Intestinal microbiota composition in patients with amyotrophic lateral sclerosis: establishment of bacterial and archaeal communities analyses

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Abstract
Background: Emerging evidences have indicated that the composition of gut microbiota was significantly influenced by central nervous system diseases. The digestion and metabolism disturbances of patients with amyotrophic lateral sclerosis (ALS) might be strongly associated with ALS; however, this has rarely been evaluated in these populations. This study was to evaluate bacterial and archaeal composition of gut flora and the corresponding metabolism performance of these micro-organisms in fecal samples of patients with ALS.

Methods: A comparative study was performed on the intestinal microbiota from eight patients with ALS and eight healthy individuals at Huadong Hospital during November 2017 to April 2018; meanwhile, the metabolite concentrations of human endotoxin, short-chain fatty acids (SCFA), NO2-N/NO3-N, and γ-aminobutyric acid were also evaluated by spectrophotometry methods. The correlations between intestinal microbiota and metabolite concentration were compared between the two groups using one-way analysis of variance; the relative abundance of beneficial and harmful micro-organisms in fecal samples was also analyzed.

Results: In general, the richness and evenness of bacterial and archaeal communities of healthy individuals were healthier than that of patients with ALS. The phylum Firmicutes/Bacteroidetes ratio, genus Methanobrevibacter showed an enhance tendency in patients with ALS, whereas the relative abundance of beneficial micro-organisms (genera Faecalibacterium and Bacteroides) presented a significant decrease tendency in patients with ALS. In addition, the average concentrations of human endotoxin, SCFA, NO2-N/NO3-N, and γ-aminobutyric acid in patients with ALS and healthy individuals were 64.2 vs 65.3 EU/mL, 57.5 vs 55.3 mg/mL, 5.7 vs 5.3 ng/mL, and 6.1 vs 5.4 μmol/L, respectively, indicating that the digestion and metabolism functions of gastrointestinal tract of patients might decline with this disease.

Conclusions: The relative abundance of beneficial and harmful micro-organisms respectively showed decrease and increase tendency in patients with ALS.

Keywords: Human gastrointestinal tract; Microbial biodiversity and composition; Beneficial and harmful microorganisms; Metabolite concentrations

Introduction
Amyotrophic lateral sclerosis (ALS), which results in a loss of neurons at all levels of the motor system, has been considered as a fatal neuromuscular disease.[1,2] It has been investigated that the crude annual incidence rate of ALS was 2.16 per 100,000 person-year in European.[3] Owing to the ALS is an age-dependent disease, as the population grows and ages, the incidence population might show an enhance trend around the world.[4] The clinical and scientific interest to study on this disease started to raise in about 1990s, the discovery of survival in ALS was mainly dependent on rate of disease progression, clinical presentation (phenotype), early presence of respiratory failure, and nutritional status of patients.[5,6] Unfortunately, there is no powerful intervention that can be used to change the course of this disease.[7] The count showed that approximately 50% of the patients could survive only below than 3 years, while only about 20% of patients could survive for 5 years and a small percentage could be alive after 10 years.[8] Hence, developing further understanding about disease progression and pathogenesis mechanism of ALS is of great significance and urgency.
The human gastrointestinal tract provides an optimal environmental condition for the growth and metabolism of bacterial and archaeal communities. It was generally accepted that the biodiversity and composition of these micro-organisms were significantly related to human metabolism, immunity and gut-brain axis diseases.[9-11] In literature, the variation of microbial biodiversity and communities showed apparent interactions with the disease progression of central nervous system.[12] In particular, the central nervous system could modify the gastrointestinal tract via the release of hormones, immune and neurotransmitters factors.[13,14] Meanwhile, the level of metabolites (short-chain fatty acids [SCFA], NO2-N/NO3-N, and γ-aminobutyric acid) generated by gut bacteria could positively and negatively influence on autism spectrum disorders.[15] It was observed that the abundance of methanogens showed a significant increase in patients with irritable bowel syndrome diseases.[16,17] However, there are large gaps in the interaction mechanism of gut flora composition and metabolism performance in gastrointestinal tract of patients with ALS.

Owing to ALS is a systemic wasting disease, the patient's weight declined with the digestive disturbances and intestinal barrier dysfunction.[18] It was generally accepted that the gut flora composition and metabolism performance were significantly related to the digestive mechanism of intestines.[19] It has been indicated that the relative abundance of harmful bacteria (genus Dorea) and beneficial micro-organisms (genus Anaerostipes, Oscillobacter, and Lachnospiraceae), respectively presented an enhancive and declined trend in the gastrointestinal tract of patients with ALS.[5] In addition, the host-bacterial interactions were apparently reduced in ALS mice treated with butyrate.[20] The SCFA could be used as the metabolic substrates by Methanobrevibacter genus to produce methane (CH4), which resulted in the weight loss of patients with intestinal diseases.[21] However, there has not been a comprehensive understanding of the interactions about bacterial and archaeal communities and metabolites composition in the human gastrointestinal tract of patients with ALS.

The objective of this study was to evaluate bacterial and archaeal composition of gut flora and the corresponding metabolism performance of these micro-organisms in fecal samples of patients with ALS. The composition of bacteria and archaea, and the concentration of human endotoxin, SCFA, NO2-N/NO3-N, and γ-aminobutyric acid were determined. We hypothesized that the biodiversity and composition of bacteria and archaea and the corresponding metabolism concentration in human gastrointestinal tract might be affected by this disease.

Methods

Ethical approval

The study on microbial community and metabolites composition of patients with ALS was approved by the Ethical Committee of Huadong Hospital of Fudan University (Shanghai) (No. 2017K058). All participants have been told research details before signed informed consent, and all experiments were done in compliance with the approved guidelines and national directives.

Patient's recruitment

Eight patients with ALS were recruited according to the revised El Escorial criteria (1998)[21] at Huadong Hospital from November 2017 to April 2018. The inclusion criteria could be concluded as follows: (1) the survival period of all patients with ALS was more than 3 months; and (2) no antibiotics were used in the last 1 month. The exclusion criteria could be concluded as follows: (1) patients with ALS were with serious heart, lung, liver, kidney, brain and tumor, blood system diseases, and other disorders of life signs; (2) other diseases (ie, cervical spondylosis, spinal cord tumors) that need to be differentiated from ALS; and (3) patients with overeating and drinking in the past 2 weeks. In addition, eight healthy individuals were recruited as the controls at about the same time. The sex, height, weight, duration of disease, and body mass index information of patients with ALS and healthy controls were collected [Table 1].

Fecal sampling and polymerase chain reaction amplification

The fecal sample of each participant was collected from the first fecal motion of 1 day after a 30-day similar diet. Then all samples were stored at –80°C. For polymerase chain reaction (PCR) amplification of bacterial communities, the universal bacterial primers of 515F (5'-GTGCCAGCM GCCGCGG-3’) and 907R (5'-CCGTAATTCCMTTTRAGTTT-3’) were used to amplify the V4–V5 hypervariable regions, while for PCR amplification of archaeal communities, the universal bacterial primers of 524F_10_ext (5'-TGYCAGCCGGCGGTTA-3’) and Arch958R_mod (5’-YCCGGCTTGTATCCAATT-3’) were used. The pre-treatment, amplification processes, pyrosequencing data, and quality control of microbial samples were performed as described in previous study.[22] All PCR amplification of microbial samples were performed on the Illumina Miseq PE300 platform (Shanghai Majorbio Bio-Pharm Technology Co. Ltd., China). The archaeal throughput sequencing in fecal sample of patients ALS6 and ALS8 was failed because of sequencing technology reasons.

Metabolites determination

The correlation coefficients ($R^2$) of calibration curves of human endotoxin, SCFA, NO2-N/NO3-N, and γ-aminobutyric acid were determined; absorbivity values on 450 nm wavelength with spectrophotometry, and correlation coefficients ($R^2$) were more than 0.99. The concentrations of human endotoxin, SCFA, NO2-N/NO3-N, and γ-aminobutyric acid of fecal sample were respectively tested with the corresponding enzyme-linked immunosorbent assay (ELISA) kits according to standard directions. Each sample was determined with a two to four repetitions.

Data and statistical analyses

To ensure the high-quality of sequence reads, all reads were checked and denoised based on the listed criteria: (1) set the
| Participant No. | Sex | Age (years) | Height (cm) | Weight (kg) | Duration of disease (months) | BMI (kg/m²) |
|-----------------|-----|-------------|-------------|-------------|-----------------------------|-------------|
| ALS1            | Male| 60          | 178         | 65.8        | 24                          | 20.8        |
| ALS2            | Male| 61          | 173         | 56.0        | 28                          | 18.7        |
| ALS3            | Male| 67          | 176         | 64.0        | 9                           | 20.7        |
| ALS4            | Male| 62          | 175         | 60.0        | 8                           | 19.6        |
| ALS5            | Female| 57         | 155         | 51.3        | 13                          | 21.4        |
| ALS6            | Female| 49         | 158         | 62.0        | 15                          | 24.8        |
| ALS7            | Female| 72         | 156         | 45.0        | 27                          | 18.5        |
| ALS8            | Female| 31         | 160         | 47.0        | 20                          | 18.4        |
| H1              | Male| 56          | 178         | 80.0        | /                           | 25.3        |
| H2              | Male| 60          | 161         | 66.2        | /                           | 25.5        |
| H3              | Male| 53          | 171         | 67.5        | /                           | 23.1        |
| H4              | Male| 48          | 168         | 70.0        | /                           | 24.8        |
| H5              | Female| 48         | 145         | 53.0        | /                           | 25.2        |
| H6              | Female| 45         | 158         | 62.0        | /                           | 24.8        |
| H7              | Female| 52         | 156         | 59.7        | /                           | 24.5        |
| H8              | Female| 45         | 163         | 70.0        | /                           | 26.5        |

BMI: Body mass index; ALS: Amyotrophic lateral sclerosis; H: Healthy control.

Distribution of micro-organisms at phylum, class, and genus level

As shown in Figure 1A and Supplementary Table S1, http://links.lww.com/CM9/A69, bacterial community of fecal sample presented a high predominance of Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Verrucomicrobia. Notably, the average relative abundance of phylum Firmicutes in patients with ALS was 4.7% higher than that of healthy individuals. On class level, the relative abundance of classes Negativicutes and Bacteroidetes in patients with ALS was on the decline, compared with that of healthy individuals [Figure 1B and Supplementary Table S2, http://links.lww.com/CM9/A69]. In Figure 1C and Supplementary Table S3, http://links.lww.com/CM9/A69, the top ten genera in patients with ALS and healthy individuals were Bacteroides, Blautia, Faecalibacterium, Escherichia-Shigella, Anaerostipes, Streptococcus, Akkermansia, Fusicatenibacter, Megamonas, and Bifidobacterium (21.1% vs. 25.9%, 15.3% vs. 9.0%, 4.8% vs. 9.9%, 5.9% vs. 3.6%, 4.4% vs. 1.9%, 4.6% vs. 1.0%, 4.5% vs. 0.4%, 1.9% vs. 2.5%, 2.6% vs. 1.4%, and 1.4% vs. 2.5%), which respectively accounted for 65.1% and 55.6% of the total reads in patients with ALS and healthy individuals. In Figure 2 and Supplementary Tables S4 to S6, http://links.lww.com/CM9/A69, although the determined archaeal community was under a relatively narrow
range, and results indicated that the relative abundance of phylum Euryarchaeota, class Methanobacteria, and genus Methanobrevibacter showed a significant enhancement in patients with ALS, compared with healthy individuals.

Metabolite concentration

In general, the average concentrations of human endotoxin, SCFA, NO₂⁻N/NO₃⁻N, and γ-aminobutyric acid in patients with ALS and healthy individuals were 64.2 vs. 65.3 EU/mL, 57.5 vs. 55.3 μg/mL, 5.7 vs. 5.3 ng/mL, and 6.1 vs. 5.4 μmol/L, respectively [Table 3]. Although there were no significant differences in these metabolites between patients with ALS and healthy individuals (P = 0.756, 0.614, 0.621, and 0.296, respectively), the results still indicated that the increase trend of SCFA, NO₂⁻N/NO₃⁻N, and γ-aminobutyric acid was observed in patients with ALS, compared with healthy individuals.

Discussion

Intestinal microbiome and their interactions in food metabolism play an important role in the nutrient digestion and human health.[24] Most of related studies indicated that the biodiversity, distribution, and metabolism of intestinal microflora showed a significant influence on the healthy and homeostatic balance of human central nervous system.[25-27] Owing to ALS is an age-dependent disease, as the population grows and ages, the incidence population might show an enhancive trend in the world.[4] In addition, ALS is a systemic wasting disease, the patient’s weight declined with the digestive disturbances and intestinal barrier dysfunction.[18] Notably, it was observed that the weight of patients with ALS was significantly lower than that of healthy individuals [Table 1]. Therefore, the intestinal flora, which has been considered as the second brain, may be significantly influenced by the ALS disease progressions.[2] Accumulating researches and findings demonstrate that the composition changes of these micro-organisms in the gastrointestinal tract possessed a strongly correlated to the neurological diseases, specifically, neurodegenerative diseases.[2,28]

In the present study, the biodiversity and distribution of intestinal microflora and the metabolite concentrations of these micro-organisms were performed by the high throughput sequencing and the corresponding ELISA kit methods, respectively. Based on the experimental findings,
the richness and evenness of bacterial and archaeal communities of healthy individuals were healthier than that of patients with ALS [Table 2]. The increase of bacterial diversity in patients with ALS is caused mainly on the decline of metabolic function, which could have a negative effect on the growth and metabolism of microorganisms. In literature, it has been indicated that the richness and evenness of bacterial communities showed a
decrease trend in patients with ALS or irritable bowel syndrome disease. In addition, the biodiversity of archaeal communities of patients with ALS was also on the decline compared with that of healthy individuals. Compared with healthy individuals, the relative abundance of bacterial communities reflected that a higher percent of phylum Firmicutes and a lower percent of phylum Bacteroidetes were found in patients with ALS [Figure 1]. It has been reported that the phylum Firmicutes to Bacteroidetes ratio could be regarded as an important index for the health of human gastrointestinal tract. The significantly promoted abundance of phylum Firmicutes and reduced abundance of phylum Bacteroidetes indicated that the gastrointestinal tract health of patients with ALS was significantly influenced by this disease. On genus level, the top ten genera in patients with ALS and healthy individuals were Bacteroides, Blautia, Faecalibacterium, Escherichia-Shigella, Anaerostipes, Streptococcus, Akkermansia, Fusicatenibacter, Megamonas, and Bifidobacterium. The relative abundance of harmful bacteria (genus Dorea) in patients with ALS was observed with an increasing tendency in previous study. However, there were no similar findings obtained in the present study. Notably, compared with the healthy individuals (9.9%), the beneficial micro-organisms (genus

Table 3: Metabolites concentration in fecal sample collected from patients with ALS and healthy individuals.

| Samples | Human endotoxin (EU/mL) | SCFA (μg/mL) | NO₂-N/NO₃-N (ng/mL) | γ-aminobutyric acid (μmol/L) |
|---------|-------------------------|------------|---------------------|----------------------------|
| ALS1    | 73.7                    | 40.9       | 8.2                 | 6.3                        |
| ALS2    | 58.6                    | 69.0       | 6.6                 | 5.9                        |
| ALS3    | 52.5                    | 49.1       | 4.7                 | 7.8                        |
| ALS4    | 67.8                    | 64.3       | 4.7                 | 7.8                        |
| ALS5    | 67.7                    | 66.3       | 4.3                 | 3.6                        |
| ALS6    | 68.7                    | 48.4       | 6.3                 | 4.8                        |
| ALS7    | 69.2                    | 64.7       | 5.6                 | 5.3                        |
| ALS8    | 55.8                    | 58.8       | 5.1                 | 7.8                        |
| H1      | 69.4                    | 54.8       | 7.3                 | 5.4                        |
| H2      | 60.8                    | 41.0       | 7.5                 | 6.4                        |
| H3      | 65.1                    | 52.4       | 6.0                 | 6.7                        |
| H4      | 53.0                    | 59.8       | 4.7                 | 4.9                        |
| H5      | 68.4                    | 58.7       | 3.4                 | 5.4                        |
| H6      | 71.4                    | 53.5       | 2.2                 | 4.1                        |
| H7      | 67.2                    | 61.8       | 5.3                 | 4.8                        |
| H8      | 67.3                    | 61.5       | 5.9                 | 5.9                        |

ALS: Amyotrophic lateral sclerosis; SCFA: Short-chain fatty acids; H: Healthy control.
Faecalibacterium) was observed with a significant decline in fecal sample of patients with ALS (4.8%). In general, this genus maintained an above level of 5% in the gut of healthy people, the decline of genus Faecalibacterium usually represented the development of some diseases like coeliac disease, irritable bowel syndrome, and asthma. In addition, for genus Bacteroides, which could reduce the potential pathogenic factors of gastrointestinal tract in the progression of host intestinal parasitism. This genus also presented an apparent decline in fecal sample of patients with ALS.

The main determined archaeal communities in fecal sample of patients with ALS and healthy individuals were phylum Euryarchaeota, class Methanobacteria, and genus Methanobrevibacter (Figure 2). It has been reported that the SCFA could be used as the substrate by genus Methanobrevibacter to produce CH₄, meanwhile the weight of host might show a decline trend with the enhancement of this genus. In addition, research on the distribution of archaeal composition indicated that the relative abundance of methanogens in irritable bowel syndrome patients was significantly higher than that of healthy individuals. Similar results were found in fecal sample of patients with ALS compared with healthy individuals. These findings demonstrated that the intestinal metabolic function of patients with ALS might be influenced by this disease.

As shown in Table 3, although there was no significant difference in metabolite concentration between patients with ALS and healthy individuals, the results still indicated an increase trend of SCFA, NO₂⁻/NO₃⁻, and γ-amino-butyric acid was observed in patients with ALS compared with healthy individuals. These metabolites have been considered as the important elements for nutrient consumption in daily diet. The concentrations of these metabolites promoted in fecal sample of patients with ALS might demonstrate that the absorptive function of human gastrointestinal tract declined with this disease.

However, there were several limitations in this study. First, no metagenomic sequencing was performed to investigate the genomic information about the microbial function variation in patients with ALS and healthy individuals. Second, to help the patients with ALS establish a healthier gastrointestinal tract, the acclimation of microbial communities might be needed for further studies to have a depth understanding about the interactions of intestinal microbiota composition with ALS.

In summary, the biodiversity and composition of intestinal microflora in patients with ALS were generally on the decline compared with healthy individuals. The relative abundance of beneficial micro-organisms (genera Faecalibacterium and Bacteroides) presented with a decrease trend in patients with ALS. In addition, the phylum Firmicutes/Bacteroidetes ratio and genus Methanobrevibacter showed an enhancement trend in patients with ALS indicated that the imbalance of gut flora composition in fecal sample collected from patients with ALS. The concentrations of these metabolites promoted in fecal sample of patients with ALS might demonstrate that the absorptive function of human gastrointestinal tract declined with this disease.

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**Conflicts of interest**

None.

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