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Metagenomic Analysis of Bacteria Phylum *Firmicutes* and *Bacteroidetes* in Women with Type 2 Diabetes

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ABSTRACT

Diabetes mellitus (DM) is a serious health problem in Indonesia. Type 2 diabetes is a metabolic disease which primarily caused by obesity-linked insulin resistance. There is a link between insulin resistance and bacterial populations in the gut. Therefore, the aims of this study was to analyze composition of bacteria belong to the phylum *Firmicutes* and *Bacteroidetes* in women with type 2 diabetes and non-diabetic persons as control. The study included five female adults type 2 diabetes patients and five healthy controls. The bacteria composition was analyzed for abundance by denaturing gradient gel electrophoresis (DGGE). Metagenomic analysis based on 16S rRNA gene was represented by 12 DGGE bands. The twelve respective bands showed the similarity ranging from 77 up to 98%. Diversity of microbial composition in women type 2 diabetes was represented by five DGGE bands are *Bacteroides fragilis, Bacteroides vulgatus, uncultured Bacteroides* sp. *clone* Lb4eF4, *uncultured bacterium clone* 16sms90-5g05, and *uncultured bacterium clone* Malaga 1F14. Four DGGE bands in women healthy controls are closely related to *uncultured bacterium isolate* DGGE *gel band* Eub42, *uncultured bacterium isolate* DGGE *gel band* K115, *uncultured bacterium clone* HFV04255, and *Enterococcus* sp. the finding shows bacterial species belong to the phylum *Bacteroidetes* was mostly found in women diabetic group, while those belong to the phylum *Firmicutes* was mostly found in healthy controls.

1. Introduction

Diabetes mellitus (DM) is a serious world health problem. According to the data of International Diabetes Federation (2015), a number of DM cases in worldwide exceeds 415 million patients. This number is expected to increase 642 million patients in 2040. Indonesia ranks 7th out of 10 countries with most DM contributors in the world. Data of KEMENKES (2013), the total of DM patients in Indonesia achieves 12 million people in 2013 and it will mount gradually. This disease actually is caused by an increase of glucose in blood that is impacted by an impaired insulin secretion. Furthermore, more than 90% of diabetes cases are DM type 2 (IDF 2015). Diabetes in this type is more in consequence of in effective insulin usage by the human body that is characterized by obesity (Harstra *et al.* 2015). Kootte *et al.* (2012) report that a composition change of microbiota diversity in the gut will lead to an unbalanced microbiota that triggers appearing some diseases, one of them is diabetes mellitus.

One of the metabolism changes is a *Short Chain Fatty Acid* (SCFA) fermentation such as butyrate, acetate, succinate, and propionate. The SCFA is able to be used for different tracks based on numbers and types of microbiota that are available in the intestines. A ratio between *Bacteroidetes* and *Firmicutes* will liven up in patients with DM type 2 which is positively correlated to decrease tolerance on glucose (Larsen *et al.* 2010). Certain bacteria species from phylum *Firmicutes* are able to produce butyrate for preserving intestinal epithelium that inflammation in the gut is avoided. The abundance of *Bacteroidetes* is highly related to decreasing body weight. The *Firmicutes* will drop into a hypocaloric condition that the *Bacteroidetes* is able to proliferate in the intestine. A high abundance of *Bacteroidetes* can convert lactate to become acetate, succinate, and propionate that are able to harm intestinal epithelium and trigger inflammation and resistance towards glucose where impacting to occur diabetes mellitus disease (Remely *et al.* 2013).

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Several pieces research have been conducted to analyze the diversity of intestinal microbiota that is contributed to the DM disease. However, a faced obstacle recently is an absence of a clear consensus relating to microbiota data. Microbiota growth in the intestine is highly affected by several factors namely genetic, diet, food type, and geographic (Kinross *et al.* 2011) where every individual has a different microbiota diversity. Based on a research that has been carried out by Wu *et al.* (2010) for six adult women of DM type 2 patients in China infer that the abundance of *Bacteroidetes* in DM patients (53.6%) is higher than healthy people (11.8%). Another research that has been conducted by Harstra *et al.* (2015) for women with DM type 2 in Sweden found that phylum *Bacteroidetes* decrease in the DM patients and the phylum *Firmicutes* increases. Metagenomic analysis of intestinal microbiota in women patients of DM type 2 is never been reported in Indonesia previously. Therefore, a study about the microbiota diversity is necessarily important to be carried out as an effort to analyze a mark of microbiota that is associated with DM type 2 disease events. Hence, this research was aimed to analyze composition of bacteria belong to the phylum *Firmicutes* and *Bacteroidetes* in women with type 2 diabetes and non-diabetic persons as control.

2. Materials and Methods

2.1. Sample Collection

Samples of feces were taken from a population that was consisted of 30 patients and 30 non-patients as respondents. Those respondents were delivered questionnaires to compile information related to several characteristics such as women with ages in range of 25 to 67 years old, willing to sign an informed consent letter, willing not to take antibiotic drugs for 6 months (Goossens *et al.* 2005) and no being suffered from diarrhea to avoid pathogenic bacteria in their feces. Patients of DM type 2 is characterized by an in the course of blood glucose rate in blood capillaries ≥200 mg/dl with body mass index (BMI) which is based on WHO (2018) namely less (≤18.5 kg/m^2), normal (18.5-24.9 kg/m^2), pre-obesity (≥25 kg/m^2), and obesity (≥30 kg/m^2). Determination of samples was based on the criteria above resulting five-woman respondents of patients with DM type 2 and five-woman respondents of non-patients. Feces of those respondents were stored in closed sterile containers under 4^{o}C condition and then they were conveyed to a laboratory for analyzing.

2.2. DNA Extraction and Quantification

An extraction of DNA from samples of feces was carried out by using a kit Zymobiomics™ DNA Miniprep Kit (USA). Concentration and DNA purification were then analyzed by using a *NanoDrop* 2000 (Thermo Scientific, Wilmington, DE, USA).

2.3. PCR Amplification of 16S rRNA Gene

Amplification step was executed by using a Polymerase Chain Reaction (PCR) method with T1-thermocycler (Biometra, Goettingen Germany). The used primers were P338F-GC (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCAC GGGGGGACTCCTACGGGAGGCAGCAG-3') and P518R (5'-ATTACCGCGGCTGCTGG-3') (Overeas *et al.* 1997). Amplification was taken by utilizing a *GoTaq Green Mastermix 2x* (Promega, Madison, USA), with a composition of PCR as follows 12.5 µl of *Gotaq Green Promega*, two primers (P338F and P518R were 2.5 µl, DNA template 0.5 µl (21 ng/µl), respectively) and an additional *nuclease-free water* until the final volume exceeded 30 µl. The PCR was conditioned for several treatments such as pre-denaturation under 94^{o}C for 5 minutes, denaturation under 94^{o}C for 30 seconds, annealing under 55^{o}C for 30 seconds, elongation under 72^{o}C for 30 seconds as many as 30 cycles. Results of the PCR then were electrophoresed in 1.5% agarose gel and furthermore were documented in a G:BOX (Syngenee Frederick, MD, USA).

2.4. DGGE Analysis of the 16S rRNA Gene

The resulted PCR of gene 16S rRNA was migrated into an 8% (w/v) polyacrylamide gel that was created from acrylamide-bisacrylamide [37.5:1] in 1x *Tris*-asetate-EDTA (TAE). The gradient of denaturant was set between 35 to 65% and the denaturant becoming 100% was formulated by adding 7M of urea and 40% (v/v) of formamide. The electrophoresis process was conditioned under temperature 60^{o}C and voltages 60 Volt as long as15 hours by using a *D Code Universal Mutation Detection System* (Bio-Rad, Hercules, CA, USA). Visualization of the resulted process was documented by using a G:BOX (Syngenee, Frederick, MD, USA). The resulted photo of gel doc was then analyzed by a Labimage ID software. The separated DNA band was cut by using a sterile scalpel knife and put in a tube containing a

100 µl ddH_2O and then eluted. The product of DGGE furthermore was amplified using a primer gene 16S rRNA without using a GC-*clamp* (Muyzer *et al.* 1993).

2.5. 16S rRNA Gene Sequencing, Bioinformatics Analysis, and Phylogeneetic Tree Construction

The cut gel containing eluted DNA band was then amplified again with the DNA which was functioned as a template. Amount of 50 ng of DNA was amplified by using a specific primer P338F/P518R without *GC clamps*. The successful amplified DNAs were then sent to a Sequencing Service Company (Macrogene Korea) for sequencing process. The sequenced DNAs were analyzed using a ChromasPro software (Technelysium, AU) for assembling process and trimming. Homology analysis was administered by comparing sequenced DGGE bands with a gene bank database through a software of *Basic Local Alignment Search Tool Nucleotide* (BLAST-N) (www.blast.ncbi.nlm.nih.gov). The gained homolog sequences were further aligned by using a MEGA 6.0 software (Tamura *et al.* 2013) with a Neighbor-Joining Method. A clustering analysis was taken based on the profile of DGGE that had been interpreted by using Labimage ID and PAST 3 software.

2.6. Ethical Considerations

The research protocol was approved by the Ethics Committee of the Faculty of Medicine, the University of Indonesia (No:17-08-0788).

3. Results

3.1. Respondent Characteristics

Respondents consisted of two groups namely DM type 2 patients and non-patients as the control. The population of this research was students and housewives who are living in around Babakan Raya, Dramaga, Bogor, West Java. Those respondents were categorized according to Body Mass Index (BMI) which patients and non-patients, pre-obesity, and obesity, were 7 persons, 1 person, and 2 persons, respectively (Table 1). Eating habit of patients (P1-P5) in consuming foodsis more similar with less time intensity per day than non-patients (C1-C5). The eating habits of control in consuming foods is more varied with more time intensity per day.

3.2. Analysis of Gene 16S rRNA in Feces Samples

Amplification of gene 16S rRNA in feces sample used a universal primer of bacteria namely P338F/P518R that specifically amplified V3 region in 16S rRNA. There were 10 samples that had been amplified and generatea single band. The product of PCR gene 16S rRNA of bacteria in feces sample was analyzed by using a nested PCR technique. This technique applies two times of PCR. The first and second steps of PCR exhibit a proper amplicon size with the allowed fragment size namely ±200 pb (Figure 1).

3.3. Metagenomic Analysis of Bacteria Community

This research used a non-culture approach (metagenomic) with DGGE technique. Based on the band distribution pattern of polyacrylamide gel, the community of feces bacteria of control is in range of 15 bands at sample K1 to 22 bands at sample K3. This result is more uniform than the patients of DM type 2 that is in range of 14 bands at sample P5 to 18 bands at sample P3 (Figure 2). Direct observation and position consideration of resulted DGGE bands point out that there are three DGGE bands which always appear for all samples (band 4, 9, 10), and five bands are only found in samples of patients (band 2, 5, 7, 8, 12), and four bands which are only discovered in samples of control (band 1, 3, 6, 11). Those twelve separated bands are successfully isolated through gel cut for further sequencing analysis of their DNA.

Table1. Characteristics of patients and non-patients of DM type 2

Sample	Age (year)	BMI (kg/m²)	RBG (mg/dl)	Eating habits					Information
				Consuming staple foods (time/day)	Consuming vegetable (time/day)	Consuming fruits (time/day)	Consuming snack (yes or not)	Consuming fast food (yes or not)	
P1	53	18.8	407	2	1	1	Not	Not	Sick
P2	67	28.6	243	1	2	1	Not	Not	Sick
P3	62	30.0	263	1	1	1	Not	Not	Sick
P4	57	31.0	210	2	1	1	Not	Not	Sick
P5	47	22.1	400	1	1	1	Not	Not	Sick
K1	25	20.3	77	3	3	2	Yes	Yes	Healthy
K2	25	23.6	90	3	3	3	Yes	Yes	Healthy
K3	26	23.1	85	3	3	3	Yes	Not	Healthy
K4	47	22.1	101	3	2	2	Yes	Yes	Healthy
K5	30	22.4	88	3	3	2	Yes	Not	Healthy

Abbreviation: p = patient, k = control (non-patient), BMI = body mass index, RBG = random blood glucose

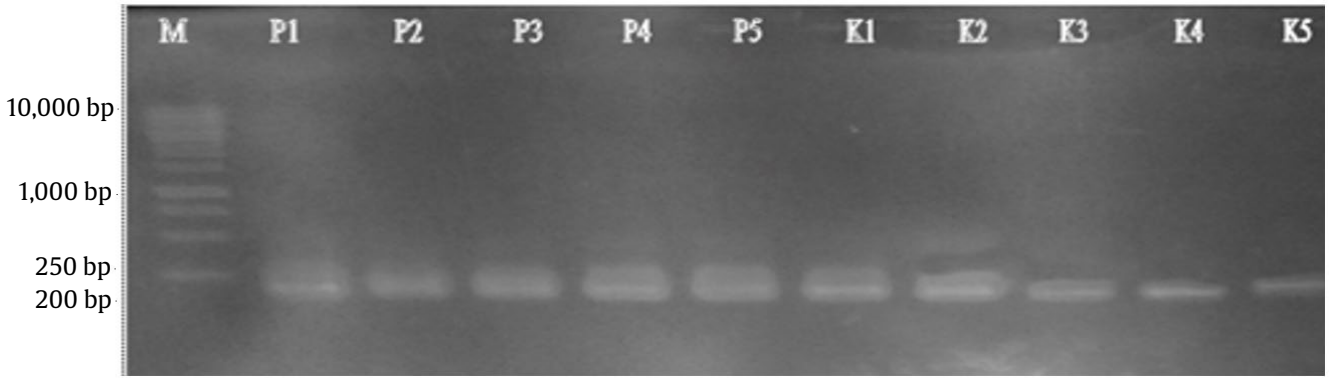

Figure 1. Visualization of resulted DNA amplification of gene 16S RNA in agarose 1.5%, M=a mark of DNA molecule; P1, P2, P3, P4, P5: feces samples of DM type 2 patients; K1, K2, K3, K4, K5: feces sample of healty respondent as control

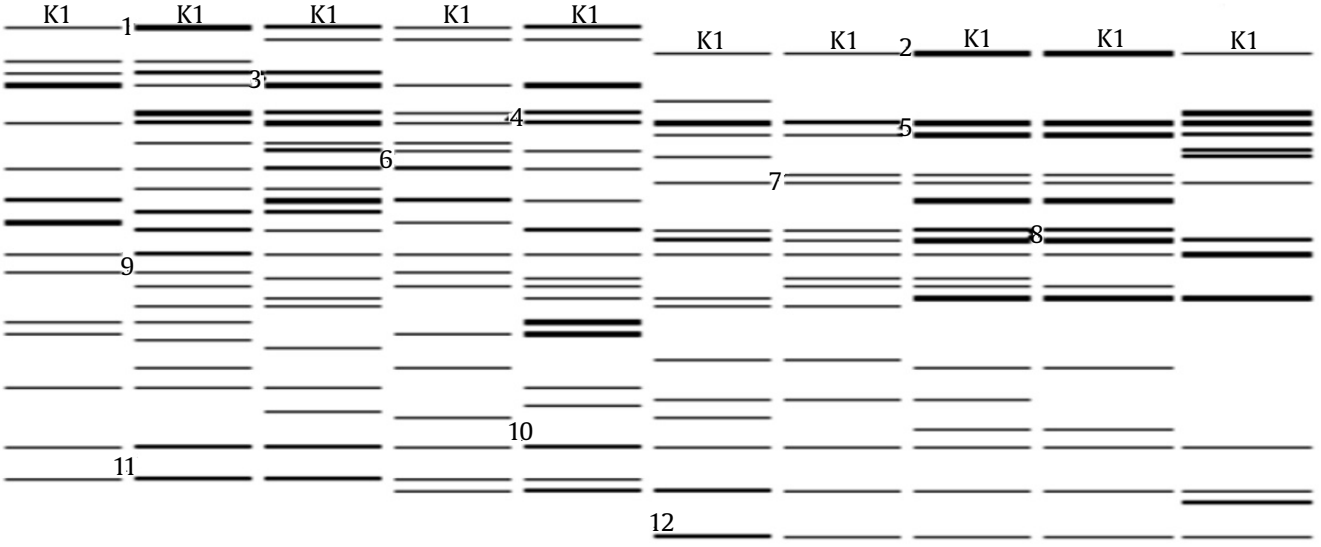

Figure 2. Ilustration of DGGE band of gene 16S rRNA using a labimage 1D software. K1, K2, K3, K4, K5: feces sample of control; P1, P2, P3, P4, P5: feces sample of patients with DM type2. The sequential numbers exhibit bands that were cut for re-PCR

A diversity analysis of Shannon-Wienner Index (H') was used to estimate bacterial diversity in each sample in both DM type 2 patients and normal respondents. The estimation results signify that the Shannon-Wienner Index (H') commonly community of bacterial feces of control based on gene 16S rRNA is in range of 2,624-2,997, while DM type 2 patients are in range of 2,476-2,829. Furthermore, the evenness index (e') is in range of 0.8422-0.9604 that is categorized into high category. A high category of this index is where e'>0.6 (Figure 3).

The advanced analysis is a sequential analysis where used a Chromas Pro software. Alignment result of nucleotide shows that nine bands of DGGE are majority identified as an uncultured bacterium, however, three bands of the identified DGGE are *Bacteroides vulgatus, Bacteroides fragilis, and Enterococcus* sp. (Table 2). In

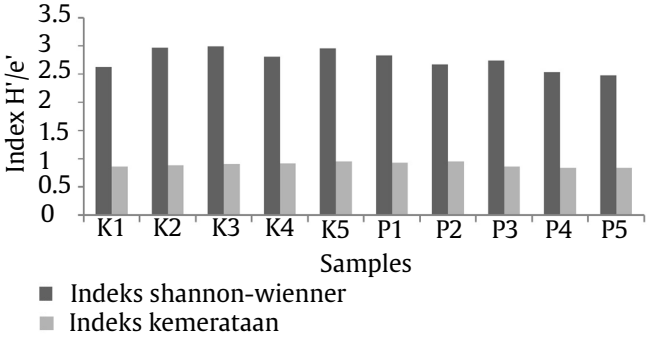

Figure 3. Diversity index of shannon-wiennerand everness index of bacterial community based on gene 16S rRNA in 10 feces samples. K1, K2, K3, K4, K5: feces samples of control; P1, P2, P3, P4, P5: feces samples of DM type 2 patients

commonly, a percentage of sequence similarity of the resulted DGGE with database genebank target is about 77-98%. Based on the phylogenetic analysis of gene 16S rRNA, grouping types of bacteria arein according with their phylum. The phylogenetic tree is constructed by a neighbor-joining tree model namely *Kimura 2-parameter model with bootstrap* 2000x (Figure 4).

Table 2. Homology analysis of bacteria metagenome based on 16S rRNA sequence

Band	Description	Query cover (%)	E-value	Identity (%)	No. access	Phylum
1	*Uncultured bacterium isolate* DGGE *gel band* Eub_42	85	3e-69	93	GQ411146	*Firmicutes*
2	*Bacteroides fragilis* strain DBT194	100	3e-79	95	MH482986	*Bacteroidetes*
3	*Uncultured bacterium isolate* DGGE *gel band* K1-15	100	1e-81	99	KF880980	*Firmicutes*
4	*Uncultured Bacteroidetes bacterium clone* SS37	100	6e-86	97	HM442603	*Bacteroidetes*
5	*Bacteroides vulgatus* strain DNF00399	100	6e-91	99	KU726654	*Bacteroidetes*
6	*Uncultured bacterium clone* HFV04_255	54	1e-22	89	GU105100	*Firmicutes*
7	*Uncultured Bacteroides* sp. *clone* Grap1_Lb4e_F4	100	4e-73	93	KP716770	*Bacteroidetes*
8	*Uncultured bacterium clone* 16sms90-5g05	100	4e-72	97	JF259225	*Firmicutes*
9	*Uncultured bacterium clone* nbw79c03c1	100	7e-10	76	GQ064768	*Firmicutes*
10	*Uncultured bacterium clone* LN07	100	1e-32	81	AY980738	*Bacteroidetes*
11	*Enterococcus* sp. strain C12	100	1e-93	99	MH754157	*Firmicutes*
12	*Uncultured bacterium clone* G02_2_2a5_Malaga_1F_14	77	7e-30	87	FJ345877	*Bacteroidetes*

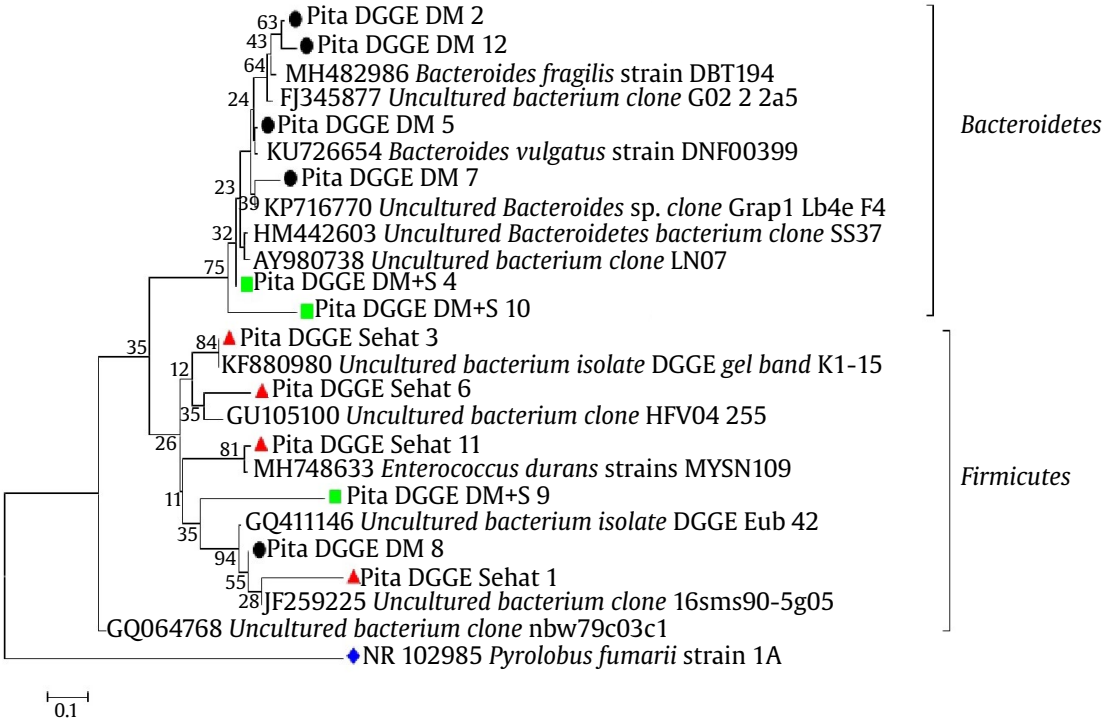

Figure 4. Phylogenetic tree (*neighbor joining tree*) of 16S rRNA using *Jukes Cantor model with boostrap* 2000x. Sequential numbers indicate sequented bands

4. Discussion

Respondents in this research are women. Yulianti *et al.* (2014) report that prevalence of women who are suffered from diabetes mellitus is higher than men due to their activities is less to produce energy. The gene 16S rRNA of feces samples were analyzed by using a universal primer namely P338F/P518R (Overeas *et al.* 1997). This primer is designed to amplify all regions of V3 in 16S rRNA. The Gene 16S rRNA has nine hypervariable regions (V1-V9). These regions are very effective for designing a mark in identifying bacteria. In geneeral, regions V2 and V3 are very appropriate in distinguishing a various type of bacteria in geneus level. The region V2 has a long alkalinity of 106 nucleotides, while V3 only has 65 nucleotides (Chakravorty *et al.* 2007). In this research, the region V3 was used due to it is able to provide phylogenetic information adequately about bacteria that are presented in a sample (Huse *et al.* 2008).

Based on a melting pattern that is caused by denaturant gradient in a polyacrylamide gel, the DNA fragment with different sequence will migrate with a different position that yield a band pattern which represent a different taxon. According to Crosby and Criddle (2003), DGGE is able to distinguish a sequence of species based on a differentiation of melting temperature (Tm) in a DNA. Melting temperature in a DNA sequence is affected by G-C alkaline compound in DNA (Muyzer *et al.* 1993). Metagenomic approach with DGGE has several advantages such as more efficient, quick, and a good result to compare diversity structure of a total community of microbiota (Fuji *et al.* 2006). Therefore, the DGGE technique is able to differentiate species based on GC composition of analyzed sequence in the analysis (Overeas *et al.* 1997). In this research, not all bands were cut and sequenced. Band selection in all samples were aimed to seek a more dominant bacteria. While the band was only found in the DM patients or only in the control (non-patients) that can be used for a mark of intestinal microbiota that are associated with metabolism process of health people and DM type 2 patients.

Results of analyzing Shannon-Wienner (H') indicates that feces samples of control have more diversity and varied of Operational Taxonomic Unit (OTU) than the diversity of OUT in feces samples of DM type 2 patients. However, overall diversity category of those sample feces was appertained into medium with Shannon index among 1>H>3.5. The similar result also has been reported by Wu *et al.* (2010) that there is no difference of Shannon-Wienner Index (H') for feces bacteria community of control and DM type 2 patients within averaged of 0.94 which is included low category. The current research also points out that the evenness index (e') of all feces samples for both control and patients is similar each other.

Furthermore, band 1 (*Uncultured Roseburia* sp. clone QY27 is found in control and band 5 (*Bacteroides vulgatus* strain NMBE-5) was in DM type 2 patients are unique bands that have the highest abundance. These both bands are able to be presumed as marks for intestinal microbiota that are associated with metabolism process in both control and patients of DM type 2. *Roseburia* sp. is a member of phylum *Firmicutes*, while *B. vulgatus* is a member of phylum *Bacteroidetes*. Both phyla are bacterial phylum that present in gut dominantly (Gill *et al.* 2006). In general, sequenced bands for DM type 2 patients come from phylum *Bacteoridetes*, and conversely, phylum *Firmicutes* is commonly for control or healthy people. Larsen *et al.* (2010) have reported that the abundance *Firmicutes* is higher in healthy people than DM type 2 patients.

Harstra *et al.* (2015) reported that *Roseburia* sp. is a type of bacteria that produce butyrate for preserving intestinal epithelium toward inflammation. The inflammation is able to disturb insulin signaling, that insulin is able to work maximally in controlling blood glucose for keeping normal. In another hand, *B. vulgatus* is able to change lactate in guts to become succinate, acetate, and propionate that can cause a disruption of intestinal permeability (Mejia-Leon and Calderon de la Barca 2015). This matter causes alipopolysaccharide (LPS) composing cell wall of gram-negative bacteria that should be in alimentary canals entering blood circulation and triggering an inflammation. This condition is able to activate *Toll-like receptor* (TLR) where the inflammation mediator like inhibitor NF-KB kinase (IKK) in metabolism track will inhibit signaling of insulin. It is impacted to targeted cells of insulin will be failed or unable to respond to insulin normally that the glucose rate is high in the blood (Wellen dan Hotamisligil 2005).

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