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COMPARISON BETWEEN METABOLIC PARAMETERS, FOOD INTAKE, AND GUT MICROBIOTA IN TYPE 2 DIABETES AND NON-DIABETIC INDONESIAN WOMEN

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ABSTRACT

Background: *Globally, the increasing incidence of type 2 diabetes mellitus (T2D) has resulted in an upsurge in research into this metabolic condition. Women, particularly in Indonesia, have a greater risk of T2D than males. The diversity of the gut microbiota (GM) in T2D is regulated by the number of carbs, protein, fat, and fiber consumed.*

Objectives: *This study examined the comparison between metabolic parameters, food intake, and GM in T2D and non-diabetic Indonesian women.*

Materials and Methods: *The cohort study included people who did not have T2D and those who did. On day 28 of observations, anthropometric, metabolic parameters, food intake, physical activity, and feces were collected. Feces were collected for pH, SCFA, and GM (L. plantarum, Bifidobacterium, and Prevotella) analysis.*

Results: *There were significant differences between non-diabetic and diabetic women in age, Waist Hip Ratio (WHR), fasting blood sugar (FBS), and HbA1c. The two groups did not differ significantly in terms of their macronutrient intake (calories, carbs, protein, and fat), total water, and dietary fiber. Fecal pH and GM did not statistically differ between the control and T2D groups. Fasting blood sugar and HbA1c were positively associated with age, duration of T2D, WHR, and total water consumption, but slightly negatively associated with dietary fiber intake. Fasting blood sugar was also slightly negatively associated with Prevotella, meanwhile HbA1c with Bifidobacterium. Carbohydrate intake were positively correlated with acetic, propionic, and butyric acid levels.*

Conclusion: *Macronutrient intake, fecal pH, SCFA, and GM did not differ because GM in T2D increased bacause metformin consumption so that SCFA similar between two group.*

Keywords *: food, gut microbiota, short-chain fatty acid, diabetes, women*

BACKGROUND

Diabetes Mellitus Type 2 (T2D) is a metabolic condition defined by elevated blood glucose levels caused by a combination of inadequate insulin production and insulin resistance [1]. The International Diabetes Federation (IDF) estimates that around 463 million (9.7%) adult persons aged 20–79 years had diabetes in 2019, with that number expected to climb to 700 million (10.9 percent) by 2045. Indonesia is one of the top ten nations in the world with the greatest prevalence of diabetes, with 10.7 million adult diabetics in 2019 and a projected increase to 16.6 million by 2045 [2]. The prevalence of diabetes among women is higher than among men in Indonesia based on the national basic health research in 2018 [3]. Compared to men, women had a greater relationship between diabetes mellitus and acute myocardial infarction, and chronic ischemic heart disease [4]

T2D in Indonesia is determined by lifestyle, eating behavior, eating patterns such as smoking, obesity, unhealthy diet, lack of physical activity, consumption of alcoholic beverages, hypertension, dyslipidemia, and risk factors that cannot change, such as age and genetic factors [5–7]. Evidence suggests that dysbiosis of the gut microbiota (GM) plays a critical role in the development and progression of T2D. Gut microbiota can disrupt the host's glucose homeostasis [8]. A prior study revealed a link between *Bifidobacterium* and diabetes mellitus development. *Bifidobacterium* were less represented in the microbiota of the diabetic group than the non-diabetic group [9–11]. The diversity of GM is regulated by the number of carbs, protein, fat, and fiber consumed, as well as the type of diet consumed [12–14]. *Prevotella*, which is primarily seen in persons in developing countries or vegetarians, is very prevalent in the gut microbiome of most Indonesians, according

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to the previous study [15]. *L. plantarum* is also a dominating bacterium in the GM of the average Indonesian [16]. Numerous metabolic products of GM, including short-chain fatty acids (SCFA) are implicated in the control of glucose metabolism [17]. Therefore, GM and its correlation with SCFA, metabolic markers, food intake has been considered as a suitable target for studying the T2D mechanisms. The purpose of this study was to compare between metabolic parameters, food intake, and GM (*L. plantarum, Bifidobacterium*, and *Prevotella*) in type 2 diabetes and non-diabetic Indonesian women.

MATERIALS AND METHODS

Study Design

This study was a cohort study compared two groups of subject participants: the non-T2D group and the T2D group. The primary outcomes in this study are food intake, GM composition, and fecal SCFA, whereas the secondary outcomes are demographic data, anthropometric data, metabolic markers, and physical activity. Demographic data including age, and duration of T2D were obtained at the start of this study. Anthropometric data, stool samples, and blood samples were collected at the end of this study. Further analysis of stool samples was performed for GM and SCFA analyses, while blood samples were for metabolic markers analysis such as HbA1c, fasting blood sugar (FBS), and cholesterol total analysis. For 28 days, food intake and physical activity data were obtained using a semi-quantitative food frequency questionnaire (SQ-FFQ) and the International Physical Activity Questionnaire (IPAQ). Based on the Indonesian Food Composition Data and Indonesians' eating patterns, the FFQ included 165 food items in 9 food categories. The individuals disclosed their consumption habits (never, daily, weekly, or monthly), as well as the quantity of every food item they had consumed. For further analysis, the stated daily consumption of each food item was translated to grams. The FFQ validated before to used. Consumption of macronutrients and micronutrients were determined using the NutriSurvey 2007 application. (http://www.nutrisurvey.de/).

Location and Time

The research was conducted in public health centers in the Sleman regency, Yogyakarta, Indonesia, and conducted from October 2019 to March 2020. FBS, HbA1c, and total cholesterol analysis were conducted in Parahita Laboratory, Yogyakarta. Preparation of DNA extraction and SCFA analysis was done in Biotechnology Laboratory and Waste Management Laboratory in the Faculty of Agricultural Technology, Universitas Gadjah Mada. The PCR analysis of gut microbiota was carried out at the Laboratorium Penelitian dan Pengujian Terpadu (LPPT), Universitas Gadjah Mada.

Subject Participants

The study compared two groups of subject participants: 12 non-diabetic women (the non-T2D group) and 12 women with T2D (the T2D group). Subjects with T2D were obtained from 133 women with T2D and 14 non-T2D women who visited 3 public health centers in Sleman regency, Yogyakarta, Indonesia. Flow diagram of study participant can be seen in Figure 1.

They were screened based on the inclusion criteria in this study. Subjects were recruited based on puskesmas visitor data. Then the data is screened based on medical records. if they are eligible, the subject is visited at his home to be interviewed and his nutritional status and fasting blood sugar are measured. if they met the inclusion criteria, subjects were asked to sign an informed consent. The inclusion criteria for the T2D group were as follows: women aged between 20 and 50 years old, BMI < 30, HbA1c ≥ 6.5%, not pregnant and/or breastfeeding, not menopausal, not smoking, not drinking alcohol, not consuming antibiotic drugs, and other drugs. The inclusion criteria for the non-T2D group were the same as for the T2D group, with except of having an HbA1c level of 6.5%. The exclusion criteria were: going through probiotic and/or antibiotic therapy within 28 days before drawing fecal samples, being pregnant, or withdrawal of consent during the study. At the end of the field study, only 22 women finished the study: 11 non-T2D women and 11 women with T2D. The subject who dropped out of the non-T2D group was excluded due to the consumption of antibiotics, while the subject from the T2D group dropped out because the subject did not consume anti-diabetic medicine.

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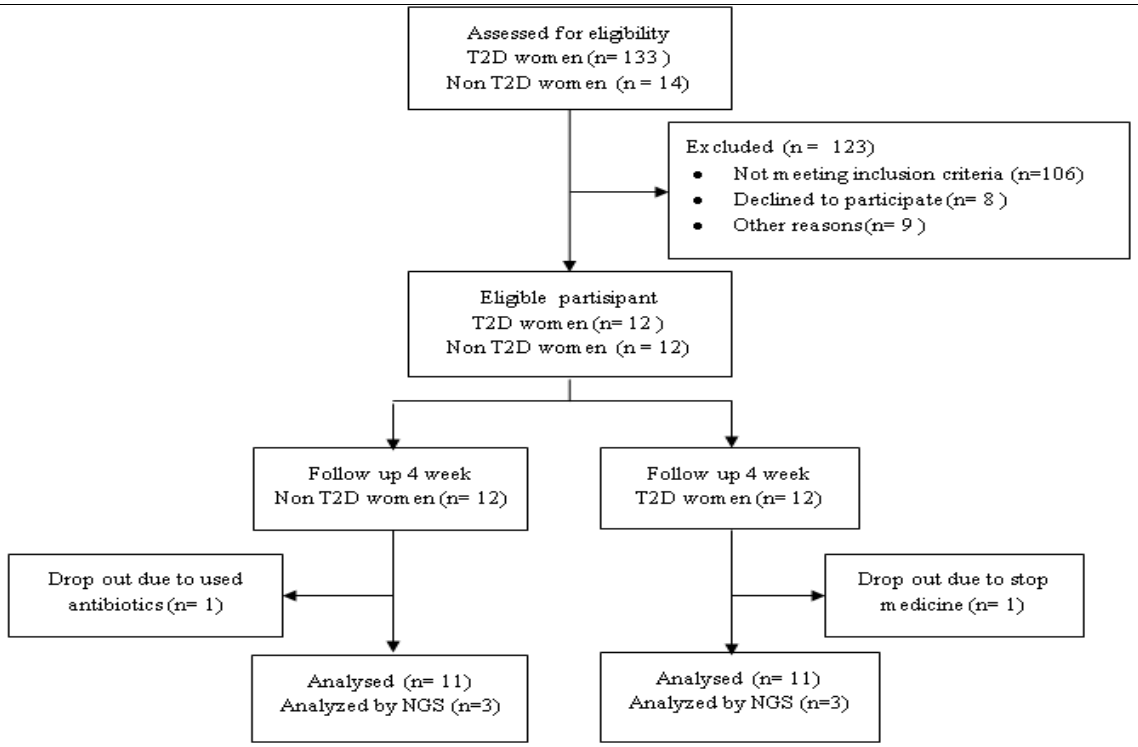

Figure 1. Flow diagram of study partisipant

Ethical Approval

As a condition of participation, all of the subjects had to provide written informed consent. The Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, authorized the study protocol, which followed the principles of the 1975 Declaration of Helsinki (Protocol number: KE/FK/1356/EC/2019; Approval date: 18 November 2019). The Ethical Committee approval document can be seen in Supplementary File 1.

Fecal Sample Collection

Before stool collection on day 28 (+1 day), each patient was given a fecal kit box and the method was described. The participants were asked to defecate and were then placed in fecal tubes. A sample in a fecal box containing ice bags was bought to the laboratory as soon as possible. After that, a fecal sample was immediately transferred into another fecal tube containing 2 mL of RNA (Sigma-Aldrich; R0901; Saint Louis, MO, USA). It was stored at frozen temperature (-18°C until -40°C) imamediately before it was used [15,18].

DNA Extraction

The sequencing process began with the extraction of DNA from the fecal sample. DNA was extracted using a modified bead-beating technique previously described by Nakayama et al [15] with adjustments as previously explained by Rustanti et al. [19].

Quantitative real-time qPCR Analysis

The microbiota analysis stage used the quantitative real-time PCR method, including DNA dilution from the results of DNA isolation, making PCR master mix, reading, making standard curves, and calculating the results of the total number of bacteria (log10 bacterial cells/g stool)[19,20]. The primers used had a DNA base sequence, as shown in Table 1.

Table 1. The specific primers used in the study

Target	Primer	Sequence (5' → 3')	
Lactobacillus plantarum	sg-Lpla-F	CTC TGG TAT TGA TTG GTG CTT GCA T	[20]
	sg-Lpla-R	GTT CGC CAC TCA CTC AAA TGT AAA	
Bifidobacterium	g-Bifid-F	CTC CTG GAA ACG GGT GG	[21]
	g-Bifid-R	GGT GTT CTT CCC GAT ATC TAC A	
Prevotella	g-Prevo-F	CACRGTAAACGATGGATGCC	[21]
	g-Prevo-R	GGTCGGGTTGCAGACC	

pH and SCFA Analysis

A pH meter was used to determine the pH of the feces (pH meter; Spear Eutech). Following calibration, the probe was dipped immediately into the feces sample and measured until a stable value was obtained [19].

Statistical Analysis

The SPSS 17 for windows was used for statistical analysis. Data presented as the mean ± SD. The data normality test was carried out using the Shapiro Wilk with a significance of 0.05 because it has a sample size of < 50. An independent t-test and non-parametric Mann-Whitney test were used to compare the groups with a significance of 0.05. Spearman correlation was used to examine a few parameters. Corrplot figure showing the correlation between two parameters analyzed using R.

RESULTS

Subject Characteristics

Subject characteristics after 4 weeks of observation are presented in Table 2.

Table 2. Characteristic of Subjects

Subject Characteristics	Non T2D (n=11) Mean ± SD	T2D (n=11) Mean ± SD	p-value
Age (years)	36.27 ± 5.69	43.82 ± 4.17	0.002[1]*
Weight (kg)	55.71 ± 5.76	5.68 ± 8.30	0.753[1]
Height (cm)	152.60 ± 4.77	150.62 ± 6.40	0.420[1]
Body Mass Index/ BMI (kg/m2)	23.94 ± 2.33	24.99 ±3.26	0.393[1]
Normal	7 (63.6 %)	6 (54.5 %)	
Overweight	4 (36.4%)	5 (45.5 %)	
Waist Circumference/WC (cm)	82.03 ± 5.70	85.96 ± 7.46	0.180[1]
Hip Circumference/HC (cm)	96.20 ± 4.67	95.03 ± 6,26	0.624[1]
WHR (Waist Hip Ratio)	0.85 ± 0.04	0.90 ± 0.05	0.011[1]*
Systolic	115.09 ± 10.80	124.45 ± 16.90	0.061[2]
Diastolic	77.73 ± 3.38	81.09 ± 9.69	0.489[2]
Fasting Blood Sugar /FBS (mg/dL)	81.09 ± 6.28	149.55 ± 53.58	0.000[2]*
HbA1c (%)	5.42 ± 0.39	8.19 ± 1.84	0.000[2]*
Total cholesterol (mg/dL)	175.64 ± 32.09	193.45 ± 39.81	0.261[1]
Duration of T2D (year)	-	2.5 ± 1.55	
< 1 year		2 (18.2%)	
1 - 3 years		6 (54.5%	
> 3 years		3 (27.3%)	
Type of drugs			
Metformin		2 (18.2%)	
Metformin & Glimepiride		8 (72.7%)	
Metformin & Gliabetes		1 (9.1%)	

[1] independent t-test; [2] Mann Whitney

Subject characteristics in T2D group had significantly higher age, FBS, HbA1c, and waist-to-hip ratio (WHR) than non-T2D group. Body mass index (BMI), cholesterol total, systolic, and diastolic were not significantly different between the two groups. The mean WHR in the T2D group (0.90 ± 0.05 cm) was higher than in the non-T2D group (0.85 ± 0.04 cm).

Food Intake and Physical Activity

The results of food intake and physical activity can be seen in Table 3. Food intakes per day, including calories, protein, fat, and dietary fiber, were not significantly different in the two groups. In both groups, only protein relative intake was in the range of WHO recommendations (protein 10% to 15%). Meanwhile, carbohydrate relative intake was lower (carbohydrate, 55% to 75%) and the fat relative ratio was higher than

the WHO recommendation (fat, 15 to 30%). However, the T2D group had a significantly higher total water daily intake (2073.36 ± 374.89 ml) than the non-T2D group (1401.13 ± 477.57 ml).

Table 3. Macronutrient Intake and Physical Activity Per Day in Two Groups

Macronutrient Intake and Physical activity	Non-T2D(n=11) Mean ± SD (% Energy)	T2D (n=11) Mean ± SD (% Energy)	p-value
Energy (kcal)	1395.66 ± 220.61	1453.55 ± 491.70	0.450[2]
Carbohydrate (g)	186.80 ± 45.94 (53.0)	186.66 ± 62.24 (51.6)	0.370[2]
Protein (g)	52.66 ± 13.87 (15.1)	46.75 ± 19.34 (12.9)	0.200[2]
Fat (g)	48.65 ± 12.15 (31.7)	61.46 ± 25.74 (37.5)	0.599[2]
Dietary Fiber (g)	13.22 ± 3.61	10.41 ± 6.87	0.061[2]
Total water (ml)	1401.13± 477.57	2073.36 ± 374.89	0.002[1]*
Physical activity (MET)	7354.6 ± 2618.8	6872.5 ± 3709.2	0.598[1]

[1] independent t-test; [2] Mann Whitney

Fecal pH and Short-Chain Fatty Acids (SCFA)

In the feces of the non-T2D and T2D groups, there was no significant variation in pH, total SCFA, acetic, propionic, and butyric acid (Table 4). Acetic acid was the most predominant SCFA. The acidity of the gut environment as a result of microbial metabolites was measured using fecal pH.

Table 4. The short chain fatty acid (SCFA) and fecal stool pH profile in two group

Characteristics	Mean ± SD		p-value
	Non T2D (n=11)	T2D (n=11)	
pH	6.17 ± 0.43	6.11 ± 0.45	0.778[1]
Total SCFA[a] (mmol/g)	21.04 ± 8.70	22.46 ± 10.42	0.732[1]
Acetic acid (mmol/g)	12.97 ± 5.36	12.89 ± 5.76	0.973[1]
Propionic acid (mmol/g)	3.87 ± 2.21	5.53 ± 4.26	0.562[2]
Butyric acid (mmol/g)	2.75 ± 1.21	2.75 ± 1.49	0.996[1]

[a]Total SCFA was the sum of acetic, propionic, iso-butyric, butyric, iso-valeric, valeric, and iso-caproic acid.
[1] independent t-test; [2] Mann Whitney

Gut Microbiota

Table 5. Gut Microbiota between Two Treatment Groups

Type of microbial	Log10 bacterial cell/g (detection rate)		p value
	Non T2D (n=11)	T2D (n=11)	
L. plantarum	4.41 ± 0.29 (100)	4.72 ± 0.54 (100)	0.107[1]
Bifidobacterium	7.11 ± 0.47 (100)	6.72 ± 0.82 (100)	0.181[1]
Prevotella	7.85 ± 0.97 (100)	7.32 ± 1.35 (100)	0.302[1]

[1] independent t-test; [2] Mann Whitney

As the bacteria of interest, qPCR analysis was used to determine the counts of *L. plantarum, Bifidobacterium*, and *Prevotella* (Table 5). Compared to the T2D group, *L. plantarum* in the non-T2D group tended to be lower, but *Bifidobacterium* and *Prevotella* tended to be greater. There was no significant difference *in L. plantarum, Bifidobacterium,* and *Prevotella* in the T2D and non-T2D groups.

Correlations Between Metabolic Markers, Food Intake, SCFA, and Gut Microbiota

The correlation of the two investigated parameters using corrplot was seen in Figure 2. Both FBS and HbA1c showed a positive correlation with age (r: 0.498, p: 0.018 and r: 0.591, p: 0.004, respectively), duration of T2D (r: 0.838, p: 0.000 and r: 0.819, p: 0.000, respectively), total water (r: 0.456, p: 0.033 and r: 0.445, p: 0.038, respectively). Meanwhile they showed slightly negatively with dietary fiber intake (r: -0.397, p: 0.067 and r: 0.-363, p: 0.097, respectively). FBS had negative correlate with *Prevotella* (r: -0.400, p: 0.065), but HbA1c with *Bifidobacterium* (r: -0.417, p: 0.054). FBS positively correlate with HbA1c (r: 0.828, p: 0.033). HbA1c was positive correlation with WC and fat ratio (r: 0.442, p: 0.039 and r: 0.401, p: 0.065, respectively).

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There was a positive correlation between total cholesterol and fat intake ratio (r: 0.502, p: 0.017), but a slightly negative correlation with carbohydrate ratio (r: -0.408, p: 0.060). Systolic displayed a positive correlation with total water (r: 0.467, p: 0.029) and a slightly positive correlation with duration of T2D, calorie and carbohydrates intake (r: 0.373, p: 0.082; r: 0.381, p: 0.080 and r: 0.387, p: 0.075, respectively).

Fecal pH displayed a negative correlation with WC (r: -0.460, p: 0.031), WHR (r: -0.430, p: 0.046), acetic acid (r: -0.501, p: 0.018), propionic acid (r: -0.574, p: 0.005), total SCFA (r: -0.493, p: 0.020). Acetic, propionic, and butyric acid acid was positively correlated with carbohydrate intake (r: 0.478, p: 0.024; r: 0.535, p: 0.010; r: 0.417, p: 0.053 respectively). Acetic acid had slightly negatively correlation with *Bifidobacterium* (r: -0.400, p: 0.065), meanwhile butyric acid had slightly positively correlation with *L. plantarum* (r: 0.363, p: 0.097) and calorie intake (r: 0.363, p: 0.097).

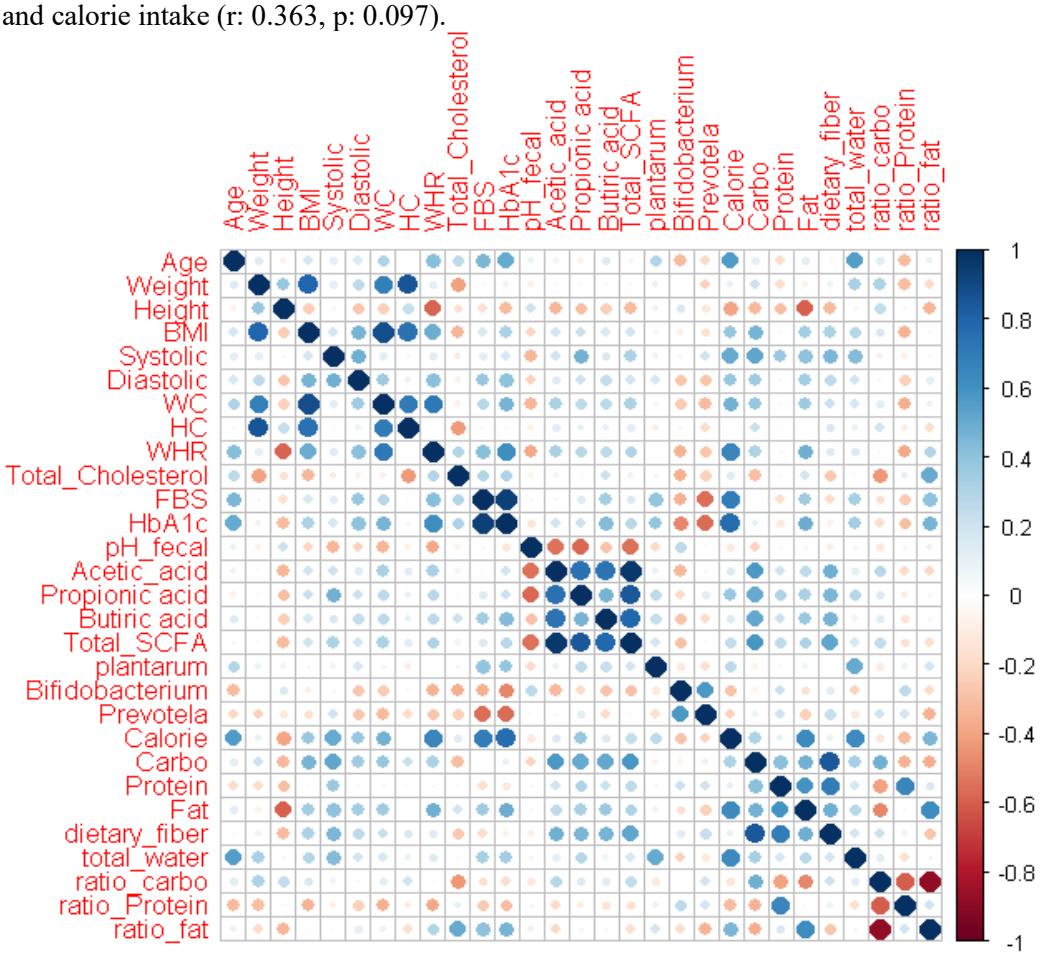

Figure 2. Corrplot Showing the Correlation between Two Parameters Analyzed using The Spearman Method. A Bigger Circle shows A Higher Correlation Coefficient. A Blue Circle Means Positive Correlation, While A Red Circle Indicates Negative Correlation.

DISCUSSION

There was no significant difference *in L. plantarum, Bifidobacterium,* and *Prevotella* in the T2D and non-T2D groups. Meanwhile, the relative abundance of Bifidobacterium, in the DM2 group was lower than the non-DM2 group. Bifidobacterium has a negative correlation with fasting blood glucose [22]. Bifidobacterium is often associated with protective properties in DM2 [10]. However, metformin significantly increased Bifidobacterium to improve glucose tolerance [23]. *Prevotella* levels tends to decreased in the T2D group. Because T2D was shown to have more *Bacteroides*, which has an antagonistic relationship with *Prevotella*, as seen in enterotypes, according to several studies [24]. Several studies have shown that there is a positive effect of metformin on the Bacteroidetes phylum, in particular increasing the abundance of Bacteroides, one of the genera within the Bacteroidetes phylum [25]. Another study shows that *Prevotella* level in lean T2D Indonesian subjects was lower than in the non-T2D subjects. In this study all T2D patients used metformin. some T2D patients in the overweight BMI category.

However, *Lactobacillus plantarum, Bifidobacterium,* and *Prevotella* in two groups were lower than *L.*

plantarum, Bifidobacterium, and *Prevotella* in the young healthy subject in Yogyakarta by about 5.0 ± 1.0 ; 9.4 ± 0.6 and 10.0 ± 1.2 log10 bacterial cell/g, respectively [16]. In the T2D group, *Lactobacillus plantarum* tended to be more prevalent than in the non-T2D group. *Lactobacillus plantarum* was shown to be the most prevalent among the higher-level *Lactobacillus* species detected in the gut.

Food intakes per day, including calories, protein, fat, and dietary fiber, were not significantly different in the two groups. Similar to another study, there were no significant differences in intake of total calories, carbohydrates, protein, or fat between the diabetes group and the control group [24]. The T2D group had a significantly higher total water daily intake (2073.36 ± 374.89 ml) than the non-T2D group (1401.13 ± 477.57 ml). Diabetic patients have symptoms including polyuria and polydipsia because of glucose homeostasis. For diabetics, drinking water can help to reduce your blood sugar (glucose) levels by diluting the amount of sugar in the bloodstream. Adequate intake of water also helps to alleviate the dehydration that comes with excess urination caused by high glucose levels[1,26]. In this study, water intake correlated positively with *L. plantarum*, but the mechanisms are still unclear.

In the feces of the non-T2D and T2D groups, there was no significant variation in pH, total SCFA, acetic, propionic, and butyric acid. These results are the same as a study in Japan which showed no significant difference in stool pH between the control group, namely 6.85 ± 0.85 and DM2, namely 6.74 ± 0.75 [27]. Stool pH under normal conditions ranges from 6.0 to 7.2 [28]. The pH of the stool indicates the acidity of the intestinal environment related to organic acids which are the result of commensal microbial fermentation in the colon such as acetic, propionic, and butyric acids [29,30]. It was proven in this study that the results of Spearman's correlation analysis showed a negative correlation between fecal pH and acetic acid (r: -0.501, p: 0.018), propionic acid (r: -0.574, p: 0.005) and total SCFA (r: -0.501, p: 0.018).

In DM2 patients who used metformin, SCFA increased again after decreasing in prediabetic and DM2 patients who did not use metformin [31,32].Propionic acid in the DM2 group, namely 5.53 ± 4.26 mmol/g, tended to be higher than non-DM2, namely 3.87 ± 2.21 mmol/g. This is because the relative abundance of Bacteroidetes which produce acetate and propionate in the DM2 group is significantly higher than the non-DM2 group. Firmicutes produces butyrate as a metabolic end product [8,29]. Acetic acid is the most dominant SCFA. The molar ratio of acetate, propionate and butyrate is approximately 60:20:20 [29]. Propionate has been demonstrated to cause the gut peptides glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) to secrete, which are implicated in hunger regulation, glucose metabolism, and inflammation [33]. The SCFA formed in the intestine is influenced by diet, the number and type of microbiota, and the transit time in the intestine. SCFA is mostly produced from anaerobic fermentation of dietary fiber and carbohydrates, especially resistant starch. r: 0.438, p: 0.042). Meanwhile, butyric acid has a positive correlation with dietary fiber (r: 0.456, p: 0.033)

A diet high in complex carbs and fiber can improve HbA1c and FBS because they can produce amount of producing bacteria short-chain fatty acids and produce mucus. The non-T2D group had a diverse gut microbiota that was enriched with short-chain fatty acid-producing bacteria and mucus-producing bacteria like *Faecalibacterium, Akkermansia, Lachnospira*, and *Roseburia*, which inhibited *Collinsella* and *Streptococcus* with proinflammatory effects and reduced inflammation in T2D. The presence of SCFA-producing bacteria can increased levels of intestinal SCFAs, reduced proliferation of dangerous bacteria, activated intestinal cells to release GLP-1, and increased insulin and HbA1c levels in patients [34].

This study has some limitations, which are listed below. The sample size was insufficient to provide significant statistical power, implying that further samples would be necessary to validate the study's findings.

CONCLUSIONS

The two groups did not differ significantly in terms of their macronutrient intake (calories, carbs, protein, and fat), total water, and dietary fiber. Fecal pH and GM did not statistically differ between the control and T2D groups. FBS and HbA1c were positively associated with age, duration of T2D, WHR, and total water consumption, but slightly negatively associated with dietary fiber intake. FBS was also slightly negatively associated with *Prevotella,* meanwhile HbA1c with *Bifidobacterium.* Carbohydrate intake were positively correlated with acetic, propionic, and butyric acid levels.

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