Gut dysbiosis and detection of “live gut bacteria” in blood of Japanese patients with type 2 diabetes

Running title: Gut dysbiosis in Japanese diabetics

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Abstract

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

Methods: Using a sensitive reverse transcription-quantitative PCR method, we determined fecal gut microbiota composition 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 *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 the control. Especially, the counts of *L. reuteri* and *L. plantarum* subgroups were significantly higher ($P<0.05$). Gut bacteria were detected in blood at a significantly higher rate in diabetic patients than the control (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 reverse transcription-quantitative PCR. The high rate of gut bacteria in the circulation suggests translocation of bacteria from the gut to the blood stream.

Key words: gut bacteria, pathophysiology of diabetes, gut bacteria translocation, fecal examination

Introduction

The gut microbiota is essential for host immune system[1], digestion, including the breakdown of complex carbohydrates, such as dietary fibers, and 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 ribosomal RNA 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 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 et al.[4, 10-13] In a series of study, these investigators demonstrated that intestinal Gram-negative bacteria-produced lipopolysaccharide (LPS), which is a well-known proinflammatory molecule, can translocate to the blood stream from a leaky gut and cause metabolic endotoxemia associated with obesity[4]. More specifically, high-fat diet enhances the disruption of the tight-junction proteins in the intestine such as Zonula Occludens-1 and Occludin involved in the gut barrier function

of 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 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 due to 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 in its population in the world[16]. However, there were no human studies on gut microbiota composition in Japanese patients with type 2 diabetes. Japanese diet is greatly different from Western diet and the gut microbiota is known to be influenced by food type[6], suggesting that gut microbiota in Japanese patients with type 2 diabetes might be different from that of Westerners. Therefore, it is important to investigate gut microbiota composition in Japanese patients with type 2 diabetes.

Intriguingly, Amar et al. [17] reported that high-fat diet induces bacterial translocation from the gut to the blood stream in human 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 present 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.

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 management of hypertension, dyslipidemia or thyroid disease, whose conditions were under good control, or who visited Juntendo University Hospital for routine medical checkup between 2011 and 2012. Fifty controls with HbA1c <6.0% (42 mmol/mol) agreed to participate. Patients with the following conditions were excluded from the study; 1) proliferative retinopathy, 2) age ≥80 years of age, 3) serious liver disease (AST and/or ALT >100 IU/L) or serious kidney disease (serum creatinine >2.0 mg/dL, 4) acute heart failure, 5) malignancy, 6) inflammatory bowel disease, and 7) history of treatment with antibiotics within three months. The study protocol was approved by the Human Ethics Committee of Juntendo University, and a 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 (RT-qPCR)

After enrollment, the participants were asked to submit fresh fecal samples. The fecal samples were directly placed into 2 tubes (approximately 1.0g/tube) by the participants or the hospital staffs; 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 minutes of excretion. Blood samples were obtained after overnight fast, within five days of submitting fecal samples. One ml 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], examined the gut microbiota composition and plasma levels of the gut bacteria by using the16S rRNA-targeted RT-qPCR, Yakult Intestinal Flora-SCAN (YIF-SCAN®). Three serial dilutions of the extracted RNA sample were used for bacterial rRNA-targeted reverse transcription-quantitative PCR [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. These 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 as an online material.

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 freezed sample was homogenized in 4 volumes of 0.15 M perchloric acid and allowed to stand at 4°C for 12 hours. The suspension was centrifuged at 20,400 x *g* at 4°C for 10 minutes. Then, the resulting supernatant was passed through a filter with a pore size of 0.45μm (Millipore Japan, Tokyo). The sample was analyzed for organic acids using the Waters HPLC system (Waters 432 Conductivity Detector; Waters Co., Milford, MA) and pH in feces was analyzed by 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), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglyceride (TG)], fasting blood glucose (FBG), and HbA1c were measured with

standard techniques. The plasma levels of high-sensitivity C-reactive protein (hs-CRP), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were measured by latex nephelometry, chemiluminescent enzyme immunoassay and enzyme-linked immunosorbent assay in a private laboratory (SRL Laboratory, Tokyo), respectively. The plasma level of lipopolysaccharide binding protein (LBP) was measured by human LBP ELISA kit (Hycult Biotech, the Netherlands).

Questionnaire of dietary intake

Original self-administered diet history questionnaire (DHQ) was developed by Sasaki et al in 1998 and its validity has been confirmed [22]. However it takes about 45-60 minutes to answer. In this study we adopted a brief-type self-administered DHQ (BDHQ) [23] because BDHQ takes about only 15-20 minutes 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 the food list very commonly consumed in Japan, with some modifications using a food list 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 yoghurt and food supplements.

Statistical analysis

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

Results

Body mass index (BMI), fasting blood glucose, HbA1c and TG were significantly higher in type 2 diabetes group than the control group, while T-CHO and HDL-C were significantly lower in type 2 diabetes group compared to the control group. Furthermore, hs-CRP and IL-6 (representing markers of inflammation) were significantly higher in type 2 diabetes group than the control. There were no significant differences in age and TNF-α between the two groups (Table 1).

The estimated total energy intake according to the BDHQ questionnaire was 1662±569 and 1749±521 kcal/day in type 2 diabetes and 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 ratio of protein intake was 17.1±3.5% and 17.5±3.0%, respectively. There were no significant differences in intakes of total energy, carbohydrate, fat and protein between the two groups. The number of the participants taking yoghurt at least once a week was similar in the two groups (diabetes: 32, control: 30).

The fecal bacterial count was not significantly different between the two groups (Table 2). However, among the obligate anaerobe, the counts of *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. Especially, the counts of *L. reuteri* and *L. plantarum* subgroups were significantly higher (P<0.05).

Metformin and alpha-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 alpha-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 with 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], *L.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 with alpha-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 the control group (Table 2). Fecal isovaleric acid concentration was significantly higher (P<0.05) in the diabetes group than the control group, but the fecal levels of other organic acids and pH value were not significantly different between the two groups. On the other hand, no difference was observed between 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 *Clostridium coccoides* group in feces correlated negatively with hs-CRP (r = -0.387), energy intake (r = -0.332), saturated fatty acid intake (r = -0.313) and BMI (r = -0.312). Furthermore, fecal counts of *Atopobium* cluster correlated negatively with hs-CRP (r = -0.392), 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 in BDHQ.

Table 3 also summarizes the associations between various clinical parameters, food items in BDHQ and fecal organic acids altered in type 2 diabetes 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 level of acetic acid correlated negatively with saturated fatty acid intake (r = -0.364), total fat intake (r = -0.327), diabetic duration(r = -0.301) and positively with carbohydrate intake (r = 0.356). Like acetic acid, the fecal level of propionic acid correlated negatively with diabetic duration (r = -0.349) and saturated fatty acid intake (r = -0.311). The high fecal levels of isovaleric acid in type 2 diabetes did not correlate with clinical parameters or food items in BDHQ.

Table 4 shows the rate of detection of gut bacteria rRNA in blood samples. The bacteria were detected in 14 of 50 subjects of the diabetes group, compared with only 2 of 50 subjects of the control group (28 vs. 4%, P<0.01). The detection rate of each bacterium in type 2 diabetes group was 14% in *Clostridium coccoides* group, 14% in *Atopobium cluster*, 4% in *Clostridium leptum* subgroup, 2% in *Streptococcus* and 2% in *Enterobacteriaceae*, and the detection rate in control group was 2% in *Clostridium coccoides* and 2% in *Streptococcus*. The bacterial species detected in the blood samples were limited and the majority was

Gram-positive anaerobic bacteria known to be a 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 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, $P<0.01$), HbA1c (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, $P<0.01$).

Discussion

The main findings of the present study were gut dysbiosis and presence of live bacteria in blood of Japanese patients with type 2 diabetes. Amar et al. [17] reported previously that the blood concentration of bacterial gene (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, we used in the present study RT-qPCR, which can detect “live bacteria” in both the gut and blood because this method is based on reverse transcription quantitative PCR using specific primers that target bacterial RNA molecules[18]. Using this technology, we identified

the presence of gut dysbiosis and possible bacterial translocation from the gut to blood in Japanese 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 quantitative PCR. Furthermore, Qin et al. [26] reported gut dysbiosis in Chinese patients with type 2 diabetes in 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 *Clostridium coccoides* group were significantly lower in type 2 diabetes, 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 diabetes, 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. Since our method cannot distinguish innate bacteria and bacteria that originate from foods such as yoghurt, we counted the number of participants who consumed yoghurt but found no significant difference between the two groups. These results suggest that the high fecal *Lactobacillus* count reflects high innate bacteria in type 2 diabetes. 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 oligosaccharide and monosaccharide and 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 glucagon-like peptide-1 secretion via the G-protein-coupled receptor (FFAR2)[28]. Thus, organic acids produced by the gut flora are closely associated with 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 type 2 diabetes were significantly lower than the controls, and the fecal concentrations of acetic acid and propionic acid correlated negatively with the diabetic duration. 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 reduction of incretin hormone secretion after a meal and increased susceptibility to infection in type 2 diabetes[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 lipopolysaccharide binding protein (LBP) levels were significantly higher in type 2 diabetes. LBP is known to be increased in obesity and reflect the level of LPS, the wall part of the Gram-negative anaerobic bacteria and binds to important cell surface pattern

recognition receptors called Toll-like receptor 4 (TLR4)[31]. In the diabetes group, the level of LBP showed positive correlations with clinical markers such as BMI, HbA1c, hs-CRP, TNF-α and IL-6. These findings are, in part, consistent with the hypothesis of Cani et al. that high fat diet triggers gut dysbiosis and subsequently lead to insulin resistance through LPS-dependent mechanism.[4, 13] In the present study, although the high rate of bacteria was detected in the blood samples of type 2 diabetes, the majority of the detected bacteria was Gram-positive anaerobic bacteria known to be part of the commensal gut flora. In experiments on 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, among the bacteria detected in blood of type 2 diabetes, more than 90% of the bacterial DNA belonged to the Gram-negative *Proteobacteria* phylum[17]. 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 be possibly due to the increased bacterial level from the other sites such as oral cavity.

The clinical significance of Gram-positive bacterial translocation in type 2 diabetes remains unknown. In this regard, the interaction between lipoteichoic acid (LTA) and Toll-like receptor 2 (TLR2) in cytokine production is important. LTA, a Gram-positive bacterial wall component binds to TLR2, a Toll-like receptor[33]. Recent studies have reported that LTA enhances IL-6 expression in various cells[34, 35], and our study demonstrated the

presence of high levels of IL-6 in type 2 diabetes. Considered together, further studies are needed to determine the significance of translocation of Gram-positive bacteria in systemic inflammation identified in obesity and type 2 diabetes.

The present study has certain limitations. First, 75g-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 non-diabetic controls. However, the HbA1c levels of all the subjects did not exceed 6.0% (42mmol/mol). Second, we were not able to confirm a causal relationship between gut dysbiosis and bacterial translocation because our study was a cross-sectional in design. Third, as type 2 diabetic patients had overweight or obesity, 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≥25) and the non-obese group (BMI<25). There were no significant differences in detection rate of blood bacteria. This data support that obesity was not a major character for the difference. Fourth, our study involved a small sample size.

Although these limitations should be taken into consideration, our findings have the potential to provide new insight to 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 type 2 diabetes. The next step in this research protocol is interventional studies to investigate whether improvement of gut dysbiosis by life style interventions or administration of probiotics can reduce the levels of circulatory inflammation markers and the rate of bacrerial translocation, with amelioration of glycemic control.

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J.S. researched data, participated in data collection, contributed to the discussion, analyzed data, wtote and edited the manuscript. A.K. researched data, participated in data collection, contributed to the discussion, analyzed data 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., 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 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 data analysis.

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Table 1. Characteristics of study subjects

	Control (n=50)	T2DM (n=50)
Sex (Male/Female)	26/24	26/24
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)**
Diabetic 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)
HbA1c (%)	5.6 (5.4-5.8)	8.7 (8.0-9.5)**
HbA1c (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	-	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)	1749 ± 521	1662 ± 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 yoghurt at least once a week	30	32

Data are mean ± SD or median (IQR; 25%-75%). *$P < 0.05$ vs. control ,**$P < 0.01$ vs. control

Coefficient of variation (CV) is followed after mean±SD.

BMI; Body mass index, T-CHO; total cholesterol, HDL-C; high density lipoprotein cholesterol, TG; triglyceride, hs-CRP; high-sensitivity C-reactive protein, IL-6; interleukin-6, TNF-α; tumor necrosis factor-α

Table 2. Comparisons of bacterial counts, organic acids, and pH between the control and type 2 diabetes

	Controls (n=50)		T2DM (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
C. 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
L. 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
L. ruminis subgroup	5.4 ± 2.1	42	5.9 ± 1.8	64
L. casei subgroup	4.8 ± 1.3	32	4.9 ± 1.3	44
L. fermentum	5.5 ± 1.1	22	6.2 ± 1.2	32
L. brevis	3.9 ± 0.8	12	4.0 ± 1.0	26
L. 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. ND: Not detected, <0.005 µmol/mL serum

*$P < 0.05$ vs. control, **$P < 0.01$ vs. control

Table 3. Correlations between fecal bacterial counts and organic acids and various clinical parameters in type 2 diabetes.

	Clinical parameter	*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
	Diabetic duration (years)	-0.301	0.0339
	Carbohydrate intake (%)	0.356	0.0112
Propionic acid	Diabetic duration (years)	-0.349	0.0130
	Saturated fatty acid intake (g/day)	-0.311	0.0282

See Table 1 for abbreviations.

Table 4. Counts of bacteria in blood samples and lipopolysaccharide binding protein (LBP) levels.

	Controls (n=50)		T2DM (n=50)	
	Detected number	Detection rate (%)	Detected number	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
C.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
	Controls (n=50)		T2DM (n=50)	
LBP (μg/mL)	10.5 ± 3.0(28.3)		12.1 ± 3.7*(30.9)	

Data are mean±SD (CV). *$P < 0.05$ vs. control, **$P < 0.01$ vs. control. The minimum detectable number of all the target bacteria by RT-qPCR was 1 bacterial cell per 1-mL blood sample.