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), NO_2-N/NO_3-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 enhancive 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, NO_2-N/NO_3-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, 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 enhancive 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.

Access this article online

Quick Response Code:

Website: www.cmj.org

DOI: 10.1097/CM9.0000000000000351

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Chinese Medical Journal 2019;132(15)
Received: 02-05-2019 **Edited by:** Xin Chen

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], NO_2-N/NO_3-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 on 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*, *Oscillibacter*, and *Lachnospiraceae*), respectively presented an enhancive and declined trend in the gastrointestinal tract of patients with ALS.[2] In addition, the host-bacterial interactions were apparently reduced in ALS mice treated with butyrate.[4] The SCFA could be used as the metabolic substrates by *Methanobrevibacter* genus to produce methane (CH_4), which resulted in the weight loss of patients with intestinal diseases.[20] 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, NO_2-N/NO_3-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 E1 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′-GTGCCAGCMGCCGCGG-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-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′-TGYCAGCCGCCGCGGTAA-3′) and Arch958R_mod (5′-YCCGGCGTTGAVTCCAATT-3′) were used. The pretreatment, 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, NO_2-N/NO_3-N, and γ-aminobutyric acid were determined; absorptivity values on 450 nm wavelength with spectrophotometry, and correlation coefficients (R^2) were more than 0.99. The concentrations of human endotoxin, SCFA, NO_2-N/NO_3-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

Table 1: Sex, age, height, weight, duration of disease, and BMI information of patients with ALS and healthy individuals.

Participant No.	Sex	Age (years)	Height (cm)	Weight (kg)	Duration of disease (months)	BMI (kg/m^2)
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.

window options of 50 base pairs (bps), if the average mass value in the window was less than 20, the back end bps was removed from the window, and reads below 50 bps after quality control was filtered to remove reads containing N base; (2) according to the relationship between overlap and paired-end reads, pair reads were merged into a sequence, the minimum overlap length was 10 bps; (3) the maximum mismatch rate of the overlap region of the merged sequence was 0.2; (4) the samples were identified according to the barcode and primers at the end of the sequence. The mismatch number of barcodes was 0 and the maximum primer mismatch number was 2. Then, a similarity of 97% was used to classify the similar 16S rRNA reads into same operational taxonomic units (OTUs) with uclust algorithm.[23] The relative abundance of phylum, class, order, family, and genus was generated according with OUTs annotation, and then determined with a proportion of all reads for each microbial sample. In addition, the biodiversity estimators (Ace, Chao, Shannon, and Simpson) were used to evaluate richness and evenness of microbial communities.[2,22]

In this study, the statistical difference of metabolites concentrations (human endotoxin, SCFA, NO_2-N/NO_3-N, and γ-aminobutyric acid) between patients with ALS and healthy individuals was compared using one-way analysis of variance (least significant difference test). The data were analyzed using software SPSS version 24.0 (IBM Corp., Armonk, NY, USA). A $P < 0.05$ was set as the significant level.

Results

Microbial biodiversity and reads coverage

The biodiversity of bacterial and archaeal communities in fecal sample of patients with ALS and healthy individuals were shown in Table 2. The coverage value of each sample maintained approximately 0.9991 to 0.9998. Average OTUs numbers in patients with ALS and healthy individuals were 141 and 138, respectively. Ace and Chao estimators included the richness information of microbial communities. Compared with healthy individuals, the richness of bacterial and archaeal communities in patients with ALS were on the increased and decreased tendencies, respectively. It was generally accepted that the Shannon and Simpson estimators were used to evaluate the richness and evenness of microbial communities. According to these analyses, the richness and evenness of bacterial and archaeal communities of healthy individuals were healthier than those of patients with ALS.

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 *Bacilli* 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

Table 2: Richness and evenness of micro-organisms in fecal sample of patients with ALS and healthy individuals.

Microbes	Sample	OTUs	Coverage	Ace	Chao	Shannon	Simpson
Bacteria	ALS1	99	0.9996	120.07	114.17	2.74	0.12
	ALS2	197	0.9991	221.63	245.33	3.65	0.06
	ALS3	87	0.9997	104.92	100.33	2.12	0.20
	ALS4	181	0.9997	196.37	195.62	3.47	0.07
	ALS5	198	0.9986	237.00	233.00	3.83	0.04
	ALS6	118	0.9995	156.37	149.67	3.25	0.06
	ALS7	84	0.9998	92.35	91.86	2.40	0.17
	ALS8	164	0.9997	175.24	170.32	3.45	0.06
	H1	188	0.9992	202.86	204.50	3.34	0.08
	H2	156	0.9991	206.29	183.08	3.45	0.06
	H3	158	0.9993	175.08	173.00	3.67	0.04
	H4	120	0.9996	129.33	128.25	3.44	0.05
	H5	95	0.9998	97.32	96.67	2.70	0.17
	H6	172	0.9995	180.72	180.57	3.66	0.04
	H7	115	0.9995	130.17	155.00	2.90	0.13
	H8	102	0.9996	111.26	111.43	2.92	0.10
Archaea	ALS1	57	0.9998	61.25	64.50	1.63	0.31
	ALS2	53	0.9999	57.78	54.43	2.63	0.10
	ALS3	72	0.9998	81.30	79.50	0.86	0.72
	ALS4	48	0.9996	88.28	65.00	0.25	0.91
	ALS5	90	0.9993	107.47	107.50	0.85	0.71
	ALS7	84	0.9997	91.24	95.00	1.32	0.48
	H1	83	0.9996	99.19	96.60	2.09	0.18
	H2	112	0.9997	119.52	118.60	1.44	0.52
	H3	68	0.9997	74.44	74.43	0.64	0.79
	H4	100	0.9997	108.04	106.50	2.02	0.22
	H5	71	0.9998	116.85	101.00	2.60	0.13
	H6	75	0.9998	81.76	82.00	2.97	0.09
	H7	51	0.9998	54.96	53.33	0.47	0.85
	H8	38	0.9998	42.64	41.00	0.48	0.84

A higher number of Ace and Chao estimators mean the richness of bacterial and archaeal communities was plentiful; a higher number of Shannon index or a lower value of Simpson estimator respectively represent the distribution (evenness and richness) of bacterial and archaeal communities was plentiful. ALS: Amyotrophic lateral sclerosis; OUT: Operational taxonomic unit; H: Healthy control.

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, NO_2-N/NO_3-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, NO_2-N/NO_3-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,

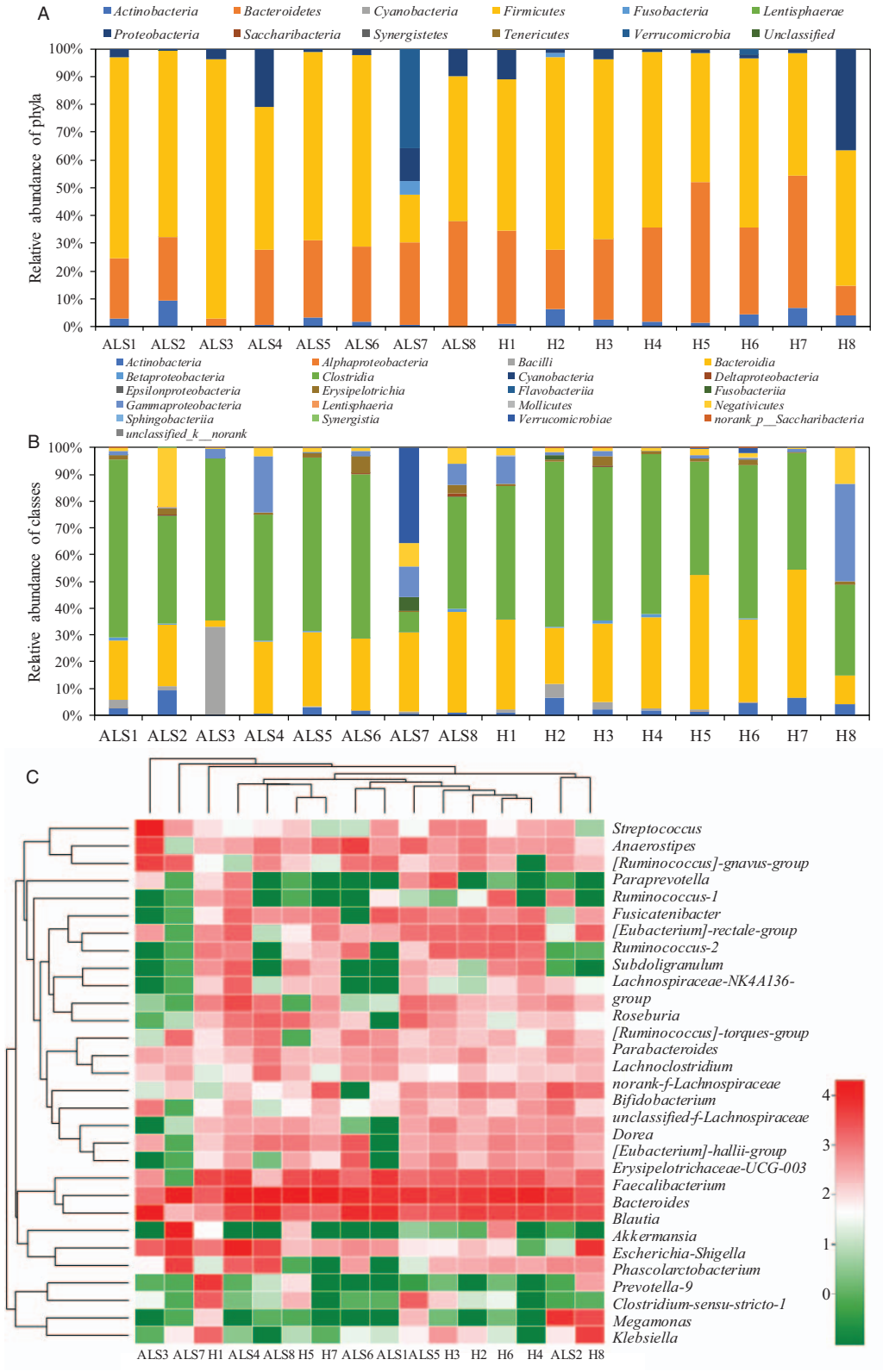

Figure 1: Taxonomic classification of the bacterial communities at phylum (A) and class (B) levels, and hierarchy cluster for the top 30 genera (C) from bacterial throughput sequencing in fecal sample of patients with ALS and healthy individuals. ALS: Amyotrophic lateral sclerosis; H: Healthy control.

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

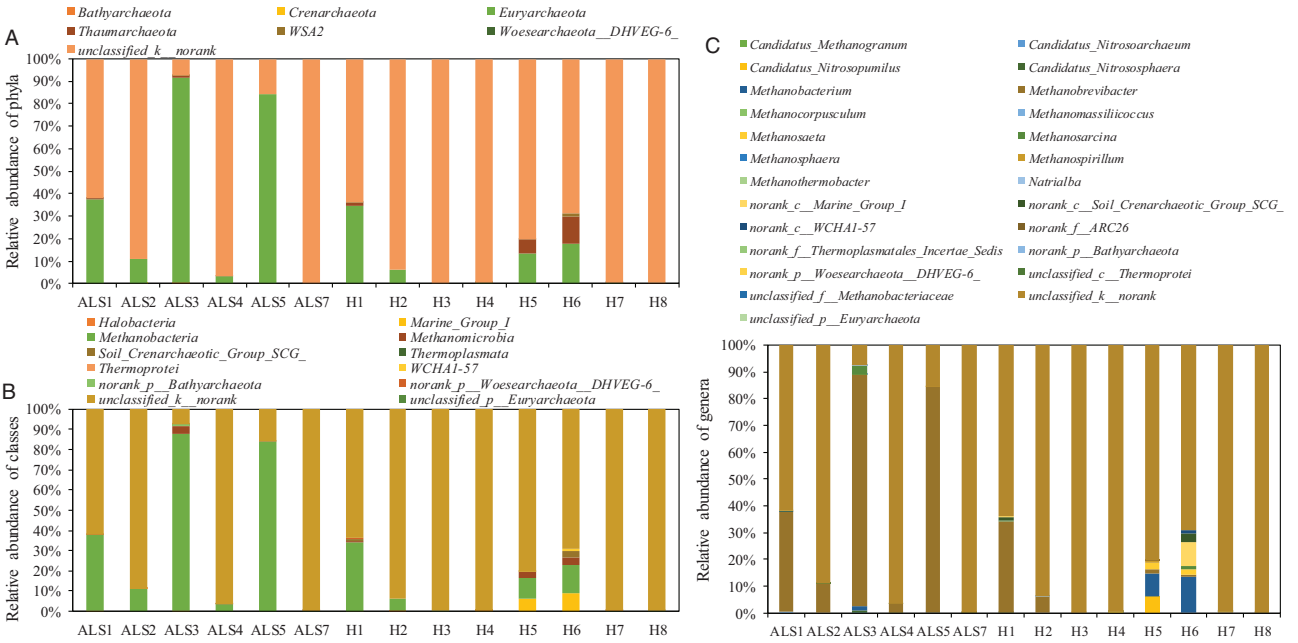

Figure 2: Taxonomic classification of the archaeal communities at phylum (A), class (B), and genus (C) levels from archaeal throughput sequencing in fecal sample of patients with ALS and healthy individuals. ALS: Amyotrophic lateral sclerosis; H: Healthy control.

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

Samples	Human endotoxin (EU/mL)	SCFA (μg/mL)	NO_2-N/NO_3-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	7.5
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.

decrease trend in patients with ALS or irritable bowel syndrome disease.[2,29,30] 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/Bacteroidetes ratio could be regarded as an important index for the health of human gastrointestinal tract.[2,31] 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.[2] 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.[2] However, there were no similar findings obtained in the present study. Notably, compared with the healthy individuals (9.9%), the beneficial micro-organisms (genus

Faecalibacterium) was observed with a significant decline in fecal sample of patients with ALS (4.8%).[32,33] 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.[32] In addition, for genus *Bacteroides*, which could reduce the potential pathogenic factors of gastrointestinal tract in the progression of host intestinal parasitism.[34,35] This genus also presented an apparent decline in fecal sample of patients with ALS.

The mainly 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 CH_4, meanwhile the weight of host might show a decline trend with the enhancement of this genus.[20] 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.[16] 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, NO_2-N/NO_3-N, 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 understand 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.

Funding

This study was supported by grants from the Discipline Construction of Important Weak Disciplines in Shanghai Health System (No. 2015ZB0402), Chinese Medicine Research Fund from Shanghai Health and Family Planning Commission (No. 2014LP006A and ZY3-RCPY-4-2005), and Dual Task Training to Prevent Fall of the Elderly Patients with Cognitive (No. SHDCI2014126).

Conflicts of interest

None.

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How to cite this article: Zhai CD, Zheng JJ, An BC, Huang HF, Tan ZC. Intestinal microbiota composition in patients with amyotrophic lateral sclerosis: establishment of bacterial and archaeal communities analyses. Chin Med J 2019;132:1815–1822. doi: 10.1097/CM9.0000000000000351