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Host genetics and gut microbiota influence lipid metabolism and inflammation: potential implications for ALS pathophysiology in SOD1^{G93A} mice

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is a devastating neurodegenerative disorder characterized by the progressive loss of motor neurons, with genetic and environmental factors contributing to its complex pathogenesis. Dysregulated immune responses and altered energetic metabolism are key features, with emerging evidence implicating the gut microbiota (GM) in disease progression. We investigated the interplay among genetic background, GM composition, metabolism, and immune response in two distinct ALS mouse models: 129Sv_G93A and C57Ola_G93A, representing rapid and slow disease progression, respectively.

Using 16 S rRNA sequencing and fecal metabolite analysis, we characterized the GM composition and metabolite profiles in non-transgenic (Ntg) and SOD1^{G93A} mutant mice of both strains. Our results revealed strain-specific differences in GM composition and functions, particularly in the abundance of taxa belonging to Erysipelotrichaceae and the levels of short and medium-chain fatty acids in fecal samples. The SOD1 mutation induces significant shifts in GM colonization in both strains, with C57Ola_G93A mice showing changes resembling those in 129 Sv mice, potentially affecting disease pathogenesis. ALS symptom progression does not significantly alter microbiota composition, suggesting stability.

Additionally, we assessed systemic immunity and inflammatory responses revealing strain-specific differences in immune cell populations and cytokine levels.

Our findings underscore the substantial influence of genetic background on GM composition, metabolism, and immune response in ALS mouse models. These strain-specific variations may contribute to differences in disease susceptibility and progression rates. Further elucidating the mechanisms underlying these interactions could offer novel insights into ALS pathogenesis and potential therapeutic targets.

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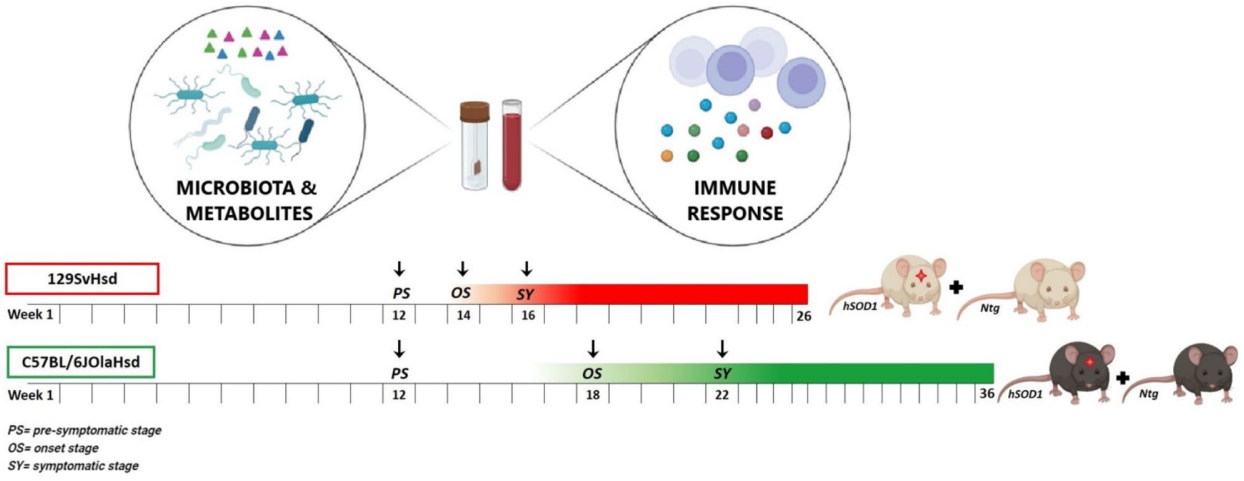
Graphical Abstract



Keywords Amyotrophic lateral sclerosis, Genetic background, SOD1, Immune response, Microbiome, MCFA, Fatty acids, Cytokines

Introduction

Amyotrophic Lateral Sclerosis (ALS), a neurodegenerative disorder characterized by the progressive loss of motor neurons, entails a multifaceted interplay of genetic and environmental factors, underpinning its diverse clinical manifestations [25]. This complexity is marked by substantial genotypic and phenotypic heterogeneity, extending beyond motor neurons. This variability underscores the complexity of the disease and highlights the need for comprehensive investigations to unravel its underlying mechanisms [29]. The pathophysiology of ALS implicates a dysregulated immune system, evidenced by neuroinflammation, involving both resident and peripheral immune cells, which contributes to disease progression [5, 18]. Central and peripheral inflammatory mechanisms are significant contributors to ALS, both in the context of specific genetic mutations and probably as a consequence of the general disease process in sporadic cases [17, 47]. However, the role of the immune system in ALS is complex; initially protective responses are eventually overridden by cytotoxic processes [5, 48]. Another significant aspect of ALS pathology involves the alteration of energetic metabolism, particularly lipid metabolism, exacerbating its complexity [48]. In many patients, there is evidence of a hypermetabolic state that correlates with a more severe lower motor neuron involvement, accelerated functional decline, and shorter survival times [10]. Skeletal muscles suggested as the origin site of this metabolic alteration, exhibit increased energy demand due to chronic denervation, further exacerbating fat mass depletion [40]. Furthermore, ALS is characterized by alterations in plasma lipid levels and changes in fatty acid β-oxidation [8, 64]. Interestingly, hyperlipidemia is associated with increased survival rates, possibly due to the protective effect of elevated lipid levels in circulation [22]. The exact mechanisms underlying hyperlipidemia in ALS remain unclear but could involve mitochondrial dysfunction [23].

Such intricacies prompt exploration into potential contributors, including the gut microbiota (GM) and its modulation of metabolism and immune responses. GM has garnered attention in neurological conditions for its capacity to influence brain function and disease progression. Evidence has implicated GM dysbiosis in driving ALS features and progression, both in patients and in animal models [7, 11, 20, 27, 53]. Notably, previous studied in superoxide dismutase 1 (SOD1) transgenic mice, an established ALS animal model, have unveiled alterations in the gut barrier, abnormal Paneth cell presence, and changes in gut microbial composition, implicating the GM in ALS [63, 69]. Moreover, alterations of microbial metabolites may affect ALS by immune cell activation or cell polarization (regulatory versus effector cells) possibly sustaining neuroinflammation and neuron degeneration [45]. Furthermore, GM contributes significantly to regulating the energetic metabolism, suggesting another potential association between GM and disease progression [45]. For instance, the GM plays a role in converting primary bile acids into secondary bile acids, which are essential for lipid digestion in the gastrointestinal tract and interact with receptors that regulate host metabolism. Additionally, GM composition influences the levels of free fatty acids (FFAs) in the body, serving as essential energy sources for tissues and regulators of the

inflammatory response [37]. Studies conducted in SOD1 mice have demonstrated significant metabolic dysfunction associated with changes in GM composition [61]. Moreover, Guo and colleagues have recently found disparities in the GM composition between ALS cases and controls, with correlations observed between specific genera and plasma metabolites, particularly lipids [32]. These insights underscore the potential significance of GM-mediated metabolic alterations in ALS pathogenesis, suggesting avenues for further exploration into the intricate interplay between gut health, metabolic dysregulation, and neurodegeneration in this devastating disease.

Transgenic mice expressing various human SOD1 mutations closely mimic clinical and pathological features of human ALS, including significant phenotypic variability in terms of onset age and disease progression speed [6, 35]. Particularly intriguing are mice carrying the SOD1^{G93A} transgene on different strains [51]. Despite expressing the same quantity of the human mutant SOD1 protein [41, 42], those on 129SvHsd genetic background (129Sv_G93A, denoted as fast-progressing) exhibit a considerably swifter disease course compared to those on the C57BL/6JOlaHsd background (C57Ola_G93A, slow-progressing), underscoring the substantial influence of genetic background on modulating ALS progression [57]. Transcriptomic analyses of laser-dissected lumbar motor neurons have further highlighted significant gene expression differences in pathways related to metabolism, protein degradation, and immune response [50]. In addition, metabolomic analysis of the spinal cord of two mouse strains evidenced substantial metabolic effects relating to genetic background which may contribute to ALS progression differences between the two SOD1G93A strains [68]. More recently, we demonstrate an important influence of the immune response in determining the different onset and progression of the disease in these mice [41, 66].

Given the bidirectional impact of immunity on the composition and function of the microbiota, our study delved into the influence of genetic background on the composition of the GM, the fatty acid metabolism and the immune response in fast and slow-progressing SOD1 ^{G93A} mouse models. Our findings unraveled the intricate interconnections of these biological processes that may contribute to the nuanced landscape of ALS progression and its variability.

Materials and methods

Animals management and sample collection

Female transgenic 129SvHsd (129Sv_G93A) (n=8), C57BL/6JOlaHsd (C57Ola_G93A) (n=8) and their corresponding non-transgenic littermates (n=10 and n=8, respectively) have been used in this study. Mice have been maintained at a temperature of 21±1 °C with a relative humidity of 55±10% and a 12 h light/dark cycle. Food (standard pellets) and water will be supplied ad libitum. Procedures involving animals and their care has been conducted according to the Mario Negri institutional guidelines, which are compliant with national (D.L. no. 116, G.U. suppl. 40, Feb.18, 1992, Circular No.8, G.U., 14 July 1994) and international policies (EEC Council Directive 86/609, OJ L 358, 1 Dec.12, 1987; NIH Guide for the Care and use of Laboratory Animals, U.S. National Research Council, 1996). All experiments and protocols will be examined by the Institutional Ethical Committee and authorized by the Italian Ministry of Health. The mice have been bred and maintained in a specific pathogen-free environment. Animals with substantial motor impairment had food on the cage bottom and water bottles with long drinking spouts.

Blood and faeces have been collected from 129Sv_G93A mice or C57Ola_G93A mice and age-matched Ntg littermates at pre-symptomatic (PS=12 weeks for both models), onset (OS=14 or 18 w age, respectively) and symptomatic (SY=16 or 22 w age, respectively) stage. To avoid any bias, mice were separated by strain (C57Ola Vs. 129 Sv) and genotype (NTg Vs. SOD1G93A) (No. 4 per cage) and sampling was done per mouse by taking fresh blood and faeces at the defined timepoints.

Faecal microbiota characterization

Total DNA was extracted using the DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany) from frozen (-80 °C) stool samples, according to the manufacturer's instructions. Briefly, 0,25 g of stool samples were added to a bead beating tube and homogenized with TissueLyser II for five minutes at 30 Hz. Genomic DNA was captured on a silica membrane in a spin column format, washed and subsequently eluted. The quality and quantity of extracted DNA were assessed with the Qubit Fluorometer (Thermo Fisher Scientific) and then frozen at -20 °C.

Subsequently, DNA samples were sent to IGA Technology Services (Udine, Italy) where amplicons of the variable V3–V4 region of the bacterial 16s rRNA gene, obtained through primers 341 F and 805R, were sequenced in paired-end (2×300 cycles) on the Illumina MiSeq platform, according to the Illumina 16 S Metagenomic Sequencing Library Preparation protocol.

The demultiplexed sequence reads were processed in QIIME2 2022.8 environment. Briefly, the sequencing primers and the reads without primers were removed using the Cutadapt tool. DADA2 was used to perform paired-end reads filtering, merging and chimeras' removal steps after trimming low-quality nucleotides from both forward and reverse reads (--p-trunc-len-f 274 --p-trunc-len-r 169). Hence, ASVs (amplicon sequence variants) were generated, and the taxonomic

assignments were performed through the Scikit-learn multinomial naive Bayes classifier re-trained on SILVA database (release 138) V3-V4 iper-variable region. Every cross-amplified host DNA has been identified and removed, aligning the ASVs to GRCm39 (murine reference genome) using Bowtie2 2.2.5. Moreover, the ASV derived from chloroplasts or mitochondria, according to SILVA, were also discarded. Finally, every ASV associated with genera with mean relative abundance across the samples under the cutoff of 0.005% have been discarded to minimize sequencing contaminants and improve statistical inferences [9, 12]. Finally, the potential expression of microbial pathways in each sample were estimated using PICRUSt2 v 2.5. Further details about the FASTQ processing are available at https://github.com/LeandroD94/Papers/tree/main/2024_SLA_mice_Fast129Sv_SlowC57_G93A.

Short and medium chain fatty acids analysis

The evaluation of short and medium chain fatty acids (SCFA and MCFA, respectively) and the standard curves' preparation was performed on stools of 12 weeks age mice, by an Agilent GC-MS system composed of a 5971 single quadrupole mass spectrometer, 5890 gas chromatograph and 7673 autosampler, through our previously described GC-MS method [52].

Immunophenotype analysis in blood

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood with Lymphosep (L0560, Biowest) according to manufacturer instructions and stained with the following antibodies: BUV 661 rat anti-mouse CD45 (612975, BD Biosciences), BUV395 rat anti-CD11b (563553, BD Biosciences), BV421 anti-mouse Ly6G (127627, Biolegend) and FITC anti-mouse Ly6C (128005, Biolegend). Events were acquired with Cytoflex LX (Beckman Coulter) using the gating strategy showed in the Figure S4. Flow cytometry data were analyzed using FlowJo™ v10.8 Software (BD Life Sciences).

Molecular inflammatory response evaluation

Serum cytokines (IL-1Beta, IL-6, IL-17, KC/IL-8, monocyte chemotactic protein 1 (MCP-1) and, tumor necrosis factor-α (TNF alpha)) were assayed in 5 mice/group using the Milliplex Map kit Mouse cytokine/chemokine magnetic bead panel for the Luminex MAGPIX detection system (Merck) following the manufacturer's instructions. Cytokine levels were estimated using a 5-parameter polynomial curve with Bio-plex Manager software (Biorad).

Statistical analyses

Continuous variables were presented as median value and interquartile range (calculated as the difference between the 75th and 25th percentiles of the data) and were compared using the non-parametric Mann–Whitney test.

The statistical analyses on bacterial communities were performed in R 4.3 with the help of the packages phyloseq 1.44.0, vegan 2.6-4, DESeq2 1.40.1 and other packages satisfying their dependencies. The packages ggplot2 3.4.2, ggh4×0.2.2 and ggpubr 0.40 were used to plot data and results. A saturation analysis on ASV was performed on every sample using the function rarecurve (step 50 reads), further processed to highlight saturated samples (arbitrarily defined as saturated samples with a final slope in the rarefaction curve with an increment in ASV number per reads < 1e-5). The observed richness and Shannon indices were used to estimate the bacterial genera alpha-diversity in each sample using the function estimate_richness from phyloseq. The Pielou's evenness index was calculated using the formula E = S/log(R), where S is the Shannon diversity index, and R is the observed ASV richness in the sample. Differences in alpha-diversity indices were inspected through the Mann-Whitney test. PCoAs were performed using the Hellinger distance on Hellinger transformed genera abundances. PERMANOVA and Betadisper were used to test the statistical significance of the beta-diversity distances and dispersions. At different taxonomic ranks, the differential analysis (DA) of the abundances has been computed with DESeq2 on raw count data, and only the results with a *p*-value (adjusted through Benjamini-Hochberg method) lower than 0.01 were considered significant. Furthermore, differentially abundant taxa with a DESeq2 baseMean value lower than 100 have been discarded from the displayed results, irrespective of their statistical significance, to limit noisy results. Finally, the algorithm Lefse v 1.1.2 was utilized to compare the expression of microbial pathways predicted by PICRUSt2 between genotypes and strains. Spearman correlation coefficients were calculated to evaluate the association between fatty acids and bacterial taxa; *p*-values were corrected for multiple comparisons using the Benjamini-Hochberg FDR procedure. Further details about the analyses regarding the microbiota are available at https://github.com/LeandroD94/Papers/tree/main/2024_SLA_mice_Fast129Sv_SlowC57_G93A.

Results

Distinct microbiota profiles in non-transgenic C57Ola and 129 sv strains

The sequencing efforts in assessing microbiota composition encompassed a total of 13,650,526 reads for 101 samples. After all the steps of pre-processing (pair merging, trimming, quality filtering, chimera detection) and decontamination, a total of 6,969,470 (51%) were available for further analysis. Saturation curves revealed that every specimen was sufficiently sampled (Figure S1).

Samples showed a Good's coverage ranging from 99 to 100%, indicating that less than 1% of the reads in a given sample came from ASVs, that appeared only once in that sample. The subsequent taxonomic analysis, detailed in Table S1, identified nine phyla, 12 classes, 26 orders, 41 families and 89 genera. In all samples, the top five phyla exhibited the following average abundances: Firmicutes (47.9%), Bacteroidota (40.2%), Campilobacterota (6.2%), Desulfobacterota (1.8%), and Actinobacteriota (1.3%) (Figure S2A). The ten most abundant genera were *Muribaculaceae* (27.2%), *Lactobacillus* (21.3%), *Helicobacter* (6.2%), *an unidentified genus* of Lachnospiraceae family, (4.3%), *NK4A136_group* of Lachnospiraceae (3.9%), *Dubosiella* (3.7%), *Alistipes* (2.9%), *Bacteroides* (2.8%), *Odoribacter* (2.5%), and an uncultured genus of Lachnospiraceae (2.1%) (Figure S2B).

The microbiota composition between non transgenic C57Ola and 129 Sv strains exhibited notable distinctions. Specifically, at 12 weeks of age, C57OlaNtg and 129SvNtg mice displayed divergent microbiota profiles. Analysis of alpha diversity indicated that in the 129 Sv strain, the microbiota did not exhibit a significantly higher ASV richness but a greater abundance uniformity as evidenced by the Evenness index ($p=0.034$) compared to the C57Ola strain (Fig. 1A). More importantly, beta diversity analysis revealed a highly significant separation in microbiota composition between the two strains, as demonstrated by a very low p-value in the PERMANOVA test ($p=0.0001$) (Fig. 1B), highlighting the distinct microbial communities associated with each genetic background.

In-depth univariate analysis disclosed differences in abundances at specific taxonomic levels. Within these disparities, the 129SvNtg mice exhibited a notable increase in Clostridia (log2FC=-1.41, padj=0.006) and genera belonging to the Lachnospiraceae family, such as *Roseburia* (log2FC=-2.52, padj=0.0006), an *unidentified genus* of Lachnospiraceae clade (log2FC=-1.9, padj=0.0015), and *A2* genus (log2FC=-5.35, $p<0.0001$) compared to C57OlaNtg. Conversely, there was a decrease in the Erysipelotrichaceae family (Bacilli) (log2FC=6.01, $p<0.0001$), especially *Dubosiella* (log2FC=14.92, $p<0.0001$) and *Faecalibaculum* (log2FC=12.46, $p<0.0001$) genera, and in the Coriobacteriaceae_*UCG-002* genus (log2FC=9.93, $p<0.0001$) (Fig. 1C). We also detect differences in the fecal levels of short and medium-chain fatty acids. In detail, the percentage of total MCFA ($p=0.041$), heptanoic acid ($p=0.049$) and dodecanoic acid ($p=0.044$) were higher

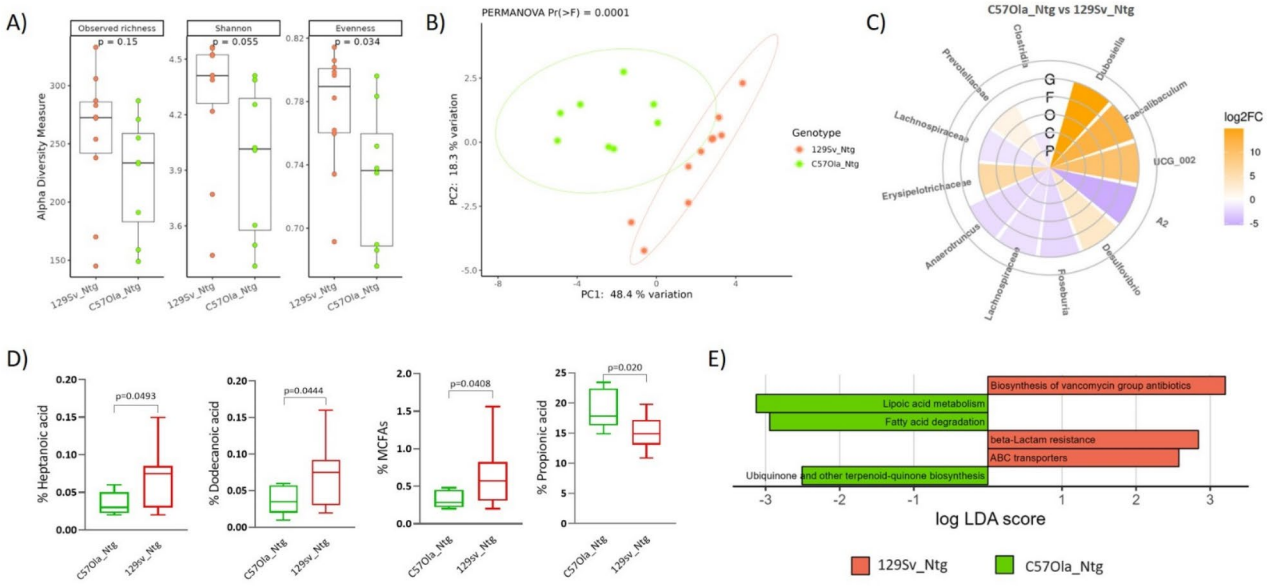


Fig. 1 Comparative Analysis of Microbiota Composition and Metabolic Profiles in C57Ola_Ntg and 129Sv_Ntg Mice at 12 weeks' age. **(A)** Boxplots showcasing alpha diversity indices (Observed Richness, Shannon index, Evenness) in fecal samples. Statistical differences were evaluated using a paired Wilcoxon signed-rank test. *P*-values less than 0.05 were considered statistically significant. **(B)** Principal coordinates analysis (PCoA) according to the Hellinger distance computed on genera abundances. Results of the permutational multivariate analysis of variance (PERMANOVA) are also shown based on the first two coordinates. **(C)** Circular heatmap representing the differentially abundant taxa in C57Ola versus 129 Sv mice samples: concentric circles represent taxonomic ranks from phylum (P) to genus (G); yellow shades indicate positive logFC values, whereas blue shades indicate negative logFC values correlations; the intensity of colors is proportional to logFC values. **(D)** Box plot reporting the statistically significant different free fatty acids among the two Ntg strains. **(E)** Computed Linear Discriminant Analysis (LDA) scores representing significantly differential KEGG Pathway Expression (LDA > 2.5) between 129Sv_Ntg and C57Ola-Ntg mice

whereas the level of propionic acid ($p=0.020$) was lower in 129SvNtg mice than C57OlaNtg, (Fig. 1D). To corroborate these findings, functional prediction analysis using PICRUSt revealed differential expression of several KEGG pathways between the two strains. Specifically, in C57OlaNtg mice, pathways related to fatty acid degradation, lipoic acid metabolism, ubiquinone and other terpenoid-quinone biosynthesis were more abundant. Conversely, in 129SvNtg mice, pathways associated with beta-lactam resistance, biosynthesis of vancomycin group antibiotics, and ABC transporters were more abundant (Fig. 1E).

Additionally, the temporal stability of the microbiota within each strain was examined over time. Supplementary figures depicting beta diversity at different ages for both C57OlaNtg and 129SvNtg mice illustrated that, in each strain, the microbiota composition remained relatively consistent as the mice aged (see Fig. 3 : Beta diversity at different ages for each strain, C57Ola Ntg, and 129 Sv Ntg).

The presence of the human mutant SOD1G93A exerts an influence on gut microbial colonization

The presence of the SOD1 mutation induced significant shifts in GM colonization before the onset of the disease in both strains, with changes particularly evident in C57-G93A mice compared to their Ntg counterparts. At 12 weeks of age, C57Ola-G93A mice exhibited significant differences in microbiota composition compared to their Ntg littermates, as indicated by beta diversity analysis (PERMANOVA=0.0001) (Fig. 2A), while alpha diversity did not show significant differences (data not shown). Furthermore, DESeq analysis unveiled a significant decrease in specific microbial taxa in G93A mice, including Erysipelotrichaceae (log2FC=5.11, padj<0.0001) family, *NK3B31_group* of Prevotellaceae (log2FC=8.89, padj<0.0001), *Dubosiella* (log2FC=11.97, padj<0.0001), and *Faecalibaculum* (log2FC=9.92, padj<0.0001) (Fig. 2B) genera. The fecal levels of total MCFA ($p=0.005$), heptanoic acid ($p=0.010$), dodecanoic acid ($p=0.013$) and decanoic acid ($p=0.039$) were higher in C57Ola_G93A mice compared to C57OlaNtg, while the propionic acid was lower ($p=0.0152$) (Fig. 2D). Interestingly, in C57Ola_G93A, we observed negative correlations between the abundance of Erysipelotrichaceae

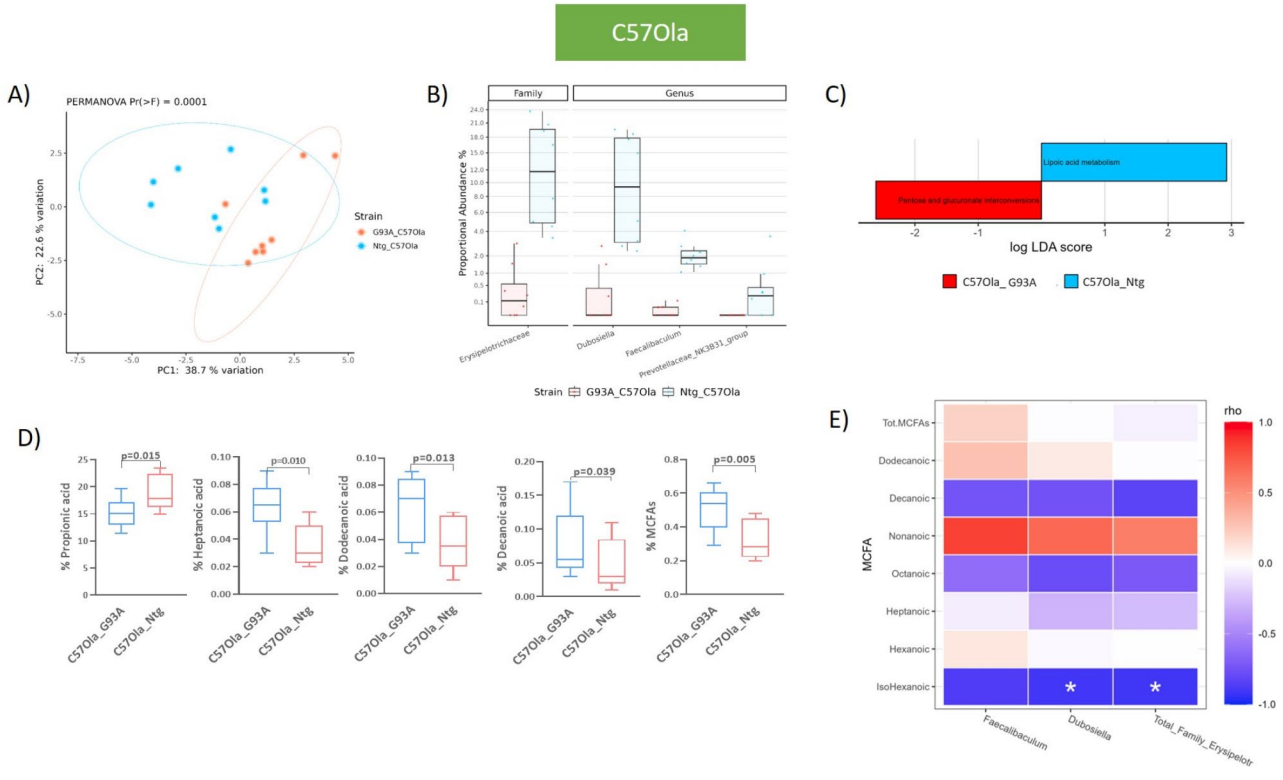


Fig. 2 Microbiota Differences and Diversity Analyses in C57Ola Strain, Comparing Non-Transgenic (Ntg) and G93A Transgenic Mice at 12 Weeks of Age. **(A)** Principal coordinates analysis (PCoA) according to Hellinger distance computed on genera abundances. **(B)** Boxplots with the differentially abundant taxa. **(C)** Computed Linear Discriminant Analysis (LDA) scores representing significantly differential KEGG Pathway Expression (LDA > 2.5). **(D)** Boxplots of the significantly different free fatty acids. **(E)** Heatmap of correlations between MCFA levels and the relative abundance of the Erysipelotrichaceae, *Dubosiella* and *Fecalibaculum* taxa MCFA = medium chain fatty acids. **p*-value < 0.05

(Rho=-0.91; padj=0.04) and *Dubosiella* (Rho=-0.91; padj=0.03) and medium-chain fatty acids (MCFA), especially isohexanoic acid (Fig. 2E). PICRUST analysis revealed that lipoic acid metabolism was higher in Ntg compared to G93A mice (Fig. 2C).

Regarding the 129 Sv strain, subtle differences emerged when comparing the microbiome of SOD1^{G93A} mice to the Ntg littermates, with a minor yet significant separation observed in the PCoA plot (PERMANOVA=0.0299) (Fig. 3A). Notably, only the genus *UCG-001* of Lachnospiraceae displayed a significant decrease in mice with the G93A mutation (log2FC=4.35, padj<0.0001) (Fig. 3B) and no difference was observed in fecal SCFA and glycosaminoglycan degradation, as well as other glycan degradation pathways, in G93A 129 Sv mice compared to their non-transgenic counterparts (Fig. 3C).

Distinct microbiota profiles in SOD1G93A mutant mice strains: strain-specific profiles across disease progression

The presence of the SOD1^{G93A} mutation does not induce a shared microbiota profile across the two strains. Both C57Ola_G93A and 129Sv_G93A mice exhibit distinct

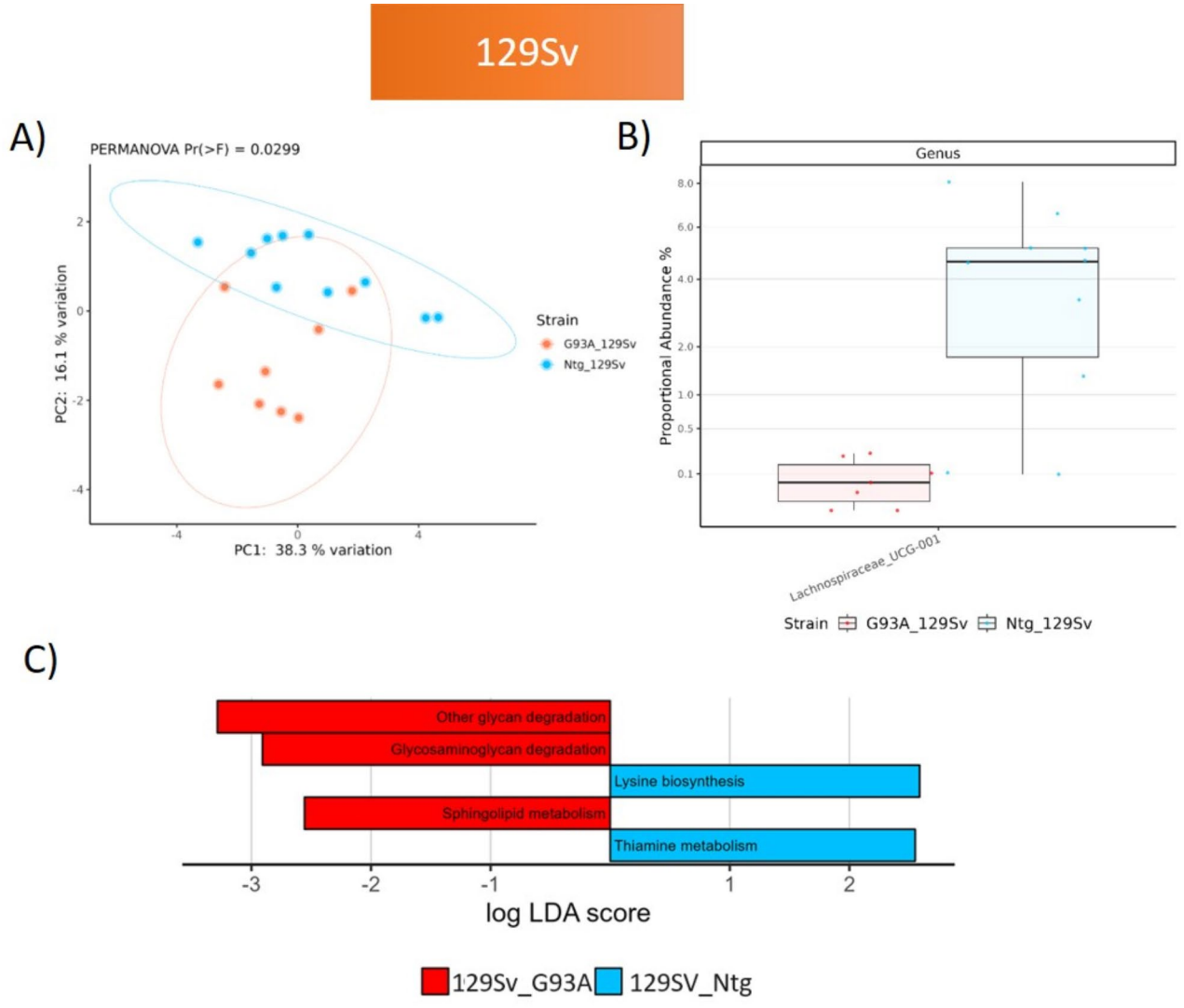


Fig. 3 Microbiota Differences and Diversity Analyses in 129 Sv Strain, Comparing Non-Transgenic (Ntg) and G93A Transgenic Mice at 12 Weeks of Age. **(A)** Principal coordinates analysis (PCoA) according to Hellinger distance computed on genera abundances. **(B)** Boxplots with the differentially abundant taxa. **(C)** Computed Linear Discriminant Analysis (LDA) scores representing significantly differential KEGG Pathway Expression (LDA > 2.5)

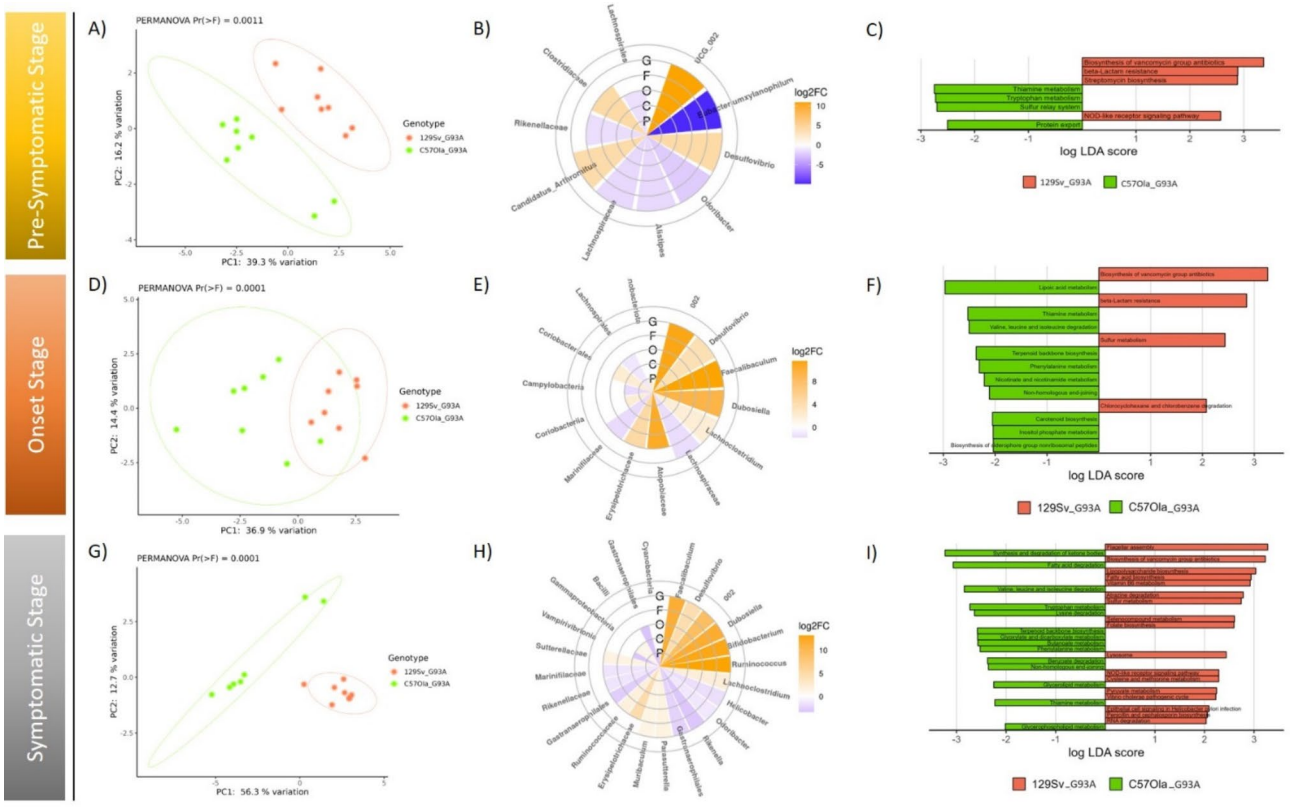


Fig. 4 Temporal analysis of microbiota between C57Ola_G93A and 129Sv_G93A mice. Beta diversity, as well as DESeq2 and PICRUSt2 analysis, were conducted at the pre-symptomatic stage (PS) in panels **A**, **B**, and **C**; at disease onset (OS) in panels **D**, **E**, and **F**; and during the symptomatic stage (SY) in panels **G**, **H**, and **I**. LDA = linear discriminant analysis

microbial profiles, characterized by significant beta diversity (PERMANOVA = 0.0009), with numerous bacterial taxa showing differential expression between the two strains, across the disease course (refer to Fig. 4). Notably, at a pre-symptomatic stage, some of the differences observed among the non-transgenic strains are present also among SOD1^{G93A} mice, with the *unidentified genus* of Lachnospiraceae clade remaining higher in 129 Sv compared to C57Ola, and the *Coriobacteriaceae_UCG-002* genus remaining more abundant in C57Ola. Conversely, members of the Erysipelotrichaceae family (*Dubosiella* and *Faecalibaculum*) and fatty acids levels no longer exhibit differential abundance among the SOD1^{G93A} mutated strains. Additionally, it is noteworthy that no pathways related to lipid metabolism exhibited differential abundance between 129Sv_G93A and C57Ola_G93A mice in the functional prediction analysis (Fig. 4C).

As disease onset and symptomatic stages progress, many differences persist in the microbiota composition between the two strains (Fig. 4). Particularly noteworthy is the resurgence of Erysipelotrichaceae in C57Ola_G93A compared to 129Sv_G93A, alongside alterations in lipid metabolism. Remarkably, at the symptomatic stage, C57Ola mice exhibit an upregulation in fatty acid degradation (LDA = 3; $p = 0.034$) and an increase in the synthesis and degradation of ketone bodies (LDA = 3.2; $p = 0.002$), while 129 Sv mice show a rise in fatty acid biosynthesis (LDA = 2.9; $p = 0.004$) (Fig. 4I).

Finally, the temporal microbiota stability was assessed over time and, in each SOD1^{G93A} strain, the microbiota composition remained relatively consistent throughout the disease stages (see Figure S3).

Systemic immunity and inflammatory response

Similar to the microbiota, the systemic immune-phenotype profile was significantly different between the two Ntg mouse strains at 12 weeks' age. Notably, in the blood, proinflammatory monocytes (Ly6C+) were significantly higher in C57Ola_Ntg versus 129Sv_Ntg mice. Conversely, the patrolling monocytes (Ly6C–) were higher in 129Sv_Ntg compared to C57Ola strain (Fig. 5A). For the SOD1G93A mice, only the 129 Sv strain showed a difference compared to their respective NTG mice, with an increase in proinflammatory monocytes and a decrease in patrolling monocytes at the presymptomatic stage (Fig. 5A). No differences were observed in the C57Ola_G93A mice compared to their respective NTG mice for

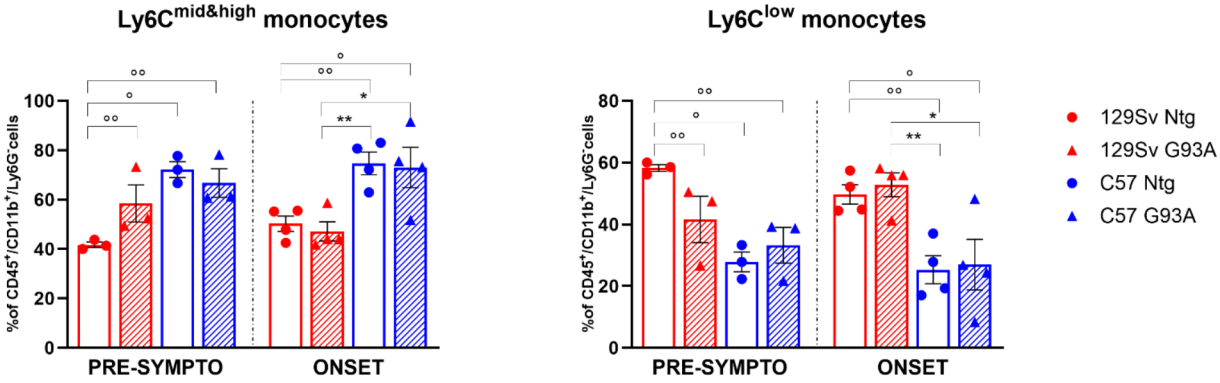


Fig. 5 Analysis of circulating monocyte populations across different time points and mouse strains. Proinflammatory monocytes are identified as CD45 + CD11b + Ly6C + cells, while patrolling monocytes are defined as CD45 + CD11b + Ly6C- cells. Data are expressed as mean ± SEM of $n = 3–4$ mice per experimental group. °$p < 0.05$, °°$p < 0.01$ (129 Sv Ntg Vs 129 Sv G93A, C57 Ntg and C57 G93A); *$p < 0.05$, **$p < 0.01$ (129 Sv G93A Vs 129 Sv Ntg, C57 Ntg and C57 G93A) by two way-ANOVA with Fisher post-analysis

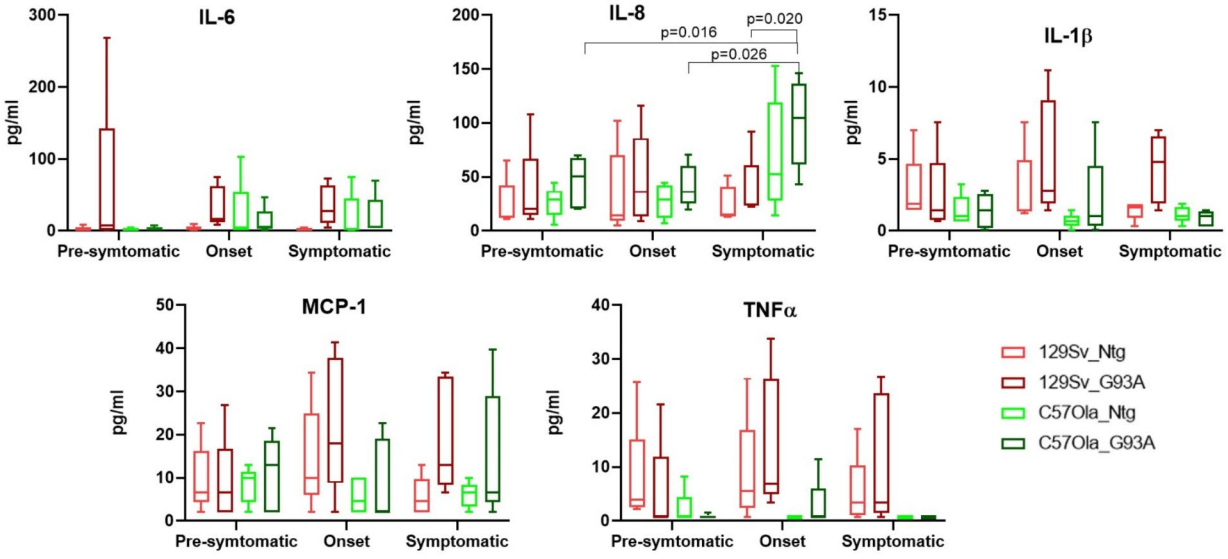


Fig. 6 Serum cytokine levels across different time points and mouse strains. Boxplot showing the serum cytokines levels. Comparison between time points and strains were performed by two-way ANOVA using Tukey's multiple comparisons post-test. * adjusted p value < 0.05

either monocyte population. At the onset age, significant differences in both monocyte populations were still present between the NTG of the two mouse strains. However, the difference between the 129Sv_G93A mice and their respective NTG mice was not maintained (Fig. 5B). Unfortunately, analysis at the symptomatic stage was hindered by a scarcity of available blood samples from the mice.

No statistically significant differences in peripheral blood cytokine levels were observed between $SOD1^{G93A}$ and Ntg littermates across all examined time points. Also, the cytokine levels in both $SOD1^{G93A}$ mice demonstrated minimal variation throughout disease progression, except for KC/IL-8 that exhibited an increase during the symptomatic stage in C57Ola_G93A mice compared to the PS ($p=0.016$) and OS ($p=0.026$) stages (see Fig. 6). When comparing $SOD1^{G93A}$ mice across strains, significant differences emerged at the symptomatic stage. Specifically, KC levels were significantly higher in C57Ola_G93A compared to 129Sv_G93A, indicating a distinct response ($p=0.020$). Additionally, a discernible, although not statistically significant trend was observed for TNFα and IL-1β, which appeared higher in 129Sv_G93A than C57Ola_G93A peripheral blood at onset and symptomatic stages. This suggests a potential strain-specific

variation in the immune response, although the significance was not retained after post-test adjustment of *p* values.

Discussion

Recent research highlights the complex relationship between gut microbiota and ALS progression, suggesting that dysbiosis and altered metabolites' profiles may contribute to disease progression through mechanisms involving the brain-gut-microbiota axis [7, 15, 27]. Despite extensive studies, the right causative factors remain unclear; however, understanding these dynamics may be critical for developing effective diagnostic and therapeutic strategies for ALS. Our investigation delved into the intricate interplay among genetic factors, GM composition, metabolism, and immune response within ALS mouse models. Specifically, we analyzed two distinct $SOD1^{G93A}$ mice strains, along with their Ntg littermates: 129Sv_G93A, characterized by rapid disease progression and C57Ola_G93A, exhibiting a slower course. This contrast highlights the substantial impact of genetic background on ALS trajectory.

The contribution of the genetic background to the microbiota and inflammation

First, we observed strain-specific nuances in microbiota composition and fecal metabolites between C57Ola and 129 Sv strains, with taxonomic analysis revealing a diverse microbial landscape dominated by *Firmicutes*, *Bacteroidota*, and *Campilobacterota* [31, 49]. Previous studies suggest that genetic background significantly influences GM composition [56]. For example, BALB/c mice exhibit greater microbial diversity and distinct compositional variations compared to other strains [24, 28, 36].

The 129 Sv strain displayed a more consistent microbial profile, characterized by an increase in butyrate-producing taxa from the Clostridia and Lachnospiraceae families, and a reduction Erysipelotrichaceae genera (*Dubosiella* and *Faecalibaculum*) and the *UCG-002* genus of Coriobacteriaceae family. Differences in fecal fatty acid levels was observed, with SCFA and MCFA playing essential roles as both energy-supplying metabolites and signalling molecules involved in regulating lipid metabolism. Abnormal levels over time can contribute to inflammation and insulin resistance, potentially leading to several diseases, including diabetes, neurodegenerative diseases, and cancer [19, 34]. MCFA, such as capric acid and lauric acid, are primarily obtained from medium-chain triglycerides in dietary sources like milk and plant oils, but can also be produced by GM [59, 60]. For instance, yeast strains, like *Saccharomyces cerevisiae*, produce caprylate (C8) via EHT1 and EEB1 genes, which facilitate medium-chain fatty acid synthesis [60]. Recent studies show that GM can rapidly metabolize MCFA to produce energy in the colon, with Erysipelotrichaceae, Peptococcaceae, and other taxa positively correlating with cecal MCFA concentrations [30]. MCFA are readily absorbed and metabolized in liver mitochondria, producing ketone bodies that can enhance cerebral energy metabolism during low glucose availability [13, 26, 55]. Daily consumption of MCFA, such as coconut oil or triglycerides, has been linked to neuroprotection and cognitive benefits in ALS models [21, 55]. SCFA, produced by GM through the fermentation of dietary fibers, modulate immune responses via G protein-coupled receptors (GPCRs) such as GPR41 and GPR43, improving intestinal barrier integrity and promoting anti-inflammatory response [58]. Additionally, SCFAs act as histone deacetylase (HDAC) inhibitors, modulating gene expression to support barrier functions and immune cell activation [46]. Conversely, MCFAs act primarily through GPR84, triggering proinflammatory responses, including chemotaxis of immune cells, and increased production of cytokines like IL-8 and TNFα in response to lipopolysaccharide (LPS) stimulation [65].

Notably, 129Sv_Ntg mice displayed higher total MCFA levels, especially heptanoic acid and dodecanoic acid, and a reduction in propionic acid compared to same-age C57Ola mice. This discrepancy may stem from inherent genetic variability influencing fatty acid metabolism, as129Sv mice have a higher metabolic rate and a fat oxidation capacity [4]. These metabolic differences likely contribute to variations in substrate utilization patterns, impacting the processing and excretion of MCFA. Furthermore, GM plays a crucial role in modulating dietary component metabolism. It is plausible that the GM of 129 Sv mice possesses specific enzymatic capabilities or preferential pathways for metabolizing MCFA, leading to alterations in fecal fatty acid profiles. Conversely, differences in microbial composition and functionality in C57Ola mice may result in distinct metabolic outcomes for these fatty acids. Functional prediction revealed that C57Ola mice have higher lipid metabolism potential possibly linked to the abundance of Erysipelotrichaceae, which correlates with MCFA levels [38].

The divergent GM compositions may also contribute to differential immune responses; for instance, lower *Faecalibaculum* levels in C57Ola mice corresponded with a higher proportion of proinflammatory monocytes compared to 129 Sv mice [71].

Given the observed differences in cytokine levels and monocytic profiles between 129 Sv and C57OlaNtg mice, we propose a hypothesis regarding the underlying metabolic and immune mechanisms. The higher fecal levels of MCFA in 129 Sv mice might indicate lower systemic levels of these fatty acids, as MCFA are rapidly absorbed and metabolized. This could lead to reduced availability

of MCFA in the bloodstream, which may favour a less proinflammatory monocytic profile. Conversely, the lower fecal levels of SCFA in 129 Sv mice might correspond to higher systemic concentrations, as SCFA are efficiently absorbed and promote the patrolling phenotype of monocytes, exerting ant-inflammatory effects. Alternatively, differential expression of fatty acid receptors could explain these observations. SCFAs and MCFAs mediate their effects through receptors such as GPR41, GPR43, and GPR84. Higher receptor expression in 129 Sv mice might enhance the anti-inflammatory signaling of SCFA, even at similar systemic concentrations. In contrast, elevated GPR84 expression could influence the proinflammatory effects of MCFAs, accounting for the higher proinflammatory monocyte levels in C57OlaNtg mice. The elevated levels of proinflammatory cytokines (e.g., TNF-α and MCP-1) in 129 Sv Ntg mice, despite a higher proportion of patrolling monocytes, may indicate a compensatory immune response attempting to counterbalance the systemic inflammatory environment. In contrast, C57BL/6 Ntg mice, with lower fecal levels of MCFA, might retain more of these fatty acids systemically, contributing to higher levels of proinflammatory monocytes and potentially a more direct proinflammatory state. Additionally, higher fecal SCFA levels in C57Ola mice could indicate lower systemic availability, diminishing anti-inflammatory effects. Therefore, the differential expression of fatty acid receptors warrants further investigation.

These findings suggest a complex interplay between gut-derived metabolites and systemic immune responses, where the balance of MCFA and SCFA may significantly influence the inflammatory status and the phenotypic distribution of monocytes. Besides, we demonstrated that genetic background may influence differences in energetic metabolism and immune response modulation capacity of the GM, potentially contributing to variable susceptibility to disease. Further studies are needed to explore the systemic levels of these fatty acids and their mechanisms in modulating immune cell function in different genetic backgrounds, potentially leading to novel therapeutic strategies for managing inflammation in ALS.

The contribution of SOD1G93A to the microbiota and inflammation

The SOD1^{G93A} mutation influenced GM colonization in both strains, albeit to varying extents. In a pre-symptomatic stage, C57Ola_G93A mice exhibited a significant microbiota shift compared to their Ntg littermates, whereas the 129 Sv G93A strain showed subtle changes, suggestinging a more stable baseline microbiota profile less susceptible to mutation-induced perturbations. Interestingly, in C57Ola_G93A mice, specific taxa, such as Erysipelotrichaceae, Prevotellaceae NK3B31_group, *Dubosiella*, and *Faecalibaculum* decreased, while total MCFA, heptanoic acid, dodecanoic acid, and decanoic acid levels increased, alongside reduced propionic acid. The mutation-induced shift led C57Ola_G93A mice to acquire similarities with 129 Sv but in some taxa and metabolite levels, such as Clostridia, Lachnospiraceae, Erysipelotrichaceae, *Dubosiella*, and *Faecalibaculum* as well as SCFA and MCFA levels. This suggests that the SOD1G93A mutation in C57Ola mice may induce microbiota changes resembling those of 129 Sv strain, potentially contributing to disease pathogenesis or progression. Erysipelotrichaceae and Coriobacteriaceae have been linked to host lipid metabolism and dyslipidemic phenotypes [16, 43, 44, 70]. While Clostridia and Lachnospiraceae are significant producers of beneficial SCFA, Lachnospiraceae has also been associated with glucose and/or lipid metabolism disturbances, indicating potential metabolic dysregulation [14, 39, 62]. Furthermore, Lachnospiraceae *genus bacterium A4* has been associated with disease progression in SOD1^{G93A} ALS mice [72], suggesting a complex role of this taxon in both host health and disease [67].

The elevation of MCFA levels and reduction of SCFA in C57Ola_G93A mice may mirror a connection between mutant SOD1 expression and fatty acid metabolism, as MCFA are associated with inflammation and higher levels in ALS patients [54]. The lower propionic acid level in both 129Sv_Ntg mice and C57Ola_G93A could indicate altered anti-inflammatory pathways. Notably, propionic acid is known to reduce inflammatory cytokines (TNF-α and IP-10) and promote metabolic health [1–3, 33].

Additionally, C57Ola_G93A strain demonstrated distinct immune responses compared to the 129Sv_G93A, with increased Erysipelotrichaceae, *Dubosiella*, and *Faecalibaculum* levels observed at disease onset and late stages. Strain-specific increases in cytokine KC/IL-8 during the symptomatic stage further reflect the immune profile changes in these mutated strains. Although we could not measure fecal fatty acid levels during the onset and symptomatic stages due to material constrains, functional pathway prediction estimated significant increases in lipoic acid metabolism and fatty acid degradation in C57Ola_G93A mice compared to 129Sv_G93A. The predicted upregulation of fatty acid degradation and ketone body metabolism in C57Ola_G93A mice may suggest an enhanced utilization of fatty acids for energy production, possibly as a compensatory mechanism for the impaired energy metabolism associated with ALS progression, which is not observed in 129Sv_G93A microbiota.

Lastly, our assessment of temporal changes within the same strains documented no significant shifts in alpha or beta diversity, suggesting that GM composition remained relatively stable throughout disease progression. This contrasts with previous studies reporting microbiota

changes over time in both ALS patient [20] and animal models [27]. Differences in study design, confounding variables (e.g., diet, environment), and the use of distinct strains likely account for these disparities. However, our findings do not exclude potential subtle alterations in specific microbial genera or functional properties. A more comprehensive approach, such as shotgun metagenomic sequencing, could reveal functionally relevant changes that 16 S rRNA sequencing may have missed.

Conclusions

Overall, these findings suggest that genetic background may influence differences in energetic metabolism and immune response modulation capacity of the GM in the two strains, potentially contributing to variable susceptibility to disease. Since the housing facility has been reported to significantly impact the mouse survival and microbiota [11, 27], the advantage of this study is that both fast and slow disease-progressing mouse cohorts are housed in the same facility and receive the same diet, so the potential diversity of their microbiota and immune/inflammatory system in relation to their survival may be due exclusively to the different genetic background.

We acknowledge that mice were analyzed at different time points (16 or 22 weeks) due to variations in disease progression, which may introduce biological variability. While we focused on the pre-symptomatic phase (12 weeks) for microbiota and fatty acid comparisons, immune responses were assessed at both onset and symptomatic stages. Age-related factors may have influenced immune outcomes, which should be considered when interpreting these results.

The functional and compositional variations in the GM, particularly in MCFA metabolism and specific bacterial taxa, such as Erysipelotrichaceae, may show predisposition to ALS. Further research is needed to clarify their role in disease progression.

Surely, the study has some limitations; firstly, we primarily relied on fecal samples which may not fully capture microbial diversity and functionality throughout the gastrointestinal tract. Additionally, we did not assess circulating free fatty acids, and our taxonomic resolution was limited to the genus level. Future studies employing metagenomic or metatranscriptomic approaches could provide more detailed taxonomic information, enhancing our understanding of GM composition in ALS.

Future mechanistic studies, including fecal microbiota transplantation (FMT) experiments, targeted interventions or genetic manipulations, could elucidate the causal relationships between genetic background, GM, and ALS pathology. In detail, FMT will allow for a more rigorous assessment of the causal GM effects on ALS progression. Additionally, research focused on elucidating the metabolic pathways and enzymatic mechanisms involved in medium-chain fatty acid metabolism in ALS models could provide valuable insights into their role in disease progression.

Abbreviations

ALS	Amyotrophic lateral sclerosis
SOD	Superoxide dismutase
GM	Gut microbiota
FFA	Free fatty acids
SCFA	Short chain fatty acids
MCFA	Medium chain fatty acids

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40478-024-01877-x.

Supplementary Material 1

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Author contributions

A.A., E.N., G.N., C.B.: Conceptualization and Methodology; E.N., G.N., S.B., M.C.T., P.F., C.M.: Investigation; L.D.G., M.C.T., S.B., C.N., E.N.: Formal Analysis; A.A., G.N., C.B.: Resources; E.N., M.C.T., L.D.G.: Writing- Original draft preparation; L.D.G., E.N., M.C.T.: Visualization; C.B., A.A., G.B., M.R.: Supervision; E.N., C.N., G.N., C.B., A.A.: Writing- Reviewing and Editing.

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Data availability

The datasets generated in this study are publicly available in NCBI Gene Expression Omnibus (GEO) repository at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi? acc= GSE278875. The data that support the findings of this study are available at https://github.com/LeandroD94/Papers/tree/main/2024_SLA_mice_Fast129Sv_SlowC57_G93A. Data not included in the repository are available upon request.

Declarations

Ethics approval and consent to participate

Procedures involving animals and their care were conducted according to the Mario Negri institutional guidelines, which comply with national (D.L. no. 116, G.U. suppl. 40, Feb.18, 1992, Circular No.8, G.U., 14 July 1994) and international policies (EEC Council Directive 86/609, OJ L 358, 1 Dec.12, 1987; NIH Guide for the Care and use of Laboratory Animals, U.S. National Research Council, 1996). All experiments and protocols were examined by the Institutional Ethical Committee and authorized by the Italian Ministry of Health.

Consent for pubblication

not applicable.

Competing interests

The authors declare no competing interests.

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