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Profile of Gut Bacteria in Hypertensive Patients Based on Terminal Restriction Fragment Length Polymorphism Analysis

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ABSTRACT

Hypertension is a severe public health problem due to its high prevalence worldwide. About 7.5 million deaths or 12.8% of all annual deaths worldwide occur due to high blood pressure. The hypertensive disease is also associated with the intestinal bacteria. To our knowledge, the diversity of the gut bacteria in hypertensive patients has not been reported yet in Indonesia. Thus, the aims of this study were to analyze profile of gut bacteria in hypertensive patients compared to non-hypertensive based on metagenomic analysis, Terminal Restriction Fragment Length Polymorphism (T-RFLP). The results of the affiliation analysis of the entire Terminal Restriction Fragments (TRF) contained 6 groups of bacteria from 26 TRFs in hypertensive and non-hypertensive respondents. Cutting the 16S rRNA gene with the BslI restriction enzyme successfully detected intestinal bacterial groups, namely *Clostridium* subcluster XIVa, *Prevotella, Clostridium* cluster IV, *Clostridium* cluster XI, *Bacteroides* and others. In hypertensive patients, a higher relative abundance of bacterial groups showed in *Clostridium* cluster XI, *Clostridium* cluster IV and *Clostridium* subcluster XIVa. The abundance of *Bacteroides* and *Prevotella* groups in hypertensive patients were lower than non-hypertensive. The abundance of enterotype I and enterotype II was lower in hypertensive patients than non-hypertensive. Contrarily to that enterotype III cluster. It is worth noting that the F/B ratio was higher in hypertensive patients than that in non-hypertensive. Our data suggest that the gut bacteria profile of hypertensive patients differs to that non-hypertensive.

1. Introduction

Hypertension is a severe public health problem due to its high prevalence worldwide. Hypertension is called a "silent killer" because it may have no warning signs or symptoms. The incidence of hypertension worldwide reaches more than 1.3 billion. High blood pressure is responsible for 7.5 million or 12.8% of the annual deaths in the world (WHO 2010). In 2018, the prevalence of hypertension nationally in Indonesia was 34.1%. A significant increase occurred when compared to the data from Riskesdas results in 2013, which was 25.8% (Kemenkes 2018).

Hypertension is a very high arterial blood pressure (WHO 2021). The hypertensive disease can be influenced by several factors such as genetic, physiology and environmental factors (Carey *et al.* 2018). Genetics can only account for less than 5% of the occurrence of hypertension (Giri *et al.* 2019). Hypertension also has an interaction between sex and age variables. Whereas under 60 years old, hypertension is more common in men, but after that, hypertension is commonly found in men or women (Dubey *et al.* 2002). Environmental and lifestyle factors have a significant influence on the occurrence of hypertension (Whelton *et al.* 2018). In addition to the above factors, hypertension is also related to the intestinal microbiota, especially bacteria.

The gut microbiota has a symbiosis of mutualism with metabolic processes that occur in the intestine to improve the host's health (Illiano *et al.* 2020). However, if the composition of the microbiota diversity changes, it can trigger the occurrence of several diseases, including hypertension. One of the metabolic changes in the synthesis of Short Chain Fatty Acids (SCFA), such as butyrate, acetate and propionic acid (Rowland *et al.* 2018). The SCFA can be

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used on different metabolic pathways depending on the type and number of gut microbiota. A significant increase in the Firmicutes (F)/Bacteroidetes (B) ratio is considered as a special parameter of gut microbiota in hypertensive patients (Mushtaq *et al.* 2019). A decrease in the number of bacteria producing butyrate and acetate in hypertensive patients (Li *et al.* 2017). Moreover, hypertension reduces the abundance and diversity of the gut bacteria by affecting gut physiology, thereby lowering the concentration of Short-Chain Fatty Acid (SCFA) (Yang *et al.* 2020).

Several factors can change gut microbiota, including the type of food, lifestyle, medicine, environment and geography (Gupta *et al.* 2017). In Japan, Yokoyama *et al.* (2020) explored the gut microbiota in 12 portal hypertensive patients using the T-RFLP method. They showed an increase in the *Lactobacillales* and *Prevotella* groups, while the *Bacteroides* group was high in normal. However, analysis of the gut bacteria in hypertensive patients in Indonesia has not been reported yet. Therefore, studying associated gut bacteria and hypertension is important to know the community profile of the gut bacteria. Such information is substantial for predicting the role of each group of bacteria in the development of microbiota-based hypertension disease control.

2. Materials and Methods

2.1. Participants and Sample Collection

Human faecal samples were collected from 30 hypertensive patients and 30 non-hypertensive as respondents. The questionnaire was given to respondents to obtain information related to the characteristics of each respondent, and respondents will also sign a sampling informed consent letter. Respondents with hypertension and non-hypertensive control must be in good health, no history of diarrhoea and do not take antibiotics for 6 months at that time of sampling (Goossens *et al.* 2009). Faecal samples were kept in a sterile container and held at 4°C or using a cool box.

2.2. Genomic Microbial DNA Extraction

TDNA was extracted from faecal samples using the Quick-DNA™ Faecal/Soil Microbe Miniprep Kit (USA). DNA purity and concentration were then analyzed using NanoDrop spectrophotometer (Thermo Scientific, USA).

2.3. PCR Amplification of 16S rRNA Gene

PCR T1-thermocycler (Biometra, Goettingen Germany) is used for amplification DNA genome. The 16S rRNA gene was amplified with a fluorescent-labeled primer of FAM-labeled 516f (5′-TGCCAGCAGCCGCGGTA-3′) and 1510r (5′-GGTTACCTTGTTACGACTT-3′). The amplification process was carried out using GoTaq Green Mastermix 2x (Promega, Madison, USA), 5 µl of each primer (concentration of 10 pmol), 4 µl of template DNA (30 ng/µl), and NFW (Nuclease Free Water) up to a total volume of 50 µl. The PCR conditions used are 30 cycles for denaturation (95°C for 30 seconds), annealing (58°C for 30 seconds), and elongation (72°C for 1 minute 30 seconds). The amplified product was further visualized by electrophoresis in 1% agarose gel.

2.4. T-RFLP Analysis

PCR resulted from 16S rRNA gene purification with a GenepHlow™ Gel/PCR Kit (Geneaid Biotech Ltd.). The purified PCR products (2 µg) were digested with 10 U of BslI at 55°C for 16 h. The fragments are visualized with a 2% agarose gel. The successful cutting DNAs with BslI were then sent to a sequencing service company, 1st Base Malaysia. ABI PRISM 310 genetic analyzer (Applied Biosystems) was used to measure the TRFs length. We used standard size markers like GS 2500 ROX (Applied Biosystems).

2.5. Statistical Analysis

The bacteria were predicted for each classification unit and the corresponding OTU was identified according to reference Human faecal microbiota T-RFLP profiling based on Nagashima *et al.* (2006). T-RFLP was used to classify gut microbes into the following 10 groups: *Bacteroides, Bifidobacterium, Prevotella, Lactobacillales, Clostridium* cluster IV, *Clostridium* subcluster XIVa, *Clostridium* cluster IX, *Clostridium* cluster XI, *Clostridium* cluster XVIII, and others. And then, we stratified the gut microbiome into three enterotypes: enterotype I, including *Bacteroides* at >30%, enterotype II including *Prevotella* at >15% and enterotype III, including the remaining bacteria, concerning the T-RFLP profile in human faecal. We further assessed the Firmicutes and Bacteroidetes (F/B) ratio. The *Lactobacillales* and *Clostridium* clusters include the phylum *Firmicutes*, whereas *Bacteroides* and *Prevotella* include the phylum *Bacteroidetes*.

Data from the T-RFLP analysis were shown with Principal Coordinate Analysis (PCoA) based on Bray-Curtis similarity matrices and dendrograms with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method to see patterns from sample. The one-way Analysis of Similarity (ANOSIM) method based on Bray-Curtis similarity matrices at a p-value of 0.05 was used to determine the significance of differences in bacterial community composition between samples with the Past3 (Hammer *et al.* 2001).

2.6. Ethical Considerations

The Ethics Committee approved the research protocol of the Faculty of Medicine, the University of Indonesia (No:21-08-0804).

3. Results

3.1. Respondent Characteristics

Based on the questionare data and requirement of sample, we found 8 hypertensive patients and 6 non-hypertensive that met the criteria as, respectively (Table 1). Non-hypertensive respondents were designated as control. Body mass index (BMI) of hypertensive patients consist of the underweight, normal, overweight and obesity were 1 person, 3 persons, 1 person and 3 persons respectively (Table 1). Based on the data obtained, 35% of people with hypertension have a high Body Mass Index (BMI) or are obese.

3.2. Analysis of Gene 16S rRNA in Faecal Samples

The 16S rRNA gene was amplified from the faecal of non-hypertensive and hypertensive patients using primer 516F labeled 6-FAM and 1510R. The amplification process of the 16S rRNA gene produces amplicons with a base length of ±1,000 bp (Figure 1).

3.3. T-RFLP Analysis

The fragment cutting with the BslI obtained different amounts of TRF (Table 2). TRF calculations in hypertensive and non-hypertensive show that the proportion of TRF in hypertensive patients is more diverse than in non-hypertensive. 16 TRFs were only found in hypertensive, 3 in non-hypertension respondents, while 7 TRFs were found in both of them.

The pattern of bacterial communities was analysed using PCoA and dendogram. The ordination plot showed that the sample consisted of two groups, namely hypertensive patients and non-hypertensive (Figure 2). Several samples had similarities with non-hypertensive samples, namely the P1, P2 and P3

Table 1. Characteristics of hypertensive and non-hypertensive respondents

Sample	Age (years)	Gender (F/M)	BMI (kg/m^2)	Blood pressure (mmHg)	Eating habits			
					Consuming staple foods (time/day)	Consuming vegetable (time/day)	Consuming fruits (time/day)	Consuming fast food (yes or not)
P1	32	F	22.2	150/90	3	1	2	Not
P2	43	F	33.3	130/95	3	2	1	Not
P3	66	F	19.0	180/80	3	1	2	Not
P4	69	M	27.3	130/80	3	1	1	Not
P5	69	M	19.6	180/90	3	1	1	Not
P6	68	F	15.3	180/80	3	1	1	Not
P7	60	M	24.6	160/87	3	1	1	Not
P8	29	M	32.7	150/80	2	2	2	Yes
N1	23	F	25.0	120/80	3	1	2	Yes
N2	24	F	22.2	120/80	3	1	1	Yes
N3	24	F	30.2	120/80	3	1	1	Yes
N4	25	F	19.5	110/80	3	1	2	Yes
N5	23	M	17.3	114/74	2	1	2	Yes
N6	24	M	22.2	120/80	2	1	1	Yes

Abbreviation: P = hypertensive, N = non-Hypertensive, F = female, M = male, BMI = body mass index

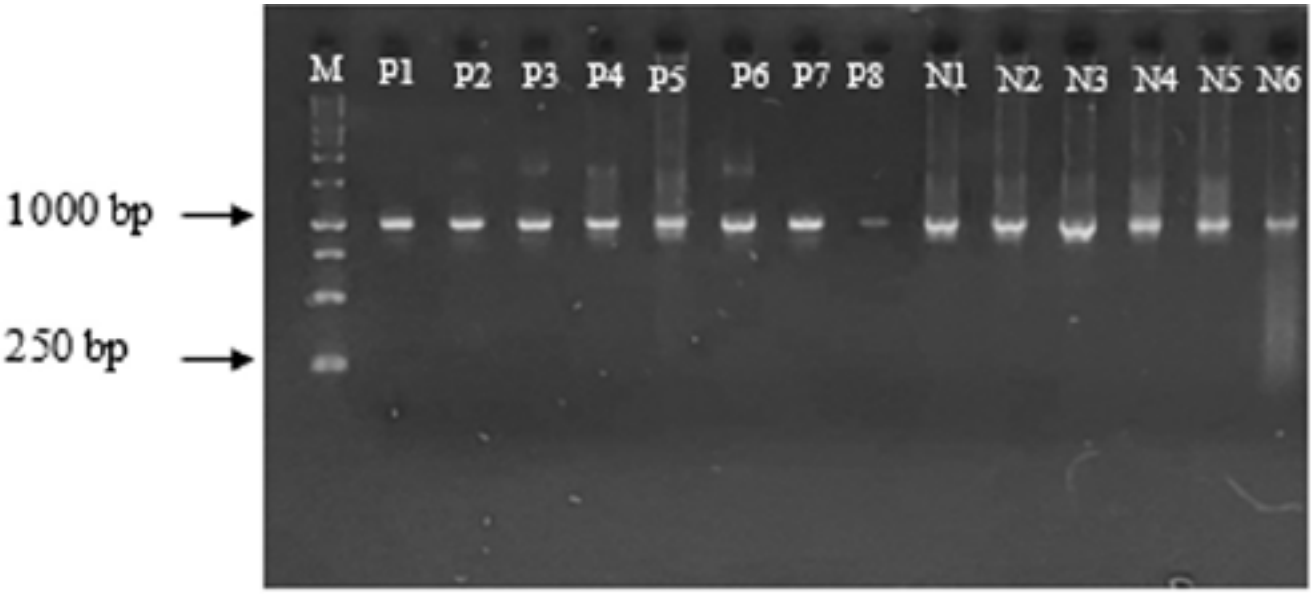

Figure 1. Visualization of 16S rRNA amplicon (±1,000 bp) in agarose 1%, M = a mark of DNA moleculer; P1, P2, P3, P4, P5, P6, P7, P8: hypertensive patients; N1, N2, N3, N4, N5, N6: non-hypertensive

Table 2. TRFs of the gut bacteria community in hypertensive patients and non-hypertensive samples with the *Bsl*l restriction enzyme

Sample	Sample	TRFs	Total TRFs	Sample	Sample	TRFs	Total TRFs
Hypertensive patients	Faecal sample P1	109, 170, 334, 366	4	Non-hypertensive	Faecal sample N1	148,170, 301, 315, 366, 462, 465	7
	Faecal sample P2	109, 334, 366	3		Faecal sample N2	109, 315, 363, 366, 462	6
	Faecal sample P3	50, 75, 91, 148, 170, 171, 462	7		Faecal sample N3	49, 148, 315, 363, 462	5
	Faecal sample P4	65, 108, 148, 170, 465	5		Faecal sample N4	148, 170, 315	3
	Faecal sample P5	65, 69, 103, 107, 110, 171, 463	7		Faecal sample N5	50, 109, 315, 363, 366, 462	6
	Faecal sample P6	108, 148, 171, 461, 485	5		Faecal sample N6	170, 315, 462	3
	Faecal sample P7	65, 70, 103, 107, 139, 143, 148, 170, 334, 464	10				
	Faecal sample P8	65, 70, 103, 107, 109, 148, 170, 171, 334, 462	10				

clusters (Figure 3). The ANOSIM test was performed to confirm the analysis of PCoA and dendrograms. ANOSIM analysis obtained p-value less than 0.05, namely 0.009, while the R-value of this analysis was obtained 0.3.

Diversity analysis of Shannon-Wienner Index (H') was used to estimate bacterial diversity in each sample based on the number of TRFs. The estimation results signify that the Shannon-Wienner Index (H') commonly community of bacterial faecal of hypertensive patients based on gene 16S rRNA is in range of 1.00-2.09, while in the healthy sample was ranged from 0.91-1.62. Additionally, the evenness index (e') belongs to the high category, with values ranged 0.70- 0.91. A high category of this index is where e'>0.6 (Figure 4).

The TRFs classification has 6 groups of bacteria from 26 TRF in the hypertensive and non-hypertensive samples (Figure 5A). BslI restriction enzyme successfully detected gut microbiota groups, namely *Clostridium* subcluster XIVa,

Prevotella, Clostridium cluster IV, *Clostridium* cluster XI, *Bacteroides* and others.

Hypertensive patients have a higher microbiota in *Clostridium* cluster XI, *Clostridium* cluster IV and *Clostridium* subcluster XIVa, but in non-hypertensive have a higher in *Bacteroides* and *Prevotella*. The number of enterotypes I and II was lower than enterotype III in hypertensive patients (Figure 5B). Ratio of Firmicutes and Bacteroidetes (F/B) increased in hypertensive patients than that in non-hypertensive (Figure 6).

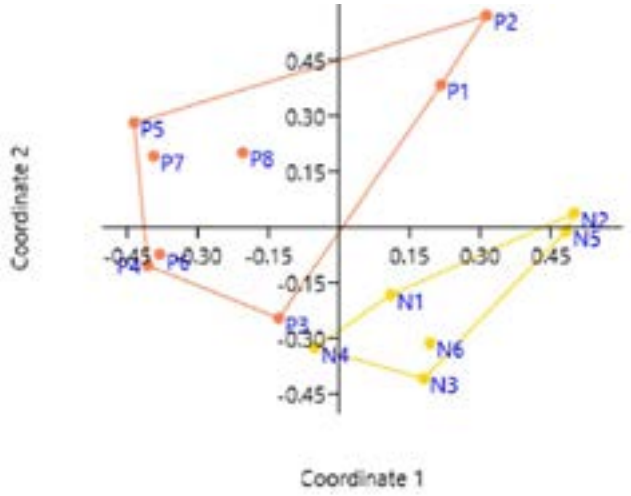

Figure 2. PCoA from DNA digesti of hypertensive patients and non-hypertensive with the *Bsl*I restriction enzyme (hypertension: o, non-hypertension: o)

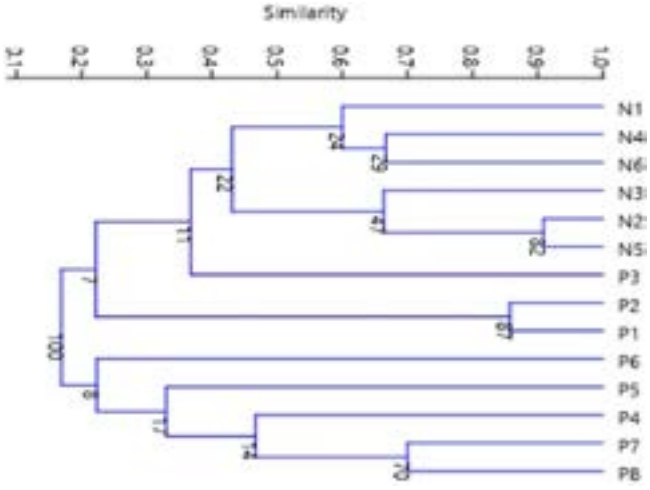

Figure 3. Clustering analysis of bacterial community similarity in faecal samples using binary data with the UPGMA PAST 3 methods (boostrap 1,000x). P: hypertensive patients; N: non-Hypertensive

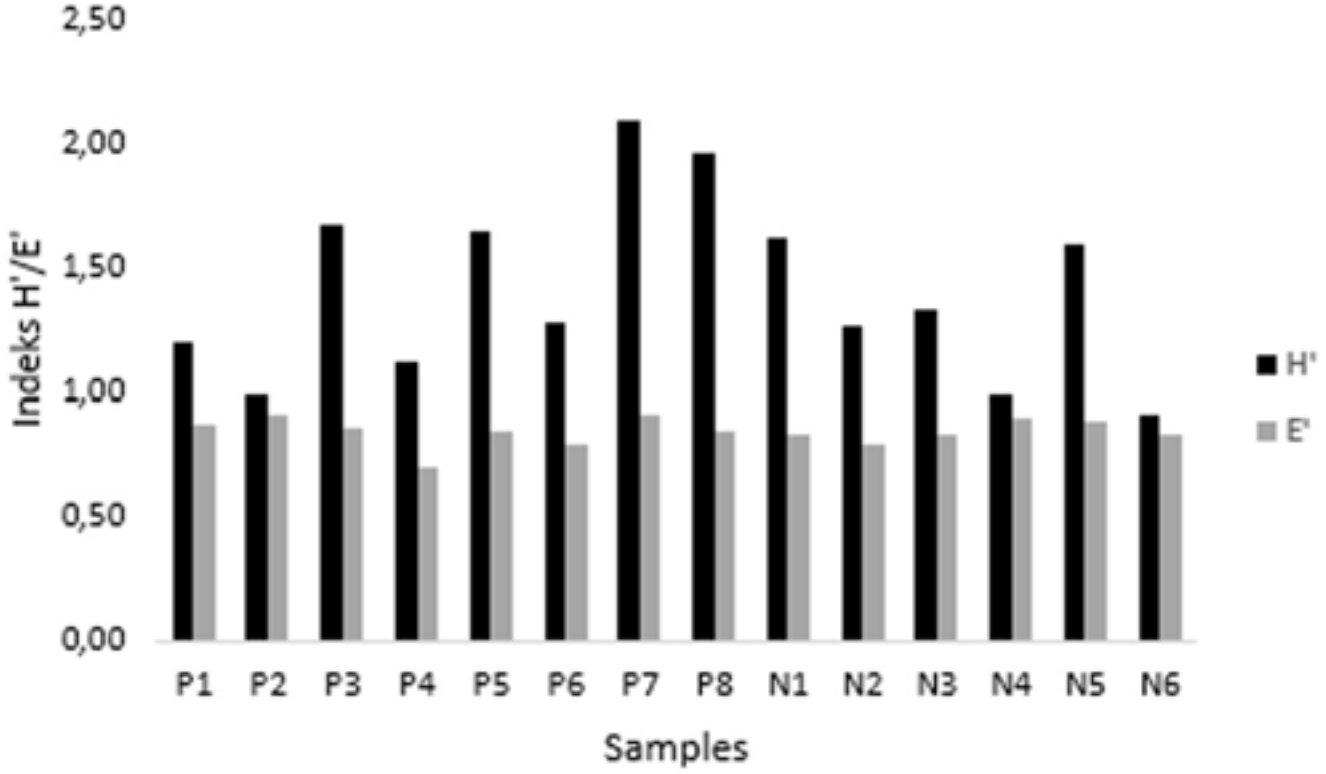

Figure 4. Shannon-Wienner diversity index and evenness index of the bacterial community based on the 16S rRNA gene in 14 Faecal samples. P1, P2, P3, P4, P5, P6, P7, P8: faecal samples hypertensive patients; N1, N2, N3, N4, N5, N6: non-hypertensive faecal samples

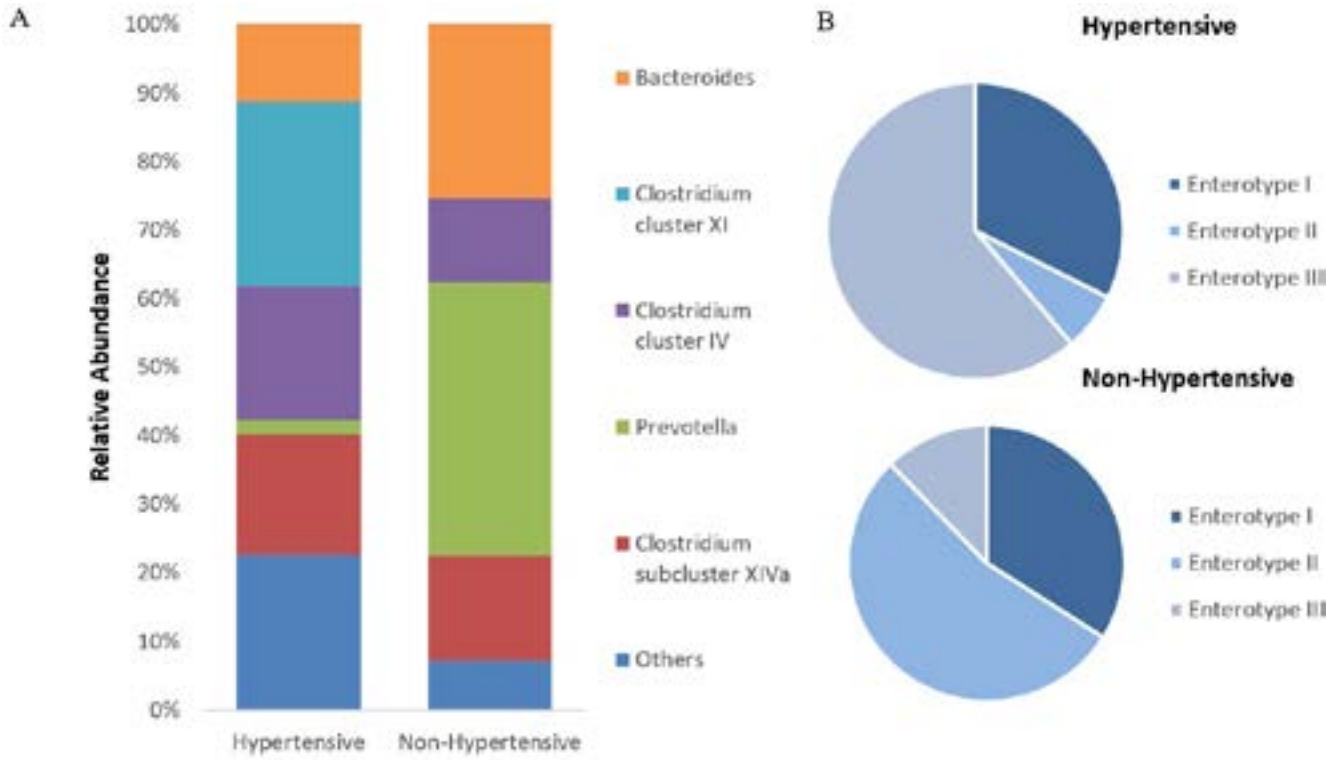

Figure 5. (A) Distribution of the gut bacteria, (B) frequency of each enterotype in the hypertensive and non-hypertensive groups. Enterotype I (Bacteroides >30%) was enriched in the non-hypertensive group

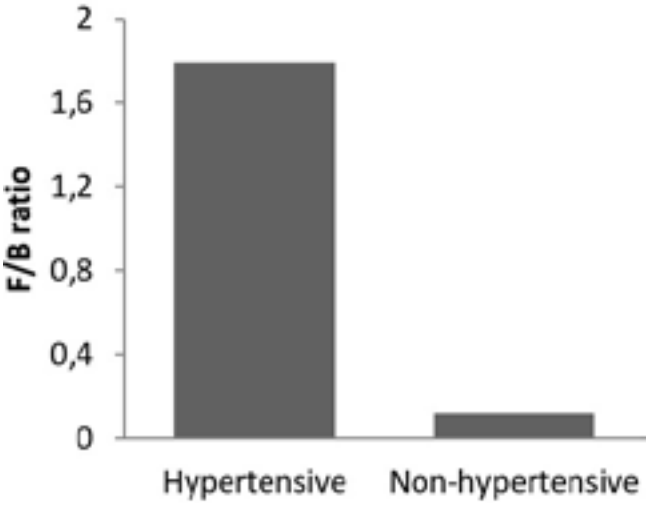

Figure 6. F/B ratio in hypertensive and non-hypertensive groups

4. Discussion

The Body Mass Index (BMI) has an effect on hypertensive. People with a high body mass index (BMI) have a systolic of 16 mmHg and diastolic of 9 mmHg which were higher than people with a low BMI category (Aronow 2017). The study showed a different ages between hypertensive patients and non-hypertension. In hypertensive patients, it has an age range of 29-69 years, while in non-hypertensive or normal it ranges from 23-25 years. Based on our study, the intestinal microbiota profile in adulthood does not differ significantly from elderly age. The initial gut microbiota of infants is a relatively unstable with a simple structure dominated by Bifidobacteria and is will continuously change to the age of three years. At this moment, the microbiota profile is established and is acquired as an adult pattern that is relatively stable over time (Porras and Rom 2021). The overall microbiota composition of the healthy aged group was similar to that of people decades younger. And then, the significant differences between groups in the gut microbiota profiles were found before age 20 (Bian *et al.* 2017).

Such phenomenon of similar microbiota profile in young and elderly group is reported elsewhere. For instance, Ottman *et al.* (2012) observed that microbiota composition was quite similar between the young and the elderly groups represented by dominant portions of Firmicutes and Bacteroidetes. Li *et al.* (2021) also reported that alpha and beta diversity from faecal samples between young adults

(25-40 years) and elderly subjects (65-77 years) were not significantly different like in Firmicutes and Bacteroides phylum. Elderly microbiota was observed to be dominated by the phylum of Bacteroidetes (57%) compared with Firmicutes (40%) (Claesson *et al.* 2011). Biagi *et al.* (2010) identified an elderly population in Italy with low levels of *Clostridium* cluster XIVa. *Prevotella* was the most abundant bacteria in the young subjects (aged 25-45 years) and elderly subjects (aged 70 years and above) of 80 volunteers from Bali and Yogyakarta (Rahayu *et al.* 2019). Such results are in line with our findings.

The 16S rRNA gene was amplified using primers 516F labeled 6-FAM and 1510R. Primers 516F and 1510R were selected based on Nagashima *et al.* (2003), who had optimized several primers for gut microbiota analysis in healthy people with faecal samples. The type of restriction enzyme used in T-RFLP analysis is important for gaining an accurate picture of microbial diversity (Engebretson dan Moyer 2003). BslI was selected based on the effectiveness of DNA the cutting which was performed by Nagashima *et al.* (2003). The BslI-based T-RFLP analysis is effective in analyzing the gut bacteria community of various diseases such as diabetes (Nakamura *et al.* 2019), dementia (Saji *et al.* 2019), cancer (Kasai *et al.* 2016) and obese people (Kasai *et al.* 2015). TRF data analysis showed that the proportion of TRF in hypertensive patients was more diverse than in non-hypertensive.

Shannon-Wiener (H') in hypertensive patients was more diversed than non-hypertension samples. With a Shannon index of 1>H>3.5, the total diversity category of those faecal samples, nevertheless, belonged to a medium category. A similar result also has been reported by Mushtaq *et al.* (2019) that there is no difference in the Shannon-Wiener Index (H') for faecal bacteria community of non-hypertensive and hypertensive patients within an average of 3.01, which is included high category. The current study also revealed that all faecal samples from patients and controls had identical evenness indices (e').

Based on the analysis of all TRF containing 6 groups of bacteria from 26 TRF in the hypertensive and non-hypertensive samples, namely *Clostridium* subcluster XIVa, *Prevotella*, *Clostridium* cluster IV, *Clostridium* cluster XI, *Bacteroides* and others. Rahayu *et al.* (2019) states that the most abundant bacteria in healthy Indonesians are *Clostridium*, *Prevotella, Atopobium, Bifidobacterium* and *Bacteroides*. Nakayama *et al.* (2015) also reported that the enterotype for Indonesians ranging from children to old age is the Prevotella enterotype.

Our results showed that the *Prevotella* and *Bacteroides* groups had a low abundance in hypertensive patients. Such phenomenon has also been reported in portal hypertension patients in japan (Yokoyama *et al.* 2020), level 3 hypertensive patients (Mushtaq *et al.* (2019) and on pulmonary hypertension patients (Kim *et al.* 2020). The low abundance of *Prevotella* group on hypertensive patients was also confirmed in the previous study (Dan *et al.* 2019).

The low abundance of *Prevotella* and *Bacteroides* in hypertensive patients was likely related with the synthesis of Short-Chain Fatty Acid (SCFA). SCFA (acetate, butyrate and propionic acid) is derived from dietary fiber (especially polysaccharides), plays an essential role in maintaining the homeostasis of the gut microbiome and host immunity (Miyamoto *et al.* 2016). A low abundance of *Prevotella* and *Bacteroides* groups will result in low availability of SCFA. Indeed, such low SCFA production may affect the inflammatory factors that further activate sympathetic nerves, resulting in changes in the structure of blood vessels (Verhaar *et al.* 2020). SCFA produced by bacteria will generally suppress the occurrence of increased blood pressure by modulating the host's physiology primarily by acting as a ligand for the G-Protein-Coupled Estrogen Receptor (Gpr41) (Natarajan *et al.* 2016).

A high microbial group found in hypertensive patients was Clostridium group, Yan *et al.* (2017) reported that in 60 hypertensive patients, the Clostridium cluster IV group was found in high percentage. The metabolic function of *Clostridium* cluster IV group in producing propionic acid support the presence of this group of bacteria in hypertensive patients (Ohue-Kitano *et al.* 2019). Indeed, Sun *et al.* (2019) informed that there was a positive association between hypertension and the *Clostridium* cluster IV group. Overall the gut microbiota plays an essential role in modulating the host's blood pressure through the production of microbial SCFA.

Microbiota analysis on hypertensive patients revealed the higher abundance of enterotype III, while enterotype I and enterotype II were higher in non-hypertensive respondents, instead. Li *et al.* (2017) also reported that Enterotype I is higher

(73.17%) in healthy people than in prehypertension and hypertension patients. Our data showed that F/B ratio in hypertensive patients was higher than that of non-hypertensives. Yang *et al.* (2015) and Joishy *et al.* (2022) have also reported that the (F/B) ratio was found increased in hypertensive patients. Given an imbalanced F/B ratio in hypertensive patient, we know that there is the alteration of gut microbiota composition towards dysbiosis correlated with this particular cardiovascular disease.

Unique composition of gut microbiota in each sample can be influenced by various things, such as food, intestinal mucosa, drug consumption/abuse, the immune system, and the microbiota itself (Maiuolo *et al.* 2022). Yokoyama *et al.* (2020) have report that blood pressure will be lower in those who consume vegetarian foods than omnivorous foods. Nakayama *et al.* (2015) report that the microbiota profiles of children up to old age in Indonesia are similar and have Prevotella enterotype. This is supported by the diet of Indonesians leading to plant-based foods, in contrast to Chinese, Taiwanese and Japanese who have *Bacteroides* or *Bifidobacterium* enterotype, which is supported by animal-based diets as well different carbohydrates.

Taken together, our data indicate that the gut microbiota profile of hypertensive patients differs to that of non-hypertensive. In hypertensive patients, a higher relative abundance of bacterial groups showed in *Clostridium* cluster XI, *Clostridium* cluster IV and *Clostridium* subcluster XIVa. Meanwhile, the abundance of *Bacteroides* and *Prevotella* groups in hypertensive patients was lower. Shannon-Wiener (H') index in hypertensive patients was more diversed than in non-hypertension samples with medium category. The F/B ratio was found higher in hypertensive patients than in non-hypertensive. Further research needs to be done to determine the relationship between SCFA produced by the gut bacteria and the incidence of hypertension.

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