Gut microbiota differences in elderly subjects between rural city Kyotango and urban city Kyoto: an age-gender-matched study

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Several outcomes have been reported on the role of gut microbiota in health promotion and disease prevention. Kyoto, one of the longevity areas with various centenarians, is a provincial city located in the northern part of Kyoto Prefecture in Japan. To understand the relationship between gut microbiota and urbanization, we compared the diversity, abundance, and function of gut microbiota in older healthy subjects between Kyotango and Kyoto cities; Kyoto is an urban city located in the southern part of Kyoto Prefecture. In total, 51 subjects at Kyotango and 51 subjects at Kyoto matched by age and gender were recruited, and their fecal samples were obtained to analyze the gut microbiota using 16S rRNA gene sequencing. Principal coordinate analysis for β-diversity revealed significant differences in the gut microbiota between two cities. In contrast, the analysis of α-diversity revealed no significant differences between the groups. On comparison at the phylum levels, the abundance of Firmicutes was decreased with the urbanization, whereas that of Proteobacteria and Bacteroidetes increased. On comparison at the genus levels, with urbanization, a significant decrease was observed in Lachnospiraceae families including genus Roseburia and Coprococcus, and significant increases was observed in Bacteroides, Oscillibacter, Parabacteroides, and Ruminococcus. The most markedly increased functional pathway with urbanization was lipopolysaccharide biosynthesis proteins and lipopolysaccharide biosynthesis, and decreased pathway was transporters and ABC transporters. In conclusion, the present findings indicate significant differences in the gut microbiota between the provincial city and urban cities at Kyoto Prefecture. These alterations in the microbiota may provide new insights to consider the relationship between longevity and gut microbiota.

Key Words: gut microbiota, 16S rRNA, Firmicutes, Bacteroidetes, Lachnospiraceae

In the twenty-first century, when the elderly people are increasing due to better medical and therapeutic facilities, it is becoming an important subject to lengthen the period of healthy, self-reliant and long-lived life (healthy longevity) as long as possible. Among the numerous pathological changes that emerge with age, the most important ones include degeneration of blood vessels and nerves. Relating to a common proverb “people grow old with blood vessels,” the pathological and functional changes in the blood vessels due to arteriosclerosis affect the blood pressure and microcirculation, causing various subsequent dis-eases and physiological abnormalities. Changes in the cranial nerves and peripheral nerves affect not only brain diseases such as dementia, but also motor function. The environmental factors are gaining immense attention in influencing such age-related change. A recent community-based prospective cohort has demonstrated that inflammation essentially triggers aging up to extreme old age in humans. Various factors lead to the inflammatory response in the host; however, in recent years, several studies have demonstrated that the gut microbiota and its metabolites are crucial in the promotion as well as the inhibition of inflammation, in particular in rodents.

In a study on gut microbiota and lifespan, Wang et al. reported that Roseburia and Escherichia were significantly abundant, whereas Lactobacillus, Faecalibacterium, Parabacteroides, Butyricimonas, Coprococcus, Megamonas, Mitsuokella, Sutterella and Akkermansia were less observed in centenarians at the genus level, in a Chinese centenarians. Nevertheless, Nishijima et al. reported that the gut microbiota of the Japanese is a characteristic bacterial flora, diverse from other countries worldwide, including China, indicating that it is necessary to assess the Japanese gut microbiota in detail and to clarify their correlation with lifestyle and various diseases in a study aimed at Japanese healthy longevity.

The average life expectancy of Japanese people has been extended to 81.08 years old for males and 87.28 years for females, which indicated a high record, in the public report at the end of 2018. While comparing 47 prefectures, the average life expectancy of Kyoto prefecture was found to be 81.40 years (ranks third place in Japan) for men and 87.35 years (ranks ninth place in Japan) for females, and is regarded as a long-lived province. Kyotango city, one of the longevity areas with various centenarians, is a provincial city located in the northern part of Kyoto Prefecture; whereas, Kyoto city is an urban city located in the southern part of Kyoto Prefecture. Provincial area Tango including Kyotango and Kyoto are almost similar in size (844.5 km² and 860.7 km², respectively); however, huge difference is observed in the proportion of centenarians (Supplemental Fig. 1*). The number of centenarians per 100,000 population is 48 people nationwide, 73 in Kyoto city, and 133 in the Tango area. Percentage of centenarians in Tango is about thrice the national average, one of the longevity areas in Japan. By comparing these two areas, we may presumably observe several factors related with

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longevity and health promotion. In collaboration with a cohort study targeting residents of Kyotango city by Department of Longevity and Regional Epidemiology, Kyoto Prefectural University of Medicine, which commenced in 2015, we conducted a comparative study in gut microbiota of healthy subjects aged above 65 years between Kyotango and Kyoto by analyzing the bacterial 16S ribosomal RNA (16S rRNA) gene pyrosequencing method.

Materials and Methods

Ethics statements. This study conformed to the code of ethics stated in the Declaration of Helsinki. The research protocol was approved by the Ethics Committee of Kyoto Prefectural University of Medicine (permission No. ERB-C-534, ERB-C-885) and written informed consent was provided by all participants prior to enrollment. The study was registered at the University Hospital Medical Information Network Center (UMIN R000041350).

Study population and data collection. We collected fecal samples from a cohort study targeting residents of Kyotango city by Department of Longevity and Regional Epidemiology, Kyoto Prefectural University of Medicine and a cohort study targeting residents of Kyoto city by Department of Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine under the approval of the Ethics Committee of Kyoto Prefectural University of Medicine. From these subjects, 51 subjects were selected from the Kyotango cohort and 51 healthy subjects from the Kyoto cohort from November 2016 to December 2017. About 51 Kyotango subjects and 51 Kyoto subjects were matched by age and gender and their details are listed in Table 1. Volunteers did not reveal any evidence of significant gastrointestinal inflammatory disease such as inflammatory bowel disease or functional gastrointestinal disorders such as irritable bowel syndrome. Additional exclusion criteria included the administration of antibiotics, corticosteroids, and immunosuppressants within the past 3 months and a history of underlying malignant disease. In addition, the patients with serious metabolic, respiratory, cardiological, renal, hepatic, hematologic, neurologic, or psychiatric functions and who regularly used medications affecting intestinal motility such as laxatives, antidepressants, opioid narcotic analgesics, anti-cholinergic, and prebiotic or probiotics were excluded. Patients with other factors that could affect the intestinal motility or gut microbiota, as evaluated by researchers, were also ineligible.

Sample collection and DNA extraction. Fecal samples were collected and the gut bacterial composition was analyzed. Genomic DNA was isolated using the NucleoSpin Microbial DNA Kit (MACHEREY-NAGEL, Düren, Germany). Approxi-

mately 500 μl of the stored fecal sample was placed in a micro centrifuge tube containing 100 μl of Elution Buffer BE. The mixture was then placed into a NucleoSpin Beads Tube with proteinase K and was subjected to beating with mechanical beads for 12 min at 30 Hz in the Tissuemizer LT. The subsequent extraction procedure was performed as per the manufacturer’s instructions. Extracted DNA samples were purified using the Agencourt AMPure XP (Beckman Coulter, Brea, CA).

Sequencing of 16S rRNA gene. Two-step polymerase chain reactions (PCRs) were performed for the purified DNA samples to obtain sequence libraries. The first PCR was performed to amplify and used a 16S (V3–V4) Metagenomic Library Construction Kit for NGS (Takara Bio Inc., Kusatsu, Japan) with primer pairs 341F(5′-TCGTCGCGACGTCAAGATTAAAGACAGCCTACGGNNGGCWGCAG-3′) and 806R(5′-GCTCTCCTGCGGCTCAGATTGTATAAGAGACAGGTGTCTAAAT-3′) corresponding to the V3–V4 region of the 16S rRNA gene. The second PCR was performed to add the index sequences for the Illumina sequencer with a barcode sequence using the Nextera XT Index kit (Illumina, San Diego, CA). The prepared libraries were subjected to sequencing of 250 paired-end bases using the MiSeq Reagent v3 kit and the MiSeq (Illumina) at the Biomedical Center at Takara Bio.

Microbiome analysis. The processing of sequence data, including chimera check, operational taxonomic unit (OTU) definition, and taxonomy assignment, was performed using QIIME ver. 1.9,(9) USEARCH ver. 8.0,(9) and UCHIME ver. 4.2.40(10) according to the work of Inoue et al.(10) Singletons were removed in this study. Taxonomy assignment of the resulting OTU was completed using an RDP classifier ver. 2.10.2 and the Greengenes database (published May 2013). Statistical differences (p<0.05) in the relative abundance of bacterial phyla and genera between groups were evaluated using Welch’s unpaired t test.

The observed Chao1 and Shannon phylogenetic diversity indices were calculated by the R “phyloseq” package and were statistically analyzed using a Wilcoxon rank-sum test. The β-diversity was estimated using the UniFrac metric to calculate the distances between the samples and was visualized by principal coordinate analysis (PCoa); it was statistically examined using permutational multivariate analysis of variance (PERMANOVA). The final figures were generated using R “phyloseq” package.

Potential changes in the microbiome at the functional level were evaluated using PICRUSt software(11) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, release 70.0,(12) The human-specific pathways were removed from the results to focus on true bacterial pathways. The PICRUSt software uses 16S rRNA sequence profiles to estimate metagenome content based on reference bacterial genomes and the KEGG pathway database. The result was further statistically analyzed by Welch’s unpaired t test using the stamp software.(13)

Table 1. Baseline characteristics of enrolled subjects

| Number of subjects | Kyotango 51 | Kyoto 51 |
|--------------------|-------------|----------|
| Age                | Male Female | Male Female |
| 65–69              | 4 7         | 4 7       |
| 70–74              | 8 11        | 10 10     |
| 75–79              | 6 9         | 4 11      |
| 80–                | 2 4         | 2 3       |
| Total              | 20 31       | 20 31     |

| Incidence of life-style related diseases | Kyotango 51 | Kyoto 51 |
|-----------------------------------------|-------------|----------|
| Hypertension (%)                        | 15 (29.4)   | 20 (39.2) |
| Hypercholesterolemia (%)                | 14 (27.5)   | 18 (35.3) |
| Diabetes mellitus (%)                   | 4 (7.8)     | 11 (21.6) |
The taxonomic changes in the microbial community were evaluated at the genus level. As presented in Fig. 3 and Table 2, the microbial changes revealed a significant decrease in the abundance of five genera and significant increase in six genera in the Kyoto group compared to the Kyotango group. These differences were characterized by an increase in the abundance of the genera *Roseburia* (p<0.01), *Coprococcus* (p<0.01), *Unclassified Lachnospiraceae* (p<0.01), *Lachnospira* (p<0.01), *Unclassified Erysipelotrichaceae* (p<0.05), and *Unclassified Peptococcaceae* (p<0.01), and by a decrease in the abundance of the genera *Bacteroides* (p<0.01), *Oscillospira* (p<0.05), *Parabacteroides* (p<0.01), *Ruminococcus* (p<0.05), and *Anaerotruncus* (p<0.05).

**Discussion**

In the present study, we used fecal DNA samples obtained from provincial Kyoto and Kyotango cities and performed an age-gender-matched study using data obtained from the sequencing of 16S rRNA gene. We described the differences of the relative abundance of gut microbiota between the Kyotango and Kyoto groups. Initially, using the unweighted and weighted UniFrac distance, we compared the overall microbial structure between these two groups. Importantly, as presented in Fig. 1, the unweighted and weighted PCoA indicated significant structural differences between these two groups. Thus, an obvious shift of the microbial community was observed. α-Diversity indices between the two groups revealed no significant differences, though there appears to be higher distribution of diversity within the Kyotango group compared with the Kyoto group subjects. Previous studies have reported that urbanization in developing countries has been linked to changes in the gut microbiota, including decreased diversity and altered composition. The differences in the gut microbial structure were first taxonomically evaluated at the phylum level (Fig. 2A). In agreement with the previous results, the microbiota composition included four predominant phyla (Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria) in both groups. The abundance of *Ruminococcus* was significantly increased in Kyoto city, whereas the relative abundance of *Roseburia* was significantly decreased in Kyotango city compared with the Kyoto group.

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Fig. 2. Comparative analyses of the taxonomic composition of the microbial community at the phylum level. (A) Each component of the cumulative bar chart indicates a phylum. (B, C) The representative phyla (Firmicutes and Bacteroidetes) were evaluated between the Kyotango and Kyoto subjects using Student’s unpaired t tests.

Fig. 3. Comparative analyses of the taxonomic composition of the microbial community at the genus level. The significant differences in genera between the Kyotango and Kyoto subjects were presented.
countries has been associated with an increasing incidence of several diseases, including obesity, diabetes mellitus, and inflammatory bowel disease.\textsuperscript{[15,16]} The different patterns of the gut microbiota composition in rural and urban areas offer an opportunity to understand the contribution of a “rural beneficial microbiome” in potentially protecting against the development of inflammatory and metabolic diseases. Most of the data assessing the role of urbanization on the gut microbiome have been derived from comparative studies of the microbiome in rural and urban areas in healthy individuals. Children from a rural Africa village of Burkina Faso exhibited a significant enrichment of Bacteroidetes and a depletion of Firmicutes compared with the children from the urban area of Florence, Italy, with a unique abundance of bacteria from the genera \textit{Prevotella} and \textit{Xylanibacter}, known to contain a set of bacterial genes for cellulose and xylan hydrolysis, completely lacking in the European children.\textsuperscript{[17]} In Korea, Park \textit{et al.}\textsuperscript{[18]} demonstrated that the ratio of Firmicutes to Bacteroidetes in the gut microbiota was greater in the urbanized town community adults than in the longevity village community adults due to an increase of Firmicutes and a reduction of Bacteroidetes.

In contrast, one of the most evident results in the present study was an increase in the Firmicutes phylum and a decline in the Bacteroidetes and Proteobacteria phyla in provincial Kyotango compared to urban Kyoto. In contrast to the previous report from Korea,\textsuperscript{[18]} Kim \textit{et al.}\textsuperscript{[19]} reported that the abundances in Firmicutes phylum were higher in subjects of longevity villages than in urbanized towns, whereas the proportions of Bacteroidetes were higher in urbanized towns, which is in accordance with our results. Furthermore, the abundances of six genera were significantly greater in Kyotango compared to Kyoto, and the top four of abundances (\textit{g} Roseburia, \textit{g} Coprococcus, \textit{g} Unclassified \textit{f} Lachnospiraceae, and \textit{g} Lachnospira) all belong to the Firmicutes class Clostridia family Lachnospiraceae. Most of these include the butyrate-producing and oxygen-sensitive anaerobes belonging to the Clostridial clusters XIVa. Recent studies have reported that Clostridial clusters XIVa derived from human feces have the potential to induce Foxp3\textsuperscript{+} regulatory T cells and are able to suppress the inflammatory conditions such as inflammatory bowel disease via production of butyrate, a short chain fatty acid.\textsuperscript{[20,21]} In particular, gut \textit{Roseburia} spp. are part of commensal bacteria producing short-chain fatty acids, in particular butyrate, affecting the colonic motility, immunity maintenance, and anti-inflammatory properties. It has been reported that the abundance of \textit{Roseburia} genus was less in several diseases (ulcerative colitis, type 2 diabetes, and neuronal diseases), and that this bacterium could serve as probiotics for restoration of beneficial flora.\textsuperscript{[22]}

Patterson \textit{et al.}\textsuperscript{[23]} have demonstrated that mono-association of mice with \textit{Roseburia hominis} bacteria results in specific bidirectional gene expression patterns. A set of genes thought to be important for host colonization are induced in \textit{Roseburia hominis}, while the host cells respond by strengthening the gut barrier function and enhancing regulatory T cell population expansion, possibly via TLR5-flagellin signaling. The anti-inflammatory action mediated by the butyric acid produced by such Lachnospiraceae family, in particular, \textit{Roseburia} genus, is known to be strongly involved in health promotion and disease prevention of residents in the Kyotango area. In future, studies exploring the cause of high abundance of Clostridia species are warranted, and it is necessary to investigate the genetic and environmental factors, diet, and so on.

Another important finding in this study is that urbanization tended to increase the abundance of Bacteroidetes and Proteobacteria phyla, and significantly increased the abundance of \textit{Parabacteroides} and \textit{Bacteroides}, both belonging to the Bacteroidetes phylum, at the genus levels in elderly people of urban Kyoto. Several studies have confirmed that fat intake tended to increase with urbanization as well as correlated positively with the Firmicutes-to-Bacteroidetes ratio.\textsuperscript{[24]} The Firmicutes-to-Bacteroidetes ratio was previously reported as a good marker for predicting obesity,\textsuperscript{[25]} however, our recent data revealed no association between this ratio and body mass index at least in healthy Japanese subjects.\textsuperscript{[14]} In addition, the significant enriched abundance of \textit{Bacteroides} in the urban Kyoto was in accordance to the previous reports, in which \textit{Bacteroides} increased with the degree of urbanization.\textsuperscript{[26-28]} A recent human study also confirmed that a high-fat diet (fat 40% energy) increased \textit{Alistipes} (\textit{p} = 0.04) and \textit{Bacteroides} (\textit{p} < 0.001), belonging to Bacteroidetes phylum, and decreased \textit{Faecalibacterium} (\textit{p} = 0.04) in a 6-month randomized controlled-feeding trial using 217 healthy young adults.\textsuperscript{[29]} These data including ours suggest that the effects of various markers of urbanization including changes in diet, in particular the increase in high-fat diet, on the gut microbiome, should be further explored.

In this study, the abundance of Actinobacteria phylum, mainly \textit{Bifidobacterium} genus, was about 10% in the both groups, indicating no difference. According to Benno \textit{et al.},\textsuperscript{[29]} \textit{Bifidobacterium} was increased in the rural area Yuzurihara compared to urban Tokyo; however, these results were not in accordance with those of the present study.

One of the major limitation of this study was that the rural and urban areas were enrolled from a limited area Kyoto Prefecture in Japan. Therefore, the results might reflect only a limited area of Japan. In addition, the Kyotango and Kyoto groups in the present study had small sample size. An analysis of gut microbiota with larger samples is necessary in future.

In conclusion, this study demonstrated that several compositional changes in the gut microbiota are associated with urbanization. Noticeably, in the present study, an increase was observed in the Clostridial clusters XIVa, that is, butyrate-producing bacteria, at the rural Kyotango city, a long-lived province with various centenarians, compared to the urban Kyoto city.

Table 2. The relative abundance of gut microbiota in the Kyotango and Kyoto subjects

| Kingdom     | Phylum     | Class   | Order      | Family          | Genus          | Kyotango | Kyoto | p values |
|-------------|------------|---------|------------|-----------------|----------------|----------|-------|----------|
| k_Bacteria  | p_Firmicutes | c_Clostridia | o_Clostridales | f_Lachnospiraceae | \textit{g} Roseburia | 5.919 ± 4.813 | 3.316 ± 3.580 | 2.8E-03  |
| k_Bacteria  | p_Firmicutes | c_Clostridia | o_Clostridales | f_Lachnospiraceae | \textit{g} Coprococcus | 4.941 ± 3.060 | 2.691 ± 2.336 | 7.8E-05  |
| k_Bacteria  | p_Firmicutes | c_Clostridia | o_Clostridales | f_Lachnospiraceae | Unclassified | 6.532 ± 4.131 | 4.421 ± 2.978 | 4.3E-03  |
| k_Bacteria  | p_Firmicutes | c_Clostridia | o_Clostridales | f_Lachnospiraceae | \textit{g} Lachnospira | 1.763 ± 1.920 | 0.856 ± 1.377 | 7.9E-03  |
| k_Bacteria  | p_Firmicutes | c_Erysipelotrichi | o_Erysipelotrichales | f_Erysipelotrichaceae | Unclassified | 0.768 ± 0.903 | 0.435 ± 0.682 | 4.0E-02  |
| k_Bacteria  | p_Firmicutes | c_Clostridia | o_Clostridales | f_Ruminococcaceae | \textit{g} Aneautotrichunus | 0.015 ± 0.016 | 0.045 ± 0.088 | 2.5E-02  |
| k_Bacteria  | p_Firmicutes | c_Clostridia | o_Clostridales | f_Lachnospiraceae | \textit{g} Lachnospira | 0.066 ± 0.012 | 0.001 ± 0.003 | 6.4E-03  |
| k_Bacteria  | p_Firmicutes | c_Clostridia | o_Clostridales | f_Ruminococcaceae | \textit{g} Aneautotrichunus | 2.303 ± 1.742 | 3.073 ± 2.103 | 4.9E-02  |
| k_Bacteria  | p_Bacteroidetes | c_Bacteroidia | o_Bacteroidales | f_Porphyrmonadaceae | \textit{g} Parabacteroides | 0.932 ± 0.757 | 1.711 ± 1.846 | 7.5E-03  |
| k_Bacteria  | p_Bacteroidetes | c_Bacteroidia | o_Bacteroidales | f_Ruminococcaceae | \textit{g} Oscillospora | 1.968 ± 1.864 | 2.998 ± 2.849 | 3.5E-02  |
| k_Bacteria  | p_Bacteroidetes | c_Bacteroidia | o_Bacteroidales | f_Bacteroidaceae | \textit{g} Bacteroides | 9.004 ± 5.640 | 13.579 ± 8.541 | 2.2E-03  |

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Fig. 4. The relative abundance of functional pathways in gut microbiota between the Kyotango and Kyoto subjects. The KEGG database functional categories are presented with the displayed histograms (left panel: means) and p value determinations (right panel: 95% confidence intervals).
Author Contributions

YN, TT, SK, and KM conceived the experiments; YN, TT, SK, KM, ST, AA, NM, YO and SM collected feces; RI, YT and HO performed analyses of fecal bacteria; RI, SK, KM, and TA analyzed the data; and YN, TT, YI and SM edited the manuscript. All authors discussed the results and commented on the manuscript.

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Conflict of Interest

YN received scholarship fund from EA Pharma. Co. Ltd. and collaboration research fund from Fujifilm Medical Co., Ltd. and has been paid lecture fees by Janssen Pharma K.K., Mylan EPD Co., Takeda Pharma Co. Ltd., Mochida Pharm. Co. Ltd., EA Pharma Co. Ltd., Otsuka Pharma Co. Ltd., and Astellas Pharma Co. Ltd. TT received lecture fees by Mochida Pharm. Co. Ltd. and Mitsubishi Tanabe Pharma Co. The other authors have no conflicts of interest to declare.

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