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ORIGINAL ARTICLE

Membrane filter method to study the effects of *Lactobacillus acidophilus* and *Bifidobacterium longum* on fecal microbiota

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ABSTRACT

A large number of commensal bacteria inhabit the intestinal tract, and interbacterial communication among gut microbiota is thought to occur. In order to analyze symbiotic relationships between probiotic strains and the gut microbiota, a ring with a membrane filter fitted to the bottom was used for *in vitro* investigations. Test strains comprising probiotic nitto strains (*Lactobacillus acidophilus* NT and *Bifidobacterium longum* NT) and type strains (*L. acidophilus* JCM1132^T and *B. longum* JCM1217^T) were obtained from diluted fecal samples using the membrane filter to simulate interbacterial communication. *Bifidobacterium* spp., *Streptococcus pasteurianus*, *Collinsella aerofaciens*, and *Clostridium* spp. were the most abundant gut bacteria detected before coculture with the test strains. Results of the coculture experiments indicated that the test strains significantly promote the growth of *Ruminococcus gnavus*, *Ruminococcus torques*, and *Veillonella* spp. and inhibit the growth of *Sutterella wadsworthensis*. Differences in the relative abundances of gut bacterial strains were furthermore observed after coculture of the fecal samples with each test strain. *Bifidobacterium* spp., which was detected as the dominant strain in the fecal samples, was found to be unaffected by coculture with the test strains. In the present study, interbacterial communication using bacterial metabolites between the test strains and the gut microbiota was demonstrated by the coculture technique. The detailed mechanisms and effects of the complex interbacterial communications that occur among the gut microbiota are, however, still unclear. Further investigation of these relationships by coculture of several fecal samples with probiotic strains is urgently required.

Key words cocultivation, fecal species, interbacterial communication, probiotic strain.

The human intestinal tract contains more than 100 trillion (10^{14}) microbial cells classified into at least 1000 different species (1–3). The majority of bacteria resident in the gut influence the host's health, physiological functions, resistance to infection, and responses to endotoxins and various other stressors (1, 2). Recently, relationships between the intestinal microbiota and systemic conditions such as inflammatory bowel disease (4, 5), Crohn's disease (6, 7), obesity (8–10), diabetes (11), and autism (12–14) have been reported. Furthermore, the composition and activity of the gut microbiota are influenced by diet (15–18), age (19), and antibiotics (20). It is necessary to decipher the detailed interactions between the diversity and functions of the microbial gut community and human health.

It has recently been reported that the bifidobacteria and lactobacilli comprising the normal gut bacteria are as important as probiotics for maintaining human health (21, 22). However, the outcomes measured to determine the effects of dietary probiotics commonly

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List of Abbreviations: BL, glucose blood liver; NT, nitto strain; TYG, trypticase yeast extract glucose.

include changes in the composition of the normal gut microbiota, such as the numbers of bifidobacteria and lecithinase-positive clostridia. Such measurements do not fully take into account the functional interactions between dietary probiotics and the gut microbiota, nor do they account for the environment of the human host. A large number of commensal bacteria inhabit the intestinal tract. Therefore, in the intestine, interactions among bacteria need to be considered. However, no robust method has been developed to study such interbacterial communications among gut bacteria.

The purpose of the present study was to investigate interbacterial communications among gut microbiota. For this purpose, we described a cocultivation technique using a simple nutrient soft agar medium that allows coculture between gut bacteria and probiotic strains, with physical separation between the strains by the membrane filter method (23). Features of this technique that we optimized included the following: (i) anaerobic conditions; (ii) incubation time; and (iii) media. We compared two different test strains (*Lactobacillus acidophilus* JCM1132^T, NT and *Bifidobacterium longum* JCM1217^T, NT) for their impact on normal gut bacteria using this technique.

MATERIALS AND METHODS

Media

TYG agar (pH 7.0) comprising trypticase (10.0 g), yeast extract (5.0 g), glucose (4.0 g), Tween® 80 (0.5 g), L-cysteine·HCl·H_2O (0.5 g), 1 M $MgCl_2$ (0.4 mL), 1 M KCl (20.0 mL), 1 M NaCl (30.0 mL), 1 M NH_4Cl (19.0 mL), 1 M Na_2SO_4 (0.8 mL), 1 M Na_2HPO_4 / 1 M NaH_2PO_4 buffer, pH 7.0 (64.0 mL), agar (15.0 g or 4.0 g), and distilled water (865.8 mL) was used (23).

Preparation of bacterial strains

L. acidophilus JCM1132^T and *B. longum* JCM1217^T were used as type strains. *L. acidophilus* JCM1132^T and NT were cultured on MRS agar (Difco Laboratories Inc., Detroit, MI, USA) at 37°C for 48 hr under anaerobic conditions using Anaero Pack-Anaero (Mitsubishi Gas Chemical, Tokyo, Japan). *B. longum* JCM1217^T and NT were cultured on BL agar (Nissui Pharmaceutical Co., Tokyo, Japan) for 48 hr under the same conditions as that specified for *L. acidophilus*.

Cultivation of bacteria from human feces

All experimental procedures were carried out in a vinyl anaerobic airlock chamber (Coy Laboratory Products, Grass Lake, MI, USA), with an atmosphere composed of N_2, H_2, and CO_2 (but depleted in oxygen). Fecal samples were obtained from two healthy volunteers (two males aged 30 and 60 years) who had provided verbal informed consent. Then, 0.5 g fecal samples were immediately suspended in 4.5 mL anaerobic phosphate-buffered solution containing KH_2PO_4 (4.5 g), Na_2HPO_4 (6.0 g), L-cysteine·HCl·H_2O (0.5 g), Tween® 80 (0.5 g), and agar (1.0 g) per 1 L distilled water. Approximately 50 μL fecal samples at 10^7- and 10^8-fold dilutions were plated on BL and TYG agar. After 2 days (BL agar) and 7 days (TYG agar) of incubation under anaerobic conditions at 37°C, the colonies on these agars were counted and selected as living cells. The percentage of recovery was calculated as (average colony count on upper layer/average total colony count) × 100.

Coculture system using a membrane filter

All experimental procedures were carried out under anaerobic conditions. In a 90-mm petri dish, 150 μL of the sample mixtures (0.4% [w/v] soft TYG agar mixed with the probiotic strain at 10^5-fold dilution [140:10 v/v]) was overlaid onto 20 mL of 1.5% (w/v) agar TYG medium (probiotic strain colony counts: 100–200 CFU). A 70-mm (inner diameter) polypropylene ring with a membrane filter (pore size, 0.22 μm; Merck Millipore, Billerica, MA, USA) (Shizuko-Industry Co. Ltd, Numazu City, Japan) at the bottom was placed on the lower soft agar layer (Fig. 1). After 100 μL fecal samples at 10^7-fold dilution were plated onto the membrane filter, 9 mL of 0.4% (w/v) soft TYG agar medium was overlaid (fecal samples colony counts: 50–100 CFU). Then, plates and Anaero Pack-Anaero (Mitsubishi Gas Chemical) were immediately placed in bags filled with oxygen-free gas. After 7 days of incubation under anaerobic conditions at 37°C, the colonies on the upper layer were counted and selected as living cells. Colonies that were too close to be selected separately or that underwent surface spreading were not selected.

16S rRNA gene sequencing

The colonies that were selected on the upper layer were used to amplify the 16S ribosomal RNA (rRNA) gene from each isolate by using the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The resulting PCR products were purified with an UltraClean PCR Clean-Up Kit (MO BIO Laboratories, San Diego, CA, USA), then directly sequenced with a Big Dye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems, Foster City, CA, USA) and the ABI 3730xl DNA analyzer system (Applied Biosystems). Sequences were matched with the GenBank database using BLAST. These isolated strains

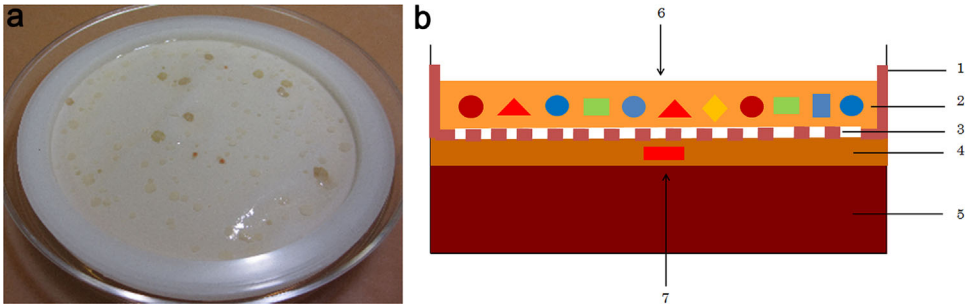

Fig. 1. Coculture system for the analysis of interbacterial interactions between probiotic or type strains and the normal gut microbiota. (a) Overview of gut bacteria colonies grown on the upper layer of the membrane filter grown from a fecal sample after 7 days of anaerobic culture at 37°C. (b) Development of detection system for the analysis of interactions between probiotic or type strains and normal gut microbiota. 1, spacer ring; 2, upper layer of soft agar medium; 3, membrane filter with 0.22 μm pore size; 4, lower layer of soft agar; 5, basal agar; 6, inoculation of fecal sample; 7, inoculation of probiotic or type strain.

shared more than 98% similarity among their 16S rRNA gene sequences.

Statistical analysis

The means of results ($n = 3$) obtained in the above tests were compared between treatment groups using Student's t-test and differences with $P < 0.05$ were considered statistically significant.

RESULTS

Colony counts

To determine the minimum number of colony counts required for accurate determination of fecal bacterial composition, we selected and counted approximately 20, 30, 50, and 70 colonies in succession under anaerobic conditions after incubation of a single fecal sample for 7 days at 37°C (Fig. 2). The number of bacterial species identified increased with the number of colonies selected until approximately 50 colonies, but the number of bacterial species and relative abundance of total bacteria were no different between analyses conducted by selecting 50 and 70 colonies. Therefore, approximately 50 colonies on the upper layer of agar were selected and counted for further analysis using this method.

Anaerobic conditions

The culture of gut bacterial species under aerobic or anaerobic conditions using the same fecal sample after

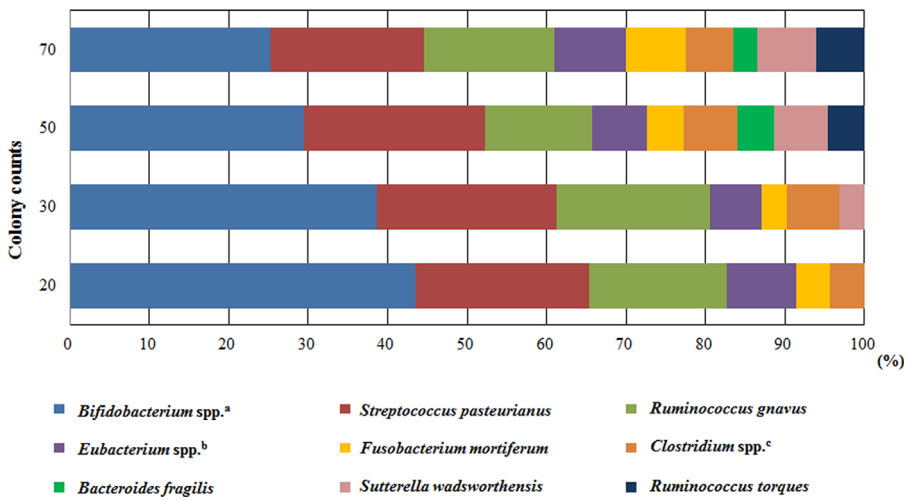

Fig. 2. Optimal number of colony counts. Comparison among the results obtained from a single fecal sample by selecting approximately either 20, 30, 50, or 70 colonies after incubation under anaerobic conditions for 7 days. Data shown represent averaged results from two replicate experiments. [a]Two species (*Bifidobacterium longum* and *Bifidobacterium pseudocatenulatum*); [b]two species (*Eubacterium limosum* and *Eubacterium callanderi*); [c]four species (*Clostridium butyricum*, *Clostridium beijerinckii*, *Clostridium roseum*, and *Clostridium diolis*).

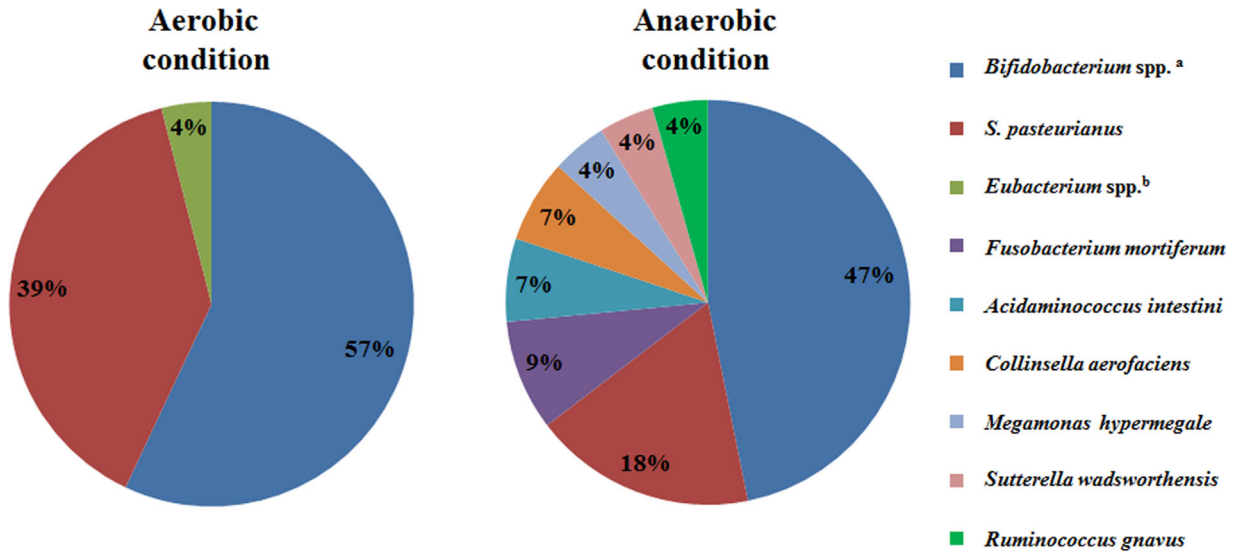

Fig. 3. Influence of aerobic and anaerobic conditions. Gut bacterial species identified from a single fecal sample following culture under either aerobic or anaerobic conditions for 7 days. Data shown represent results averaged from two replicate experiments. [a]Three species (*Bifidobacterium longum*, *Bifidobacterium pseudocatenulatum*, and *Bifidobacterium adolescentis*); [b]two species (*Eubacterium limosum* and *Eubacterium callanderi*).

incubation for 7 days at 37°C is shown in Figure 3. *Bifidobacterium* spp. and *Streptococcus pasteurianus* constituted more than 95% of the bacteria observed under aerobic incubation conditions, whereas higher proportions of anaerobic bacteria such as *Fusobacterium mortiferum*, *Acidaminococcus intestini*, *Collinsella aerofaciens*, *Megamonas hypermegale*, *Sutterella wadsworthensis*, and *Ruminococcus gnavus* were observed under anaerobic conditions.

Incubation time

Gut bacterial species identified following culture of a single fecal sample under anaerobic conditions for either 2 or 7 days at 37°C are shown in Figure 4. *Bifidobacterium* spp. and *S. pasteurianus* were the most abundant gut bacteria observed after a 2-day incubation period, whereas the number and detection rate of anaerobic bacteria such as *S. wadsworthensis*, *Eubacterium* spp., *Bacteroides ovatus*, *Eggerthella lenta*, and *M. hypermegale* tended to be higher after 7 days of incubation compared with those observed after 2 days of incubation. The results indicated that these anaerobic bacteria must have been able to grow gradually between 2 and 7 days of incubation under anaerobic conditions.

Media

In order to investigate whether different species of gut bacteria would be detected on different nutrient agars, we cultivated bacteria from human feces under relatively simple nutrient conditions (TYG agar, 7-day incubation period) and compared the growth of gut bacteria with that on BL agar (2-day incubation period), previously used to isolate gut bacteria (Fig. 5). *Bacteroides* spp. on BL agar and *Bifidobacterium* spp. on TYG agar were the most abundant gut bacteria observed, comprising more than 25% of total bacteria. To further investigate the effects of different nutrient conditions on bacterial growth, we examined the impacts of adding horse blood and Fildes Enrichment Solution. The horse blood digested by pepsin (41) was added to TYG agar (5% v/v) on the gut species obtained from culture of a single fecal sample (Fig. 6). The results showed that the relative abundance of *Bacteroides* spp. tended to increase following inoculation of the horse blood. Additionally, the relative abundance of *Bacteroides* spp. increased to more than 90% after inoculation of the Fildes Enrichment Solution.

Effects of coculture with NT and type strains of *L. acidophilus* and *B. longum* on the diversity of human gut microbiota

Changes in the gut bacterial species observed following coculture with NT and type strains (*L. acidophilus* JCM1132^T, NT, and *B. longum* JCM1217^T, NT) are shown in Table 1. The findings showed that coculture with these strains significantly promoted the growth of *R. gnavus*, *R. torques*, and *Veillonella* spp. and inhibited

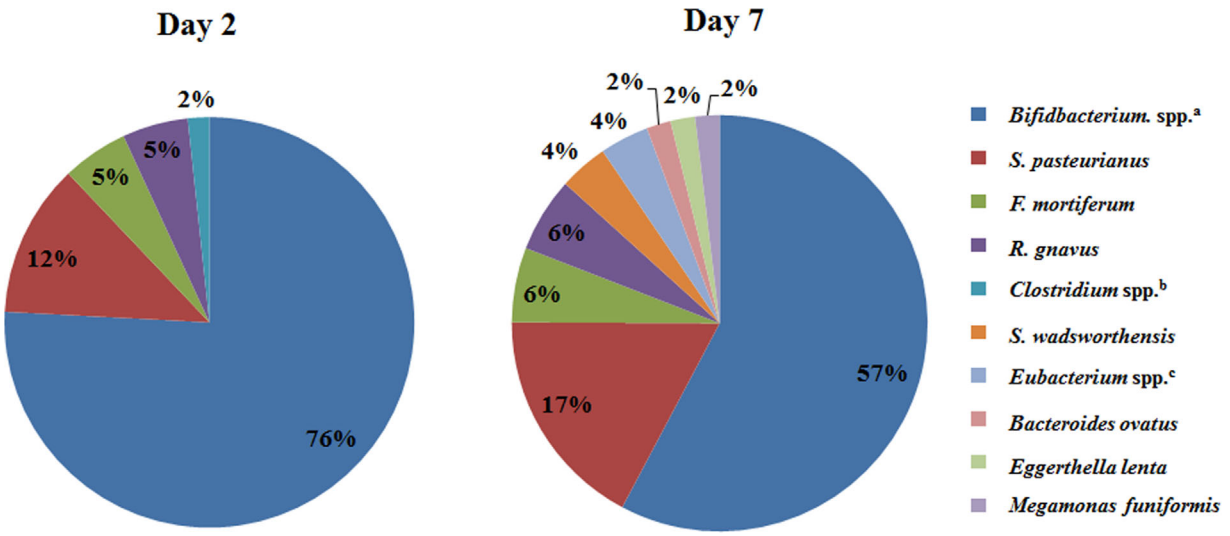

Fig. 4. Influence of cultivation time. Gut bacterial species identified from a single fecal sample following culture for either 2 or 7 days under anaerobic conditions. Data shown represent results averaged from two replicate experiments. [a]Two species (*Bifidobacterium longum* and *Bifidobacterium pseudocatenulatum*); [b]four species (*Clostridium butyricum*, *Clostridium beijerinckii*, *Clostridium roseum*, and *Clostridium diolis*); [c]two species (*Eubacterium limosum* and *Eubacterium callanderi*).

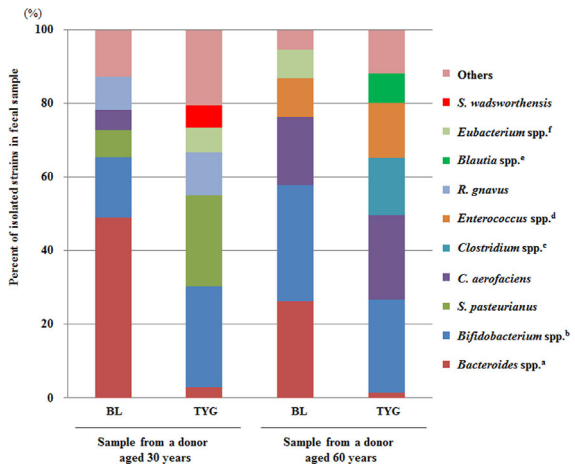

Fig. 5. Effects of different nutrient agar types on bacterial growth. Gut bacterial species identified on either BL or TYG agar under anaerobic conditions (representing fecal samples from two donors aged 30 and 60 years, respectively). Data shown represent results averaged from two replicate experiments. [a]Eight species (*Bacteroides dorei*, *Bacteroides ovatus*, *Bacteroides uniformis*, *Bacteroides fragilis*, *Bacteroides eggerthii*, *Bacteroides coprocola*, *Bacteroides vulgatus*, and *Bacteroides plebeius*); [b]three species (*Bifidobacterium longum*, *Bifidobacterium pseudocatenulatum*, and *Bifidobacterium adolescentis*); [c]four species (*Clostridium butyricum*, *Clostridium beijerinckii*, *Clostridium roseum*, and *Clostridium diolis*); [d]four species (*Enterococcus durans*, *Enterococcus faecium*, *Enterococcus lactis*, and *Enterococcus faecalis*); [e]two species (*Blautia luti* and *Blautia wexlerae*); [f]two species (*Eubacterium limosum* and *Eubacterium callanderi*). 'Others' includes strains present at proportions of <5.0%.

that of *S. wadsworthensis* ($P < 0.05, 0.01$). Coculture with the probiotic strains increased the detection rate of *R. gnavus* more than did coculture with each type strain. Although the detection rate of *S. wadsworthensis* was almost the same following coculture with each of *B. longum* NT and JCM1217^T, coculture with *L. acidophilus* NT significantly decreased the detection rate of *S. wadsworthensis*, compared with that observed following coculture with JCM1132^T. Additionally, the *Bifidobacterium* spp. that were observed on the upper layer of agar following incubation were not affected by coculture with these strains.

DISCUSSION

The human intestinal tract contains more than 100 trillion (10^{14}) microbial cells classified into at least 1000 different species (1, 2). Advances in culture-independent molecular techniques have suggested a relationship between the gut microbiota and the pathogenesis of various diseases (4–11). However, over 70–80% of the total number of gut bacterial species have not been cultivated in spite of the development of culture and molecular culture-independent techniques (3, 24). The reasons as-yet-uncultured bacteria remain unclear are speculated to include the following: (i) anaerobic culture techniques require special training for investigators (25, 26); (ii) most of the microorganisms in the human intestinal tract are highly oxygen-sensitive anaerobes (27–29); and (iii) multiple interbacterial communications exist in the gut microbiota. Ohno

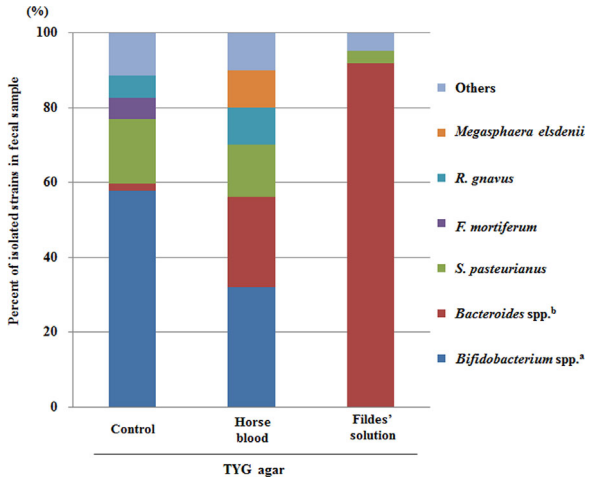

Fig. 6. Effects of different nutrient conditions on bacterial growth. Effects of the addition of horse blood and Fildes Enrichment Solution to TYG agar (5% v/v) on gut species obtained from culture of a single fecal sample. Data shown represent results averaged from two replicate experiments. [a]Two species (*Bifidobacterium longum* and *Bifidobacterium pseudocatenulatum*); [b]six species (*Bacteroides dorei*, *Bacteroides ovatus*, *Bacteroides uniformis*, *Bacteroides fragilis*, *Bacteroides thetaiotaomicron*, and *Bacteroides caccae*). 'Others' includes strains present at proportions of <5.0%.

et al. (30) reported that the growth of *Symbiobacterium thermophilum* is accelerated by the addition of supernatants from *Bacillus* cultures. Watanabe *et al.* (31) reported that the growth of *Phascolarctobacterium* spp. is stimulated by succinate-producing *Paraprevotella* spp., and the metabolic products of these bacteria have been considered important factors in interbacterial communication, being capable of promoting or inhibiting the growth of other bacteria. Recently, novel cultivation devices have been developed to facilitate the study of cell–cell symbiosis in soil, sludge, and aquatic environments (32–34). However, these cultivation methods have not yet been applied to the isolation of gut bacteria. Therefore, no robust method has been developed specifically for the study of interbacterial communications among gut bacteria.

In the present study, we investigated the interactions between probiotic strains and normal intestinal bacteria. For this purpose, we used the *in vitro* membrane filter method (23) to reveal interbacterial communications between gut bacteria and probiotic strains.

Anaerobic conditions

Most of the microorganisms in the human intestinal tract are highly oxygen-sensitive anaerobes (29, 35). Duncan *et al.* (36) reported that *Roseburia intestinalis* strains, butyrate-producing anaerobic bacteria from human feces, all failed to grow after a minimum exposure time of 2 min to air, whereas these same strains maintained continuously under anaerobic conditions showed good growth. Total colony counts for human and animal fecal samples incubated using the roll tube and glove box methods of anaerobic culture were approximately two- to 10-fold greater than those for the same samples incubated on agar plates using the more widely reported anaerobic jars method (26, 37). Furthermore, the colony counts for fecal samples incubated using the plate-in-bottle method, which enables the growth of strictly fastidious anaerobes possible, were often slightly higher than those for samples grown using the roll tube method (38, 39). Therefore, the precise methods used for the anaerobic culture of fecal samples are considered an important factor in determining the bacterial species obtained. In order to examine the influence of the anaerobic condition on the bacterial species obtained following the culture of fecal samples in the present study, the relative abundances of gut bacterial species were detected following culture of a single fecal sample under either aerobic or anaerobic conditions. The findings showed that there were many highly oxygen-sensitive anaerobes on the upper layer of agar, which were detected at a higher rate following culture under anaerobic conditions than under aerobic conditions (Fig. 3). This result indicates that the growth of highly oxygen-sensitive gut bacteria was inhibited by the presence of oxygen during the experimental procedure. Therefore, this technique needs to be carried out under anaerobic conditions.

Incubation time

As the gastrointestinal tract in humans contains more than 100 trillion commensal microbes classified into at least 1000 different species (1), the different growth rates of gut bacterial species in culture need to be considered for their study. Moore and Holdeman (29) conducted an investigation into the gut microbiota of 20 healthy Japanese-Hawaiian males using the anaerobic roll tube method and characterized a total of 1147 isolates comprising 113 fast- and slow-growing gut bacterial species after a total of 5 days of incubation at 37°C. Additionally, Tanaka and Benno (23) cultivated a single sample of human feces using TYG agar and Eggerth-Gagnon (EG) agar, which has been used previously to isolate gut bacteria. Over 70% of the maximum number of colonies detected on EG agar was obtained after 2 days of incubation, with the number of colonies reaching a plateau after 7 days of incubation. In contrast, the colony counts on TYG agar increased until 14 days of incubation; however, the final colony counts of both media were nearly equivalent.

In order to examine the effects of different incubation times the present study, we cultivated a single fecal sample for either 2 or 7 days at 37°C under anaerobic conditions. Detection of anaerobic gut bacteria on the upper layer of agar was higher after 7 days of incubation than after 2 days of incubation (Fig. 4). In fact, the gut bacteria obtained after a 2-day incubation period mostly comprised *Bifidobacterium* spp. We surmised that *Bifidobacterium* spp. used the glucose, which was the sole source of energy in TYG agar, and therefore grew more rapidly, thus dominating the cultures after the first 2 days of incubation. In addition, the growth rates of slow-growing gut bacteria were gradually accelerated by the metabolites (lactate and acetate etc.) produced by *Bifidobacterium* spp., resulting in a high detection rate of these slow-growing species after 7 days of incubation. We observed interbacterial interactions among gut bacteria through bacterial metabolites, and we suggest that our coculture technique can play a major role in examining the diversity of gut microbiota.

Media

TYG agar is a nutrient-restricted and high-buffer-capacity medium that can limit the changes in pH caused by the metabolites produced by fast-growing gut bacteria (23). We compared the gut bacterial species detected following incubation of a fecal sample using BL and TYG agar (Fig. 5). The results showed that *Bacteroides* spp. on BL agar and *Bifidobacterium* spp. on TYG agar were the most abundantly detected gut bacteria (comprising more than 25% of total bacteria). BL agar contains horse blood at the final concentration of 5% (v/v). Horse blood contains hemoglobin, vitamins, and minerals (40). We surmised that the growth of *Bacteroides* spp. in the fecal samples was accelerated by the addition of the horse blood. In conformity with this hypothesis, we examined the impacts of adding horse blood and Fildes Enrichment Solution; the horse blood was digested with pepsin (41) and added to TYG agar at a final concentration of 5% (v/v) on the gut bacterial species detected following the culture of a single fecal sample (Fig. 6). The results showed that the relative abundance of *Bacteroides* spp. was increased by the addition of these agents. It has been reported that the growth of most *Bacteroides* spp. is stimulated by the addition of hemin, vitamin K1, and bile (42–45). Although the growth of most *Bacteroides* spp. was promoted, the growth rates of other gut bacteria, such as some *Clostridium*, *Eubacterium*, *Bifidobacterium* species, were inhibited in the presence of bile (44). Therefore, we did not include bile as an additive in the present study, given that the objective was to obtain as high a diversity of gut bacteria as possible. The specific growth factors in horse blood that promoted the growth of *Bacteroides* spp. in the present study are still unclear, and further research is required to identify these.

Effects of NT and type strains of *L. acidophilus* and *B. longum* on the diversity of human gut microbiota

Changes in gut bacterial species detected following coculture with NT and type strains of *L. acidophilus* and *B. longum* are shown in Table 1. After coculture with these strains, the relative abundances of *R. gnavus*, *R. torques*, and *Veillonella* spp. were significantly increased, whereas that of *S. wadsworthensis* was significantly decreased. The bacterial species whose growth was increased by coculture with these strains were selected and passaged on TYG agar under anaerobic conditions. *R. gnavus* and *R. torques* formed colonies, but *Veillonella* spp. did not. It has been reported that *Ruminococcus* spp. produce acetate and formate from peptone-yeast (PY) glucose broth cultures (46), whereas *Veillonella* spp. produce acetic and propionic acids, CO_2, and H_2 from lactate (47). Therefore, we surmised that *R. gnavus* and *R. torques* formed colonies by using glucose in TYG agar, whereas *Veillonella* spp. could not colonize TYG agar because it does not include lactate. Additionally, the growth of *Veillonella* spp. on the upper layer of agar was accelerated significantly by coculture with these strains. As hypothesized, this observation indicates that the metabolites of these strains, such as lactate and acetate, may influence the growth rates of gut bacteria. However, the interbacterial interactions among gut bacteria on the upper layer of agar through bacterial metabolites should also be considered. Tanaka and Benno (23) reported that the growth of *Bacteroides fragilis*, which promoted the growth of *Phascolarctobacterium* sp. BL377, was itself stimulated by *Sutterella* sp. BL252 through multistep interbacterial interactions among gut bacteria. Although some possibilities have been discussed, the specific factors influencing the growth of *Veillonella* spp. could not be identified with certainty based on these results. Further research is required for a satisfactory explanation.

Additionally, the *Bifidobacterium* spp. that were the most abundantly detected gut bacteria on the upper layer of agar following incubation of fecal samples without coculture were not changed by coculture with the NT or type strains. It has been discussed that the changes in the number and incidence of *Bifidobacterium* spp. in the human intestinal tract were used as the main index to improve gut microbiota following the dietary administration of probiotic strains. Benno and Mitsuoka (48)

Table 1. Effects of coculture with NT and type strains of *Lactobacillus acidophilus* and *Bifidobacterium longum* on species composition of human gut microbiota

Sample	Bacterial species	Control	*L. acidophilus*		*B. longum*	
			NT	JCM1132^T	NT	JCM1217^T
30-year-old donor	*Ruminococcus gnavus*	9.64 ± 0.09[§] (3/3)[¶]	9.90 ± 0.05** (3/3)	9.81 ± 0.03* (3/3)	9.83 ± 0.03* (3/3)	9.77 ± 0.03 (3/3)
	Ruminococcus torques	9.31 ± 0.10 (3/3)	9.64 ± 0.09* (3/3)	9.70 ± 0.05** (3/3)	9.69 ± 0.12* (3/3)	9.62 ± 0.06** (3/3)
	Sutterella wadsworthensis	9.37 ± 0.10 (3/3)	8.95** (1/3)	9.11 ± 0.28 (3/3)	9.05 ± 0.17* (3/3)	9.06 ± 0.17* (3/3)
60-year-old donor	*Ruminococcus gnavus*	9.15 ± 0.17 (3/3)	9.51 ± 0.07* (3/3)	9.47 ± 0.07* (3/3)	9.47 ± 0.07* (3/3)	9.45 ± 0.17 (3/3)
	Veillonella spp.[†]	0 (0/3)	9.51 ± 0.07** (3/3)	9.41 ± 0.15** (3/3)	9.37 ± 0.10** (3/3)	9.31 ± 0.10** (3/3)
	Enterococcus spp.[‡]	9.79 ± 0.10 (3/3)	9.51 ± 0.07* (3/3)	9.54 ± 0.11 (3/3)	9.60 ± 0.16 (3/3)	9.62 ± 0.06 (3/3)

Asterisks (*[$P < 0.05$] and **[$P < 0.01$]) indicate significant differences in species composition following coculture with probiotic strains and type strains, compared with the control.
Change in the number and incidence of bacterial species isolated from NT and type strains of *L. acidophilus* and *B. longum* and gut microbiota using the membrane filter method (representing fecal samples from two donors aged 30 and 60 years, respectively). Data shown represent results averaged from three replicate experiments.
[†]Two species (*V. parvula* and *V. dispar*); [‡]four species (*E. durans*, *E. faecium*, *E. lactis*, and *E. faecalis*); [§]bacterial count expressed as mean ± SD of log10 per gram of wet feces; [¶]detection rate (no. subjects yielding the organism/no. subjects examined).

investigated the effectiveness of giving oral *B. longum* to five healthy volunteers (average age: 31.5 years) by examination at the species level and confirmed an increase in the number of *Bifidobacterium* spp. in the gut microbiota. Matsumoto *et al.* (49) administered yogurt including *Bifidobacterium lactis* LKM512 to 11 elderly volunteers (average age: 76.9 years), and reported that LKM 512 temporarily colonized the gut, resulting in an increased frequency of defecation and an increased number of *Bifidobacterium* spp. in the gut microbiota. However, the mechanisms of interaction between orally given probiotic strains and gut microbiota are not explained by the present data and further research is required to determine why *Bifidobacterium* spp. in the intestinal tract are increased by giving probiotic strains. As mentioned earlier, our intestinal tract contains more than 100 trillion (10^{14}) microbial cells, and complex interbacterial communications among gut bacteria have been suggested (50). These results suggest that these probiotic strains do not directly produce an increase in the number of *Bifidobacterium* spp. inhabiting the intestinal tract, but that complex interbacterial communications occur between these probiotics and the gut microbiota.

We have reported here on the use of a novel cocultivation technique in which probiotic or type strains are physically separated from gut bacteria by using the membrane filter method *in vitro*. We observed interbacterial communications between probiotic strains and gut microbiota through bacterial metabolites by using this technique. However, the detailed mechanisms and effects of the complex interbacterial communications that occur among gut microbiota are still unclear. Further study of these relationships is urgently required by investigating cocultures between several fecal samples and probiotic strains.

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DISCLOSURE

No authors have any conflicts of interest to declare.

REFERENCES

1. Qin J., Li R., Raes J., Arumugam M., Burgdorf K.S., Manichanh C., Nielsen T., Pons N., Levenez F., Yamada T., Mende D.R., Li J., Xu J., Li S., Li D., Cao J., Wang B., Liang H., Zheng H., Xie Y., Tap J., Lepage P., Bertalan M., Batto J.M., Hansen T., Le Paslier D., Linneberg A., Nielsen H.B., Pelletier E., Renault P., Sicheritz-Ponten T., Turner K., Zhu H., Yu C., Li S., Jian M.,

Zhou Y., Li Y., Zhang X., Li S., Qin N., Yang H., Wang J., Brunak S., Doré J., Guarner F., Kristiansen K., Pedersen O., Parkhill J., Weissenbach J.; MetaHIT Consortium, Bork P., Ehrlich S.D., Wang J. (2010) A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* **464**: 59–65.
2. Egert M., de Graaf A.A., Smidt H., de Vos W.M., Venema K. (2006) Beyond diversity: functional microbiomics of the human colon. *Trends Microbiol* **14**: 86–91.
3. Kurokawa K., Itoh T., Kuwahara T., Oshima K., Toh H., Toyoda A., Takami H., Morita H., Sharma V.K., Srivastava T.P., Taylor T.D., Noguchi H., Mori H., Ogura Y., Ehrlich D.S., Itoh K., Takagi T., Sakaki Y., Hayashi T., Hattori M. (2007) Comparative metagenomics revealed commonly enriched gene sets in human gut microbiomes. *DNA Res* **14**: 169–181.
4. Tannock G.W. (2008) Molecular analysis of the intestinal microflora in IBD. *Mucosal Immunol* **1**: S15–18.
5. Png C.W., Lindén S.K., Gilshenan K.S., Zoetendal E.G., McSweeney C.S., Sly L.I., McGuckin M.A., Florin T.H. (2010) Mucolytic bacteria with increased prevalence in IBD mucosa augment *in vitro* utilization of mucin by other bacteria. *Am J Gastroenterol* **105**: 2420–8.
6. Li Q., Wang C., Tang C., Li N., Li J. (2012) Molecular-phylogenetic characterization of the microbiota in ulcerated and non-ulcerated regions in the patients with Crohn's disease. *PLoS ONE* **7**: e34939.
7. Shiga H., Kajiura T., Shinozaki J., Takagi S., Kinouchi Y., Takahashi S., Negoro K., Endo K., Kakuta Y., Suzuki M., Shimosegawa T. (2012) Changes of faecal microbiota in patients with Crohn's disease treated with an elemental diet and total parenteral nutrition. *Dig Liver Dis* **44**: 736–42.
8. Ley R.E., Bäckhed F., Turnbaugh P., Lozupone C.A., Knight R. D., Gordon J.I. (2005) Obesity alters gut microbial ecology. *Proc Natl Acad Sci USA* **102**: 11,070–5.
9. Turnbaugh P.J., Bäckhed F., Fulton L., Gordon J.I. (2008) Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. *Cell Host Microbe* **3**: 213–23.
10. Yoshimoto S., Loo T.M., Atarashi K., Kanda H., Sato S., Oyadomari S., Iwakura Y., Oshima K., Morita H., Hattori M., Honda K., Ishikawa Y., Hara E., Ohtani N. (2013) Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. *Nature* **499**: 97–101.
11. Wen L., Ley R.E., Volchkov P.Y., Stranges P.B., Avanesyan L., Stonebraker A.C., Hu C., Wong F.S., Szot G.L., Bluestone J.A., Gordon J.I., Chervonsky A.V. (2008) Innate immunity and intestinal microbiota in the development of Type 1 diabetes. *Nature* **455**: 1109–13.
12. Parracho H.M., Bingham M.O., Gibson G.R., McCartney A.L. (2005) Differences between the gut microflora of children with autistic spectrum disorders and that of healthy children. *J Med Microbiol* **54**: 987–91.
13. Finegold S.M., Dowd S.E., Gontcharova V., Liu C., Henley K.E., Wolcott R.D., Youn E., Summanen P.H., Granpeesheh D., Dixon D., Liu M., Molitoris D.R., Green J.A. 3rd. (2010) Pyrosequencing study of fecal microflora of autistic and control children. *Anaerobe* **16**: 444–53.
14. Heberling C.A., Dhurjati P.S., Sasser M. (2013) Hypothesis for a systems connectivity model of autism spectrum disorder pathogenesis: Links to gut bacteria, oxidative stress, and intestinal permeability. *Med Hypotheses* **80**: 264–70.
15. De Filippo C., Cavalieri D., Paola M.D., Ramazzotti M., Poullet J.B., Massart S., Collini S., Pieraccini G., Lionetti P. (2010) Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc Natl Acad Sci USA* **107**: 14,691–6.
16. Wu G.D., Chen J., Hoffmann C., Bittinger K., Chen Y.Y., Keilbaugh S.A., Bewtra M., Knights D., Walters W.A., Knight R., Sinha R., Gilroy E., Gupta K., Baldassano R., Nessel L., Li H., Bushman F.D., Lewis J.D. (2011) Linking long-term dietary patterns with gut microbial enterotypes. *Science* **334**: 105–8.
17. Claesson M.J., Jeffery I.B., Conde S., Power S.E., O'Connor E.M., Cusack S., Harris H.M., Coakley M., Lakshminarayanan B., O'Sullivan O., Fitzgerald G.F., Deane J., O'Connor M., Harnedy N., O'Connor K., O'Mahony D., van Sinderen D., Wallace M., Brennan L., Stanton C., Marchesi J.R., Fitzgerald A.P., Shanahan F., Hill C., Ross R.P., O'Toole P.W. (2012) Gut microbiota composition correlates with diet and health in the elderly. *Nature* **488**: 178–84.
18. David L.A., Maurice C.F., Carmody R.N., Gootenberg D.B., Button J.E., Wolfe B.E., Ling A.V., Devlin A.S., Varma Y., Fischbach M.A., Biddinger S.B., Dutton R.J., Turnbaugh P.J. (2014) Diet rapidly and reproducibly alters the human gut microbiome. *Nature* **505**: 559–63.
19. Biagi E., Nylund L., Candela M., Ostan R., Bucci L., Pini E., Nikkïla J., Monti D., Satokari R., Franceschi C., Brigidi P., De Vos W. (2010) Through ageing, and beyond: gut microbiota and inflammatory status in seniors and centenarians. *PLoS ONE* **5**: e10667.
20. Dethlefsen L., Relman D.A. (2011) Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *Proc Natl Acad Sci USA* **108**: 4554–61.
21. Kondo J., Xiao J.Z., Shirahata A., Baba M., Abe A., Ogawa K., Shimoda T. (2013) Modulatory effects of *Bifidobacterium longum* BB536 on defecation in elderly patients receiving enteral feeding. *World J Gastroenterol* **19**: 2162–70.
22. Lidbeck A., Allinger U.G., Orrhage K.M., Ottova L., Brismar B., Gustafsson J.Å., Rafter J.J., Nord C.E. (1991) Impact of *Lactobacillus acidophilus* supplements on the faecal microflora and soluble faecal bile acids in colon cancer patients. *Microbial Ecol Health Dis* **4**: 81–8.
23. Tanaka Y., Benno Y. (2015) Application of a single-colony coculture technique to the isolation of unculturable gut bacteria. *Microbiol Immunol* **59**: 63–70.
24. Eckburg P.B., Bik E.M., Bernstein C.N., Purdom E., Dethlefsen L., Sargent M., Gill S.R., Nelson K.E., Relman D.A. (2005) Diversity of the human intestinal microbial flora. *Science* **308**: 1635–8.
25. Bryant M.P. (1972) Commentary on the Hungate technique for culture of anaerobic bacteria. *Am J Clin Nutr* **25**: 1324–8.
26. Arank A., Syed S.A., Kenney E.B., Freter R. (1969) Isolation of anaerobic bacteria from human gingiva and mouse cecum by means of a simplified glove box procedure. *Appl Microbiol* **17**: 568–76.
27. Spears R.W., Freter R. (1967) Improved isolation of anaerobic bacteria from the mouse cecum by maintaining continuous strict anaerobiosis. *Exp Biol Med* **124**: 903–9.
28. Gordon D.F., Stutman M., Loesche W.J. (1971) Improved isolation of anaerobic bacteria from the gingival crevice area of man. *Appl Microbiol* **21**: 1046–50.
29. Moore W.E.C., Holdeman L.V. (1974) Human fecal flora: the normal flora of 20 Japanese-Hawaiians. *Appl Environ Microbiol* **27**: 961–79.
30. Ohno M., Okano I., Watsuji T., Kakinuma T., Ueda K., Beppu T. (1999) Establishing the independent culture of a strictly symbiotic bacterium *Symbiobacterium thermophilum* from its

supporting *Bacillus* strain. *Biosci Biotechnol Biochem* **63**: 1083–90.
31. Watanabe Y., Nagai F., Morotomi M. (2012) Characterization of *Phascolarctobacterium succinatutens* sp. nov., an asaccharolytic, succinate-utilizing bacterium isolated from human feces. *Appl Environ Microbiol* **78**: 511–18.
32. Aoi Y., Kinoshita T., Hata T., Ohta H., Obokata H., Tsuneda S. (2009) Hollow-fiber membrane chamber as a device for in situ environmental cultivation. *Appl Environ Microbiol* **75**: 3826–33.
33. Kaeberlein T., Lewis K., Epstein S.S. (2002) Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. *Science* **296**: 1127–9.
34. Zengler K., Toledo G., Rappe M., Elkins J., Mathur E.J., Short J.M., Keller M. (2002) Cultivating the uncultured. *Proc Natl Acad Sci USA* **99**: 15,681–6.
35. Rosenblatt J.E., Fallon A., Finegold S.M. (1973) Comparison of methods for isolation of anaerobic bacteria from clinical specimens. *Appl Microbiol* **25**: 77–85.
36. Duncan S.H., Aminov R.I., Scott K.P., Louis P., Stanton T.B., Flint H.J. (2006) Proposal of *Roseburia faecis* sp. nov., *Roseburia hominis* sp. nov. and *Roseburia inulinivorans* sp. nov., based on isolates from human faeces. *Int J Syst Evol Microbiol* **56**: 2437–41.
37. Vervaeke I.J., Van Nevel C.J. (1972) Comparison of three techniques for the total count of anaerobes from intestinal contents of pigs. *Appl Microbiol* **24**: 513–15.
38. Mitsuoka T., Morishita Y., Terada A., Yamamoto S. (1969) A simple method ("Plate-in-Bottle Method") for the cultivation of fastidious anaerobes. *J Micro* **13**: 383–5.
39. Mitsuoka T., Ohno K., Benno Y., Suzuki K., Namba K. (1976) Die Faekalflora bei Menschen. IV. Mitteilung: Vergleich des neu entwickelten Verfahrens mit dem bisherigen üblichen Verfahren zur Darmfloraanalyse. *Zentralbl Bakteriol Orig A* **234**: 219–33.
40. Mitsuoka T., Sega T., Yamamoto S. (1965) Eine verbesserte Methodik der qualitativen und quantitativen Analyse der Darmflora von Menschen und Tieren. *Zentralbl Bakteriol Orig* **195**: 455–69.
41. Fildes P. (1920) A new medium for the growth of *B. influenza*. *Br J Exp Pathol* **1**: 129–30.
42. Macy J., Probst I., Gottschalk G. (1975) Evidence for cytochrome involvement in fumarate reduction and adenosine 5′-triphosphate synthesis by *Bacteroides fragilis* grown in the presence of hemin. *J Bacteriol* **123**: 436–42.
43. Sperry J.F., Appleman M.D., Wilkins T.D. (1977) Requirement of heme for growth of *Bacteroides fragilis*. *Appl Environ Microbiol* **34**: 386–90.
44. Holdeman L.V., Moore W.E.C. (1975) Gram-negative/positive nonsporing rods and Clostridia. *Anaerobe Laboratory Manual*, 3rd edn. Blacksburg, VI: Virginia Polytechnic Institute and State University, pp. 20–89.
45. Whitman W.B., Parte A.C. (2009) *Bergey's Manual of Systematic Bacteriology*, 2nd edn. New York: Springer.
46. Moore W.E.C., Johnson J.L., Holdeman L.V. (1976) Emendation of *Bacteroidaceae* and *Butyrivibrio* and descriptions of *Desulfornonas* gen. nov. and ten new species in the genera *Desulfomonas, Butyrivibrio, Eubacterium, Clostridium*, and *Ruminococcus*. *Int J Syst Bacteriol* **26**: 238–52.
47. Rogosa M., Bishop F.S. (1964) The genus *Veillonella* II. Nutritional Studies. *J Bacteriol* **87**: 574–80.
48. Benno Y., Mitsuoka T. (1992) Impact of *Bifidobacterium longum* on human fecal microflora. *Microbiol Immunol* **36**: 683–94.
49. Matsumoto M., Tadenuma T., Nakamura K., Kume H., Imai T., Kihara R., Watanabe M., Benno Y. (2000) Effect of *Bifidobacterium lactis* LKM 512 yoghurt on fecal microflora in middle to old aged persons. *Microbial Ecol Health Dis* **12**: 77–80.
50. Friedman J., Alm E.J. (2012) Inferring correlation networks from genomic survey data. *PLoS Comput Biol* **8**: e1002687.