J. Korean Dairy Technol. Sci. 23(2) : 125~134, 2005

# Culture-independent Analysis of Human Gut Microbiota and New Probiotic Effect for Human Health

Yoshimi Benno

JCM, RIKEN BioResource Center

A portion of these bacteria, more than 500 species of human gut microbiota, with an estimated total weight of approximately 1.5 kg, is excreted with the feces - nearly one trillion per gram of dry feces. The whole analysis of gut microbiota is gradually coming into focus through the efforts of our laboratory. Recently, an association has been found between gut microbiota and many diseases beyond those of the gastrointestinal system, including today's three leading causes of death - cancer, heart disease, and cerebrovascular disease - as well as allergies and dementia. Our group is trying to capture the whole analysis of gut microbiota, which constantly changes with age and lifestyle-related factors such as diet, and apply it to preventive medicine.

Probiotics have been defined in several ways, depending on our understanding of the mechanisms of action of their effects on health and well-being of humans. At present, the most commonly used definition is that of Fuller (1989): *Probiotoics are live microbial feed supplements, which beneficially affect the host animal by improving its intestinal microbial balance.* Recently a European expert group (Salminen et al., 1998, Salminen & von Wright, 1998) widened the definition to include mechanisms other than just microbiota mediated ones. Now, the definition was as follows: Pro- biotics are live microbial; food ingredients that have a beneficial effect on human health (Salminen et al., 1999).

To include the current application and scientific data on proven effects of probiotics, we proposed the following definition: Probiotoics are microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host.

In my presentation, I will introduce a new culture-independent analysis of human gut microbiota and the new function of probiotics. Then, I also need to describe my research history of human intestinal microbiota in RIKEN for above 30 years.

Corresponding author : Yoshimi Benno, JCM, RIKEN BioResource Center, Wako, Saitama 3510198, Japan, E-mail: benno@jcm.riken.jp

## Ⅰ. We were only seeing 20% of whole gut microbiota ?

In the mid-19th century, Theodor von Escherich of the University of Vienna found bacteria in feces - the species that would eventually be called *Escherichia coli*. However, until the mid-20th century, few fecal bacteria other than *E. coli* had been successfully cultured, leading most to believe that the majority of bacteria existing in the intestines were dead. The dispeller of this long-held belief was Dr. T. Mitsuoka, a RIKEN scientist who, in the 1950s, began to reveal that there are many living bacteria in the gut tract using some agar media and an anaerobic culture procedure. It turned out that most gut bacteria are anaerobic bacteria that cannot survive in the presence of oxygen.

I spent my younger days with gut microbiota and joined Dr. Mitsuoka's laboratory at RIKEN approximately 30 years ago. Our work can be likened to artisanship - creating agar medium by trial and error, and counting individual culturable bacteria to record bacterial species and numbers as

given in Table 1. Numerous microbial species consist of the gut microbiota in 30 health Japanese. Microbial species with high numbers and incidence in human gut microbita are *Bacteroides vulgatus, B. thetaiotaomicron, B. disitasonis, Collinsella aerofaciens, Ruminococcus* spp., *Faecalbacterium prausnitzii* and *Bifidobacterium adolescentis.* Then, fecal microorganisms with low number and high incidence were *Clostridium clostridioforme, C. innoccum, C. ramosum, C. perfringens, Enterococcus faecalis, E. faecium* and *Esherichia coli.*

Not only does it require a great deal of perseverance and energy, but also the stool specimens are malodorous and pose a constant risk of bacterial infection. No one is excited to take on this assignment.

Dr. Mitsuoka received the Japan Academy Award in 1988 for his work on systematic research on gut microbiota utilizing original culturing techniques. "Everyone thought that we knew all there was to know about gut microbiota, and that Dr. Mitsuoka's work was complete." However, we could not help but wonder if the culturing methods would be available at that time had really taught us everything. Culture-independent analysis, which became available for the study of gut microbiota in the mid-1980s, enabled researchers to determine the presence of gut microbiota without culturing. In 1996, we came across a stunning paper produced by a group of molecular biologists who analyzed DNA extracted from bacteria isolated from human feces, reporting that 10% to 25% of the bacteria can be cultured but the remaining 75% are either extremely difficult or impossible to culture, and that the gut microbiota that had been thought to comprise approximately 100 species are in fact 500 to 1000 species. The gut microbiota that seemingly had been almost fully elucidated by the culturing methods available at that time, which were the sum total of the efforts of many researchers, were merely around 20~30% of the extant bacteria.

Table 1. Composition of fecal microbiota in 30 Japanese

| High counts<br>High occurrence | Low counts<br>High occurrence | High counts<br>Low occurrence |
|---|---|---|
| *Bacteroides fragilis* group | | *Bacteroides ovatus* |
| *Bacteroides vulgatus, Bacteroides distasinis,* | | *Bacteroides splanchnicus* |
| *Bacteroides thetaiotaomicron, Bacteroides uniformis* | | *Bacteroides ureolyticus* |
| *Bacteroides* spp. | | *Bacteroides putredinis* |
| *Prevotella buccae* | | *Prevotella veroralis* |
| *Prevotella oris* | | *Fusobacterium naviforme* |
| *Prevotella* spp. | | *Fusobacterium nucleatum* |
| *Fusobacterium prausnitzii* | | *Fusobacterium mortiferum* |
| *Fusobacteru \ium russii* | | *Fusobacterium varium* |
| | *Lactobacillus catenforme* | *Mitsuokella multiacida* |
| *Bifidobacterium* | | |
| *Bifidobacterium adolescentis* | | |
| *Bifidobacterium longum* | | *Bifidobacterium breve* |
| *Bifidobacterium* spp. | | |
| *Collinsella aerofaciens* | | |
| *Eubacterium rectale* | | *Eubacterium moniliforme* |
| *Eubacterium* spp. | | |
| | | |
| *Ruminococcus productus* | | |
| *Peptostreptococcus* spp. | *Closotridium perfringens* | *Peptostreptococcus anaerobius* |
| *Ruminococcus* spp. | *Closotridium beijerinckii* | *Peptostreptococcus prevotii* |
| *Veillonella parvula* | *Closotridium coccoides* | |
| | *Closotridium butyricum* | |
| *Clostridium innocuum* | *Closotridium paraputrificum* | |
| *Clostridium ramosum* | | |
| *Clostridium clostridioforme* | | |

## Ⅱ. Capturing the whole analysis of human gut microbiota

In 1998, we shifted his research focus to elucidating the whole analysis of gut microbiota, including those "Yet-unexploit" bacteria, by incorporating DNA analysis with conventional culturing methods. Seven hundredand forty-fourth DNA clones bacteria isolated from the fecal specimens of three healthy Japanese subjects, and found that 75% were novel gut bacteria, and that there are great individual variations in the composition of gut microbiota.

The human gut microbiota from three healthy subjects and elderly persons were compared using sequence analysis of 16S rDNA libraries (Hayashi et al., 2002, Hayshi et al., 2003). Direct counts ranged from $1.9\times10^{11}$ to $4.0\times10^{11}$cells/g (wet weight) while plate counts totaled $6.6\times10^{10}$ to $1.2\times10^{11}$ CFU/g (wet weight). Sixty to seventy percent of the bacteria in human intestinal tract cannot be cultured with currently available methods. The 16S rDNA libraries from three healthy subjects and three elderly subjects were generated from total community DNA *in* the *intestinal* tract, using universal primers sets. Randomly selected clones were partially sequenced. All purified colonies detected from the surface of the agar plate were used for partial sequencing of 16S rDNA. On the basis of sequence similarities, the clones and colonies were classified into several clusters corresponding to the major phylum of the domain *Bacteria*. Among a total of 984 clones (744 clones from three healthy subjects and 240 clones from elderly subjects) obtained, approximately 25% of the clones belonged to 31 known species for healthy subjects and 46% belonged to 27 known species for elderly subjects. About 75% in healthy subjects and 54% in elderly persons of the remaining clones were novel "phylotypes" (at least 98% similarity of clone sequence). The predominant intestinal microbial community consisted of 130 species or phylotypes for healthy subjects and 56 novel phylotypes according to the sequence data in our study. The 16S rDNA libraries (Table 2) included the *Bacteroides* group, the Streptococcus group, the *Bifidobacterium* group, and *Clostridium* rRNA clusters IV(Fig. 1), IX, XIVa (Fig. 2), and

Table 2. Composition of fecal microbiota in three healthy subjects and three elderly subjects as reveled by 16S rRNA gene libraies (%)

| | Healthy subjects | | | Elderly subjects | | |
|---|---|---|---|---|---|---|
| | O | B | S | A | B | C |
| *Clostridium Cluster I* | 0 | 1.1 | 0 | 0 | 0 | 0 |
| *Clostridium Cluster IV* (Clostridium leptum group) | 22.7 | 12.4 | 11 | 34.7 | 16.1 | 9.5 |
| *Clostridium Cluster IX* | 0 | 9.8 | 34 | 0 | 35.8 | 14.3 |
| *Clostridium Cluster XI* | 0 | 0.4 | 0.8 | 0 | 1.2 | 0 |
| *Clostridium subcluster XIVa* (Clostrodium coccoides group) | 58.8 | 23.7 | 29 | 25.3 | 2.5 | 3.5 |
| *Clostridium subcluster XIVb* | 0.5 | 0 | 0 | 0 | 0 | 0 |
| *Clostridium Cluster XVI* | 0 | 4.1 | 0 | 4 | 0 | 0 |
| *Clostridium Cluster VXII* | 0 | 8.3 | 0 | 0 | 2.5 | 0 |
| *Clostridium Cluster XVIII* | 0 | 0 | 0.4 | 0 | 0 | 0 |
| *Bifidobacterium* | 0 | 0.4 | 5.3 | 0 | 0 | 0 |
| *Lactobacillus* | 0 | 0 | 0 | 0 | 1.2 | 0 |
| *Cytophaga-Flabobacter-Bacteroides* | 5 | 9.4 | 16.3 | 20 | 8.6 | 15.4 |
| *Streptococcus* | 3.7 | 28.8 | 0.4 | 2.7 | 1.2 | 0 |
| *Gammaproteobacteria* | 0.5 | 0.8 | 1.6 | 5.3 | 17.3 | 54.8 |
| Others | 8.8 | 0.8 | 1.2 | 8 | 13.6 | 2.4 |

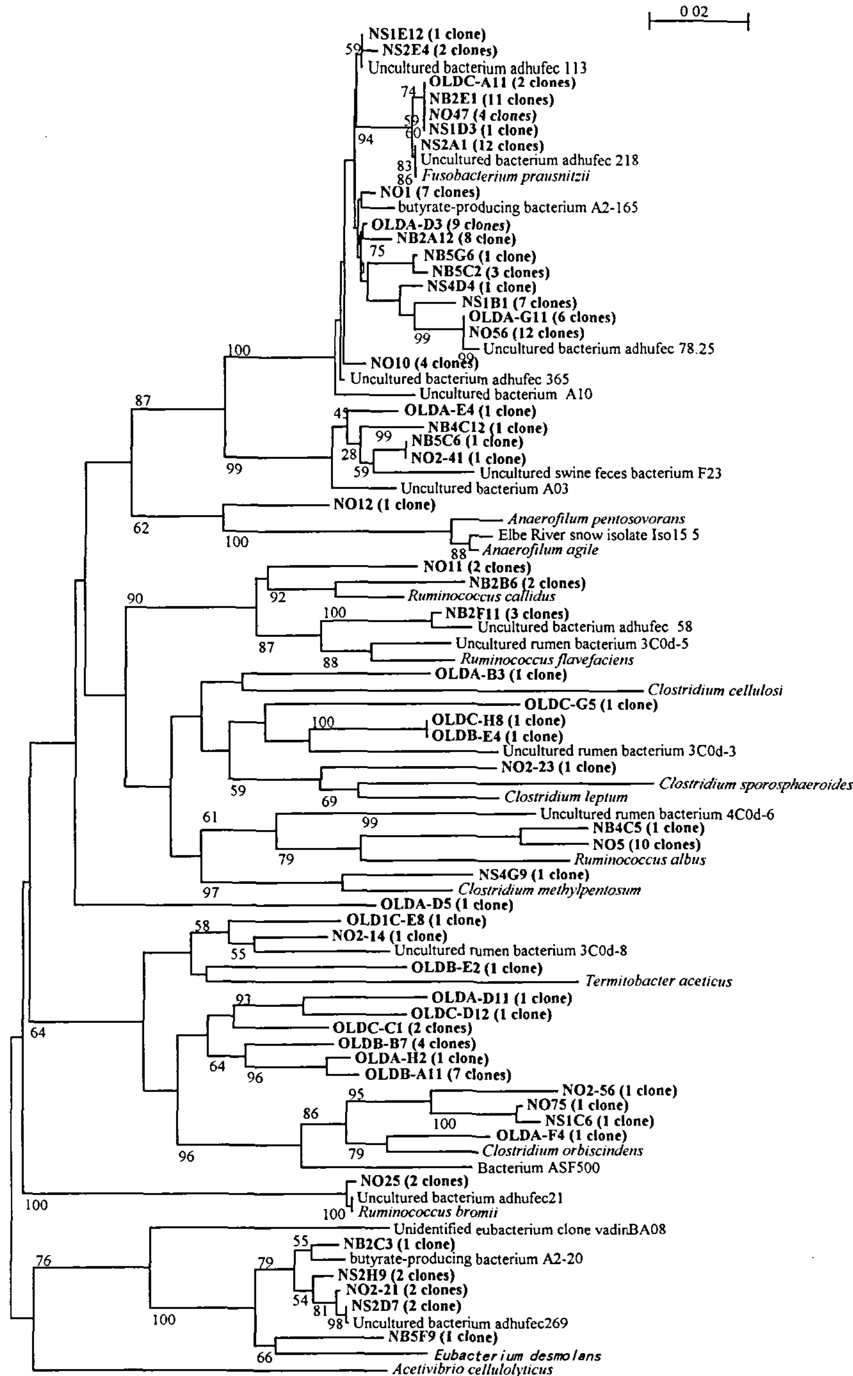


Fig. 1. Phylogenetic tree showing the relationship of 16S rDNA sequences from fecal samples of healthy subjects and elderly subjects within the *Clostridium* rRNA cluster IV (*Clostridium leptum* group). The tree was constructed by the use of neighbor-joining analysis based on 16S rDNA sequences. Bootstrap values (n=100 replicates) of $\geq 50$ are considered pereparentages. The scale bar represents 0.01 substitution per nucleotide position. Clone from the 16S rDNA library are shown by each character (NO, sample O; NB, sample B; NS, sample S; OLDA, sample A, OLDB, sample B; OLDC, sample C).

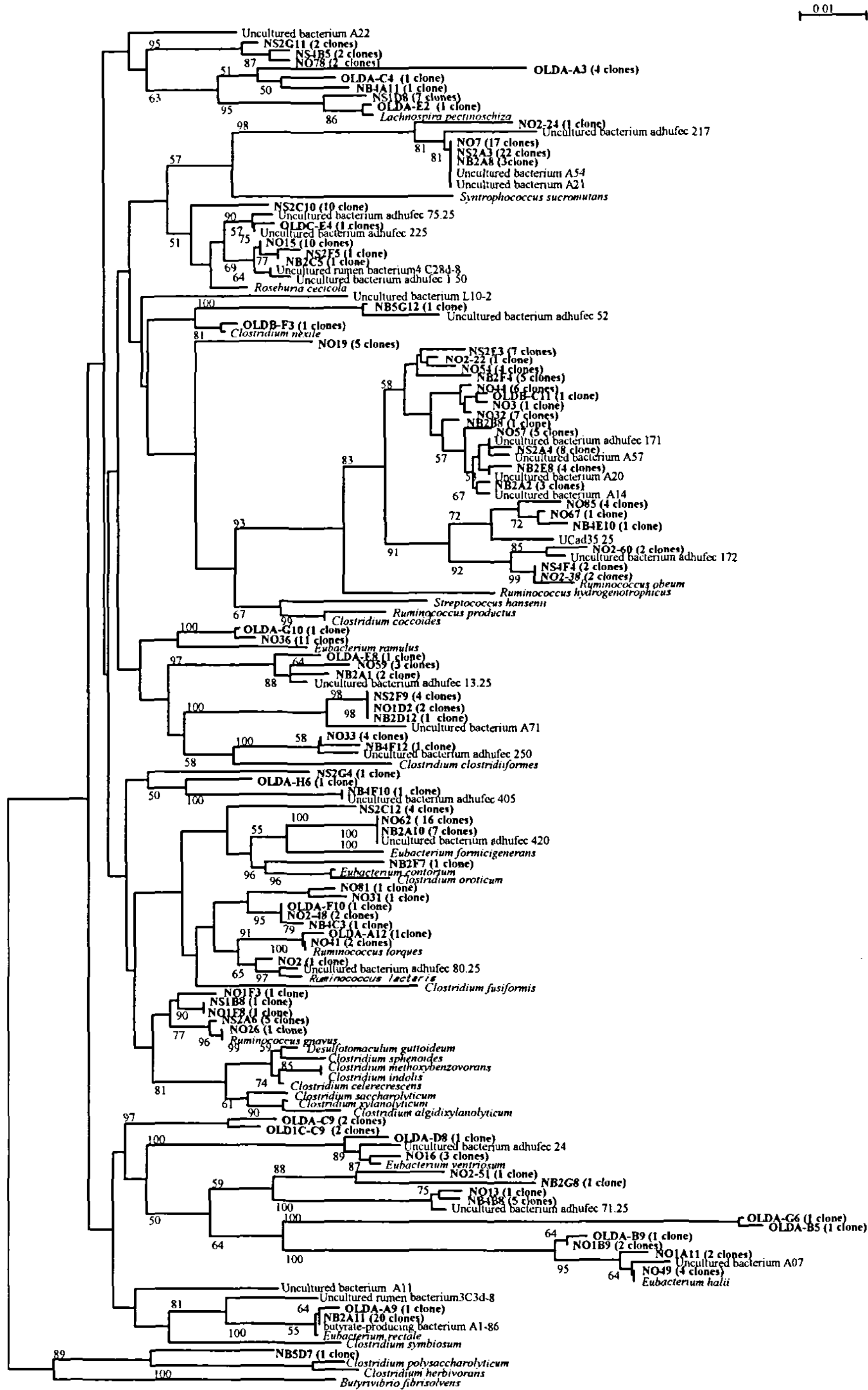


Fig. 2. Phylogenetic tree showing the relationship of 16S rDNA sequences from fecal samples of healthy subjects and elderly subjects within the *Clostridium* rRNA subcluster XIVa (*Clostridium coccoides* group). The tree was constructed by the use of neighbor-joining analysis based on 16S rDNA sequences. Bootstrap values (n=100 replicates) of ≧ 50 are considered pereparentages. The scale bar represents 0.01 substitution per nucleotide position. Clone from the 16S rDNA library are shown by each character (NO, sample O; NB, sample B; NS, sample S; OLDA, sample A, OLDB, sample B; OLDC, sample C).

XVIII in healthy subjects and the *Bacteroides* group, *Clostridium* rRNA cluster IV(Fig. 1), IX, *Clostridium* rRNA subcluster XIVa (Fig. 2) and "*Gammaproteobacteria*" in elderly subjects. In addition, a number of previously uncharacterized and uncultured microorganisms were recognized in clone libraries. Our results also showed marked individual differences in the composition of intestinal microbiota.

Subsequently, around 2000, we began to use T-RFLPs (terminal restriction fragment length polymorphisms) to facilitate determination of the compositional patterns of human gut microbiota (Sakamoto et al., 2003). T-RFLP analysis consists of extraction of genes for 16S ribosomal RNA gene from various gut bacteria in feces, amplification using primers (molecules that provide a starting point for DNA synthesis) labeled with fluorescent dyes, and digestion with two different restriction enzymes (*Msp*I and *Hha*I). Restriction enzymes cleave DNA, which is composed of four different bases, at specific sequences, generating DNA fragments of various lengths depending on the number of bases. The amount of each DNA fragment is determined based on the intensity of its fluorescence signal, and aligning the DNA fragments by the number of bases provides a gut microbiota profile reflecting the composition of gut microbiota and their amounts illustrated in Fig 3.

## Ⅲ. Novel phylogenetic assignment database for terminal-restriction fragment length polymorphism analysis of human colonic microbiota

Various molecular-biological approaches using the 16S rRNA gene sequence have been used for the analysis of human colonic microbiota. Terminal-restriction fragment length polymorphism (T-RFLP) analysis is suitable for a rapid comparison of complex bacterial communities. Terminal-restriction fragment (T-RF) length can be calculated from a

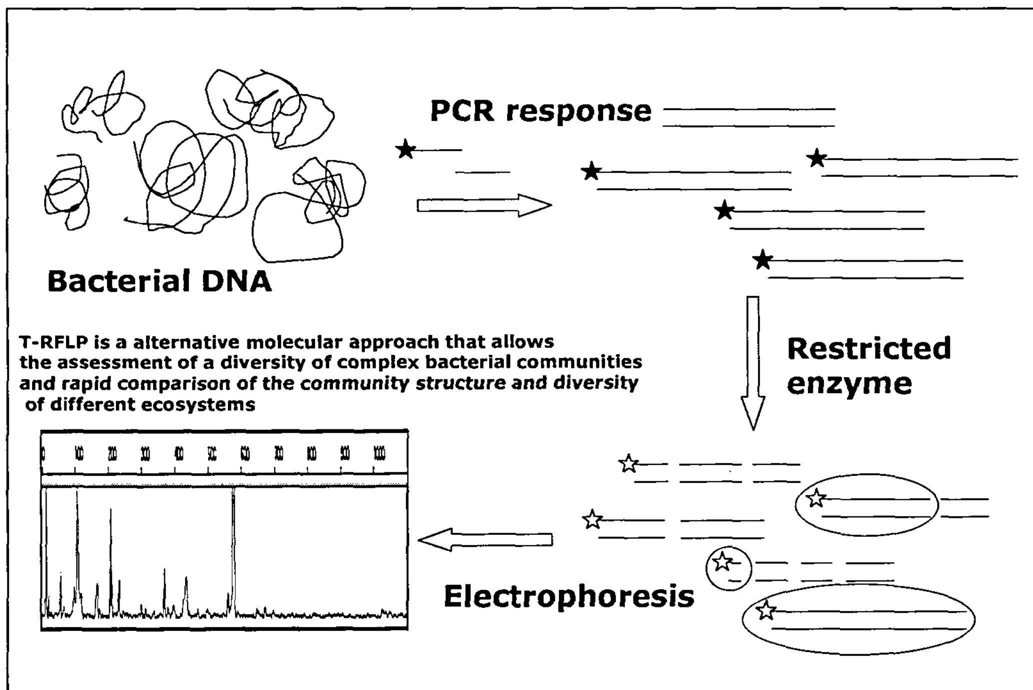


Fig. 3. Terminal restriction fragment length polymorphism (T-RFLP) method.

known sequence, thus one can predict bacterial species on the basis of their T-RF length by this analysis. Finally, we can build a phylogenetic assignment database for T-RFLP analysis of human colonic microbiota (PAD-HCM), and demonstrate the effectiveness of PAD-HCM compared with the results of 16S rRNA gene clone library analysis. PAD-HCM was completed to include 342 sequence data obtained using four restriction enzymes (Matsumoto et al., 2005). Approximately 80% of the total clones detected by 16S rRNA gene clone library analysis were the same bacterial species or phylotypes as those assigned from T-RF using PAD-HCM (Table 3). Moreover, large T-RFs consisted of common species or phylotypes detected by both analytical methods. All pseudo-T-RFs identified by mung bean nuclease digestion could not be assigned to a bacterial species or phylotype,

Table 3. Bacterial species and phylotypes detected from a Japanese feces by 16S rDNA clone library analysis

| Species or phylotype | No. of clones | (%) | |
|---|---|---|---|
| **Uncultured firmicute clone N062** | 13 | 14.1 | AB064740 |
| **Human intestinal firmicute clone CB17** | 6 | 6.5 | AB064890 |
| **Uncultured bacterium clone HuCB21** | 5 | 5.4 | AJ408996 |
| Phylotyoe 41A10 | 1 | 1.1 | |
| Uncultured human intestinal bacterium clone JW1A10 | 1 | 1.1 | AB080866 |
| Uncultured firmicute clone NB4F10 | 1 | 1.1 | AB064778 |
| *Clostrisium nexile* | 2 | 2.2 | AF499909 |
| ***Eubacterium rectale*** | 6 | 6.5 | AY169438 |
| Uncultured firmicute clone NO16 | 2 | 2.2 | AB064715 |
| Phylotype 41F10 | 1 | 1.1 | |
| Uncultured human intestinal bacterium clone JW1A1 | 3 | 3.3 | AB080865 |
| Phylotype 41F09 | 1 | 1.1 | |
| Uncultured bacterium clone OLDA-F10 | 2 | 2.2 | AB099739 |
| **Uncultured bacterium clone p-2431-55G5** | 8 | 8.7 | AF371596 |
| Uncultured firmicute clone NB5F9 | 1 | 1.1 | AB064783 |
| Uncultured firmicute clone N02-21 | 1 | 1.1 | AB064784 |
| Uncultured firmicute clone NS2D7 | 1 | 1.1 | AB064710 |
| **Butyrate-producing bacterium A2-165** | 15 | 16.3 | AJ270469 |
| ***Faecalibacterium prausnitzii*** | 6 | 6.5 | AY169427 |
| **Uncultured *Clostridium* sp. clone NO6** | 3 | 3.3 | AB064860 |
| ***Streptococcus mitissalivarius*** | 1 | 1.1 | AF393762/ AY188352 |
| Uncultured *Ruminococcus* sp. clone NO44 | 3 | 3.3 | AB064754 |
| Uncultured *Ruminococcus* sp. clone NB4C1 | 1 | 1.1 | AB064765 |
| **Uncultured bacterium clone p-2746-24E5** | 2 | 2.2 | AF371546 |
| ***Ruminococcus obeum*** | 3 | 3.3 | AB064755 |
| ***Uncultured Ruminococcus* sp. clone NO3** | 1 | 1.1 | AB064755 |
| *Ruminococcus* sp. CO12 | 1 | 1.1 | AB064896 |
| ***Eubacterium halii*** | 1 | 1.1 | L34621 |
| Total | 92 | 100.0 | |

Bacterial species and phylotypes detected from both of T-RFLP and 16S rRNA gene clone liblary analysis in red

and this finding shows that pseudo-T-RFs can also be predicted using PAD-HCM. We conclude that PAD-HCM built in this study enables the prediction of T-RFs at the species level including difficult-to-culture bacteria, and that it is very useful for the T-RFLP analysis of human colonic microbiota.

## Ⅳ. Exploring the relationship between gut microbiota and disease

Gut bacteria live in the colon, which is a "wellspring of disease"; in fact, the colon is associated with the greatest number of different diseases of any human organ. The gastrointestinal tract is also on the "front line of immunity", as it is connected to the outside world. Certain gut bacteria generate putrefactive products such as ammonia and hydrogen sulfide, bacteriotoxins, and carcinogens. These toxic substances damage the gut tract and induce colon cancer and a variety of other colonic diseases, and some of them are absorbed and circulated throughout the body by the blood, causing damage to various organs. Thus, there is an increasing body of evidence that gut bacteria can be the causes of carcinogenesis, aging, and various pathological conditions, including arteriosclerosis resulting from cholesterol deposition, liver damage, dementia, autoimmune diseases, and weakened immunity.

For example, *Clostridium* species are found at high levels in the feces of persons suffering from senile dementia. Once the toxic substances produced by these bacteria are spread throughout the body, the functions of neurotransmitters etc. are inhibited, resulting in impaired brain function. Recent molecular biological studies suggest that toxic substances produced by gut bacteria may promote cholesterol deposition in blood vessels, inducing arteriosclerosis, which in turn causes heart and cerebrovascular disease. The gut bacteria have also been reported to convert bile acids into secondary bile acids, thereby promoting the development of colon cancer. To date, six species of bacteria that produce secondary bile acids have been identified, three of which were identified and proposed by our laboratory. Thus our lifespan is evidently controlled by the endogenous bacteria in our bodies.

## Ⅴ. Developing an gut environment database

While the relationship between gut microbiota and disease is becoming clearer, we strongly feels that conventional research methods require significant modification if we are to further advance gut microbiota research and to apply the knowledge gained to actual preventive medicine and other fields. "Previously, researchers primarily targeted bacteria that can be cultured, and studied bacterial dynamics in patients with particular diseases, which did not produce the intended results. Now we need to focus on studying the interactions between bacteria as a whole and our bodies and food, and identify the substances they produce. The first step is to develop an gut environment database integrating gut microbiota profiles that reflect the composition of gut microbiota as a whole, with gut metabolic profiles that reflect the composition of the products they generate, namely metabolites. The accumulation of lifestyle-related data on diet, medical condition, and so on, and a study of the correlations present provide information about healthy gut environmental states and gut environments associated with particular diseases. We can then utilize this gut environment data for prevention and early detection of diseases. Our laboratory is leading the way in this area through research aimed at elucidating the whole analysis of gut microbiota and linking the data obtained to preventive medicine.

The gut environment is unique to each individual, and changes depending on lifestyle factors such as diet and age. Therefore, the development of a gut environment database requires both samples from a broad range of areas and age groups and quick analysis of large numbers of

samples. We are attempting to expand the routes to acquire large numbers of samples (100,000+) from throughout Japan. Recently, the number of colon cancer patients, including young people, has been increasing nationwide. If we could use the gut microbiota patterns associated with high susceptibility to colon cancer to screen for individuals at high risk, we could implement prevention and early detection at the national level and greatly reduce medical costs. Colon cancer has some of the highest associated medical costs among diseases. The gut microbiota research will bring us closerto order-made medicine, which allows selection of drugs suitable for an individual.

## Ⅵ. Connecting medicine and diet with gut microbiota

The Japanese like to say 'Food is medicine', but the door between food and medicine can only be opened with gut microbiota. Development of gut microbiota research has brought with it progress in our understanding of the mechanisms of lactic acid bacteria, which confer beneficial effects on our health. These advances have triggered a boom in probiotics - functional foods incorporating these bacteria. The best-known examples of probiotics are fermented milk and yogurt, which are both listed as "food for specified health uses (FOSHU)". The "FOSHU" designation is permitted by the Japanese government on labels for foods expected to confer health effects based on data obtained in medical and/or nutritional studies. Japan became the first country to adopt this system in 1991.

"Health claim" for FOSHU utilizing lactobacilli and bifidobacteria is limited to regulation of gut function at present, but there are data suggesting the potential for future enhancement of health claims to include reduction of cancer risk via immune reactivity, prevention of atopic dermatitis, prevention of respiratory infection, regulation of blood glucose level, blood pressure, and cholesterol, prevention and improvement of gastric ulcers, and inhibition of the causative bacteria of diseases such as periodontal disease. The boom in FOSHU is not merely a transient fad, but is founded on solid evidence presented by the elucidation of the functions of microorganisms such as lactobacilli and bifidobacteria along with the whole analysis of gut microbiota. The keys are individual efforts to improve one's lifestyle, including diet, thereby altering the overall balance of gut microbiota, and to practice health promotion and disease prevention.

## Ⅶ. Impact of *Bifidobacterium lactis* Bb-12 yogurt on improvement of intestinal environment of the elderly persons

The health effects of probiotics seem to be induce by improvement of intestinal environment, which depends on metabolites produced by probiotics or improved intestinal microbiota composition. *Bifidobacterium lactis* Bb-12 is a probiotic strain with ideal properties including acid-tolerance and adhesion to human intestinal mucosa (Matsumoto et al., 2004, Matsumoto et al., 2002). The improvement of intestinal environment by intake of *B. lactis* Bb-12 yogurt was examined using polyamine (Fig. 4), haptogloblin (Fig. 5) and mutagenecity (Fig. 5) as indexes, which directly reflect the health condition of the host (Matsumoto et al., 2001). The concentration of spermine in feces increased significantly by 3-hold ($P<0.05$) at week 2 of the Bb-12 yogurt intake compared with before administration, and that of putrscine and spermidine also tended to increase with the yogurt intake. The haptogloblin content in feces decreased significantly ($P<0.05$) at week 2 of the yogurt intake. Fecal mutagenecity was measured using fecal extract and fecal precipitate. Both preparations showed similar significant decrease ($P<0.05$) by the Bb-12 yogurt intake, as well as a negative correlation with polyamine content. Theseresult indicates that antimutagenecity due to the Bb-12 yogurt intake was not based on binding of the mutagen to the bacterial cell wall. Many reports

have suggested that polyamines increased by the Bb-12 yogurt intake led to inhibition of inflammation and mutagenecity in the human intestinal tracts.

## Ⅷ. Reference Literature


1. Fuller R. 1989. Probiotics in man and animals. J. Appl. Bacteriol., 66:365-378.
2. Hayashi H., Sakamoto M. and Benno Y. 2002. Phylogenetic analysis of the human gut micrbiota using 16S rDNA clone libraries and strictly anaerobic culture-based method. Microbiol. Immunol., 46:535-548.
3. Hayashi H., Sakamoto M. and Benno Y. 2003. Molecular analysis of fecal microbiota in elderly individuals using 16S rDNA libraries and T-RFLP. Microbiol. Immunol., 47:557-570.
4. Matsumoto M., Tadanuma T., Kume H., Imai T., Kihara R., Ohishi H., Tani H. and Benno Y. 2000. Effects of *Bifidobacterium lactis* Bb-12 on fecal microflora in the aged persons with chronic constipation. Microbial Ecol. Health Dis., 12:77-80.
5. Matsumoto M., Ohishi H. and Benno Y. 2001. Impact of LKM512 yogurt on improvement of intestinal environment of the elderly. FEMS Immunol. Med. Microbiol., 31:181-186.
6. Matsumoto M., Tani H., Ohishi H., Watanabe M. and Benno Y. 2004. H-ATPase activity in bifidobacteria with special refference to acid tolerance. Int. J. Food Microbiol., 93:109-113.
7. Matsumoto M., Tani H., Ono H., Ohishi H. and Benno Y. 2002. Adhesive property of *Bifidobacterium lactis* LKM512 and predominant bacteria of intestinal microflora to human intestinal mucin. Curr. Microbiol., 45:212-215.
8. Matsumoto M., Sakaomoto M., Hayashi H. and Benno Y. 2005. Novel phylogenetic assignment database for terminal restriction fragment length polymorphism analysis of human colonic microbiota. J. Microbol. Method, 61:305-319.
9. Sakamoto M., Hayashi H. and Benno Y. 2003. Terminal restriction fragment length polymorphism analysis for human fecal microbiota and its application for qualitative analysis of complex bifidobacterial communities. Microbiol. Immunol., 47:133-142.
10. Salminen S., Bouley M. C., Boutron-Rualt M. C., Cummings J., Franck A., Gibson G., Isolauri E., Moreau M. C., Roberfroid M. and Rowland I. 1998. Functiona food science and gastrointestinal physiology and function. Br. J. Nutr. S1:147-171.
11. Salmine S. and von Wright A. 1998. Current human probioticssaftey assured?, Microbial Ecol. Health Dis., 10:68-77.
12. Salminen S., Ouwehand A., Benno Y. and Lee, Y. K. 1998s. Probiotics - How should they be defined? Food Sci. Technol., 10:107-110.