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Correlations of Fecal Bacterial Communities with Age and Living Region for the Elderly Living in Bama, Guangxi, China

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Bama County (Guangxi, China) is famous for its longevous population. In this study, intestinal microflora of 17 healthy elderly subjects of different ages and from different regions (rural and urban) in Bama, were analyzed by denaturing gradient gel electrophoresis (DGGE). Significant effects of age and living region on the whole intestinal bacterial communities were observed by redundancy analysis (RDA). A total of 11 bacterial strains that were correlated with age and living region were identified using a t-value biplot combined with band sequencing. Four bacterial strains were correlated with both age and living region of the elderly in Bama. Two *Bacteroides* strains and one Ruminococcaceae strain were abundant in the rural, younger elderly; conversely, one *Desulfovibrio* strain was high in the urban, older elderly. Another Bacteroidetes strain was only correlated with the participant's age, and its abundance increased with the age of the elderly. The richness of one *Clostridium sordellii* strain, which was only correlated with the elderly living region, was high in the urban elderly. The study also found five other novel bacterial strains that were correlated with the age or living region of the elderly in Bama. These results expand our understanding of age- and region-effects on the intestinal microflora of the elderly and raise the possibility of developing probiotics originating from centenarians.

Keywords: intestinal microflora, elderly people, DGGE, redundancy analysis, age, living region

Human intestinal microflora play an important role in host health and disease and dynamic changes in the intestinal bacterial composition can be observed throughout a person's lifespan (Mitsuoka, 1992; O'Toole and Claesson, 2010). Previous studies suggested that a person's genotype, diet, age and living region can affect intestinal bacterial communities (Zoetendal *et al.*, 2001; Hayashi *et al.*, 2002; Mueller *et al.*, 2006; O'Toole and Claesson, 2010). In particular, age is associated with major changes in the human intestinal microflora (O'Toole and Claesson, 2010). Studies on the differences in intestinal microflora between adult and elderly subjects have yielded contradictory results, especially in the number of *Bifidobacterium*, *Bacteroides*, and *Ruminococcus* present (He *et al.*, 2003; Woodmansey, 2007; Zwielehner *et al.*, 2009; Mäkivuokko *et al.*, 2010). Regional effects on intestinal microflora were also observed in elderly Japanese and Europeans and the changes in microflora of one population were different from another (Mitsuoka, 1992; Mueller *et al.*, 2006). However, these results were based on the differences between adults and the elderly, and the intestinal microfloral variation of the elderly with different ages and from different living regions were not widely studied.

In some famous longevous regions, the effects of age or region on the elderly intestinal microflora were also observed. The elderly living in Japanese or Russian longevous regions had higher numbers of Bifidobacteria and *Lactobacillus*, and lower numbers of Clostridia, compared with the elderly in another region (Komai and Nanno, 1992; Mitsuoka, 1992). In China, the initial research on centenarian intestinal microflora was done in Bama County, in which numbers as high as 50% of Bifidobacteria in total intestinal anaerobes were detected (Zhang *et al.*, 1994). Bama County (Guangxi, China) is famous for its longevous population. China's 2000 population census showed that 76 centenarians lived in Bama County, giving it a ratio of 30.98 centenarians per 1×10^5 persons (Zou, 2002). Like the other longevous regions, most centenarians lived in the rural area of the county. In Jiazhuan, a village of 500 people in the rural part of Bama, there lived eight centenarians. It is therefore possible for us to study the intestinal microflora of the senior elderly.

Based on a culture-independent method, our earlier study showed significant effects of age and living region on the number of specific intestinal bacterial groups in the elderly of Bama (Zhao *et al.*, 2010). Together with research Zhang *et al.* (1994) previous results had been limited to bacterial groups detected by selective cultivation or real-time PCR. Any correlations of the intestinal bacterial communities with age and region were not determined in the elderly of Bama. The aim of this study was to assess the effects of age and living region on the whole intestinal bacterial communities of the elderly living in Bama and to characterize the bacterial communities correlated with age and region. Denaturing gradient gel electrophoresis (DGGE) was employed to analyze

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the fecal microflora of 17 elderly people of different ages and from different regions (rural and urban areas of the same metropolitan region). Multivariate statistical analysis was used for assessing the age and regional effects, and the related bacteria were characterized by the band sequencing.

Materials and Methods

Subjects

This research was approved by the Municipality of Bama Yao Autonomous County, Guangxi, China. A total of 17 elderly volunteers (aged 75-109 years) were recruited from communities within Bama County. All participants were confirmed as healthy, and signed the informed consent prior to the sample collecting. According to the age and living region, the volunteers were divided into three groups: Group RL-the rural longevous people, aged 103-109 years, from the Jiazhuan village, a Bama suburb (mean age=106 years; n=6); Group RE-the rural younger elderly people, aged 75-83 years, also from the Jiazhuan village (mean age=79 years; n=4); Group UE-the urban elderly people, aged 76-83 years, living in urban areas of Bama (mean age=79 years; n=7). There was no difference in mean age between Group RE and UE (Student's t-test, $P>0.05$), but the mean age of Group RL was significantly higher than that of the other two groups (Student's t-test, $P<0.01$). All volunteers were asked to maintain their usual diets prior to fecal sample collection; they were instructed not to use gastric acid inhibitors, laxatives, anti-diarrhea medication, antibiotics, probiotics, or prebiotics for more than 2 weeks prior to sample collection.

Fecal sample collection and bacterial genomic DNA extraction

Each participant was told to collect three independent fecal samples on different days within one week. All the samples were collected into 50 ml sterilized tubes. Participants were instructed to store the tubes in an ice box until these were collected by a team member (within 4 h). The tubes were transported from the donors' homes in containers (with dry ice) to the lab and stored at -80°C for later analysis. Three fecal samples from the same participant were mixed well as one sample, then bacterial genomic DNA was extracted from 17 fecal samples using QIAamp Stool Mini kit (QIAGEN, Germany) in accordance with the manufacturer's instructions (Li *et al.*, 2003). DNA was dissolved to a final volume of 200 μl.

PCR amplification of 16S rDNA V3 region

The V3 variable region of 16S rDNA was amplified by PCR using primers described by Muyzer *et al*. (1993). The primers 341F-GC (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG) and 534R (5′-ATTACCGCGGCTGCTGG) were synthesized by Invitrogen (China) Biotech Co. Ltd. The PCR reaction mixture contained 2.5 μl template DNA, 0.25 μM (final concentration) of each primer, 4 μl of dNTP mixture, 5 μl of 10× Ex Taq buffer, and 0.25 μl of Ex Taq polymerase (TaKaRa, Japan). The final volume of the reaction mixture was adjusted to 50 μl with sterile deionized water. A "touchdown" PCR was performed in a PTC-200 Thermo Cycler (Bio-Rad, USA) following the program described by Lubbs *et al*. (2009). The length of amplicons was approximately 200 bp as determined by electrophoresis in 2% (w/v) agarose gels and ethidium bromide staining. 10 μl of DGGE gel loading buffer (0.05% bromophenol blue, 0.05% xylene cyanol, 70% glycerol (w/v) in H_2O) was added to 20 μl PCR product and the mixture was then stored at -20°C until DGGE analysis.

DGGE analysis

DGGE was performed using a DCode Universal Mutation Detection System (Bio-Rad). Amplicons were separated on 8% (w/v) polyacrylamide gels (acrylamide-bisacrylamide stock solution 37.5:1, Amresco, USA) in 0.5× TAE buffer (20 mM Tris-acetate, pH 7.4, 10 mM sodium acetate, 0.5 mM Na_2-EDTA) (Muyzer *et al.*, 1993), with 30-60% linear denaturing gradient (A 100% denaturant corresponds to 7 M urea and 40% (vol/vol) deionized formamide). Electrophoresis was performed in 0.5× TAE at 85 V at 60°C for 16 h. Gels were silver stained (White *et al.*, 2004) and scanned using a CanoScan Lide 100 Scanner (Canon, Japan).

Gel images were analyzed using Quantity One software (version 4.2, Bio-Rad) according to the user guide. To visualize the interrelationships of fecal microflora among subjects, a dendrogram of samples was constructed based on Dice's coefficient using the unweighted-pair group method with the arithmetic average (UPGMA) algorithm, as implemented in the software (Fuentes *et al.*, 2008). The total intensity of all bands in each lane was defined as 100%, and relative intensities of each band in the same lane were calculated. The band type table with relative intensity values was exported to Excel (Microsoft, USA).

Multivariate statistical analysis

To assess the effects of age and living region on fecal microflora of the elderly in Bama, multivariate statistical analysis was performed using Canoco 4.5 (Biometrics, Netherlands). The band type table was imported as species data; simultaneously, subject age and living region were used as environmental variables – rural region and urban region were defined as 1 and 0 respectively. Because the longest gradient resulting from detrended correspondence analysis (DCA) was 1.627, the linear model of redundancy analysis (RDA) with the focus scaling on interspecies distances was employed (Lepš and Šmilauer, 2003; Janczyk *et al.*, 2010). Unrestricted Monte Carlo permutation tests were applied to test the significance of the microfloral response with environmental variables (499 random permutations, $P<0.05$). To investigate which bacterial communities correlated significantly with host age or living region, t-value biplots for each environmental variable were graphed based on RDA by CanoDraw (one module of Canoco 4.5). Species fit ranges were set according to the variability of species data explained by the first RDA axis (Lepš and Šmilauer, 2003). Species vectors (band types) enclosed in Van Dobben circles indicated the significant relationship with age or living region (regression coefficient <-2 or $>+2$) (Lepš and Šmilauer, 2003). Bands in positive circles indicated the abundance of bands increased with environmental variables and the opposite effect could be concluded in negative circles (Lepš and Šmilauer, 2003). The bands significantly affected by age and region were marked on DGGE gel images to identify them for later sequencing.

Excision and sequencing of selected bands from DGGE gels

Bands of interest were excised with a sterile scalpel from DGGE gels and the DNA was extracted according to the procedures described by Sanguinetti *et al.* (1994). The DNA was subjected to PCR reaction as templates with 341F (without GC-clamp, the sequence was 5′-CCTACGGGAGGCAGCAG) and 534R primers according to the following program: 3 min at 94°C, 30 cycles consisting of 15 sec at 94°C, 20 sec at 55°C, and 20 sec at 72°C, and finally 5 min at 72°C. The resulting PCR products were cleaned with the Universal DNA Purification kit (Tiangen, China) and cloned into *Escherichia coli* TOP10 competent cells (Tiangen) with the pMD18-T Simple

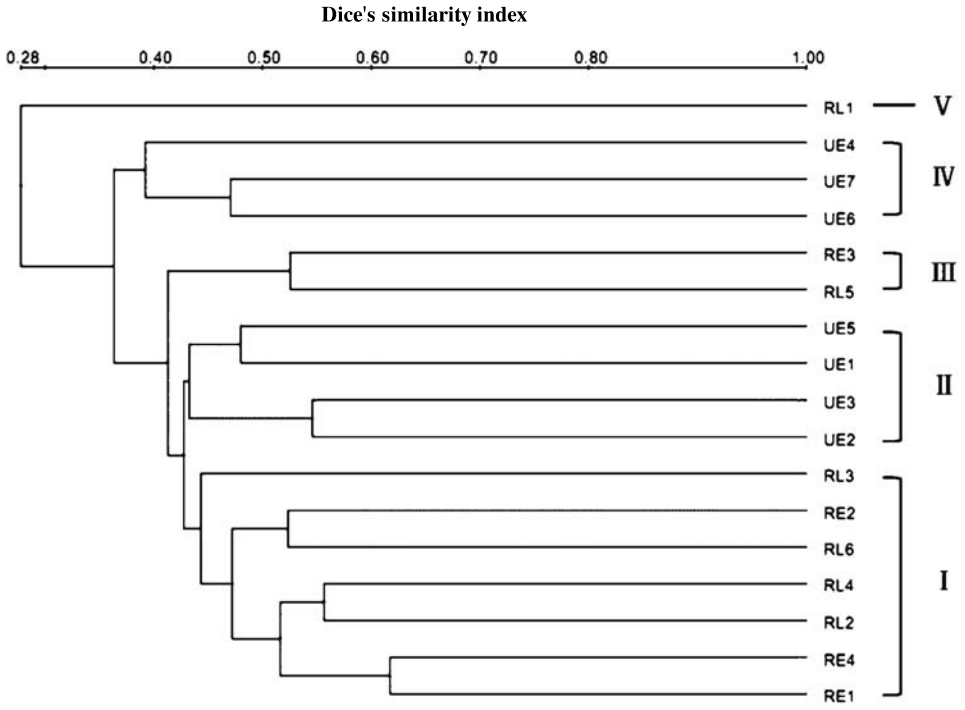

Fig. 1. Dendrogram derived from DGGE analysis of fecal microflora of elderly participants based on Dice's similarity index and the UPGMA clustering algorithm. RL1-6, six subjects of Group RL; RE1-4, four subjects of Group RE; UE1-7, seven subjects of Group UE. I-V, five clusters were observed.

Vector (TaKaRa). Plasmid DNA was isolated from the *E. coli* cells using the TIANprep Mini Plasmid kit (Tiangen), and subjected to PCR (341F-GC and 534R primers) as described previously. The PCR product was checked by DGGE to confirm the purity and the migrating position of the excised band (Tannock *et al.*, 2004; Licht *et al.*, 2006). The plasmids with desirable inserts were sequenced by Invitrogen (China) Biotech Co. Ltd using M13F primer. The obtained sequences were compared with known sequences in the GenBank database with the BLASTn algorithm.

Results

Comparison of fecal microflora among subjects

In this study, the fecal microflora of 17 elderly subjects was successfully represented by DGGE, and a total of 87 band types were recognized in Quantity One software. A dendrogram was obtained from DGGE analysis of the fecal microflora of the elderly participants using the UPGMA clustering algorithm. The subjects grouped into five distinct clusters (Fig. 1). Each cluster (ignoring Cluster V) contained subjects from the same region, indicating the effects of living region on fecal microflora. Participants from Group RL and RE were distributed into three clusters (Cluster I, III, and V) which were not dependent on the age-related differences between two rural elderly groups. Seven of the ten rural participants (Group RL and RE) were in Cluster I from which RE3, RL5, and RL1 were apart. Fecal microflora of RE3 and RL5 formed an isolated branch (Cluster III) and were different from that of the other rural participants. RL1 was alone in Cluster V and had the highest level of individual difference (0.28 similarity to other clusters). Similar to three clusters of rural subjects, Group UE was divided into two clusters (Cluster II and IV). The Dice's similarity range was between 0.28 and 0.62, indicating a highly variable fecal microflora among the elderly subjects in Bama.

Effects of age and living region on fecal microflora

In the RDA, both species-environment correlations and Monte Carlo permutation tests showed that the response of the species data was dependent on the environmental variables. This indicated significant effects of host age and living region on the fecal microflora in the elderly subjects in Bama. When both age and living region were treated as environmental variables, the first axes of the RDA explained 10.1% of total variation and the first and the second axes together explained 18.1%. Species-environment correlations for axes 1 and 2 were 0.943 and 0.934 respectively, indicating a high correlation between microflora and environmental variables (age and living region). Significant effects of age and living region on microflora were confirmed by Monte Carlo permutation tests (P= 0.002). When age or living region was used as environmental variable separately (the other one used as a covariable) in RDA, significant effects on microflora were also detected (P=0.016 and P=0.002 respectively, Monte Carlo permutation tests). This indicated that both age and living region were correlated with the fecal microflora of the participants in Bama.

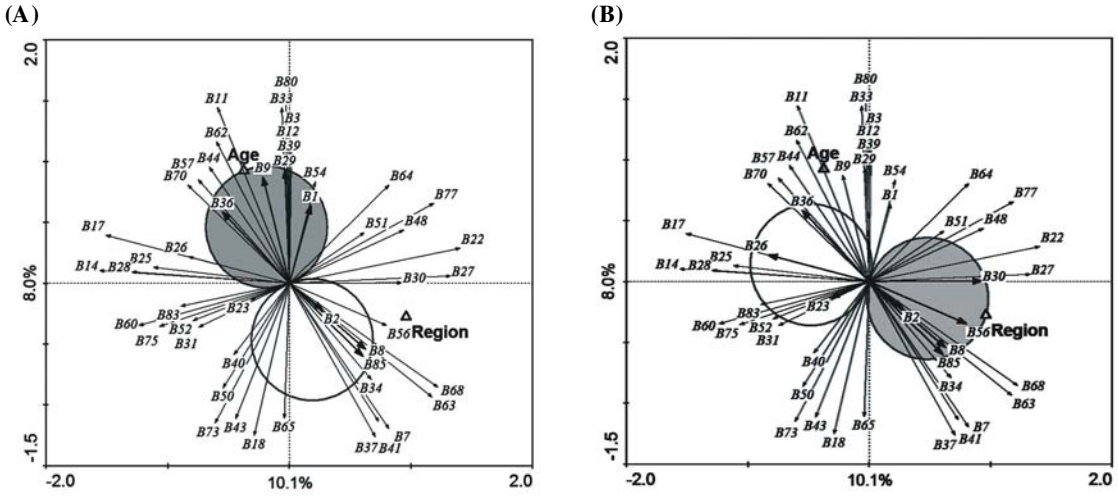

Fig. 2. T-value biplot for age (A) and region (B) obtained from CanoDraw (one module of Canoco 4.5). The first and the second axes explained 10.1% and 8.0% of total variation respectively. Gray line circles indicated Van Dobben circles. The circles filled with gray color were positive correlation circles while the transparent ones were negative correlation circles. Vectors indicated species (band types in DGGE profile). Bold vectors indicated the bands significantly correlated with age or region. Hollow triangles indicated environmental variables (age and living region).

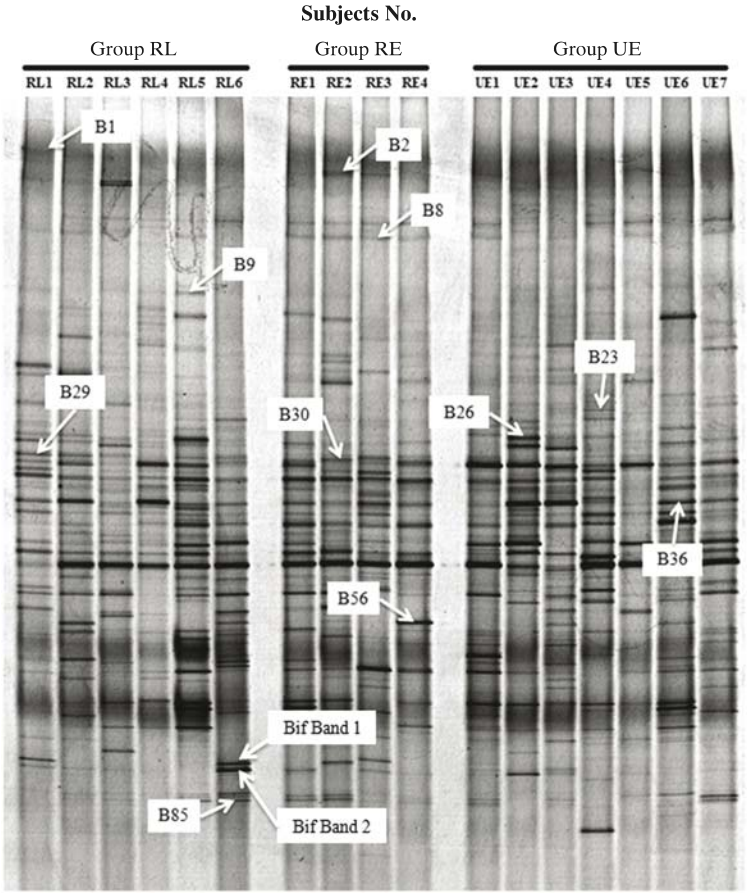

Fig. 3. DGGE profile of fecal microflora of 17 elderly subjects in Bama. Bands significantly correlated with age and region were marked as "B" followed by band type number. Bif Band 1 was most related to *Bifidobacterium longum* strain THT-010301 (99%, Accession no. EF370991.1), and the relative intensity was 3.10%. Bif Band 2 was most related to Uncultured *Bifidobacterium* sp. clone PP187-b15 (98%, Accession no. GU902754.1), and the relative intensity was 6.23%.

Table 1. Summary of band identification and significant bacterial associations with host age and living region for elderly participants in Bama

Band No.[a]	Closest relative	% identity	Accession No.	Higher in the older elderly?	Higher in the younger elderly?	Higher in the rural eldely?	Higher in the urban eldely?
B1	Uncultured Bacteroidetes bacterium clone SS298	100%	HM442832.1	Yes[b]	-	-	-
B2	Uncultured *Bacteroides* sp. clone Sew1-210	100%	FJ219852.1	-	Yes	Yes	-
B8	*Bacteroides faecis* JCM 16477	98%	AB547641.1	-	Yes	Yes	-
B9	Uncultured bacterium clone nby346e01c1	96%	HM818391.1	Yes	-	-	-
B23	Uncultured bacterium clone ST13_500m_clone20	94%	HQ015509.1	-	-	-	Yes
B26	Uncultured Firmicutes bacterium clone TF2-82	100%	GU957941.1	-	-	-	Yes
B29	Uncultured bacterium clone 6-1B21	98%	FJ684789.1	Yes	-	-	-
B30	Uncultured Firmicutes bacterium clone LI2-114	100%	GU957675.1	-	-	Yes	-
B36	Uncultured *Desulfovibrio* sp. L4M2 enrichment clone 196. H01	96%	DQ308601.1	Yes	-	-	Yes
B56	*Clostridium sordellii* JCM 3814	98%	AB550230.1	-	-	-	Yes
B85	Uncultured Ruminococcaceae bacterium clone E203A02	98%	HM080349.1	-	Yes	Yes	-

[a] Bands significantly correlated with age or living region were listed in this table.
[b] "Yes" indicated that the abundance of the bacteria in feces in response to age/living region could be detected based on t-value biplot in this study.
- indicated that the abundance of the bacteria in feces in response to age/living region could not be detected in this study.

Characterization of the fecal bacterial communities correlated with age and living region

A total of 11 bands that correlated significantly with age or living region were observed in t-value biplots for each environmental variable based on RDA (Fig. 2). The positions of these bands in the DGGE profile are represented in Fig. 3, and the microbial identification of the bands are summarized in Table 1. The abundance of four bacterial strains was correlated with both age and living region of the elderly in Bama. Two *Bacteroides* strains (B2 and B8) and one Ruminococcaceae strain (B85) "preferred to live in" the rural, younger elderly; conversely, the abundance of one *Desulfovibrio* strain (B36) was high in the urban, older elderly. Another Bacteroidetes strain (B1) and two uncultured bacterial strains (B9 and B29) were only correlated with participants' age, and the abundance of these strains increased with the age of the elderly. Another four bacterial strains were only correlated with the elderly hosts' living region. One Firmicutes strain (B30) "preferred" the rural elderly; conversely, the abundance of one uncultured bacterial strain (B23), one uncultured Firmicutes strain (B26) and one *Clostridium sordellii* strain (B56) was higher in the urban elderly.

Discussion

The fecal microflora of 17 elderly persons living in Bama, a famous county for its longevous population, were analyzed using DGGE in this study. A high inter-individual variation of fecal microflora in the elderly was observed in DGGE profiles (the Dice's similarity range was from 0.28 to 0.62). Similar results were obtained in Zwielehner and colleagues' research (2009), in which the elderly subjects had higher inter-individual variations of microflora than the adults. In RDA, the first and the second axes only explained up to 18.1% of the variances, indicating the effects of other factors on the elderly fecal microflora. Dietary factors and host genotype may contribute these effects (Zoetendal *et al.*, 2001; Hayashi *et al.*, 2002). The relatively low percentage of variance explained by the RDA is commonly found in the literature (Fuentes *et al.*, 2008; Janczyk *et al.*, 2010). In this study, we focused on the age- and region-effects on the microflora, which were detected as significant by RDA. A total of 11 fecal bacterial strains that correlated significantly with age and living region were identified in t-value biplots based on RDA. These results add to previous research on the response of intestinal bacterial communities to the elderly hosts' age and living region (Mitsuoka, 1992; Mueller *et al.*, 2006).

Age could have major effects on human intestinal microflora, which changes throughout life (Mitsuoka, 1992; O'Toole and Claesson, 2010). In old age, decreased gastrointestinal physical function, unbalanced nutrition and increased frequency of antibiotic treatment could change intestinal microflora significantly (Woodmansey, 2007; O'Toole and Claesson, 2010). Differences in the intestinal microflora between the elderly and adults have mostly been studied using culture-dependent and -independent approaches (O'Toole and Claesson, 2010; Tiihonen *et al.*, 2010). However, contradictory results obtained from different studies indicated there was no clear marker change in the composition of fecal microbiota of the elderly (Tiihonen *et al.*, 2010). In this study, we observed that two *Bacteroides* strains, one Bacteroidetes strain, one *Desulfovibrio* strain and one Ruminococcaceae strain were correlated with the age of the elderly. Especially, we observed that different Bacterodetes strains had different age-responses – two *Bacteroides* strains (B2 and B8) "preferred" the younger elderly people; however, the abundance of another strain of Bacteroidetes (B1) increased with host age. Compared with the adults, a reduced or increased number of bacteroides has been observed in the elderly in different studies (Hopkins and Macfarlane, 2002; Woodmansey *et al.*, 2004), which may be related to different age-responses of stains detected in different studies, as shown in this study. The family Ruminococcaceae contains a large proportion of bacterial genera known to be butyrate producers in the colon (Collins *et al.*, 1994; Mäkivuokko *et al.*, 2010). A different level of *Ruminococcus* (one genus of Ruminococcaceae) between adults and the elderly was observed in previous studies and contradictory results have also been obtained (He *et al.*, 2003; Mäkivuokko *et al.*, 2010). Our

study showed the abundance of one Ruminococcaceae strain decreased with the age of the elderly, which may be related to a low level of butyrate in the elderly intestine (Woodmansey, 2007). We also detected one *Desulfovibrio* strain in the older elderly. In the human gut, the *Desulfovibrio* reduce sulfate to produce hydrogen sulphide (Dzierżewicz *et al.*, 2003), which is potentially harmful to the host (Mitsuoka, 1992). A high content of sulphide may be a characteristic of the senior elderly intestines.

Geography also played an important role in intestinal microflora – subjects living in different cities or countries had large variations in intestinal bacterial communities, and age-related differences of microflora varied in different locations (Mitsuoka, 1992; Lay *et al.*, 2005; Mueller *et al.*, 2006). Similar results were also obtained in this study: living in the rural or urban region of Bama County could significantly affect the intestinal bacterial communities of the elderly. Two *Bacteroides* strains, one *Desulfovibrio* strain, one *Clostridium sordellii* strain and one Ruminococcaceae strain were correlated with the living region of the elderly. In the colon, Bacteroides is an important bacterial genus, because these species digest polysaccharides and are able to utilize a wide variety of carbon sources (Woodmansey, 2007). The Ruminococcaceae strain was a potential butyrate producing bacterium, which may play an important role in protection of the intestine (Lupton, 2004). Although the abundance of these strains (two *Bacteroides* strains and one Ruminococcaceae strain) decreased with host age, they "preferred living" in the rural elderly in Bama. Similar results were also obtained in our previous study: a high level of *Bacteroides-Prevotella* was observed in the rural longevous participants of Bama (Zhao *et al.*, 2010). These results suggested that living in the rural region of Bama could "enrich" these potential beneficial strains, and "keep" them from decreasing with the age of the elderly. On the other hand, we also observed that one *Desulfovibrio* strain and one *Clostridium sordellii* strain were high in the urban elderly. *Clostridium sordellii* has bile-acid-7α-dehydroxylating activity, and could produce secondary bile acids in the human gut (Kitahara *et al.*, 2001). Together with hydrogen sulfide produced by *Desulfovibrio*, these metabolic products in the intestine can be harmful to the host (Mitsuoka, 1992), and may accelerate the aging of the urban elderly in Bama (Metchnikoff, 1907; Mitsuoka, 1992). These results gave a possible explanation why the most longevous people are living in the rural region of Bama.

Bifidobacteria are regarded as a beneficial species in the large intestine (Woodmansey, 2007), and high numbers of Bifidobacteria have been observed in longevous people living in a specific region including Bama County (as deduced by a cultivation methodology) (Komai and Nanno, 1992; Mitsuoka, 1992; Zhang *et al.*, 1994). In this study, the Bifidobacteria were not detected in the t-value biplots, indicating a relatively stabilized abundance among the subjects, as shown in a previous study (Zhao *et al.*, 2010). However, we observed two *Bifidobacterium* bands with high relative intensity in the sample from Subject RL6 (Fig. 3), who was a longevous female living in the rural region of Bama. This is consistent with our previous study, in which as high as 9.5% of *Bifidobacterium* in total intestinal bacteria was observed in the same subject (Zhao *et al.*, 2010). Although not each longevous participant in Bama has such high abundance of *Bifidobacterium* in their intestine, an unusually high level of these bacteria in several longevous people suggest an interesting relationship between *Bifidobacterium* and human aging. Research on the composition of the elderly intestinal microflora might lead to modulating it to improve elderly health. Our studies raise the possibility of developing probiotics derived from centenarians.

Two uncultured Firmicutes strains (B26 and B30) and three uncultured bacterial strains (B9, B23, and B29) that correlated with age and living region were also observed in this study. This suggested that some novel bacterial strains in the intestine may play an important role in host aging. Previous studies suggested that approximately 75% of bacteria in intestines were novel phylotypes (Suau *et al.*, 1999) and further research should focus on the large fraction of novel bacteria in the intestine and their functions in host aging.

In conclusion, significant effects of host age and living region on the whole intestinal bacterial communities were observed in the elderly people in Bama, a longevous area. Four bacterial strains were correlated with both age and living region of the elderly in Bama, and two strains were only correlated with one of these. Potential beneficial strains were high in the rural, younger elderly, while strains causing putrifaction were high in the urban or older elderly. Living in the rural region of Bama could slow the age-related decrease of some beneficial strains. This study expands the understanding of age- and region-effects on the intestinal microflora of the elderly and suggests the possibility of developing probiotics originating from centenarians.

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