学 位 論 文

機能的身体独立性との関連における八十歳代および百歳以上のヒト口腔および腸内細菌叢の比較

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松本歯科大学大学院歯学独立研究科博士（歯学）学位申請論文

Comparison of oral and gut microflora in octogenarians and centenarians in relation to functional independence

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ABBREVIATIONS

BI	Barthel index
MMSE	Mini Mental State Examination
MNA	Mini Nutritional Status Assessment
PCR	Polymerase chain reaction
OTUs	Operational taxonomic units
LEfSe	Line Discriminant Analysis Effect Size

1. ABSTRACT

This study compared the differences in salivary and fecal bacterial flora between 80- and 100-year-old people in different independent states to provide a reference for the bacterial composition of the digestive tract in older people of different ages. Saliva and fecal samples were collected from eight centenarians and 10 octogenarians whose Barthel index (BI) is less than 60, which means poor independence, as well as 10 independent octogenarians from nursing homes. A dentist counted the number of teeth, and evaluated the denture and nutritional status. Bacterial DNA was extracted and multiplex 16S rRNA (V3–V4) sequencing was performed using the Illumina MiSeq platform. Composition and diversity were analyzed by LEfSe, student's t-test, weighted PcoA distance, and Anosim analysis. Older people who were more independent had significantly better nutritional intake than those who were less independent, and this difference was not reflected in age. The digestive tract of well independent seniors has more normal flora, which also reflects the link between general health and gut flora. While the saliva of less independent seniors contains more pathogenic bacteria, which may be a result of their inability to effectively clean their oral hygiene due to their poor functional independence. In addition, *Akkermansia*, a genus of bacteria commonly considered to be probiotics, was more frequently found in the stools of the less independent eighty-year-olds. There was no significant difference in the intestinal microbiota of 80-year-old and 100-year-old individuals with low functional independence in the ß diversity that constitutes the microbiota. On the other hand, there was a significant difference in ß diversity in the salivary flora. The reason for this was thought to be the difference in the number of remaining teeth between 80-year-old and 100-year-old subjects. In addition, there was a significant difference in ß diversity in both the

intestinal and salivary microbiota between the 80-year-old group with low functional independence and the normal 80-year-old group.

From the above, it was clarified that functional independence affects the diversity that constitutes the salivary and gut microbiota. In addition, it was suggested that functional independence affects the diversity of salivary and intestinal microbiota more than age.

2. INTRODUCTION

As human life expectancy increases and population growth rates decline, population aging is likely to become one of the most important social trends of the 21st century. It is expected that by 2050, twice as many people will be over 80 years of age as there are today; and as a proxy for the limits of human longevity, the number of centenarians worldwide has already reached 593,000 in 2021[1]. The pursuit of longevity is one of the enduring topics of human research, and research into the limits of human lifespan may help to better understand how long-lived individuals avoid age-related risk factors and thus live longer[2].

Complex genetic or environmental factors as well as stochasticity can affect human lifespan to the extent that universal models are difficult to establish[2]. However, through its effects on human metabolism and immunology, the stability and homeostasis of the gut microbiota plays an important role in the regulation of host lifespan[3, 4]. For example, gut microbial homeostasis can help older adults resist inflammation, maintain good intestinal permeability, and delay bone loss and cognitive decline[5-8]. However, factors that accompany ageing such as various systemic diseases can destabilize the intestinal flora[9]. Furthermore, clinical studies have demonstrated that the gut microbiota of centenarians are also somewhat different from those of the general elderly[10, 11], suggesting that even in old age, the human body flora changes as age limits are breached.

As the entrance to the digestive tract, the flora in the oral cavity has been shown to be closely linked to the intestinal flora[12, 13], a link that becomes even closer as the oral-gastrointestinal barrier weakens in older people. Reduced mobility in older people makes it difficult to maintain oral hygiene, and changes in their dietary habits due to tooth loss or disease can affect both oral and intestinal flora[14]. As older people generally have fewer teeth, especially in the centenarians, the edentulous jaw is more common, which means that most oral bacteria live in the mucosa and saliva rather than on the surface of the

teeth and in the gingival sulcus, and the bacteria in saliva can accompany food directly into the gastrointestinal tract, so saliva plays an important role in oral-intestinal flora exchange[15]. However, compared to the intestinal flora, the number of studies on the oral flora of the elderly is relatively small, and the oral flora composition of centenarians in particular is almost uncharted[16]. Understanding the composition of the oral flora in centenarians will not only help in oral care and disease prevention for centenarians in the future ageing society, but also have a complementary role in the maintenance of digestive tract health in the elderly.

Functional independence in the elderly may have a greater impact on the microflora of the body than age. From mild cognitive frailty to Alzheimer's disease, the loss of functional independence affects the digestive flora to a greater or lesser extent. This 'gut-brain axis' effect has been extensively studied in animal models[17-19], but in human samples it has been more observational and a comprehensive analysis of the composition and function of the gut microbiota is lacking[20]. When the functional independence of the elderly declines, changes in diet, acute or chronic illness, chronic constipation and medication can induce dysbiosis of the gut microflora[21]. This dysbiosis in turn produces pro-inflammatory cytokines that lead to the activation of brain microglia and promote neuroinflammation, neuronal apoptosis and ß-amyloid deposition[22]. In contrast, the impact of oral flora on functional independence in the elderly is mainly reflected in the association of *Porphyromonas.gingivalis* which is recognized as the main causative agent of periodontitis, with Alzheimer's disease, and exploring the composition of oral flora in the elderly could help in the early prevention and detection of cognitive decline in the elderly[23-25].

Whether longevity is the result of the development of its gut microbiome, or the cause of changes to the human gut microbiome is unclear. But what is certain is that, because longevity is inseparable from the flora of the digestive tract, it is a direction worth to explore the composition of the flora of the digestive tract of the elderly to help

the elderly improve their health and prolong life. In this study, we collected saliva and fecal samples from 20 octogenarians and 8 centenarians with different functional independence. All of them are from nursing homes in Nagano prefecture, Japan. High-throughput sequencing of 16S rRNA by metagenomics method, described the highly abundant species in the sample, and compared the diversity, so as to explore the oral and intestinal flora of the elderly in different age groups and different independent ability states compositional differences.

3. METHODS

3.1 Study subjects and clinical data collection

This cross-sectional study in 2020 July to October was conducted at 3 nursing homes in Nagano Prefecture, Japan. A total of 20 octogenarians (aged 81 to 89 years; average ± S.D.：85.9 ± 2.6) and 8 centenarians (aged 100 to 103 years; average ± S.D.：101.1 ± 1.1) were included in the study. The exclusion criteria are as follows: (1) Those taking antibiotics, antifungal agents, or antibody drugs. (2) Those who are undergoing treatment or visiting the hospital due to illness. (3) Those who need assistance in eating. The study was conducted with approval from the Clinical Research Institutional Review Board of Matsumoto Dental University (No.0302) and written informed consent was obtained from all respondents. Functional independence was assessed by one trained dentist using the Barthel Index (BI, Supporting Table 1), cognitive level by the Mini Mental State Examination (MMSE, Supporting Table 2), nutritional intake by the Mini Nutritional Status Assessment (MNA, Supporting Figure 1), and record their dental status, including the number of remaining teeth and the number of people using dentures (Supporting Table 3). Finally, according to BI score and age, they were divided into independent group (BI ≥ 60) and dependent group (BI < 60). All centenarians had BI scores below 60 in this study.

3.2 Sample collection and DNA extraction

Participants had saliva and stool samples collected by dentist and trained staff in nursing homes. Samples were immediately stored in a freezer at -20°C, collected by laboratory staff and subjected to DNA extraction using the MORA-EXTRACT kit (KYOKUTO, Nishinomiya,Japan) according to the instructions.

3.3 Sequencing data quality assessment and analysis

After extracting total DNA of samples, primers were designed according to the conserved region. Sequencing adapters were added to the end of primers; The target sequences were amplified by polymerase chain reaction (PCR) method and its products were purified, quantified and homogenized to get a sequencing library; Then library quality control was performed for constructing libraries. Qualified libraries were sequenced on Illumina Novaseq 6000. The original image data files obtained by high-throughput sequencing were converted into Sequenced Reads by Base Calling analysis. Raw reads were firstly filtered by Trimmomatic (ver.0.33, Usadellab). Then the primer sequences were identified and removed by cutadapt (ver.1.9.1, TU Dortmund University, Germany), which finally generated high-quality reads without primer sequences. Based on overlapping sequences, high-quality reads were assembled by FLASH (ver.1.2.11, The University of Maryland Center for Bioinformatics and Computational Biology, MD), which generated clean reads. A total of 6,216,117 raw reads were generated from 55 samples. Upon that, 6,121,725 clean reads were obtained after reads quality control and assembly. Usearch (ver.10.0,Drive5) was applied to cluster reads with similarity above 97.0%, generating 620 operational taxonomic units (OTUs).

The basic information of subjects was analyzed using t-test and Fisher's exact test. Biomarkers with statistical differences between groups were analyzed using LEfSe (Line Discriminant Analysis Effect Size). α-diversity was described by Chao1 and Shannon index, Student's t-test was used to detect their differences. Weighted PCoA distances were used to describe β-diversity, and anosim analysis was used to detect differences in distances. The above sequencing data quality assessment and analysis work was assisted by Kechang Biotechnology Co., Ltd, Nanchang, China.

4. RESULTS

4.1 The independence of older people affects nutritional status.

The gender, number of teeth and denture were collected from 20 octogenarians and eight centenarians. The ability to live independently, cognitive function and nutritional status were assessed by questionnaire. (Table 1,2)

Of the 28 older people, the number of women was overwhelmingly higher. Although centenarians have fewer teeth(mean ± S.D: 5.1 ± 5.2) remaining than those in their eighties （mean ± S.D: 10.7 ± 8.8）, seven of eight centenarians (87.5%) use dentures to restore chewing function. Even though the number of teeth and the number of people using dentures are similar, there are significant changes in the nutritional intake of the elderly as their independence improves(Table.2 $P < 0.001$).

4.2 Species Annotation and Taxonomic Analysis

A total of 56 samples of saliva and faces were collected from 28 elderly people separately. One saliva sample of centenarian was deleted because of the low number of reads, and 620 OTUs were obtained （Fig.1A）. Some centenarians had fewer OTUs detected in their saliva samples than others, and all of them had edentulous jaws or only a few front teeth left (Supporting Table 3).

A total of two cases were found at the kingdom level, 16 at the phylum level, 23 at the class level, 50 at the order level, 92 at the family level, 233 at the genus level and 402 at the species level in all samples (Supporting Table 4). The overlapping and characteristic OTUs between and within different groups are shown in the Supporting Figures 2-3.

For an optimal appearance, top 10 species in genes level were selected and the rest were combined as Others. Unclassified represents the species that has not been taxonomically annotated. Differences in species distribution at the genus level

between groups are depicted using bar charts (Fig.1B) and differences in species distribution at the genus level between samples are depicted in heat maps (Fig.1C).

Species with statistical differences between groups were identified by LEFSE analysis (Fig.2). When they were all in a state of being unable to live independently, the octogenarians had more types of bacteria than the centenarians, and even the centenarians did not show characteristics in the stool samples. Elderly people with good independence have more normal flora, while those with poor independence have more opportunistic pathogens.

4.3 α Diversity Analysis

α diversity reflects richness and diversity of species within single sample and can be accessed by several metrics. Although some of the centenarian saliva samples contained low levels of OTUs, the dilution curves indicated that the they were largely adequate for data analysis (Fig 3A). We used Chao1 index to counts for species richness and Shannon index to reflects species diversity. Student's *t*-test was used to analyses differences in α diversity between groups (Fig.3B, C). However, the final results showed no significant differences in α diversity between the groups for either age or independent status comparisons.

4.4 ß Diversity Analysis

ß diversity analysis was processed by QIIME software to compare species diversity between different samples. We used weighted unifrac PCoA analysis for ß diversity. PCoA graphs were drawn by R language tool. In the coordinate system, the dots with smaller distance share higher similarity (Fig.4A). The anosim analysis has been used to numerically differentiate the differences in spacing between points in PCoA analysis so that differences in ß diversity can be visually compared. The closer the R value is to 1, the higher the difference between groups; no significant difference is between them if R value less than 0. *P*-value less than 0.05 indicates

high reliability of the test (Fig.4B). The results showed that octogenarians with good independence had the most similar flora structure in their saliva and fecal samples. Centenarians had a more dispersed flora structure in saliva than octogenarians, but fecal samples were not significantly different.

Table 1. Basic information and indicators for older people in different age groups.

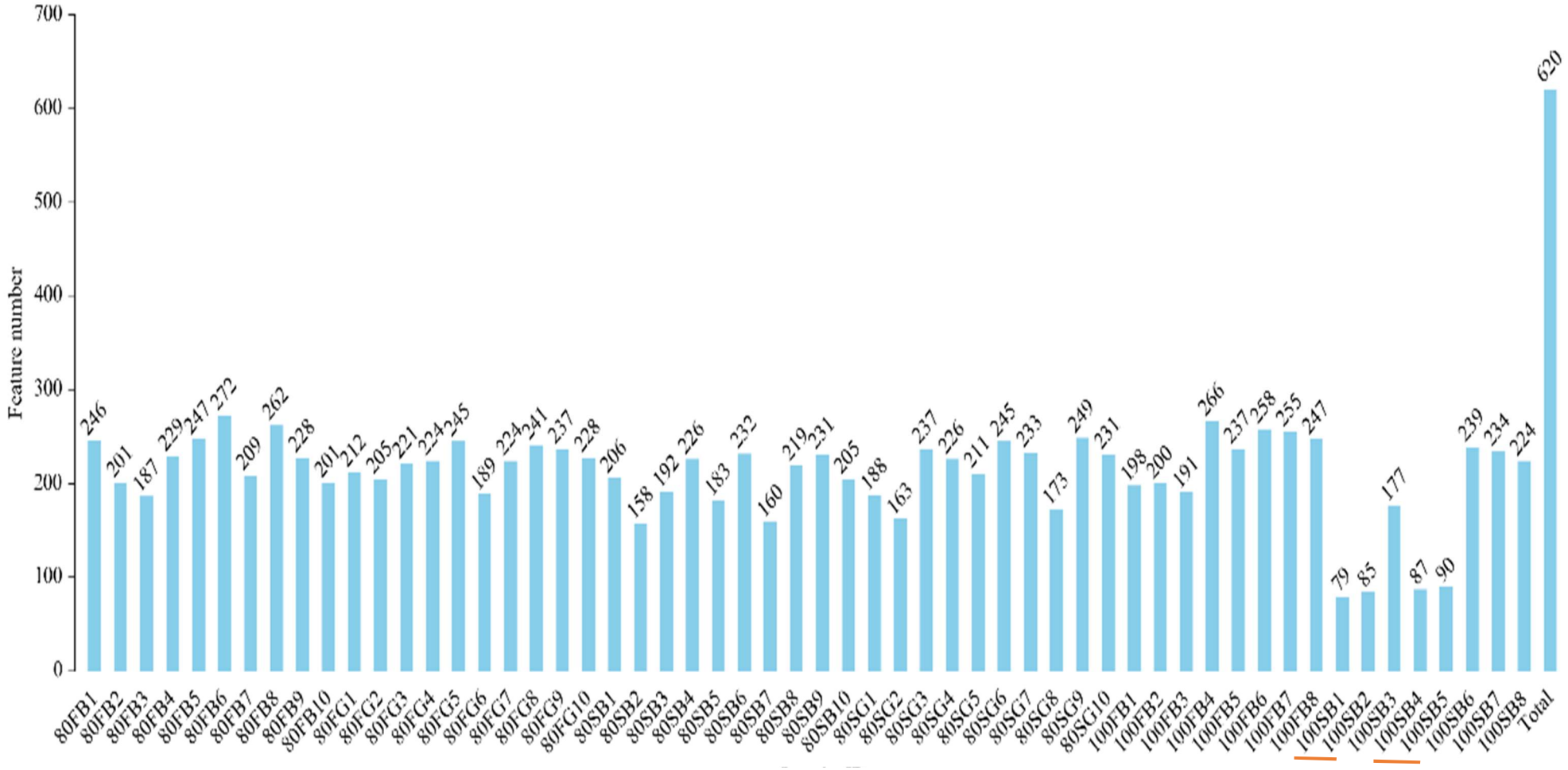
	Octogenarians (n=10)	Centenarians (n=8)	*P*-value
The number of teeth	10.7 ± 8.8	5.1 ± 5.2	0.059
Sex: Female	9(90.0%)	7(87.5%)	1.000[a]
Using denture	6(60.0%)	7(87.5%)	0.314[a]
Bathel index	31.5 ± 14.5	18.1 ± 21.2	0.132
Mini nutritional assessment short Form	8.2 ± 1.9	7.8 ± 1.5	0.596
Mini-Mental state examination	10.6 ± 4.3	4.6 ± 9.1	0.082

Student's t-test and [a]Fisher's exact test. Mean ± Standard deviation or N(%)

Table 2. Basic information and indicators for older people in different independent groups.

	Octogenarians with BI scores≤60 (n=10)	Octogenarians with BI scores > 60 (n=10)	*P*-value
The number of teeth	10.7 ± 8.8	10.6 ± 7.5	0.978
Sex: Female	9(90.0%)	9(90.0%)	1.000[a]
Using denture	6(60.0%)	7(70.0%)	1.000[a]
Bathel index	31.5 ± 14.5	78.0 ± 9.5	< 0.001
Mini nutritional assessment short Form	8.2 ± 1.9	11.9 ± 1.3	< 0.001
Mini-Mental state examination	10.6 ± 4.3	18.4 ± 6.2	0.004

Student's t-test and [a]Fisher's exact test. Mean ± Standard deviation or N(%)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500
400
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200
100
0
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80FB1 80FB2 80FB3 80FB4 80FB5 80FB6 80FB7 80FB8 80FB9 80FB10 80FG1 80FG2 80FG3 80FG4 80FG5 80FG6 80FG7 80FG8 80FG9 80FG10 80SB1 80SB2 80SB3 80SB4 80SB5 80SB6 80SB7 80SB8 80SB9 80SB10 80SG1 80SG2 80SG3 80SG4 80SG5 80SG6 80SG7 80SG8 80SG9 80SG10 100FB1 100FB2 100FB3 100FB4 100FB5 100FB6 100FB7 100FB8 100SB1 100SB2 100SB3 100SB4 100SB5 100SB6 100SB7 100SB8 Total

Figure.1A

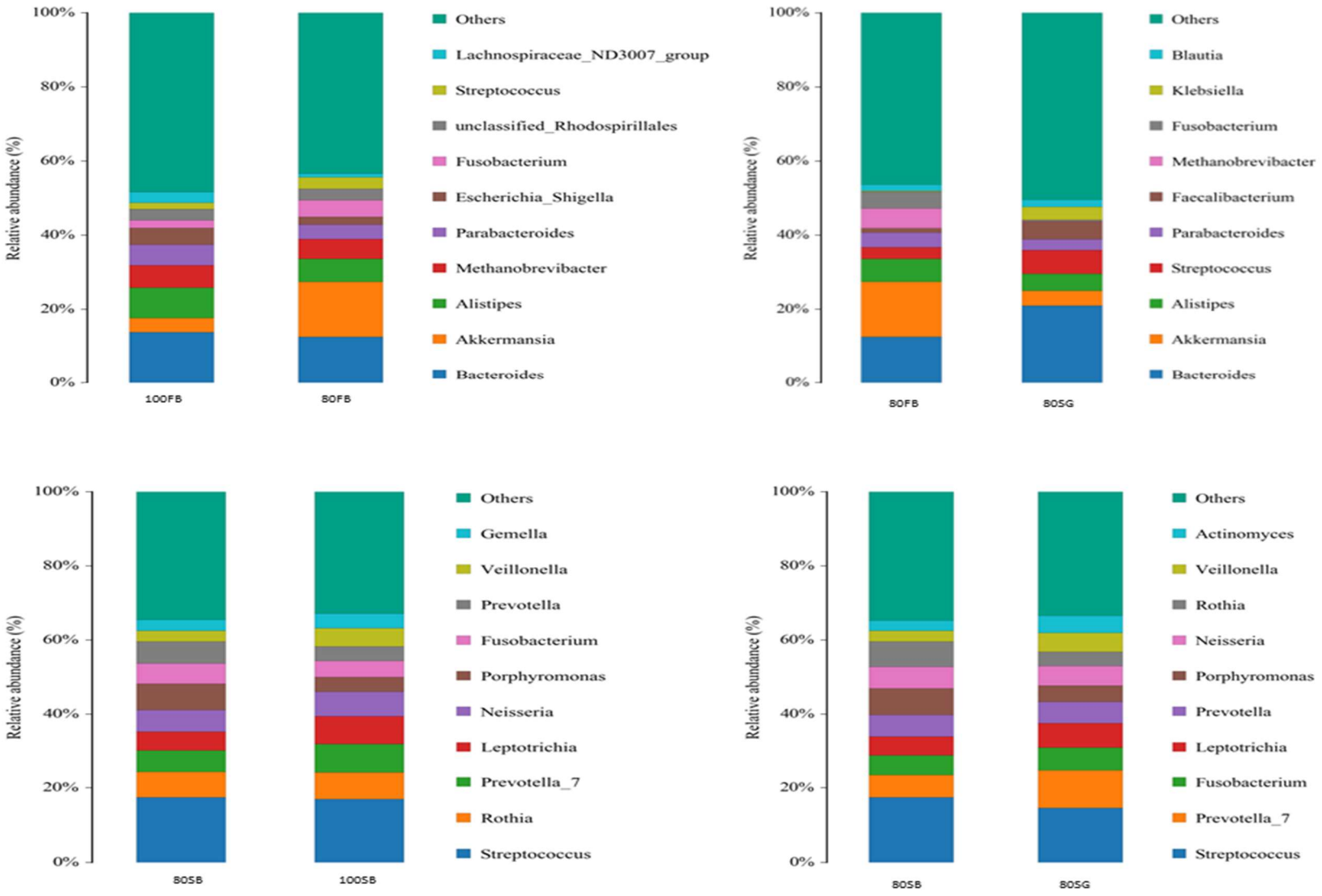
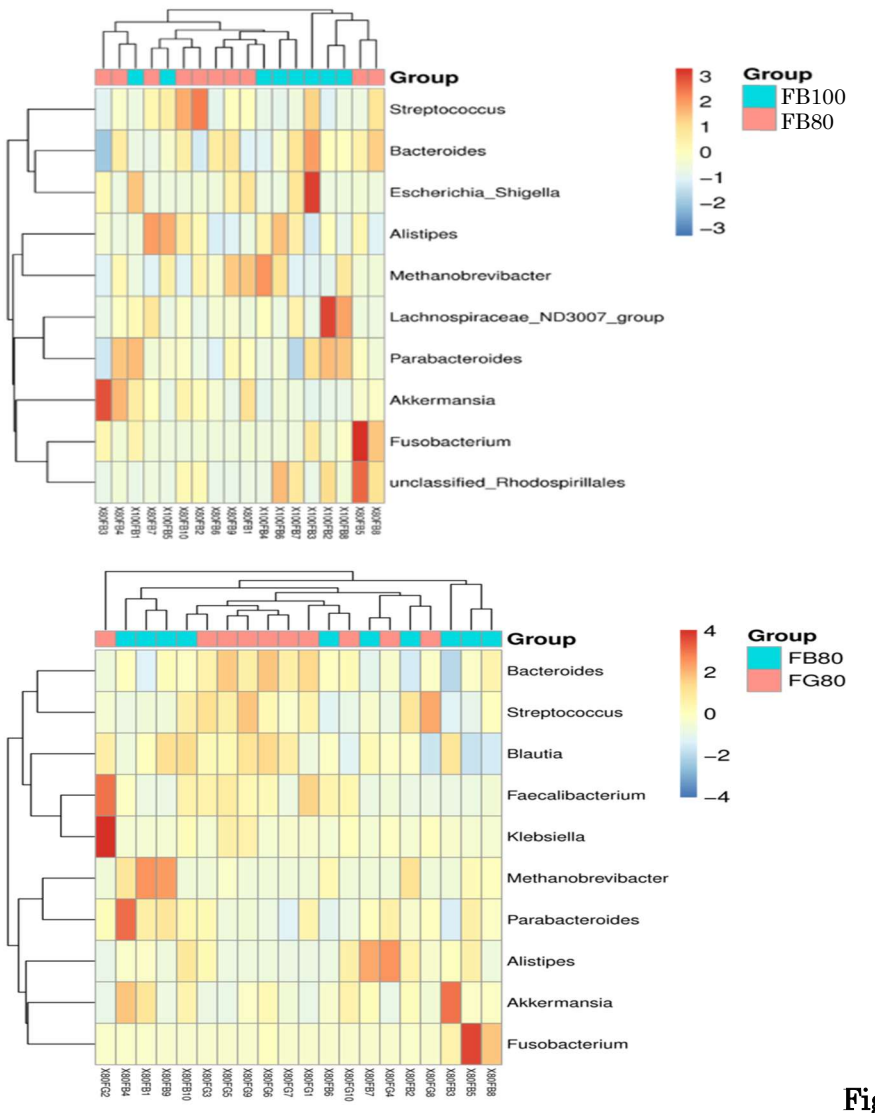
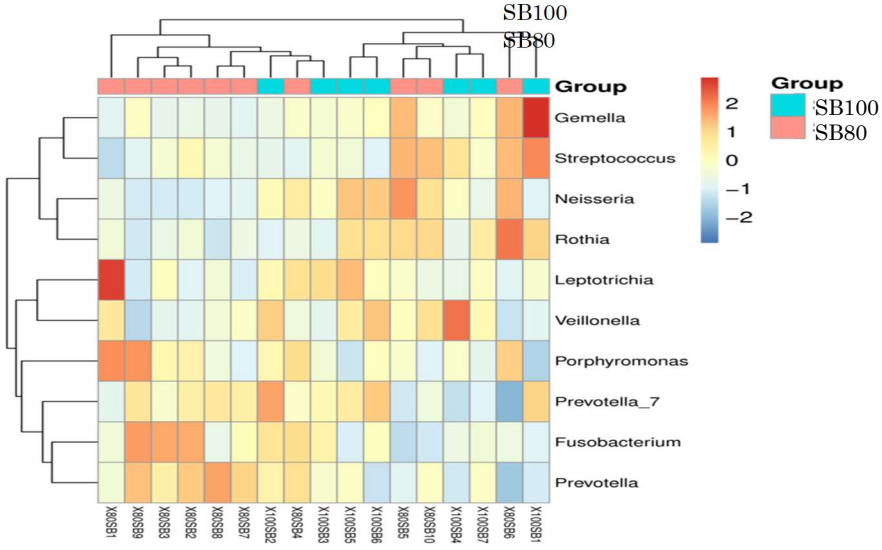

Relative abundance (%)
100%
80%
60%
40%
20%
0%
100FB
80FB
Others
Lachnospiraceae_ND3007_group
Streptococcus
unclassified_Rhodospirillales
Fusobacterium
Escherichia_Shigella
Parabacteroides
Methanobrevibacter
Alistipes
Akkermansia
Bacteroides
80FB
80SG
Others
Blautia
Klebsiella
Fusobacterium
Methanobrevibacter
Faecalibacterium
Parabacteroides
Streptococcus
Alistipes
Akkermansia
Bacteroides
80SB
100SB
Others
Gemella
Veillonella
Prevotella
Fusobacterium
Porphyromonas
Neisseria
Leptotrichia
Prevotella_7
Rothia
Streptococcus
80SB
80SG
Others
Actinomyces
Veillonella
Rothia
Neisseria
Porphyromonas
Prevotella
Leptotrichia
Fusobacterium
Prevotella_7
Streptococcus

Figure.1B


Group
Streptococcus
Bacteroides
Escherichia_Shigella
Alistipes
Methanobrevibacter
Lachnospiraceae_ND3007_group
Parabacteroides
Akkermansia
Fusobacterium
unclassified_Rhodospirillales
3
2
1
0
−1
−2
−3
Group
FB100
FB80

Group
Bacteroides
Streptococcus
Blautia
Faecalibacterium
Klebsiella
Methanobrevibacter
Parabacteroides
Alistipes
Akkermansia
Fusobacterium
4
2
0
−2
−4
Group
FB80
FG80


SB100
SB80
Group
Gemella
Streptococcus
Neisseria
Rothia
Leptotrichia
Veillonella
Porphyromonas
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Fusobacterium
Prevotella
2
1
0
−1
−2
Group
SB100
SB80

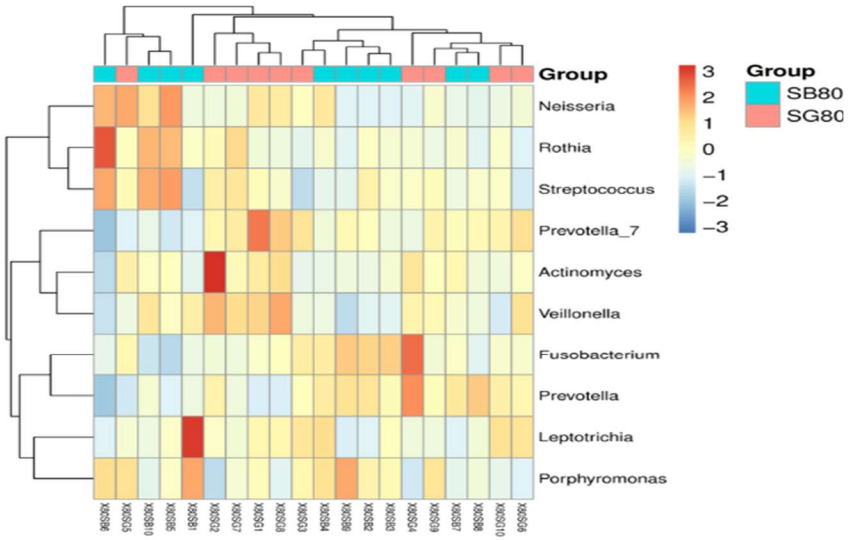

Group
Neisseria
Rothia
Streptococcus
Prevotella_7
Actinomyces
Veillonella
Fusobacterium
Prevotella
Leptotrichia
Porphyromonas
3
2
1
0
−1
−2
−3
Group
SB80
SG80

Fig. 1 C

Fig. 1 Clustering results display and description.

A. Statistics of OTUs in each sample. OTU counts marked on top of each bar.

B. Histogram of species distribution at the genus level. Top 10 genus were selected and the rest were combined as others. Unclassified represents the species that has not been taxonomically annotated.

C. Heat map of species abundance clustering at the genus level.

100FB: non-independent centenarian stool samples;
100SB: non-independent centenarian saliva samples;
80FB: non-independent octogenarian stool samples;
80SB: non-independent octogenarian saliva samples;
80FG: independent octogenarian stool samples;
80SG: independent octogenarian saliva samples.

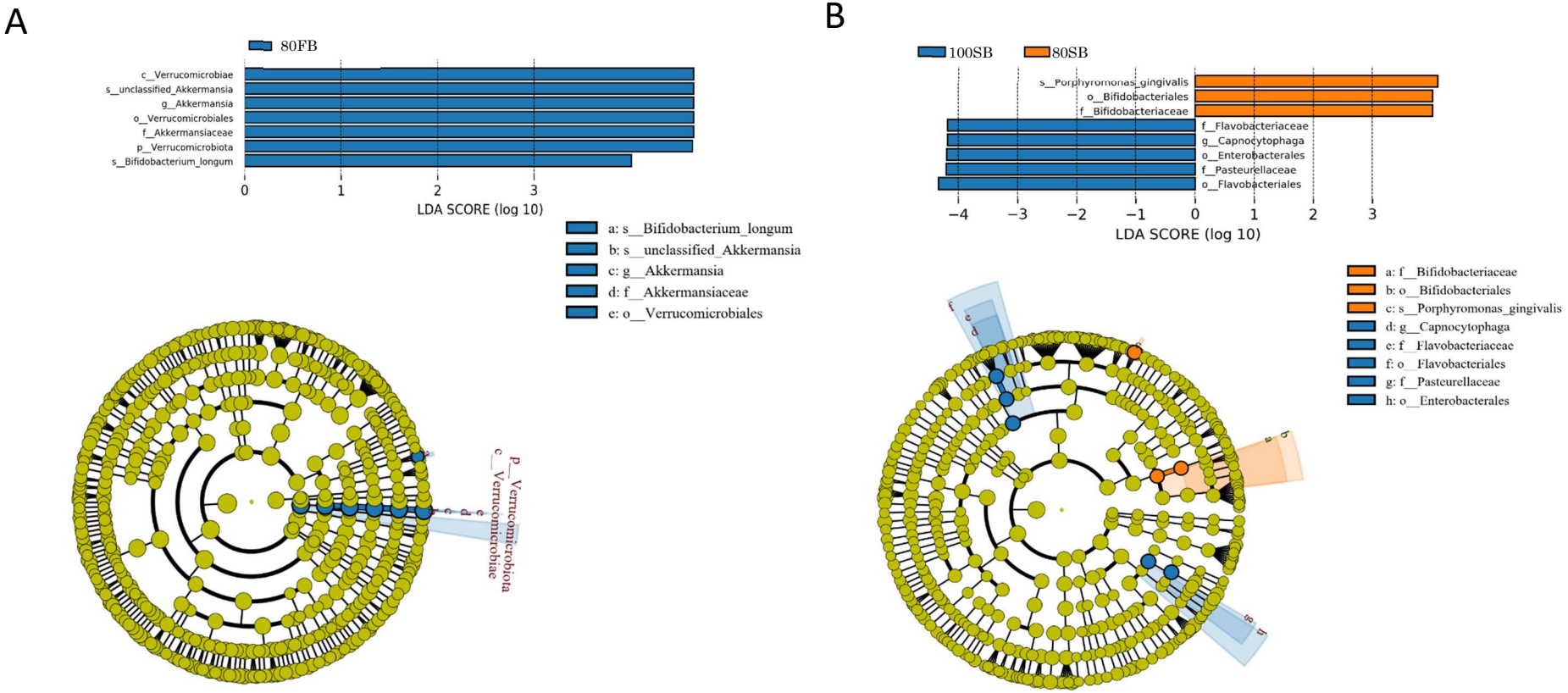
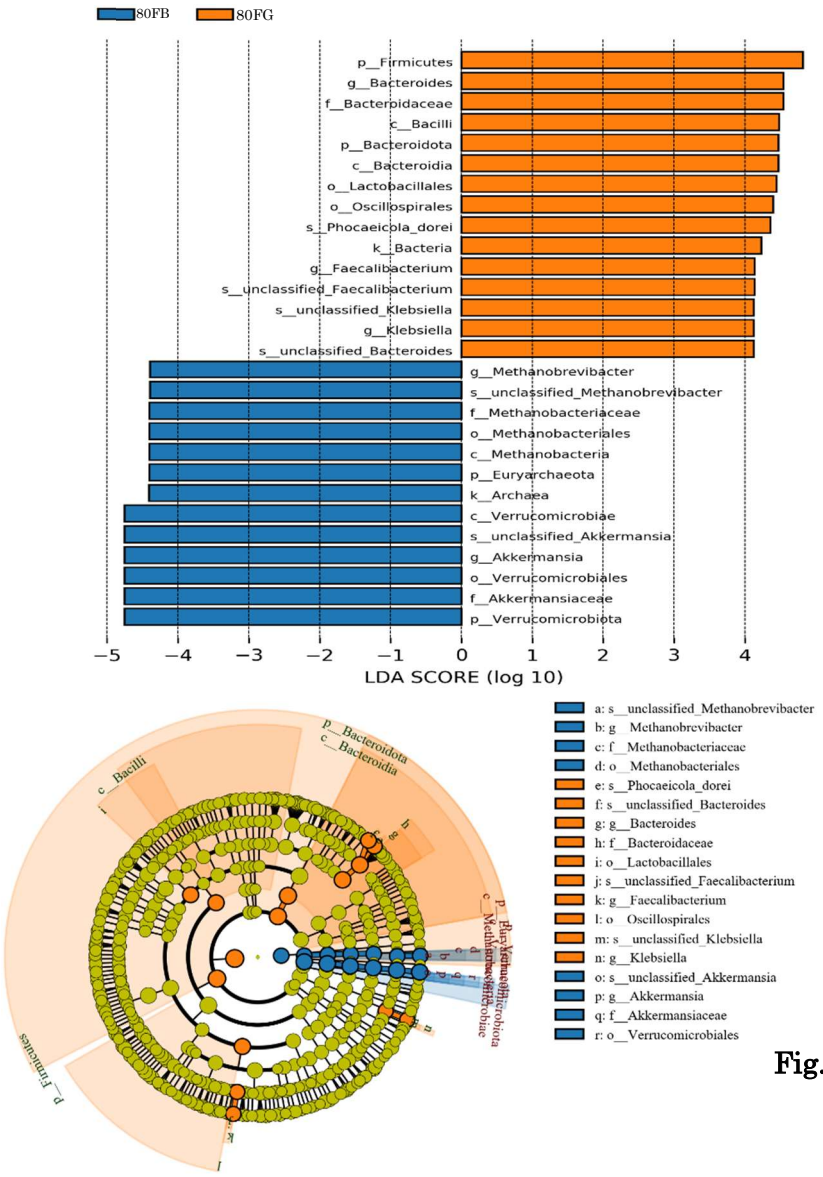
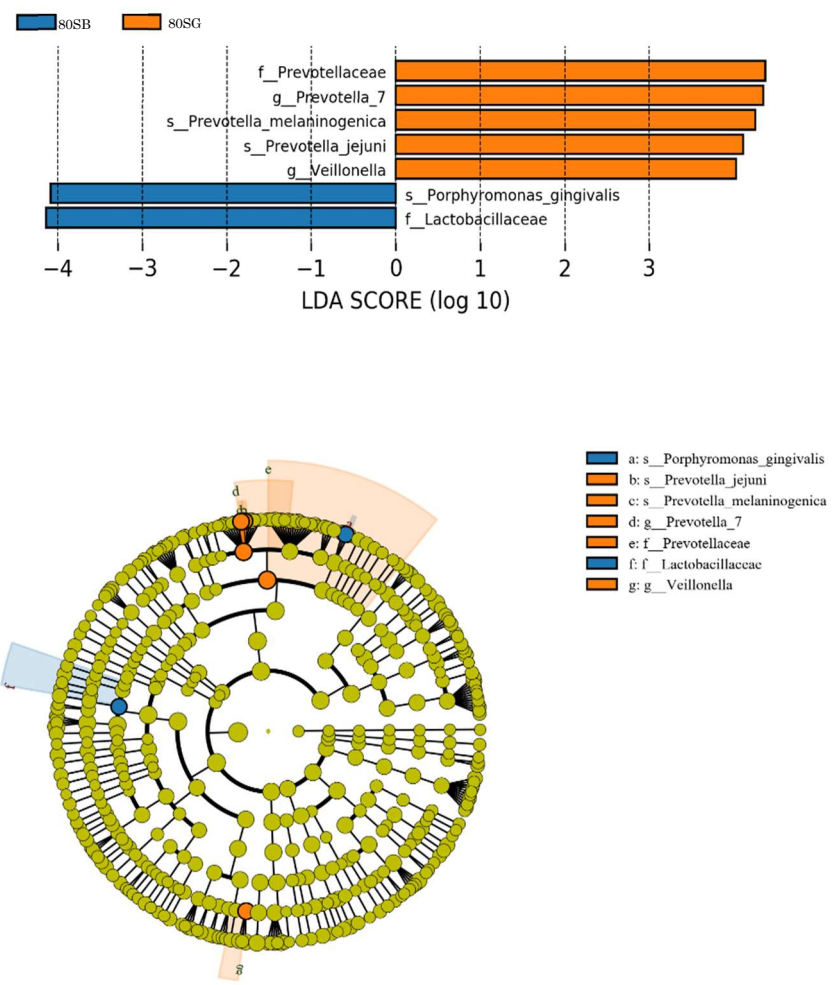

A
80FB
c__Verrucomicrobiae
s__unclassified_Akkermansia
g__Akkermansia
o__Verrucomicrobiales
f__Akkermansiaceae
p__Verrucomicrobiota
s__Bifidobacterium_longum
0
1
2
3
LDA SCORE (log 10)
a: s__Bifidobacterium_longum
b: s__unclassified_Akkermansia
c: g__Akkermansia
d: f__Akkermansiaceae
e: o__Verrucomicrobiales
p__Verrucomicrobiota
c__Verrucomicrobiae
B
100SB
80SB
s__Porphyromonas_gingivalis
o__Bifidobacteriales
f__Bifidobacteriaceae
f__Flavobacteriaceae
g__Capnocytophaga
o__Enterobacterales
f__Pasteurellaceae
o__Flavobacteriales
−4
−3
−2
−1
0
1
2
3
LDA SCORE (log 10)
a: f__Bifidobacteriaceae
b: o__Bifidobacteriales
c: s__Porphyromonas_gingivalis
d: g__Capnocytophaga
e: f__Flavobacteriaceae
f: o__Flavobacteriales
g: f__Pasteurellaceae
h: o__Enterobacterales

Fig. 2 A, B

C


80FB
80FG
p__Firmicutes
g__Bacteroides
f__Bacteroidaceae
c__Bacilli
p__Bacteroidota
c__Bacteroidia
o__Lactobacillales
o__Oscillospirales
s__Phocaeicola_dorei
k__Bacteria
g__Faecalibacterium
s__unclassified_Faecalibacterium
s__unclassified_Klebsiella
g__Klebsiella
s__unclassified_Bacteroides
g__Methanobrevibacter
s__unclassified_Methanobrevibacter
f__Methanobacteriaceae
o__Methanobacteriales
c__Methanobacteria
p__Euryarchaeota
k__Archaea
c__Verrucomicrobiae
s__unclassified_Akkermansia
g__Akkermansia
o__Verrucomicrobiales
f__Akkermansiaceae
p__Verrucomicrobiota
−5 −4 −3 −2 −1 0 1 2 3 4
LDA SCORE (log 10)
a: s__unclassified_Methanobrevibacter
b: g__Methanobrevibacter
c: f__Methanobacteriaceae
d: o__Methanobacteriales
e: s__Phocaeicola_dorei
f: s__unclassified_Bacteroides
g: g__Bacteroides
h: f__Bacteroidaceae
i: o__Lactobacillales
j: s__unclassified_Faecalibacterium
k: g__Faecalibacterium
l: o__Oscillospirales
m: s__unclassified_Klebsiella
n: g__Klebsiella
o: s__unclassified_Akkermansia
p: g__Akkermansia
q: f__Akkermansiaceae
r: o__Verrucomicrobiales

D


80SB
80SG
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g__Prevotella_7
s__Prevotella_melaninogenica
s__Prevotella_jejuni
g__Veillonella
s__Porphyromonas_gingivalis
f__Lactobacillaceae
−4 −3 −2 −1 0 1 2 3
LDA SCORE (log 10)
a: s__Porphyromonas_gingivalis
b: s__Prevotella_jejuni
c: s__Prevotella_melaninogenica
d: g__Prevotella_7
e: f__Prevotellaceae
f: f__Lactobacillaceae
g: g__Veillonella

Fig. 2 C, D

Fig. 2 Histogram of LDA value distribution and evolutionary branch graph of LEfSe analysis.

A. Comparison of fecal samples from different age groups.

B. Comparison of saliva samples from different age groups.

C. Comparison of fecal samples from different independent groups.

D. Comparison of saliva samples from different independent groups.

100FB: non-independent centenarian stool samples;

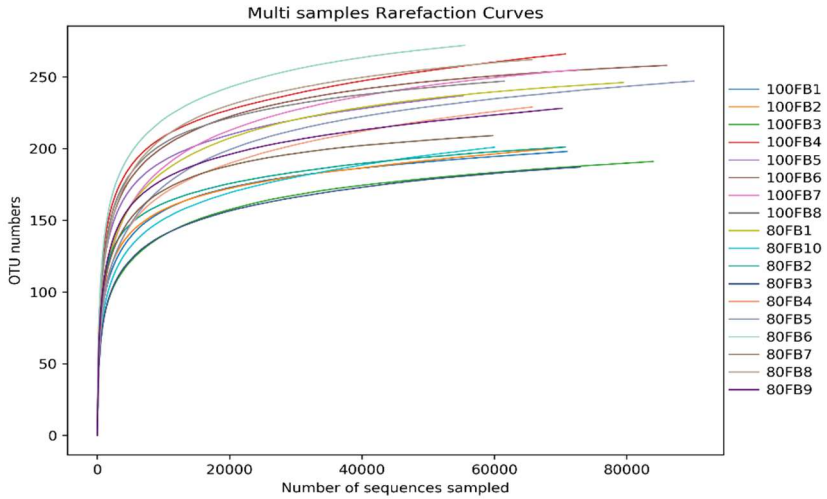
100SB: non-independent centenarian saliva samples;

80FB: non-independent octogenarian stool samples;

80SB: non-independent octogenarian saliva samples;

80FG: independent octogenarian stool samples;

80SG: independent octogenarian saliva samples.


Multi samples Rarefaction Curves
OTU numbers
Number of sequences sampled
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20000
40000
60000
80000
0
50
100
150
200
250
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100FB3
100FB4
100FB5
100FB6
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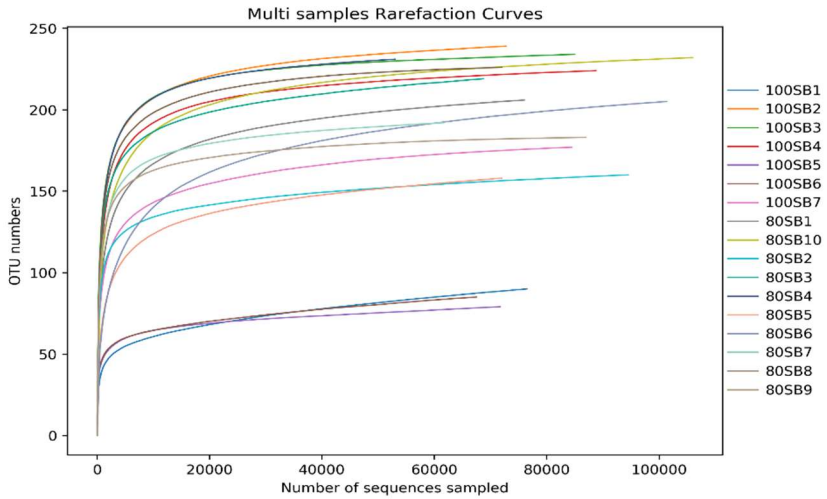
Multi samples Rarefaction Curves
OTU numbers
Number of sequences sampled
0
20000
40000
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80000
100000
0
50
100
150
200
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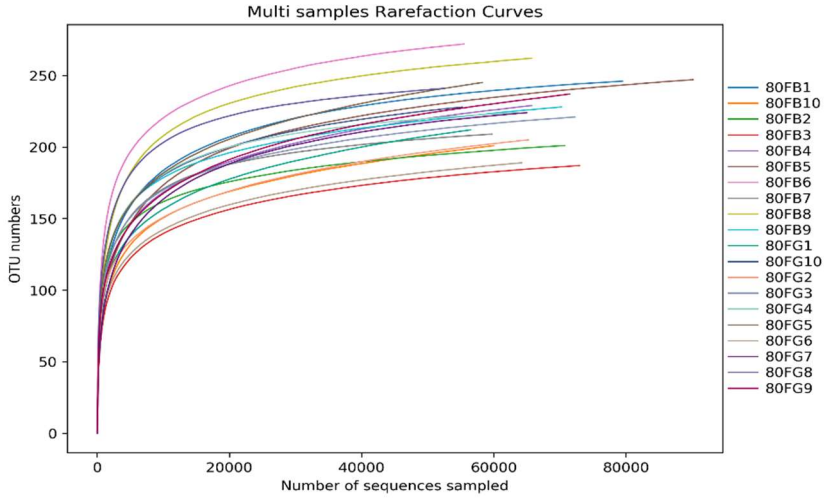
100SB2
100SB3
100SB4
100SB5
100SB6
100SB7
80SB1
80SB10
80SB2
80SB3
80SB4
80SB5
80SB6
80SB7
80SB8
80SB9


Multi samples Rarefaction Curves
OTU numbers
Number of sequences sampled
0
20000
40000
60000
80000
0
50
100
150
200
250
80FB1
80FB10
80FB2
80FB3
80FB4
80FB5
80FB6
80FB7
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80FG4
80FG5
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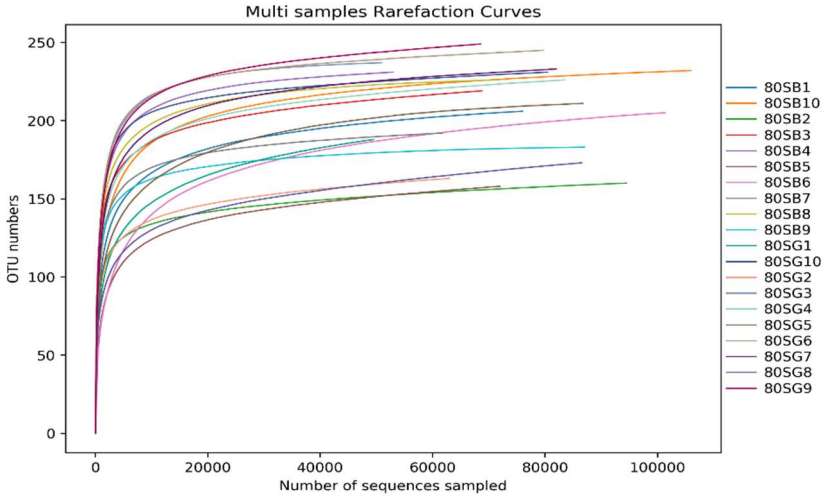
80FG9


Multi samples Rarefaction Curves
OTU numbers
Number of sequences sampled
0
20000
40000
60000
80000
100000
0
50
100
150
200
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80SB10
80SB2
80SB3
80SB4
80SB5
80SB6
80SB7
80SB8
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80SG8
80SG9

Fig.3 A

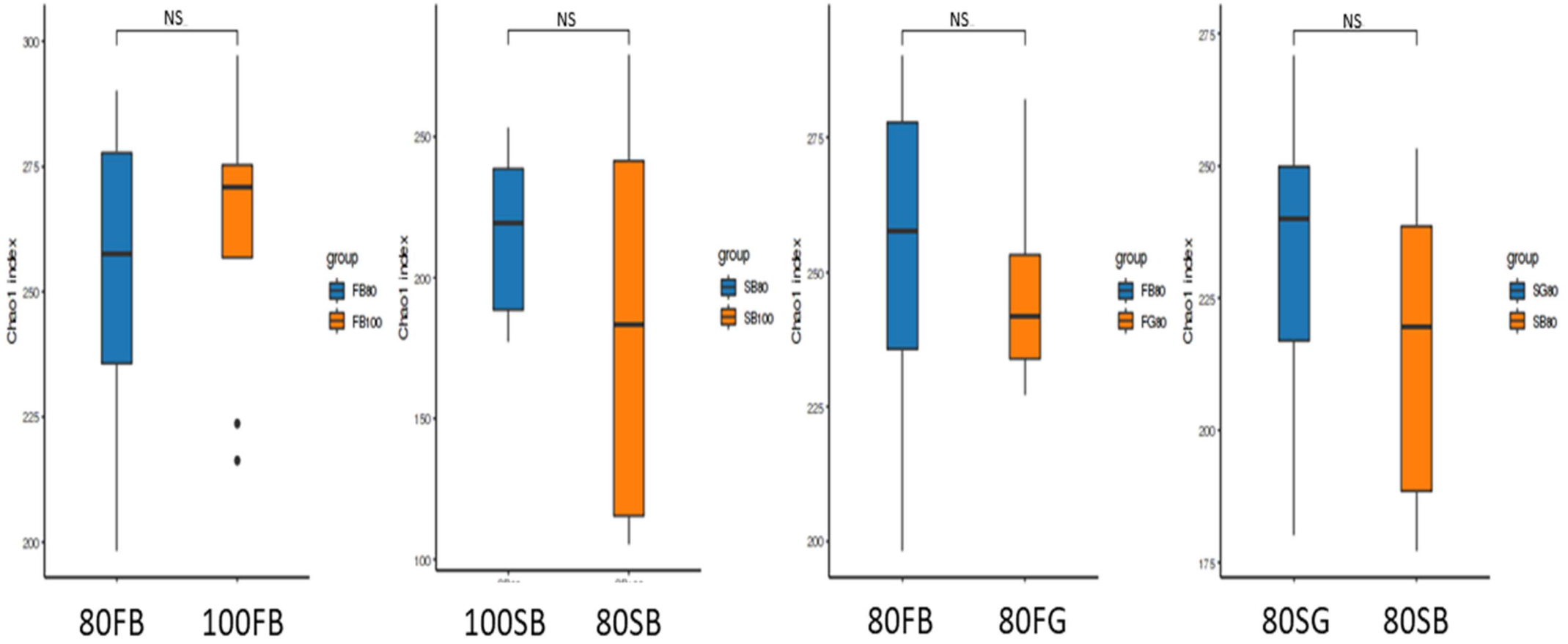

Fig.3 B

NS: No significance

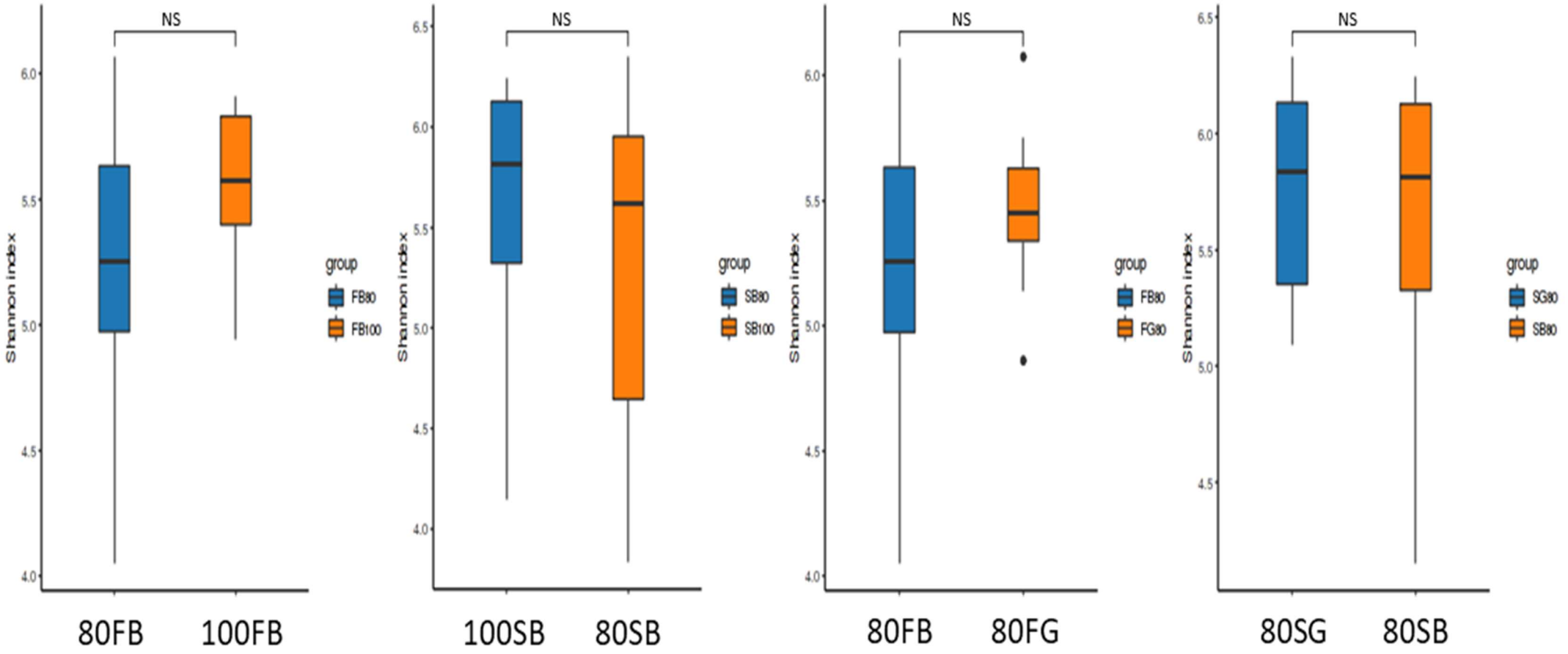

Fig.3 C

NS: No significance

Fig.3 α diversity analysis.

A. Rarefaction curve gradually become flat, which indicates that sequencing data is adequate to present most species in the sample.

B. Chao1 index counts for species richness. No significant difference in comparison between groups.

C. Shannon index reflects species diversity. No significant difference in comparison between groups.

100FB: non-independent centenarian stool samples;
100SB: non-independent centenarian saliva samples;
80FB: non-independent octogenarian stool samples;
80SB: non-independent octogenarian saliva samples;
80FG: independent octogenarian stool samples;
80SG: independent octogenarian saliva samples.

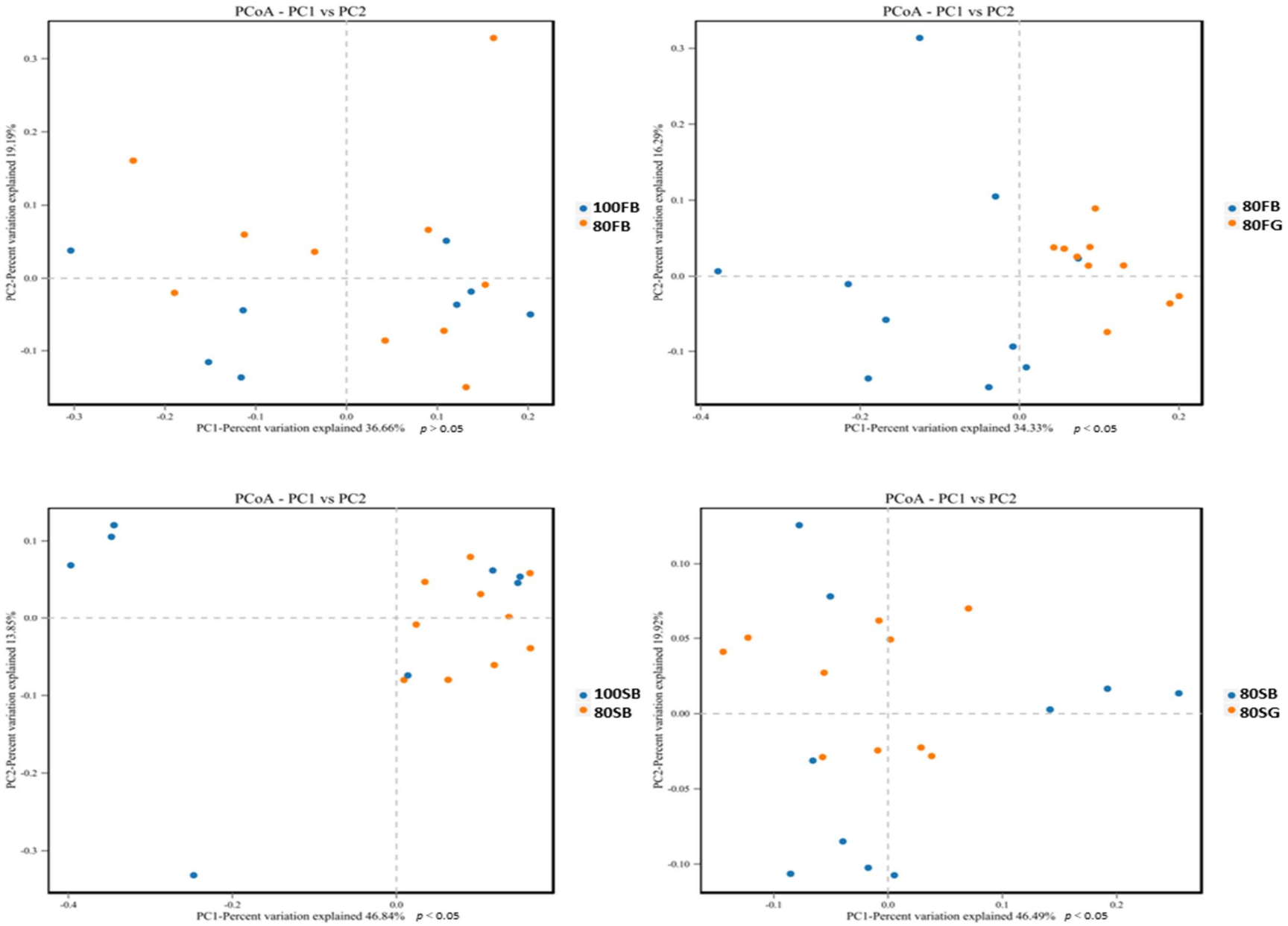
PCoA - PC1 vs PC2
PC2-Percent variation explained 19.19%
PC1-Percent variation explained 36.66% p > 0.05
100FB
80FB
PCoA - PC1 vs PC2
PC2-Percent variation explained 16.29%
PC1-Percent variation explained 34.33% p < 0.05
80FB
80FG
PCoA - PC1 vs PC2
PC2-Percent variation explained 13.85%
PC1-Percent variation explained 46.84% p < 0.05
100SB
80SB
PCoA - PC1 vs PC2
PC2-Percent variation explained 19.92%
PC1-Percent variation explained 46.49% p < 0.05
80SB
80SG

Fig.4 A

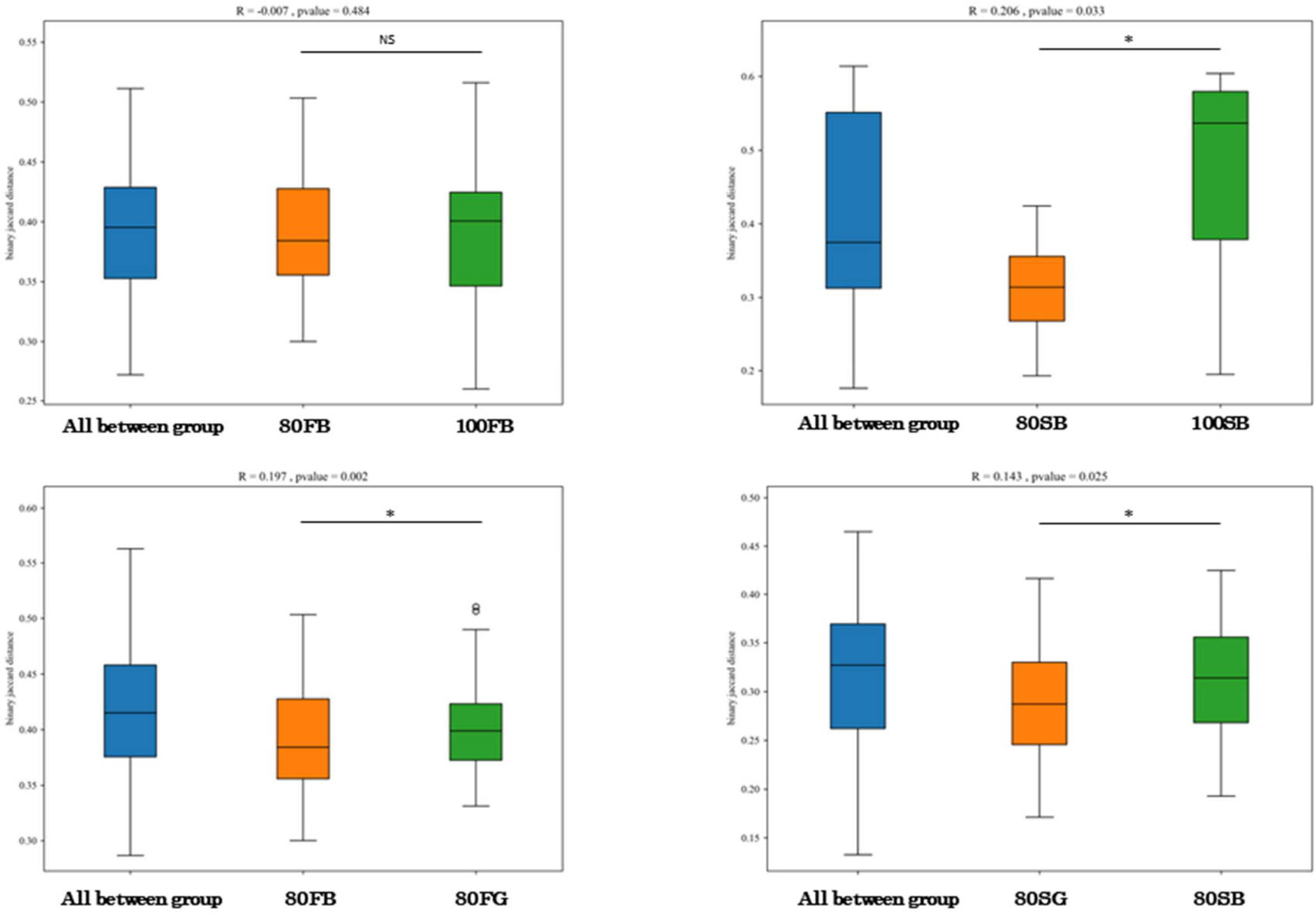

Fig.4 B

*$p < 0.05$, NS: No significance

Fig.4 ß diversity analysis

A. PCoA Graph. Each dot represents a sample. Samples in different groups are presented in different color. X-axis and Y-axis represent two eigenvalues that could maximize difference between samples. The influence of each eigenvalue was measured in percentage.

B. Anosim analysis box plot. Y-axis represents ß distance; The closer the R value (obtained from anosim analysis) is to 1, the higher the difference between groups. *P*-value less than 0.05 indicates high reliability of the test.

100FB: non-independent centenarian stool samples;
100SB: non-independent centenarian saliva samples;
80FB: non-independent octogenarian stool samples;
80SB: non-independent octogenarian saliva samples;
80FG: independent octogenarian stool samples;
80SG: independent octogenarian saliva samples.

5. Discussion

Since there are many factors affecting the composition of the digestive tract flora of the elderly, the sample size of studies on the elderly, especially the centenarians are generally small, which leads to slight differences in the results of studies on the composition of the digestive tract flora of the elderly in different regions[26, 27]. At the phylum level, the first dominant bacteria in our all groups are *Firmicutes*, which is basically consistent with previous studies[15, 28-30], and it is considered to dominate the healthy gut microbiota with *Bacteroidetes* following closely[31], but there are also studies that suggest that the proportion of *Firmicutes* decreases with age[32].

In genus-level between-group comparisons of fecal samples, some studies suggest that *Akkermansia* tends to be considered more common in centenarians[11, 29, 33]. But we observed the opposite: the relative abundance of *Akkermansia* in 80-year-old independent group and centenarians, is only 4%, but it is as high as 15% in the eighty-year-old group with poor independence(Fig.1B), which is similar to the research results of centenarians in Sardinia and other places in Italy[34, 35]. *Akkermansia* is thought to degrade mucin and promote gut health by reducing toxicity levels associated with high-fat diets[36]. But there are also studies suggesting that it plays a contributing role in the development of inflammatory bowel disease, especially when other harmful bacteria cause damage to the intestinal mucosal barrier[37]. Recent studies have reported the effect of dietary fibre on *Akkermansia*, and perhaps the dietary structure of the elderly may have contributed to the increased abundance of *Akkermansia*[38]. But because of conflicting findings, whether *Akkermansia* reduction in centenarians is related to host aging and longevity requires further research. In addition, *Bacteroids*, *Faecalibacterium* and *Klebsiella*, which are characteristically present in the feces of octogenarians with better independence in our study(Fig.2C), are consistent with their positioning as healthy gut resident bacteria[38-41].

Porphyromonas gingivalis is characteristically present in the saliva of octogenarians who cannot live independently in our study. Not only is this bacterium a major causative agent of adult periodontitis, it is also associated with rheumatoid arthritis[42], diabetes[43], cardiovascular disease[44], Alzheimer's disease[45], nonalcoholic fatty Liver disease[46], and other common diseases in the elderly are related. This bacterium tends to be negatively correlated with oral hygiene, and elders with poor functional independence are unable to maintain good oral hygiene, providing favorable conditions for their growth. However, because its living environment is highly dependent on natural teeth (in the gingival sulcus or periodontal pocket), the decrease in the number of remaining teeth in the centenarians actually decreased the abundance of *P. gingivalis* in this group[43].

The only genus characteristic of centenarian saliva is *Capnocytophaga*, and most studies on this genus have focused on infections in humans caused by the bite of bacteria-carrying animals[47]. It is usually considered to be a commensal bacterium in the human oral cavity, but it may also be associated with oral and systemic infections in the case of weakened immune function. For example, Cao et al. found that the abundance of *Capnocytophaga* in the oral cavity of IgA nephropathy patients with periodontitis was significantly higher than that of ordinary periodontitis patients[48]. However, this may be the first time that *Capnocytophaga* has been found characteristically in the saliva of centenarians. The reason for its characteristic existence is unclear, as there has been less research on this genus in the past. Given that the immune function of centenarians declines with age, this genus may be a risk factor for the future health of older adults.

α-diversity reflects the species abundance and species diversity of a single sample, and host genes, environment (including drugs and diet), and lifestyle have important effects on microbial diversity in the digestive tract[49, 50]. Kelley et al. found that, for adults, age was positively associated with gut microbiota diversity, but this trend stopped around age 40[51]. Diversity can be maintained for a long time in healthy elderly people until the body's bacterial diversity declines when physical health declines[52-54]. However, in the study of

centenarians, some scholars observed that the α-diversity of the long-lived people is higher[33, 55, 56], and some studies believe that the α-diversity does not change significantly due to longevity[29, 35]. Our results support the latter, namely that α diversity was not significantly different (Fig.3). There is also controversy over the relationship between the diversity of oral flora and the health of the elderly. Some scholars have found that the diversity of oral flora in patients with dental caries or periodontitis is not much different from that of healthy people[57-59], and some studies suggest that more diverse flora may correspond to a healthier ecosystem[60-62]. However, it is certain that microbes in different ecological niches will show significant differences, and even in the oral cavity, the composition of the flora in dental plaque and saliva is very different[59, 63]. Due to the limited number of teeth of centenarians in this study, we only collected saliva samples, and further analysis of other ecological sites such as dental plaque may provide a clearer understanding. In addition, all the subjects in this study were from nursing homes, and their lifestyles and diets were relatively simple, which may have an impact on the diversity of digestive tract flora[4, 29, 64]. In summary, we believe that the reason for this result may be that the diversity of elders' flora is affected by a variety of unfavorable factors, resulting in overall low diversity level, which in turn leads to a small difference between groups.

Our study found that although the diversity of gut microbiota was similar in the elderly, the variation between individuals (i.e., ß-diversity) with different functional independence status was significantly increased. The similarity in the composition of the microbiota of each sample in the group reflects the stability of the group's microbiota. Significant differences in the ß-diversity of the intestinal microbiota between the elderly and young have been confirmed by previous studies[33, 35, 65]. However, Biagi found similar ß-diversity of gut microbiota in centenarians and semi-supercentenarians (i.e., 105-109 years old)[11], and our study extended this similarity to octogenarians. From this, we speculate that the effect of age on the diversity of gut microbiota in the population seems to be weakened after entering old age. The presence or absence of long-term care may also have an impact on gut flora ß-

diversity, with Jeffery et al. comparing the flora composition of community residents and nursing home residents and finding that ß-diversity in community-dwelling older people was even more similar to that of younger people. However, this similarity is predicated on maintaining good health, otherwise the dispersion of the community flora of elderly people with unstable microbiota is more skewed towards long-term care residents[4, 66]. A small number of studies have focused on changes in the ß-diversity of the oral flora. The oral environment is less able to self-regulate than the gut and is more susceptible to host status, and several studies have shown that the oral flora of older adults with cognitive impairment is more likely to be disrupted than normal, thereby increasing the degree of dispersion of diversity within the group[67, 68]. Schwartz et al. compared salivary flora in humans of different ages and identified six of seven variables including age and oral health status that could significantly influence oral flora ß-diversity, however the study did not include the systemic health status of older adults[69]. Wu et al. found no significant differences in ß-diversity between the oral flora of centenarians and the general elderly in their study of the Italian Sardinian population[35], which contrary to our results. However, as their study excluded unhealthy older people, perhaps this is another indication of the effect of health on individual variation in oral flora.

In conclusion, our results show that differences in age and functional independence status lead to variability in the oral and intestinal flora of older adults. As the flora in older people is more susceptible to changes in host and environmental factors, research show regional variability across the globe. As the sample size of future studies increases, some consensus may emerge. The number of natural teeth in the centenarians included in this study was significantly less than in octogenarians, and although almost all of the sampled elderly used a denture to restore masticatory function, it has also been suggested that a low number of teeth or even edentulous has an effect on the digestive flora of the elderly[70, 71]. Thus, the extent to which the age factor affects the digestive flora in the elderly population remains to be tested. In addition, as a cross-sectional study, this experiment did not consider

the effect of time on the flora of the elderly, and future studies may have different results by looking at the changes in the flora of the elderly over time.

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