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Comparative Study on the Detection of Short Chain Fatty Acids in Fecal Samples from Patients with Type 2 Diabetes Mellitus: An Insight into Diabetic Kidney Disease Compared with Normal Kidney Function

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Abstract:

Background: The role of the gut microbiota in metabolic health was increasingly recognized. Short-chain fatty acids (SCFAs) – including propionic acid, acetic acid and butyric acid – are vital metabolites produced by gut microbiota. These SCFAs play a pivotal role in energy metabolism, glucose homeostasis, and systemic inflammation. The link between gut microbiota and human health, especially in the context of metabolic disorders like Type 2 Diabetes Mellitus (T2DM) and Chronic Kidney Disease (CKD), has garnered significant attention in recent years.

Objective: This study aimed to compare the levels of SCFAs in fecal samples from three distinct patient groups: 1) Type 2 diabetic patients with diabetic kidney disease, 2) Type 2 diabetic patients with normal kidney function, and 3) Hypertensive patients without diabetes with normal kidney function.

Materials and Method: A total of thirty-nine participants were involved in this research, categorized into three distinct groups. Fecal samples were collected, immediately frozen, and processed for the extraction of SCFAs. The concentrations of SCFAs were determined using a gas chromatography-mass spectrometry (GC/MS) system.

Results: The study evaluated the concentrations of three pivotal SCFAs in stool samples across the three patient groups. While there were nuanced variations in the concentrations of acetic, butyric and propionic acids among the groups, an ANOVA test revealed no statistically significant differences in SCFA concentrations among them.

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Conclusion: While this study provides foundational insights into the SCFA landscape across specific health conditions, the absence of significant disparities prompts contemplation. The multifactorial nature of SCFA production, influenced by disease states, diet, genetics and other environmental factors, underscores the need for further investigative depth. Future research might delve deeper into the potential therapeutic implications of modulating gut microbiota and SCFA profiles in managing metabolic disorders.

Keywords: Diabetic Kidney Disease, Gut Microbiota, Short-chain Fatty Acid, Type 2 Diabetes Mellitus

Introduction

The link between gut microbiota and human health has been a subject of interest in recent years. The gut microbiota, often referred to as the "hidden organ" plays a significant role in various metabolic disorders, including Type 2 Diabetes Mellitus (T2DM) and chronic kidney disease (CKD).[1,2] One key area of focus is on short-chain fatty acids (SCFAs) - primarily propionic, acetic, and butyric acids - as these bacterial metabolites are associated with a wide range of physiological functions.[3]

Recent studies have shown that SCFAs play a crucial role in maintaining gut barrier integrity, modulating immune responses, and influencing metabolic processes, which can have significant implications for systemic health and disease.[4,5] Furthermore, alterations in the gut microbiota and SCFA profiles have been linked to the development and progression of T2DM and CKD.[6] In addition to these roles, emerging research has highlighted the potential of gut microbiota as a therapeutic target in T2DM. Certain anti-hyperglycemic agents and traditional Chinese medicine have been found to exert hypoglycemic effects by altering the gut microbiota composition, thereby improving glucose metabolism.[2] Moreover, studies have identified a particular ganglioside profile associated with early diabetic kidney disease in T2DM patients, further emphasizing the complex interplay between metabolic disorders and gut microbiota.[7]

In light of these findings, this study seeks to further explore this association by comparing the levels of these SCFAs in the feces of patients in three distinct groups: 1) Type 2 diabetic patients with diabetic kidney disease, 2) Type 2 diabetic patients with normal kidney function, and 3) Hypertensive patients with normal kidney function without diabetes.

Materials and method

This research involved forty-five participants, divided into three distinct categories. *Group A (DN)* was made up of fifteen individuals diagnosed as Type 2 Diabetes Mellitus with diabetic kidney disease. The definition of diabetic kidney disease is the diabetic patient who has a spot urine sample for microalbumin equal to or greater than 30 mg/g creatinine, and an estimated glomerular filtration rate (eGFR) less than 60 mL/min/1.73 m^2.[8] *Group B (DM)*, which served as a control group, comprised of fifteen patients with Type 2 Diabetes Mellitus with normal kidney function. The definition of normal kidney function is a spot urine sample for microalbumin less than 30 mg/g and an eGFR greater than 60 mL/min/1.73 m^2.[8] *Group C (HT)*, consisted of fifteen hypertensive patients without diabetes and having normal kidney function. The participants were a diverse group of men and women who resided in Chiang Rai province, Thailand. The recruitment phase ranged from March 2022 to February 2023.

Inclusion Criteria: Group A (DN) included individuals with type 2 diabetes mellitus, aged 35-70, who had a spot urine sample for microalbumin equal to or greater than 30 mg/g creatinine, and an estimated glomerular filtration rate (eGFR) less than 60 mL/min/1.73 m^2. Group B (DM) consisted of individuals with type 2 diabetes mellitus, aged 35-70, who had a spot urine sample for microalbumin less than 30 mg/g and an eGFR greater than 60 mL/min/1.73 m^2. Group C (HT) included hypertensive individuals, aged 35-70, who had a spot urine sample for microalbumin less than 30 mg/g creatinine, and an eGFR greater than 60 mL/min/1.73 m^2.

Exclusion Criteria included the recent use of antibiotics within 30 days prior to 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.

Withdrawal Criteria included the use of antacid or proton pump inhibitors and the use of prebiotics and/or probiotics during the study period.

Informed Consent and Ethical Approval: The study was conducted following the principles outlined in the Declaration of Helsinki posing minimal health risks to the participants. All participants in the study gave their informed consent prior to enrollment and participation. The study protocol received approval from The Mae Fah Luang University Ethics Committee on Human Research, as evidenced by a certificate of approval (COA: 045/2022).

Sample Preparation

Fecal samples were collected from each participant using sterile containers. The samples were immediately frozen at -80°C until further processing. For the extraction of SCFAs, approximately 1 gram of each fecal sample was weighed and homogenized in 1 mL of distilled water. The homogenate was then centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatant was collected and acidified with 25% metaphosphoric acid to a final concentration of 5%. The acidified samples were kept at 4°C for 30 minutes to allow for the precipitation of proteins and were then centrifuged again under the same conditions. Two-methyl-butyric-acid (Sigma-Aldrich, USA) was added to each vial to reach a final concentration of 0.001 M. 2-methyl-butyric-acid was added as an internal standard (IS) to correct for injection variability between samples, and minor changes in the instrument response. All vials were stored at – 20°C before GC analysis.

GC/MS Analysis

The analysis of SCFAs was performed using a gas chromatography-mass spectrometry (GC/MS) system. The GC/MS was equipped with a capillary column (30 m x 0.25 mm, 0.25 μm film thickness). The oven temperature was initially set at 80°C, then increased to 180°C at a rate of 10°C/min, and finally to 200°C at a rate of 5°C/min. The temperatures of the injector and detector were set at 250°C and 280°C, respectively. Helium was used as the carrier gas at a flow rate of 1 mL/min. The mass spectrometer was operated in electron impact mode at 70 eV. SCFAs were identified by comparing their retention times and mass spectra with those of standards. The concentrations of SCFAs were calculated based on the peak area ratios of the SCFAs to the internal standard. The mass spectrometer was set to scan mode at m/z 100–300 and selected ion monitoring mode at m/z 60 for acetate, butyrate maintaining for 4.72 min, 7.34 min respectively, as well as m/z 73 for propionate for 5.90 min. The results were then normalized to the dry weight of the fecal samples.

Statistical analysis

All statistical analyses were performed by GraphPad Prism 8.0. The results were expressed as means with standard deviation (SD). ANOVA was used for normally distributed continuous variables. The correlation difference between variables was analyzed by Spearman's R coefficient. The association between fecal level with the clinical index was examined via binary logistic regression analysis, based on the median level of fecal or serum SCFAs. P value < 0.05 was considered statistically significant.

Results

Baseline characteristics among the three groups

Although 45 participants were initially enrolled in the study - with 15 participants in each group, some stool sample were not qualified for processing. There were 39 stool samples in remain, with 13 participants in Group A (DN) and 12 in Group B (DM) and 14 in Group C (HT). The mean age of 39 total participants was 55.0 years, ranging from 39 to 67 years. The average blood pressure was 138/78 mmHg and the average BMI was 26.46 kg/m^2.

Both Group A (DN) and Group B (DM) had poor glycemic control (HbA1C were 8.4% and 8.2% respectively. All three groups were obese (average BMI was 26.5 kg/m^2). In comparisons between groups, the Group A (DN) was rather older than the Group B (DM). Significant differences were observed in creatinine, eGFR and urine microalbumin levels among the three groups, based on the study's design and enrollment criteria. However, blood pressure, BMI and inflammatory marker (CRP) levels did not differ significantly among the three groups (Table 1)

Comparisons of fecal and serum SCFAs among the three groups

Our study evaluated the concentrations of three pivotal short-chain fatty acids (SCFAs) - acetic, butyric and propionic - in stool samples across three distinct patient groups (Table 2). For acetic acid concentrations (Figure 1), Group A (DN), consisting of individuals with Type 2 Diabetes Mellitus with diabetic kidney disease, exhibited a concentration of 1111.791 ± 179.119 μmol/g dry weight. In comparison, the control group, Group B (DM), which includes patients with Type 2 Diabetes Mellitus with normal kidney function, showed a slightly lower concentration of 1061.101 ± 148.689 μmol/g dry weight. Meanwhile, Group C (HT), made up of hypertensive patients with normal kidney function, had concentrations closely mirroring Group A (DN) at 1115.751 ± 177.345 μmol/g dry weight. For butyric acid (Figure 2), Group A (DN) had a concentration of 18.378 ± 4.173 μmol/g dry weight, Group B (DM) displayed 17.333 ± 4.725 μmol/g, and Group C (HT) showed 18.019 ± 3.879 μmol/g. Lastly, for propionic acid (Figure 3), in Group A (DN), the concentration was 11.624 ± 3.418 μmol/g dry weight, Group B (DM) ehibited 12.299 ± 5.157 μmol/g, and Group C (HT) aligned closely with Group A (DN) at 11.557 ± 4.728 μmol/g. Notwithstanding these nuanced variations, an ANOVA test revealed no statistically significant differences in SCFA concentrations among the groups.

Table 1 Comparison of clinical and biochemical features between 3 groups: Group A (DN), Group B (DM), Group C (HT)

	Group	Mean	S.D.	p-value
Age	A	58.2	7.4	
	B	51.3	6.7	0.039*
	C	56.6	6.9	
BMI	A	27.2	3.8	
	B	26.1	3.9	0.886
	C	26.1	4.2	
s-BP	A	138	13	
	B	139	17	0.936
	C	138	22	
d-BP	A	79	9	
	B	80	7	0.840
	C	77	11	
eGFR	A	44	11	
	B	102	14	0.001*
	C	92	13	
Cr	A	1.6	0.7	
	B	0.7	0.2	0.001*
	C	0.8	0.2	
UMA	A	574	841	
	B	27	38	0.001*
	C	10	6	
CRP	A	2.4	1.9	
	B	2.2	2.9	0.493
	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 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 (mg/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)

Table 2 The concentration of short-chain fatty acids (SCFAs) in different groups

Fecal SCFAs	DN (13)	DM (12)	HT (14)	P
Acetate (μmol/g)	1111.79 ± 179.12	1061.10 ± 148.68	1115.75 ± 177.34	NS
Propionate (μmol/g)	11.62 ± 3.41	12.29 ± 5.15	11.55 ± 4.72	NS
Butyrate (μmol/g)	18.37 ± 4.17	17.33 ± 4.72	18.01 ± 3.87	NS

SCFAs concentration is expressed as Mean± SD (μmol/g); NS represents that the difference is not significant ($p > 0.05$)

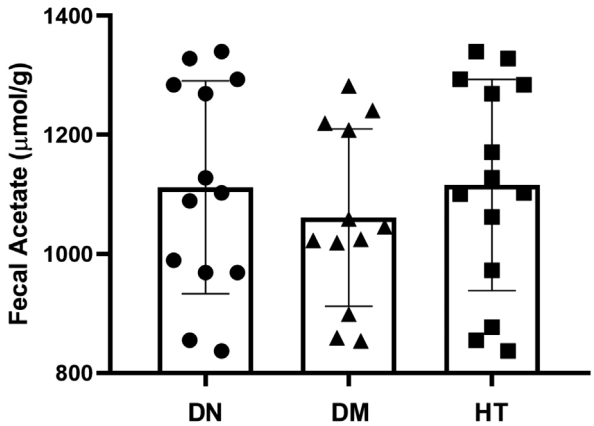

Figure 1 Acetic acid concentrations across patient groups. The figure illustrates the concentrations of acetic acid (in μmol/g dry weight) in fecal samples from three distinct patient groups: DN (Type 2 diabetic patients with diabetic kidney disease), DM (Type 2 diabetic patients with normal kidney function) and HT (Hypertensive patients with normal kidney function without diabetes). The bars represent mean concentrations, and the error bars denote standard deviations.

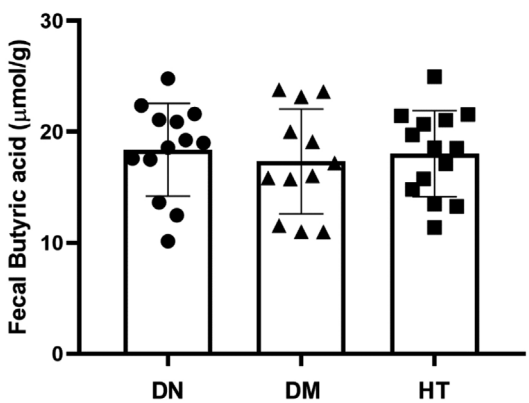

Figure 2 Butyric acid concentrations across patient groups. The figure displays the concentrations of butyric acid (in μmol/g dry weight) in fecal samples from the three patient groups: DN, DM, and HT. The bars indicate mean concentrations, while the error bars represent standard deviations.

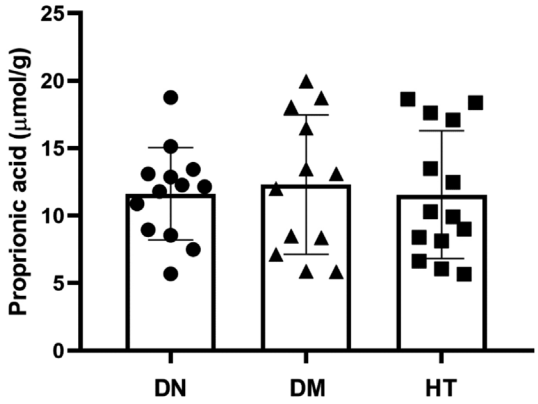

Figure 3 Propionic acid concentrations across patient groups. The figure showcases the concentrations of propionic acid (in μmol/g dry weight) in fecal samples from the three patient groups: DN, DM and HT. The bars depict mean concentrations, and the error bars indicate standard deviations.

Discussion

The role of short-chain fatty acids (SCFAs) in modulating gut and metabolic health has been a subject of increasing scientific scrutiny. Our findings, which revealed nuanced yet statistically non-significant variations in SCFA concentrations across distinct clinical cohorts, align with the broader scientific discourse on this topic.

SCFAs, primarily produced from the microbial fermentation of dietary components, play a pivotal role in shaping the biochemical profile of the diet, influencing both gut and host metabolism.[9] Acetic acid concentrations across our cohorts, particularly the similarity between the DN and HT groups, resonate with previous studies suggesting shared gut microbial signatures between conditions like hypertension and diabetes.[10] Butyric acid, renowned for its anti-inflammatory properties and its role in maintaining gut barrier integrity, showed consistent concentrations across our cohorts. This observation is in line with the understanding that SCFAs, especially butyric acid, are essential for gut integrity, regulating luminal pH, mucus production and providing fuel for epithelial cells.[11] The consistency in propionic acid concentrations across the groups further underscores the multifaceted role of SCFAs in metabolic health. SCFAs, including propionic acid, have been implicated in a range of physiological processes, from glucose homeostasis to immunomodulation.[12]

Several factors may explain the lack of significant differences in SCFAs concentration in our study: (1) *Renal Function Variations:* participants in the diabetic kidney disease (DN) and diabetes mellitus (DM) group showed a slight difference in their estimated glomerular filtration rate (e-GFR). The DN group had an e-GFR of 44 mL/min/1.73 m^2, while the DM group had a higher e-GFR of 102 mL/min/1.73 m^2. Additionally, the average creatinine level in the DN group was 1.62 mg/dL, in comparison with 0.72 mg/dL in the DM group. These subtle differences in renal function could have influenced on the SCFAs concentration. If the study had involved participants with more advanced kidney damage, such as those in stage 5 chronic kidney disease (CKD) with an e-GFR below 15 mL/min/1.73 m^2, we may have observed more pronounced differences in SCFAs concentration (2) *Dietary Patterns:* The study did not assess the dietary pattern in

all participants, despite the fact that most lived in the same geographical area. This region, located in northernmost part of Thailand, is home to a diverse, multicultural population consisting the people originated from Laos, Myanmar, Chinese, Hill tribes people and people from central and northeastern part of Thailand which made it very difficult to evaluate participants dietary habits accurately.[13-14] Future studies should include more detailed dietary assessments for each participant that need laborious effort and time for preparation, considering both household and individual factors. Such information would provide a valuable insight into how dietary patterns could influence the SCFAs concentration between groups.

Conclusion

While our study provides foundational insights into the SCFA landscape across specific health conditions, the absence of significant disparities prompts contemplation. The multifactorial nature of SCFA production, influenced by disease states, diet, genetics, and other environmental factors, underscores the need for further investigative depth.

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Conflict of Interest

The authors declare no conflict of interest of this study.

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