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Gut Microbiome Ecological Network Destabilization in Amyotrophic Lateral Sclerosis: Insights from Integrated Oral–Gut Analysis

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Abstract

The pathogenesis of amyotrophic lateral sclerosis (ALS) involves multiple systems, among which the "gut-brain axis" has been extensively studied. However, the characteristics of intestinal microbiota alterations and their potential association with the oral microbiota remain unclear. This cohort study aims to characterize the ecological destabilization of intestinal microbiota in ALS patients. On this basis, this study further explored the potential association between the intestinal and oral microbiotas. In this study, paired oral and fecal samples from ALS patients and fecal samples from healthy controls were collected and subjected to 16S rRNA amplicon sequencing. The results showed that the intestinal microbiota in ALS patients exhibited significant compositional shifts, characterized by enrichment of pro-inflammatory genera and depletion of butyrate-producing genera, along with reduced alpha diversity. Critically, quantitative analysis using a potential landscape model revealed that this altered community was in a metastable state (comprehensive stability index: HC + 0.00110 vs. ALS − 0.0118), indicating ecological destabilization. this study examined oral-gut microbial interactions and identified 20 significant correlations ($|r| \geq 0.6$), with the strongest between oral Prevotella salivae and intestinal Dorea ($r = 0.937$). Notably, the intestinal genera involved in these associations (*Dorea*, *Ruminococcus*, and *Blautia*) were also differentially abundant in ALS patients, suggesting that oral-gut microbial interactions may contribute to intestinal dysbiosis. This study demonstrates that the intestinal microecosystem in ALS patients exhibits features of ecological destabilization, and identifies oral-gut microbial interactions may as potential drivers of intestinal dysbiosis.

1.Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease with an irreversible and rapidly progression [1], characterized by the progressive and selective loss of upper and lower motor neurons in the brain and spinal cord. This disease leads to skeletal muscle atrophy, paralysis and eventually respiratory failure. Although the exact cause of ALS remains unclear, it is widely accepted that ALS results from a combination of genetic susceptibility and environmental factors [2], including lifestyle and microbial alterations. The global incidence and prevalence of ALS show significant heterogeneity, influenced by factors such as age, sex, and ethnicity [3]. Especially with the global trend of population aging, the disease burden of ALS is projected to increase continuously [4]. Current clinical treatments are very limited, with only a few medicines such as riluzole [5] and edaravone [6] are available, These medicines offer modest efficacy, slightly delaying the progression of disease, failing to significantly improve neurological function or reverse the progression of disease. This situation underscores the urgent need to explore non-neuronal pathogenesis of ALS and to identify new therapeutic targets.

In recent years, the "brain-gut axis" has gained extensive attention as a key pathway in the pathogenesis of neurological diseases. The intestinal microbiota and its metabolites can profoundly influence the function, neuroinflammation and homeostasis of the central nervous system through immune [7], endocrine [8], and neural [9] pathways. In the field of ALS research, accumulating evidence indicate that patients exhibit characteristic intestinal microbial dysbiosis. This dysregulation is closely associated

with the severity of disease [10], rate of the progression and systemic inflammatory status [11]. A study by Gotkine et al. [12] further confirmed that ALS patients have characteristic dysbiosis of the intestinal microbiota, aiming to indicated that it may be related to the disease progression and systemic inflammation, providing a new entry point for understanding the pathology of ALS. A recent Mendelian randomization study was published in 2025 [13] further strengthened the causal link between the two, demonstrating that specific intestinal bacterial genera, such as *Bifidobacterium*, may be risk factors for ALS, while others, such as *Enterobacter*, may have protective effects. Furthermore, animal model studies have shown that changes in the intestinal microbiota may occur before the appearance of neuromuscular symptoms [14], indicating its potential role in the initial mechanisms of the disease.

Although significant progress has been made in understanding the "brain-gut axis", there are still major gaps in understanding the microbial dysbiosis in ALS. First, most studies have focused solely on describing compositional changes of the fecal microbiota, lacking quantitative assessment of microbial community ecological stability. Microbial communities are not simply collections of independent microorganisms; their internal networks of synergy and competition are crucial for understanding community function and ecological stability. However, this ecological dynamics perspective remains underexplored in ALS research. Second, the oral cavity, as the entrance of the digestive tract, is anatomically and functionally connected to the intestine. The oral microbiota may continuously influence intestinal microbiota composition and function through daily swallowing, yet the role of oral-gut microbial interactions in intestinal dysbiosis in ALS remains unclear.

To systematically figure out these scientific issues and fill the existing knowledge gaps, this study was designed. 16S rRNA amplicon sequencing was usedfor detecting paired oral and fecal samples from ALS patients in a cohort (to achieve direct comparison between body parts), as well as fecal samples from healthy controls (as a baseline for the intestinal microbiota). Utilizing high-throughput 16S rRNA amplicon sequencing, combined with a series of multivariate bioinformatics analyses—including α/β diversities analysis, identification of differential abundance species, construction of microbial co-occurrence network, and algorithm of random forest. this study aims to characterize the ecological destabilization of the intestinal microbiota in ALS and to explore whether oral-gut microbial interactions contribute to this destabilization.

2. Methods

2.1. Study Subjects and Sample Collection

Participant Recruitment, Time and Location

A cohort study (Figure 1) was conducted at Suzhou Municipal Hospital (Taihu Campus) from December 2024 to December 2025. A total of 22 participants were enrolled,including 11 patients with amyotrophic lateral sclerosis (ALS) and 11 healthy controls.All ALS patients met the revised El Escorial diagnostic

criteria, and diagnoses were independently confirmed by two neurologists. Clinical trial number: not applicable

Exclusion Criteria

Use of antibiotics, probiotics, or prebiotics within one month before enrollment. Presence of other systemic diseases (e.g., diabetes, autoimmune diseases, inflammatory bowel disease, liver cirrhosis, rheumatoid arthritis). other infectious diseases (e.g., respiratory tract infection, genitourinary infection). long-term use of immunosuppressants or glucocorticoids. pregnancy or lactation. other neurodegenerative or psychiatric disorders

Diagnosis and Grouping

ALS group: Patients diagnosed with amyotrophic lateral sclerosis. Healthy control group: Healthy volunteers with no history of neurodegenerative diseases and met none of the above exclusion criteria.

Sample Collection

ALS patients: Oral swabs and fecal samples were collected at enrollment. Oral samples: Used sterile oral swabs,bilateral buccal mucosa and dorsal tongue surfaces were collected before brushing tooth in the morning. Swabs were immediately placed in sterile PBS tubes and stored at − 80°C. Fecal samples: Used sterile collection tubes to collect the middle portion of the first stool sample in the morning and immediately stored at − 80°C. Healthy controls: Fecal samples were collected at the same time and stored under identical conditions, oral samples were not collected from healthy controls;

Finally, among 33 samples from 22 participants (11 oral samples and 11 fecal samples from ALS patients; 11 fecal samples from healthy controls) were subjected to high-throughput 16S rRNA amplicon sequencing.

2.2. Microbial DNA Extraction and Library Construction

2.2.1. DNA Extraction

Fecal samples: Bacterial genomic DNA was extracted by using the QIAamp DNA Stool Mini Kit (QIAGEN, Germany) according to the manufacturer’s instructions.Oral samples: Bacterial DNA from oral swabs was extracted by using the QIAamp DNA Microbiome Kit (QIAGEN, Germany). DNA concentration was measured by using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, USA), and DNA integrity was verified by 1% agarose gel electrophoresis. Samples with concentration < 1 ng/μL or obvious degradation were excluded.

2.2.2. 16S rRNA Gene Amplification

The V3–V4 hypervariable region of the bacterial 16S rRNA gene was amplified by PCR by using the primer pair 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-

GGACTACHVGGGTWTCTAAT-3'). The PCR reaction system (25μL) contained: 10 ng DNA template, 12.5μL 2× Phusion High-Fidelity PCR Master Mix (New England Biolabs, USA), 0.5μM (final concentration) of each forward and reverse primer,and sterile ddH_2O up to 25μL. PCR thermal cycling conditions were as follows: Initial denaturation at 98°C for 1 min; 30 cycles of denaturation at 98°C for 10s, annealing at 50°C for 30s, extension at 72°C for 30s; followed by a final extension at 72°C for 5 min. Amplicons were purified by using Agencourt AMPure XP beads (Beckman Coulter, USA).

2.2.3. Library Construction and Sequencing

Sequencing libraries were constructed by using the Nextera XT DNA Library Preparation Kit (Illumina, USA). Unique dual-index barcodes were added to each sample by PCR for splitting the sample. Purified libraries were assessed at size and quality by using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and quantified with the Qubit.All libraries were pooled in equimolar amounts and diluted to 4 nM. The pooled library was denatured, diluted to 8 pM, and mixed with 30% PhiX control library. Paired-end sequencing was performed on the Illumina MiSeq platform by using the MiSeq Reagent Kit v3 (600 cycles), with a read length of 2 × 300 bp.

2.3. Bioinformatic Analysis

2.3.1. Raw Data Processing

Adapter and primer sequences were removed from raw sequencing reads using Cutadapt (v1.2.1). Quality filtering, denoising, paired-end merging, and chimera removal were performed using the DADA2 plugin in QIIME2 (v2020.11) to generate amplicon sequence variants (ASVs). Representative sequences of each ASV were taxonomically annotated against the SILVA database (v138) using a naive Bayes classifier with a confidence threshold of 0.8.

2.3.2. Diversity Analysis

Diversity analyses were performed using the vegan package (v2.5-3) in R software (v4.0.3). α-diversities: The Shannon index, Chao1 index, and observed species index were calculated to reflect richness and evenness of within-sample species. Between-group comparisons were performed using the Wilcoxon rank-sum test. β-diversities: Community dissimilarity was calculated based on Bray–Curtis distance. Principal Coordinate Analysis (PCoA) was used for dimensionality reduction and visualization. Between-group differences were tested by permutational multivariate analysis of variance (PERMANOVA,999 permutations). t-SNE (perplexity = 30) and Principal Component Analysis (PCA) were used for supplementary validation.

A heatmap was generated to visualize the top 50 most abundant genera across all samples. Wilcoxon rank-sum tests were used to compare relative abundances at the phylum and genus levels between groups. Boxplots for the top 20 differentially abundant genera were generated to show the distribution of these taxa between groups. A separate heatmap displaying the relative abundance of key differentially

abundant genera was also generated to visualize the distinguishing patterns between ALS patients and healthy controls.

Correlations among genera were calculated using the SparCC algorithm (implemented in Python, 100 iterations). Genus pairs with|correlation coefficient| > 0.3 and $p < 0.05$ were used for network construction. Networks were visualized in Gephi (v0.9.2). In the generated network diagram, nodes represent different bacterial genera, and node size is proportional to the degree (the number of edges connected to the node). Edges indicate significant correlations between two bacterial genera. To visually distinguish the direction of the correlation, using different colors to mark the edges: red edges represent positive correlations, and blue edges represent negative correlations. The thickness of an edge is proportional to the absolute strength of the correlation ($|r|$).

2.3.3. Potential Landscape Analysis

The ecological stability of the microbial ecosystem was quantitatively assessed using a potential landscape model based on the framework previously described by Li et al. [15] and Wang et al. [16]. This approach conceptualizes the microbial community as a dynamic system moving across an energy landscape, where stable states correspond to valleys (attractors) and unstable states correspond to peaks.

The analysis was performed using custom R scripts with the following steps:

First, dimensionality reduction was conducted using Principal Coordinate Analysis (PCoA) based on Bray-Curtis distances from the genus-level relative abundance data. The first two principal coordinates (PCo1 and PCo2) were selected to represent the major axes of community variation, capturing the most significant ecological gradients.

Second, energy landscape construction involved creating a two-dimensional grid over the range of PCo1 and PCo2 scores. For each grid point, the local density of samples was estimated using a Gaussian kernel function. The potential energy U at each grid point was then calculated as the negative logarithm of the sample density: $U = -\ln(\rho)$, where ρ is the estimated density. This transformation results in regions with high sample density (frequently occupied community states) corresponding to low energy valleys, while regions with low sample density correspond to high energy peaks.

Third, stability parameters were derived from the constructed potential landscape to characterize ecosystem stability:

Valley depth (ΔU): The difference in potential energy between the bottom of a valley and the surrounding ridge. A deeper valley indicates a more stable attractor state, requiring more energy to perturb the system out of that state.

Valley area (A_b): The size of the basin of attraction. A larger area indicates that the system can tolerate wider fluctuations while remaining within the same stable state.

Comprehensive stability index (S): A composite index integrating both valley depth and area, calculated as $S = \Delta U \times A_b$. A positive S value indicates a stable ecosystem with deep, well-defined attractor basins, while a negative S value suggests an unstable or metastable ecosystem prone to state transitions under perturbation.

Finally, comparative analysis of the potential landscape parameters was performed separately for the healthy control group and the ALS patient group based on their respective sample distributions in the PCoA space. The stability indices (ΔU, A_b, and S) were compared between groups to quantify the shift in ecosystem stability associated with ALS.

2.4. Machine Learning Model Construction

A classification model for ALS disease status was constructed using the randomForest package (v4.6-14) in R. Input features were the relative abundances of differential gut genera identified by LEfSe. The dataset was split into training (70%) and testing (30%) sets. The number of trees (ntree) and the number of randomly selected features per node (mtry) were tuned by 10-fold cross-validation.

The pROC package (v1.16.2) was used to plot the receiver operating characteristic (ROC) curve and calculate the area under the curve (AUC), sensitivity, and specificity. Feature importance was evaluated by mean decrease accuracy and mean decrease Gini index.

2.5. Statistical Analysis

All statistical analyses were performed using R software (v4.0.3) and GraphPad Prism (v8.0). Continuous variables are expressed as mean ± standard deviation or median (interquartile range, IQR).

Between-group comparisons for continuous variables: Student's t-test (normal distribution) or Wilcoxon rank-sum test (non-normal distribution). Multiple groups: Kruskal–Wallis test, with post-hoc pairwise comparisons by Dunn's test. A two-sided $p < 0.05$ was considered statistically significant. The Benjamini-Hochberg method was used to control the false discovery rate (FDR) in differential taxa analysis.

3. Results

3.1. intestinal microbial diversity is reduced and community structure is altered in ALS

As illustrated in Fig. 1, the study workflow included paired oral and fecal samples collected from ALS patients and fecal samples from healthy controls, followed by 16S rRNA sequencing and multi-dimensional bioinformatics analyses. The two groups were well-matched in age (median: HC 54 years, IQR 49–65; ALS 54.5 years, IQR 48–66; P = 0.78) and gender composition (54.5% female in the HC group vs. 63.6% female in the ALS group; P = 0.45) (P > 0.05), thereby minimizing potential confounding effects from demographic factors (Fig. 2a).

To compare the species richness and evenness of the microbial communities between the two groups, an alpha diversity analysis was first performed. The analysis showed that the Shannon index of the intestinal microbiota in ALS patients was significantly lower than that of the healthy controls (mean ± SD: ALS 3.110 ± 0.595 vs. HC 3.479 ± 0.354; IQR: ALS 0.292 vs. HC 0.325), indicating a comprehensive decline in species richness and evenness (Fig. 2b). The reduction in microbial diversity is a typical characteristic of ecological dysbiosis, indicating that the intestinal ecosystem of ALS patients has diminished functional redundancy and the ability to resist external disturbances (e.g., infections, medications, dietary changes) has weakened. This result is consistent with several previous studies on the intestinal microbiota of ALS patients [17], all of which have reported significant ecological imbalance in the disease state.

Subsequently, analysis of beta diversities were conducted to explore differences in the overall structure of microbial communities between the two groups. Principal Coordinate Analysis (PCoA) based on Bray-Curtis distance clearly revealed a significant separation in the intestinal microbial structure between the ALS group and the control group (PERMANOVA, $R^2 = 0.15$, $p = 0.002$) (Fig. 2c). This finding indicates that the overall intestinal microbial community undergoes directional changes in ALS. This structural change may reflect the selective pressure exerted by the disease state on the intestinal ecosystem, possibly driven by systemic inflammation, altered intestinal motility, the use of medication, and are consistent with the consensus that disease state is associated with overall microbial community structure [9, 18]. The t-SNE results further supported this conclusion, two methods of dimensionality reduction showed a consistent grouping trend (Fig. 2d), enhancing the robustness of the finding.

3.2. ALS is characterized by depletion of butyrate-producers and enrichment of pro-inflammatory taxa

Systematical assessment of the compositional changes in the intestinal microbiota of ALS patients, analyzing differences at the phylum and genus levels. At the phylum level, the relative abundance of Firmicutes was significantly decreased in ALS patients compared to healthy controls (ALS: 88.45% vs. HC: 93.65%, $p = 0.047$). Firmicutes includes many butyrate-producing bacteria indicates. Conversely, the relative abundance of Bacteroidetes was significantly increased (ALS: 3.75% vs. HC: 0.65%, $p = 0.012$) (Fig. 3a). The Bacteroidetes phylum is rich in LPS-producing bacteria, and its increase may exacerbate intestinal inflammation. This trend is consistent with the findings of Zeng et al. [19] and Wu et al. [20] in ALS cohorts, further confirming the prevalence of "phylum-level imbalance" in ALS.

Following the phylum-level observations, examining the top 50 most abundant genera across all samples (Fig. 3b). The bacterial genera that were significantly enriched in ALS patients mainly included *Rothia, Prevotella, Veillonella, Porphyromonas*, and *Actinomyces*. Some genera (such as *Prevotella* and *Veillonella*) have been reported to be associated with pro-inflammatory phenotypes in studies related to neuroinflammation [21, 22]. Genera enriched in healthy controls were predominantly beneficial butyrate-producing bacteria, including *Faecalibacterium, Roseburia, Blautia*, and *Dorea.* This binary pattern of

"beneficial microbe depletion and pro-inflammatory microbe enrichment" represents one of the core findings of this study.

This binary pattern has important implications for ALS pathogenesis. Depletion of butyrate-producing bacteria (e.g., *Faecalibacterium, Roseburia*) can lead to damage to the intestinal barrier function [23], increasing the entry of inflammatory substances such as lipopolysaccharide (LPS) into the blood circulation, activating systemic immune responses, and exacerbating neuroinflammation through the "gut-brain axis" [24]. Sokol et al. previously demonstrated the anti-inflammatory effects of *Faecalibacterium* and its reduction in inflammatory bowel disease [25]; its reduction in ALS patients may similarly contribute to disease pathology. Conversely, enrichment of pro-inflammatory bacteria such as *Prevotella* can activate the Toll-like receptor 4 (TLR4) signaling pathway by producing LPS, promoting the release of pro-inflammatory factors including Tumor necrosis factor-alpha (TNF-α) and Interleukin-6 (IL-6) [26]. While the precise pathogenic mechanisms of these bacteria in ALS remain to be elucidated, their increased abundance in the intestine of ALS patients is evident.

Rothia, an opportunistic pathogen in the oral cavity [27], differs from the intestinal "inhabitants" such as butyrate-producing bacteria and *Prevotella.* and can cause systemic infections such as infectious endocarditis and pneumonia in immunocompromised patients [28]; its enrichment in the intestinal tract of ALS patients may suggest oral-gut microbial translocation. This finding is consistent with reports of periodontal pathogen enrichment in Alzheimer's disease [29]. *Prevotella* and *Veillonella* are known as pro-inflammatory genera, closely related to periodontitis and systemic inflammation [30], further supporting the characteristic shift of the intestinal microbial community towards a pro-inflammatory phenotype in ALS patients. In particular, the increase of *Rothia* has been reported in previous studies linking oral microbiota to neurodegenerative diseases [31], suggesting a potential cross-site association with neuroinflammation. This finding is consistent with the conclusions of the seminal study by Atarashi et al. [32] published in Science, which demonstrated that oral bacteria can ectopically colonize the intestine and induce Type 1 T helper cell (Th1) mediated immune responses, which exacerbate local inflammation.

Subsequently, this study explored microbial characteristics at multiple levels. First, at the single bacterial genus level, box plots were used to display the 20 bacterial genera with the most significant differences in the intestinal samples, further verifying the changes in abundance of the aforementioned anti-inflammatory and pro-inflammatory bacteria (Fig. 3c). Second, at the overall pattern level, heatmap showing the relative abundance of the top 20 differentially abundant genera between ALS patients and healthy controls clearly delineated their distinguishing patterns (Fig. 3d). Genera enriched in ALS patients included the pro-inflammatory genera *Prevotella*, *Rothia*, and *Veillonella*, as well as *Streptococcus* and *Actinomyces*. Genera enriched in healthy controls were predominantly butyrate-producing bacteria, including *Faecalibacterium*, *Roseburia*, *Blautia*, and *Dorea*. Finally, a functional classification of the top 20 most abundant genera was performed based on their reported functions (Fig. 3e). Butyrate-producing genera, including *Anaerostipes hadrus* and *[Eubacterium] hallii*, accounted for 30% of the high-abundance taxa, whereas pro-inflammatory genera (e.g., *Streptococcus salivarius*) and dual-function genera (e.g.,

Ruminococcus faecis) were also prominently represented. This functional distribution suggests a shift towards a pro-inflammatory milieu in the intestinal ecosystem of ALS patients. These observations are consistent with the "disease-related synergistic microbial module" concept proposed by Turnbaugh et al. [33], indicating that the disease state may restructure the interactions among microorganisms.

3.3. Oral-gut microbial interactions are associated with intestinal dysbiosis in ALS

In healthy controls (Fig. 4a), pro-inflammatory taxa (e.g., certain Proteobacteria and Bacteroidetes) and anti-inflammatory, butyrate-producing Firmicutes were interwoven within a single integrated cluster, reflecting an ecological balance between opposing functional groups. In contrast, the ALS network (Fig. 4b) split into two distinct clusters. One cluster was heavily enriched with oral-derived pro-inflammatory microbes (purple nodes), while the other showed a marked reduction of Firmicutes. This segregation indicates a disruption of the pro-inflammatory and anti-inflammatory balance, which likely contributes to the metastable state of the intestinal ecosystem in ALS.

Quantitative assessment of the overall stability of the intestinal ecosystem was performed using a potential landscape model (Fig. 4c). The results revealed that the healthy state occupied a deep and concentrated potential basin ($\Delta U = + 0.082$; basin area = 0.345), while the ALS state was distributed on a shallow and scattered potential plateau ($\Delta U = -0.357$; basin area = 0.85). The comprehensive stability index (S) further quantified this difference: the healthy state was positive (+ 0.00110), while the ALS state was negative (-0.0118), confirming that the intestinal microecosystem in ALS patients is in a metastable state.

3.4. Oral-gut microbial coordination and diagnostic model in ALS patients

Cross-site correlation analysis revealed that observed species richness (Fig. 5a: $r = 0.147$, $p = 0.666$) and Simpson diversity (Fig. 5c: $r = 0.475$, $p = 0.140$) were not significantly correlated between oral and fecal samples. Shannon diversity showed a moderate positive correlation that approached statistical significance (Fig. 5b: $r = 0.589$, $p = 0.056$), suggesting a potential trend toward coordinated regulation of overall microbial diversity across the two body sites. However, the limited sample size may have been insufficient to detect weak but genuine correlations. Therefore, these findings are preliminary, and require validation in independent cohorts with larger sample sizes.

Unlike alpha diversity metrics, which reflect community-level richness and evenness, network centrality captures the topological importance of individual nodes within the co-occurrence network. Centrality correlation analysis revealed a significant positive correlation between degree centrality and betweenness centrality for oral and gut microbes in ALS patients (Fig. 5d: $R^2 = 0.3379$, $p = 0.0010$), supporting the existence of structural coordination between oral and gut microbial networks.

Cross-site network analysis identified 20 significant associations between oral and intestinl genera ($|r| \geq 0.6$, $p < 0.05$), and the abundance levels of these bacteria are visualized (Fig. 5e). The three strongest associations were: (1) oral *Prevotella salivae* and intestinal *uncultured Dorea sp.* (r = 0.937); (2) oral *Lactobacillus salivarius* and intestinal *Blautia sp*. (r = 0.911); (3) oral *Lactobacillus salivarius* and intestinal *Blautia obeum* (r = 0.881). Notably, the intestinal genera involved in these 20 strong associations (Dorea, Ruminococcus, and Blautia) also showed significant differential abundance between ALS patients and healthy controls in the intestinal microbiota analysis (Fig. 3). This convergence suggests that oral-gut microbial interactions may constitute an important driver of intestinal dysbiosis in ALS.

A random forest classification model was constructed based on differential intestinal genera to evaluate whether the observed intestinal microbiota alterations could serve as biomarkers for ALS. Feature importance analysis revealed that the top 10 intestinal genera contributing to the model included *Blautia*, *Dorea*, and *Ruminococcus* (Fig. 5f).

Phylum-level ROC curve analysis was performed to evaluate the diagnostic value of intestinal microbiota at the phylum level. The results showed that AUC values of major phyla were generally low (Fig. 5g). Firmicutes exhibited the highest AUC of 0.72 (95% CI: 0.58–0.86), followed by Bacteroidetes (AUC = 0.68, 95% CI: 0.52–0.84), Proteobacteria (AUC = 0.65, 95% CI: 0.48–0.82), and Actinobacteria (AUC = 0.61, 95% CI: 0.44–0.78). AUC values of other phyla were below 0.60. These findings indicate that phylum-level intestinal microbiota has limited capacity to discriminate ALS patients from healthy controls. A random forest classification model was constructed at the species level. The model demonstrated good discriminatory performance, with an area under the curve (AUC) of 0.93 (95% CI: 0.85–0.99), a sensitivity of 88.9%, and a specificity of 90.0% (Fig. 5h). This AUC value was substantially higher than the phylum-level AUC (maximum 0.72), suggesting that ALS-associated intestinal microbiota alterations occur primarily at the species level. Due to the limited sample size and the absence of an independent validation cohort, the diagnostic value of this model requires further validation in larger populations.

4. Discussion

The core of this study lies in systematically characterizing the ecological destabilization of the intestinal microbiota in ALS patients and revealing, through integrated oral-gut analysis, that oral-gut microbial interactions may serve as important drivers of intestinal dysbiosis.

4.1. Oral-gut microbial interactions as potential drivers of intestinal dysbiosis in ALS

The abnormal associations of bacterial genera such as *Prevotella, Streptococcus*, and *Ruminococcus* identified in this study are highly consistent with existing research of ALS patients usually exhibit characteristic shifts, including a reduction in butyrate-producing bacteria and an enrichment of pro-inflammatory bacteria [34]. Building on this, the present study further revealed that the strong positive correlation between oral *Prevotella salivae* [35] and intestinal *Dorea* [36] (r = 0.937) is consistent with the

hypothesis of a "pro-inflammatory synergy module" that, if functionally validated, could contribute to intestinal inflammation.

Prevotella, as a common oral genus, has been widely confirmed to be associated with periodontitis and systemic inflammation. Larsen (2017) [30] studied that *Prevotella* mainly activates Toll-like receptor 2 (TLR2) to promote antigen-presenting cells to produce T helper 17 (Th17) polarizing cytokines, activating epithelial cells to produce Interleukin-8(IL-8), IL-6, and C-C motif chemokine ligand 20 (CCL20), thereby promoting mucosal Th17 immune response and neutrophil recruitment. The mucosal inflammation caused by *Prevotella* can lead to the systemic spread of inflammatory mediators, bacteria, and bacterial products. Chen et al. (2025) [37] further confirmed that *Prevotella* can accelerate alveolar bone destruction in periodontitis, accelerates neuronal necrosis and abnormal morphology of hippocampal neurons in mice, and reduce the cognitive function of mice. *Dorea* shows increased abundance in patients with inflammatory bowel disease and can produce pro-inflammatory metabolites such as hydrogen sulfide. The strong positive correlation between the oral *Prevotella salivae* and the intestinal *Dorea* (r = 0.937) indicates the potential formation of an "oral-gut pro-inflammatory synergistic module," jointly promoting the systemic inflammatory state in ALS.

Although the present study sampled the oral mucosa rather than subgingival plaque, it is noteworthy that enrichment of periodontal pathogens in Alzheimer's disease has been reported in multiple studies. Ciccotosto et al. (2024) found that inoculation with the Porphyromonas gingivalis alone significantly increased neuronal damage, astrocyte and microglial activation, inflammatory cytokine expression, as well as amyloid plaque deposition and hyperphosphorylated tau protein production in the mouse brain [38]. The abnormal association of oral *Prevotell* and intestinal microbiota in ALS patients in this study indicated that the oral-gut axis might play a role in ALS that is similar to that in AD-oral pathogens enter the intestine through swallowing or blood circulation, activate the intestinal immune system, and then affect the central nervous system.

Lactobacillus salivarius is identified as a probiotic, its strong association with *Blautia* species (r = 0.911) in the disease might reflect the adaptive reorganization of the "beneficial microbial synergistic network." Notably, strong positive correlations were observed" between oral *streptococci* and intestinal *Ruminococcus-Streptococcus mitis* with fecal *Ruminococcus* (r = 0.848), and oral *Streptococcus parasanguinis* and untrained *Ruminococcus* (r = 0.838), which have clear associations with ALS. The enrichment of *Ruminococcus torques* has been proven to be associated with the worsening of symptoms in an ALS mouse model [39], suggesting a potential association between oral streptococci and the abundance of opportunistic pathogens like *Ruminococcus* through regulating the intestinal microenvironment, participating in the neuroinflammatory process in ALS. This is consistent with the reports of two previous studies on the dysbiosis of the oral microbiota in ALS patients [18, 40]. It is hypothesized that these oral microbiota could potentially translocate to the intestine via daily swallowing or, if barrier function is impaired, through the bloodstream. Once in the intestine, they might interact with the resident microbiota [32], Furthermore, their antigens or metabolites could influence the host's

systemic immune status [41], potentially creating a microenvironment conducive to the growth of pro-inflammatory bacteria.

The association of *Ruminococcus* with ALS has been supported by animal experiments: a review by Boddy et al. (2021) highlighted that *Ruminococcus* torques is associated with exacerbated symptoms in ALS mouse model [39]. The positive correlations between oral streptococci and intestinal *Ruminococcus* in this study (r = 0.848 and 0.838) indicates that oral streptococci might promote the expansion of *Ruminococcus* by altering the intestinal microenvironment. The Inflammatory polysaccharides produced by *Ruminococcus* can activate dendritic cells, promoting TNF-α secretion, which may be related to the elevated inflammatory factor levels in the serum of ALS patients [42].

The intestinal genera involved in the 20 strong oral-gut associations *(Dorea, Ruminococcus*, and *Blautia*) were precisely those that differed significantly between ALS patients and healthy controls in our intestinal microbiota analysis (Fig. 3). Dorea and Ruminococcus were enriched in ALS patients, *Blautia* was depleted. This convergence suggests that oral-gut microbial interactions may contribute to the intestinal dysbiosis observed in ALS, providing a potential mechanistic link between oral microbial alterations and the characteristic shift in gut microbial composition toward a pro-inflammatory phenotype.

4.2. Potential Landscape Model: Quantitative Evidence of Intestinal Ecosystem Destabilization

The potential landscape analysis revealed a striking contrast between the two groups. The healthy state occupied a deep and concentrated potential basin (ΔU = + 0.082; basin area = 0.345), analogous to a marble at the bottom of a deep bowl—stable and self-correcting after perturbation. In contrast, the ALS state was distributed on a shallow and scattered potential plateau (ΔU = -0.357; basin area = 0.85), resembling a marble on a flat surface, where even a slight disturbance causes it to drift away with little tendency to return. The comprehensive stability index (S) quantified this difference: healthy controls were positive (+ 0.00110), while ALS patients were negative (-0.0118), indicating a metastable ecosystem prone to state transitions under perturbation.

This finding carries several implications. First, it provides quantitative evidence that the intestinal ecosystem in ALS is not merely compositionally altered but fundamentally destabilized at the systems level. Second, the metastable nature may explain the heterogeneous clinical progression observed in ALS, This may explain why ALS patients show differential symptomatic progressions under different disease stages or different environmental exposures [43]. The fragility of the microecosystem also indicate that microecological interventions for ALS may require continuous treatment rather than a one-time treatment.

4.3. Limitations and Future Directions

This study has several limitations: First, the sample size is limited, and the conclusions require validation in larger cohorts. Second, oral samples from healthy controls were not collected, which represents a

significant limitation. This precludes our ability to determine whether the observed oral microbiota alterations in ALS patients are disease-specific or reflect secondary factors associated with motor dysfunction, such as altered oral hygiene practices, reduced salivary flow, or dysphagia-common features in ALS that can independently affect oral microbial communities. Consequently, the oral microbiota findings presented here should be considered preliminary and hypothesis-generating. Third, the cross-sectional design cannot determine causality between changes in oral-gut axis and ALS patients. Third, 16S rRNA amplicon sequencing only can identify to the genus level, and cannot distinguish among different strains. Fourthly, it is important to emphasize the correlational nature of this study. While strong associations were observed between the oral and intestinal microbiota in ALS patients, the cross-sectional design precludes any determination of causality. The directional relationships proposed in the discussion, whether microbial dysbiosis is a cause or a consequence of disease progression, remain speculative. Future studies employing longitudinal cohorts, germ-free animal models colonized with ALS-derived microbiota, and interventional trials (e.g., probiotics, fecal microbiota transplantation) are urgently needed to establish causal links and to determine whether modulating the oral-gut axis represents a viable therapeutic strategy for ALS. Finally, Beyond the ecological insights, the intestinal microbial alterations identified in this study demonstrated potential clinical utility. A random forest model based on differential gut genera achieved an AUC of 0.93 in distinguishing ALS patients from healthy controls, suggesting that gut microbial features may serve as potential biomarkers. Notably, the top contributing genera included *Blautia*, *Dorea*, and *Ruminococcus*, which were also central to the oral-gut associations identified above, reinforcing their potential importance in ALS pathogenesis. However, given the limited sample size and the absence of an independent validation cohort, these results should be considered preliminary.

5. Conclusion

This cohort study revealed that the intestinal microbial ecosystem in ALS is in a metastable state (stability index: HC + 0.00110 vs. ALS − 0.0118) using a potential landscape model, providing quantitative evidence of ecological destabilization. Additionally, 21 significant oral-gut microbial associations ($|r| \geq 0.6$) were identified through paired oral-intestinal sampling, with the strongest between oral *Prevotella salivae* and intestinal *Dorea* ($r = 0.937$). The intestinal genera involved in these associations *(Dorea*, *Ruminococcus*, and *Blautia*) were also differentially abundant in ALS patients (Fig. 3), suggesting that oral-gut microbial interactions may contribute to intestinal dysbiosis. However, the absence of oral samples from healthy controls limits the interpretation of disease-specific oral microbiota changes, and these findings should be considered hypothesis-generating rather than definitive.

Declarations

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Declaration of generative AI and AI-assisted technologies in the manuscript preparation process.

Statement: During the preparation of this work, the authors used RStudio, Adobe Illustrator 2025 and Adobe Photoshop 2026 in order to Polishing the manuscript and figures. After using these tools, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Declaration of competing interests

Authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Ethics approval and consent to participate

Informed consent was obtained from all participants for this research, which was approved by the Ethics Committee of the Affiliated Hospital of Tongji University (2023-013).

Authorship contribution statement

Zelong Yang: Conceptualization, Formal analysis, Visualization, Writing – original draft, Writing – review & editing.

Wenhao Liu: Resources, Investigation, Data curation.

Bo Yan: Investigation, Validation.

Zhan Cao: Methodology, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Jingxin Li: Methodology, Funding acquisition, Project administration, Writing – review & editing.

Zhongwei Chang: Data curation.

Xixin Jin: Investigation, Project administration.

Xinjun Wang: Conceptualization, Supervision, Writing – review & editing.

Haoyu Wang: Supervision, Supervision，Writing – review & editing.

Data Availability Statement

The raw sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1425920. The data are currently private but are accessible to reviewers via the following secure link:

https://dataview.ncbi.nlm.nih.gov/object/PRJNA1425920?reviewer=gk215bgekcemo3j10m2hm3qofg

The data will be made publicly available upon publication of the manuscript.

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