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Gut Microbiota in Diabetic Kidney Disease in Northern Thailand: A Preliminary Study

Kaset Chimplee[1]*, Kamonnaree Chotinantakul[2], Narudol Teerapattarakan[3], Pattranuch Chusri[2] and Seiji Okada[4]

[1]Department of Internal Medicine, School of Medicine, Mae Fah Luang University, Chiang Rai, Thailand
[2]Department of Microbiology, School of Medicine, Mae Fah Luang University, Chiang Rai, Thailand
[3]Department of Pharmacology, School of Medicine, Mae Fah Luang University, Chiang Rai, Thailand
[4]Devision of Hematopoiesis, Joint Research Center for Human Retrovirus Infection, Kumamoto University, Kumamoto 860-0811, Japan

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***Corresponding author**:
Kaset Chimplee
Department of Internal Medicine, School of Medicine, Mae Fah Luang University, Chiang Rai, Thailand

E-mail:
kaset.chi@mfu.ac.th

ABSTRACT

Introduction: The risk factors that lead to the development of kidney damage in type 2 diabetic patients were known, such as poor glycemic control and poor blood pressure control. Data on clinical studies from Thailand and many countries show alteration in composition of gut microbiota in diabetes compared with non-diabetes. Studies from certain countries showed alteration in variation and composition of gut microbiota in patients with chronic kidney disease and end-stage kidney disease. There is no information in Thailand on the alteration in variation and composition of gut bacteria in patients with diabetic kidney disease. The main purpose of the study was to compare the difference in diversity and compositions of gut microbiota between three groups of patients: group A, type 2 diabetic patients with diabetic kidney disease (DN); group B, type 2 diabetic patients with normal kidney function (DM); and group C, hypertensive patients with normal kidney function who did not have diabetes (HT).

Methods: After screening and selecting by inclusion and exclusion criteria, 15 type 2 diabetes patients with chronic kidney disease were enrolled as the study participants (group A), 15 type 2 diabetic patients with normal kidney function were enrolled as the controls (group B), and 15 hypertensive patients with normal kidney function were enrolled as another control group (group C). Stool samples were sent for DNA extraction and 16S metagenome sequencing. For bioinformatic analysis, the Alpha-diversity metric, beta-diversity metric, and Principal Coordinate Analysis (PCoA) were applied. Taxonomy was developed for ASVs using the classify-sklearn native Bayes taxonomy classifier against the Greengenes 13_8 99% Operating taxonomy unit (OTUs) reference sequences. Statistical tests of alpha and beta diversity were performed using Kruskal-Wallis and PERMANNOVA.

Results: The study could not demonstrate the difference in community diversity of gut microbiota in all three groups. The Principal co-ordinate analysis (PCoA) based on Bray Curtis dissimilarity at the OTU level is the main method for the beta diversity analysis. PCoA showed no difference in gut microbiota composition among the three groups (p-value 0.544). PCoA using Jaccard, unweighted unifrac, and weighted unifrac distance analysis all of these methods also showed no difference in the microbial composition among the three groups.

Conclusion: The study could not find the differences in diversity and variation in compositions of gut microbiota in comparison among three groups of participants: type 2 diabetes mellitus with diabetic kidney disease, type 2 diabetes mellitus with normal kidney function, and hypertensive patients with normal kidney function. However, this study confirmed the features of gut microbiota compositions in type 2 diabetes mellitus with diabetic kidney disease from many previous studies, for example, the lower ratio of Firmicutes over Bacteroides in the diabetes group compared with the non-diabetes group. In addition, certain factors such as dietary

profiles, lifestyle, and ethical investigation of the participants need to be considered in further study.

Keywords: Amplicon sequence variants, Diabetic kidney disease, Gut microbiota, Type 2 Diabetes Mellitus, 16S metagenome sequencing

Introduction

The prevalence of diabetes mellitus in Thailand has been gradually increasing, and diabetes is known to be a major cause of chronic kidney disease [1]. Patients with end-stage kidney disease require regular hemodialysis or peritoneal dialysis, which have a significant impact on their quality of life and result in economic burdens, including healthcare costs at both individual and national levels. Several predisposing factors for diabetic kidney disease have been identified, such as poor glycemic control, poor blood pressure control, and dyslipidemia. Interestingly, a study from Thailand has revealed an alteration in the composition of gut microbiota in individuals with diabetes compared to those without diabetes [2]. Studies from certain countries have also demonstrated changes in the variation and composition of gut microbiota in patients with chronic kidney disease and end-stage kidney disease [3].

However, there is currently no information available on the alterations in the variation and composition of gut bacteria, specifically in Thai patients with diabetic kidney disease. Therefore, our research group is interested in studying the gut microbiota in type 2 diabetic patients with diabetic kidney disease (DKD) in order to investigate the relationship between the alterations in gut bacteria and the development of diabetic-related complications, particularly the deterioration of kidney function. Previous studies in Asia have found that diabetic patients exhibit a lower diversity of gut microbiota, measured as operational taxonomy units (OTUs), compared to healthy individuals. Certain bacteria have been found to be associated with clinical biomarkers in type 2 diabetic patients. For example, Acinetobacter and Bifidobacterium have shown a positive correlation with fasting plasma glucose (FPG) and glycosylated hemoglobin (HbA1C), while Prevotella has shown a negative correlation with FPG and HbA1C. Escherichia and Shigella have been positively correlated with body mass index (BMI) [4].

The main objective of this study was to compare the diversity and composition of gut microbiota among three groups of patients: (i) type 2 diabetic patients with diabetic kidney disease (DN), (ii) type 2 diabetic patients with normal kidney function (DM), and (iii) hypertensive patients with normal kidney function without diabetes (HT). These findings could enhance our understanding of the roles of gut bacteria in the pathogenesis of diabetic kidney disease, and this can serve as valuable preliminary information for further studies on the gut microbiota in Thai diabetic patients.

Methods

Study designs and participants

The study was conducted at The Mae Fah Luang University Medical Center Hospital in Chiang Rai, Thailand. Three groups of participants were enrolled. A total of 15 cases of type 2 diabetic patients with diabetic kidney disease (DN) were recruited into group A, and another 15 cases of type 2 diabetes patients with DKD were recruited for group B (DM); additionally, 15 hypertensive cases with normal kidney function were recruited into group C (HT). The enrollment period spanned from March 2022 to the end of February 2023.

Inclusion criteria: Group A members were diabetic kidney disease (DN) with type 2 diabetes mellitus, age from 35-70, spot urine sample for microalbumin more than or equal to 30 mg/g creatinine, estimated glomerular filtration rate (eGFR) less than 60 ml/min/1.73 m². Group members were diabetes mellitus with normal kidney function (DM) and type 2 diabetes mellitus, age from 35-70, spot urine sample for microalbumin less than 30 mg/g, and estimated glomerular filtration rate (eGFR) more than 60 ml/min/1.73 m². Group C members were hypertension (HT), age from 35-70, spot urine sample for microalbumin less than 30 mg/g creatinine, and estimated glomerular filtration rate (eGFR) more than 60 ml/min/1.73 m².

Exclusion criteria: Those who recently used antibiotics within 30 days before stool sample collection, current gastrointestinal symptoms such as diarrhea, bloody diarrhea, melena, chronic abdominal pain, nausea, or vomiting, use of antacid or proton pump inhibitors to reduce gastric acid secretion, and use of prebiotics and/or probiotics were excluded from the study.

Withdrawal criteria: Those who used antacid or proton pump inhibitors or used of prebiotics and/or probiotics during stool collection were considered to withdraw from the study.

Stool sample collection and metagenome sequencing

Stool samples were collected from all participants following a written guideline for stool collection preparation. The samples were sent to the laboratory department of Mae Fah Luang Medical Center Hospital in the early morning and immediately frozen and stored at -80°C until DNA extraction and analysis.

According to the manufacturer's guidelines, a total of 0.2 grams of stool sample was extracted using the PureLink™ microbiome DNA purification kit (Invitrogen, USA). The extracted DNA was stored at -20°C until further processing for 16S metagenome sequencing.

The V3 and V4 regions of the 16S ribosomal DNA (rDNA) gene were amplified using polymerase chain reaction (PCR) [5]. The purified amplicons were pooled and subjected to paired-end sequencing (2x300) on the Illumina MiSeq platform (Illumina, San Diego, USA) following standard protocols by Marcrogen (Seoul, South Korea).

Statistical and bioinformatics analysis

The baseline demographic data of the three groups of study participants were compared by non-parametric statistics (Kruskall-Wallis). The parameters included age, creatinine, eGFR, urine microalbumin, blood pressure, BMI, CRP. For HbA1C and FPG, only group A and group B were compared.

Bioinformatics analysis: Microbiome analysis was performed using QIIME 2 2022.2 by first demultiplexing and quality assessing the raw sequence data using the q2-Demux plugin and performing noise reduction using DADA2 (q2-dada2) [6]. Subsequently, the DNA sequence matching the chloroplast sequences was removed. The q2-diversity was used to compute alpha and beta diversity metrics and Principle Coordinate Analysis (PCoA), where the samples were rarefying to 16,756 reads. Amplicon sequence variants (ASVs) were assigned to a taxonomy using the Classify-Sklearn Naïve Bayes Taxonomy Classifier compared to the Greengenes (version 13_8) 99% OTUs database. Alpha diversity was analyzed using Kruskal-Wallis, while beta diversity was tested using PERMANNOVA (number of permutations = 999). Significantly different taxa abundances between all three groups were examined using the linear discriminant analysis (LDA) algorithm for effect size (LEfSe) [7], which is available in the Galaxy calculation tool (http://huttenhower.sph.harvard.edu/galaxy).

Kruskal−Wallis sum-rank tests were used to compare feature differences between classes (p-value <0.050). To estimate effect sizes, LDA was added and supported by bootstrapping (default 30-fold, cutoff = LDA score of ≥ 1.0). In addition, the Venn diagram was visualized using InteractiVenn [8].

Results

General characteristics of participants

A total of 45 participants were enrolled in the study, with 15 participants in each of group A (DN), group B (DM), and group C (HT). During the process of stool DNA extraction, some samples did not qualify for genome sequencing. Therefore, group A had 13 qualified samples, group B had 12 qualified samples, and group C had 14 qualified samples. In total, 39 qualified samples were processed for metagenome sequencing.

The mean age of the participants was 55.0 years (ranging from 39 to 67 years), and their average blood pressure was 138/78 mmHg, with an average BMI of 26.46 kg/m². Participants in group A and group B had poor glycemic control (HbA1C > 8.0%) and were obese, with an average BMI of 26.46 kg/m². When comparing the three groups, there was a slight difference in age, with the group A being slightly older than the group B. However, both groups were in the same middle-age range. Creatinine, eGFR, and urine microalbumin levels showed significant differences between the three groups, as per the study's design and enrollment criteria. Blood pressure, BMI, and inflammatory markers (CRP) did not differ significantly among the three groups. When comparing the two diabetes groups (group A and group B), fasting plasma glucose and HbA1C levels were similar (Table 1).

Table 1 Comparison of clinical and biochemical characteristics between 3 groups: group A, group B, and group C

Characteristics	Group	Mean	SD	p-value
Age	A	58.2	7.4	0.039*‡
	B	51.3	6.7	
	C	56.6	6.9	
Cr	A	1.6	0.7	0.001*‡
	B	0.7	0.2	
	C	0.8	0.2	
eGFR	A	44	11	0.001*‡
	B	102	14	
	C	92	13	
UMA	A	574	841	0.001*‡
	B	27	38	
	C	10	6	
s-BP	A	138	13	0.936*
	B	139	17	
	C	138	22	

Characteristics	Group	Mean	SD	p-value
d-BP	A	79	9	0.840*
	B	80	7	
	C	77	11	
BMI	A	27.2	3.8	0.886*
	B	26.1	3.9	
	C	26.1	4.2	
CRP	A	2.4	1.9	0.493*
	B	2.2	2.9	
	C	2.1	1.1	
HbA1c	A	8.4	2.1	0.683**
	B	8.2	1.5	
FPG	A	176	114	0.242**
	B	175	51	

** Kruskal-Wallis statistics for analysis of the difference of median between 3 groups*
*** Mann-Whitney statistics for analysis of difference of median between 2 groups (DN and DM)*
‡ Statistical significance
Note: *Cr = creatinine (md/dl), eGFR = estimated glomerular filtration rate (ml/min/1.73 m^2), UMA = urine microalbumin (mg/gCr), s-BP = systolic blood pressure (mmHg), d-BP = diastolic blood pressure (mmHg), BMI = body mass index (kg/m^2), CRP = c-reactive protein (mg/L), HbA1c = glycosylated hemoglobin (%), FPG = fasting plasma glucose (mg/dl)*

Analysis of gut microbiota composition among DN, DM, and HT

In total, 1,365,797 high-quality sequence reads from 39 samples were selected and clustered into 1,100 features of amplicon sequence variants (ASVs) processed with the QIIME 2 pipeline. The Venn diagram (Fig 1) displayed the distribution of ASVs; group A had 340 ASVs, group B had 325 ASVs, and group C had 317 ASVs. Groups A and B shared 281 ASVs. Groups A and C shared 259 ASVs. Groups B and C shared 249 ASVs. Additionally, 230 ASVs were common to all three groups. Furthermore, group A had 30 specific ASVs, group B had 25 specific ASVs, and group C had 39 specific ASVs. The comparison of microbial diversity in terms of evenness, Faith's PD, observed species, and Shannon index did not differ significantly in all three groups (Table 2). Comparison of the gut bacterial composition among the three groups was not a significant difference. In addition, the principal coordinates analysis (PCoA) plot, based on Bray-Curtis distances, did not demonstrate the difference in composition in microbial communities across the three groups (p-value = 0.544) (Fig 2).

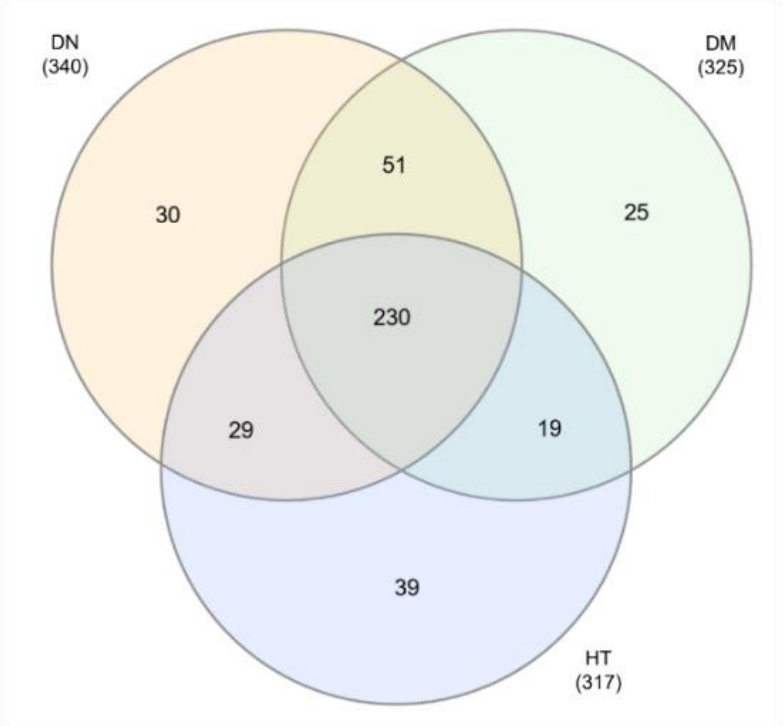

Figure 1. Venn diagrams of shared and unique ASVs in three groups of patients: group A (DN), group B (DM), and group C (HT)

Table 2 Comparison of alpha diversity indexes by groups.

Group	Evenness	Faith's PD	Observed species	Shannon
A	0.64 ± 0.12	8.72 ± 2.34	128.8 ± 42.5	4.71 ± 1.08
B	0.66 ± 0.10	8.05 ± 2.35	115.8 ± 38.6	4.52 ± 0.93
C	0.68 ± 0.07	7.89 ± 1.47	120.3 ± 32.1	4.67 ± 0.59

Note: Means ± SD (Kruskal Wallis sum rank test)

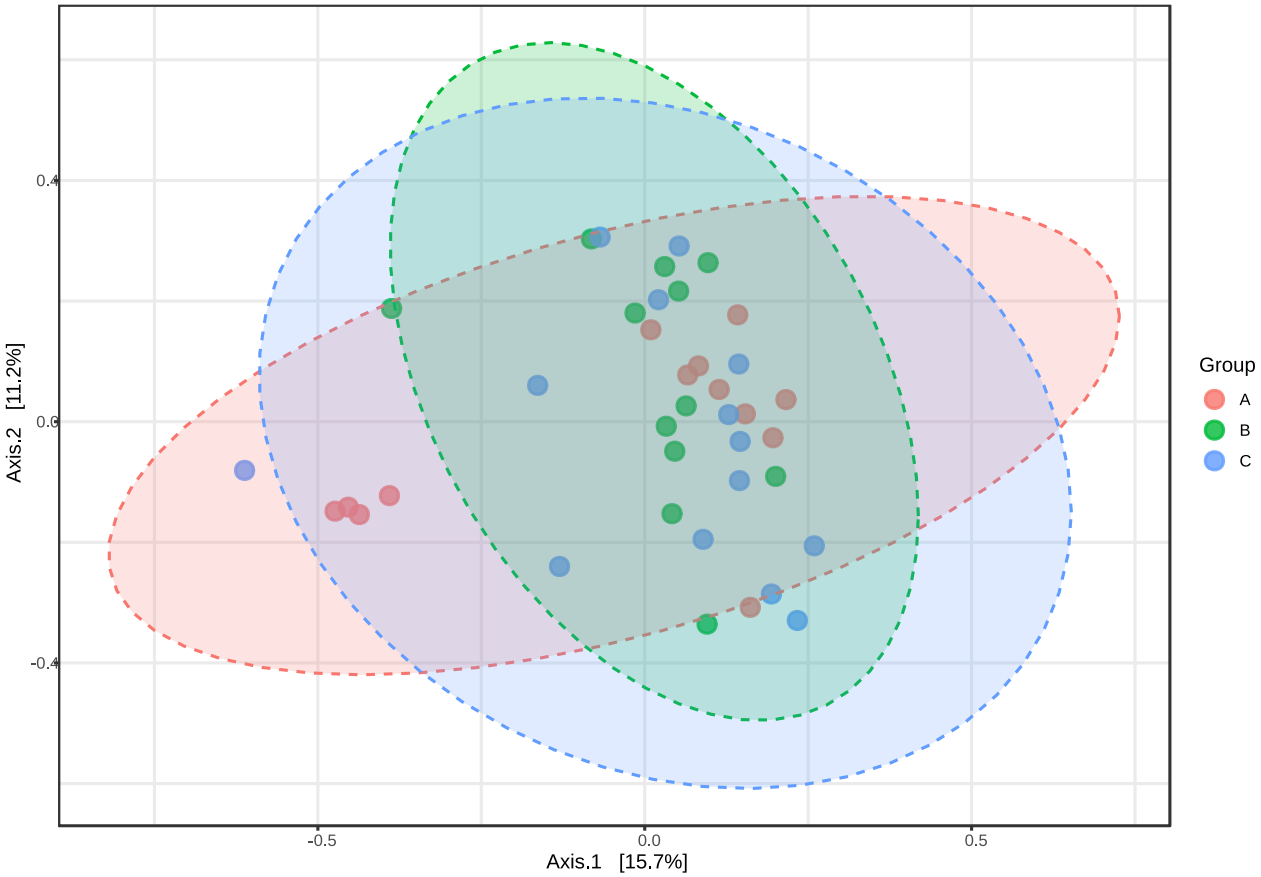

Figure 2. Principal coordinates analysis (PCoA) of microbial communities by groups

In terms of taxonomic profiling, the most abundant phylum in all three groups was Firmicutes A (Fig 3), and the most abundant class was Clostridia. The most abundant family was Lachnospiraceae, and at the genus level, *Streptococcus* was the most abundant genus in the group A, while *Blautia* was the most abundant genus in both groups B and C. The effect differentiating phenotype, as analyzed by LEfSe, demonstrated that the most differentially abundant bacteria taxa in group A belong to phyla Firmicutes D (Fig 4A). The most likely observed genus differences in group A were *Senegalimassilia*, while in group B were *Clostridium* and *Ruminococcus*. In group C, the most differential abundance was the genus *Klebsiella* (Fig 4B). Interestingly, *Roseburia inulinivorans* were more abundant in group A than in groups B and C (Fig 4C).

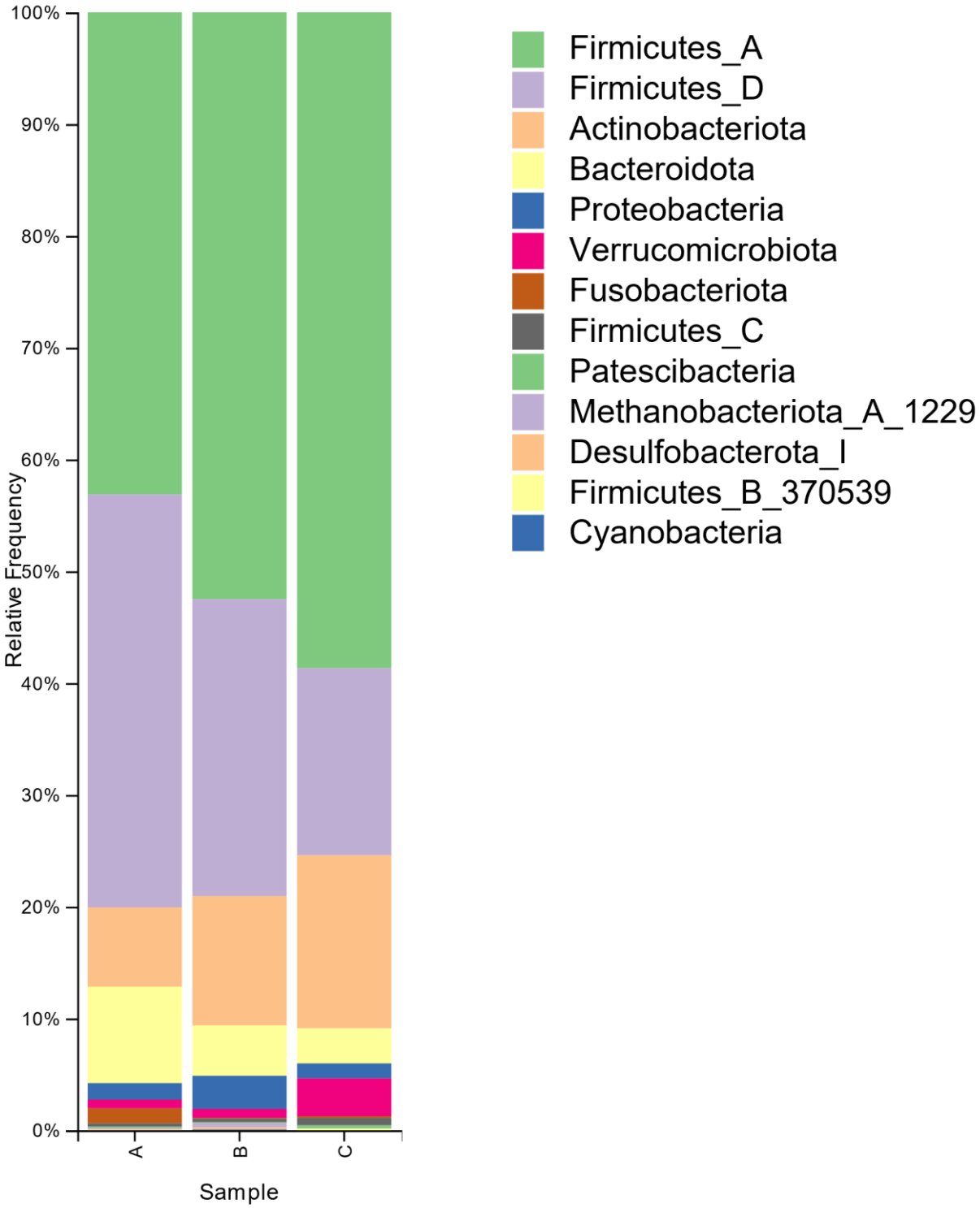

Figure 3. Taxonomy composition of bacterial community in all three groups

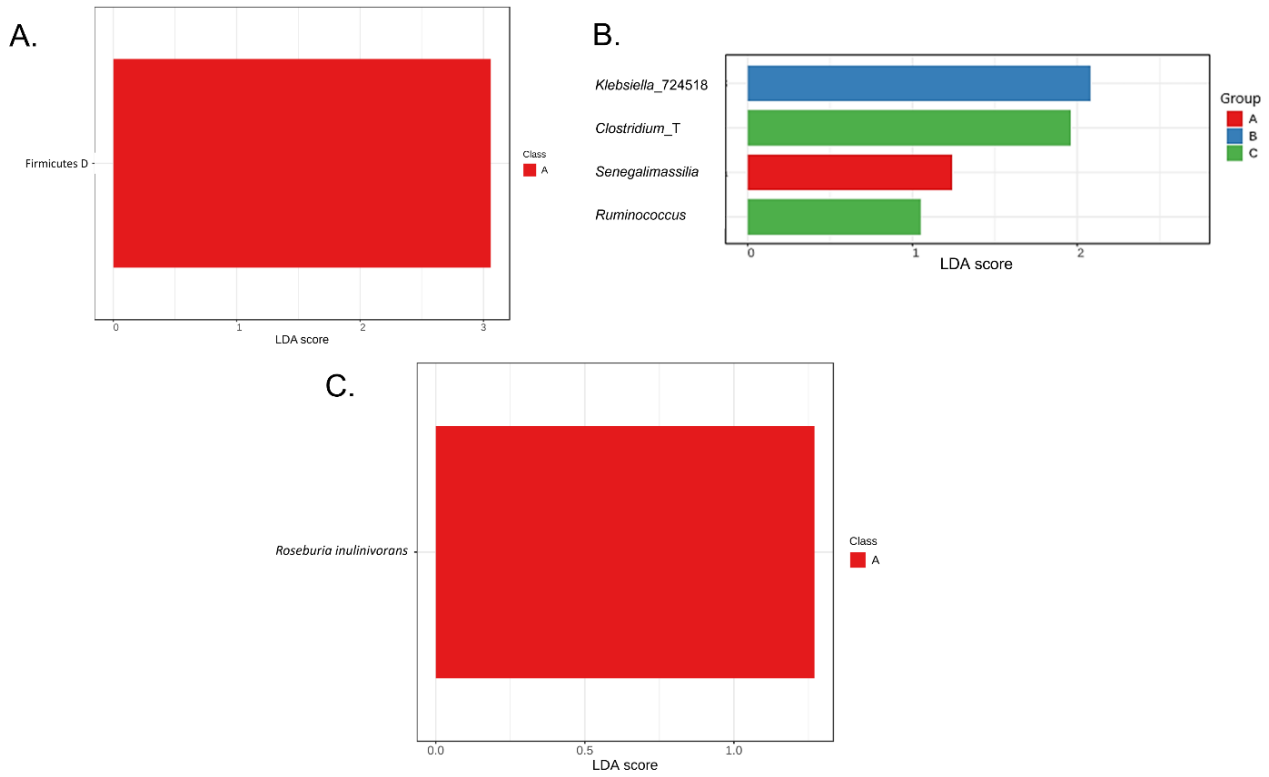

Figure 4. Linear discriminant analysis of the effect size of microorganisms in different groups of patients showing a significant threshold score (p-value> 0.05) at (A) phylum, (B) genus and (C) species levels

Discussion

This study focused on investigating the differences in gut microbiota variation and composition among patients with diabetes kidney disease (DN), type 2 diabetes mellitus (DM), and hypertensive patients (HT) with normal kidney function. The study is the first of its kind in Thailand and addresses an important knowledge gap in the region. However, there are certain limitations that need to be considered. The study was conducted in the northernmost area of Thailand, which has a multicultural and multiracial population. Some participants in the study may belong to racial minorities and have different dietary styles and compositions that can influence gut microbiota composition. The study team attempted to control for known factors that can alter gut microbiota composition by excluding participants using antibiotics, prebiotics, probiotics, and drugs that affect the intestinal acid-base status.

Regarding sample collection, stool samples were collected in the early morning and immediately frozen at -80°C to preserve the bacterial components. Initially, the study aimed to have 15 participants in each group, but due to the exclusion of samples that did not qualify for genome sequencing, the final numbers were 13 samples in the DN group, 12 samples in the DM group, and 14 samples in the HT group. Comparing the demographic data, age showed a slight difference, while the DN group being slightly older than the DM and HT groups. Creatinine, eGFR, and urine microalbumin were significantly different among the groups by the study's design. Blood pressure, BMI, and C-reactive protein (CRP) did not differ significantly among the groups to avoid confounding factors. Both diabetic groups (DN and DM) had poor glycemic control, with HbA1C levels exceeding 8.0%, and all groups were obese based on the Asian criteria for obesity classification (BMI > 25 kg/m^2).

Alpha diversity analyses of the bacterial community did not differ among all 3 groups (DN, DM, and HT) as determined by Faith's PD, observed species, evenness, and Shannon. These data indicate the species richness and uniformity in the community ecology. Based on the amplicon sequence variants (ASVs), there was no significant difference in the numbers of ASVs among the groups, and there was a substantial overlap of ASVs between the two comparison groups. In contrast to our study, a previous study in 2013 found evidence of poor diversity in the gut microbiome in obesity, insulin resistance, and hepatic steatosis [9]. At the phylum level, our study found a lower ratio of *Firmicutes* to *Bacteroidetes* in the diabetes group compared to the non-diabetes group, which is consistent with a study by Yoo et al. [10]. The ratio of *Actinobacteria* was decreased in the DN group compared to the DM and HT groups, which aligns with a study by Eckburg et al in 2005 [11]. In human life span, the development of gut microbiota composition undergoes three consecutive stages: a developmental stage (3-14 months of age), a transition stage (15-30

months of age), and a stable period (31-46 months of age). After the stable period, the gut microbiome pattern remains relatively stable for life and contributes to the development of various diseases [12]. In this study, Principal Coordinate Analysis (PCoA) for beta diversity revealed no significant differences in gut microbiota composition among three groups at various taxonomic levels (phylum, class, order, family). However, a significant difference was observed at the genus level based on the Linear Discriminant Analysis (LDA) score. Despite this finding, the impact on clinical applications was limited.

There are a few possible explanations for the lack of difference in gut microbiome compositions in our study. (i) *Differences in renal function:* even the participants in the DN (diabetic nephropathy) and DM (diabetes mellitus) groups exhibited statistical differences in the estimated glomerular filtration rate (e-GFR) that the e-GFR of the DN group was 44 ml/min/1.73 m², while that of the DM group was 102 ml/min/1.73 m². In the clinical practice, the patients with e-GFR were classified as chronic kidney disease (CKD) stage 3B [13], and there were no clinical symptoms of CKD in comparison with type 2 diabetic patients with normal kidney function (DM group). Additionally, the average creatinine level in the DN group was 1.62 mg/dl, compared to 0.72 mg/dl in the DM group. These slight differences in renal function might have contributed to the lack of significant differences in gut microbiota composition. If the study had included participants with significant kidney damage (e.g., CKD stage 5, e-GFR less than 15 ml/min/1.73 m²), it is possible that differences in gut microbiota compositions would be different from our study. (ii) *Dietary patterns:* the research team did not assess the dietary patterns of all participants despite the majority of them residing in the same geographical area. This area, located in the northernmost part of Thailand, is composed of a multicultural and multiethnic society of people originating from Myanmar, Laos, Southern Chinese, Hill tribes, and people from the central and northeastern parts of Thailand, which made it very difficult to assess of participants dietary habits precisely [14,15]. Evaluating the lifestyle and dietary patterns of participants in such a region can be challenging. Conducting further studies that include detailed assessments of the dietary patterns of each participant, with household and individual controls matched for each participant, would greatly benefit the analysis of gut microbiome composition.

While no significant differences in gut microbiota composition were observed among the three groups at various taxonomic levels, a significant difference was found at the genus level. The slight differences in renal function and the lack of comprehensive dietary assessments may have contributed to the overall non-significant findings. Further studies considering these factors, especially detailed dietary patterns and renal function in participants, would enhance the understanding of gut microbiome composition in relation to the studied conditions.

Conclusion

This study is designed to investigate the differences in gut microbiome composition among patients with diabetic kidney disease, type 2 diabetes mellitus, and hypertensive patients with normal kidney function. The study implemented various measures to control confounding factors and utilized standard methods for DNA extraction and metagenome sequencing. However, despite these efforts, the study did not find significant differences in the diversity and composition of gut microbiota among the three groups. Nevertheless, the study did confirm certain features of gut microbiome composition in patients with type 2 diabetes mellitus and diabetic kidney disease, such as the lower ratio of *Firmicutes* to *Bacteroides* compared to non-diabetic individuals. The study highlighted the importance of considering factors such as dietary profiles, lifestyle, and ethnicity in future research.

Ethical approval

All study participants provided informed consent before enrolling in the study. The study protocol was approved by The Mae Fah Luang University Ethics Committee on Human Research with a certificate of approval (COA: 045/2022).

Acknowledgments

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Conflicts of Interest

The authors declared no conflict of interest.

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