



Intestinal Microbiota Composition in Iranian Diabetic, Pre-diabetic and Healthy Individuals

Farahnaz Ghaemi¹ · Abolfazl Fateh^{2,3} · Abbas Akhavan Sepahy⁴ · Mehrangiz Zangeneh⁵ · Mostafa Ghanei⁶ · Seyed Davar Siadat^{2,3,7}

Received: 18 April 2020 / Accepted: 25 August 2020 / Published online: 31 August 2020
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Abstract

Background Type 2 diabetes, as the most prevalent metabolic disorder, is one of the leading causes of death worldwide. Recent studies showed a significant association between intestinal microbiota and type 2 diabetes. These studies have shared evidences that alteration in the composition of intestinal microbiota can disrupt the balance of the host homeostasis and lead to metabolic disorders such as type 2 diabetes. In the present study, we compared the intestinal microbiota composition in three groups of type 2 diabetes patients, pre-diabetic patients and healthy individuals of Iranian population.

Methods After obtaining informed consent, stool samples were collected from 90 individuals of three studied groups. The DNA was extracted using column-based method. Intestinal microbiota composition was evaluated by quantitative real-time PCR using specific bacterial 16S rRNA primers. The difference of bacterial load was compared between three groups.

Results The prevalence of *Akkermansia muciniphila* and *Bifidobacteria* species in healthy group was higher than type 2 diabetes group (*P Value* 0.006 and 0.001, respectively). In contrast, the load of *Lactobacillus* (*P Value* 0.044), *Escherichia coli* (*P Value* 0.005), and *Bacteroides fragilis* (*P Value* 0.017) in type 2 diabetes group, and the frequency of *E. coli* (*P Value* 0.001) and *Bacteroides fragilis* (*P Value* 0.004) in pre-diabetic group was significantly higher than healthy group. Moreover, the frequency of *Faecalibacterium prausnitzii* in healthy group was significantly higher compared to two other groups (*P Value* 0.005).

Conclusion There is a correlation between intestinal microbiota composition and type 2 diabetes. Determination and restoration of this microbiota composition pattern may have a possible role in prevention and control of type 2 diabetes in a certain population.

Keywords microbiota · gut · type 2 diabetes · pre-diabetes

✉ Seyed Davar Siadat
siadat@pasteur.ac.ir

¹ Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran

² Department of Mycobacteriology and Pulmonary Research, Microbiology Research Center, Pasteur Institute of Iran, No. 358, 12th Farvardin Ave, Jomhhoori St, Tehran 1316943551, Iran

³ Microbiology Research Center, Pasteur Institute of Iran, Tehran, Iran

⁴ Department of Microbiology, Tehran North Branch, Islamic Azad University, Tehran, Iran

⁵ Department of Infectious Diseases, Tehran Medical Sciences Branch, Islamic Azad University, Tehran, Iran

⁶ Chemical Injuries Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

⁷ Endocrinology & Metabolism Research Center, Endocrinology & Metabolism Clinical Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran

Background

Gut flora, also known as gut microbiota, differs widely among individuals. It is estimated that there are about 100 trillion bacteria in human's gastrointestinal tract, while 70 to 80% of them cannot be cultured [1]. Gastrointestinal flora composition also changes with diet [2], age [3], and other human factors [4]. The role of microbes in human health is not fully understood. But in recent years, studies have shown that microbes play an important role in the health, disease, and even treatment [5]. Intestinal microbiota is considered as a key factor in metabolism of carbohydrates, lipids, and amino acids and thus influences several metabolic disorders, including obesity; type 2 diabetes, and atherosclerosis [6, 7]. At the same time, Intestinal microbiota regulate inflammatory status by producing short-chain fatty acids (SCFAs) via carbohydrate fermentation and Lipopolysaccharide (LPS) of microbial membranes [8, 9].

Diabetes mellitus is a chronic and debilitating disorder characterized by persistent hyperglycemia. It may be due to dysfunction in insulin secretion (type 1 diabetes) or resistance to peripheral actions of insulin (type 2 diabetes). Type 2 diabetes is a metabolic disease with multiple predisposing factors, including obesity, genetic, physical inactivity and stress [10, 11]. A number of studies found out that intestinal microbiota and its metabolites can regulate the inflammatory responses, energy homeostasis, and lipid and glucose metabolism [12–14]. In this concern, it is documented that stimulation of glucagon-like peptide-1 release is a potential mediating mechanism for the effects of SCFAs on glucose homeostasis (SCFA-producing bacteria, such as *Bifidobacterium*). Hence, strategies have usually focused on a type 2 diabetes intestinal bacteria with anti-inflammatory properties and propensity to produce SCFAs. The clinical trials indicate that the modulation of the intestinal microbiota could be effective in diabetes management [15]. There is also evidences that intestinal microbiota has a significant role in triggering the chronic, low-grade inflammation that underpins insulin resistance and type 2 diabetes [16–18].

Numerous Factors like diet, hygiene and antibiotic consumption may alter composition of microbiota [19] and Contrary to studies carried out recently to explore the gut microbiota in diabetic patients and to identify the probable connection between type 2 diabetes and microbiota, there is still need for evidence supporting this hypothesis. Therefore, in the present study we tried to identify and compare the intestinal microbiota composition in three groups of type 2 diabetes patients, pre-diabetic patients and healthy individuals in Iranian population.

Materials and Methods

Study Design and Population

Ninety individuals referred to university hospitals in Tehran, Iran in 2018 were included in this case-control study. The inclusion criteria were age between 40 to 60 years. The exclusion criteria were suffering from chronic diseases (including inflammatory bowel diseases, liver cirrhosis, and chronic kidney diseases), smoking, alcohol consumption, cancer, current infectious diseases, receiving antibiotics and corticosteroids within the last month. Total sample size considering the standard effect size and normal approximation using the *Z* statistic, derived from published studies.

The subjects recruited into three groups. Group 1) thirty type 2 diabetes patients with A1c Hemoglobin less than 10%; group 2) thirty pre-diabetic patients identified with high fasting glucose ($100 \leq \text{IFG} < 126$ mg/dl) or increased glucose

within two hours after glucose injection ($140 \leq \text{IGT} < 200$ mg/dl) and with A1c Hemoglobin less than 6.4; and group 3) thirty healthy individuals.

Method

After obtaining a written informed consent, the stool samples collected in sterile laboratory dishes containing ice and transferred to the Tuberculosis and Lung Research Department in Pasteur Institute, Tehran Iran. The samples preserved in -20 °C until analysis. DNA from the 200 mg of each stool sample was extracted using QIAamp® DNA Stool Mini Kit according to the manufacturer's instructions method [20]. The concentration and purity of extracted DNA evaluated by a Nanodrop spectrophotometer (Thermo scientific, USA). The extracted DNA samples were stored at -20 for further analysis.

Quantitative real-time PCR was used in order to determine and quantify different bacterial populations. Genus-specific sequences of primers targeted the bacterial 16SrRNA genes used in the current study. Specificity of the primers was evaluated using the nucleotide BLAST in NCBI. The specific primers sequences are listed in Table 1 [19–27]. PCR reactions were performed in duplicate using Roche LightCycler® 96 system (Roche, Switzerland). Each 20 μ l PCR reaction contained 1 μ l DNA, 10 μ l syber green master mix (Takara, Japan), 8 μ l distilled water, and 0.5 μ l of forward and reverse primers (10 pmol/L). The mixture was heated for 1 min at 95 °C, followed by 40 amplification cycles: denaturation (95 °C for 5 s), annealing (55 °C for 30 s), and extension (72 °C for 30 s). After amplification, the final melting curve analysis was performed by cooling down PCR product from 95 °C to 60 °C.

To calculate the concentration of bacteria, 10 fold serial dilutions of extracted DNA from standard strain *E. coli* were prepared. This standard curve allowed calculating DNA concentration of each bacterium from stool samples.

Statistical Analysis

The normality of data distribution was evaluated using Kolmogorov-Smirnov test. For data with normal distribution, One-way ANOVA and Fisher's Least Significant Difference Test (LSD) were used. For non-normally distributed data, non-parametric Kruskal-Wallis test and Man-Whitney Test were applied. Mann-Whitney Test was used to analyze the correlation between gender and bacterial abundance. *P* Value less than 0.05 considered statistically significant. Statistical analyses performed using SPSS version 25.0 (SPSS Inc., Chicago, IL, USA).

Table 1 16S rRNA specific primers used in this study

Bacteria	Forward	Reverse	Amplicon size (bp)	Reference
<i>Akkermansia</i>	CAGCACGTGAAGGTGGGGAC	CCTTGCGGTTGGCTTCAGAT	327	[19]
<i>Bacteroides</i>	CTGAACCAGCCAAGTAGCG	CCGCAAACCTTTCACAACCTGACTTA	230	[20, 21]
<i>Lactobacillus</i>	AGCAGTAGGGAATCTTCCA	CACCGCTACACATGGAG	341	[22, 23]
<i>E. coli</i>	CATTGACGTTACCCGCAGAAGAAGC	CTCTACGAGACTCAAGCTTGC	195	[24, 25]
<i>Bifidobacterium</i>	TCGCGTCYGGTGTGAAAG	CCACATCCAGCRTCCAC	128	[25, 26]
<i>Faecalibacterium</i>	GGAGGAAGAAGGTCTTCGG	AATTCCGCTACCTCTGCACT	248	[25, 27]

Results

The mean age of patients with type 2 diabetes (53.3 years) was significantly higher than pre-diabetics (48.63 years) and healthy controls (51.63 years) (P Value = 0.024). Gender, Weight and BMI were not significantly different between studied groups (P Value >0.05). The concentration of FBS (mean \pm SD) was significantly higher in pre-diabetic (100 ± 13.3) and type 2 diabetes patients (136 ± 17.03) compared to healthy controls (85.6 ± 9.9) (P Value <0.001).

The load of the studied bacteria differs between the studied groups (Fig. 1). The bacterial load of *E. coli* was higher in fecal samples of pre-diabetic and type 2 diabetic patients compared to healthy controls (P -Values 0.001 and 0.005, respectively). The quantity of *Bacteroides fragilis* was also higher in pre-diabetic and type 2 diabetes patients compared to healthy controls (P Values 0.004 and 0.017, respectively).

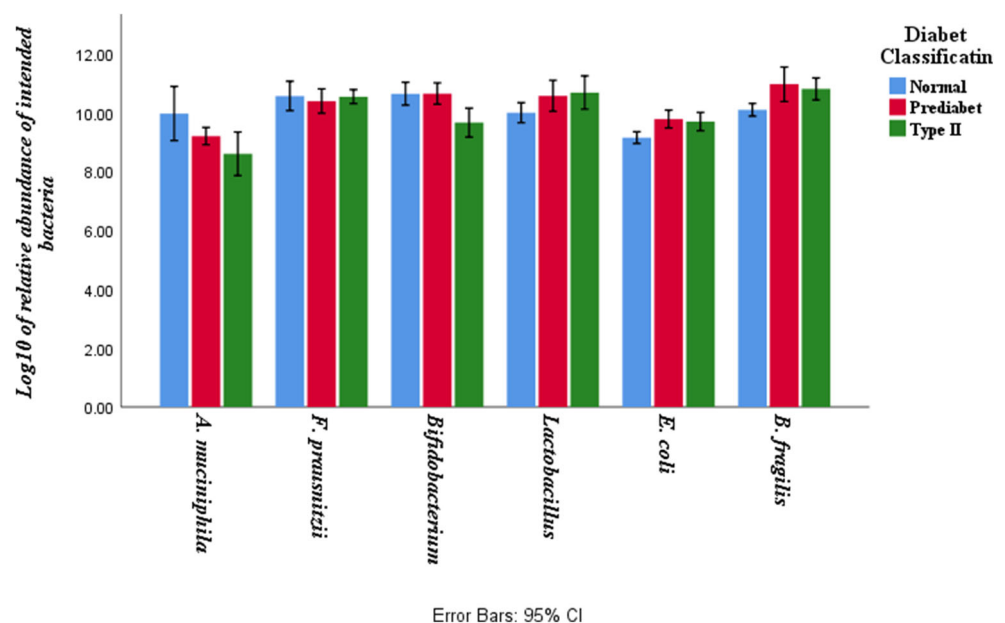
Faecalibacterium prausnitzii, a butyrate-producing genus, was significantly higher in healthy volunteers compared to pre-diabetic (P - Value 0.05) and type 2 diabetes patients (P Value 0.05).

The difference between the load of *Akkermansia muciniphila* in healthy group compared to pre-diabetic group was not significant but it was statistically significant compared to type 2 diabetes patients (P Values 0.11 and 0.006, respectively). In contrast, the bacterial load of *lactobacillus* was higher in type 2 diabetic patients than healthy group (P Value 0.006). The difference between pre-diabetic group and healthy controls was not statistically significant (P Value >0.05).

The number of *Bifidobacterium* was significantly higher in healthy subjects compared to type 2 diabetic patients (P Value 0.001). The pre-diabetic group also had a higher load of *Bifidobacterium* compared to type 2 diabetic patients (P Value 0.001).

The study further explored the correlation between subjects' gender and bacterial abundance. The load of *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *E. coli*, and *Bacteroides fragilis* was higher in male subjects whereas female participants had a larger number of *Bifidobacterium* and *Lactobacillus* but the results were not statistically significant (P Value 0.1).

Fig. 1 Bacterial load of fecal samples in pre-diabetic, type 2 diabetic patients and healthy controls. Values are calculated based on Log₁₀ CFU/g stool and are presented as mean \pm SD



Discussion

Previous studies showed that gut microbiota composition is different in populations with different ethnicities. It is hypothesized that gut microbiota including *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *Bifidobacterium*, *Lactobacillus*, *E. coli* and *Bacteroides fragilis* has a role on regulating metabolic profile and immune system and in triggering the chronic, low-grade inflammation which leads to insulin resistance and type 2 diabetes [21, 22].

In the present study we showed that the frequency of *Akkermansia muciniphila* and *Bifidobacterium* in healthy group was higher than that in type 2 diabetes group. In contrast, the load of *Lactobacillus*, *E. coli*, and *Bacteroides fragilis* in type 2 diabetes group and the load of *E. coli* and *Bacteroides fragilis* in pre-diabetes group were significantly higher than healthy group. Also, the frequency of *Faecalibacterium prausnitzii* in the healthy group was significantly greater than the other two groups.

Karlsson et al. study as one of the most comprehensive studies on the gut microbiota composition, indicated lower abundance of butyrate-producing bacteria in type 2 diabetes patients [23]. In another similar study, Qin et al. found that the frequency of *Faecalibacterium prausnitzii* decreased in patients with type 2 diabetes, while opportunistic pathogens such as *Bacteroides* and *E. coli* increased. They also reported that the prevalence of *Akkermansia muciniphila* in patients with Type 2 diabetes was higher than healthy individuals [24]. In the present study, we also showed a significant decrease in the frequency of *Faecalibacterium prausnitzii* bacteria in patients with type 2 diabetes compared to the control group. Furthermore, we showed that the prevalence of *Bacteroides fragilis* and *E. coli* was significantly higher in pre-diabetic and type 2 diabetes patients than in normal subjects. Interestingly, in contrast to Qin et al., the results of our studies showed that the frequency of *Akkermansia muciniphila* was significantly higher in healthy individuals than in those with type 2 diabetes. Different geographical and epidemiological factors may cause these differences.

Consistent with the present results, Le et al. showed that the frequency of *Lactobacillus* in fecal samples of patients with type 2 diabetes was significantly higher than healthy subjects, while *Bifidobacterium* was lower [25].

In previous investigations, Wu et al. observed a lower load of *Bifidobacterium* in patient with type 2 diabetes. [26]. Also, Moghaddam et al. reported a significant decrease in frequency of *Faecalibacterium prausnitzii* in Iranian type 2 diabetic patients compared to control group but there was no significant difference in the number of *Bifidobacterium* and *Bacteroides fragilis* between type 2 diabetic and healthy individuals [27]. Consistent with these studies, we showed a significant decrease in the frequency of *Faecalibacterium prausnitzii* and

Bifidobacterium in type 2 diabetes patients compared to the control group. Additionally, we showed that the frequency of *Bacteroides fragilis* in Pre-diabetic and type 2 diabetic patients were significantly higher than that in healthy subjects.

Sedighi et al. studied the association between frequency of *Lactobacillus* and *Bifidobacterium* and type 2 diabetes in Iranian population. They reported that in patients with type 2 diabetes the frequency of *Lactobacillus* was significantly higher than healthy individuals. In contrast, the frequency of *Bifidobacterium* in patients with type 2 diabetes was significantly lower than healthy individuals [28]. In contrast, Le et al. and Halawa and colleagues, reported significantly lower *Lactobacillus* in stool sample among type 2 diabetic patients when compared to control group [25, 29]. The present study indicated that the prevalence of *Lactobacillus* bacteria in the gastrointestinal tract of patients with type 2 diabetes was significantly higher than healthy individuals. Our study also confirmed the reduction of *Bifidobacterium* load in type 2 diabetes patients and showed that there was a significant correlation between *Bifidobacterium* frequency and type 2 diabetes.

Ejtahed et al. reported that bacterial load of *Bifidobacterium* and *Bacteroides* was higher in healthy control group in Iranian population [30]. Since long-term diet affects *Bacteroides*, It has been reported that *Bacteroides* was associated with a diet rich in protein and animal fat [31].

In the present study we found that the bacterial load of *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* in healthy group is higher than type 2 diabetes group. In contrast with the present evidences, Remely et al. reported that the frequency of *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* also increased in type 2 diabetes group [32].

Controversial results regarding the gut microbiota composition in type 2 diabetes can be seen in different studies. These controversies may be due to the affecting co-factors including, ethnicity, different locations, life style, various diet, drug consumption history and even the methodology to evaluate the microbiota.

In this study we were able to evaluate the association between gut microbiota and type 2 diabetes in Iranian population. Because of time and financial limitations, we only selected several bacteria to evaluate in this research. Conducting a multi-centric national study on individuals from most parts of Iran and evaluation of more bacterial genera in gut microbiota (more representative sample) could increase the reliability of these findings.

The considered time of cessation of antibiotic prior to sampling in this study was one month. We were not able to consider longer time (three months). We were not also able to rule out all situations that may lead to chronic inflammation and use them as exclusion criteria.

Conclusion

The correlation between gut microbiota and different medical conditions like type 2 diabetes has been widely established in several studies, with differences in findings and attributable strains. It is important that possible interventions for changing the composition of intestinal microbiota in order to prevent or control inflammatory conditions should be based on regional habits and dietary factors.

Acknowledgements This work was supported by the National Institute for Medical Research Development (NIMAD), Tehran, Iran (942995). The authors would like to thank the colleagues at Mycobacteriology and Pulmonary Research Department and Microbiology Research Center at Pasteur Institute of Iran, Tehran, Iran.

Compliance with Ethical standards

Conflict of interests The authors declare that they have no conflict of interest.

Informed consent Prior to sampling, a written informed consent obtained from all participants.

Abbreviations IFG, Impaired Fasting Glucose; IGT, Impaired Glucose Tolerance; A1c, Glycated Hemoglobin; PCR, Polymerase Chain Reaction; DNA, Deoxyribonucleic Acid; CFU, Colony Forming Unit

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