European Review for Medical and Pharmacological Sciences 2020; 24: 12983-12992

Structural difference analysis of adult's intestinal flora basing on the 16S rDNA gene sequencing technology

H.-J. YU[1], C. JING[1], N. XIAO[2], X.-M. ZANG[1], C.-Y. ZHANG[3], X. ZHANG[1], Y.-N. QU[1], Y. LI[1], Q.-W. TAN[1]

[1]College of Acupuncture and Massage, Shandong University of Traditional Chinese Medicine, Jinan, China
[2]State Key Laboratory of Cardiovascular Disease, Fuwai Hospital, Chinese Academy of Medical Science and Peking Union Medical College, Beijing, China
[3]Department of Treatment and Prevention Center, Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan, China

Abstract. – OBJECTIVE: Through 16S rDNA technology, we aimed at separating adults aging 20-50 years old into a few groups and processing the high-throughput sequencing analysis, in order to explore the features and differences of intestinal flora in each age group in a microcosmic perspective.

PATIENTS AND METHODS: 120 stool specimens were collected strictly in accordance with acceptance criteria and exclusion criteria. 49 subjects aging 20-29 years old (Group AGE1), 51 subjects aging 30-39 years old (Group AGE2), and 20 subjects aging 40-49 years old (Group AGE3) were divided into 3 groups. Bacteria DNA from fresh stool specimens of 3 groups were abstracted. Illumina MiSeq high-throughput sequencing platform was applied to process 16S rDNA sequencing in Area 338F_806R for intestinal flora detection. I-Sanger Bio-cloud platform was applied for the analysis of intestinal flora structure changes in phylum level and genus level.

RESULTS: Among the age of 20-50, with older age, the abundance of intestinal flora decreased among healthy adults more than 40 years old. In addition, the diversity and sample dispersion of intestinal flora is significantly different from people among 20-40 years old. The decrease ratio of *Firmicutes*/*Bacteroidetes* indicated that as the age grows, glucose tolerance might decrease. Comparing with people among 20-40 years old, the amount of *Bifidobacterium* and *Eubacterium* in people over 40 years old have significantly decreased. The decrease of *Bifidobacterium* and *Eubacterium* may increase the risks of cognitive impairment and lower the anti-inflammation and anti-cancer efficacy in human body, respectively. *Subdoligranulum* relates to poor metabolism and chronic inflammation and it happens more in people aged over 40 than young people who are among 20-40 years old.

CONCLUSIONS: There are differences in the intestinal flora of healthy adults aged 20-50. Effective intervention of the intestinal flora may play a role in delaying aging and preventing diseases.

Key Words:
Intestinal flora, Bacteria DNA, Adult, 16S rDNA.

Introduction

Intestinal microorganism is an active participant of host physiology and relates to the changes of gastrointestinal microorganism, as well as human and animal's disease, including cystic fibrosis[1], inflammatory bowel disease (irritable bowel syndrome, Crohn's disease and colon cancer)[2-4], nervous system disease (Parkinson's disease, Alzheimer's disease, and multiple sclerosis)[5-7], metabolic disease (obesity and diabetes)[8,9] and disease related to muscle and skeleton (weakness, osteoporosis, rheumatoid arthritis and gout)[10-13].

Age is an important factor that affects the structure and constitution of intestinal microorganism. Human intestinal flora is different in species abundance according to different ages[14-16]. The age division is general and most of the studies concentrate on the comparison of aged 20-50 and people over 60[17-22]. The structure and constitution of adult's intestinal flora in different ages still needs further detailed research. Epidemiol-

Corresponding Author: Qiwen Tan, MD; e-mail: 60050011@sdutcm.edu.cn

ogy research indicated that people over 50 years old have significantly increased percentage to have type 2 diabetes, hypertension, brain stroke, non-alcoholic fatty liver, and obesity[23-25]. To study the structure and constitution features of adults among 20-50 years old, we can make targeted adjustment to intestine, which can play an important role in anti-aging and disease prevention, as well as match the traditional Chinese medicine's idea of "cure before illness". 16S rDNA, existing in all organism, is the most useful and commonly used molecular clock in systematic classification of bacteria. With its appropriate size, it can reflect the differences among bacteria and can gain their sequence by sequencing technology. So, it is widely accepted by bacteriologists and taxonomists.

Through the 16S rDNA technology, the study separated adults aging 20-50 years old into a few groups and processed the high-throughput sequencing analysis, and explored the features and differences of intestinal flora in each age group in a microcosmic perspective.

Patients and Methods

Research Object and Grouping

This study was approved by the Ethics Committee of Affiliated Hospital of Shandong University of Traditional Chinese Medicine. Signed written informed consents were obtained from all participants before the study. The study objects are from Curing Before Illness Center of the Affiliated Hospital of the Shandong University of Traditional Chinese Medicine. Ethical batch number is 2018-033-KY. Questionnaire investigation method was adopted to collect the age, gender, living habit information (including smoking, drinking and diet) and human body measurement information (including height, weight, blood pressure, physical quality index, waist circumference, hip circumference, and body fat rate). 120 stool specimens were collected strictly in accordance with the acceptance criteria and exclusion criteria. 49 subjects (25 male and 24 female) aging 20-29 years old (Group AGE1), 51 subjects (29 male and 22 female) aging 30-39 years old (Group AGE2) and 20 subjects (6 male and 14 female) aging 40-49 years old (Group AGE3) were divided into 3 groups. Bacteria DNA from fresh stool specimens of 3 groups were abstracted. Inclusion criteria are shown as follows: (1) age among 20-50 years old; (2) living in Jinan more than 1 year and no antibiotics were taken within 1 month; (3) no serious illness and who signed the Informed Consent Form. Exclusion criteria are shown as follows: (1) take each kind of medicine or accept other therapies; having other serious diseases; (2) women in pregnancy and lactation period.

Stool Specimens Collection

Enrolled subjects adopted their stool specimens in outpatient department. Subjects with special situations, such as diarrhea and female menstruation should postpone to adopt the sample. After emptying the bladder, the subject adopted fresh and natural discharge around 2 g and put it in a 2 mL test tube (Biologix, Jinan, China) immediately. Specimens were kept under liquid nitrogen condition.

Abstraction of Stool's DNA and 16S rDNA Sequencing Analysis

Detection of the specimens were completed by Shanghai Majorbio Technology Co., Ltd. (Shanghai, China). Database was created for qualified DNA specimens. PCR amplification was processed according to 338F_806R region. Passing the quantified detection, we built up Miseq library and performed sequencing. PE (paired-end) acronym readings from Miseq sequencing were jointed together according to overlap relationship. Operational Taxonomic Unit (OTU) cluster analysis and taxonomic analysis were processed in all sequencing. We calculated specimens community structure mainly in phylum level and genus level.

Bioinformatics Analysis

PE reads from Miseq sequencing were jointed together according to overlap relationship. According to different similarity level, processed OTU cluster analysis for all sequencing. We adopted a RDP classifier Bayesian algorithm to conduct taxonomic analysis and calculated each sample's community composition on each classification level. Based on OTU and its cluster analysis results as well as the taxonomic information, in-depth analysis of community structure in each classification level, such as PLS-DA, Partial Least Squares Discriminant Analysis, cluster composition analysis and Wilcoxon rank sum test can be conducted.

Statistical Analysis

We adopted Statistical Product and Service Solutions (SPSS) 25.0 software (IBM Corp., Armonk, NY, USA) to process statistical analysis.

Table I. General Comparison Among Three Groups (mean ± SD).

General Situation	AGE1 (n = 49)	AGE2 (n = 51)	AGE3 (n = 20)	*p*-value
BMI (kg/m^2)	24.8078 ± 0.7155	25.8743 ± 0.68981	25.545 ± 0.95698	$p = 0.543 > 0.05$
Waist-to-Hip Ratio (WHR)	0.8833 ± 0.01022	0.9106 ± 0.01039	0.8945 ± 0.01778	$p = 0.187 > 0.05$

The measurement data used the *t*-test of independent samples. The data were expressed as mean ± standard deviation, and $p<0.05$ indicated that the difference was statistically significant.

Results

General Comparison

Body Mass Index (BMI) and Waist-to-Hip Ratio (WHR) among the three groups showed no significant difference but could be comparable. $p>0.05$ (Table I)

Diversity Index Analysis

Diversity index means sobs (the species number in actual observation). When the curve tends to be flat, it means that the sequencing quantity is abundant. More sequencing quantity (abscissa) does not contribute a lot to the numbers of OTU (ordinate). It indicates that current sequencing quantity can reflect the species diversity of the specimens (Figure 1).

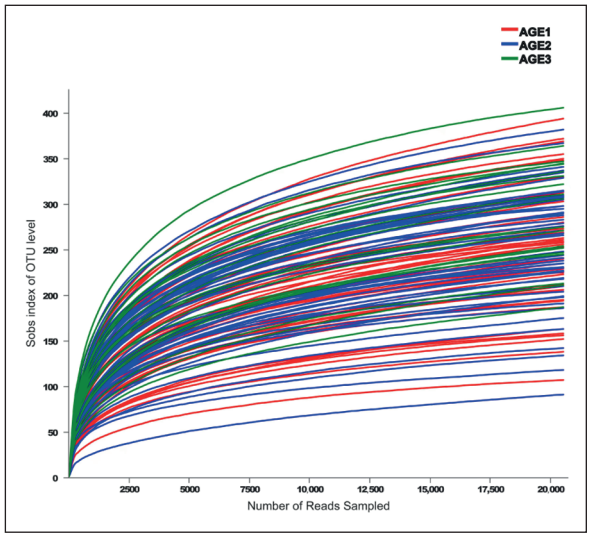

Figure 1. AGE1, AGE2, AGE3 species rarefaction curve. Each color represents one group. The abscissa is the number of sampling sequences and ordinate is the OTU number of specimens. Different color indicated different specimens.

Venn Diagram of OUT Distribution Differences Among Bacterial Groups at Different Ages

OUT number of Group AGE1 was 891, Group AGE2 was 919, and Group AGE3 853. The OUT number of Group AGE2 was more than Group AGE1 and Group AGE3 (Figure 2).

Alpha Adversity Analysis of Bacteria Community in Three Groups

Alpha diversity of the three groups was calculated based on the OTU species and abundant in the specimens (Figure 3). The figure revealed the significant difference of specimens between the two selected groups. Two groups with significant differences were marked ($0.01 < p \leq 0.05$ marked

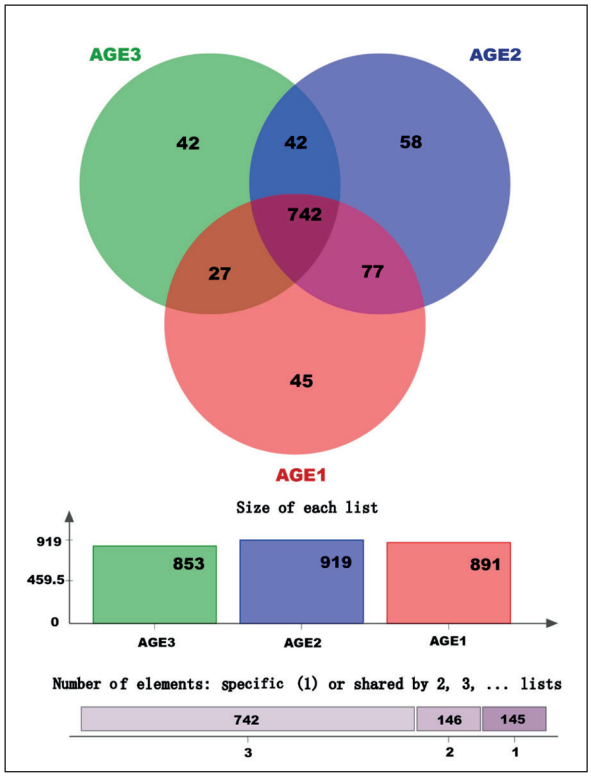

Figure 2. Three Groups' OUT Number. Each color represents one group. The crossing part is the shared OTU with the adjacent group. Each number represents the independent or shared OTU number.

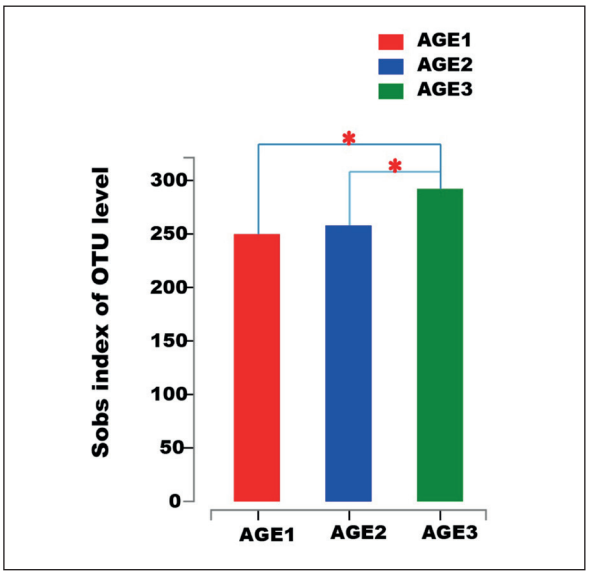

Figure 3. Bacteria diversity analysis among three specimens. This figure shows the significant differences between the three groups of samples selected. The test method is student's t-test, and the two groups with significant differences are marked. (*$0.01 < p \leq 0.05$). The abscissa is the group's name while the ordinate is the index average of each group.

*). Significant differences in bacteria diversity existed between Group AGE1 and Group AGE3, as well as between Group AGE2 and Group AGE3. No significant difference of bacteria diversity existed between Group AGE1 and Group AGE2. The result indicated that as the age grows, the bacteria diversity gradually increased.

Partial Least Squares-Discriminant Analysis (PLS-DA Analysis)

Figure 4 indicated that according to PLS-DA analysis, three groups could be separated and clustered into groups. It revealed that intestinal flora in adults of different ages had significantly differences in OUT level. In addition, the dispersion degree of the specimen point distribution in the PLS-DA analysis can also be reflected that in the OTU level, the dispersion degree of the specimen point distribution in Group AGE1 and Group AGE2 was less than Group AGE3. The intestinal flora Group AGE1 and Group AGE2 had small differences. The difference of intestinal flora dispersion degree in Group AGE1 and Group AGE3, as well as Group AGE2 and Group AGE3 was big.

Community Composition of Three Groups in Phylum Level and Kruskal-Wallis Test

The dominant bacteria of intestinal flora in the three groups were *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria*. The abundance in the genus level was different. The ratio of *Bac-*

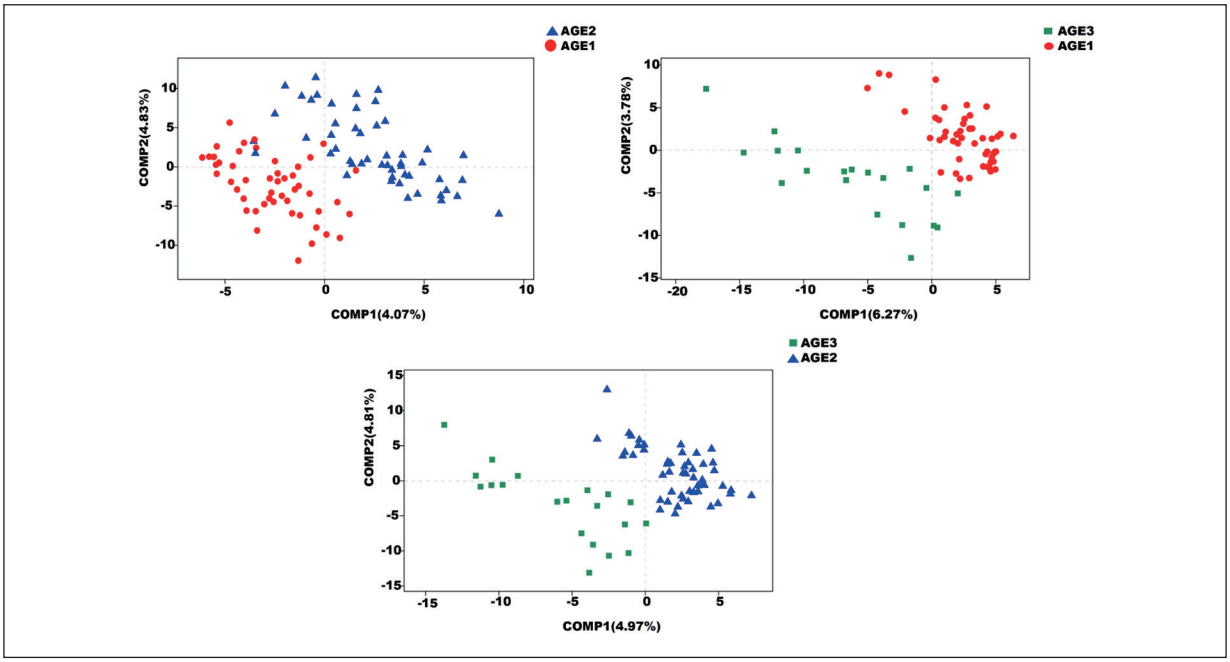

Figure 4. According to PLS-DA analysis, three groups could be separated significantly and clustered into groups. It revealed that intestinal flora in adults of different ages had significantly differences in OUT level.

Table II. Analysis of the proportion of three specimens in phylum level.

Group	*Firmicutes*	*Bacteroidetes*	*Proteobacteria*	*Actinomycota*	*Bacteroidetes/Firmicutes*
AGE1	72.67%	8.95%	9.57%	7.70%	0.123159
AGE2	77.18%	7.52%	6.80%	7.97%	0.097434
AGE3	80.54%	7.15%	6.42%	4.23%	0.088775

teroidetes/Firmicutes (B/F) in Group AGE1 was the highest, and Group AGE2 was the second and Group AGE3 the third. (Table II and Figure 5).

From Kruskal-Wallis Test (Figure 6), *Firmicutes* and *Actinobacteria* were the bacteria in phylum level that had significant difference in the intestinal floral of Group AGE1, Group AGE2, and Group AGE3 ($p<0.05$).

Community Composition of Three Groups in Genus Level and Kruskal-Wallis Test

The dominant bacteria of intestinal flora in the three groups were different. In Group AGE1, the first five were *Faecalibacterium*, *Blautia*, *Subdoligranulum*, *Eubacterium* and *Bifidobacterium*. The first five in Group AGE2 were *Faecalibacterium*, *Eubacterium*, *Blautia*, *Bifidobacterium* and *Eubacterium*. The first five dominant bacteria were *Blautia*, *Subdoligranulum*, *Faecalibacterium*, *Eubacterium* and *Dialister*. Different bacteria has different relative abundance (Table III and Figure 7).

It was indicated by Kruskal-Wallis Test (Figure 8) that *Bifidobacterium*, *Escherichia-shigella*, *Eubacterium* and *Subdoligranulum* were the bacteria that had significant differences in genus level.

Discussion

Adult human intestines are colonized by large and complex microbiota, which have each vital

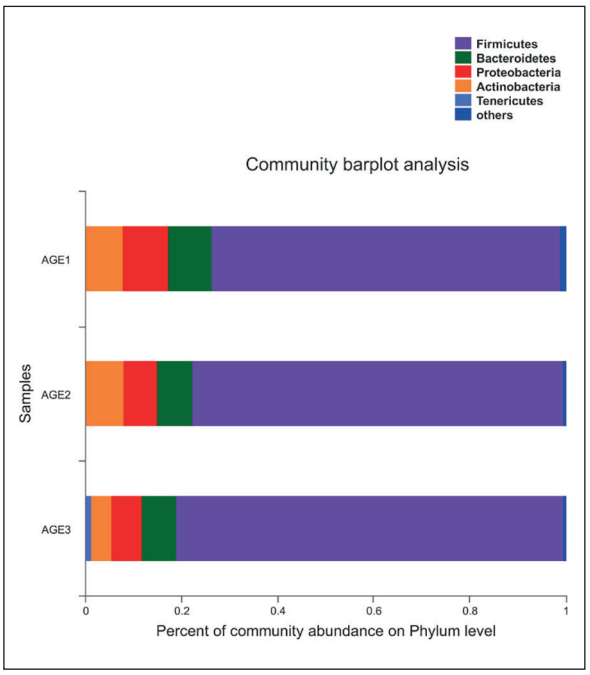

Figure 5. Column diagram of relative abundance of three groups of phylum level. The ordinate is the sample name, and the abscissa is the proportion of the species in the sample. The pillars of different colors represent different species, and the length of the pillars represents the proportion of the species.

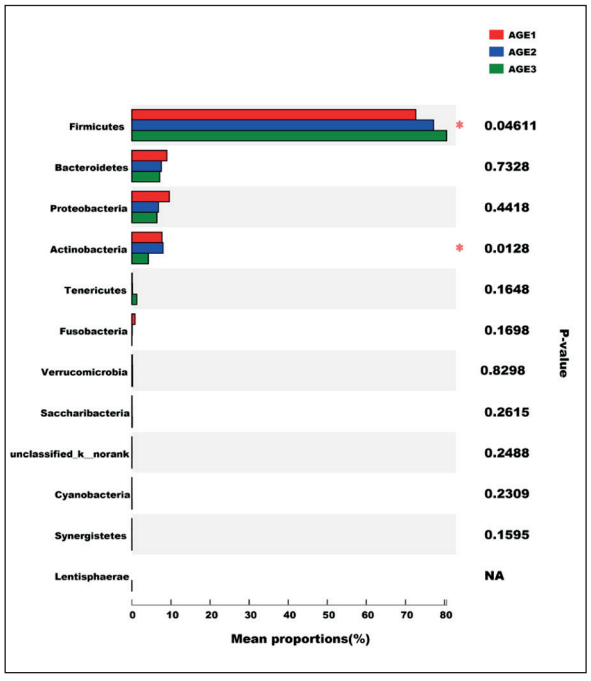

Figure 6. Kruskal-Wallis Test of three groups in phylum level. The ordinate is the group's name in phylum level. The corresponding length of the column is the average relative abundance of that species in each specimen group. Different colors indicated different grouping. The rightmost side was p value. * $0.01 < p \leq 0.05$, ** $0.001 < p \leq 0.01$, *** $p \leq 0.001$.

Table III. Analysis of the proportion of three specimens in genus level.

Group	*Faecalibacterium*	*Blautia*	*Subdoligranulum*	*Eubacterium*	*Bifidobacterium*
AGE1	12.227%	8.24%	6.85%	5.84%	5.47%
AGE2	12.18%	7.43%	5.25%	7.95%	5.86%
AGE3	10.29%	10.52%	10.44%	3.97%	1.87%

effects in the host system[26-30]. The host's genetic heritage, healthy condition, age, gender, diet habit, living environment, and social background all have co-influence in the colonization ability of intestinal flora and diversity of its composition[31-34].

The subjects of the study were health adults with appropriate gender percentage and disperse in the same place of residence. It focused on how age affects intestinal flora. From the study result, it is indicated that as the adults' age increased, the abundance of intestinal flora decreased and the diversity index among each groups had significant difference. The PLS-DA analysis result also presented that the dispersion degree of the specimen point distribution was higher in Group AGE3 (40-49 years old) than Group AGE1 and Group AGE2 and it had significant difference. This is consistent with the literature[35]. The diversity of intestinal flora and relative abundance change as age increases. The diversity of elder's intestinal flora is higher than adults. Mueller et al[36] concluded that the structure and features of the intestinal flora relate to age. According to the classification criteria of the World Health Organization (WHO), the subjects from Group AGE3 are not old people, but the changes of the abundance and diversity of intestinal flora were significant and it even tended to the changes of the old.

From the community composition in phylum level of three groups and Kruskal-Wallis test, we found that the *Firmicutes* and *Actinobacteria* had significant differences in three groups. *Firmicutes* and *Bacteroidetes* were the dominant bacteria[37] that had the most intestinal flora. That is the reason why the ratio of *Firmicutes* and *Actinobacteria* (F/B) can reflect the changes of the bacteria community in certain degree. Some research results revealed that the higher

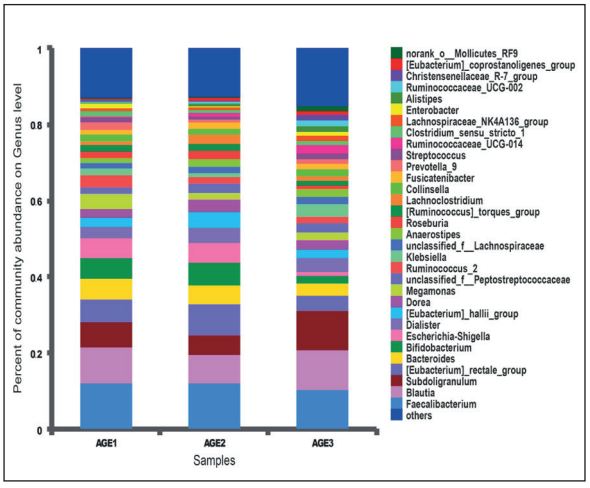

Figure 7. Column diagram of relative abundance of three groups of genus level. The abscissa is the sample name, and the ordinate is the proportion of the species in the sample. The pillars of different colors represent different species, and the length of the pillars represents the proportion of the species.

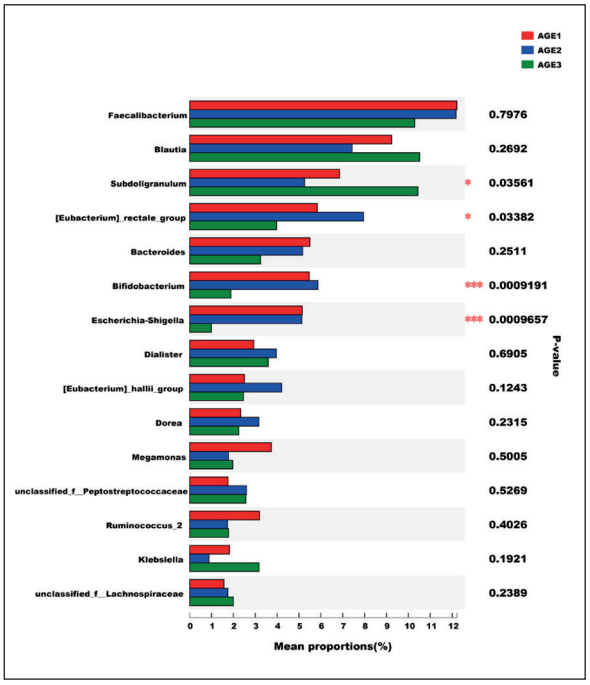

Figure 8. Kruskal-Wallis Test of three groups in genus level. The ordinate is the group's name in genus level. The corresponding length of the column is the average relative abundance of that species in each specimen group. Different colors indicated different grouping. The rightmost side was p-value. * $0.01 < p \leq 0.05$, ** $0.001 < p \leq 0.01$, *** $p \leq 0.001$.

glucose intolerance, the less ratio of F/B. From our research result, it indicated that it is tended to have more *Firmicutes* and less *Actinobacteria* as the age increases. The change regularity of F/B ratio is in accordance with it. Among the three groups of healthy subjects, the ratio of F/B increased as the age increased. It indicated that as the age grows, the glucose tolerance may decrease and diabetes may process in some degree. The research conclusion about *Firmicutes* and *Actinobacteria* in the elder people's intestinal flora from other countries were different[19,39-42]. The clostridium cluster XIVa in *Firmicutes* of German increased while Italians, Finns, and Japanese decreased. The German and Finns's *Actinobacteri*a increased while the Italians and North Europeans decreased. Regional, living habits, and a few factors may influence the intestinal flora. *Actinobacteria*, a vital source of natural medicine, can produce rich bioactive substance. Its metabolic substances have the features, such as antibacterial, antitumor and antiviral activities. The result in the study also indicated that *Actinobacteria* in Group AGE3 decreased significantly with less antibacterial, antitumor, and antiviral activities. This reflected the effect of age to intestinal flora.

From the community composition in phylum level of three groups and Kruskal-Wallis test, we found that the *Bifidobacterium*, *Escherichia-shigella*, *Eubacterium* and *Subdoligranulum* were the bacteria that had significant differences in genus level. Some researches revealed that *Bifidobacterium* relates closely to human health. To keep *Bifidobacterium* stable or increased is an important sign to stay healthy. Some researchers[43] found that increased *Bifidobacterium* can alleviate the cognitive impairment among Alzheimer's patients. *Escherichia-shigella* is a compound known to produce a very important anti-inflammatory and anti-tumor factor, butyrate, which plays a key protective role in inflammation[44-46]. The reduced abundance of the bacteria has negative correlation with the pro-inflammatory molecules in our specimens, which indicated its increased sensitivity to inflammatory processes. Butyrate regulates the beneficial bacteria of human intestinal microecological balance, which have important anti-inflammatory and anti-tumor effects. This is consistent with recent evidence demonstrating that increased rectal abundance of *Eubacterium* is associated with less inflammation. The increased *Eubacterium* also foreshows that the response to anti-TNF-α treatment in patients with inflammatory bowel diseases is positive. The content of *Subdoligranulum* in Group AGE3 is remarkably higher than in Group AGE1 and Group AGE 2. Some researches revealed that *Subdoligranulum* relates to poor metabolic control and chronic inflammation. The changes of its abundance may lead to the disorder of the host metabolism. *Escherichia-shigella* is a conditional pathogen and is associated with a pro-inflammatory state[47]. The abundance of proinflammatory *Escherichia/shigella* was higher in the stools of subjects with cognitive impairment and cerebral amyloidosis. The high abundance of *Escherichia-shigella* shows that young people aged 20-40 are susceptible to digestive diseases such as diarrhea.

Conclusions

In summary, with the increase of age, the abundance of intestinal flora of healthy adults over 40 years of age decreases, and the diversity and the degree of specimens' dispersion are significantly different from those of 20-40 years old. The decrease in the ratio of *Firmicutes* and *Actinobacteria* indicates that glucose tolerance may decrease with age; the beneficial bacteria *Bifidobacterium* and *Eubacterium* are significantly lower than those of 20-40 years old, and the decrease in *Bifidobacterium* may increase the incidence There is a risk of cognitive impairment and a reduction in *Eubacterium* may reduce the body's anti-inflammatory and anti-tumor effects. Subdoligranulum is associated with poor metabolic control and chronic inflammation, and it is significantly increased compared with young people aged 20-40. Buford[48] shows that the gut microbiome may represent a new type of intervention site for the prevention and/or treatment of a disease. Therefore, effective intervention against the intestinal flora can play a role in delaying aging and preventing diseases.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding Acknowledgments

Research and Innovation Team of the Prevention and Health Care Center of the Shandong University of Traditional Chinese Medicine.

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