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Gut microbiota variations over the lifespan and longevity in rabbit's maternal lines

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In rabbit breeding, selection for production efficiency traits has been successful but has reduced rabbit functional longevity. The gut microbiota, which influences host health, is linked to longevity and undergoes significant changes with age. While previous studies have focused on young rabbits, research on gut microbiota changes in adult rabbits is limited. Understanding how gut microbiota evolves with age and its impact on longevity of does during reproductive life could offer insights into improving productivity, health and welfare. This study aims to investigate the evolution of gut microbiota through age and to compare different functional longevity groups between and within two maternal rabbit lines with different longevities; a standard commercial line (A) and another founded using longevity criteria (LP). Our analysis demonstrated a significant impact of age on the gut microbiome of does during their reproductive lifespan, with a decline in alpha diversity and change in beta diversity composition as age progressed. Differential abundance analysis revealed that 20% and 16% of taxa in lines A and LP, respectively, were influenced by age, predominantly showing a negative correlation. In terms of functional longevity, differences in abundance between groups were more pronounced within line A, with up to 16% of taxa differing between high-longevity HL (females with more than 10 parities) and low-longevity LL (females died/culled before 5th parity) groups, compared to only 4% within line LP, highlighting the role of genetic background in shaping microbiota composition and its potential influence on longevity. Finally, differences in microbiome between the two lines A and LP were consistent and maintained through their lifespan independently from their longevity. This study reveals that age significantly influences gut microbiome diversity and composition in adult female rabbits, leading to decreased alpha diversity and notable shifts in composition. Microbiome also differs according to functional longevity, with differences varying by genetic line. This suggests that using microbiome through selection or using specific taxa within it as biomarkers could be a promising avenue for improving longevity. Moreover, microbiome differences between genetic lines persist throughout life, even among animals with the same longevity.

Keywords Gut microbiota, Rabbits, Reproductive lifespan, Longevity.

Historically, animal breeding programs have primarily focused on enhancing economic efficiency by increasing production traits through directional selection. However, populations selected for high production efficiency led to individuals with more risk for physiological and immunological problems which means a deterioration of longevity[1–3]. For instance, dairy cows selected for high milk yield have reduced productive longevity, typically to less than four years, despite their natural lifespan of 20 years[2]. Similarly, reproductive sows face high culling rates, with up to 50% removed before reaching the third or fourth parity, failing to recover initial replacement costs[1]. A similar pattern is observed in rabbit breeding, where selection for high litter size at weaning in maternal lines has been successful[4]. However, selection for this trait, along with other economically important traits such as feed efficiency[5], has negatively impacted functional traits in rabbit such as longevity. This has led to higher replacement rates[3] and increased early culling[6].

The gut microbiota, comprising diverse microorganisms such as bacteria, fungi, and viruses, plays a critical role in host health through complex interactions with the host's physiology[7]. These microbial communities influence key physiological processes, including digestion, immune function, disease resistance, and mortality[8,9]. Studies involving germ-free animals have demonstrated that interactions between gut microbiota and the host immune

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system are essential for proper immune development and maturation[10]. Furthermore, a balanced gut microbiota can protect the host against pathogens via colonization resistance, reducing infection risks and enhancing survival rates[11]. Conversely, dysbiosis (an imbalance in the gut microbiota) can impair immune function which leads to an increase in disease susceptibility[12]. In rabbits, evidence indicates a relationship between gut microbial composition and longevity[13,14]. For instance, in a related study, gut microbiome profiles measured at early life stages (first parity) have been found to accurately predict whether females belong to low-longevity groups (dying or culled before third parity) or high-longevity groups (surviving until at least 15 parities), highlighting the microbiome's potential for longevity prediction[14]. However, the gut microbiome is highly dynamic, and its composition and diversity are influenced by various factors, including diet, host genetics, and age[15,16]. The gut microbiome undergoes significant changes with age, including alterations in microbial diversity, community structure, and function[17]. These age-related changes have been associated with a variety of health outcomes, such as inflammation, immunosenescence, and the development of age-related diseases[18]. In rabbits, research in age-related changes in gut microbiota were mainly focused on young rabbits transitioning form weaning to subadulthood[19–21]. To our knowledge, Savietto et al. is the only study to investigate changes in the gut microbiota of adult female rabbits throughout their reproductive lifespan (up to 78 weeks)[22]. While their research provided insights into diversity changes with age, it did not address shifts in taxa composition or differences associated with longevity. This gap is significant, as changes in the gut microbiome with age have been linked to various health outcomes[18]. The relationship between the gut microbiome, age, and longevity remains complex and not fully understood. Exploring this relationship could help us better understand how microbiome can be related to the host's longevity.

While longevity has traditionally received less emphasis than economically relevant traits like litter size at weaning[23], advancements in gut microbiota research offer new opportunities to better understand and improve longevity. These insights could lead to important benefits, including reduced replacement costs, enhanced animal welfare, and improved productivity for both females and their kits[24]. Furthermore, the findings from this study could have broader applications, potentially informing strategies to address longevity challenges in other livestock species as well.

Therefore, the aim of this study is to perform a longitudinal analysis in two rabbit maternal with different longevity to, first, investigate the evolution of the gut microbiota through age, specifically during reproductive adulthood (from first parity to death or culling). Second, to investigate the relationship between gut microbiota changes and longevity by comparing gut microbiota evolution in different longevity groups between and within lines A and LP.

Methods

Animals

A total of 164 females from two rabbit lines A and LP were used in this experiment. Line A comprised 48 females from the 50 th generation of selection, where, in each generation, females with superior genetic merit for litter size at weaning were selected based on breeding values estimated using a Best Linear Unbiased Prediction (BLUP) statistical model[25]. The LP line included 116 females from the 18 th generation, resulting in a final dataset of 319 samples (Supplementary Material 1, Figure S1). The A line is a standard commercial maternal line founded in 1981; sampling New Zealand White rabbits[26]. The LP line is a robust maternal line founded in 2002 using longevity criteria; females with a minimum of 30 parities (reproductive event or litter) and having an average prolificacy (average number of offspring per litter) per parity close to 9, which is the Spanish commercial population average[26]. Since its foundation, line LP has exhibited nearly double the longevity of line A at the population level[27]. Both lines LP and A are currently selected for litter size at weaning.

All females were housed at the farm of the *Universitat Politècnica de València* in individual cages (flat-deck) with an extractable nest box with isolated plastic, and under a photoperiod of 16-h light: 8-h dark and controlled temperature and ventilation. Access to feed was ad libitum through the experimental period and a standardized diet composed only of commercial feed was served to all animals. All females were bred using natural mating and they were allowed to reach the maximum number of parities possible before death or culling. The study measured functional longevity, defined here as the length of the productive lifespan until death or involuntary culling due to infertility or health-related issues[26–28]. Animals were either culled for reproductive reasons (six failed attempts of mating; a failed attempt is defined as mating occurring without successful pregnancy or mating refusal) or culled for health reasons (presenting symptoms indicative of various diseases, such as respiratory issues, digestive, mammary gland inflammation (mammitis), eye inflammation (conjunctivitis), abscesses and others). At the end of the experiment, the females of both lines A and LP were catalogued into three longevity groups based on their functional longevity: Low longevity (LL): females died/culled before 5 th parity, medium longevity (ML): died/culled between 5 th parity and 10 th parity, and high longevity (HL): animals with more than 10 parities (Table 1).

All animal experimental procedures conducted in this study were reviewed and approved by the Committee of Ethics and Animal Welfare of the *Universitat Politècnica de València* according to the Council Directives 98/58/EC and 2010/63/EU. All methods were performed in accordance with the guidelines and regulations. The animal management and experimental procedures were carried out in compliance with the ARRIVE guidelines to ensure transparency and reproducibility in animal research.

Sample collection and DNA extraction

In total, 319 samples were collected for metataxonomic analysis covering the lifespan of the females, and they were sampled as follows: During the second week after parity, daily attempts were made to collect faecal samples from the anus of females by applying gentle pressure to the perianal area. Three separate collections were attempted each day of the second week after parity until successfully obtaining a sample. Once the faecal

	LL[1]	ML[2]	HL[3]	Total
A[4]	21 (44%)	17 (35%)	10 (21%)	48
LP[5]	38 (33%)	30 (26%)	48 (41%)	116

Table 1. Distribution of females from lines A and LP across groups based on functional longevity. 1: Low longevity (LL), died/culled before 5 th parity. 2: Medium longevity (ML), between 5 th and 10 th parity. 3: High longevity (HL), more than 10 parities. 4: Rabbit´s standard maternal line A. 5: Rabbit´s high longevity maternal line LP. Values between parentheses are relative values within line.

samples were available, they were frozen at −72ºC until DNA extraction. Bacterial genomic DNA was isolated from the frozen faecal samples using the DNeasy PowerSoil kit (QIAGEN Inc, Hilden, Germany), according to the protocol described in in Biada et al. 2024.

PCR amplification, barcoding, and DNA sequencing

Microbial genomic DNA was amplified and purified following the 16 S Metagenomic Sequencing Library Preparation protocol by Illumina, as described in detail in Biada et al.[13]. In short, the V3 and V4 regions of the 16 S rRNA gene were amplified using the recommended primers. Multiplexing was performed using Nextera XT Index Kit dual indices, and the PCR products were verified with a Bioanalyzer DNA 1000 chip.

Bioinformatic analyses

Primary processing was carried out on the raw sequencing reads, including quality control filtering using fastp[29] and removal of primers using Cutadapt[30]. Then, the sequences were processed using DADA2 pipeline[31] in R version (4.3.2)[32]. Forward and reverse reads were trimmed to 260 and 220 bp, respectively, to remove the low-quality portion of the sequences. Removal of chimeras was performed. Amplicon Sequence Variants (ASVs) were inferred after denoising, and the result was the construction of ASV tables. Following this, the ASV table was imported into QIIME2 software, version 2021.11[33]. Taxonomic classification was performed using a trained Silva-based 16 S classifier (classifier was trained on the primers used for amplification and the length of the sequence reads). Final ASVs were filtered to remove potential artifacts; sequences not classified under the kingdom Bacteria and those with a relative abundance below 0.01% were excluded[34].

Alpha-diversity analysis

Three alpha-diversity metrics: Observed richness, Shannon, and Pielou evenness were computed using phyloseq package[35]. Each alpha-diversity index was modelled using linear mixed model. The model considered fixed effects including the animal's genetic line (A or LP) and longevity group which categorizes animals based on functional longevity (Low longevity or LL: females died/culled before 5 th parity, medium longevity or ML: died/culled between 5 th parity and 10 th parity, and high longevity HL: animals with more than 10 parities), age represents the animal's age in weeks at the sample collection, and finally temperature-humidity index (THI) the day of sample collection[36] divided into two categories: thermal comfort or C (THI $\leq$ 28) and heat stress or H (THI > 28). Random effects for individual females were incorporated to address repeated measurements. Sequencing depth per sample (N) was included to control variation in sequencing depth across samples. The model equation was:

$$y_{ijkls} = Line_j + Longevity_k + THI_s + Age_{ijkls} + N_{ijkls} + Female_{jkl} + e_{ijkls} \quad (1)$$

Where y_{ijkls} represents the alpha-diversity measurement for the sample i of line j (A = 85 or LP = 234), longevity group k (LL = 74, ML = 92, or HL = 153), female l (164 levels), under THI category s (C = 166 or H = 153) and sampled at age $ijkls$ (ranging from 25 to 186 weeks). N_{ijkls} donates the total sampling depth and e_{ijkls} represents the error term. To ensure robustness in the analysis, the same model was repeated using rarefied tables instead of including N_{ijkls} for sequencing depth control.

Beta-diversity analysis

To account for compositionality of microbiome data[37], prior to computing Aitchison dissimilarity matrix, the data was transformed using the centred log-ratio (CLR). Then Principal Component Analysis (PCA) was computed using Aitchison dissimilarity to visually represent between-samples dissimilarity according to the predictors[38]. Then, Permutational Multivariate Analysis of Variance (PERMANOVA) tests was used to assess the effect of the predictors on the Aitchison distance between samples, using 10,000 permutations[39]. The model included: the effect of line, longevity, age, and THI. Individual identity was included as a blocking factor to control for repeated female's measures.

Differential abundance analysis

Zero Inflated Negative binomial mixed models (ZINBMM) were used to examine how the predictors are associated with the abundance of the taxa. Negative binomial mixed models allow the handling of over-dispersed and zero-inflated distributions that are characteristic of microbiome data[40]. Moreover, they can handle longitudinal designs by including random effects.

Effect	Levels	Dataset	
		A[6]	LP[7]
Longevity (j)	LL[1]	25	49
	ML[2]	35	57
	HL[3]	25	128
THI Category (l)	C[4]	53	113
	H[5]	32	121
Range of age in weeks (ijkl)	-	25–154	25–186
Female (k)	-	48	116

Table 2. Summary of samples distribution per effect according to the model in Eq. (2). 1: Low longevity (LL), died/culled before 5 th parity. 2: Medium longevity (ML), between 5 th and 10 th parity. 3: High longevity (HL), more than 10 parities. 4: Comfort thermal condition based on Temperature Humidity index (THI) $\leq$ 28. 5: Heat stress thermal condition based on Temperature Humidity index (THI) $>$ 28. 6: Rabbit´s standard maternal line A. 7: Rabbit´s high longevity maternal line LP.

Effect	Levels	Dataset		
		LL[5]	ML[6]	HL[7]
Line (j)	A[1]	25	35	25
	LP[2]	49	57	128
THI Category (l)	C[3]	31	44	91
	H[4]	43	48	62
Age in weeks (ijkl)	-	25–56	25–68	25–186
Female (k)	-	59	47	58

Table 3. Summary of samples distribution per effect according to model in Eq. (3). 1: Rabbit´s standard maternal line A. 2: Rabbit´s high longevity maternal line LP. 3:Comfort thermal condition based on Temperature Humidity index (THI) $\leq$ 28. 4: Heat stress thermal condition based on Temperature Humidity index (THI) $>$ 28. 5: Low longevity (LL), died/culled before 5 th parity. 6: Medium longevity (ML), between 5 th and 10 th parity. 7: High longevity (HL), more than 10 parities.

Age and longevity effects

Since differences in longevity[27] and gut microbiome composition[13] between the two lines A and LP were already established, ZINBMM were used separately for each line to test for differential abundance of ASVs univariately. Only ASVs present in at least 10% of the samples were tested. For each ASV, the count per sample was modelled using fixed effects; functional longevity group of the animal (LL: Low longevity, ML: Medium longevity or HL: High longevity), animal's age in weeks at sample collection and THI category (C or H) during the day of sample collection. A random effect was used to account for repeated female measurements. Finally, the log-transformed number of reads per sample (N) was included as an offset term to control variation in sequencing depth across samples.

The full model equation is specified as follows, and it was applied separately to samples from lines A and LP (Table 2):

$$Y \sim ZINB(\mu)$$

$$\log\left(\mu_{ijkl}\right) = Longevity_j + THI_l + Age_{ijkl} + \log(N_{ijkl}) + Female_{jkl} + e_{ijkl} \quad (2)$$

Here, μ_{ijkl} represents the count for sample i from longevity group j, female k, under THI category l, and sampled at age $ijkl$. N_{ijkl} denotes the total sequencing depth for each sample, and e_{ijkl} represents the error term.

Genetic line effect

ZINBMM was used to compare lines A and LP across three datasets, each corresponding to different longevity group (Table 3). This will allow to compare the two lines while controlling for longevity. First, the two lines were compared within the low longevity LL group, consisting of females that died or were culled before the 5 th parity. Then lines were compared in the medium longevity group (ML), comprising those that died or were culled between parity 5 and 10. Finally, differences within the high longevity (HL) group, comprising females with 11 or more parities, were analysed. The counts of each ASV were modelled using fixed effects, including the animal's genetic line (A or LP), age in weeks at sample collection, and the THI category (C or H) on the day of collection. To account for repeated measurements from the same females, a random effect was incorporated. Additionally,

the log-transformed total number of reads per sample (N) was included as an offset term to control for variation in sequencing depth across samples. The full model equation is specified as follows:

$$Y \sim ZINB(\mu)$$

$$\log(\mu_{ijkl}) = Line_j + THI_k + Age_{ijkl} + \log(N_{ijkl}) + Female_{jkl} + e_{ijkl} \quad (3)$$

Where μ_{ijkl} represents the count for sample i within line j, female k, under THI category l, and sampled at age $ijkl$. Here, N_{ijkl} denotes the total sequencing depth per sample, and e_{ijkl} represents the error term.

Statistical analysis

Linear mixed models for the alpha diversity index and ZINBMM for differential abundance were analysed using Bayesian statistics. The inference was made with marginal posterior densities estimated with Markov Chain Monte Carlo (MCMC) using the brms package[41]. All models (Eqs. 1–3) were run by four chains with a length of 50,000 iterations, a lag of 10, and a burn-in of 5000 iterations. Default priors provided by brms package were used[41] and they were as follows: the intercept was assigned a weakly informative Student's t-distribution with 3 degrees of freedom and a scale parameter that depends on the standard deviation of the response (after applying the link function in case of ZINBMM) with a minimum of 2.5. The rest of fixed effects coefficients were assigned flat priors. The female random effect and residuals were assumed to follow independent normal distributions $N(b_f, \sigma_f^2)$ and $N(0, \sigma_e^2)$ respectively, where the female random effect intercept b_f, standard deviation σ_f and the error standard deviation σ_e have weakly informative as described for the initial intercept. Additionally, for ZINBMM, the intercept for the zero-inflation component was assigned a logistic distribution with a mean of 0 and a scale of 1 and the shape parameter was given a gamma distribution with shape and rate parameters both set to 0.01. The R-hat statistic was checked for convergence[42]. The marginal posterior distribution of the differences was used to estimate the posterior mean and the probability of the difference being higher (if the difference is positive) or lower (if negative) than 0 for each effect (P0). The posterior mean of the differences was indicated as standard deviations units (PM) of each variable. Variables with an PM higher than 1/3 and a P0 higher than 0.95 were considered the most relevant. It is important to mention that the Bayesian framework used here interprets the 95% probability (P0 > 0.95) as the actual probability of differences, rather than as a p-value threshold as in frequentist hypothesis testing[43].

Results

Rabbit gut microbiota composition

The 319 samples used in this experiment yielded 1382 ASVs (mean ± SD = 557 ± 132 ASVs per sample) and 24,137,896 reads (75,667 ± 46,759 reads per sample). The taxonomic assignment resulted in a total of 7 phyla (100% of the ASVs were classified to phylum), 9 classes (99.8%), 20 orders (99.6%), 34 families (99.5%), and 70 genera (81%). Out of the 1382 ASVs, 97 ASVs (7% of total) were prevalent in at least 80% of the samples with a minimum relative abundance of 1%, thus forming the core microbiota of female rabbits (Supplementary Material 2, table S1).

The taxonomic composition of rabbit´s gut microbiome at phylum and family levels are presented in Fig. 1 and they were as follows: The most abundant phyla were *Firmicutes* (89%), *Bacteroidota* (4%), *Verrucomicrobiota*

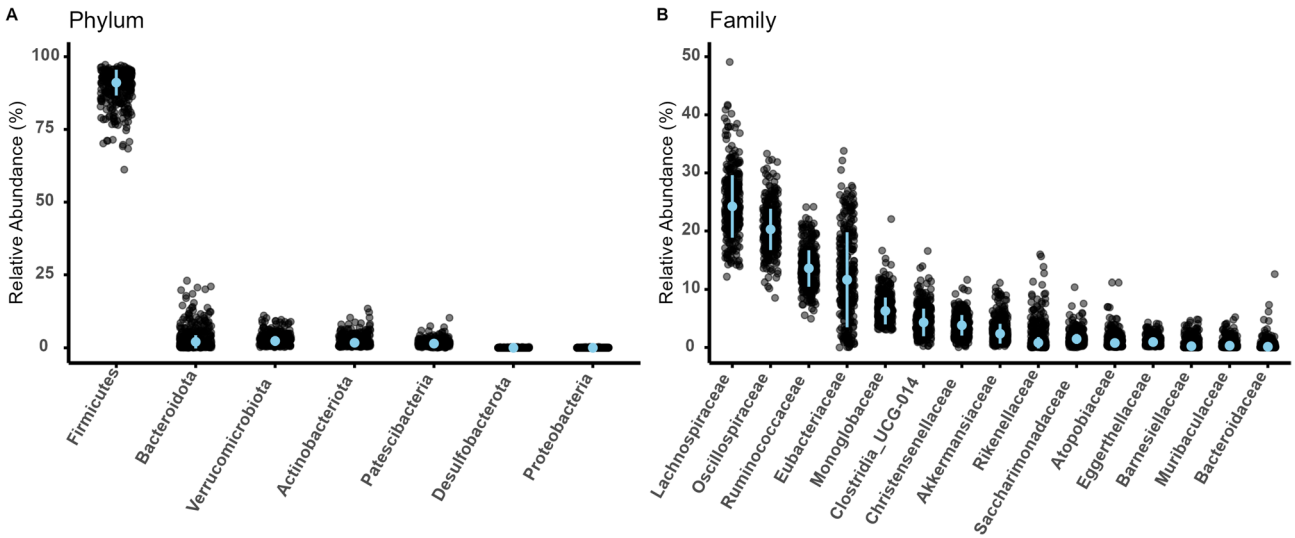

Fig. 1. Taxonomic composition of the female rabbit´s gut at the phylum and family levels. **(A)** Relative abundance of all bacterial phyla and **(B)** Relative abundance of the 15 most abundant families in the faeces. The median and median absolute deviation (error limit) are represented in blue. Each dot represents an Amplicon Sequence Variant (ASV).

	Index	PM[1]	HPD95%[2]	P0(%)[3]
Age	Shannon	−1.4	[−0.34: −0.28]	100
	Pielou evenness	−1.1	[−0.03: −0.02]	100
	Observed richness	−1.7	[−94: −77]	100
HL[6] – LL[4]	Shannon	−0.37	[−0.152: −0.004]	98
	Pielou evenness	−0.42	[−0.019: −0.002]	98
	Observed richness	−0.12	[−26: 15]	71
HL[6] – ML[5]	Shannon	−0.21	[−0.11: 0.03]	90
	Pielou evenness	−0.28	[−0.016: 0.002]	92
	Observed richness	−0.01	[−20: 21]	52
ML[5]- LL[4]	Shannon	−0.16	[−0.08: 0.08]	83
	Pielou evenness	−0.13	[−0.012: 0.006]	81
	Observed richness	−0.10	[−7: 63]	69
LP[6] - A[7]	Shannon	0.35	[0.02: 0.14]	99
	Pielou evenness	0.24	[−0.001: 0.014]	94
	Observed richness	0.40	[3: 38]	99

Table 4. Summary of the main effect estimates for alpha-diversity indices Shannon, Pielou evenness and observed richness obtained with the repeatability linear model. 1: Marginal posterior mean in standard deviation units. 2: Highest posterior density interval at 95% probability. 3: Probability of the difference being higher (if the difference is positive) or lower (if negative) than zero. 4: Low longevity (LL), died/culled before 5 th parity. 5: Medium longevity (ML), between 5 th and 10 th parity. 6: High longevity (HL), more than 10 parities. 6: Rabbit´s high longevity maternal line LP. 7: Rabbit´s standard maternal line A.

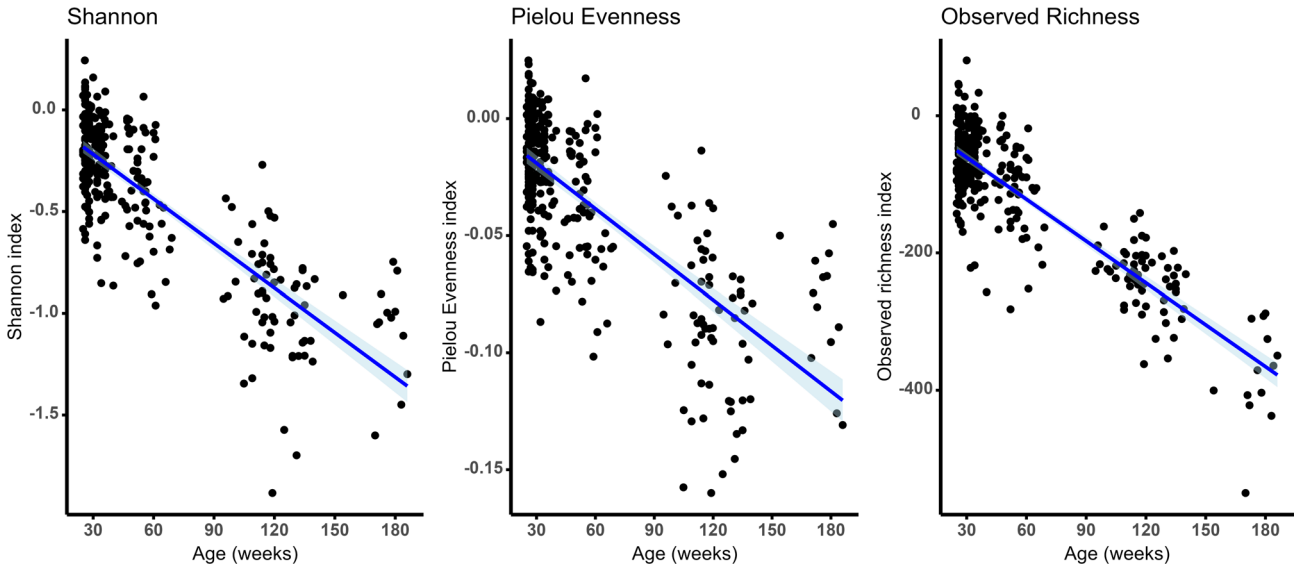

Fig. 2. Plot of alpha-diversity metrics according to age (in weeks). Black dots represent the partial residuals from the linear model (i.e., showing the association between age and alpha diversity, while controlling for all other predictors). The blue line and confidence intervals come from a linear regression (for representation only).

(3%) and *Actinobacteria* (2%) (Supplementary Material 2, table S2). *Firmicutes* was previously reported as most abundant phylum in rabbit faeces followed by *Bacteroidota* and *Actinobacteria*[44] due to its importance in cellulose degradation and digestion[45]. The *Verrucomicrobiota* was solely composed of the genus *Akkermansia*. At family level, the most abundant families were: *Lachnospiraceae* (25%), *Oscillospiraceae* (20%), *Ruminococcaceae* (14%) and *Eubacteriaceae* (12%) (Additional file1, table S3). *Lachnospiraceae* and *Ruminococcaceae* are cellulolytic/fibrolytic families, previously reported by Cotozzolo et al. as highly abundant in rabbits[46].

Changes in gut microbiota composition with age

Concerning alpha-diversity results (within sample diversity), age was negatively associated with all three alpha-diversity metrics (Shannon, Pielou evenness and observed richness) with a probability of 100% (Table 4; Fig. 2). These differences were highly relevant for all three indices (P0 ≥ 95% and mean difference in standard deviation

units higher than 1/3). Specifically, observed richness and Shannon indices suggest a decrease in richness over time, and Pielou evenness suggests that community homogeneity also decreases with age during the reproductive life of females. Similar trends were found when using rarefication (Supplementary Material 1). Concerning beta diversity, PERMANOVA results showed that age significantly explained the highest proportion of the total dissimilarity between samples; 6% measured by Aitchison distance. Principal Component Analysis (PCA) based on Aitchison showed clear gradual separation between the bacterial communities while age is increasing (Fig. 3a). Moreover, the first principal component, which explained 45% of variation was positively associated with age (R-square = 0.5, Fig. 3b).

To investigate how specific bacterial taxa vary with age, we employed ZINBMM as in methods section (formula 2). Age was found to be an important factor affecting the gut microbiota, showing association with 20% and 16% of ASVs analysed, specifically 265 and 209 within lines A and LP, respectively (P0 $\geq$ 95% and posterior mean in standard deviation units higher than 1/3). Of the identified ASVs associated with age, 76% (202) and 71% (149) exhibited a negative correlation with age in lines A and LP, respectively. Notably, 94 ASVs were shared between the two lines (Fig. 4). ASVs from families *Butyricicoccaceae*, *Clostridia_UCG-014*, *Erysipelotrichaceae*, *Eubacteriaceae*, *Lachnospiraceae*, *Monoglobaceae*, *Muribaculaceae Oscillospiraceae* and *Ruminococcaceae* were mostly negatively correlated with age. In contrast, ASVs from the families *Atopobiaceae* and *Bacteroidaceae* were generally positively correlated with age in both lines A and LP. Interestingly, ASVs from the *Akkermansiaceae* family showed opposite trends, being positively correlated with age in line A but negatively correlated in line LP.

Changes in gut microbiota composition between longevity groups

Concerning alpha-diversity results, the only relevant differences were between the two distant groups LL (Low longevity) and HL (High longevity) for Shannon and Pielou evenness (Mean difference in standard deviation units higher than 1/3 and P0 higher than 95%) (Table 4). For other contrasts, although not relevant, differences were found between Shannon and Pielou evenness indices and not in observed richness (Table 4). HL group exhibited lower alpha diversity values when compared to ML (Medium longevity) (P0 = 90% and 92% for Shannon and Pielou evenness respectively). The ML also showed lower alpha-diversity than LL, though the differences were less pronounced (P0 = 83% and 81% for Shannon and Pielou evenness respectively). These results indicate that lower longevity groups exhibited higher alpha diversity (when considering relative abundances) but did not have higher absolute numbers of taxa (richness). Beta diversity results analysed using PERMANOVA from Aitchison dissimilarity matrix showed that longevity significantly explained 3% of the overall Aitchison dissimilarity between samples, which was the second highest after age effect.

Concerning the ZINBMM differential abundance models (see Eq. 2), when comparing the HL group to the LL group within line A, differences were identified in 15% of the total ASVs analysed (196 ASVs). This percentage far exceeds the 4% observed in line LP, were only 55 ASVs showed differences between HL and LL (Fig. 4). Comparing HL and ML groups, differences remained higher in line A, with 16% of ASVs analysed (210 ASVs) showing differences, compared to 4% (59 ASVs) in line LP (Fig. 4). Finally, between ML and LL groups, differences were found in 7% of ASVs (90 ASVs) in line A, and 6% (79 ASVs) in line LP. Overall, these results indicate a higher number of ASVs found differentially abundant between longevity groups in line A, particularly when contrasting the high longevity (HL) group with the other groups. Additionally, for all contrasts HL-LL,

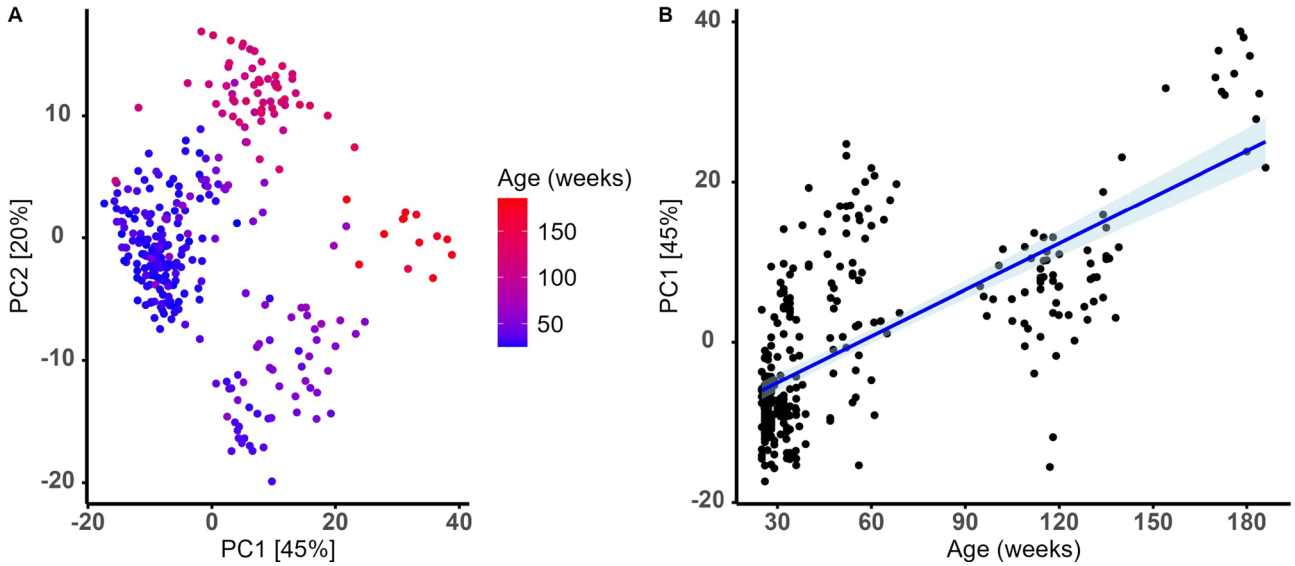

Fig. 3. Visualization of two first principal components representing the dissimilarity between samples (based on Aitchison distance) and its relationship with age of animals (in weeks). **(A)** Visualization of two first principal components representing the dissimilarity between samples, samples are coloured by age. **(B)** Visualization of relationship between first principal component and age effect.

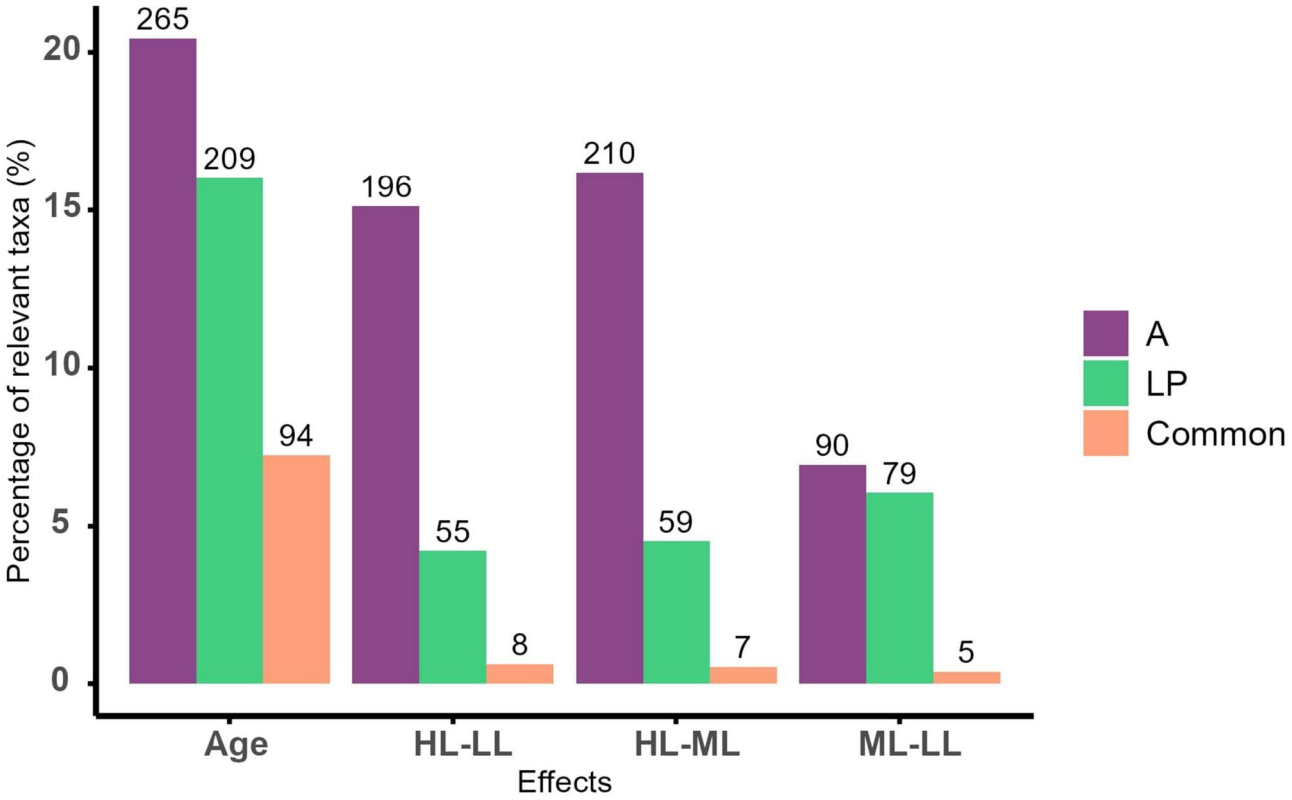

Fig. 4. Percentage of Amplicon Sequence Variants (ASVs) differentially abundant for age, and longevity groups. LL (low longevity, females that died/culled before 5 th parity), ML (medium longevity, died/culled between 5 th parity and 10 th parity), and HL (high longevity, more than 10 parities), analysed using a zero-inflated negative binomial model. ASVs differentially abundant by age within line A are indicated in purple, while those in line LP are shown in green; ASVs common to both lines are represented in orange and are also counted in both the line A (purple) and line LP (green) bars. Only taxa with a posterior mean (PM) in standard deviation units greater than 0.3 and P0 (probability that the difference is higher, if positive, or lower, if negative, than 0) above 95% were considered relevant. The numbers above the bars depict the absolute number of relevant ASVs differentially abundant.

HL-ML and ML-LL only a small number of ASVs (8, 7 and 5 respectively) were common between the two lines A and LP.

Changes in gut microbiota composition between genetic lines

In terms of differences between the two genetic lines, line LP consistently exhibited higher alpha diversity values across all three indices compared to line A, with a minimum probability of 94%. However, differences in evenness between the lines were not relevant (mean difference in standard deviation units lower than 1/3) (Table 4). Similar results were found when using rarefication. PERMANOVA results revealed that the genetic line explained only 0.9% of dissimilarity between samples (Aitchison), a proportion lower than the effects of age and longevity.

Concerning differential abundance results, the line effect was compared across animals with the same longevity (see Eq. 3). The line effect was found to be associated with the abundance of 9% (118 ASVs), 6% (76 ASVs), and 12% (155 ASVs) of the total ASVs within low (LL), medium (ML) and high (HL) longevity groups, respectively (Fig. 5). When focusing on the 155 ASVs with relevant differences between lines A and LP within the HL group, a high proportion of these ASVs did not overlap with those showing differences when comparing HL to LL (196 ASVs, with only 61 differing between lines in HL) and to ML within line A (210 ASVs, with only 20 differing between lines in HL) (Fig. 4). This result indicates that there are differences between the two lines microbiome independently from differences in longevity. When examining the differentially abundant taxa across the three longevity groups, only four ASVs were found in common (Fig. 5). However, 90%, 97% and 88% of ASVs that were differentially abundant between the two lines in LL, ML and HL belonged to 11 families that were consistently differentially abundant between lines across the three groups. These families were: *Akkermansiaceae*, *Atopobiaceae*, *Christensenellaceae*, *Clostridia_UCG-014*, *Erysipelatoclostridiaceae*, *Eubacteriaceae*, *Lachnospiraceae*, *Monoglobaceae*, *Muribaculaceae*, *Oscillospiraceae* and *Ruminococcaceae.*

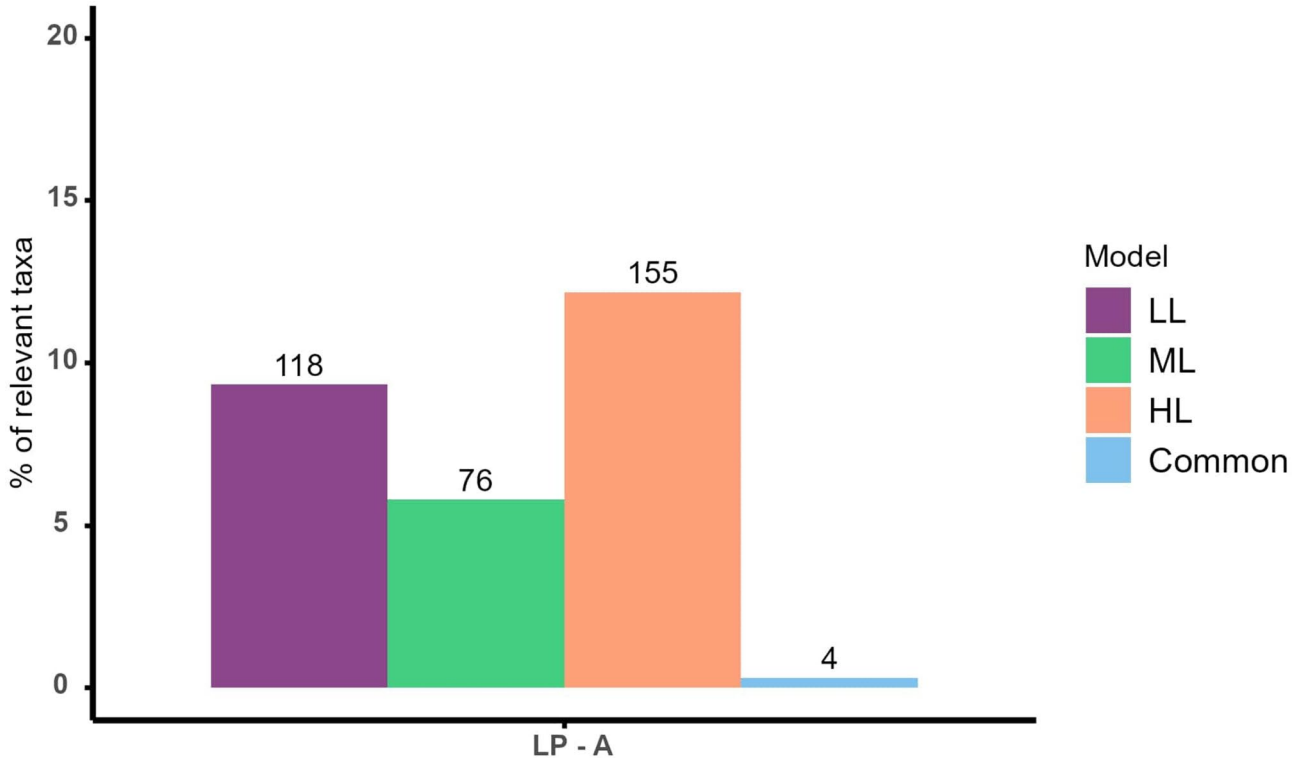

Fig. 5. Percent of Amplicon Sequence Variants (ASVs) that are associated with line effect within each longevity group dataset. LL (low longevity, females that died/culled before 5 th parity), ML (medium longevity died/culled between 5 th parity and 10 th parity), and HL (high longevity more than 10 parities), and those shared across the three longevity groups. Only taxa with a posterior mean (PM) in standard deviation units greater than 0.3 and P0 (probability that the difference is higher, if positive, or lower, if negative, than 0) above 95% were considered relevant. The numbers above the bars depict the number of relevant ASVs differentially abundant.

Discussion

The results of diversity analysis showed clear and relevant age effect influencing the microbiome of female rabbits during their reproductive lifespan. Previous research on the gut microbiota changes through age in rabbits has primarily focused on young (subadult) individuals[19–21]. They observed an increase in alpha diversity with age, which was attributed to the establishment process of the gut microbiota[19,21]. In our study examining changes in diversity among adult female rabbits, we observed the opposite trend, with a decrease in both the richness and evenness of bacterial communities as age progressed. This decrease aligns with the results of Savietto et al. who reported a similar decline (based on observed richness, Shannon and inverse Simpson indices) in adult female rabbits (up to 78 weeks)[22]. Other mammalian studies have shown varied patterns; for instance, in humans, alpha diversity generally increases from infancy to young adulthood but stabilizes and sometimes increased again in older adults[47,48] or decreased in others[49]. Studies in adult pigs and mice did not find a negative correlation of diversity with age in adults, and they reported no differences in alpha diversity with increasing age[50,51]. Concerning beta diversity results, we found that age explained the highest proportion of the variation in beta diversity dissimilarity matrix. Savietto et al. also found differences in beta-diversity metrics, reflecting differences between samples as age progressed[22]. Moreover, same results were found in other species, with age explain a significant proportion of variance in different distances matrices in humans, mice and pigs[47,50,51]. The decline in microbial diversity with age, despite controlled diet and environmental conditions for all animals raises questions about the underlying mechanisms and potential health implications, as reduced diversity is often associated with poor health in other species[15,52–54]. Several interconnected factors intrinsic to aging, particularly during the reproductive lifespan of female rabbits, may contribute to this phenomenon. Age-related physiological changes, such as altered gut motility and decreased nutrient absorption, could significantly impact the gut microbial environment[55]. Additionally, cumulative physiological stress from successive reproductive cycles, combined with immunosenescence (the age-related decline in immune function) and the onset of low-grade chronic inflammation (inflammaging), may create selective pressures that progressively diminish microbial diversity[56]. However, further research is needed to test these hypotheses, identify specific mechanisms, and clarify the extent to which these factors directly influence gut microbial communities and overall health outcomes.

Beyond diversity results, abundance analysis demonstrated a strong age effect in a high proportion (27%) of ASVs regardless of the genetic line. The abundance of most of these ASVs (> 71% in both lines) was negatively associated with age. This negative association likely contributes to the observed decrease in alpha diversity, as

a reduction in the abundance of numerous taxa leads to decreased richness and evenness. Additionally, a high proportion of ASVs was found in common between the two genetic lines A and LP (123 ASVs), suggesting that these ASVs are more regulated and affected by the environment than by host genetics. To our knowledge, no studies have investigated changes in taxa abundance with age in rabbits. However, in other species age changes in microbiome compositions are evident and described as part of the process of aging[47,50,51]. In pigs, similarly to our findings, differences in bacterial taxa have been reported with increasing age, with higher proportion of significant bacterial taxa being negatively associated with age[51]. Moreover, in humans and mice, variation in bacterial abundance with age were detected at phylum level[47,50].

Our analysis of alpha diversity across longevity groups revealed that the high longevity (HL) group exhibited lower evenness in bacterial community abundance compared to the medium (ML) and low longevity (LL) groups. This means that while the observed richness, or the absolute number of unique taxa remained consistent across all longevity groups, the relative abundances of these taxa were more evenly distributed in the LL and ML groups. In contrast, the HL group showed a less even distribution, suggesting that a few taxa might be dominating the microbial community, reducing overall evenness. This finding contrasts with some studies in humans, where alpha diversity was either unchanged between longevity groups[57,58] or increased in high longevity groups compared to younger adults[57,59,60]. Our results suggest that the microbial communities in high longevity rabbits may be structured differently, with fewer taxa being dominant. In terms of beta diversity, the longevity effect explained 3% of the overall dissimilarity between samples. Although this proportion is relatively small, it is the second highest proportion explained by any factor, following age. Consistent with our findings, previous studies have also reported significant differences in beta diversity between high and low longevity groups[47].

The differential abundance analysis provided detailed insights into the microbial differences between longevity groups. We found a higher percentage of ASVs showing differences between the high (HL) and low (LL) longevity groups in line A (15%) compared to line LP (4%). A similar pattern was observed between the HL and medium longevity (ML) groups (16% in line A and 4% in line LP). However, when comparing ML and LL groups, the percentage of differing ASVs was low and similar in both lines (7% in line A and 6% in line LP). Moreover, only a small fraction of relevant ASVs were common between both lines. This suggests that the different genetic background between the two lines A and LP may influence the differences observed, since the environment was the same for all animals. Line A is a commercial maternal line[26]. Conversely, line LP was founded using longevity criteria, specifically from females with an average number of parities of 30, which is six times higher than the average longevity[26]. Since foundation, the LP line has consistently demonstrated higher longevity compared to line A[27]. This distinction implies that the foundation criteria for line LP has potentially exerted a selective pressure on the microbiome or specific taxa within it that are associated with longevity. Conversely, line A, not founded nor selected for longevity, retained greater variability in its gut microbiome associated with longevity. Hence, we are observing much higher differences when comparing groups with high and low longevity within line A in comparison to line LP. Moreover, in the comparison between medium and low longevity groups in line A, the differences were not as high, and they were similar to line LP. Other studies in rabbit maternal lines found that the selection for other functional traits, such as resilience (using environmental variance as a proxy) can shift the gut microbiome[61]. In summary, these results highlight the important influence of genetic background on microbiome composition and its relationship to longevity. This suggests the presence of a genetic component affecting longevity and that could be used to improve this complex trait in rabbit breeding.

Alpha diversity results showed a consistent genetic line effect, with line LP having higher values across all diversity indices compared to line A. Although differences in beta diversity were observed, the line effect accounted for a small proportion of the variance in dissimilarity. Similar findings of both alpha (Line LP with higher alpha diversity values than line A) and beta diversity were reported when comparing these two lines at first parity in another study[13]. Based on these findings, we conclude that the differences in alpha diversity between the two lines are maintained throughout the reproductive lifespan of female rabbits.

The goal of the differential abundance analysis was to evaluate changes between the two genetic lines while accounting for longevity. The results revealed differences between the two lines, A and LP, across all longevity groups. This suggests that the microbiomes of these lines differ independently of their longevity. Several taxa identified as differentially abundant in this study were also reported in Biada et al. such as *Akkermansiaceae*, *Christensenellaceae*, *Clostridia_UCG-014*, *Eubacteriaceae*, *Lachnospiraceae*, *Monoglobaceae*, *Muribaculaceae*, *Oscillospiraceae*, and *Ruminococcaceae*[13]. Notably, the highest proportion of differentially abundant taxa was observed in the high longevity group. This may be due to greater inter-individual variation in the microbiota of long-lived groups, as seen in humans[18]. In summary, both diversity and differential abundance analyses revealed consistent differences found between the genetic lines LP and A. This shows that differences in microbiome are maintained between the two lines during reproductive life and underscore a clear genetic influence on the microbiome of the two lines.

Conclusions

This study provides valuable insights into the evolution of the gut microbiome in adult female rabbits, a topic that has not been sufficiently studied. Our findings demonstrate that age is a crucial factor influencing microbiome diversity and composition, marked by a decrease in alpha diversity and shifts in the microbiome, with a substantial proportion of ASVs being affected by age. Regarding longevity, we observed that animals with different longevity exhibit distinct microbiome diversity and compositions. Notably, these differences are influenced by host´s genetics, suggesting that using microbiome through selection or using specific taxa within it as biomarkers could be a promising avenue for improving longevity. Furthermore, the microbiome differences between genetic lines persist throughout the animals' lifespan, with differences observed even among animals with same longevity.

Data availability

The data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB71513 (https://www.ebi.ac.uk/ena/browser/view/PRJEB71513).

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Author contributions

I.B analysed the data and wrote the manuscript. M.A.S conceived and designed the study, contributed to the analysis and discussion of the results and edited the manuscript. A.B conceived and designed the study and edited the manuscript. R.P.S prepared the laboratory samples and edited the manuscript. N.I.E conceived and designed the study, contributed to the analysis and discussion of the results and edited the manuscript. All authors read and approved the final manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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