Study of *Firmicutes* and *Bifidobacterium phyla* in Patients with Type 2 Diabetes mellitus

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Abstract

Background: Gastrointestinal microbiota may play a vital role in pathogenesis of type 2 diabetes mellitus (DM). *Firmicutes* phyla and *Bifidobacterium* phyla represents the major components of the microbiota in gastrointestinal tract.

Aim: The aim of this study was to investigate the ratio of phyla *Firmicutes* and *Bifidobacterium* in patients with type 2 DM compared to apparently healthy control subjects by real time polymerase chain reaction (PCR).

Method: The study was a retrograde case control study that included 100 patients with type 2 DM and 100 control subjects. *Firmicutes* phyla and *Bifidobacterium* phyla were determined in the stool samples by real-time PCR.

Results: Real time PCR study revealed significant increase in the phyla of *Firmicutes* in the patients with type 2 DM (5.2 ±1.0 $x10^9$/gram, P <0.001) compared to control subjects (4.2±0.84$x10^9$/gram), and significant decrease in phyla *Bifidobacterium* in patients with type 2 DM (10.02± 0.94, P<0.001) compared to control subjects (12.4 ±1.2, P<0.001) with significant increase of *Firmicutes* / *Bifidobacterium* ratio in patients

(0.52±0.1, P<0.001) compared to control subjects (0.34± 0.1). There were statistically significant correlations between Firmicutes/ Bifidobacterium ratio and fasting blood glucose, HB1C, cholesterol and LDL-cholesterol (P<0.001 for each).

Conclusion: The present study highlights the disturbance of the ratio of the *Bifidobacterium* phyla and *Firmicutes* phyla in patients with type 2 DM compared to control subjects that revealed an increase in *Firmicutes phyla* with decrease in *Bifidobacterium* phyla with its subsequent effect on different metabolic pathways that needs more multicentric studies on a large scale of patients to signify these results.

Keywords: *Bifidobacterium*, *Firmicutes*, Diabetes mellitus, real time PCR

Introduction

The microbiota in the gut is composed of trillions of microorganisms that weigh about 1.5 kilogram and represents an organ with physiological function both in health and diseases (1). The alteration of microbiota composition is associated with alteration of intestinal permeability leading to endotoxin release and metabolic changes that can lead to obesity and type 2 DM (2, 3).

There are four families (phyla) that represent the major components of gut microbiota namely *Firmicutes, Bacteroidetes*, *Proteobacteria* and *Actinobacteria* (4-6). There are minor phyla that include the *Verrucomicrobia* and *Fusobacteria* (7). In normal physiological conditions, *Firmicutes* make up the greatest proportion of the gut microbiota (64%), followed by the *Bacteroidetes* (23%), *Proteobacteria* (8%) and lastly *Actinobacteria* (3%).

Therefore, the major components of gut microbiota are the Phyla *Firmicutes* and *Bacteroidetes* that represent more than 80% of total gut microbiota (8). There is evidence that the ratio of phyla *Firmicutes* to

Bacteroidetes (phyla F/B ratio) is decreased in patients with type 2 DM (9, 10).

There is a hypothesis that Phyla *Firmicutes* assists in the production of butyrate from dietary fiber. Butyrate maintains the tight junction of intestinal barrier and reduces the inflammation and increase the sensitivity to insulin (11-14). The butyrate producing bacteria such as *Bifidobacterium* species are proportionally decreased in patients with type 2 DM (15). The predominant microbiota in patients with type 2 DM is affected by numerous factors such as geographical location, culture, diet, health status and medication-use (16). There is a study from Taiwan reported a decrease in Phyla *Firmicutes* and Phyla *Bifidobacterium* in patients with type 2 DM (17).

The development of molecular techniques for detection of microorganisms had facilitated the study of gut microbiota using real-time polymerase chain reaction (PCR) with specific 16S ribosomal RNA (rRNA) gene-based oligonucleotide primers (18). Previous study revealed that the use of real time PCR for detection and quantitation of *Firmicutes* and *Bacteroidetes* was specific and sensitive (19).

Therefore, the aim of this study was to investigate the ratio of phyla *Firmicutes* and *Bifidobacterium* in patients with type 2 DM compared to apparently healthy control subjects by real time PCR.

Material and Method

This study is a retrograde case control study that includes 100 control subjects and 100 patients with type 2 DM who were recruited from Mansoura University hospital during the period between August 2021 till May 2022.

The diagnosis of type 2 DM was based upon the criteria of the American Diabetes Association 2021. The patient was diagnosed type 2 DM with

the presence of one of the following: a fasting plasma glucose level of ≥126 mg/dl, a 2-hour plasma glucose level of ≥ 200 mg/dL during a 75-g oral glucose tolerance test, a random plasma glucose ≥ 200mg/dl with classic symptoms of hyperglycemia or hyperglycemic crisis, A hemoglobin A1c (HbA1c) level of ≥ 6.5% (20). The exclusion criteria for the patients included those with previous antibiotics intake within 30 days prior to the study, patients with irritable bowel syndrome, inflammatory bowel diseases, Celiac disease, patients with malignancies, patients with previous surgeries in the gastrointestinal tract within the last year prior to the study and patients with significant signs or symptoms suggesting gastrointestinal problem.

The study was approved by the ethical committee of the faculty of the medicine. Informed written consent was obtained from each participant. Each participant was subjected to full clinical examination.

Laboratory Study

Blood Samples

Ten milliliters blood samples were obtained from each participant and divided to two tubes one plain tube and other over EDTA. The plain sample was used for the separation of serum by centrifugation at 3000 rpm for 15 minutes. This serum was used for the measurement of fasting blood glucose level, total cholesterol, triglycerides, and high-density lipoprotein cholesterol (HDL-cholesterol) by autoanalyzer Dialab 48 (USA). The level of low-density lipoprotein cholesterol (LDL-cholesterol) was determined by Friedewald's formula: LDL = Total Cholesterol - (Triglyceride / 5) – HDL-cholesterol. The blood sample with EDTA was used for measurement of HB1c by enzymatic assay of ready to use Crystal chem kit (Crystal Chem- USA).

Stool Sample

Stool sample was obtained from each subject in clean container and transported rapidly to the laboratory within 30 minutes.

DNA extraction

The microbial DNA was extracted from 220 mg of fecal sample using QIAamp® DNA stool mini kit (Qiagen, Hilden, Germany) following the manufacturer´s protocol instruction. The extracted DNA was kept frozen at -20°C till the time of the amplification procedures.

Real time PCR for *Lactobacillus and Bifidobacterium*

The real time PCR estimated the amount of 16S ribosomal RNA (rRNA) genes of *Lactobacillus and Bifidobacterium* by separate amplification procedures.

The primers and the probes used for the real time PCR was summarized in **table 1**.

Table (1): The primers and the probes used in real time PCR

Phyla	primers and the probes
Bifidobacterium	5′-CGCGTCYGGTGTGAAAG-′3 5′CCCCACATCCAGCATCCA′ MGB-AACAGGATTAGATACCC
Lactobacillus	5′GAGGCAGCAGTAGGGAATCTTC -′3 5′GGCCAGTTACTACCTCTATCCTTCTTC -′3 MGB-ATGGAGCAACGCCGC

The amplification reactions were performed by the use of a total volume of 25 µl containing 1×TaqMan universal PCR master mix (Applied Biosystems-USA), with adding primers of 300 nM concentration and 200 nM TaqMan MGB probe, 60 ng of the extracted DNA, and BSA at the final concentration of 0.1 µg/µl . Amplification was performed by the following cycles: 2 minutes at 50 °C, 10 minutes at 95 °C, then 45 cycles of 15 seconds at 95 °C and 1 minute at 60°C at thermal cycle of the Applied Biosystems. The amount of genomic DNA extracted was determined by ultraviolet spectrophotometry at 260 nm in comparison to TagMan. TaqMan™ comprehensive microbiota Control
catalog number: A50382- Applied Biosystems). Number of bacteria was expressed as numbers of 16S rRNA genes/g wet weight of feces (21-23).

Results

The study included 100 patients with type 2 DM with mean age SD 51.8 ± 10.3 years. They were 55 males and 45 females. There was no statistically significant difference regarding age and sex distribution among both groups (P=0.32, P=0.4 respectively). Patients had statistically significant elevated levels of fasting blood glucose level (239.3 ± 60.8, P<0.001), HB1c level (10.73 1.6 P<0.001), cholesterol (209.1 ± 43.3, P<0.001), LDL-cholesterol (138.5 ± 46.7, P<0.001) compared to control subjects, **table 2**.

Real time PCR study revealed significant increase in the phyla of *Firmicutes* in the patients with type 2 DM (5.2 1.0 x10^9/gram, P <0.001) compared to control subjects (4.2 0.84x10^9/gram). There was statistically

significant decrease in phyla *Bifidobacterium* in patients with type 2 DM (10.02 ± 0.94, P<0.001) compared to control subjects (12.4 ± 1.2, P<0.001) with significant increase of *Firmicutes* / *Bifidobacterium (F/B)* ratio in patients (0.52 ± 0.1, P<0.001) compared to control subjects (0.34 ± 0.1, P<0.001), **table 3.**

In the correlation study between the Firmicutes phyla and biochemical data of the studied subjects, there was statistically significant correlation with fasting blood glucose level, triglycerides and LDL-cholesterol (P<0.001, P<0.001, P=0.001 respectively). Bifidobacterium phyla had significant negative correlation with fasting blood glucose, HB1c, cholesterol, HDL-cholesterol, LDL-cholesterol and Firmicutes phyla (P<0.001, P<0.001, P=0.039, P<0.001, P<0.001, P<0.001 respectively). There was statistically significant correlation between *F/B* ratio and fasting blood glucose, HB1C, cholesterol and LDL-cholesterol (P<0.001 for each), **table 4**.

Table (2): Comparison of demographic and laboratory findings between patients and control subjects.

Parameter	Control (n=100	Patients (n=100)	P
Age	51.8± 10.3	50.6± 5.9	0.32
Sex Male Female	 55 45	 58 42	0.4
Fasting blood glucose	89.01± 24.7	239.3± 60.8	<0.0(
HB1c	3.7± 0.9	10.73 ±1.6	<0.0(
Triglycerides	129.8± 19.7	131.7± 61.7	0.8
Cholesterol	155.4± 22.5	209.1± 43.3	<0.0(
HDL-cholesterol	38.9± 6.0	41.6± 10.1	0.07
LDL-cholesterol	90.6± 24.2	138.5 46.7	<0.0(

Table (3): Comparison of Firmicutes phyla, Bifidobacterium phyla and Firmicutes/ Bifidobacterium ratio between patients and control

	Control (n=100)	Patients (n=100)	P
Firmicutes	4.2 0.84	5.2 ±1.0	<0.001
Bifidobacterium	12.4 ±1.2	10.02± 0.94	<0.001
Firmicutes / *Bifidobacterium* ratio	0.34± 0.1	0.52 ±0.1	<0.001

Table (4) Correlation of *Firmicutes* phyla, *Bifidobacterium* phyla and *Firmicutes/ Bifidobacterium* ratio, age and biochemical laboratory findings in the studied subjects

	Firmicutes	*Bifidobacterium*	Firmicutes / *Bifidobacterium* **ratio**
Age R P	0.0.13 0.059	0.12 0.094	0.12 0.094
Fasting blood glucose	0.427 P<0.001**	-0.16 P<0.001**	0.59 P<0.001**
HB1c R P	-.09 0.93	-0.67 P<0.001**	0.66 P<0.001**
Triglycerides R P	0.465 P<0.001**	-0.045 0.53	0.065 0.36
Cholesterol R P	0.052 0.46	-0.432 P<0.001**	0.38 P<0.001**
HDL-cholesterol R P	-.66 0.35	-0.146 0.039	0.012 0.87
LDL-cholesterol R P	0.23 0.001**	-0.37 P<0.001**	0.34 P<0.001**
Firmicutes R P		-0.32 P<0.001**	

Discussion

There are a lot of evidence that link changes of the gut microbiota with glucose intolerance, metabolic disorders and type 2 DM. These findings result in the appearance of therapeutic approaches to control metabolic diseases by the modification of gut microbiota (24).

In the present study, there was significant increase in the base line biochemical laboratory results regarding fasting blood glucose level, total cholesterol, LDL- cholesterol and HBA1c in the patients compared to the

control subjects. Firmicutes phyla was increased in diabetic patients in comparison to control subjects. This finding was similar to a study from China showed an increase in the abundance of Firmicutes in type 2 DM (25). On contrary, other studies reported a decrease in the Firmicutes phyla in patients with type II DM compared to control subjects in a study from urban West Africans (26) and from Denmark (27). These conflicting findings are attributed to the difference in the ancestry, geographic regions, difference in the included number of the subjects, eating habits, and the method of the study (28), However, the results indicates that bacterial composition of the microbiota in the gut act as a signature of glucose tolerance status (25).

Regarding *Bifidobacterium phyla* they were decreased in type 2 DM compared to control subjects. This finding agreed with previous reports (22,27,29). Previous study from China with advanced metagenome technology showed moderate degree of alteration in the gut microbial composition and a decrease in the universal butyrate-producing bacteria with an increase in various opportunistic pathogens (30).

The increase in the *Bifidobacterium* phyla in the gut microbiota leads to the improvement of high fat diet induced glucose intolerance, improve the secretion of insulin and reduce the low-grade inflammation. The increase of this phylum can be approached by a prebiotic dietary fiber intervention (31). Another study has shown that the abundance of Bifidobacterium is significantly decreased in the intestines of T2D patients (7), and supplementation with Bifidobacterium (B. bifidum or B. adolescentis) alleviates gut microbiota disorders and lowers blood glucose concentration (8,9).

The pathogenesis of type 2 DM is linked to dietary habits and the modification of the gut microbiota may play a role in the development of

DM. Therefore, further studies are required for causal relationships assessment.

The difference in gut microbiota could presumably reflects the difference in the dietary habits with high intake of carbohydrate and fat with low fiber intake. There was significant positive correlation between the phyla of Firmicutes and fasting blood glucose level, cholesterol and LDL-cholesterol. This finding agreed with previous study by Ahmad et al., 2019 (32).

There is a known relation between the Firmicutes phyla, fat ingestion and obesity with production of short-chain fatty acids (SCFAs). It is also suggested that both *Firmicutes* and *Bacteroidetes* increase the absorption of monosaccharide from the host gut that leads to increase production of hepatic triglycerides and results in the insulin resistance (25, 33). Moreover, the dysbiosis of the gut microbiota may be associated with low-grade inflammation through the activation of SCFA-linked G-protein-coupled receptors (GPCR) which leads to metabolic disorders (34-36). Also, the resistance to resistin due to prolonged gut dysbiosis increase the susceptibility to insulin resistance (37). Previous study revealed a difference in the susceptibility to inflammation and insulin resistance among different races. Genetic factors and ethnicity may be associated with an increase in the susceptibility to insulin resistance, this needs further studies in Egyptian subjects (38).

This study revealed statistically significant increase in *F/B* ratio among diabetic patients compared to control subjects. Previous experimental study by Qian et al., 2022 revealed that mice with type 2 DM showed an increase in the F/B ratio compared to normal mice (39).

In this study there was a significant negative correlation between Bifidobacterium and fasting blood glucose, HB1c, cholesterol, HDL-cholesterol, LDL-cholesterol. Previous studies on gestational DM found

that, *Bifidobacterium* was found to be lower in women with DM than in normoglycemic pregnant women (40, 41). Moreover, reduced *Bifidobacterium* was reported to be associated with hyperlipidemia in previous study (42).

These findings suggest that gut microbiota could serve as a potential predictive biomarker of glucose intolerance. Many findings suggest that the gut microbiota has the capacity to alter blood lipid composition in particular cholesterol, through their role in bile acid metabolism and the generation of microbial products (43).

CONCLUSION

The present study highlights the disturbance of the ratio of the *Bifidobacterium* phyla and *Firmicutes* phyla in patients with type 2 DM compared to control subjects with subsequent effects on fasting blood glucose, HB1c, cholesterol, HDL-cholesterol, LDL-cholesterol. These findings need further multicenter studies on large scale of patients.

Author contributions

MESZ had shared in the laboratory study, the draft preparation of the article and data analysis of the study. AGA and EIME shared in the laboratory study draft preparation of the article. MESZ designed the study, and writing the article. MMM shared in the study design and writing of the article. All authors read and approved the final manuscript.

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Availability of data and materials

The data of the present study is available at

https://data.mendeley.com/drafts/db7kkcjg9f

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The Mansoura Faculty approved the study of the Medicine Ethical Committee (R.21.11.1536).

HUMAN AND ANIMAL RIGHTS

The study was performed according to the declaration of Helsinki.

CONSENT FOR PUBLICATION

The informed written consent was obtained from each patient.

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AVAILABILITY OF DATA AND MATERIALS

Not applicable.

CONFLICT OF INTEREST
None.

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