Research Article

Comparison of Gut Microbiota between Sasang Constitutions

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The Sasang constitutional medicine has long been applied to diagnose and treat patients with various diseases. Studies have been conducted for establishment of scientific evidence supporting Sasang Constitutional (SC) diagnosis. Recent human microbiome studies have demonstrated individual variations of gut microbiota which can be dependent on lifestyle and health conditions. We hypothesized that gut microbial similarities and discrepancies may exist across SC types. We compared the difference of gut microbiota among three constitutions (So-Yang, So-Eum, and Tae-Eum), along with the investigation of anthropometric and biochemical parameters. Firmicutes and Bacteroidetes were predominant phyla in all SC types. The median plot analysis suggested that Firmicutes and Bacteroidetes appeared more abundant in SE and TE, respectively, in the male subjects of 20–29 years old. At the genus level, Bifidobacterium and Bacteroides manifested the difference between SE and TE types. For anthropometry, body weight, body mass index, and waist circumference of the TE type were significantly higher than those of the other types. Overall, findings indicated a possible link between SC types and gut microbiota within a narrow age range. Further investigations are deemed necessary to elucidate the influences of age, gender, and other factors in the context of SC types and gut microbiota.

1. Introduction

Individualized treatment according to the patients’ “syndrome” pattern is one of the characteristics of traditional oriental medicine in the East Asian countries. The Sasang constitutional medicine, a holistic approach based on the unique constitutional typology, has long been applied to diagnose and treat patient with various diseases. Characteristics pertaining one’s physical, psychosomatic, and emotional aspects are integrated in the determination of Sasang constitutional (SC) types. The SCM deals with biological and psychological traits in individuals and explains individual differences in behavioral tendencies, physical characteristics, and varying levels of vulnerability to disease and responsiveness to environmental stimuli. Diagnosis under the SC typology divides human beings into four categories (Tae-Yang, Tae-Eum, So-Yang, and So-Eum) according to their inherited traits, including appearance, physiology, susceptibility to disease, and personality. Traditional Korean medical doctors classify patients using SC for diagnosis and treatment in clinics. However, like other forms of complementary and alternative medical systems worldwide, the identification of SC has been subjective and fallible because of lack of standardization and scientific evidence for a long time [1]. Therefore, with development of new biomedical technologies, various attempts at scientific identification of SC have been made. Several studies
2. Materials and Methods

2.1. Study Subjects. Subjects were recruited to Dongguk University Ilsan Hospital by advertisements in the local newspaper or by posters in the hospital with approval of the Institutional Review Board of Dongguk University Ilsan Hospital (approval no. 2012-SR-27). For qualification, subjects were normal weight (BMI < 25 kg/m²), male or female, between the ages of 19 and 65. They had been weight-stable within ±10% during the last six months. Any antibiotics, probiotics, or drugs that might have an impact on their weight were not used for the last three months. Subjects with weight-influencing diseases, including hyper/hypothyroidism, heart disease, psychogenic disease, or other chronic systemic disease, were excluded. Smokers or pregnant women confirmed by positive screening on the hCG test were also excluded. A total of 40 subjects were recruited (14 Tae-Eum, 13 So-Yang, and 13 So-Eum) for this study. Subjects underwent physical examinations, body composition analysis, anthropometry, blood test, and Sasang constitutional diagnosis.

2.2. General Characteristics of Subjects. Blood pressure and heart rate were measured using an automatic digital sphygmomanometer. Body weight and height were measured using an automatic scale (G-tech, Uijeongbu, Korea), wearing a hospital gown (the nearest 0.1 kg and 0.5 cm, resp.). Body mass index was calculated by weight in kilograms and divided by height in meters squared. Waist circumference was measured three times according to the WHO instruction [21]. Body composition was measured using the bioelectrical impedance analysis method (InBody 3.0, Biospace, Seoul).
This device measures impedance through eight tactile electrodes placed on palms, thumbs, heels, and soles. Each subject stood upright stepping on the foot electrodes and loosely gripping the pipe-shaped hand electrodes with arms held vertically. Values for lean body mass, fat mass, fat percentage, and waist-to-hip ratio were obtained using the device mentioned above. Blood tests, including fasting glucose, HDL-cholesterol, triglyceride, total cholesterol, and AST/ALT, were performed using the Cobas 8000 modular analyzer (Roche, Branford, CT, USA).

2.3. Statistical Analyses. Continuous data are expressed as mean ± standard deviation (SD) and categorical data were described by frequency. Statistical calculations were performed using a statistical analysis package (SAS, version 9.3; SAS Institute; Cary, NC, USA). One-way analysis of variance (ANOVA) was performed for determination of the significance of the difference at the baseline of each constitution. Tukey’s HSD test was used as a post hoc analysis. For the ANOVA method, the data were considered that there were four basic assumptions: the expected values of
kit (MP Biomedicals, Santa Ana, CA, USA), and the 16S rRNA gene (V1–V3 regions) was amplified from extracted DNA. Amplifications were performed as in previous reports using a barcoded fusion primer [22, 23] (http://oklb.ezbiocloud.net/content/1001), using a C1000 Touch thermal cycler (Bio-Rad, Hercules, CA, USA). The amplified products were confirmed by 2% agarose gel electrophoresis and visualized using the Gel Doc system (Bio-Rad). Amplicons were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and quantified using a PicoGreen dsDNA Assay kit (Invitrogen, Carlsbad, CA, USA). Equimolar concentrations of each amplicon from different samples were pooled and purified using an AMPure bead kit (Agencourt Bioscience, Beverly, MA, USA) and then amplified on sequencing beads by emulsion PCR. Sequencing reactions were performed using a Roche/454 GS Junior system according to the manufacturer’s instructions.

2.6. Sequence Data Analysis. The analysis of pyrosequence data was conducted according to previous descriptions [23, 24]. Briefly, raw data for each sample were sorted by a unique barcode in the demultiplexing step, and low quality reads (average quality score <25 or read length <300 bp) were removed for further analysis. Pairwise sequence alignment and the hmm-search program of the HMMER 3.0 package [25] were used for trimming of primer sequences based on the profile of the 16S RNA V1–V3 regions. For correction of sequencing errors, representative sequences in clusters of trimmed sequences were selected and considered for taxonomy identification. Individual reads were assigned their taxonomic positions according to the highest pairwise similarity among the top five BLASTN hits against the EzTaxon-e database [26]. Chimera sequences were removed with UCHIME [27]. The read numbers in each sample were normalized by random subsampling, and the diversity indices were calculated using the Mothur program [28]. Pyrosequencing reads obtained from this study are available in the EMBL SRA database under study number ERP002551 (http://www.ebi.ac.uk/ena/data/view/ERP002551).

3. Results and Discussion

3.1. General Characteristics of Subjects. Sasang constitution of 40 subjects was determined by the SCDS, and characteristics and clinical markers were compared among SC groups (Table 1). SE, SY, and TE accounted for 32.5%, 32.5%, and 35%, respectively, of the total subjects. Expectedly, the TY type was not included in our study. Individuals having the TY type have been reported to be rare in the south part of Korean peninsula. Ninety-two percent of SY group (n = 13) were female, and the average age of this group (42.6 years old) was higher than that of other SC groups (SE: 32.9 versus TE: 29.1 years old) (P < 0.05). The height of SY group was lower than that of other groups, which may be partly due to the higher ratio of female and older ages in this group. Systolic and diastolic blood pressure as well as fasting blood sugar were not significantly different across groups. Significant differences were observed for all of the anthropometric and
body composition associated variables: height, weight, waist circumference, BMI, fat percent, lean body mass, and fat percentage ($P < 0.05$). The height (159.8 cm) and lean body mass (21.3 kg) of SY group were significantly lower than those of the other groups ($P < 0.001$). The weight (67.5 kg), BMI (22.7), and waist circumference (850.1 mm) of TE group were significantly higher compared with the other groups ($P < 0.001$). Because a higher level of these parameters is often associated as risk factor of abdominal obesity, subjects in the TE group could be most vulnerable to obesity and metabolic syndrome [29]. The waist-hip ratio of SE group was significantly lower than that of the other groups ($P < 0.05$). Fat mass was the only variable that all three groups showed the difference of gut microbiota among constitutions. The richness of bacterial communities obtained from TE constitution was relatively higher than that of SY and SE. The slopes of rarefaction curves from bacterial communities of SY and SE varied and those of SE varied more than those of SY, which could indicate the difference of gut microbiota in three constitutions.

The average phyla compositions of bacterial communities from three constitutions were analyzed and compared (Figure 1). The average compositions of phyla were similar among constitutions; two phyla of Firmicutes (ranging from 53.2% to 59.4% of total reads) and Bacteroidetes (from 27.9% to 35.6%) predominated, followed by phylum of Actinobacteria (ranging from 6.8% to 8.0%). The proportion of Tenericutes was more than 3.6% in samples of SY and SE, whereas it is under 0.6% in samples of TE. The proportion of Verrucomicrobia in SE was (0.1%) lower than those of SY (1.0%) and TE (1.3%). Although the proportion of each phylum varied, the average compositions of predominant bacteria from three constitutions were similar. This could be because all of the tested subjects were healthy persons and the gut microbiota play a normal role in their body. However, the compositions of bacterial communities from individuals varied within constitutions (Supplementary Figure 1). The richness of bacterial communities obtained from TE constitution was relatively higher than that of SY and SE. The slopes of rarefaction curves from bacterial communities of SY and SE varied and those of SE varied more than those of SY, which could indicate the difference of gut microbiota in three constitutions.

3.2. Identification of Gut Microbiota in Subjects. A total of 99,622 sequences were obtained and analyzed from fecal samples of 40 subjects, which consisted of 14 Tae-Eum, 13 So-Yang, and 13 So-Eum constitutions; 32,518 sequences (452.9 ± 9.6 bp) obtained from TE subjects, 30,048 sequences (453.4 ± 9.3 bp) from SY, and 37,056 sequences (454.2 ± 7.1 bp) from SE were analyzed for comparison of gut microbiota. Calculated diversity indices after normalization of read sizes in each sample were compared (Table 2). The read numbers of TE, 12, 13, 14, SY, 1, 5, 13, and SE, 9 samples were less than those of normalized reads. However, the number of observed OTUs and Chao 1 was not influenced by a lower read number. For example, the number of observed OTUs of TE, 13 was 463 by 1,677 total reads, while that of the observed OTUs of TE, 9 was 138 by 2,000 normalized reads. This indicated that the statistical analyses of samples were not affected by read number of samples. The values of Good's coverage exceeded 0.77 in all samples. Estimated values in Table 2 varied within constitution type. Although averages of Shannon indices of three constitutions were similar, ranging from 3.66 to 3.87, the rarefaction curves showed the difference of gut microbiota among constitutions (Supplementary Figure 1). The richness of bacterial communities from bacterial communities of SY and SE varied and those of SE varied more than those of SY, which could indicate the difference of gut microbiota in three constitutions.

A table showing the proportion of genera in different constitutions is included. For example, the proportion of Blautia in SY is higher than in TE and SE. The proportion of Roseburia in SE is higher than in SY and TE. The proportion of Anaerostipes in TE is higher than in SY and SE. The proportion of Dorea in SY is higher than in TE and SE. The proportion of Eubacterium in SE is higher than in SY and TE. The proportion of Clostridium in SE is higher than in SY and TE. The proportion of Dialister in SE is higher than in SY and TE. The proportion of Bifidobacterium in SY is higher than in TE and SE. The proportion of Bacteroides in SY is higher than in TE and SE. The proportion of Prevotella in SY is higher than in TE and SE. The proportion of Alistipes in SY is higher than in TE and SE. The proportion of Faecalibacterium in SY is higher than in TE and SE. The proportion of Oscillibacter in SY is higher than in TE and SE. The proportion of Ruminococcus in SY is higher than in TE and SE. The proportion of Eum, 13 So-Yang, and 13 So-Eum constitutions; 32,518 sequences (452.9 ± 9.6 bp) obtained from TE subjects, 30,048 sequences (453.4 ± 9.3 bp) from SY, and 37,056 sequences (454.2 ± 7.1 bp) from SE were analyzed for comparison of gut microbiota. Calculated diversity indices after normalization of read sizes in each sample were compared (Table 2). The read numbers of TE, 12, 13, 14, SY, 1, 5, 13, and SE, 9 samples were less than those of normalized reads. However, the number of observed OTUs and Chao 1 was not influenced by a lower read number. For example, the number of observed OTUs of TE, 13 was 463 by 1,677 total reads, while that of the observed OTUs of TE, 9 was 138 by 2,000 normalized reads. This indicated that the statistical analyses of samples were not affected by read number of samples. The values of Good's coverage exceeded 0.77 in all samples. Estimated values in Table 2 varied within constitution type. Although averages of Shannon indices of three constitutions were similar, ranging from 3.66 to 3.87, the rarefaction curves showed the difference of gut microbiota among constitutions (Supplementary Figure 1). The richness of bacterial communities obtained from TE constitution was relatively higher than that of SY and SE. The slopes of rarefaction curves from bacterial communities of SY and SE varied and those of SE varied more than those of SY, which could indicate the difference of gut microbiota in three constitutions.

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![Table showing the proportion of genera in different constitutions](image-url)
3.4. Comparison of Gut Microbiota between SE and TE. For comparison of bacterial communities in the gut among constitutions without considerable variation factors, male samples in the 20-year age group (range from 20 to 29 years old) in SE (n = 8) and TE (n = 8) were selected and analyzed using median plots (Figure 4). Differences of gut microbiota between SE and TE became clearer when age and gender were adjusted for the analysis. The relative abundance of Firmicutes in SE (61.7% of median value) samples was higher than that in TE (42.1%), whereas the proportions of Bacteroidetes...
### Phylum Composition

<table>
<thead>
<tr>
<th>Phylum</th>
<th>So-Eum</th>
<th>Tae-Eum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Firmicutes</td>
<td>23.7%</td>
<td>17.1%</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>47.4%</td>
<td>34.2%</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>71.8%</td>
<td>30.8%</td>
</tr>
<tr>
<td>Tenericutes</td>
<td>94.9%</td>
<td>68.4%</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>11.6%</td>
<td>12.2%</td>
</tr>
<tr>
<td>Verrucomicrobia</td>
<td>0.0%</td>
<td>0.0%</td>
</tr>
</tbody>
</table>

### Genus Composition

<table>
<thead>
<tr>
<th>Genus</th>
<th>So-Eum</th>
<th>Tae-Eum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bifidobacterium</td>
<td>5.9%</td>
<td>13.1%</td>
</tr>
<tr>
<td>Bacteroides</td>
<td>3.1%</td>
<td>4.4%</td>
</tr>
<tr>
<td>Prevotella</td>
<td>11.8%</td>
<td>6.3%</td>
</tr>
<tr>
<td>Alistipes</td>
<td>4.7%</td>
<td>17.2%</td>
</tr>
<tr>
<td>Faecalibacterium</td>
<td>63.1%</td>
<td>52.3%</td>
</tr>
<tr>
<td>Oscillibacter</td>
<td>0.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>Subdoligranulum</td>
<td>4.9%</td>
<td>4.9%</td>
</tr>
<tr>
<td>Ruminococcus</td>
<td>0.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>GQ897654</td>
<td>0.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>Blautia</td>
<td>0.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>Roseburia</td>
<td>0.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>Dorea</td>
<td>0.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>Eubacterium</td>
<td>0.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>Clostridium</td>
<td>0.0%</td>
<td>0.0%</td>
</tr>
</tbody>
</table>

Figure 4: Bacterial communities obtained from the 20-year male group of So-Eum and Tae-Eum were compared using median plots. (a) Differences of phyla composition were compared between two constitutions. (b) Detailed differences of genera composition were analyzed in two constitutions. The identified genus name of GQ897654 indicates that these sequences have the highest similarity to uncultured bacteria GQ897654 (GenBank accession number).

(44.4%) and Actinobacteria (5.6%) were higher in TE than in SE (29.2% of Bacteroidetes and 3.7% of Actinobacteria) samples. Detailed differences were observed at the genus level. Proportions of Bifidobacterium within Actinobacteria were higher in TE, compared with SE samples. Ratios of Bacteroides within Bacteroidetes showed similar median values in both constitutions, while the relative abundance of Prevotella and Alistipes was different in SE and TE samples. The median values of Faecalibacterium and Ruminococcus within Firmicutes were the most different genera between two constitutions. Other genera within Firmicutes are shown in Figure 4.

The difference of bacterial communities between SE and TE was also investigated in PCoA plots based on UniFrac distance (Figure 5). The communities of SE and TE were distributed and mixed without consideration for gender and age (Figure 5(a)). This result was consistent with difference of average bacterial community (Figure 2 and Supplementary Figure 2). Bacterial communities originating from male samples of similar age (ranging from 20 to 29 years old) in SE and TE groups showed distinguished pattern according to their constitution (Figure 5(b)), consistent with the median plot analysis shown in Figure 4. This result indicates that gender and age were considerable factors in comparison of gut bacterial communities between SC groups. In general, SE constitution was known to have a tendency of dyspepsia and diarrhea due to a weak gastrointestinal system and “cold” nature, whereas few gastrointestinal problems are associated with TE constitution due to the relatively strong digestion and adsorption function in traditional medicine [36]. These differences could be related to the different microbial communities in the gut and their different functions.
4. Conclusion

In this study, we analyzed the gut microbiota in three SC types (SY, SE, and TE) using 16S rRNA gene-based pyrosequencing as a pilot study. Individual variations of gut microbiota in each SC as well as gut microbial variation across SC were investigated. Firmicutes and Bacteroidetes were the common, predominant phyla followed by Actinobacteria in all SC types. The median plot analysis suggested that Firmicutes and Bacteroidetes may be more abundant in SE and TE, respectively, in the male subjects of 20–29 years old. At the genus level, Bifidobacterium and Bacteroides manifested the difference between SE and TE types. For anthropometry, body weight, body mass index, and waist circumference of the TE type were significantly higher than those of the other types. Although we could not confirm the associations between gut microbial variation with SC types in conjunction with age and gender thoroughly, the gut microbiota could be used one of the scientific factors in deciding SC type in traditional Korean medicine. Findings in this study should be investigated in depth with a larger sample size to increase our understanding for the association between gut microbial variation and SC types.

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References

Evidence-Based Complementary and Alternative Medicine


