Gut Dysbiosis and Detection of "Live Gut Bacteria" in Blood of Japanese Patients With Type 2 Diabetes

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OBJECTIVE

Mounting evidence indicates that the gut microbiota are an important modifier of obesity and diabetes. However, so far there is no information on gut microbiota and "live gut bacteria" in the systemic circulation of Japanese patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS

Using a sensitive reverse transcription–quantitative PCR (RT-qPCR) method, we determined the composition of fecal gut microbiota in 50 Japanese patients with type 2 diabetes and 50 control subjects, and its association with various clinical parameters, including inflammatory markers. We also analyzed the presence of gut bacteria in blood samples.

RESULTS

The counts of the *Clostridium coccoides* group, *Atopobium* cluster, and *Prevotella* (obligate anaerobes) were significantly lower ($P < 0.05$), while the counts of total *Lactobacillus* (facultative anaerobes) were significantly higher ($P < 0.05$) in fecal samples of diabetic patients than in those of control subjects. Especially, the counts of *Lactobacillus reuteri* and *Lactobacillus plantarum* subgroups were significantly higher ($P < 0.05$). Gut bacteria were detected in blood at a significantly higher rate in diabetic patients than in control subjects (28% vs. 4%, $P < 0.01$), and most of these bacteria were Gram-positive.

CONCLUSIONS

This is the first report of gut dysbiosis in Japanese patients with type 2 diabetes as assessed by RT-qPCR. The high rate of gut bacteria in the circulation suggests translocation of bacteria from the gut to the bloodstream.

The gut microbiota are essential for the host immune system (1), digestion, including the breakdown of complex carbohydrates such as dietary fibers, and the production of organic acids to maintain an appropriate pH environment in the gut (2). The study of gut microbiota is rapidly progressing, and it is no exaggeration to say that the introduction of culture-independent approaches based on 16S rRNA analysis has led to a paradigm shift in this field (2,3). In addition to its physiological importance, gut dysbiosis is associated with obesity through the increased availability of energy-rich foods such as Western diet (4–6). Together with previous data, new information on the pathophysiological roles of the gut and blood microbiota in

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the development of atherosclerosis have been reported (7–9). The important concept of the role of gut microbiota in insulin resistance was first described by Cani and colleagues (4,10–13). In a series of studies, these investigators demonstrated that intestinal Gram-negative bacteria produced lipopolysaccharide (LPS), which is a well-known proinflammatory molecule, can translocate to the bloodstream from a leaky gut, and causes metabolic endotoxemia, which is associated with obesity (4). More specifically, a high-fat diet enhances the disruption of the tight junction proteins in the intestine, such as zonula occludens-1 and occludin, that are involved in the gut barrier function in mouse models (13). This effect is directly dependent on the gut microbiota because antibiotic treatment abolished diet-induced gut permeability (4). The above studies suggest that gut dysbiosis and the related increased permeability of the gut could serve as environmental factors for the development of obesity before the development of diabetes. While data from mice are convincing, the functional links between human gut microbiota and disease are less well-understood because of various confounding factors, including age, sex, diet, genetics, and race (14,15).

In Japan, the estimated number of patients with type 2 diabetes has increased to 10.7 million, the sixth largest such population in the world (16). However, there were no human studies on gut microbiota composition in Japanese patients with type 2 diabetes. The Japanese diet is very different from the Western diet, and gut microbiota are known to be influenced by food type (6), suggesting that gut microbiota in Japanese patients with type 2 diabetes might be different from those of Western individuals. Therefore, it is important to investigate gut microbiota composition in Japanese patients with type 2 diabetes.

Intriguingly, Amar et al. (17) reported that a high-fat diet induces bacterial translocation from the gut to the bloodstream in humans by detecting bacterial genomic DNA encoding 16S rRNA. However, there is so far no evidence that "live bacteria" are translocated from the gut to the systemic circulation.

In the current study, we analyzed fecal gut microbiota composition and plasma levels of gut bacteria in 50 Japanese patients with type 2 diabetes and 50 control subjects by detecting 16S rRNA. We also investigated the relation among various clinical parameters, food intake, and gut microbiota to determine the clinical significance of gut microbiota in Japanese patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS

Study Subjects

We recruited Japanese type 2 diabetic patients who regularly visited the Outpatient Clinic of Juntendo University Hospital between 2011 and 2012, and 50 patients agreed to participate in the study. We also recruited control subjects who regularly visited the Outpatient Clinic of Juntendo University Hospital for the management of hypertension, dyslipidemia, or thyroid disease, whose conditions were under good control or who visited Juntendo University Hospital for routine medical checkups between 2011 and 2012. Fifty control subjects with HbA_{1c} levels $<$6.0% (42 mmol/mol) agreed to participate. Patients with the following conditions were excluded from the study: *1*) proliferative retinopathy; *2*) age $\geq$80 years; *3*) serious liver disease (aspartate aminotransferase and/or alanine aminotransferase level $>$100 IU/L) or serious kidney disease (serum creatinine level $>$2.0 mg/dL; *4*) acute heart failure; *5*) malignancy; *6*) inflammatory bowel disease; and *7*) a history of treatment with antibiotics within 3 months of study participation. The study protocol was approved by the Human Ethics Committee of Juntendo University, and written informed consent was obtained from each patient before enrollment in the study.

Determination of the Bacterial Count by 16S rRNA-Targeted Reverse Transcription–Quantitative PCR

After study enrollment, the participants were asked to submit fresh fecal samples. The fecal samples were placed directly into two tubes (~1.0 g/tube) by the participants or hospital staff members; one tube contained 2 mL of RNA*later* (an RNA stabilization solution; Ambion, Austin, TX), and the other was empty. The samples were placed in a refrigerator at 4°C (for analysis of fecal microbiota) or in a freezer at −20°C (for analysis of fecal organic acid concentration and fecal pH) within 30 min of excretion. Blood samples were obtained after overnight fast, within 5 days of submitting fecal samples. One milliliter of blood was added to 2 mL of RNAprotect bacterial reagent (Qiagen, Hilden, Germany) immediately after collection. The samples were stored at −80°C. Both the fecal and blood samples were transported at −20°C to the Yakult Central Institute for Microbiological Research.

To quantify the bacteria present in the samples, we extracted total RNA fractions from feces and blood by the method previously described (18–21), and examined the gut microbiota composition and plasma levels of the gut bacteria by using the 16S rRNA-targeted RT–quantitative PCR (qPCR) using the Yakult Intestinal Flora-SCAN. Three serial dilutions of the extracted RNA sample were used for bacterial rRNA-targeted RT-qPCR (18–21), and the threshold cycle values in the linear range of the assay were applied to the standard curve to obtain the corresponding bacterial cell count in each nucleic acid sample. Those data were then used to determine the number of bacteria per sample. The specificity of the RT-qPCR assay using the group-, genus-, or species-specific primers was determined as described previously (18–21). The sequences of the primers are listed in Supplementary Table 1.

Measurement of Organic Acids and pH

The concentrations of organic acids in feces and serum were determined using methods described previously (21) with slight modifications. Briefly, the frozen sample was homogenized in four volumes of 0.15 mol/L perchloric acid and allowed to stand at 4°C for 12 h. The suspension was centrifuged at 20,400 × *g* at 4°C for 10 min. Then, the resulting supernatant was passed through a filter with a pore size of 0.45 μm (Millipore Japan, Tokyo, Japan). The sample was analyzed for organic acids using a high-performance liquid chromatography system (432 Conductivity Detector; Waters Co., Milford, MA), and pH in feces was analyzed using the IQ 150 pH/Thermometer (IQ Scientific Instruments, Inc., Carlsbad, CA).

Biochemical Assays

Blood samples were obtained after overnight fast. Serum lipids (total cholesterol [T-CHO], HDL cholesterol [HDL-C], LDL cholesterol, and triglyceride [TG]), fasting

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blood glucose, and HbA_{1c} were measured with standard techniques. The plasma levels of high-sensitivity C-reactive protein (hs-CRP), interleukin-6 (IL-6), and tumor necrosis factor (TNF)-α were measured by latex nephelometry, chemiluminescent enzyme immunoassay, and ELISA in a private laboratory (SRL Laboratory, Tokyo, Japan), respectively. The plasma level of LPS binding protein (LBP) was measured by human LBP ELISA kit (Hycult Biotech, the Netherlands).

Questionnaire of Dietary Intake

The original self-administered diet history questionnaire (DHQ) was developed by Sasaki et al. (22) in 1998, and its validity has been confirmed. However, it takes ~45–60 min to answer. In this study, we adopted a brief-type self-administered DHQ (BDHQ) (23), because BDHQ takes only ~15–20 min to answer. The validity of BDHQ is also confirmed as described previously (23). The questionnaire was completed when participants visited the hospital to provide blood samples. It is a four-page, structured, self-administered questionnaire that assessed dietary habits during the preceding month. Most food and beverage items were selected from a list of foods that are very commonly consumed in Japan, using a food list that, with some modifications, was used in the National Health and Nutrition Survey of Japan. In addition to the BDHQ, the participants provided further written information about the frequency of consumption of specific foods known to affect gut microbiota such as yogurt and food supplements.

Statistical Analysis

All statistical analyses were conducted using the JMP statistical software package, version 10.0.2 (SAS Institute, Cary, NC). Data were expressed as the mean ± SD, for normally distributed data, and median (interquartile range) for data with skewed distribution. The Mann-Whitney *U* test was used for data analysis. Spearman correlation analysis was used to determine the association between fecal bacterial counts/organic acids, LBP, and clinical parameters. The detection rate was analyzed using the Fisher direct probability test. $P < 0.05$ was considered to be statistically significant.

RESULTS

BMI, and fasting blood glucose, HbA_{1c}, and TG levels were significantly higher in the type 2 diabetes group than in the control group, while T-CHO and HDL-C were significantly lower in the type 2 diabetes group compared with the control group. Furthermore, hs-CRP and IL-6 (representing markers of inflammation) were significantly higher in the type 2 diabetes group than in the control group. There were no significant differences in age and TNF-α level between the two groups (Table 1).

The estimated total energy intake according to the BDHQ questionnaire was 1,662 ± 569 and 1,749 ± 521 kcal/day in the type 2 diabetes group and the control group, respectively. The ratio of carbohydrate intake to total energy intake was 56.1 ± 7.9% in the diabetes group and 53.9 ± 6.6% in the control group, while the ratio of fat intake was 26.8 ± 5.7% and 28.6 ± 5.1%, respectively, and the ratio of protein intake was 17.1 ± 3.5% and 17.5 ± 3.0%, respectively. There were no significant differences in the intake of total energy, carbohydrates, fat, and protein between the two groups. The number of the participants ingesting yogurt at least once a week was similar in the two groups (diabetes group 32 participants; control group 30 participants).

The fecal bacterial count was not significantly different between the two groups (Table 2). However, among the obligate anaerobe, the counts of the *Clostridium coccoides* group, *Atopobium* cluster, and *Prevotella* were significantly lower ($P < 0.05$), and the counts of total *Lactobacillus* among facultative anaerobe was significantly higher ($P < 0.05$) in the diabetes group compared with the control group. The counts of *Lactobacillus reuteri* and *Lactobacillus plantarum* subgroups were especially significantly higher ($P < 0.05$).

Metformin and α-glucosidase inhibitors are known to affect the gastrointestinal system. Thus, we investigated the difference in the gut microbiota between diabetic patients with and without metformin and α-glucosidase inhibitors. The levels of *Enterobacteriaceae* (7.5 ± 0.9 [$n = 18$] vs. 6.7 ± 1.1 $\log_{10}$ cells/g [$n = 31$], $P < 0.05$) and *Staphylococcus* (4.9 ± 0.9 [$n = 17$] vs. 4.4 ± 0.9 $\log_{10}$ cells/g [$n = 30$], $P < 0.05$) were significantly higher in the patients receiving metformin. The level of *Bifidobacterium* (9.7 ± 0.6 [$n = 9$] vs. 9.1 ± 0.8 $\log_{10}$ cells/g [$n = 41$], $P < 0.05$), total *Lactobacillus* (8.3 ± 1.1 [$n = 9$] vs. 6.7 ± 1.4 $\log_{10}$ cells/g [$n = 41$], $P < 0.01$), *Lactobacillus gasseri* (7.7 ± 1.1 [$n = 9$] vs. 5.8 ± 1.6 $\log_{10}$ cells/g [$n = 39$], $P < 0.01$), and *Enterococcus* (7.8 ± 1.2 [$n = 9$] vs. 6.3 ± 1.2 $\log_{10}$ cells/g [$n = 41$], $P < 0.01$) were significantly higher in the patients receiving α-glucosidase inhibitors.

The fecal concentrations of total organic acids ($P < 0.05$), acetic acid ($P < 0.01$), and propionic acid ($P < 0.05$) were significantly lower in the diabetes group than in the control group (Table 2). Fecal isovaleric acid concentration was significantly higher ($P < 0.05$) in the diabetes group than in the control group, but the fecal concentrations of other organic acids and pH values were not significantly different between the two groups. On the other hand, no difference was observed between the blood concentration of organic acids in the diabetes group and that in the control group.

Next, we examined the associations between various clinical parameters (listed in Table 1), food items in BDHQ, and fecal bacteria with low counts in the diabetes group. As shown in Table 3, the counts of the *C. coccoides* group in feces correlated negatively with hs-CRP levels ($r = -0.387$), energy intake ($r = -0.332$), saturated fatty acid intake ($r = -0.313$), and BMI ($r = -0.312$). Furthermore, fecal counts of the *Atopobium* cluster correlated negatively with hs-CRP level ($r = -0.392$) and BMI ($r = -0.321$), and positively with HDL-C ($r = 0.353$). In contrast, the counts of *Prevotella* did not correlate with clinical parameters or food items in BDHQ. The total *Lactobacillus* count, which was higher in the diabetes group, did not correlate with the clinical parameters or food items listed in the BDHQ.

Table 3 also summarizes the associations among various clinical parameters, food items listed in the BDHQ, and fecal organic acids altered in type 2 diabetic patients. Fecal total organic acids correlated negatively with saturated fatty acid intake ($r = -0.325$) and total fat intake ($r = -0.324$), and positively with carbohydrate intake ($r = 0.281$) in patients with type 2 diabetes. The fecal acetic acid level correlated negatively with saturated fatty acid intake ($r = -0.364$), total fat intake ($r = -0.327$), and diabetes duration ($r = -0.301$), and positively with carbohydrate intake

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Table 1—Characteristics of the study subjects

Characteristics	Control subjects (n = 50)	Type 2 diabetic patients (n = 50)
Sex (n)		
Male	26	26
Female	24	24
Age (years)	60.2 ± 12.9	62.5 ± 10.8
BMI (kg/m^2)	21.7 (20.9–23.5)	25.5 (23.5–30.8)*
Diabetes duration (years)	—	9.0 (5.0–21.5)
Fasting blood glucose (mg/dL)	94.1 ± 12.3 (13.1)	155.3 ± 44.7* (28.8)
HbA_{1c} (%)	5.6 (5.4–5.8)	8.7 (8.0–9.5)*
HbA_{1c} (mmol/mol)	38 (36–40)	72 (64–80)*
T-CHO (mg/dL)	212.9 ± 28.1 (13.2)	190.3 ± 45.5* (23.9)
HDL-C (mg/dL)	61.5 ± 16.3 (26.5)	46.8 ± 13.9* (29.8)
TG (mg/dL)	109.5 ± 83.3 (76.1)	124.9 ± 59.1† (47.4)
hs-CRP (mg/dL)	0.05 (0.02–0.12)	0.08 (0.04–0.40)*
TNF-α (pg/mL)	1.1 (0.9–1.4)	1.3 (1.0–1.6)
IL-6 (pg/mL)	1.2 (0.9–1.8)	2.2 (1.6–2.9)*
Medication for diabetes (n)	—	43
No medication	—	7
Insulin only or with oral therapy	—	12
Oral therapy only	—	31
SU	—	23
Metformin	—	17
α-GI	—	7
Thiazolidine	—	3
DPP-4 inhibitor	—	16
Glinide	—	3
GLP-1 receptor agonist	—	0
Medications for other diseases	32	42
No medication	18	8
Antihypertensive drugs	10	26
Lipid-lowering drugs	6	22
Drugs for thyroid diseases	12	0
Total energy intake (kcal/day)	1,749 ± 521	1,662 ± 569
Ratio of carbohydrate intake (%)	53.9 ± 6.6	56.1 ± 7.9
Ratio of fat intake (%)	28.6 ± 5.1	26.8 ± 5.7
Ratio of protein intake (%)	17.5 ± 3.0	17.1 ± 3.5
Participants taking yogurt at least once a week (n)	30	32

Data are mean ± SD (coefficient of variation) or median (interquartile range), unless otherwise indicated. α-GI, α-glucosidase inhibitors; DPP-4, dipeptidyl peptidase-4; SU, sulfonylurea. *$P < 0.01$ vs. control. †$P < 0.05$ vs. control.

(r = 0.356). Like acetic acid, the fecal level of propionic acid correlated negatively with diabetes duration (r = −0.349) and saturated fatty acid intake (r = −0.311). The high fecal levels of isovaleric acid in type 2 diabetic patients did not correlate with clinical parameters or food items listed in the BDHQ.

Table 4 shows the rate of detection of gut bacteria rRNA in blood samples. The bacteria were detected in 14 of 50 subjects in the diabetes group, compared with only 2 of 50 subjects in the control group (28% vs. 4%, $P < 0.01$). The detection rate of each bacterium in the type 2 diabetes group was 14% in the *C. coccoides* group, 14% in the *Atopobium* cluster, 4% in the *Clostridium leptum* subgroup, 2% in the *Streptococcus* group, and 2% in the *Enterobacteriaceae* group, and the detection rate in the control group was 2% in the *C. coccoides* group and 2% in the *Streptococcus* group. The bacterial species detected in the blood samples were limited, and the majority were Gram-positive anaerobic bacteria, which are known to be part of the commensal gut flora, with the exception of Gram-negative *Enterobacteriaceae*. Serum levels of LBP, which is known to play a role in LPS-mediated inflammation response (24), were significantly higher in the diabetes group than in the control group (12.1 ± 3.7 vs. 10.5 ± 3.0 μg/mL, $P < 0.05$). Moreover, in the diabetes group, the level of LBP showed positive correlations with BMI (r = 0.499, $P < 0.01$), HbA_{1c} (r = 0.356, $P < 0.05$), hs-CRP (r = 0.724, $P < 0.01$), TNF-α (r = 0.313, $P < 0.05$), and IL-6 (r = 0.595, $P < 0.01$).

CONCLUSIONS

The main findings of the current study were gut dysbiosis and the presence of live bacteria in the blood of Japanese patients with type 2 diabetes. Amar et al. (17) reported previously that the blood concentration of bacterial genes (using bacterial 16S rDNA) could predict the onset of diabetes, indicating for the first time the clinical significance of blood microbiota in the development of type 2 diabetes. However, analysis of bacterial 16S rDNA cannot discriminate whether the targeted bacteria in the gut and blood are alive or dead. For this reason, in the current study we used RT-qPCR, which can detect live bacteria in both the gut and blood, because this method uses specific primers that target bacterial RNA molecules (18). Using this technology, we identified the presence of gut dysbiosis and the possible bacterial translocation from the gut to blood in Japanese patients with type 2 diabetes.

Recently, Larsen et al. (25) identified the presence of gut dysbiosis in Danish patients with type 2 diabetes using tag-encoded pyrosequencing and qPCR. Furthermore, Qin et al. (26) reported gut dysbiosis in Chinese patients with type 2 diabetes in a metagenome-wide association study. The methods used in the above two studies were different from those used in our study and probably explain the differences in the results of the three studies. However, we confirmed some similar results in Japanese patients with type 2 diabetes. Also, the fecal counts of the *C. coccoides* group were significantly lower in type 2 diabetic patients, a finding that is in agreement with the results of Qin et al. (26). Furthermore, the fecal counts of total *Lactobacillus* were significantly higher in type 2 diabetic patients, which is in agreement with the data presented by Larsen et al. (25) The reason for the high count of *Lactobacillus*, a probiotic, is not clear at present. Because our method cannot

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Table 2—Comparisons of bacterial counts, organic acids, and pH between the control subjects and type 2 diabetic patients

	Control subjects (n = 50)		Type 2 diabetic patients (n = 50)	
	Fecal bacterial count (log_{10} cells/g)	Detection rate (%)	Fecal bacterial count (log_{10} cells/g)	Detection rate (%)
Total bacteria	10.4 ± 0.4	100	10.3 ± 0.5	100
Obligate anaerobe				
C. coccoides group	9.8 ± 0.5	100	9.4 ± 0.8*	100
C. leptum subgroup	9.7 ± 0.6	100	9.4 ± 1.1	100
Bacteroides fragilis group	9.5 ± 0.6	100	9.2 ± 0.9	100
Atopobium cluster	9.3 ± 0.5	100	9.0 ± 0.7*	100
Bifidobacterium	9.2 ± 0.8	100	9.2 ± 0.8	100
Prevotella	8.3 ± 1.6	54	7.4 ± 1.5*	84
Clostridium perfringens	5.1 ± 1.6	36	4.3 ± 1.2	62
Facultative anaerobe				
Total *Lactobacillus*	6.4 ± 1.2	100	7.0 ± 1.5*	100
L. gasseri subgroup	5.9 ± 1.2	96	6.2 ± 1.7	96
Lactobacillus sakei subgroup	4.3 ± 1.4	86	4.5 ± 1.2	92
L. reuteri subgroup	4.6 ± 1.1	74	5.6 ± 1.6*	90
L. plantarum subgroup	3.7 ± 0.7	60	4.2 ± 0.8*	60
Lactobacillus ruminis subgroup	5.4 ± 2.1	42	5.9 ± 1.8	64
Lactobacillus casei subgroup	4.8 ± 1.3	32	4.9 ± 1.3	44
Lactobacillus fermentum	5.5 ± 1.1	22	6.2 ± 1.2	32
Lactobacillus brevis	3.9 ± 0.8	12	4.0 ± 1.0	26
Lactobacillus fructivorans	< 2.3	0	< 2.3	0
Enterobacteriaceae	6.7 ± 1.2	86	7.0 ± 1.1	98
Enterococcus	6.6 ± 1.3	84	6.6 ± 1.3	100
Staphylococcus	4.5 ± 0.8	82	4.6 ± 0.9	94
Aerobe				
Pseudomonas	4.8 ± 1.1	18	5.6 ± 0.8	16
	Fecal organic acids (μmol/g) and pH	Detection rate (%)	Fecal organic acids (μmol/g) and pH	Detection rate (%)
Total organic acids	93.5 ± 30.2	100	77.8 ± 35.2*	100
Acetic acid	61.0 ± 18.1	100	48.0 ± 21.1†	100
Propionic acid	18.3 ± 6.9	100	15.5 ± 9.1*	100
Butyric acid	10.9 ± 7.6	92	9.2 ± 7.4	94
Isovaleric acid	2.5 ± 1.3	56	3.2 ± 1.5*	62
Valeric acid	2.5 ± 0.9	48	2.7 ± 1.4	40
Succinic acid	2.7 ± 6.9	44	2.0 ± 4.4	36
Formic acid	0.7 ± 0.2	38	1.2 ± 1.4	54
Lactic acid	1.5 ± 0.6	16	5.1 ± 5.7	26
pH	6.8 ± 0.6	100	6.8 ± 0.6	100
	Blood organic acids (μmol/mL serum)	Detection rate (%)	Blood organic acids (μmol/mL serum)	Detection rate (%)
Total organic acids	1.8 ± 0.6	100	2.1 ± 0.8	100
Lactic acid	1.8 ± 0.6	100	2.1 ± 0.8	100
Acetic acid	0.02 ± 0.02	56	0.02 ± 0.02	64
Formic acid	0.007	2	0.007 ± 0.002	6
Butyric acid	ND	0	ND	0
Isovaleric acid	ND	0	ND	0
Propionic acid	ND	0	ND	0
Succinic acid	ND	0	ND	0
Valeric acid	ND	0	ND	0

Data are mean ± SD, unless otherwise indicated. ND, not detected (<0.005 μmol/mL in serum). *P < 0.05 vs. control. †P < 0.01 vs. control.

distinguish innate bacteria and bacteria that originate from foods such as yogurt, we counted the number of participants who consumed yogurt but found no significant difference between the two groups. These results suggest that the high fecal *Lactobacillus* count reflects high innate bacteria levels in type 2 diabetic patients. Further studies are needed to investigate the roles of *Lactobacillus* in type 2 diabetes.

Complex carbohydrates, such as dietary fibers, are metabolized by the colonic microbiota into oligosaccharides and monosaccharides, and are fermented to short-chain fatty acids, such as butyric acid, acetic acid, and propionic acid (2). Butyric acid provides energy for colonic epithelial cells (27). In addition, it was reported that short-chain fatty acids stimulate GLP-1 secretion via the G-protein–coupled receptor (FFAR2) (28). Thus, organic acids produced by the gut flora are closely associated with

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Table 3—Correlations among fecal bacterial counts, organic acids, and various clinical parameters in type 2 diabetic patients

	Clinical parameters	*r*	*P*
C. coccoides group	hs-CRP (mg/dL)	−0.387	0.0066
	Energy intake (kcal/day)	−0.332	0.0184
	Saturated fatty acid intake (g/day)	−0.313	0.0268
	BMI (kg/m^2)	−0.312	0.0272
Atopobium cluster	hs-CRP (mg/dL)	−0.392	0.0058
	BMI (kg/m^2)	−0.321	0.0231
	HDL-C (mg/dL)	0.353	0.0119
Total organic acids	Saturated fatty acid intake (g/day)	−0.325	0.0215
	Total fat intake (%)	−0.324	0.0219
	Carbohydrate intake (%)	0.281	0.0477
Acetic acid	Saturated fatty acid intake (g/day)	−0.364	0.0093
	Total fat intake (%)	−0.327	0.0204
	Diabetes duration (years)	−0.301	0.0339
	Carbohydrate intake (%)	0.356	0.0112
Propionic acid	Diabetes duration (years)	−0.349	0.0130
	Saturated fatty acid intake (g/day)	−0.311	0.0282

energy harvest from the diet and glucose homeostasis by incretin hormone. Interestingly, in our study, the fecal concentrations of total organic acid, acetic acid, and propionic acid in patients with type 2 diabetes were significantly lower than those in the control subjects, and the fecal concentrations of acetic acid and propionic acid correlated negatively with the duration of diabetes. In addition, organic acids in feces promote elimination of *Escherichia coli O*-157 (29). Considering these roles of organic acids in feces and our findings, the low fecal levels of organic acids might be harmful by causing deterioration of glycemic control through the reduction of incretin hormone secretion after a meal and increased susceptibility to infection in type 2 diabetic patients (28,30).

Our data showed that the level of fecal total organic acids correlated closely with carbohydrate intake, and negatively with total fat intake and saturated fatty acid intake. In addition, plasma LBP levels were significantly higher in type 2 diabetic patients. LBP is known to be increased in obesity and to reflect the level of LPS, the wall part of the Gram-negative anaerobic bacteria and binds to an important cell surface pattern recognition receptors called Toll-like receptor (TLR)-4 (31). In the diabetes group, the level of LBP showed positive correlations with clinical markers such as BMI, HbA_{1c}, hs-CRP, TNF-α, and IL-6. These findings are, in part, consistent with the hypothesis of Cani et al. (4,13) that a high-fat diet triggers gut dysbiosis and subsequently leads to insulin resistance through an LPS-dependent mechanism. In the current study, although the high rate of bacteria was detected in the blood samples of type 2 diabetic patients, the majority of the detected bacteria were Gram-positive anaerobic bacteria, which are known to be part of the commensal gut flora. In experiments with mice, high-fat food increased the translocation of live Gram-negative bacteria through intestinal mucosa to blood and mesenteric adipose tissue, which was in turn linked to low-grade inflammation (32). In one study (17), among the bacteria detected in the blood of type 2 diabetic patients, >90% of the bacterial DNA belonged to the Gram-negative *Proteobacteria* phylum. However, in this study, we found the increased level of LBP without the increased level of the Gram-negative bacteria from the gut. This could possibly be due to the increased bacterial level from the other sites, such as the oral cavity.

The clinical significance of Gram-positive bacterial translocation in type 2 diabetes

Table 4—Counts of bacteria in blood samples and LBP levels

	Control subjects (*n* = 50)		Type 2 diabetic patients (*n* = 50)	
	Detected/total (*n*)	Detection rate (%)	Detected/total (*n*)	Detection rate (%)
Total bacteria	2/50	4	14/50	28*
Obligate anaerobe				
C. coccoides group	1/50	2	7/50	14
Atopobium cluster	0/50	0	7/50	14†
C. leptum subgroup	0/50	0	2/50	4
Bacteroides fragilis group	0/50	0	0/50	0
Bifidobacterium	0/50	0	0/50	0
Prevotella	0/50	0	0/50	0
Clostridium perfringens	0/50	0	0/50	0
Facultative anaerobe				
Streptococcus	1/50	2	1/50	2
Enterobacteriaceae	0/50	0	1/50	2
Staphylococcus	0/50	0	0/50	0
Enterococcus	0/50	0	0/50	0
Aerobe				
Pseudomonas	0/50	0	0/50	0
LBP (μg/mL)	10.5 ± 3.0 (28.3)		12.1 ± 3.7† (30.9)	

The minimum number of all target bacteria detectable by RT-qPCR was 1 bacterial cell per 1-mL blood sample. *$P < 0.01$ vs. control. †$P < 0.05$ vs. control.

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patients remains unknown. In this regard, the interaction between lipoteichoic acid (LTA) and TLR-2 in cytokine production is important. LTA, a Gram-positive bacterial wall component binds to TLR-2 (33). Recent studies (34,35) have reported that LTA enhances IL-6 expression in various cells, and our study demonstrated the presence of high levels of IL-6 in type 2 diabetic patients. Considered together, further studies are needed to determine the significance of the translocation of Gram-positive bacteria in systemic inflammation identified in obese and type 2 diabetic patients.

The current study has certain limitations. First, a 75-g oral glucose tolerance test was not performed in control subjects to completely exclude the presence of diabetes. Therefore, control subjects in our study cannot be labeled with certainty as nondiabetic control subjects. However, the HbA_{1c} levels of all the subjects did not exceed 6.0% (42 mmol/mol). Second, we were not able to confirm a causal relationship between gut dysbiosis and bacterial translocation because our study was cross-sectional in design. Third, because type 2 diabetic patients were overweight or obese, it cannot be excluded that gut dysbiosis and bacterial translocation were due to adiposity. In this regard, we divided the diabetic patients into two groups, the obese group (BMI $\geq$25 kg/m^2) and the nonobese group (BMI $<$25 kg/m^2). There were no significant differences in the detection rate of blood bacteria. These data support the idea that obesity was not a major cause of the differences. Fourth, our study had a small sample size.

Although these limitations should be taken into consideration, our findings have the potential to provide new insight into the pathophysiological mechanisms of type 2 diabetes. Further large-scale studies on gut and blood microbiota are needed in Japanese patients with type 2 diabetes.

In conclusion, our results demonstrated gut dysbiosis and possible blood bacterial translocation in patients with type 2 diabetes. The next step in this research protocol is to perform interventional studies to investigate whether improvement of gut dysbiosis by lifestyle interventions or the administration of probiotics can reduce the levels of circulatory inflammation markers and the rate of bacterial translocation, with amelioration of glycemic control.

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Author Contributions. J.S. and A.K. researched the data, participated in data collection, analyzed the data, contributed to the discussion, and wrote and edited the manuscript. F.I., T.Y., H.G., H.A., K.K., M.K., T.S., T.O., Y.T., Y.S., R.Y., T.M., Y.F., and H.F. participated in data collection and contributed to the discussion. K.N., T.T., and T.A. designed the study, analyzed data, and edited the manuscript. T.H. designed the study and contributed to the discussion. S.N. contributed to the discussion. Y.Y. designed the study, contributed to the discussion, and edited the manuscript. H.W. researched the data, participated in data collection, contributed to the discussion, and edited the manuscript. A.K. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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