Comparison of *Bifidobacterium* spp. and *Lactobacillus* spp. Count in Faeces of Patients with Type 2 Diabetes Mellitus and healthy people

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Abstract

Type 2 diabetes mellitus is a metabolic disease that is associated with factors such as nutrition and lifestyle. Recent studies have shown a relationship between normal intestinal flora and diabetes. The aim of this study was to compare the number of different species of *Lactobacillus* and *Bifidobacterium* in Type 2 diabetic patients and healthy individuals. From February to October 2015, 20 patients with Type 2 diabetes mellitus and 20 healthy individuals as controls were selected. To distinguish these two groups, 2-hour postprandial glucose test, fasting blood sugar and glycosylated hemoglobin in both groups were measured. Fresh fecal samples were cultured on selective mediums for identification of 4 species of *Lactobacillus* (*L. acidophilus, L. salivaricus, L. fermentum*, and *L. reuteri*), and 3 species of *Bifidobacteria* (*B. langum, B. bifidum*, and *B. adolescentis*). To determine the absolute number of Bifidobacterium and Lactobacillus spp., in gut microbiota, pour plate assay was used.

Interestingly, comparison of *Bifidobacterium* and *Lactobacillus* spp. in the two groups showed a significant decrease in most species except *B. adolescentis* in Type 2 diabetic patients . In studied samples, *L. reuteri* was not found in diabetic patients. The study shows that imbalance in the intestinal microbiota can be one of the risk factors for Type 2 diabetes mellitus. Also, it suggests that suitable species of *Lactobacillus* and *Bifidobacterium* can be used in the diet of Type 2 diabetes mellitus as a treatment approach.

Key words: *Bifidobacterium; Lactobacillus*; Probiotic, Microbiota; Type 2 diabetes mellitus; Gut

Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disease characterized by increased blood glucose and therefore emergence of related diseases [1]. Complications such as cardiovascular disease, nephropathy and retinopathy can lead to conflict and high mortality [1, 2]. According to the World Health Organization (WHO), there are 346 million people with diabetes in the world. Without any intervention, it is likely to double by 2030. Furthermore, current treatments are expensive and painful, and investigators are looking for easier and cheaper ways to combat this disease [2].

Recently, meta-genetics have defined a hypothesis. More than 100 trillion microorganisms in the human gut and feces make up nearly 60% and 150 times the mass of their eukaryotic genome [3, 4]. This profile shows that more than 99% of microbiota are anaerobes and 98% contain several families including *Firmicutes* (64%), *Bacteroidetes* (23%), *Proteobacteria* (8%) and *Actinobacteria* spp. (3%) [3, 5]. Normal intestinal flora can improve the intestinal peristalsis, food intake, drug metabolism, vitamins and hormones endogen, and also prevent toxic and carcinogenic effects. The changes in the intestinal flora, towards Gram-negative bacteria, cause the release of endotoxins. Thus, unusual changes in diet can have a significant impact on the endotoxin cycle, and this is evident in the case of diabetics [6]. Probiotics such as *Bifidobacteria* and *Lactobacilli* are microorganisms that live in the small intestine and colon. In fact, adding probiotics and prebiotics in the diet, may be a non-drug alternative to treat chronic inflammatory diseases such as T2DM [7]. According to the data, the difference between *Lactobacillus* and *Bifidobacterium* spp. among diabetic patients was observed in two studies [8, 9]. In this case, it is clear that there is need for further studies to investigate this further. The aim of the present study is to determine the difference between *Bifidobacterium* and *Lactobacillus* spp. in T2DM and healthy people.

Materials and methods

1. Subjects

During February to October 2015, 20 patients with T2DM and 20 healthy subjects, of similar body mass index (BMI), were selected from Imam Reza Hospital in Tabriz, Iran; confirmed by Endocrinologist. All patients and healthy individuals were confirmed by testing of fasting blood sugar (FBS), 2-hour postprandial blood glucose test (2-HPPBGT) and glycosylated hemoglobin (HbA1c) by a standard enzymatic assay (Randox Laboratories Ltd., and UK) [4, 8, 10]. Participants did not take probiotics or prebiotics before sampling, and were between 30 to 80 years old (Table-1). The eating habits/nutrition of the both groups was similar. The exclusion criteria were antibiotics use, the presence of severe infections, acute gastrointestinal disorders such as constipation, diarrhea and abdominal pain 2 weeks prior to fecal sampling; and chronic intestinal inflammatory disease, heart failure, kidney diseases, autoimmune diseases, and hepatitis B and C. This study was approved by the Ethical Committee of Tabriz University of Medical Sciences (9371, 4 Aug 2014).

2. Stool sample collection

Stool sterile containers were given to the participants. The fresh stool samples were collected in the morning and were promptly transferred to the microbiology laboratory. One gram of each sample was diluted in 9 ml of sterile saline solution (0.85% (m/v) NaCl) supplemented with 0.05% L-cysteine (MERCK, Germany) and mixed thoroughly. From each sample, serial dilutions (10 fold) were made [11].

3. Culture and Identification of Lactobacilli and Bifidobacteria spp.

To obtain the absolute count of Lactobacillus and Bifidobacterium spp., standard quantitative plating was used. For *Lactobacilli* and *Bifidobacterium* count, MRS agar medium (MERCK, Germany) and Bifidobacterium agar medium (MERCK, Germany) were used, respectively. The plates were placed in anaerobic jars and favorable conditions (0% = O2, 10% = CO2, 10% = H2) with anoxomat (MART, Netherland) and specimens were created. The plates were incubated at 37°C for 72 hours.

Generally, identification of *Lactobacillus* and *Bifidobacterium* spp. are based on cell morphology, biochemical tests, enzyme activity and ability to use carbohydrates [11, 12]. Finally, to detect *Bifidobacterium* spp., the determination of fructose-6-phosphate phosphoketolase enzymes described by Scardovi (1986) and also resistance to mupirocin disk, the most important phenotypic confirmatory tests were used [13]. To establish the *Lactobacillus* and *Bifidobacterium* spp., carbohydrate fermentation tests were performed [14].

4. Statistical analysis

All data were analyzed by SPSS software version 20.0 (SPSS Inc, Chicago, IL, USA). Differences between groups were compared using t- test and ANOVA test. The two-tailed $\chi 2$ test and Fisher's exact test were used for categorical data. A p-value of ≤ 0.05 was considered to be significant.

Results

In this study, 20 patients with T2DM and 20 healthy controls were studied. Anthropometric characteristics showed that FBS in T2DM patients is significantly different from the control group (158.4±37.02 vs. 85.3±6.7, p-value <0.000), and the HbA1c also has a great difference between the two groups (5.54±.42vs. 7.7±.63, p-value <0.029). These differences between the two groups are reasonable [Table-1 - next page].

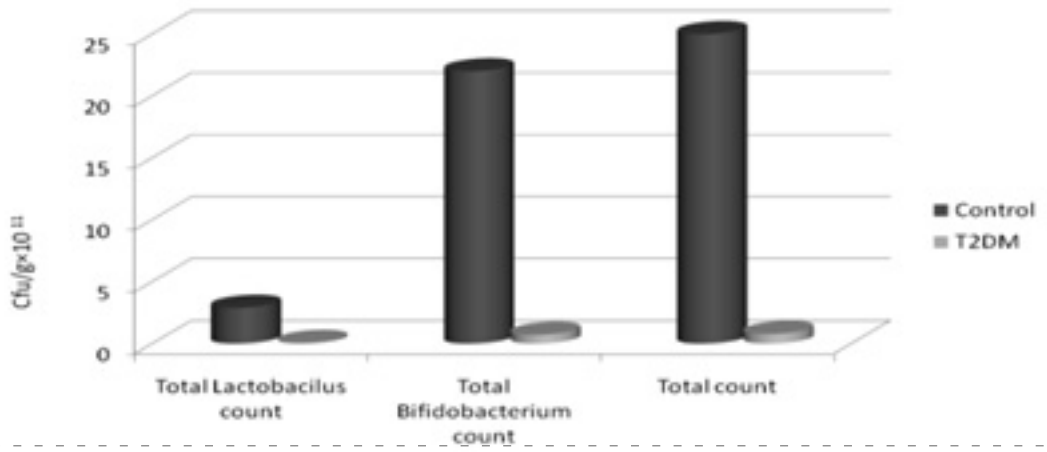
Four species of Lactobacillus spp. (*L. acidophilus, L. salivaricus, L. fermentum* and *L. reuteri*), and 3 *Bifidobacteria* spp. (*B. langum, B. bifidum* and *B.adolescentis*) were identified in the fecal samples. In the studied samples, *L. reuteri* was not observed in diabetic patients. The analysis of *Lactobacillus* and *Bifidobacterium* spp. count between the two groups show the differences in the most species

Table: 1. Demographic and laboratory data

Parameter	Control	T2DM	*P-value*
Sex (M/F)	10/10	10/10	1.00
Age (years)	54 ± 12	55 ± 12.50	0.93
BMI (kg/m²)	24 ± 3.1	24.60 ± 3.50	0.46
2-HPPBGT (mg/dL)	102.20 ± 12.0	212.40 ±48.90	0.004
FBS (mg/dL)	85.3 ± 6.7	158.4 ± 37.02	0.001
HgA1c (%)	5.54 ± 0.42	7.7 ± 0.63	0.02

M, male; F, female; BMI , body mass index; 2-HPPBGT, 2-hour postprandial blood glucose test; FBS, fasting blood sugar; HbA1c, glycosylated hemoglobin A1c

Figure 1: The total Lactobacillus and Bifidobacterium spp. Count in the stool of patients with Type 2 Diabetes Mellitus and healthy people



[Figure-1]. No significant changes in *B. adolescentis* numbers were noted. The results of the study are presented in Table 2. The total number of *Lactobacillus* and *Bifidobacterium* ssp. in patients with T2DM was lower than the healthy group; there was a significant difference between the two groups (P-value ≤0.05).

Discussion

In the present study, we examined the differences between the counts of *Lactobacillus* and *Bifidobacterium* spp. in stool samples of T2DM and healthy groups. This study showed that there was a significant decrease in the count of *Lactobacillus* and *Bifidobacterium* spp. (except B. adolescentis) in patients with T2DM.

Currently, the reason for gut microbiota difference between healthy and T2DM patients is uncertain; however the influence of intestine microbiota on metabolism and nutrition absorption has been suggested as a potential mechanism. Otherwise, an imbalance in gut microorganisms may alter the contact to diabetogenic environmental chemical compounds. The studies in mice with high fat diet has shown the insulin resistance and inflammatory reactions associated with lipopolysaccharide of Gram-negative bacteria [15]. Additionally, current reports have also shown that probiotics and prebiotics have a significant role in the treatment of gastrointestinal disorders due to immune-inflammatory effects and prevent some infectious diseases. It also is a promising hypothesis that the use of probiotics in order to prevent T2DM, can be effective [16]. However, few studies have been undertaken in order to determine the differences in the number of beneficial intestinal bacteria between diabetic and healthy individuals. In a study conducted on 16 Type 1 diabetic patients and 16 healthy children, the combination of intestinal bacteria was studied by quantitative Real-time PCR. Similar to our study, the beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* spp were observed. In diabetes patients the level was significantly lower than in healthy children [9]. In a study conducted by Le et al., 50 patients with T2DM and 30 healthy controls were selected from south China. *Lactobacillus* spp. levels in diabetic patients were significantly more than in the control group. In contrast, in type 2 diabetic patients, total count of *Bifidobacterium* genus was 7%, and B. adolescentis was less than 12% of the control individuals [8]. Although, in comparing the two groups the reduced level of *Bifidobacterium* spp. in both studies [8, 9] was shown, in some species of *Lactobacillus*, there was a significant difference. As in our study, unlike the Le et al study [8] (except L. casei which was decreased), the reduction of *Lactobacillus* spp. can be seen. The administration of L. casei in obese mice has reduced tolerance to glucose, however exact *Lactobacillus* spp. role in metabolic diseases still remains unknown [17].

Increased blood sugar in people with diabetes is normally associated with insulin resistance and it is as one of the risk factors in the pathophysiology of T2DM. Several animal

Table: 2. Comparison of bacteria count between controls and diabetic patients. CFU, colony forming unit; gr, gram; SD, standard deviation; T2DM, type 2 diabetes mellitus

Bacterial counts	Control cfu/gr ± SD	T2DM cfu/gr ± SD	*P-value*
L. acidophilus $\times 10^{10}$	22 ± 8.1	0.54 ± 0.33	0.000
L.salivaricus $\times 10^{10}$	3.3 ± 1.8	0.15 ± 0.09	0.001
L.fermentum $\times 10^{10}$	3.9 ± 1.5	0.07 ± 0.05	0.000
L.reuteri $\times 10^{9}$	1.8 ± 1.1	0	0.001
B.longum $\times 10^{11}$	22 ± 8.1	0.61 ± 0.33	0.000
B.bifidum $\times 10^{10}$	4.2 ± 2.6	1.0 ± 0.74	0.036
B.adolescentis $\times 10^{8}$	15 ± 1.0	2.0 ± 1.9	0.58
Total *Lactobacillus* spp. $\times 10^{10}$	29 ± 10	0.76 ± 0.45	0.000
Total *Bifidobacterium* spp. $\times 10^{11}$	22 ± 8.2	0.72 ± 0.40	0.000
Total *Lactobacillus* and *Bifidobacterium* spp.$\times 10^{11}$	25 ± 8.8	0.79 ± 0.41	0.000

studies on the effect of probiotics for reducing insulin resistance have had conflicting results [18-21]. In human studies, the results were not satisfactory after administration of probiotics in patients with insulin resistance [22-24], but in some studies it was satisfactory [6, 25]. This difference in results may be due to using the different species of probiotics, as well as the duration and amount of probiotic administration. It would seem that researchers who have reduced the normal intestinal flora and have used different species of probiotics have achieved a better result. These studies also suggest that pro-inflammatory factors play an important role in regulating insulin resistance, which is influenced by probiotics [25, 26].

Since the importance of T2DM is increasing in the world, T2DM management is one of the main problems of human health. It seems that appropriate use of probiotics, significantly reduces the inflammatory effects of metabolic diseases like T2DM, and can be used as a convenient and inexpensive therapeutic strategy. Also, it can be used as a prophylactic agent in metabolic diseases by adding it to the diet of healthy people.

Here, solely *Bifidobacterium* and *Lactobacillus* spp. were studied, but the gut has several different microbiota. Thus, it would be extremely advisable that other normal flora should be studied in order to determine the effect of normal flora on T2DM. We also suggest the use of more accurate methods such as molecular assays, because some microbiota are fastidious or non-cultivable. We recommend performing more study particularly on the mechanisms, more in vivo and randomized clinical trial searches, may help to identify the pathogenesis and design a program to control T2DM.

Conclusion

In the present study, *Lactobacillus* and B*ifidobacterium* spp. were predominant in the healthy group, and the number of probiotic bacteria in the gut had a significant reduction in type 2 diabetic patients. The reduction was the same in the most of *Lactobacillus* and *Bifidobacterium* species. These results point towards a potential role for *Lactobacillus* and *Bifidobacterium* spp. in the pathophysiology of diabetes.

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