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RESEARCH ARTICLE

Molecular Study of *Lactobacilli* Species in Patients with Type 2 Diabetes Mellitus

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Abstract:

Background:

Diabetes mellitus type 2 (T2DM) is a metabolic disorder of multiple etiologies due to disturbances in carbohydrate, protein, and fat metabolism. Egypt is among the top 10 countries with a high prevalence of T2DM (15.56% of adults). There are studies that show a link between the diversity of the gut microbiota and the development of T2DM. There are species of *Lactobacilli* that inhabit the gut that might differ in patients with T2DM compared to healthy subjects.

Objective:

The aim of the present research is to study the presence of *Lactobacilli* species in gut microbiota by multiplex PCR in patients with T2DM compared to healthy controls as a preliminary approach to open the way for future treatment with the help of probiotics or diet modulation.

Methods:

A retrograde case-control study was conducted on 79 patients with T2DM and 100 healthy controls cross-matched with age and sex. All patients were subjected to full clinical examination and laboratory tests, including identification of stool Lactobacillus species by multiplex polymerase chain reaction (PCR).

Results:

Certain species of *L. acidophilus*, and *L. rhamnosus* were found to be significantly increased in patients with T2DM (67.1%, 50.6% respectively) compared to control subjects (35%, P=0.001, OR 3.8, 95% CI:2.1-7.1, 25%, P=0.001, OR 3.1, 95% CI:1.64-5.8 respectively). Other species as determined by multiplex PCR, namely, *L. gasseri*, *(70%, P=0.001, OR 0.16, 95% CI: 0.1-0.3), L. reuteri (74%, P=0.001, OR 0.28, 95% CI: 0.5-0.53),* and *L. plantarum (69%, P=0.003, OR 0.4, 95% CI: 0.073-0.22)* were significantly higher in prevalence in control compared to patients with T2DM.

Conclusion:

The present study highlights the significant prevalence of certain species of *Lactobacilli* in gut as determined by multiplex PCR, namely *L. gasseri*, *L. reuteri* and *L. plantarum* in controls compared to patients with T2DM. These species may have a role in the reduction of certain risk factors associated with the development of T2DM. Moreover, certain species of *L. acidophilus, L. delbrueckii* and *L. rhamnosus* were significantly increased in prevalence in patients with T2DM. The findings of this preliminary study need further verification by a larger longitudinal study.

Keywords: Type 2 diabetes, Lactobacillus, Multiplex PCR, *L. gasseri*, *L. delbrueckii*, *L. rhamnosus*.

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1. INTRODUCTION

Diabetes mellitus type 2 (T2DM) is a metabolic disorder of multiple etiologies due to disturbances in carbohydrate, fat, and protein metabolism [1]. Diabetes mellitus (DM) represents a global health burden as it is estimated that around 425 million adults worldwide are living with diabetes. The disease represents a burden upon economic society and government besides patients [2]. Egypt is among the top 10 countries in terms of the high prevalence of DM and it is estimated that around 15.56% of adults in Egypt with an age group between 20 and 79 have DM [3].

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There are a lot of well-known risk factors that are associated with the development of T2DM, as socioeconomic status, genetic susceptibility, age, sex, and other environmental factors which are considered to be uncontrollable, while controllable risk factors include obesity [4], hypertension [5], smoking [6], and dyslipidemia [7].

There are studies that show a link between the diversity of the gut microbiota and the development of type 2 diabetes mellitus (T2DM) [8]. The microbiota in the gastrointestinal tract plays a role in the immune and inflammatory systems in the host [9, 10]. Moreover, there is a link between dysbiosis, which refers to the change in the composition of the microbiota, and the host diseases related to metabolic disorders [11]. These findings led to the use of the gut microbiota as a target for treatment in patients with T2DM [12].

Among the different microorganisms that inhabit the gastrointestinal tract, *lactobacilli* represent a common probiotic inhabitant along with *Bifidobacteria* [13, 14]. There are different species of *Lactobacilli* that inhabit the gut that was studied in patients with T2DM compared to control groups with controversial results.

For example, there were studies that found an increase in *L. acidophilus* [15], *L. gasseri*, and *L. salivarius* [16] in patients with T2DM with a decrease in other species such as *L. amylovorus* [17]. These findings suggest a correlation between bacterial diversity and the host metabolism in patients with T2DM.

The laboratory identification of *Lactobacilli* species using culture methods is a tedious and time-consuming method with limited accuracy [18, 19]. The common method used now for accurate identification of *Lactobacilli* depends upon the use of molecular techniques [20]. The molecular methods depend mainly upon the use of 16S rDNA or 23S rDNA targeted primers by the polymerase chain (PCR) [21, 22]. These primers can be used in the multiplex polymerase chain reaction (PCR) with accurate results.

There are limited studies about the *Lactobacilli* species in Egyptian patients compared with control subjects in some disorders such as obesity [23], and DM [24].

Therefore, the aim of the present research was to study the presence of *Lactobacilli* species, *L. acidophilus*, *L. casei*-group, *L. delbrueckii, L. gasseri, L. reuteri, L. Plantarum,* and *L. rhamnosus* in the gut microbiota by multiplex PCR in patients with T2DM compared to healthy control.

2. MATERIALS AND METHODS

The study is a retrograde case-control study that includes 79 patients with T2DM and 100 healthy controls cross-matched with age and sex. The patients were recruited from Mansoura University Internal Medicine Hospital from December 2020 to June 2021. All patients were adults above 18 years old, diagnosed to have T2DM according to the criteria of the American Diabetes Association [25], which included patients with T2DM diagnosed upon any of the following criteria; a fasting plasma glucose level of ≥126 mg/dL, a two-hour plasma glucose level of ≥200 mg/dL (random plasma glucose of ≥200 mg/dL in a patient with classic symptoms of hyperglycemia or hyperglycemic crisis), and glycosylated hemoglobin (HbA1c) level of ≥ 6.5%. The patients should indicate no previous antibiotics therapy during the last month before recruitment in the study. Each patient was subjected to a full clinical examination. The control subjects were cross-matched according to age and sex with no previous antibiotics therapy during the last month or manifestations related to gastrointestinal tract disorders. The study was approved by the Mansoura Faculty of Medicine ethical committee (R.21.08.1403) and approval was obtained from each participant in the study.

2.1. Laboratory Investigation

Fasting blood samples after 12 hours of fasting were drawn and divided into two aliquots, one without anticoagulant and one with EDTA anticoagulant. Plain aliquots were used for measurement of fasting blood glucose, creatinine, uric acid, HDL-cholesterol, LDL-Cholesterol, triglycerides, and total cholesterol using a fully automated system. Quantitative turbidimetric determination of HbA1c was performed using a commercial spectrum assay kit, Spectrum Diagnostics (Block 20008 - 19AIndustrial Zone PO. Box 30Obour CityEgypt11811).

2.2. Stool Sample

Stool samples were collected in clean containers and transported to the laboratory for DNA extraction. The stool samples were kept frozen at -80°C until DNA extraction.

2.3. DNA Extraction from Stool

Bacterial DNA was extracted from the stool samples using a QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol with prior handling as previously described [26]. At first, one gram of stool was added to 100 ml of ice water for homogenization with a glass rod, and then centrifuged at 1000 × g for 1 minute to remove the large particles. Then, 300 microliters of the supernatants was transferred to a tube and 200 µL of TE buffer (Tris-HCl [10mM]: EDTA 10 mM], lysozyme [20mg/mL]; pH 8.0) was added. The tubes were vortexed for 1minute and incubated at 37° C for 1 hour. Then the manufacturer protocol was continued with the increase of the lysis temperature to 95°C. The extracted DNA was kept frozen at −20 °C until use.

2.4. Multiplex PCR for *Lactobacilli* Species

The used primers for multiplex PCR for *Lactobacilli* species are listed in Table **1**. The used reaction mixture was taken from Qiagen by adding 50 pmol for each primer and 5 microns of extracted DNA. PCR amplification was performed in a thermal cycler from Thermofisher. The amplification process sequences performed included initial heating at 94 °C for 2 minutes, then thirty-five cycles including denaturation at 94 °C for 20 seconds, followed by annealing at 51 °C for 40 seconds, extension at 68 °C for 30 seconds, and then final extension for 7 minutes at 68 °C. The amplified products were subjected to electrophoresis by using 1.5% agarose gel stained with ethidium bromide [27].

Table 1. The used primers and the base pair length (bp) of the amplified products of *Lactobacillus* species by multiplex PCR .

Lactobacillus species	Primers Sequences	Bp
Lactobacillus	AGGGTGAAGTCGTAACAAGTAGCC CCACCTTCCTCCGGTTTGTCA	
L. casei-group	TGGTCGGCAGAGTAACTGTTGTCG	727
L. acidophilus	AACTATCGCTTACGCTACCACTTTGC	606
L. delbrueckii	CTGTGCTACACCTAGAGATAGGTGG	184
L. gasseri	ATTTCAAGTTGAGTCTCTCTCTC	272
L. reuteri	ACCTGATTGACGATGGATCACCAGT	1105
L. plantarum	CTAGTGGTAACAGTTGATTAAAACTGC	428
L. rhamnosus	GCCAACAAGCTATGTGTTCGCTTGC	448

2.5. Statistical Analysis

The data of the study were analyzed using SPPS22. The numerical data was expressed as mean and standard deviation (SD) and the qualitative data was expressed as numbers and percentages. The comparison between mean SD was carried out by T test and the comparison between qualitative data was carried out by chi-square. P was considered significant if it was less than .05.

3. RESULTS

The study included 79 diabetic patients and 100 cross-matched age and sex control subjects. The mean age ± SD of the diabetic patients was 58.8±16.1 and there were 50 males and 29 females. The associated clinical conditions on admission were chronic liver disorders in 44 patients, diabetic ketoacidosis in 31, and miscellaneous conditions such as cerebral hemorrhage in 4 patients. There was a significant elevation of fasting blood glucose (P=0.001), total cholesterol (P=0.003), triglycerides (P=0.001), LDL-cholesterol (P=0.001) and HbA1c (P=0.001) in patients compared to control subjects, (Table **2**).

On the other hand, the most prevalent species in the diabetic patients were *L. acidophilus*, and *L. rhamnosus* (67.1, 50.6% respectively) compared to control subjects (35%, P=0..001, OR 3.8, 95%CI:2.1-7.1, 25%, P=0.001, OR 3.1, 95%CI: 1.64-5.8 respectively), (Table **3**).

According to the study of *Lactobacilli* species by Multiplex PCR, the *L. gasseri*, *L. reuteri* and *L. Plantarum were the most prevalent species in the control subjects* compared to patients with T2DM *L. gasser (70%, P=0.001, OR 0.16, 95%CI 0.1-0.3), L. reuteri (74%, P=0.001, OR 0.28, 95%CI: 0.5-0.53), L. Plantarum (69%, P=0.003, OR 0.4, 95CI%:0.073-0.22).*

Table 2. Comparison of demographic and biochemical laboratory findings in patients with T2DM compared to control subjects.

Investigated parameter	Patients (n=79)	Control (n=100)	P
Age (mean± SD)	58.8± 16.1	55.8± 7.6	0.1
Sex Male (No.- %) Female (No.- %)	50 63.3% 29 36.7%	60 60% 40 40%	0.8
Fasting blood glucose (mean± SD)	243.8± 48.7	96.8± 15.8	0.001
Total cholesterol (mean± SD)	213.4± 63.2	189.0± 44.1	0.003
HDL-cholesterol (mean± SD)	43.9± 7.1	45.4 ± 5.4	0.12
Triglycerides	205.7± 54.3	155.9± 33.0	0.001
LDL-cholesterol (mean± SD)	119.5± 11.7	95.3± 11.0	0.001
HbA1c (mean± SD)	10.9± 1.6	4.3± 1.03	0.001

P value <0.05 is considered as statistically significant.

Table 3. Comparison of the *Lactobacilli* species prevalence between patients with T2DM and control subjects.

Lactobacilli species	Patients (n=79) No. %	Control (n=100) No. %	P	OR	95%CI
L.casei-group	26 32.9%	45 45%	0.07	0.6	0.32-1.11
L. acidophilus	53 67.1%	35 35%	0.001	3.8	2.1-7.1
L. delbrueckii	32 40.5%	43 43%	0.8	0.9	0.5-1.6
L. gasseri	21 26.6%	70 70%	0.001	0.16	0.1-0.3
L. reuteri	35 44.3%	74 74%	0.001	0.28	0.5-0.53

(Table 3) contd.....

Lactobacilli species	Patients (n=79) No. %	Control (n=100) No. %	P	OR	95%CI
L. plantarum	37 46.8%	69 69%	0.003	0.4	0.073-0.22
L. rhamnosus	40 50.6%	25 25%	0.001	3.1	1.64-5.8

Table 4. Comparison of the prevalence of *Lactobacilli* species between patients with different underlying clinical conditions.

Lactobacilli species	Patients with associated chronic liver disorders (n=44) No. %	Patients with T2DM complications (n=31) No. %	Patients with miscellaneous conditions (n=4) No. %	P
L.casei-group	14 31.8%	10 32.3%	2 50%	0.8
L. acidophilus	26 59.1%	25 80.6%	2 50%	0.11
L. delbrueckii	23 52.3%	6 19.4%	3 75%	0.01
L. gasseri	9 20.5%	11 35.5%	1 25%	0.35
L. reuteri	20 45.5%	12 38.7%	3 75%	0.38
L. plantarum	20 45.5%	15 48.4%	2 50%	0.96
L. rhamnosus	20 45.5%	18 58.1%	2 50%	0.6

The comparison between patients with different underlying clinical conditions revealed a significant increase in *L. delbrueckii* in patients with underlying chronic liver disorders (52.3%) compared to patients with T2DM complications (19.4%), P=0.01, (Table **4**).

4. DISCUSSION

The gut microbiota is a bacterial inhabitant of the gastrointestinal tract from the mouth to the large intestine [28]. A molecular study of *Lactobacillus* species confirms its presence as ≤ 1% of the total bacterial population in the large intestine. The *Lactobacilli* species are associated with the maintenance of health [29]. The difference in the composition of *Lactobacilli* species is associated with metabolic regulations, and the imbalance of these species with other microbiota may lead to obesity and metabolic disorders [30].

In the present study, *L. gasseri*, *L. reuteri* and *L. Plantarum* were the most prevalent species in the control subjects being (70%, P=0.001, OR 0.16, 95%CI 0.1-0.3), (74%, P=0.001, OR 0.28, 95%CI: 0.5-0.53) and (69%, P=0.003, OR 0.4, 95CI%:0.073-0.22), respectively, compared to patients with T2DM. There are species of lactobacillus that are isolated frequently from the stool of normal population [31]. These species are *L. casei, L. delbruckeii, L. murinus, L. plantarum, L. rhamnosus*, and *L. ruminus.* Some of these species (*e.g*., *L. rhamnosus* and *L. murinus*) are rarely isolated from environments outside the intestine and are considered gut-autochthonous microorganisms. There are species-specific to the host such as *L. reuteri* [32]. *Lactobacilli* function differs according to species. *L. reuteri* that has been proven in experiments to prevent abdominal fat pathology independent of baseline diet. These effects are immune-mediated as shown by the study of purified CD4+ T cells function depending mainly upon active immune tolerance through the induction of Foxp3+ regulatory T cells and IL-10 [33]. That study showed that the therapeutic intake of *L. reuteri* can restore a beneficial balance in the Th17/Treg in the immunity that is manifested even with the intake of the fast-food diet that provokes a proinflammatory immune state and chronic inflammation. In an experimental study conducted on mice, the administration of this species resulted in the reduction of epididymal fat mass and adipocyte size when mice were fed with a high fat and sucrose diet with no difference in body weight [34]. *Lactobacillus Plantarum* is known for its ability to reduce body weight, body mass index, and fat mass in obese hypertensive patients [35]. In an experimental study on mice, *L. gasseri* had shown to result in a significant reduction in the adipocyte size, as well as decreased leptin and cholesterol [36]. Also, experimental use of strain BNR17 of the *L. gasseri* in mice twice daily for 12 weeks fed on a high carbohydrate diet, has shown anti-obesity effects with a reduction of the weight of the body and the white adipose tissue, and significant reduction of serum leptin, and insulin levels [37]. There is a difference in the *Lactobacillus* species' association with weight gain or weight loss [38], with variation in carbohydrate metabolism and the production of fermentation end-products, such as lactate [39]. Therefore, the higher prevalence of isolated *Lactobacilli* species in the control subjects may play a role in protection from risk factors associated with T2DM.

In the present study, the most prevalent species in the diabetic patients were *L. acidophilus*, *L. delbrueckii* and *L. rhamnosus*. It is reported that experimental administration of *L. acidophilus* KLDS1.0901 or *L. rhamnosus* in mice leads to an increase in body weight. Although experimental administration of *L. acidophilus KLDS1*.0901 leads to a reduction in the fasting blood glucose, glycosylated hemoglobin, and insulin in serum and an increase in the level of glucagon-like peptide 1 in serum compared with diabetic mice. This action may be dose-dependent [40]. The variation in the association of *Lactobacilli* species with prevention of the disease or contribution to the pathogenic role in the disease might be attributed to the variation in intestinal abundance of this genus between healthy and diseased and may indicate that certain species or genotypes of *Lactobacillus* can be useful as a gut biomarker [41].

In the present study, the *L. delbrueckii* was present in patients with underlying chronic liver disorders (52.3%)

compared to patients with T2DM complications (19.4%), P=0.01. Many studies showed that various factors, including age, genetics, diet, antibiotics, and host immune system can modify the gut microbiome from the normal state [42].

CONCLUSION

The present study highlights the significant prevalence of certain species of *Lactobacilli* in the gut as determined by multiplex PCR, namely *L. gasseri*, *L. reuteri* and *L. Plantarum* in controls compared to patients with T2DM. These species may have a role in the reduction of certain risk factors associated with the development of diabetes mellitus type 2. In contrast, some other *Lactobacilli* species, *L. acidophilus, L. delbrueckii* and *L. rhamnosus* were significantly increased in patients with diabetes mellitus type 2. The findings of this preliminary study need further verification by a larger longitudinal study.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by Mansoura Ethical committee – Mansoura Faculty of Medicine-Egypt-Number R.21.08.1403.

HUMAN AND ANIMAL RIGHTS

No animals were used in this research. All human research procedures followed were in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2013.

CONSENT FOR PUBLICATION

Consent was obtained from each participant in the study.

STANDARDS OF REPORTING

STROBE guidelines were followed in this study.

AVAILABILITY OF DATA AND MATERIALS

The data of the study is available at https://data.mendeley.com/v1/datasets/publish-confirmation/s856dkc2rr/1.

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None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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