**). These microbiome profiles represent a subset of a data set previously described in Grieneisen et al. (50), Björk et al. (51) and Roche et al. (52), filtered to include only the baboon hosts whose ages were known precisely (within a few days' error). Important to human aging, baboons share many developmental similarities with humans, including an extended juvenile period, followed by sexual maturation and non-seasonal breeding across adulthood (**Figures 1C** and **1D**; [51–54]).

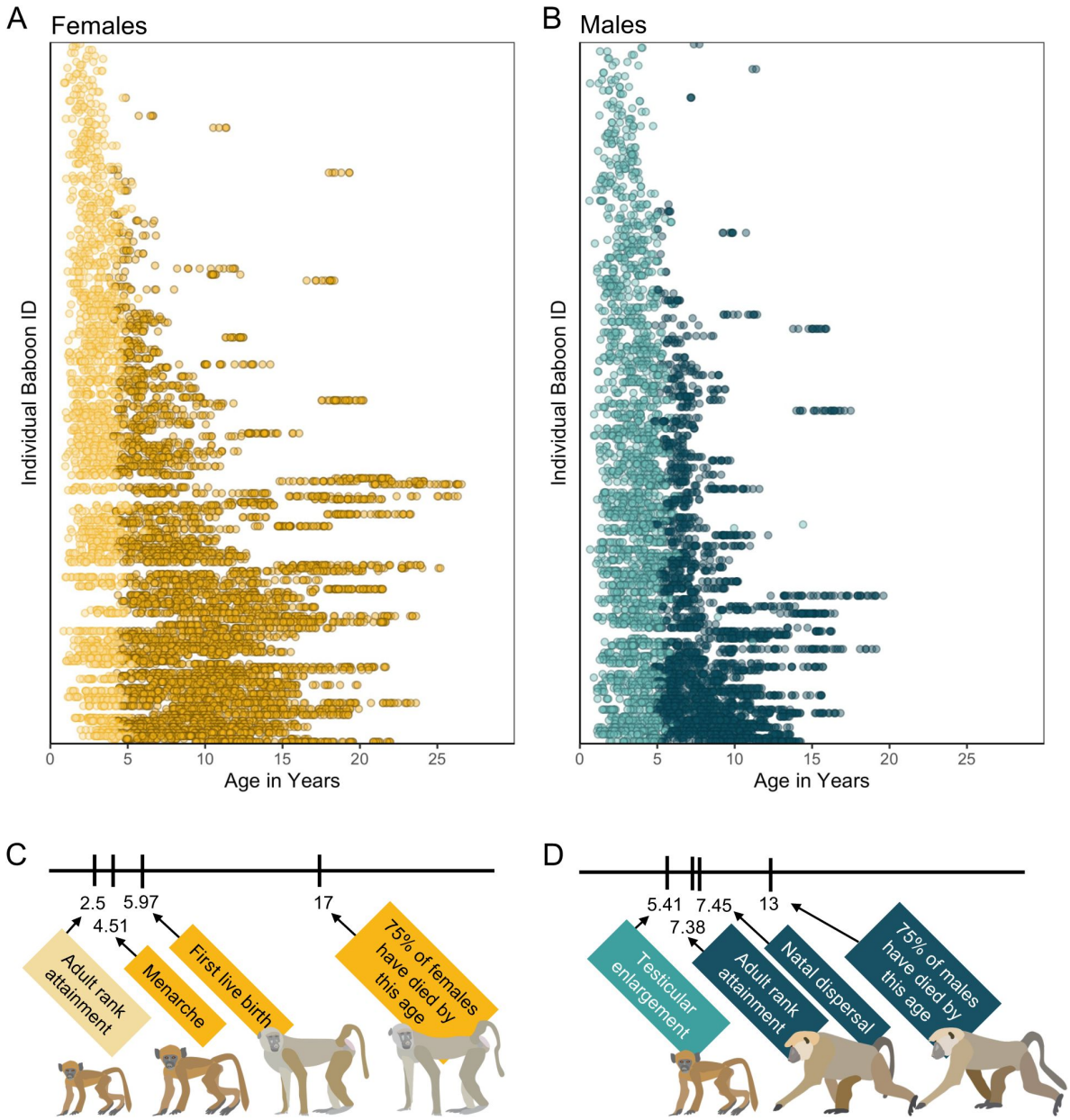

Figure 1.

Microbiome sampling time series and developmental milestones for the Amboseli baboons.

Plot (A) shows microbiome samples from female baboons, and plot (B) shows samples from male baboons. Each point represents a microbiome sample from an individual subject (y-axis) collected at a given host age in years (x-axis; n=8,245 samples from 234 females shown in yellow; n=5,231 samples from 197 males shown in blue). Light and dark point colors indicate whether the baboon was sexually mature at the time of sampling, with lighter colors reflecting samples collected prior to menarche for females (n=2,016 samples) and prior to testicular enlargement for males (n=2399 samples). Due to natal dispersal in males, we have fewer samples after the median age of first dispersal in males (n=1,705 samples, 12.6% of dataset) than from females after the same age (n=4408 samples, 32.6% of dataset). The timelines below the plots indicate the median age in years at which (C) female baboons attain the developmental milestones analyzed in this paper—adult rank, menarche and first live birth—and (D) males attain adult rank, testicular enlargement, and disperse from their natal groups (55, 56). The age at which 75% of animals in the population have died is shown to indicate different life expectancies for females versus males (63). Baboon illustrations courtesy of Emily (Lee) Nonnamaker.

The baboons in our data set were members of the well-studied Amboseli baboon population in Kenya, which has continuous, individual-based data on early-life environments, maturational milestones, social relationships, and mortality (57–62). Relevant to measuring biological age, prior research in Amboseli has identified several demographic, environmental, and social conditions that predict physical condition, the timing of development, or survival, including sex, season, social status (i.e., dominance rank), and early-life adversity (53–55, 63–69). Consistent with the possibility that these associations arise from causal effects of harsh or stressful conditions on biological aging (70–72), and with the idea that microbiomes serve as a marker of biological age (7–10), we tested whether socio-environmental conditions predicted microbiome age estimates. Our predictions about the direction of these effects varied depending on the socio-environmental condition and the developmental stage of the animal (i.e., juvenile or adult).

In terms of sex, adult male baboons exhibit higher mortality than adult females. Hence, we predicted that adult male baboons would exhibit gut microbiomes that are old-for-age, compared to adult females (by contrast, we expected no sex effects on microbiome age in juvenile baboons).

In terms of season, the Amboseli ecosystem is a semi-arid savannah with a 5-month long dry season during which little rain falls, often leading to nutritional hardship (73). We predicted that samples from the dry season might appear to be old-for-age, compared to samples from the wet season due to nutritional stress in this difficult season.

In terms of social status, baboons experience linear, sex-specific hierarchies. Female ranks are nepotistic, with little social mobility, and low-rank is linked to low priority of access to food (55, 68, 74–76). In contrast, adult male rank is determined by strength and fighting ability and is dynamic across adulthood (77). High-ranking males experience high energetic costs of mating effort, have altered immune responses, and exhibit old-for-age epigenetic age estimates compared to low-ranking males (45, 64, 65). We expected that individuals who pay the largest energetic costs—low-ranking adult females and high ranking adult males (45)—would appear old-for-age.

In terms of early-life adversity, prior research in Amboseli has identified six conditions whose cumulative, and sometimes individual, effects predict adult female mortality, including maternal loss prior to age 4 years, drought in the first year of life, birth into an especially large social group, the presence of a close-in-age competing younger sibling, and having a low-ranking or socially isolated mother (64, 65, 67). For adult female baboons, experiencing multiple sources of adversity in early life is the strongest socio-environmental predictor of mortality; hence, we expected that these individuals would have old-for-age clock estimates in adulthood (58, 59, 64, 66, 67, 78). However, we also expected that some sources of early-life adversity might be linked to young-for-age gut microbiomes in juvenile baboons. For instance, famine is linked to gut microbial immaturity (35–38), and maternal social isolation might delay gut microbiome development due to less frequent microbial exposures from conspecifics. In contrast to the expectation that harsh early-life conditions are linked to old-for-age microbiomes in adult baboons, a viable alternative is that gut microbiome age might be better predicted by an individual's current environmental or social conditions (e.g., season or social status), rather than past events. Indeed, gut microbiomes are highly dynamic and can change rapidly in response to host diet or other aspects of host physiology, behavior, or environments (79–81). Such results would support recency models for biological aging (82, 83) and would be consistent with findings from a recent epigenetic clock study in Amboseli (45).

We began our analyses by identifying gut microbiome features that change reliably with host age. We then constructed a microbiome clock by comparing the performance of several supervised machine learning algorithms to predict host age from gut microbial composition in each sample from each host. We evaluated the clock's performance for male and female baboons and tested

whether deviations from clock performance were predicted by the baboons' social and environmental conditions (guided by the predictions outlined above). Lastly, we tested whether baboons with young-for-age gut microbiomes have correspondingly late developmental timelines or longer lifespans. In general, our results support the idea that a baboon's current socio-environmental conditions, especially their current social rank and the season of sampling, have stronger effects on microbiome age than early-life events—many of which occurred many years prior to sampling. As such, the dynamism of the gut microbiome may often overwhelm and erase early-life effects on gut microbiome age. Our work highlights the diversity of ways that social and environmental conditions shape microbiome aging in natural systems.

Results

Many microbiome features change with age

Before creating the microbiome clock, we characterized microbiome features that change reliably with the age of individual hosts. Our subjects were 479 known-age baboons (264 females and 215 males) whose microbiome taxonomic compositions were characterized using 13,476 fecal-derived 16S rRNA gene sequencing profiles over a 14-year period (**Figures 1A** and **1B**; baboon age ranged from 7 months to 26.5 years; 8,245 samples from females; 5,231 samples from males; range=3-135 samples per baboon; mean=35 samples per female and 26 samples per male).

We tested age associations for 1,440 microbiome features, including: (i) five metrics of alpha diversity; (ii) the top 10 principal components of Bray-Curtis dissimilarity (which collectively explained 57% of the variation in microbiome community composition); and (iii) centered log-ratio transformed abundances (84) of each microbial phylum (n=30), family (n=290), genus (n=747), and amplicon sequence variance (ASV) detected in >25% of samples (n=358; Table S1; see methods for details on centered log-ratio transformations). We tested these different taxonomic levels in order to learn whether the degree to which coarse and fine-grained designations categories were associated with host age. For each of these 1,440 features, we tested its association with host age by running linear mixed effects models that included linear and quadratic effects of host age and four other fixed effects: sequencing depth, the season of sample collection (wet or dry), the average maximum temperature for the month prior to sample collection, and the total rainfall in the month prior to sample collection (50, 51, 62). Baboon identity, social group membership, hydrological year of sampling, and sequencing plate (as a batch effect) were modeled as random effects.

We found that many aspects of microbiome community composition changed with host age (**Figure 2**; **Figure S1**). All alpha diversity metrics, except richness, the only unweighted metric, exhibited U-shaped relationships with age, with high values in early life and old age, and low values in young adulthood. While we should interpret this pattern with caution due to the small sample size beyond age 20 (n=18 females), this U-shaped pattern differs somewhat from patterns in humans and chimpanzees: most human populations exhibit an asymptotic increase in alpha diversity with age (85, 86) while in chimpanzees alpha diversity is highest in early life (23) (FDR < 0.05; **Figures 2C** and **2E**; **Table S1**). Further, seven of the ten principal components (PCs) of microbiome composition exhibited linear, and in some cases quadratic, relationships with age, with PC1, PC2, PC4 and PC6 exhibiting the strongest age-associations (FDR < 0.05; **Figures 2C** and **2F**; **Table S1**).

51.6% of the 1,440 features exhibited significant linear or quadratic relationships with host age (**Figure S1 and S2**; **Table S1**; FDR < 0.05). 60% of phyla (18 of 30) decreased proportionally with age, while only three phyla—Kiritimatiellaeota, Firmicutes, and Chlamydiae—increased proportionally with age (FDR < 0.05; **Table S1**). Similarly, 66% (66 of 100) of age-associated families and 55% (115 of 209) of age-associated genera exhibited declining proportions with age

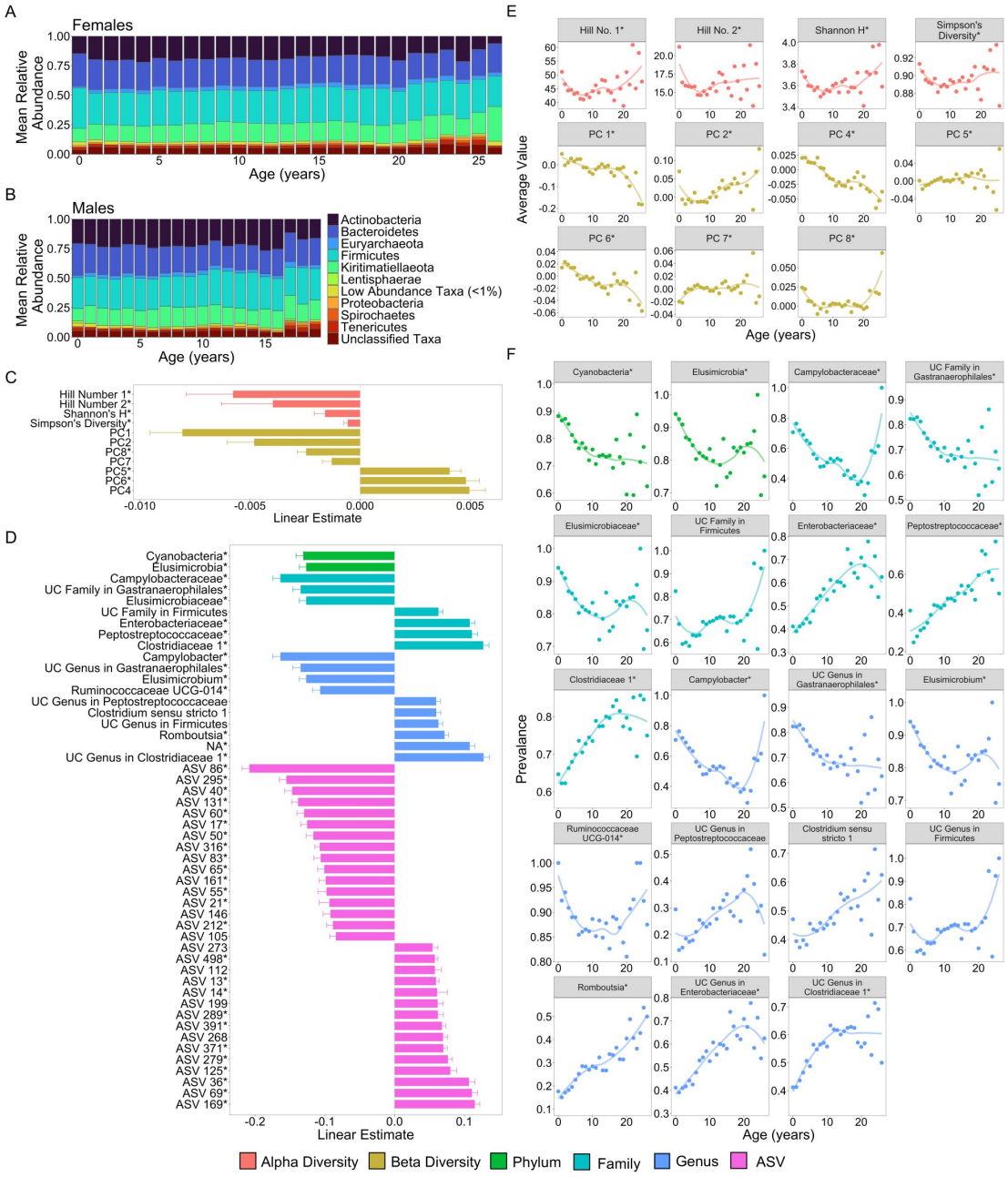

Figure 2

Microbiome features change with age.

(A) and (B) show the percent mean relative abundance of microbial phyla across life for females and males respectively. Panel (C) shows the estimates of the linear associations between mean-centered age for metrics of microbiome alpha diversity and principal components of microbiome compositional variation that exhibited significant associations with age (FDR < 0.05). Positive values are more abundant in older hosts. Panel (D) shows the estimate of the linear association between mean-centered age and the top 50 microbiome features that exhibited significant associations with age. Positive values are more abundant in older hosts. Panel (E) shows the average value of the microbiome features from (C) as a function of age, across all subjects. Note that sample sizes for patterns beyond age 20 years rely on 256 samples from just 18 females; hence, we interpret the pattern in these oldest animals with caution. Panel (F) shows the average prevalence of the higher taxonomic designations from (D) as a function of age, across all subjects. In (C-F) points are colored by the category of the feature (see legend). UC is an abbreviation for uncharacterized. Features that had a significant quadratic age term are indicated by * (see also **Figure S2; Table S1**).

(**Table S1**). Consistent with the idea that age-related taxa differ across host populations and host taxa, none of the taxa that changed in this baboon population were commonly age-associated in humans (85). The taxa most consistently linked to human aging include *Akkermansia, Faecalibacterium*, *Bacteroidaceae*, and *Lachnospiraceae* (34, 85) while in our sample of baboons, the strongest age-related changes were seen in the families *Campylobacteraceae, Clostridiaceae*, *Elusimicrobiaceae Enterobacteriaceae*, *Peptostreptococcaceae*, and an uncharacterized family within Gastranaerophilales (**Figure 2D** and **2F**; **Table S1**). The genera that had the strongest relationships with age included *Camplylobacter*, *Catenibacterium, Elusimicrobium*, *Prevotella, Romboutsia*, and *Ruminococcaceae UCG-011* (**Figure 2D** and **2F**; **Table S1**).

Microbiome clock calibration and composition

We next turned our attention to building a microbiome clock using all 9,575 microbiome compositional and taxonomic features present in at least 3 samples (**Table S2**; the 1,440 features discussed above only included taxa present in >25% of samples). We included all 9,575 microbiome features in our age predictions, as opposed to just those that were statistically significantly associated with age because removing these non-significant features could exclude features that contribute to age prediction via interactions with other taxa. In developing the clock, we compared the performance of three supervised machine learning methods to predict the chronological age of individual hosts at the time each microbiome sample was collected. The three machine learning methods were elastic net regression, Random Forest regression, and Gaussian process regression (see Supplementary Methods and Results). Because gut microbiomes are highly personalized in ours and other host populations (51), at least one sample from each host was always present in the training sets for these models (see Methods).

We found that the most accurate age predictions were produced by a Gaussian process regression model with a kernel customized to account for heteroscedasticity (**Figure 3**; **Figure S3**). This model predicted host chronological age (age_c), with an adjusted R^2 of 48.8% and a median error of 1.96 years across all individuals and samples (**Figure 3A**, **Table 1**). As has been observed in some previous age clocks (e.g., *39*, *44*, *46*), microbial age estimates (age_m) were compressed relative to the x=y line, leading the model to systematically over-predict the ages of young individuals and under-predict the ages of old individuals (**Figure 3A**).

When we subset our age_m estimates by sex, we found that the microbiome clock was slightly more accurate for males than for females (**Figure 3B**, **Table 1**). The adjusted R^2 for the correlation between age_c and age_m for males was 50.0%, with a median prediction error of 1.71 years as compared to an adjusted R^2 of 48.9% and median error of 2.15 years for female baboons (**Table 1**). Male baboons also exhibit significantly older gut microbial age than females (**Figure 3B**, chronological age by sex interaction: β=0.18, p<0.001, **Table S3**). Across the lifespan, males show a 1.4-fold higher rate of change in age_m as a function of age_c compared to females (relationship between age_c and age_m in males: β=0.63, p< 0.001; relationship between age_c and age_m in females: β=0.45, p < 0.001; **Table S4**). Similar to patterns from a recent epigenetic age-predicting clock developed for this population (45), this effect was only present after sexual maturity: when we subset the age_m estimates to microbiome samples collected prior to the median age at sexual maturity (5.4 years for testicular enlargement in males and 4.5 years for menarche in females; 54), we found no significant interaction between sex and age (age_c by host sex interaction prior to median age of maturity: β= -0.09, p=0.203; age_c by host sex interaction after median age of maturity: β=0.15, p<0.001; Table S3). After maturity, we recapitulate the 1.4-fold higher rate of change in males compared to females observed in the full data set (relationship between age_c and age_m in males only: β=0.53, p<0.001; relationship between age_c and age_m in females only: β=0.38, p<0.001; Table S4).

Figure 3.

Microbiome clock age predictions in wild baboons.

Panels (A) and (B) show predicted microbiome age in years (age_m) from a Gaussian process regression model, relative to each baboon's true chronological age in years (age_c) at the time of sample collection. Each point represents a microbiome sample. Panel (A) shows linear fit for all subjects in the model; (B) shows separate linear fits for each sex (**Table S4**). Dashed lines show the 1-to-1 relationship between age_c and age_m. Panel (A) also shows the measurement of sample-specific microbiome Δage compared to chronological age. Whether an estimate is old- or young-for-chronological age is calculated for each microbiome sample as the difference between age_m and age_c. Because of model compression relative to the 1-to-1 line, we correct for host chronological age by including chronological age in any model. An example of an old-for-age sample is shown as a red point, with dashed lines showing the value of age_c for a given sample with its corresponding age_m.

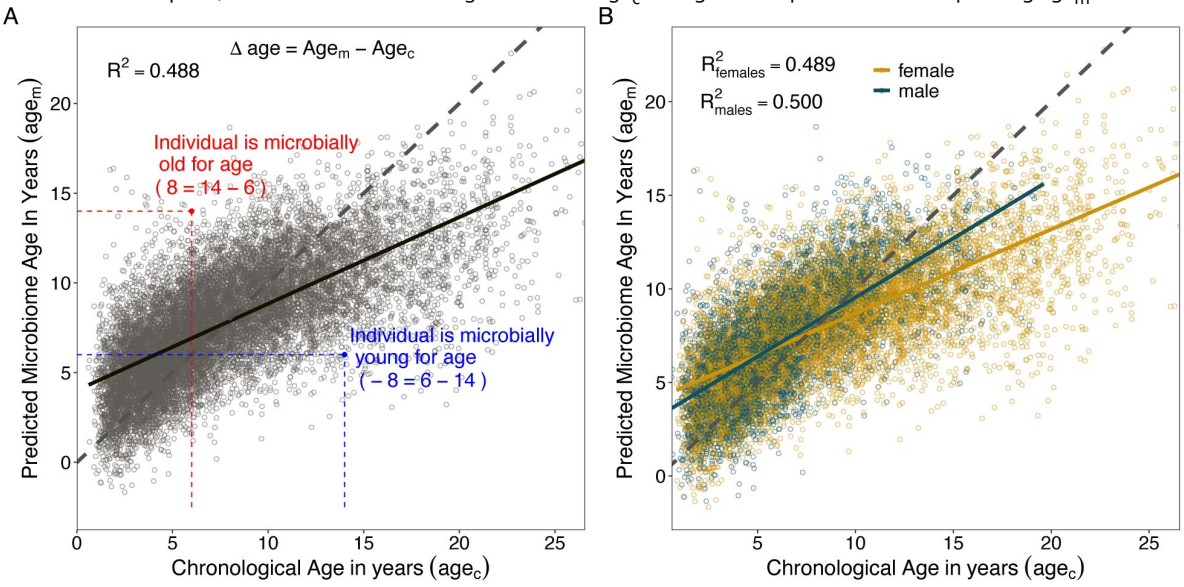

Table 1.

Comparison of Gaussian process regression model performance between sexes. Model accuracy was determined based on the correlation between known chronological age (age_c) and predicted age (age_m), the variance explained in age_c by age_m (R^2), and the median absolute difference between age_c and age_m

(40).

Subset	Sample Size	R^2	Pearson's R	Median Error (years)
All Subjects	13,476	48.8%	0.698	1.962
Females Only	8,245	48.9%	0.699	2.150
Males Only	5,231	50.0%	0.707	1.706

Overall, age_m estimates performed reasonably well compared to other known predictors of age in the Amboseli baboons (Table S5; [44]). Age_m performed similarly to the non-invasive physiology and behavior clock (NPB clock) recently developed for this population (87); R^2=51%; median error=2.33 years). Age_m was a stronger predictor of host chronological age than body mass index (BMI; except juvenile male BMI), blood cell composition from flow cytometry, and differential white blood cell counts from blood smears (Table S5; 45, 54, 87–89). However, age_m was a less accurate predictor of chronological age than both dentine exposure (males, females respectively: adjusted R^2=73%, 85%; median error=1.11 years, 1.12 years; Table S5) and an epigenetic clock based on DNA methylation data (males, females respectively: adjusted R^2=74%, 60%; median error=0.85 years, 1.62 years; Table S5; 44).

Social and environmental conditions predict variation in microbiome age

To test whether deviations in microbiome age for a given chronological age are correlated with socio-environmental predictors of health and mortality risk, we calculated whether microbiome age estimates from individual samples were older or younger than their hosts' known chronological ages (Δage in years; **Figure 3C**). We then tested whether several social and environmental variables predicted individual variation in microbiome Δage in years (**Table S7**; note that whether microbiome ages are old- or young-for-age is correlated with host age, hence our models always included host chronological age as a covariate). Overall, we expected that adult baboons who experienced harsh conditions in early-life adversity (the strongest socio-environmental predictor of adult mortality in Amboseli) would tend to look old-for-age based on the microbiome clock (58, 59, 64, 66, 67, 78). Alternatively, microbiome deviations from chronological age might be best predicted by an individual's current social status or season, rather than past events. These results would support recency models of biological aging (82, 83) and would be consistent with findings from a recent epigenetic clock study in Amboseli (45).

We found that individual baboons varied considerably in gut microbiome Δage. For instance, in mixed effects models, individual identity explained ~25% to ~50% of the variance in Δage for females and males respectively over the course of their lives (Table S6). Further, we found that season, dominance rank, and some aspects of early-life adversity (large group size, drought, and maternal social isolation) were linked to small deviations from chronological age. In support of our expectation that microbiome samples collected in the dry season are old-for-chronological age, we found that age estimates based on microbiome samples collected from female baboons in the dry season were ~2 months older than the host's true chronological age (β=-0.180, p=0.021, **Table 2**; **Table S6C; Figure S6A**). However, season did not significantly predict the difference between microbiome age and known age in male baboons.

In terms of social status, we expected to observe that low-ranking females and high-ranking males would be old-for-chronological age (45). In support, we found that estimates from high-ranking males were old-for-age compared to estimates from low-ranking males, but these effects were relatively weak and noisy (rank effect: β=0.033, p<0.001; **Figure 4A**; **Table 2**; **Tables S6B and D**). Specifically, controlling for chronological age, alpha male gut microbiomes (ordinal rank=1) appeared to be approximately 4 months older than microbiomes sampled from males with an ordinal rank of 10 (**Table 2**). However, contrary to our expectations, high-ranking female baboons also had old-for-age estimated when compared to low-ranking females (rank effect: β=1.745, p<0.001; **Figure 4B**; **Table 2**; **Tables S6A and C**). Specifically, controlling for chronological age, the microbiome of an alpha female (proportional rank=1) appeared to be approximately 1.75 years older than the lowest-ranking females in the population (proportional rank=0; **Table 2**).

Fixed Effect	β	p-value	Interpretation
Predictors of microbiome Δage in females (n=6,743 samples from 192 females)			
Chronological age	-0.551	<0.001	Included to control for the correlation between chronological age and microbiome Δage (Fig. 3)
Season	-0.180	0.021	Dry season samples are microbially old-for-age
Proportional rank*	1.745	<0.001	Low-ranking females are microbially young-for-age
Predictors of microbiome Δage in males (n=4,355 samples from 168 males)			
Chronological age	-0.404	<0.001	Included to control for the correlation between chronological age and microbiome Δage (Fig. 3)
Ordinal rank**	0.033	<0.001	Low-ranking males are microbially young-for-age
Born in a drought	-0.451	0.021	Males born during a drought are microbially young-for-age
Born into a large group	0.471	0.033	Males born into large groups are microbially old-for-age
Socially isolated mother	-0.395	0.006	Males with a socially isolated mother are microbially young-for-age

*proportional rank ranges from 0 to 1, with higher values reflecting higher social status
**ordinal rank is an integer ranking, with lower values reflecting higher social status; we have inverted the sign of the coefficient so higher numbers reflect higher rank to facilitate comparison to females

Table 2.

Social and environmental factors predicting variation in microbiome Δage in female and male baboons. Models below only show variables that minimize the Akaike information criterion (AIC) for each model; see Table S6 for full models. Coefficients for social dominance rank are transformed so higher values reflect higher rank/social status (see footnotes).

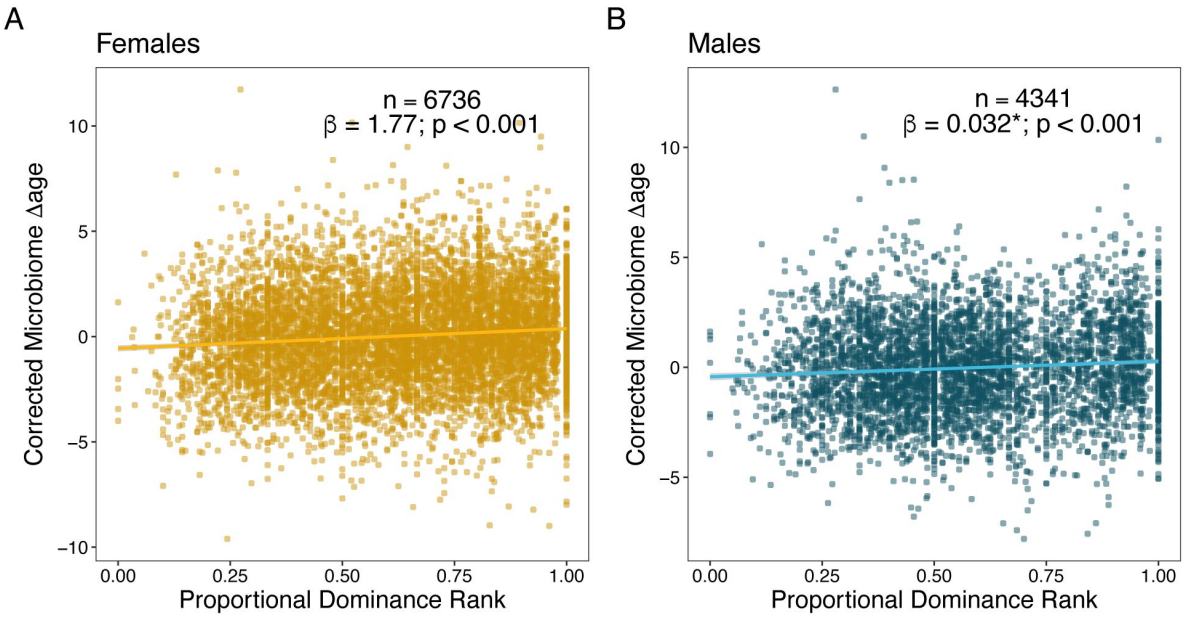

Figure 4.

Social dominance rank predicts gut microbiome Δage in male and female baboons (corrected for confounders).

Panels (A) and (B) show the relationship between host proportional dominance rank and corrected gut microbiome Δage in (A) males (blue points) and (B) females (yellow points). Each point represents an individual gut microbiome sample. Corrected microbiome Δage is calculated as the residuals of age_m correcting for host chronological age, season, monthly temperature, monthly rainfall, and social group and hydrological year at the time of collection.

Some forms of early-life adversity also predicted variation in microbiome Δage, but only in males, and in inconsistent directions. For instance, males born into the highest quartile of observed group sizes had old-for-age estimates. Males experiencing this source of early-life adversity had gut microbiomes that were predicted to be ~5.4 months older than males not experiencing this source of adversity (β=0.471, p=0.033, **Table 2**; **Table S6D; Figure S6C**). However, early-life drought and maternal social isolation were linked to young-for-age gut microbiomes in males (drought effect: β=-0.451, p=0.021; maternal social isolation effect: β=-0.395, p=0.006, **Table 2**; **Table S6D; Figure S6D**). Probably as a result of these conflicting effects, we found no effect of cumulative early adversity on microbiome Δage in males (**Table S6B**).

Microbiome age does not predict the timing of development or survival

Finally, we tested whether variation in microbiome Δage predicted the timing of individual maturational milestones or survival using Cox proportional hazards models (**Table S8**). Maturational milestones for females were the age at which they attained their first adult dominance rank, reached menarche, or gave birth to their first live offspring (**Figure 1C**). Male maturational milestones were the age at which they attained testicular enlargement, dispersed from their natal social group, or attained their first adult dominance rank (**Figure 1D**). We also tested if microbiome Δage predicted juvenile survival (in females and males) or adult survival (females only). We did not test adult survival in males because male dispersal makes it difficult to know age at death for most males (90).

Contrary to our expectations, microbiome Δage did not predict the timing of any baboon developmental milestone or measure of survival (Tables S9, S10). However, these patterns should be treated with caution, as reflected by the large number of censored animals, large hazard ratios, and small sample sizes for some tests.

Discussion

We report three main findings. First, similar to humans and other animals (11–24), baboon gut microbiomes show age-related changes in taxonomic composition that produce a dependable microbiome-based age predictor—a microbiome clock. This clock explains nearly half the variance in true host chronological age, and variation in its age predictions recapitulate well-known patterns of faster male senescence in humans and other primates (91). Second, parallel to a recent epigenetic clock in the Amboseli baboons (45), deviations from microbiome age predictions are explained by socio-environmental conditions experienced by individual hosts, especially recent conditions, although the effect sizes are small and are not always directionally consistent. Notably, recent social competition as reflected in social dominance rank predicts both microbiome and epigenetic age. Third, microbiome age did not predict the timing of individual development or survival (but caution is warranted because sample sizes were small for some tests). Hence, in our host population, gut microbial age reflects current social and environmental conditions, but not necessarily the pace of development or mortality risk.

To date, at least five other microbiome clocks have been built—all in human subjects—that predict host chronological age (46–49). Compared to these clocks, our clock in baboons has comparable or better predictive power, with a median error of 1.96 years, compared to 6 to 11 years in human age-predicting clocks ((46–49); baboon lifespans are approximately one third of a human lifespan). Age prediction from gut microbial composition may be more successful in baboons than humans for at least three reasons. First, signs of age in the baboon gut microbiome may be more consistent across hosts, perhaps because of the relative homogeneity in host environments and lifestyles in baboons compared to humans (51). Second, the baboon clock relies on dense longitudinal sampling for each host, and because the training data set included at

least one sample from each host, the microbiome clock may be better able to address personalized microbiome compositions and dynamics than clocks that rely on cross-sectional data (e.g. 46–48). However, because our training data set was not naïve to information from the host being predicted, this approach could leak information between the training and test set. Third, our Gaussian process regression approach allowed us to account for non-linear and interactive relationships between microbes with age, leveraging a wider variety of age-related signatures in the microbiome than other machine learning approaches (e.g., elastic net regression or random forest regression).

Despite the relative accuracy of the baboon microbiome clock compared to similar clocks in humans, our clock has several limitations. First, the clock's ability to predict individual age is lower than for age clocks based on patterns of DNA methylation—both for humans and baboons (40–43, 45). One reason for this difference may be that gut microbiomes can be influenced by several non-age-related factors, including social group membership, seasonal changes in resource use, and fluctuations in microbial communities in the environment. Second, gut microbiomes are highly personalized: each host species, population, and even host individuals within populations have distinctive, characteristic microbiomes, which likely limits the utility of our clock beyond our study population (51, 60, 62, 92). To make a more generalizable clock, an important next step would be to train the clock on data from many more host populations and incorporate features of the gut microbiome that are broader and more universal across host species and populations. Third, the relationships between potential socio-environmental drivers of biological aging and the resulting biological age predictions were inconsistent. For instance, some sources of early-life adversity were linked to old-for-age gut microbiomes (e.g., males born into large social groups), while others were linked to young-for-age microbiomes (e.g., males who experienced maternal social isolation or early-life drought), or were unrelated to gut microbiome age (e.g., males who experienced maternal loss; any source of early-life adversity in females).

The most consistent socio-ecological driver of baboon microbiome age was individual dominance rank. Microbiome samples from high-ranking males and females both appeared old-for-age. These results are interesting considering a growing body of evidence that finds rank-related differences in immunity and metabolism, including costs of high rank, especially for males (45, 64, 65, 93–95). For instance, in Amboseli, high social status in males is linked to old-for-age epigenetic age estimates (45), differences in immune regulation (65, 93), and, for alpha males, elevated glucocorticoid levels (64). These patterns, together with our evidence that high-ranking males tend to look 'old-for-age', are consistent with the idea that high-ranking males pursue a "live fast die young" life history strategy (45).

However, we also found evidence for old-for-age microbiome age estimates in samples from high-ranking females who do not seem to be "living fast" in the same sense as high-ranking males (indeed alpha females have *lower* glucocorticoid hormones than other females (96)). This outcome points towards a shared driver of high social status in shaping gut microbiome age in both males and females. While it is difficult to identify a plausible shared driver, one benefit shared by both high-ranking males and females is priority of access to food. This access may result in fewer foraging disruptions and a higher quality, more stable diet. At the same time, prior research in Amboseli suggests that as animals age, their diets become more canalized and less variable (50). Hence aging and priority of access to food might both be associated with dietary stability and old-for-age microbiomes. However, this explanation is speculative and more work is needed to understand the relationship between rank and microbiome age.

While some social and environmental conditions associated with baboon development or mortality predict microbiome age, our microbiome clock predictions do not themselves predict baboon development or mortality. This finding supports the idea that microbiome age is sensitive to transient social and environmental conditions; However, these patterns do not have long-term

consequences for development and mortality. One reason for this may be that the biological drivers of development and mortality are too diverse to be well reflected in gut microbial communities. For instance, animals in Amboseli die for many reasons, including interactions with predators and humans, conflict with conspecifics (97), and disease, and the biological predictors of these events in the gut microbiome are likely weaker or more diverse than the biological signals that predict developmental milestones (i.e., sex steroids, growth hormones, metabolic status, and physical condition).

Three important future directions of our work will be to test: (i) whether microbiome age is correlated with other hallmarks of biological age in this population; (ii) whether it is possible to build a microbiome-based predictor of individual lifespan (i.e., *remaining* lifespan as opposed to years already lived); and (iii) the relationships between microbiome compositional features and individual survival. While several metrics of biological age have been developed for this population (45, 54, 87–89), testing the correlations between them is complex because these metrics were measured for different sets of subjects at different ages and with different sampling designs (e.g., longitudinal versus cross-sectional measures). We also hope to measure epigenetic age in fecal samples, leveraging methods developed in Hanski et al. (98).

In sum, our findings support the hypothesis that the gut microbiome is a biomarker of some aspects of host age. By leveraging microbial, social, environmental, and life history data on host individuals followed from birth to death, we bolster the validity of microbiome clock studies in humans and find some socio-environmental predictors of microbiome age. Future work may benefit from searching for more universal aspects of the microbiome that may predict host aging across populations and even host species.

Materials and methods

Study population and subjects

Our study subjects were 479 wild baboons (215 males and 264 females) living in the Amboseli ecosystem in Kenya between April 2000 to September 2013. The Amboseli baboon population is primarily composed of yellow baboons (*Papio cynocephalus*) with some admixture from nearby anubis baboon (*Papio anubis*) populations (99–101). Prior research in our population finds no link between host hybrid ancestry and microbiome composition (92). Since 1971, the Amboseli Baboon Research Project (ABRP) has been collecting continuous observations of the baboons' demography, behavior, and environment (73). The baboons are individually identified by expert observers who visit and collect data on each social group 3 to 4 times per week (the subjects lived in up to 12 different social groups over the study period). During each monitoring visit, the observers conduct group censuses and record all demographic events, including births, maturation events, and deaths, allowing us to calculate age at maturity and lifespan with precision. This research was approved by the IACUC at Duke University, University of Notre Dame, and Princeton University and the Ethics Council of the Max Planck Society and adhered to all the laws and guidelines of Kenya.

Sample collection, DNA extraction, and 16S data generation

The 13,476 gut microbiome compositional profiles in this analysis represent a subset of 17,277 profiles, which were previously described in (50, 51). The 13,476 samples in our analyses include those from baboons whose birthdates, and hence individual ages, were known with just a few days' error. Each baboon had on average 33 samples collected across 6 years of their life (**Figure 1A** and **1B**; range=3-135 samples per baboon; median days between samples=44 days).

Samples were collected from the ground within 15 minutes of defecation. For each sample, approximately 20 g of feces was collected into a paper cup, homogenized by stirring with a wooden tongue depressor, and a 5 g aliquot of the homogenized sample was transferred to a tube containing 95% ethanol. While a small amount of soil was typically present on the outside of the fecal sample, mammalian feces contains 1000 times the number of microbial cells in a typical soil sample (102, 103), which overwhelms the signal of soil bacteria in our analyses (92). Samples were transported from the field in Amboseli to a lab in Nairobi, freeze-dried, and then sifted to remove plant matter prior to long term storage at -80°C.

DNA from 0.05 g of fecal powder was manually extracted using the MoBio (Catalog No. 12955-12) and QIAGEN (Catalog No. 12955-4) PowerSoil HTP kits for 96-well plates using a modified version of the MoBio PowerSoil-HTP kit. Specifically, we followed the manufacturers' instructions but increased the amount of PowerBead solution to 950 μL/well and incubated the plates at 60°C for 10 minutes after the addition of PowerBead solution and lysis buffer C1. We included one extraction blank per batch, which had significantly lower DNA concentrations than sample wells (t-test; t=-50, p < 2.2×10-16; 50). We also included technical replicates, which were the same fecal sample sequenced across multiple extraction and library preparation batches. Technical replicates from different batches clustered with each other rather than with their batch, indicating that true biological differences between samples are larger than batch effects.

Following DNA extraction, a ~390 bp segment of the V4 region of the 16S rRNA gene was amplified and libraries prepared following standard protocols from the Earth Microbiome Project (104). Libraries were sequenced on the Illumina HiSeq 2500 using the Rapid Run mode (2 lanes per run). Sequences were single indexed on the forward primer and 12 bp Golay barcoded. The resulting sequencing reads were processed following a DADA2 pipeline (105), with the following additional quality filters: we removed samples with low DNA extraction concentrations (< 4x the plate's blank DNA extraction concentration), samples with <1,000 reads, and amplicon sequence variants that appeared in one sample (see (50) for details). ASVs were assigned to microbial taxa using the IdTaxa(…) function in the DECIPHER package, against the Silva reference database SILVA_SSU_r132_March2018.RData (106, 107). The final set of samples had 1,017 to 427,454 reads (median =51,839 reads), with 8,492 total ASVs.

Identifying microbiome features that contribute to age predictions and that change with age

To identify microbiome features that change with host age, we ran linear mixed models on 1,440 microbiome features (Table S1). Models were run using the R package *lme4*, with p-value estimates from *lmerTest* (108, 109). These features included: (i) five metrics of alpha diversity; (ii) the top 10 principal components of microbiome compositional variation; (iii) centered log ratio (CLR) transformed abundances (i.e., read counts) of each microbial phyla (n=30), family (n=290), genus (n=747), and amplicon sequence variance (ASV) detected in >25% of samples (n=358). CLR transformations are a recommended approach for addressing the compositional nature of 16S rRNA amplicon read count data (84). Alpha diversity metrics were calculated using the R package *vegan* and principal components of microbiome compositional variation were calculated using the R package *labdsv* (110, 111).

For each feature, we modeled its relationship to host chronological age using both linear and quadratic terms. To make our quadratic terms more interpretable, we centered our age estimates on zero by subtracting the average age in the dataset from each age value. Specifically, when a quadratic term is negative, the curve is concave, whereas when the term is positive, the curve is convex. We also included season (wet or dry) and z-scored read count, rainfall, and temperature as fixed effects, and individual identity, social group at time of collection, hydrological year, and the DNA extraction/PCR plate identity were modeled as random effects. We did not model individual social network position because prior analyses of this data set find no evidence that close social

partners have more similar gut microbiomes, probably because we lack samples from close social partners sampled close in time (50, 51). All community features (i.e., alpha diversity and principal components) and all taxa present in >25% of samples were modeled using a Gaussian error distribution. We extracted the coefficient, standard error, and p-value for the age term, then corrected for multiple testing using the false discovery rate approach of Benjamini and Hochberg (112).

Building the gut microbiome clock

We created a microbiome clock by fitting a Gaussian process (GP) regression model (with a kernel customized to account for heteroskedasticity) to predict each baboon's chronological age at the time of sample collection using 9,575 microbiome compositional and taxonomic features present in at least 3 samples (Table S2; i.e., we did not restrict the features in the clock to the 1,440 most abundant features used in the age-association analyses described above). The GP regression model with heteroskedasticity correction was the best performing of four supervised machine learning approaches we considered, including elastic net, random forest, and Gaussian process regression with and without the heteroskedasticity kernel (Figure S3; Table S11; See Supplemental Methods for a comparison of other algorithms). Pearson's correlations between age predictions across the four methods ranged from 0.69 between the random forests and the GP regression model without the heteroskedasticity kernel to 0.96 between the two GP regression models (with and without the heteroskedasticity kernel; Table S11).

Gaussian process regressions were conducted in Python 3 using scikit-learn (113, 114). As a nonparametric, Bayesian approach that infers a probability distribution over all the potential functions that fit the data, the Gaussian process regression does not assume a linear relationship between chronological and predicted age (115). For the prior distribution in the Gaussian process regression, we used a radial basis function as our kernel and set the scale parameter to the mean Euclidean distance of the dataset, as calculated in the R package vegan (110). Because initial, exploratory models exhibited heteroskedasticity (Figure S7), we multiplied the variance in the training data by the radial basis function, which distributed the higher variance in later life more evenly across lifespan.

To calculate a microbial age estimate for every sample, and to estimate generalization error, we used nested five-fold cross validation. In each of the five model runs, we used 80% of the data to train the model, and the remaining 20% of the dataset as the test data. Because host identity has a strong effect on microbiome composition in our population (51), we distributed samples from each host across the five test/training data sets by randomly assigning each sample a test set without replacement. Hence, the training data set was not naïve to information from the given host being predicted as some samples for that host were included in the training set. For each model run, four of the test datasets were treated altogether as training data and the 5th set was the validation test set. We then took the estimates from all five model runs and estimated global model accuracy on the aggregated estimates.

We assessed the accuracy of our microbiome clock by regressing each sample's chronological age (age_c) against the model's predicted microbial age (age_m) and determining the R^2 value and Pearson's correlation between age_c and age_m. We also calculated the median error of the model fit as the median absolute difference between age_c and age_m across all samples (40).

Calculating microbiome Δage estimates

To characterize patterns of microbiome age from our microbiome clock, we calculated sample-specific microbiome Δage in years as the difference between a sample's microbial age estimate, age_m from the microbiome clock, and the host's chronological age in years at the time of sample collection, age_c. Higher microbiome Δages indicate old-for-age microbiomes, as $age_m > age_c$, and lower values (which are often negative) indicate a young-for-age microbiome, where $age_c > age_m$

(see **Figure 3**). Because the microbiome clock systematically over predicted the ages of young animals and under predicted the ages of old animals), we also calculated a "corrected microbiome Δage" as the residuals of age_m correcting for host chronological age, season, monthly temperature, monthly rainfall, and social group and hydrological year at the time of collection. This measure is used for visualizations of the predictors of microbiome age and for testing whether average microbiome Δage predicts developmental milestones or survival.

Testing sources of variation in microbiome Δage

Many social and environmental factors have been shown to predict fertility and survival in the Amboseli baboons (54, 59, 67, 68, 75). To test if some of the most important known factors also predict patterns of microbiome age, we used linear mixed models to test predictors of microbiome age in individual samples separately for males and females.

In these models, the response variable was the sample-specific measure of Δage (age_m - age_c). All models included the following fixed effects: individual chronological age at the time of sample collection, to correct for model compression; the average maximum temperature during the 30 days before the sample was collected, total rainfall during the 30 days before the sample was collected, and the season (wet or dry) during sample collection. Every model also included, as fixed effects, measures of early-life adversity the individual experienced prior to 4 years of age (Table S7). These were modeled as either six, individual, binary variables, reflecting the presence or absence of each source of adversity in the first four years of life, or as a cumulative sum of the number of sources of adversity the individual experienced, also in the first four years of life (67). Social rank at the time of sampling was also modeled as a fixed effect. For males we used ordinal rank, and for females we used proportional rank (116). To make model interpretation more intuitive (high rank corresponds to higher values), we multiplied the coefficients for ordinal rank and maternal rank by -1. Random effects included individual identity, the social group the individual lived in at the time of collection, and hydrological year. In models of microbiome age in females, the number of adult females in the group at the time of sample collection was included as female-specific measure of resource competition.

Testing whether microbiome Δage predicts baboon maturation and survival

We used Cox proportional hazards models to test whether microbiome Δage predicted the age at which females and males attained maturational milestones and the age at death for juveniles and adult females (Table S8). We only measured adult survival in females because males disperse between social groups, often repeatedly across adulthood, making it is difficult to know if male disappearances are due to dispersal or death (90). For females, the maturational milestones of interest were the age at adult rank attainment (median age 2.24 in Amboseli), age at menarche (median age 4.51 in Amboseli), and the age at first live birth (median age 5.97 in Amboseli). For males, these milestones were the age of testicular enlargement (median age 5.38 in Amboseli), the age of dispersal from natal group (median age 7.47 in Amboseli), and the age of first adult rank attainment (i.e., when a male first outranks another adult male in his group's dominance hierarchy; median age 7.38 in Amboseli) (55, 56). See full descriptions of each milestone in Table S8. To be included in these analyses, animals must have reached the milestone after the onset of sampling (April 2000) and had at least three samples available in the timeframe of interest. We verified that none of our models violated the proportional hazards assumption of a Cox regression.

The variables we modeled differed based on the event of interest. However, all models included as fixed effects corrected Δage as the residuals of gut microbiome Δage averaged over the timeframe. All models of developmental milestones also included variables tested in Charpentier et al. 2008 (55, 56): (iii) maternal presence at the time of the milestone, (iv) the number of maternal sisters in the social group, averaged over the timeframe, (v) rainfall averaged over the timeframe,

and (vi) whether the subject's mother was low ranked (was in the lowest quartile for female ordinal rank). For female-specific milestones, we also included (vii) the average number of adult females in the group averaged over the timeframe, and for male-specific milestones we included the number of excess cycling females in the group averaged over the timeframe, or the difference between the number of cycling females and the number of mature males within a subject's social group. Last, we included (viii) the subject's hybrid score, which is an estimation of the proportion of an individual's genetic ancestry attributable to anubis or yellow baboon ancestry (101).

All juvenile survival models included as fixed effects the residuals of microbiome Δage averaged over the timeframe and measures the cumulative number of sources of early-life adversity each individual experienced (67). Additionally, we ran three versions of the juvenile survival analysis: two subset to each sex, and one version that included both sexes. In the model including both sexes, we included sex as a predictor.

Adult female survival models included the same variables as for juvenile survival, but additionally included average lifetime dyadic social connectedness to adult females, average lifetime dyadic social connectedness to adult males, and average lifetime proportional rank. Full descriptions of all predictors are available in Table S12.

Data and materials availability

All data for these analyses are available on Dryad at *https://doi.org/10.5061/dryad.b2rbnzspv*. The 16S rRNA gene sequencing data are deposited on EBI-ENA (project ERP119849) and Qiita (study 12949). Code is available at the following GitHub repository: *https://github.com/maunadasari/Dasari_etal-GutMicrobiomeAge*

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Additional files

Supplementary Text

Supplementary Tables

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Reviewer #1 (Public review):

Summary:

The authors used a subset of a very large, previously generated 16S dataset to: 1) assess age-associated features; and 2) develop a fecal microbiome clock, based on extensive longitudinal sampling of wild baboons for which near-exact chronological age is known. They further seek to understand deviation from age-expected patterns and uncover if and why some individuals have an older or younger microbiome than expected, and the health and longevity implications of such variation. Overall, the authors compellingly achieved their goals to discover age-associated microbiome features and develop a fecal microbiome clock. They also showed clear and exciting evidence for sex and rank-associated variation in the pace of gut microbiome aging and impacts of seasonality on microbiome age in females. These data add to a growing understanding of modifiers of the pace of age in primates, and links among different biological indicators of age, with implications for understanding and contextualizing human variation. However, in the current version there are gaps in the analyses with respect to the social environment, and in comparisons with other biological indicators of age. Despite this, I anticipate this work will be impactful, generate new areas of inquiry and fuel additional comparative studies.

Strengths:

The major strengths of the paper are the size and sampling depth of the study population, including ability to characterize of the social and physical environments, and the application of recent and exciting methods to characterize the microbiome clock. An additional strength was the ability of the authors to compare and contrast the relative age-predictive power of the fecal microbiome clock to other biological methods of age estimation available for the study population (dental wear, blood cell parameters, methylation data). Furthermore, the writing and support materials are clear and informative and visually appealing.

Revisions made following initial review have further improved the content and clarity.

Weaknesses:

Revisions to the manuscript clarified some of the analysis decisions and limitations regarding drawing comparisons between the microbiome clock and other metrics of biological age, and on the impact of sociality on microbiome metrics. Hopefully these interesting topics will be further addressed in forthcoming publications.

https://doi.org/10.7554/eLife.102166.2.sa2

Reviewer #2 (Public review):

Summary:

Dasari et al present an interesting study investigating the use of 'microbiota age' as an alternative to other measures of 'biological age'. The study provides several curious insights into biological ageing. Although 'microbiota age' holds potential as a proxy of biological age, it comes with limitations considering the gut microbial community can be influenced various non-age related factors, and various age-related stressors may not manifest in changes in the gut microbiota.

Strengths:

The dataset this study is based on is impressive, and can reveal various insights into biological ageing and beyond. The analysis implemented is extensive and of high level.

Weaknesses:

The key weakness is the use of microbiota age instead of e.g., DNA-methylation based epigenetic age as a proxy of biological ageing, for reasons stated in the summary. DNA methylation levels can be measured from faecal samples, and as such epigenetic clocks too can be non-invasive.

In the first round of review, I provided authors a list of minor edits, which they have implemented in the revised version of the manuscript.

https://doi.org/10.7554/eLife.102166.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *The authors used a subset of a very large, previously generated 16S dataset to: (1) Assess age-associated features; and (2) develop a fecal microbiome clock, based on an extensive longitudinal sampling of wild baboons for which near-exact chronological age is known. They further seek to understand deviation from age-expected patterns and uncover if and why some individuals have an older or younger microbiome than expected, and the health and longevity implications of such variation. Overall, the authors compellingly achieved their goals of discovering age-associated microbiome features and developing a fecal microbiome clock. They also showed clear and exciting evidence for sex and rank-associated variation in the pace of gut microbiome aging and impacts of seasonality on microbiome age in females. These data add to a growing understanding of modifiers of the pace of age in primates, and links among different biological indicators of age, with implications for understanding and contextualizing human variation. However, in the current version, there are gaps in the analyses with respect to the social environment, and in comparisons with other biological indicators of age. Despite this, I anticipate this work will be impactful, generate new areas of inquiry, and fuel additional comparative studies.*

Thank you for the supportive comments and constructive reviews.

> *Strengths:*

> *The major strengths of the paper are the size and sampling depth of the study population, including the ability to characterize the social and physical environments, and the application of recent and exciting methods to characterize the microbiome clock. An additional strength was the ability of the authors to compare and contrast the relative age-predictive power of the fecal microbiome clock to other biological methods of age estimation available for the study population (dental wear, blood cell parameters, methylation data). Furthermore, the writing and support materials are clear, informative and visually appealing.*
>
> *Weaknesses:*
>
> *It seems clear that more could be done in the area of drawing comparisons among the microbiome clock and other metrics of biological age, given the extensive data available for the study population. It was confusing to see this goal (i.e. "(i) to test whether microbiome age is correlated with other hallmarks of biological age in this population"), listed as a future direction, when the authors began this process here and have the data to do more; it would add to the impact of the paper to see this more extensively developed.*

Comparing the microbiome clock to other metrics of biological age in our population is a high priority (these other metrics of biological age are in Table S5 and include epigenetic age measured in blood, the non-invasive physiology and behavior clock (NPB clock), dentine exposure, body mass index, and blood cell counts (Galbany et al. 2011; Altmann et al. 2010; Jayashankar et al. 2003; Weibel et al. 2024; Anderson et al. 2021)). However, we have opted to test these relationships in a separate manuscript. We made this decision because of the complexity of the analytical task: these metrics were not necessarily collected on the same subjects, and when they were, each metric was often measured at a different age for a given animal. Further, two of the metrics (microbiome clock and NPB clock) are measured longitudinally within subjects but on different time scales (the NPB clock is measured annually while microbiome age is measured in individual samples). The other metrics are cross-sectional. Testing the correlations between them will require exploration of how subject inclusion and time scale affect the relationships between metrics.

We now explain the complexity of this analysis in the discussion in lines 447-450. In addition, we have added the NPB clock (Weibel et al. 2024) to the text in lines 260-262 and to Table S5.

> *An additional weakness of the current set of analyses is that the authors did not explore the impact of current social network connectedness on microbiome parameters, despite the landmark finding from members of this authorship studying the same population that "Social networks predict gut microbiome composition in wild baboons" published here in eLife some years ago. While a mother's social connectedness is included as a parameter of early life adversity, overall the authors focus strongly on social dominance rank, without discussion of that parameter's impact on social network size or directly assessing it.*

Thank you for raising this important point, which was not well explained in our manuscript. We find that the signatures of social group membership and social network proximity are only detectable our population for samples collected close in time. All of the samples analyzed in Tung et al. 2015 (“Social networks predict gut microbiome composition in wild baboons”) were collected within six weeks of each other. By contrast, the data set analyzed here spans 14 years, with very few samples from close social partners collected close in time. Hence, the effects of social group membership and social proximity are weak or undetectable. We described these findings in Grieneisen et al. 2021 and Bjork et al. 2022, and we now explain this logic on line 530, which states, “We did not model individual social

network position because prior analyses of this data set find no evidence that close social partners have more similar gut microbiomes, probably because we lack samples from close social partners sampled close in time (Grieneisen et al. 2021; Björk et al. 2022)."

We do find small effects of social group membership, which is included as a random effect in our models of how each microbiome feature is associated with host age (line 529) and our models predicting microbiome Dage (line 606; Table S6).

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *Dasari et al present an interesting study investigating the use of 'microbiota age' as an alternative to other measures of 'biological age'. The study provides several curious insights into biological aging. Although 'microbiota age' holds potential as a proxy of biological age, it comes with limitations considering the gut microbial community can be influenced by various non-age related factors, and various age-related stressors may not manifest in changes in the gut microbiota. The work would benefit from a more comprehensive discussion, that includes the limitations of the study and what these mean to the interpretation of the results.*

We agree and have text to the discussion that expands on the limitations of this study and what those limitations mean for the interpretation of the results. For instance, lines 395-400 read, "Despite the relative accuracy of the baboon microbiome clock compared to similar clocks in humans, our clock has several limitations. First, the clock's ability to predict individual age is lower than for age clocks based on patterns of DNA methylation—both for humans and baboons (Horvath 2013; Marioni et al. 2015; Chen et al. 2016; Binder et al. 2018; Anderson et al. 2021). One reason for this difference may be that gut microbiomes can be influenced by several non-age-related factors, including social group membership, seasonal changes in resource use, and fluctuations in microbial communities in the environment"

In addition, lines 405-411 now reads, "Third, the relationships between potential socio-environmental drivers of biological aging and the resulting biological age predictions were inconsistent. For instance, some sources of early life adversity were linked to old-for-age gut microbiomes (e.g., males born into large social groups), while others were linked to young-for-age microbiomes (e.g., males who experienced maternal social isolation or early life drought), or were unrelated to gut microbiome age (e.g., males who experienced maternal loss; any source of early life adversity in females)."

> *Strengths:*
>
> *The dataset this study is based on is impressive, and can reveal various insights into biological ageing and beyond. The analysis implemented is extensive and high-level.*
>
> *Weaknesses:*
>
> *The key weakness is the use of microbiota age instead of e.g., DNA-methylation-based epigenetic age as a proxy of biological ageing, for reasons stated in the summary. DNA methylation levels can be measured from faecal samples, and as such epigenetic clocks too can be non-invasive. I will provide authors a list of minor edits to improve the read, to provide more details on Methods, and to make sure study limitations are discussed comprehensively.*

Thank you for this point. In response, we have deleted the text from the discussion that stated that non-invasive sampling is an advantage of microbiome clocks. In addition, we now

propose a non-invasive epigenetic clock from fecal samples as an important future direction for our population (see line 450).

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations for the authors):***
>
> *Abstract - The opening 2 sentences are not especially original or reflective of the potential value/ premise of the study. Members of this team have themselves measured variation in biological age in many different ways, and the implication that measuring a microbiome clock is easy or straightforward is not compelling. This paper is very interesting and provides unique insight, but I think overall there is a missed opportunity in the abstract to emphasize this, given the innovative science presented here. Furthermore, the last 2 sentences of the abstract are especially interesting - but missing a final statement on the broader significance of research outside of baboons.*

We appreciate these comments and have revised the Abstract accordingly. The introductory sentences now read, “Mammalian gut microbiomes are highly dynamic communities that shape and are shaped by host aging, including age-related changes to host immunity, metabolism, and behavior. As such, gut microbial composition may provide valuable information on host biological age.” (lines 31-34). The last two sentences of the abstract now read, “Hence, in our host population, gut microbiome age largely reflects current, as opposed to past, social and environmental conditions, and does not predict the pace of host development or host mortality risk. We add to a growing understanding of how age is reflected in different host phenotypes and what forces modify biological age in primates.” (lines 40-43).

> *If possible, it would be highly useful to present some comments on concordance in patterns at different levels. Are all ASVs assessed at both the family and genus levels? Do they follow similar patterns when assessed at different levels? What can we learn about the system by looking at different levels of taxonomic assignment?*

The section on relationships between host age and individual microbiome features is already lengthy, so we have not added an analysis of concordance between different taxonomic levels. However, we added a justification for why we tested for age signatures in different levels of taxa to line 171, which reads, “We tested these different taxonomic levels in order to learn whether the degree to which coarse and fine-grained designations categories were associated with host age.”

> *To calculate the delta age - please clarify if this was done at the level of years, as suggested in Figure 3C, or at the level of months or portion months, etc?*

Delta age is measured in years. This is now clarified in lines 294, 295, and 578.

> *Spelling mistake in table S12, cell B4 (Octovber)*

Thank you. This typo has been corrected.

> *Given the start intro with vertebrates, the second paragraph needs some tweaking to be appropriate. Perhaps, "At least among mammals, one valuable marker of biological aging may lie in the composition and dynamics of the mammalian gut microbiome (7-10)." Or simply remove "mammalian".*

We have updated this sentence based on your suggestions in line 54. It reads, “In mammals, one valuable marker of biological aging may lie in the composition and dynamics of the gut

microbiome (Claesson et al. 2012; Heintz and Mair 2014; O'Toole and Jeffery 2015; Sadoughi et al. 2022)."

> *A rewrite at the end of the introduction is needed to avoid the almost direct repetition in lines 115-118 and 129-131 (including lit cited). One potentially effective way to approach this is to keep the predictions in the earlier paragraph and then more clearly center the approach and the overarching results statement in the latter paragraph. (I.e., "we find that season and social rank have stronger effects on microbiome age than early life events. Further, microbiome age does not predict host development or mortality.").*

Thank you for pointing this out. We have re-organized the predictions in the introduction based on your suggestion. The alternative "recency effects" model now appears in the paragraph that starts in line 110. The final paragraph then centers on the overall approach and the results statement (lines 128-140)

> *Be clear in each case where taxon-level trends are discussed if it's at Family, Genus, or other level. It's there most, but not all, of the time.*

We have gone through the text and clarified what taxa or microbiome feature was the subject of our analyses in any places where this was not clear.

> *In the legend for Figure 2, add clarification for how values to right versus left of the centered value should be interpreted with respect to age (e.g. "values to x of the center are more abundant in older individuals").*

We now clarify in Figure 2C and 2D that "Positive values are more abundant in older hosts".

> *Figure 3 - Are Panels A, B, and C all needed - can the value for all individuals not also be overlaid in the panel showing sex differences and the same point showing individuals with "old" and "young" microbiomes be added in the same plot if it was slightly larger?*

We agree and have simplified Figure 3. We reduced the number of panels from three to two, and we added the information about how to calculate delta age to Panel A. We also moved the equation from the top of Panel C to the bottom right of Panel A.

> ***Reviewer #2 (Recommendations for the authors):***
>
> *Dasari et al present an interesting study investigating the use of 'microbiota age' as an alternative to other measures of 'biological age'. The study provides several curious insights which in principle warrant publication. However, I do think the manuscript should be carefully revised. Below I list some minor revisions that should be implemented. Importantly, the authors should discuss in the Discussion the pros and cons of using 'microbiota age' as a proxy of 'biological age'. Further, the authors should provide more information on Methods, to make sure the study can be replicated.*

Thank you for these important points. Based on your comments and those of the first reviewer, we have expanded our discussion of the limitations of using microbiota age as a proxy for biological age (see edits to the paragraph starting in line 395).

We have also expanded our methods around sample collection, DNA extraction, and sequencing to describe our sampling methods, strategies to mitigate and address possible contamination, and batch effects. See lines 483-490 and our citations to the original papers where these methods are described in detail.

> *(1) Lines 85-99: I think this paragraph could be revisited to make the assumptions clearer. For instance, the last sentence is currently a little confusing: are authors expecting males to exhibit old-for-age microbiomes already during the juvenile period?*

This prediction has been clarified. Line 96 now reads, “Hence, we predicted that adult male baboons would exhibit gut microbiomes that are old-for-age, compared to adult females (by contrast, we expected no sex effects on microbiome age in juvenile baboons).”

> *(2) Lines 118-121: Could the authors discuss this assumption in relation to what has been observed e.g., in humans in terms of delays in gut microbiome development? Delayed/accelerated gut microbiome development has been studied before, so this assumption would be stronger if related to what we know from previous studies.*

This comment refers to the sentence which originally stated, “However, we also expected that some sources of early life adversity might be linked to young-for-age gut microbiota. For instance, maternal social isolation might delay gut microbiome development due to less frequent microbial exposures from conspecifics.” We have slightly expanded the text here (line 117) to explain our logic. We now include citations for our predictions. We did not include a detailed discussion of prior literature on microbiome development in the interest of keeping the same level of detail across all sections on our predictions.

> *(3) As the authors discuss, various adversities can lead to old-for-age but also young-for-age microbiome composition. This should be discussed in the limitations.*

We agree. This is now discussed in the sentence starting at line 371, which reads, “… deviations from microbiome age predictions are explained by socio-environmental conditions experienced by individual hosts, especially recent conditions, although the effect sizes are small and are not always directionally consistent.” In addition, the text starting at line 405 now reads, “Third, the relationships between potential socio-environmental drivers of biological aging and the resulting biological age predictions were inconsistent. For instance, some sources of early life adversity were linked to old-for-age gut microbiomes (e.g., males born into large social groups), while others were linked to young-for-age microbiomes (e.g., males who experienced maternal social isolation or early life drought), or were unrelated to gut microbiome age (e.g., males who experienced maternal loss; any source of early life adversity in females).”

> *(4) In various places, e.g., lines 129-131, it is a little unclear at what chronological age authors are expecting microbiota to appear young/old-for-age.*

This sentence was removed while responding to the comments from the first reviewer.

> *(5) Lines 132-133: this statement could be backed by stating that this is because the gut microbiota can change rapidly e.g., when diet changes (or whatever the authors think could be behind this).*

We have added an expository sentence at line 123, including new citations. This sentence reads, “Indeed, gut microbiomes are highly dynamic and can change rapidly in response to host diet or other aspects of host physiology, behavior, or environments”.

We now cite:

· Hicks, A.L., et al. (2018). Gut microbiomes of wild great apes fluctuate seasonally in response to diet. Nature Communications 9, 1786.

· Kolodny, O., et al. (2019). Coordinated change at the colony level in fruit bat fur microbiomes through time. Nature Ecology & Evolution 3, 116-124.

· Risely, A., et al. (2021) Diurnal oscillations in gut bacterial load and composition eclipse seasonal and lifetime dynamics in wild meerkats. Nat Commun 12, 6017.

> *(6) Lines 135-137: current or past season and social rank? This paragraph introduces the idea that it could be past rather than current socio-environmental factors that might predict microbiota age, so the authors should clarify this sentence.*

We have clarified the information in this sentence. line 135 now reads, “In general, our results support the idea that a baboon’s current socio-environmental conditions, especially their current social rank and the season of sampling, have stronger effects on microbiome age than early life events—many of which occurred many years prior to sampling.”

> *(7) Lines 136-137: this sentence could include some kind of a conclusion of this finding. What might this mean?*

We have added a sentence at line 138, which speculates that, “…the dynamism of the gut microbiome may often overwhelm and erase early life effects on gut microbiome age.”

> *(8) Use 'microbiota' or 'microbiome' across the manuscript; currently, the terms are used interchangeably. I don't have a strong opinion on this, although typically 'microbiota' is used when data comes from 16S rRNA.*

We have updated the text to replace any instance of “microbiota” with “microbiome”. We use the term microbiome in the sense of this definition from the National Human Genome Research Institute, which defines a microbiome as “the community of microorganisms (such as fungi, bacteria and viruses) that exists in a particular environment”.

> *(9) Figure 1 legend: make sure to unify formatting; e.g., present sample sizes as N= or n=, rather than both, and either include or do not include commas in 4-digit values (sample sizes).*

We have checked the formatting related to sample sizes and the use of commas in 4-digits in the main text and supplement. The formats are now consistent.

> *(10) Line 166: relative abundances surely?*

Following Gloor et al. (2017), our analyses use centered log-ratio (CLR) transformations of read counts, which is the recommended approach for compositional data such as 16S rRNA amplicon read counts. CLR transformations are scale-invariant, so the same ratio is obtained in a sample with few read versus many reads. We now cite Gloor et al. (2017) at line 169 and in the methods in line 517, which reads “centered log ratio (CLR) transformed abundances (i.e., read counts) of each microbial phyla (n=30), family (n=290), genus (n=747), and amplicon sequence variance (ASV) detected in >25% of samples (n=358). CLR transformations are a recommended approach for addressing the compositional nature of 16S rRNA amplicon read count data (Gloor et al. 2017).”

> *(11) Lines 167-172: were technical factors, e.g., read depth or sequencing batch, included as random effects?*

Thank you for catching this oversight in the text. We did model sequencing depth and batch effects. The sentence starting at line 173 now reads, “For each of these 1,440 features, we

tested its association with host age by running linear mixed effects models that included linear and quadratic effects of host age and four other fixed effects: sequencing depth, the season of sample collection (wet or dry), the average maximum temperature for the month prior to sample collection, and the total rainfall in the month prior to sample collection (Grieneisen et al. 2021; Björk et al. 2022; Tung et al. 2015). Baboon identity, social group membership, hydrological year of sampling, and sequencing plate (as a batch effect) were modeled as random effects."

> *(12) Lines 175-180: When discussing how these alpha diversity results relate to previous findings, the authors should be clear about whether they talk about weighted or non-weighted measures of alpha diversity. - also maybe this should be included in the discussion rather than the results? Please consider this when revisiting the manuscript (see how it reads after edits).*

Richness is the only unweighted metric, which we now clarify in line 181. We opted to retain the interpretation in the text in its original location to maintain the emphasis in the discussion on the microbiome clock results.

> *(13) Table S1 is very hard to interpret in the provided PDF format as columns are not presented side-by-side. It is currently hard to check model output for e.g., specific families. This needs to be revisited.*

We agree. We believe that eLife's submission portal automatically generates a PDF for any supplementary item. However, we also include the supplementary tables as an Excel workbook which has the columns presented side-by-side.

> *(14) Line 184: taxa meaning what? Unclear what authors refer to with this sentence, taxa across taxonomic levels, or ASVs, or what does the 51.6% refer to?*

We have edited line 191 to clarify that this sentence refers to taxa at all taxonomic levels (phyla to ASVs).

> *(15) Line 191: a punctuation mark missing after ref (81).*

We have added the missing period at the end of this sentence.

> *(16) Lines 189-197: this should go into the discussion in my opinion.*

We have opted to retain this interpretation, now at line 183.

> *(17) Lines 215-219: Not sure what this means; do the authors mean features were not restricted to age-associated taxa, ie also e.g., diversity and other taxa-independent patterns were included? If so, the rest of the highlighted lines should be revisited to make this clear, currently to me it is very unclear what 'These could include features that are not strongly age-correlated in isolation' means. Currently, that sounds like some features included were only age-associated in combination with other features, but unclear how this relates to taxa-dependency/taxa-independency.*

We agree this was not clear. We have revised line 224 to read, "We included all 9,575 microbiome features in our age predictions, as opposed to just those that were statistically significantly associated with age because removing these non-significant features could exclude features that contribute to age prediction via interactions with other taxa."

> *(18) Line 403-407: There is now a paper showing epigenetic clocks can be built with faecal samples, so this argument is not valid. Please revisit in light of this publication: https://onlinelibrary.wiley.com/doi/epdf/10.1111/mec.17330*

Thank you for bringing this paper to our attention. We deleted the text that describes epigenetic clocks as invasive, and we now cite this paper in line 450, which reads, “We also hope to measure epigenetic age in fecal samples, leveraging methods developed in Hanski et al. 2024.”

> *(19) Line 427: a punctuation mark/semicolon missing before However.*

We have corrected this typo.

> *(20) Lines 419-428: I don't quite understand this speculation. Why would the priority of access to food lead to an old-looking gut microbiome? This paragraph needs stronger arguments, currently unclear and also not super convincing.*

We agree this was confusing. We have revised this text to clarify the explanation. The text starting at line 424 now reads, “This outcome points towards a shared driver of high social status in shaping gut microbiome age in both males and females. While it is difficult to identify a plausible shared driver, one benefit shared by both high-ranking males and females is priority of access to food. This access may result in fewer foraging disruptions and a higher quality, more stable diet. At the same time, prior research in Amboseli suggests that as animals age, their diets become more canalized and less variable (Grieneisen et al. 2021). Hence aging and priority of access to food might both be associated with dietary stability and old-for-age microbiomes. However, this explanation is speculative and more work is needed to understand the relationship between rank and microbiome age.”

> *(21) Line 434: remove 'be'.*

We have corrected this typo.

> *(22) Line 478: add information on how samples were collected; e.g., were samples collected from the ground? How was cross-contamination with soil microbiota minimised? Were samples taken from the inner part of depositions? These factors can influence microbiota samples quite drastically so detailed info is needed. Also what does homogenisation mean in this context? How soon were samples freeze-dried after sample collection?*

We have expanded our methods with respect to sample collection. This text starts in line 483 and reads, “Samples were collected from the ground within 15 minutes of defecation. For each sample, approximately 20 g of feces was collected into a paper cup, homogenized by stirring with a wooden tongue depressor, and a 5 g aliquot of the homogenized sample was transferred to a tube containing 95% ethanol. While a small amount of soil was typically present on the outside of the fecal sample, mammalian feces contains 1000 times the number of microbial cells in a typical soil sample (Sender, Fuchs, and Milo 2016; Raynaud and Nunan 2014), which overwhelms the signal of soil bacteria in our analyses (Grieneisen et al. 2021). Samples were transported from the field in Amboseli to a lab in Nairobi, freeze-dried, and then sifted to remove plant matter prior to long term storage at -80°C.”

> *(23) Line 480 onwards: were negative controls included in extraction batches? Were samples randomised into extraction batches?*

Yes, we included extraction blanks. These are now described in lines 495-500. This text reads, "We included one extraction blank per batch, which had significantly lower DNA concentrations than sample wells (t-test; t=-50, p < 2.2x10-16; Grieneisen et al. 2021). We also included technical replicates, which were the same fecal sample sequenced across multiple extraction and library preparation batches. Technical replicates from different batches clustered with each other rather than with their batch, indicating that true biological differences between samples are larger than batch effects."

> *(24) Were extraction, library prep, and sequencing negative controls included? Is data available?*

We included extraction blanks (described above) and technical replicates, which were the same sample sequenced across multiple extraction and library preparation batches. Technical replicates from different batches clustered with each other rather than with their batch, indicating that true biological differences between samples are larger than batch effects.

We have updated the data availability statement to read, "All data for these analyses are available on Dryad at https://doi.org/10.5061/dryad.b2rbnzspv. The 16S rRNA gene sequencing data are deposited on EBI-ENA (project ERP119849) and Qiita (study 12949). Code is available at the following GitHub repository: https://github.com/maunadasari/Dasari_etal-GutMicrobiomeAge".

> *(25) Line 562: how were corrected microbiome delta ages calculated? Currently, the authors state x, y and z factors were corrected for, but it is unclear how this was done.*

The paragraph starting at line 577 describes how microbiome delta age was calculated. We have made only a few changes to this text because we were not sure which aspects of these methods confused the reviewer. However, briefly, we calculated sample-specific microbiome Dage in years as the difference between a sample's microbial age estimate, age_m from the microbiome clock, and the host's chronological age in years at the time of sample collection, age_c. Higher microbiome Dages indicate old-for-age microbiomes, as $age_m > age_c$, and lower values (which are often negative) indicate a young-for-age microbiome, where $age_c > age_m$ (see Figure 3).

> *(26) Line 579: typo 'as'.*

We have corrected this typo.

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