Nutrition 63–64 (2019) 51–56

Contents lists available at ScienceDirect

Nutrition

journal homepage: www.nutritionjrnl.com

Applied nutritional investigation

# The alteration of gut microbiota in newly diagnosed type 2 diabetic patients

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ARTICLE INFO

*Article History:*
Received 30 April 2018
Received in revised form 4 October 2018
Accepted 17 November 2018

*Keywords:*
Human gut microbiota
Type 2 diabetes
Dysbiosis
*Lactobacillus*
*Clostridium*

ABSTRACT

*Objectives:* Gut microbiota dysbiosis is known to be associated with diabetes; however, the findings of previous studies are conflicting. To clarify the association between type 2 diabetes and the gut microbiota, the present study analyzed the composition of fecal gut microbiota and its correlation with specific clinical parameters in newly diagnosed, treatment-naive diabetic patients and healthy controls.
*Methods:* A total of 50 patients with newly diagnosed type 2 diabetes and 50 healthy control participants were enrolled in the study. Fecal samples, blood samples, and food diaries were collected from the diabetic patients before and 3 mo after the start of their antidiabetic treatment. These samples were also collected from the healthy controls. The gut microbiota was characterized by 16S ribosomal RNA analysis using quantitative polymerase chain reaction.
*Results:* The fecal count of *Lactobacillus* was significantly higher, whereas *Clostridium coccoides* and *Clostridium leptum* were significantly lower in the diabetic patients compared with the healthy controls. *Lactobacillus* was significantly positively correlated with glucose, glycated hemoglobin, and the homeostatic model assessment, whereas *C. coccoides* and *C. leptum* were significantly negatively correlated with the diabetic parameters. In addition, the newly diagnosed diabetic patients had a significant decrease in the presence of *C. coccoides* and *C. leptum* after 3 mo of treatment compared with before treatment.
*Conclusions:* The amount of fecal *Lactobacillus*, *C. coccoides*, and *C. leptum* was significantly different between the patients with type 2 diabetes and the healthy controls. The levels of *Clostridium* were also significantly changed after 3 mo of treatment in the diabetic patients. Further research is needed to clarify the correlation or causal relationship between the gut microbiota dysbiosis and type 2 diabetes.
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## Introduction

Type 2 diabetes mellitus is a global public health problem, the prevalence of which is rapidly increasing, including in Taiwan, where the prevalence was 6.38% in 2009 and is expected to increase [1]. Type 2 diabetes is a metabolic disease with a complicated etiology, which is characterized by insulin resistance and low-grade inflammation [2]. Together the microbiota that resides in the human intestine has more genetic material than the human genome and offers a range of physiological functions to the host. Therefore changes to the composition of the microbiota, known as dysbiosis, might fail to provide the host with these beneficial properties and result in various diseases [3]. Recent studies have suggested that the gut microbiota is associated with metabolic diseases, such as obesity and diabetes [4], and several studies have investigated the mechanisms underlying these associations [5,6]. Possible mechanisms include the modulation of energy metabolism by increasing energy harvest from the diet and an association between bacterial lipopolysaccharide-induced endotoxemia and subsequent inflammation related to insulin resistance [7]. Obese and lean participants have been found to have different bacterial populations [8], and differences in the gut microbiota of diabetic patients have also been reported [9–13]. Studies have identified certain relevant differences in the gut microbiota of individuals with type 2 diabetes; however, the results remain controversial and inconclusive. Among these cohort studies, most of the diabetic patients have been prescribed antidiabetic drugs for different

The present study was supported by a research grant (grant no. SKH-8302-102-DR-08) from the Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan.
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https://doi.org/10.1016/j.nut.2018.11.019
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treatment durations. A recent study found that control of metformin treatment led to elimination of the previously identified increase in *Lactobacillus*; however, the decrease in butyrate producers remained [14]. Recent studies also revealed that certain changes in the gut microbiota are shared across multiple diseases and medications, indicating the complicated interconnectivity of diseases, medications, and the gut microbiota [15,16].

In addition, the methods used to characterize the gut microbiota were different across the various studies. The development of culture-independent methods of bacterial identification has revived interest in the microbiota. The introduction of molecular techniques using 16S ribosomal RNA (rRNA) gene sequencing has provided phylogenetic information, which may be used to distinguish microbial groups into phylotypes, although it lacks the specificity to define the bacterial species [17]. Advanced techniques such as shotgun metagenomic sequencing are becoming more accessible, and metagenomic-wide association studies, which combine metagenomics data with clinical features, are providing a more specific view of a broader range of the microbiome. However, analysis of 16S rRNA nucleotide sequencing remains a widespread and cost-effective method [18]. Therefore to determine alterations to the gut microbiota identified in patients with type 2 diabetes, the fecal gut microbiota composition was analyzed in healthy control participants and patients with newly diagnosed, treatment-naive type 2 diabetes before and after 3 mo of medical treatment. Various clinical parameters and dietary records were also investigated to establish their correlation with the gut microbiota.

## Methods

### *Study participants and sample collection*

Between May 2014 and March 2015 patients from Shin-Kong Wu Ho-Su Memorial Hospital with newly diagnosed type 2 diabetes who were aged 20 to 80 y and had not previously taken any antidiabetic drugs were enrolled in the present study. Healthy control participants without any known diseases or any regular medication use were recruited through written advertisements. Participants with renal disease (serum creatinine level >1.5 mg/dL), liver disease (serum alanine aminotransferase [ALT] activity >200 IU/L), heart failure, malignancy, pregnancy, or a history of antibiotic treatment within 3 mo of study participation were excluded. In total, 50 diabetic patients and 50 healthy participants were enrolled into the study for analysis. Body weight and body height were measured to calculate the body mass index (BMI). Blood samples were collected after an 8-h fasting period. Fecal samples and dietary records were collected for analysis from healthy controls and the diabetic patients before initiation of oral antidiabetic medication and 3 mo after they had started the treatment. The present study was approved by the Human Ethics Committee of Shin-Kong Wu Ho-Su Memorial Hospital (Institutional Review Board number: 20120711R) and written informed consent was obtained from each participant before their inclusion in the study.

### *Biochemical analysis of blood*

Biochemical analysis of the blood was performed using standard techniques at the Department of Pathology and Laboratory Medicine of Shin-Kong Wu Ho-Su Memorial Hospital. The patient's serum was isolated and the levels of insulin (DxI800, Beckman Coulter, Brea, CA, USA), glucose, cholesterol, triglyceride, high-density lipoprotein (HDL), low-density lipoprotein (LDL), creatinine, alanine aminotransferase (ALT) (AU680/AU5800, Beckman Coulter, USA), and glycated hemoglobin A1c (HbA1c; HLC-723 G8, Tosoh Corp., Tokyo, Japan) were analyzed using automated biochemical analyzers. Insulin resistance was quantified using the homeostatic model assessment (HOMA-IR) and calculated as glucose (mg/dL) × insulin (mU/L) / 405. The plasma levels of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), glucagon-like peptide-1 (GLP-1), and adiponectin were measured using enzyme-linked immunosorbent assay at a private laboratory (Yu-Shing Biotech., Taipei, Taiwan). These biomarker measurements were performed simultaneously using the Bio-Plex Pro human cytokine assay kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. In brief, 50 μL beads were added to the well and washed. A 50-μL sample was then added and incubated with antibody-coupled beads for 60 min at room temperature. After washing to remove any unbound materials the beads were incubated with 25 μL biotinylated detection antibodies for 30 min at room temperature. Then, after washing away the unbound biotinylated antibodies, the beads were incubated with 50 μL streptavidin-PE for 10 min at room temperature. After removal of the excess streptavidin-PE the beads were resuspended in 125 μL assay buffer. Finally, the beads were read on a Bio-Plex suspension array system and the data were analyzed using Bio-Plex Manager software.

### *Collection of fecal samples*

All participants were asked to provide fresh stool samples. The fecal samples were collected in two to three tubes (0.2 g/tube) and then stored at −20°C before analysis. Bacterial DNA extraction was performed using a QIAamp Fast DNA Stool Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The concentration of extracted DNA was measured using PowerWave XS2 (BioTek, Winooski, VT, USA).

### *Quantitative polymerase chain reaction analysis of fecal microbiota*

Quantitative polymerase chain reaction (qPCR) experiments were performed using an Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The primers used to target specific bacterial genera in the present study are shown in Table 1. Each PCR test had a total volume of 25 μL, containing 1 μL of each fecal DNA sample (10 ng/μL) as the template DNA, 1 μL of each paired primer (10 μM), 12.5 μL Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific, Waltham, MA, USA), and 9.5 μL nuclease free water.

**Table 1**
The primers used to target specific bacterial genera

| Target bacteria (standard strain) | Primer | Sequence (5′–3′) |
|---|---|---|
| *Bifidobacterium* | g-Bifid-F | CTCCTGGAAACGGGTGG |
| (*Bifidobacterium longum* ATCC 15707 T) | g-Bifid-R | GGTGTTCTTCCCGATATCTACA |
| *Lactobacillus* | sg-Lgas-F | GATGCATAGCCGAGTTGAGAGACTGAT |
| (*Lactobacillus gasseri* DSM 20243 T) | sg-Lgas-R | TAAAGGCCAGTTACTACCTCTATCC |
| *Clostridium perfringens* | s-Clper-F | GGGGGTTTCAACACCTCC |
| (*Clostridium perfringens* JCM 1290 T) | ClPER-R | GCAAGGGATGTCAAGTGT |
| *Enterobacteriaceae* | En-lsu-3F | TGCCGTAACTTCGGGAGAAGGCA |
| (*Escherichia coli* JCM 1649 T) | En-lsu-3′R | TCAAGGACCAGTGTTCAGTGTC |
| *Enterococcus* | g-Encoc-F | ATCAGAGGGGGATAACACTT |
| (*Enterococcus faecium* ATCC 19434 T) | g-Encoc-R | ACTCTCATCCTTGTTCTTCTC |
| *Prevotella* | g-Prevo-F | CACRGTAAACGATGGATGCC |
| (*Prevotella melaninogenica* ATCC 25845 T) | g-Prevo-R | GGTCGGGTTGCAGACC |
| *Bacteroides fragilis* | g-Bfra-F2 | AYAGCCTTTCGAAAGRAAGAT |
| (Bacteroides vulgatus ATCC 8482T) | g-Bfra-R | CCAGTATCAACTGCAATTTTA |
| *Atopobium* cluster | g-Atopo-F | GGGTTGAGAGACCGACC |
| (*Collinsella aerofaciens* DSM 3979 T) | g-Atopo-R | CGGRGCTTCTTCTGCAGG |
| *Clostridium leptum* | sg-Clept-F | GCACAAGCAGTGGAGT |
| (*Faecalibacterium prausnitzii* ATCC 27768 T) | sg-Clept-R3 | CTTCCTCCGTTTTGTCAA |
| *Clostridium coccoides* | g-Ccoc-F | AAATGACGGTACCTGACTAA |
| (*Blautia (Ruminococcus) productus* JCM 1471 T) | g-Ccoc-R | CTTTGAGTTTCATTCTTGCGAA |

F, forward; R, reverse.

Samples from the negative controls were also analyzed using the same reaction mixture without the template DNA to confirm that no amplification product was detected. Standard curves were established using bacterial genomic DNA with a known concentration regulated to $1 \times 10^9$ cells/mL, $10^8$ cells/mL, $10^7$ cells/mL, $10^6$ cells/mL, $10^5$ cells/mL, $10^4$ cells/mL, and $10^3$ cells/mL. Bacterial genomic DNA was kindly provided by Dr. Tsai Ying-Chieh at the Institute of Biochemistry and Molecular Biology, National Yang-Ming University. The 10 different strains came from 3 locations: The American Type Culture Collection (ATCC) including *Bacteroides vulgatus* ATCC 8482T, *Bifidobacterium longum* ATCC 15707 T, *Faecalibacterium prausnitzii* ATCC 27768 T, *Prevotella melaninogenica* ATCC 25845 T, and *Enterococcus faecium* ATCC 19434 T; the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM) including *Lactobacillus gasseri* DSM 20243 T and *Collinsella aerofaciens* DSM 3979 T; and the Japan Collection of Microorganisms (JCM) including *Clostridium perfringens* JCM 1290 T, *Blautia (Ruminococcus) productus* JCM 1471 T, and *Escherichia coli* JCM 1649 T. The qPCR thermocycling conditions were as follows: uracil-DNA glycosylase pretreatment stage at 50°C for 2 min, initial denaturation stage at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 sec, and annealing/extension at 60°C for 1 min. Melting curve analysis was performed for 1 cycle at 95°C for 15 sec, 60°C for 30 sec, and 95°C for 15 sec to confirm that no primer dimers occurred. The amplification efficiency ranged from 90% to 110%, which corresponded to slope values from −3.58 to −3.10. The concentration of bacteria in each sample was determined by comparing the threshold cycle values obtained from standard curves using ABI 7300 System Sequence Detection Software Version 1.3 (Thermo Fisher Scientific). Using the concentration of the sample, it was possible to calculate the cell counts from the amount of each extracted sample DNA to bacterial cells per gram of stool of each bacteria and take the log value to present.

### *Dietary intake assessment*

The participants were asked to complete 3-d food records, including at least 1 weekend day and 1 weekday. The dietary records were collected from the diabetic patients before and after 3 mo of antidiabetic treatment. The food records were analyzed using Nutritionist Professional software (Nutritionist Edition, Enhancement plus 1 version 2013; E-Kitchen Business Corp., Taiwan). The mean value of the 3 d was used for statistical comparisons between the two groups.

### *Statistical analysis*

Data are expressed as the mean ± standard deviation or the median and interquartile range as appropriate for continuous data and number (%) for categorical data. The Mann-Whitney *U* test was used to compare the means of continuous variables, and the Wilcoxon rank sum test was used to compare dependent data as a result of violations of normal distribution assumptions. The $\chi^2$ test was used for categorical variables. Multivariate linear regression analysis was performed with microbiota as the dependent variable with adjustments for sex, age, and BMI in model 1, with further adjustments for daily fiber intake in model 2. Spearman rank correlation coefficient analysis was conducted to determine the association between gut microbiota and clinical parameters. All statistical analyses were performed using SPSS software (version 20; IBM Corp., Armonk, NY, USA), and a two-tailed *P* value of <0.05 was considered to indicate a statistically significant difference.

## Results

The characteristics of the newly diagnosed diabetic patients and healthy controls are presented in Table 2. There were no significant differences in sex and age between the two groups, whereas the average BMI was significantly increased in the diabetic patients compared with the healthy controls. Regarding food intake, there were no significant differences in the daily energy, protein, carbohydrate, and cholesterol intake between the two groups; however, the daily fiber intake was significantly lower and the fat intake was significantly higher in the diabetic patients compared with the healthy controls. However, the energy intake from fat (%) in the diabetic patients still maintained in the recommended range (20%∼30%).

Table 3 details the blood analysis of the newly diagnosed diabetic patients and the healthy controls. Glucose, insulin, HOMA-IR, ALT, HbA1c, triglycerides, and TC/HDL were significantly increased in the diabetic patients compared with the healthy controls, whereas the HDL was significantly decreased. There were no significant differences in the creatinine levels, total cholesterol, and LDL between the two groups. IL-1β, which is a marker of inflammation, was significantly increased in the diabetic patients compared with the controls, whereas adiponectin was significantly decreased. There were no significant differences in the levels of TNF-α and GLP-1 between the two groups.

Table 4 presents the fecal microbiota analysis of the newly diagnosed diabetic patients and the healthy controls. Bacterial values per gram of stool of *Clostridium leptum* and *Clostridium coccoides* were consistent with findings in previous literature [10,11]; however, the other bacterial groups varied between studies. *Lactobacillus* was significantly increased in the diabetic patients compared with the healthy controls, whereas *C. leptum* and *C. coccoides* were significantly lower in the patients than in the controls. There were no significant differences in *Bifidobacterium, Clostridium perfringens, Enterobacteriaceae, Enterococcus, Prevotella, Bacteroides fragilis*, and *Atopobium* clusters between the two groups. Regression analysis model 1, which included age, sex, and BMI, had similar microbiota results. However, multivariate model 2, which comprised model 1 and daily fiber intake, found no significant differences in *Lactobacillus* and *Clostridium leptum* between the diabetic patients and the healthy controls.

Table 5 presents the associations among fecal microbiota, clinical factors, and food intake. The *Lactobacillus* count was positively correlated with diabetic parameters, including glucose, HOMA-IR, and HbA1c, and significantly negatively correlated with

**Table 2**
Characteristics of newly diagnosed diabetic patients and healthy controls

| Variable | Diabetic patients ($n = 50$) | Healthy controls ($n = 50$) | *P* |
|---|---|---|---|
| Male (%) | 36 (72) | 28 (56) | 0.096 |
| Age (y) | 51 ± 12 | 52 ± 10 | 0.989 |
| Body mass index (kg/m$^2$) | 27 ± 4.3 | 23± 2.5 | <0.001 |
| Daily energy intake (× 10$^3$ kcal) | 1.6 ± 0.6 | 1.7 ± 0.4 | 0.476 |
| Protein intake (%) | 16.7 ± 4.5 | 16.8 ± 3.3 | 0.259 |
| Fat intake (%) | 28.6 ± 8.4 | 25.2 ± 5.4 | 0.032 |
| Carbohydrate intake (%) | 54.6 ± 10.2 | 57.9 ± 6.9 | 0.094 |
| Daily fiber intake (g) | 11 ± 6.7 | 17 ± 13 | 0.006 |
| Daily cholesterol intake (mg) | 228 ± 158 | 264 ± 130 | 0.650 |

Values are expressed as the number (%) of patients or the mean ± standard deviation.

**Table 3**
Blood analysis of newly diagnosed diabetic patients and healthy controls*

| Variable | Diabetic patients ($n = 50$) | Healthy controls ($n = 50$) | *P* |
|---|---|---|---|
| Glucose (mg/dL) | 206 ± 87 | 88 ± 9 | <0.001 |
| Insulin (μIU/mL) | 7.3 ± 4.5 | 4.9 ± 2.6 | 0.003 |
| HOMA-IR (IQR) | 2.9 (1.7–5.2) | 0.03 (0.02–0.04) | <0.001 |
| ALT, U/L (IQR) | 30 (17–49) | 18 (13–23) | 0.014 |
| Creatinine (mg/dL) | 0.7 ± 0.1 | 0.7 ± 0.1 | 0.580 |
| HbA1c (%) | 10.7 ± 2.5 | 5.4 ± 0.3 | <0.001 |
| Total cholesterol (mg/dL) | 198 ± 66 | 194 ± 31 | 0.679 |
| Triglyceride, mg/dL (IQR) | 130 (92–222) | 102 (79–138) | 0.006 |
| HDL (mg/dL) | 44 ± 15 | 54 ± 19 | 0.001 |
| LDL (mg/dL) | 110 ± 33 | 121 ± 29 | 0.175 |
| TC/HDL ratio | 5.0 ± 2.6 | 3.8 ± 1.1 | 0.002 |
| IL-1β, pg/mL (IQR) | 7 (2–17) | 0.6 (0.2–1.4) | <0.001 |
| TNF-α, pg/mL (IQR) | 11 (6–13) | 7 (4–26) | 0.203 |
| GLP-1, pg/mL (IQR) | 20 (11–28) | 14 (11–25) | 0.122 |
| Adiponectin, × 10$^6$ pg/mL (IQR) | 1.9 (1.1–2.9) | 2.9 (1.4–5.0) | 0.007 |

ALT, alanine aminotransferase; GLP, glucagon-like peptide; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; IL, interleukin; IQR, interquartile range; LDL, low-density lipoprotein; TC, total cholesterol; TNF, tumor necrosis factor.
*Values are expressed as the mean ± standard deviation or median (interquartile range).

**Table 4**
Fecal microbiota comparison between newly diagnosed diabetic patients and healthy controls

| | Raw | | | Model l | | Model 2 | |
|---|---|---|---|---|---|---|---|
| Microbiota(log10 cell/g) | Diabetics ($n = 50$) | Healthy controls ($n = 50$) | *P* | Diabetics vs. Healthy: Estimate (95% CI) | *P* | Diabetics vs. Healthy: Estimate (95% CI) | *P* |
| *Bifidobacterium* | 6.8 ± 1.5 | 7.0 ± 1.1 | 0.482 | −0.4 (−1.0 to 0.1) | 0.183 | −0.2 (−0.9 to 0.4) | 0.474 |
| *Lactobacillus* | 4.9 ± 1.1 | 4.2 ± 1.2 | 0.005 | 0.7 (0.08–1.42) | 0.029 | 0.3 (−0.3 to 1.1) | 0.333 |
| *Clostridium perfringens* | 3.0 ± 1.2 | 2.9 ± 1.2 | 0.544 | 0.09 (−0.5 to 0.7) | 0.765 | 0.07 (−0.6 to 0.7) | 0.827 |
| *Enterobacteriaceae* | 8.1 ± 0.8 | 8.2 ± 1.2 | 0.469 | −0.09 (−0.6 to 0.40) | 0.696 | 0.11 (−0.4 to 0.6) | 0.695 |
| *Enterococcus* | 3.7 ± 0.9 | 4.0 ± 1.1 | 0.238 | −0.1 (−0.7 to 0.5) | 0.731 | −0.06 (−0.84 to 0.72) | 0.877 |
| *Prevotella* | 7.5 ± 1.6 | 7.7 ± 1.5 | 0.858 | −0.08 (−0.8 to 0.7) | 0.829 | −0.82 (−1.7 to 0.1) | 0.081 |
| *Bacteroides fragilis* | 10 ± 0.5 | 10 ± 0.4 | 0.751 | −0.04 (−0.28 to 0.19) | 0.705 | 0.07 (−0.20 to 0.35) | 0.606 |
| *Atopobium* cluster | 7.6 ± 0.7 | 7.9 ± 0.6 | 0.079 | −0.16 (−0.4 to 0.15) | 0.303 | −0.21 (−0.5 to 0.16) | 0.265 |
| *Clostridium leptum* | 9.6 ± 0.9 | 10 ± 0.3 | 0.008 | −0.3 (−0.6 to −0.01) | 0.040 | −0.3 (−0.7 to 0.006) | 0.054 |
| *Clostridium coccoides* | 9.2 ± 0.3 | 9.5 ± 0.4 | <0.001 | −0.19 (−0.37 to −0.006) | 0.044 | −0.24 (−0.4 to −0.02) | 0.029 |

CI, confidence interval.
Multivariate model 1 was adjusted for age, sex, and body mass index. Multivariate model 2 comprised model 1 plus daily fiber intake.

LDL. *C. leptum* was negatively correlated with glucose and HOMA-IR and significantly positively correlated with HDL, LDL, and daily cholesterol intake. *C. coccoides* was also negatively correlated with BMI, glucose, HOMA-IR, HbA1c, triglycerides, TC/HDL, IL-1β, and GLP-1 and significantly positively correlated with HDL and daily cholesterol intake. In addition, *Bifidobacterium* was negatively correlated with age and positively correlated with daily fiber intake; *Enterobacteriaceae* was positively correlated with age and negatively correlated with TC/HDL and daily fat and carbohydrate intake; *Enterococcus* was negatively correlated with glucose and total fat intake; and *Bacteroides fragilis* was positively correlated with daily energy and cholesterol intake.

The differences in clinical parameters and fecal microbiota were also examined after 3 mo of treatment in the diabetic patients. The treatments included diet and exercise education, antidiabetic agents, antihypertensive medication, and statins for hyperlipidemia if clinically needed. The results are shown in Table 6. Glucose, HbA1c, total cholesterol, triglycerides, LDL, and TC/HDL were significantly decreased after 3 mo of treatment, whereas insulin was significantly increased. BMI, HOMA-IR, HDL, IL-1β, TNF-α, GLP-1, and adiponectin did not significantly change. *Bifidobacterium, C. perfringens, Enterobacteriaceae, Enterococcus, Prevotella, Bacteroides fragilis,* and *Atopobium* cluster were also not significantly changed after 3 mo of treatment. *Lactobacillus* tended to decrease after treatment but not significantly ($P = 0.052$). *C. leptum* and *C. coccoides* were significantly decreased after 3 mo of treatment. All the diabetic patients were educated by a nutritionist about their diet. However, the daily intake of energy, protein, fat, carbohydrates, fiber, and cholesterol did not significantly change after 3 mo of treatment. Regarding oral antidiabetic agents, 97.4% of the patients received metformin, 71.8% received sulphonylureas, 5.1% received α-glucosidase inhibitors, and 2.6% received dipeptidyl peptidase-4 inhibitors. In addition, 33.3% of the patients were prescribed antihypertensive medications and 59.0% were prescribed statins for hyperlipidemia. However, correlation analysis revealed no significant changes in the fecal microbiota associated with the use of medications (data not shown).

## Discussion

In the present study the clinical parameters and fecal microbiota of patients with newly diagnosed, treatment-naive type 2 diabetes and healthy controls were examined. Diabetic patients had significantly increased glucose, insulin, HOMA-IR, and HbA1c compared with the healthy controls. The fecal count of *Lactobacillus* was also significantly higher in the diabetic patients compared with the healthy controls. This result is consistent with previous studies [10–13]. A positive correlation was found between the *Lactobacillus* count and diabetic parameters, including glucose, HBA1c, and HOMA-IR, which is similar to the findings of previous studies [11,12]. A significantly higher fecal count of *Lactobacillus* was also found after adjusting for age, sex, and BMI; however, this significance was lost if there was an additional adjustment for fiber. Furthermore, in the diabetic patients treated for 3 mo, all clinical parameters, including glucose, HbA1c, and lipid profiles, were improved, and the *Lactobacillus* count tended to decrease, although the change was not significant. The reason for this result is not clear. Taken together these findings suggest that the increase in *Lactobacillus* may be a consequence of diabetes or sugar control. The casual relationship between diabetes and *Lactobacillus* still requires further research to confirm.

*C. coccoides* and *C. leptum* were significantly decreased in the diabetic patients compared with the healthy controls. *C. leptum* and *C. coccoides* are considered to be the two most dominant bacterial groups of butyrate-producing bacteria and are believed to significantly contribute to butyrate production [19]. Butyrate plays an important role in the health of the gut because it is the preferred energy source for epithelial cells in the colon. It has also been reported to improve the gut barrier function, reduce oxidative stress in the colon, and even play a protective role against colon cancer and colitis [20]. Regarding its metabolic effect, butyrate may have beneficial effects on a host's metabolism by maintaining intestinal integrity, which prevents lipopolysaccharide-related endotoxemia and further inflammatory responses [21]. Butyrate has also been reported to improve insulin sensitivity and increase energy expenditure in dietary obese mice [22]. Therefore these butyrate-producing bacteria are potentially beneficial for glucose homeostasis. A significantly reduced *C. coccoides* count was identified in diabetic patients compared with the healthy controls even after adjusting for age, sex, BMI, and daily fiber intake. This result is consistent with the findings of previous studies [9–12]. A negative correlation between *Clostridium* and markers of type 2 diabetes has been previously reported [10,23]. The present study also found a similar result in that *C. coccoides* and *C. leptum* were inversely related to glucose and HOMA-IR. Although these results suggest that the decreased levels of butyrate-producing bacteria identified in patients with type 2 diabetes may contribute to the disease, these causal relationships are still unconfirmed.

Unexpectedly, *C. leptum* and *C. coccoides* counts were significantly decreased in diabetic patients treated with medications for 3 mo, although the majority of the metabolic parameters were improved. Several previous studies have found that animal protein may decrease butyrate-producing bacteria, whereas high levels of resistant starch or carbohydrate fermentation may increase

**Table 5**
Association among fecal microbiota, clinical factors, and food intake

| Fecal microbiota | Age | BMI | Glucose | HOMA-IR | HbA1c | TC | TG | HDL | LDL | TC/HDL | IL-1β | TNF-α | GLP-1 | APD | Daily intake | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | | | Energy | Protein | Fat | Carb | Fiber | TC |
| *Bifidobacterium* | −0.238* | 0.082 | −0.028 | −0.009 | 0.021 | 0.136 | 0.043 | −0.037 | 0.161 | 0.167 | −0.092 | −0.065 | 0.008 | −0.081 | 0.157 | 0.022 | −0.036 | −0.068 | 0.249* | 0.023 |
| *Lactobacillus* | 0.078 | 0.131 | 0.349† | 0.255* | 0.312† | −0.110 | 0.200 | −0.083 | −0.269* | 0.030 | 0.197 | 0.159 | 0.144 | −0.107 | −0.036 | 0.026 | 0.006 | −0.008 | −0.078 | −0.070 |
| *Clostridium perfringens* | 0.049 | −0.007 | 0.087 | 0.076 | 0.135 | −0.084 | 0.139 | −0.002 | −0.134 | −0.086 | 0.068 | 0.109 | 0.032 | −0.175 | 0.057 | −0.020 | −0.132 | −0.143 | 0.102 | 0.037 |
| Enterobacteriaceae | 0.214* | −0.111 | −0.181 | −0.134 | −0.052 | −0.182 | −0.111 | 0.112 | −0.097 | −0.199* | 0.045 | −0.008 | 0.080 | 0.176 | −0.120 | −0.152 | −0.370† | −0.392† | 0.072 | 0.059 |
| *Enterococcus* | 0.132 | −0.182 | −0.255* | −0.228 | −0.050 | −0.014 | −0.196 | 0.152 | −0.009 | −0.138 | 0.183 | −0.079 | 0.053 | 0.169 | −0.139 | −0.199 | −0.293* | −0.116 | 0.034 | 0.050 |
| *Prevotella* | 0.027 | −0.075 | −0.042 | −0.089 | 0.018 | −0.154 | 0.093 | −0.127 | −0.022 | 0.070 | −0.150 | −0.073 | 0.029 | −0.069 | −0.085 | −0.122 | 0.026 | −0.030 | −0.005 | 0.158 |
| *Bacteroides fragilis* | −0.084 | −0.033 | −0.091 | −0.031 | −0.137 | 0.097 | −0.003 | 0.054 | 0.161 | −0.048 | 0.196 | 0.189 | 0.086 | −0.037 | 0.217* | 0.078 | 0.202 | −0.023 | 0.207 | 0.222* |
| *Atopobium* cluster | 0.031 | −0.184 | −0.168 | −0.151 | −0.087 | 0.038 | −0.106 | 0.125 | 0.130 | −0.039 | −0.124 | −0.128 | −0.102 | 0.179 | 0.104 | 0.082 | 0.115 | 0.051 | 0.115 | 0.057 |
| *Clostridium leptum* | 0.043 | −0.188 | −0.231* | −0.255* | −0.104 | 0.139 | −0.131 | 0.229* | 0.208* | −0.161 | −0.187 | −0.229 | −0.161 | −0.002 | 0.128 | 0.188 | 0.090 | −0.013 | 0.129 | 0.331† |
| *Clostridium coccoides* | 0.080 | −0.334† | −0.402† | −0.374† | −0.331† | 0.022 | −0.276† | 0.256* | 0.168 | −0.287† | −0.238* | −0.139 | −0.220* | 0.103 | 0.093 | 0.091 | 0.049 | −0.096 | 0.109 | 0.359† |

APD, adiponectin; BMI, body mass index; Carb, carbohydrate; GLP, glucagon-like peptide; HOMA-IR, homeostatic model assessment of insulin resistance; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; IL, interleukin; LDL, low-density lipoprotein; TC, total cholesterol; TG, triglyceride; TNF, tumor necrosis factor.
*$P < 0.05$ (two-tailed).
†$P < 0.01$ (two-tailed).

**Table 6**
Differences in clinical parameters and fecal microbiota after 3 mo of treatment in diabetic patients

| Variable | Mean difference (95% CI)(post-pre, $N = 39$) | $P$ |
|---|---|---|
| Body mass index (kg/m$^2$) | 0.1 (−0.1 to 0.4) | 0.353 |
| Glucose (mg/dL) | −76 (−106 to −46) | <0.001 |
| Insulin | 3.8 (0.2–7.4) | 0.003 |
| HOMA-IR | 0.5 (−1.1 to 2.3) | 0.635 |
| HbA1c (%) | −3.6 (−4.5 to −2.8) | <0.001 |
| Total cholesterol (mg/dL) | −37 (−51 to −23) | <0.001 |
| Triglyceride (mg/dL) | −113 (−187 to −38) | 0.001 |
| HDL (mg/dL) | 4.0 (−2.7 to 10.7) | 0.203 |
| LDL (mg/dL) | −22 (−39 to −4) | 0.014 |
| TC/HDL | −1.0 (−1.4 to 0.6) | <0.001 |
| Urine microalbumin (mg/g) | −27 (−48 to −6) | 0.001 |
| IL-1β (pg/mL) | 0.006 (−13 to 13) | 0.820 |
| TNF-α (pg/mL) | −0.03 (−4.9 to 4.8) | 0.859 |
| GLP-1 (pg/mL) | 3.6 (−3.8 to 11.1) | 0.557 |
| Adiponectin (× 10$^3$ pg/mL) | 65.7 (−391 to 522) | 0.665 |
| *Bifidobacterium* | −0.39 (−0.81 to 0.01) | 0.103 |
| *Lactobacillus* | −0.41(−0.84 to 0.01) | 0.052 |
| *Clostridium perfringens* | −0.28 (−0.90 to 0.33) | 0.704 |
| *Enterobacteriaceae* | 0.13 (−0.22 to 0.48) | 0.372 |
| *Enterococcus* | −0.57 (−0.61 to 0.49) | 0.821 |
| *Prevotella* | 0.08 (−0.29 to 0.46) | 0.993 |
| *Bacteroides fragilis* | −0.13 (−0.36 to 0.08) | 0.247 |
| *Atopobium* cluster | −0.16 (−0.37 to 0.05) | 0.065 |
| *Clostridium leptum* | −0.23 (−0.56 to 0.09) | 0.011 |
| *Clostridium coccoides* | −0.22 (−0.36 to −0.07) | 0.003 |
| Daily energy intake (× 10$^3$ kcal) | 246 (−172 to 665) | 0.434 |
| Protein intake (%) | 3.2 (−0.3 to 6.7) | 0.566 |
| Fat intake (%) | 0.3 (−3.0 to 3.6) | 0.131 |
| Carbohydrate intake (%) | −2.6 (−11 to 5.7) | 0.972 |
| Daily fiber intake (g) | 1.74 (−2.36 to 5.86) | 0.217 |
| Daily cholesterol intake (mg) | 116 (−97 to 330) | 0.821 |

CI, confidence interval; GLP, glucagon-like peptide; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; HbA1c, glycated hemoglobin; IL, interleukin; TC, total cholesterol; TNF, tumor necrosis factor.

butyrate-producing bacteria [24,25]. However, in the present study no significant changes in diet composition were identified after 3 mo of treatment in the diabetic patients. Antidiabetic drugs have been found to modify the gut microbiota composition and diversity [26]. In the present study, 97% of diabetic patients received metformin, which is currently used as a first-line treatment for type 2 diabetes. Metformin treatment has been found to be associated with an increased abundance of short chain fatty acid–producing bacteria, and to result in an increase of butyrate and propionate [12,14,27–29]. De la Cuesta-Zuluaga et al. [28] reported that some butyrate-producing bacteria were more abundant in metformin-treated type 2 diabetic patients, whereas others, including *C. coccoides* and *C. leptum*, were unaffected. In the present study, *C. coccoides* and *C. leptum* were significantly decreased after antidiabetic treatment, including metformin. The reason for this controversial result is not clear because these butyrate-producing bacteria were thought to be beneficial. However, a high level of butyrate is also thought to provide additional energy and play a role in adipose tissue expansion; it could even lower the gut barrier function as a result of an increase in the apoptosis rate. Therefore butyrate overproduction seems to be a risk factor for the development of obesity into type 2 diabetes [30–32]. This suggests that balanced levels of butyrate-producing bacteria may be crucial to health. Although it has not been extensively addressed in the present study, the authors suggest that metformin treatment may be related to the adjustment of the "energy harvest hypothesis" by decreasing some of the butyrate-producing bacteria to avoid additional energy harvest. The majority of type 2 diabetic patients are also overweight or obese. Whether the decrease in *C. coccoides* and *C. leptum* after treatment is helpful or relevant should be further investigated in future studies.

A strength of the present study compared with previous studies is that samples were collected from treatment-naïve diabetic patients, so the influence of medications on the microbiota could be excluded. In addition, changes in the fecal microbiota were investigated before and after diabetic treatment was started, and although it was not possible to confirm a causal relationship between treatment and further decreased *C. coccoides* and *C. leptum* counts, it has highlighted it for further investigation. Limitations of the present study may be the relatively small sample size, and although we investigated before and after diabetic treatment, the short treatment duration of 3 mo. Furthermore, recent metagenomics analysis has provided a broader view of microbiome diversity and richness, which could not be revealed by 16S rRNA sequencing in the present study. It is difficult to define the importance of the abundance of one bacterial species for the host metabolism because there may be "functional" or "unbalanced" dysbiosis of the gut microbiota in patients rather than a specific microbial species that has a direct association with disease.

## Conclusions

The results of the present study indicated the differences in gut microbiota between newly diagnosed, treatment-naive patients with type 2 diabetes and healthy controls. We also investigated the changes in gut microbiota after 3 mo of treatment in diabetic patients. The *Lactobacillus* count was higher in diabetic patients compared with the healthy controls, whereas *C. leptum* and *C. coccoides* counts were lower. After 3 mo of medical treatment, *C. leptum* and *C. coccoides* counts decreased in the diabetic patients, whereas most of their clinical parameters improved. Increasing evidence suggests that the composition of the gut microbiota plays an important role in glucose metabolism. Therefore further research is needed to define meaningful differences in microbial composition and its effect on metabolic function and to separate the cause of microbiota changes from its correlation with type 2 diabetes.

## Acknowledgments

The authors would like to acknowledge the technological support of the lab team of Dr. Suh-Ching Yang at the School of Nutrition and Health Sciences, Taipei Medical University, and thank all team members for their support in performing the sample analysis.

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