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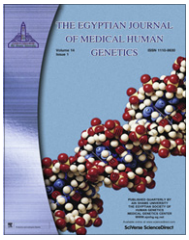

ORIGINAL ARTICLE

Role of intestinal microflora (*Lactobacillus Acidophilus*) in phagocytic function of leukocytes in type 2 diabetic patients

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Abstract The prevalence of obesity, insulin resistance and type 2 diabetes has steadily increased in the last decades. In addition to the genetic and environmental factors, gut microbiota may play an important role in the modulation of intermediary phenotypes leading to metabolic disease. Infection is an important cause of morbidity and mortality in diabetic patients. Chronic hyperglycemia impairs host defense mechanism such as cell mediated immunity, polymorphonuclear leukocyte function, and antibody formation. So we aimed to study the association between intestinal microflora (*Lactobacillus acidophilus*) count and phagocytic activity of polymorphonuclear leukocytes in humans with type 2 diabetes.

The study included 20 type 2 diabetic patients with good glycemic control and 20 type 2 diabetic patients with poor glycemic control. In addition, 20 normal healthy subjects were included as normal controls. The fecal composition of *L. acidophilus* was detected using de Man Rogosa Sharp agar followed by further confirmation using the polymerase chain reaction technique. Phagocytic function of polymorphonuclear leukocytes was assessed using the phagocytosis index %. Fecal *L.* acidophilus count was significantly increased among uncontrolled diabetic patients, while the phagocytosis index % was significantly reduced among the same patients. In uncontrolled diabetics, a significant positive correlation was observed between fecal *L. acidophilus* count and HbA1c and a

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significant negative correlation between phagocytic activity and *L. acidophilus* count. In conclusion, type 2 diabetes is associated with compositional changes in fecal *L. acidophilus* especially in the uncontrolled diabetes. The levels of glucose tolerance or severity of diabetes should be considered while linking the level of intestinal microbiota with a phagocytosis index of leukocytes.

1. Introduction

Type 2 diabetes is the most common form of diabetes that accounts for 85–95% of all diabetes in developed countries with a higher proportion in developing countries [1]. Infection in diabetic patients is severe, prolonged and difficult to treat so it is an important cause of morbidity and mortality in those patients. Infection is responsible for 6.0% of diabetic deaths [2].

Intestinal microbiota is the largest source of microbial stimulation that exerts both harmful and beneficial effects on human health. It therefore acts as a primary agent as it participates in the development of the postnatal immune system as well as oral tolerance and immunity [3].

The recently explored impact of the microbiota on energy metabolism, gut hormone regulation and the gut–brain axis was judged to be a fascinating topic and of great value in the future; however, the current limited human data do not allow the suggestion that probiotics can have a clinical role in the management of obesity or diabetes [4].

The Genus *Lactobacillus* represents a heterogeneous group with well documented immunomodulating properties and might potentially contribute to chronic inflammation in diabetic subjects [5].

Probiotics increase neutrophil bactericidal activity, phagocytosis, and oxidative burst. Bacteria, such as *Lactobacillus acidophilus*, increase lymphocyte numbers in the gut [6]. Other studies, also, report that supplementation with *L. acidophilus* in healthy subjects significantly increased the phagocytic activity of polymorphonuclear cells [7].

Most of the published studies described the differences between gut microbiota in obese compared to lean persons, while type 2 diabetes is generally considered as an attribute to obesity and thus left far behind as a focus of research. So, the objective of this study was to evaluate the role of intestinal microflora in the phagocytic activity of polymorphonuclear leukocytes in diabetic patients.

1.1. Subjects and methods

This case–control study was conducted on 40 patients with type 2 diabetes who were treated in the out-patient clinics of Ain Shams University hospitals from June 2009 to June 2010. Furthermore, 20 healthy age and sex matched subjects were considered as control group. The diagnosis of type 2 diabetes was based on the World Health Organization criteria [8].

Selected patients were classified into two groups: group A included 20 patients with type 2 diabetes with good glycemic control (HbA1c: 5.5–6.8%) and group B included 20 patients with poor glycemic control (HbA1c > 6.8%).

None of the patients had a history of gastrointestinal diseases nor received antibiotics, probiotics and prebiotics, within 30 days prior to sampling. Patients presenting with symptoms of other associated systemic illness or ICU admission were also excluded.

The Human Research Ethics Committee of Ain Shams University hospital approved this study and informed consent was obtained from each patient.

All of the patients underwent complete history taking and physical examination. The body mass index (BMI) was calculated from the formula: weight (kg)/height $(m)^2$. All subjects were investigated for the following: complete blood picture, serum fasting glucose and serum 2 h postprandial glucose levels. HbA1c levels (%) were measured at the time of enrollment using the fast ion exchange resin separation method (TECO Diagnostic, Lakeview Ave., Anaheim, CA, USA) and the methodology was done following the manufacturer's instructions.

1.2. Phagocytic index test

Quantitative determination of leukocyte phagocytosis was performed using heparinized venous blood. After centrifugation, 50 ml of the separated buffy coat was mixed with 50 ml of NBT dye (nitro blue tetrazolium dye) solution. This mixture was then incubated for one hour followed by fixing and staining of blood film using leishman's stain. The number of granulocytes showing phagocytosis under microscope was counted (the normal range 25–60%) [9].

1.2.1. Stool culture for L. acidophilus

Stool culture on de Man Rogosa Sharp agar (MRS) samples was collected in the early morning. Quantitative culture for *L. acidophilus* was done by weighing part (1 g) of the stool specimen which was diluted in 2 ml of sterile saline. Serial dilutions of this suspension were prepared in sterile saline solution; 1 ml of each dilution was inoculated into MRS agar plates using a standard loop. The plates were incubated anaerobically at 37 °C for 2–3 days. Colony forming unit per gram was detected for each stool specimen using the following formula:

$$\mathrm{CFU/Gm} = \frac{N \times \mathrm{D} \times 2}{\mathrm{W}}$$

where CFU: colony forming unit, Gm: gram, *N*: the number of colonies on the plate, D: dilution, W: the weight of the stool specimen in Gm, and 2: the original dilution of the stool specimen.

–Bacterial count normal (up to 100,000 CFU/Ml).

1.2.2. Identification of L. acidophilus

(1) *Colonial morphology: Typical* colonies of *L. acidophilus* on MRS agar appear as round, small, white, rough, raised, translucent colonies and catalase negative.
(2) *Gram' staining: L. acidophilus* appears gram positive bacilli or rods arranged in short chains (Image 1).

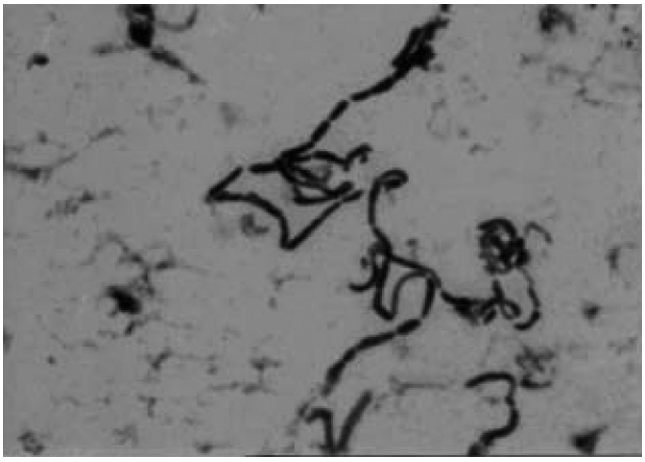

Image 1 Gram stained film of *Lactobacillus acidophilus* by oil immersion lens.

(3) *Biochemical reactions: A part* of colonial morphology *L. acidophilus* is distinguished from other lactobacilli by its inability to ferment lactose, mannitol and sorbitol.

1.2.3. Step2
Further identification of *L. acidophilus* by the PCR technique.

1.2.3.1. DNA isolation. DNA from bacterial colony that grows in MRS broth was extracted using proteinase K protocol after centrifugation of the broth for 2 min. Briefly, bacterial cells were resuspended in 467 µl of freshly prepared (50 mM Tris–HCl, 10 Mm EDTA, PH 7.5) 30 UL of 10% SDS and 3 µl of 20 mg/ml proteinase-k, Sigma Aldrich. Following incubation at 65°C for 45 min with shaking, the DNA was obtained by phenol/chloroform/isoamyl alcohol (25:24:1) precipitated with isopropanol washed with ethanol (70%) and dried under vacuum [10]. DNA pellet was resuspended in 25 µl of sterile distilled water and stored at −20 °C.

1.2.3.2. PCR primer. DNA pellet was confirmed to be of bacterial origin using the universal 16-S primer. F (5-AGA GTT TCC TGG CTC TSA AG-3) and R (5-ACG ACC TTG TTA GMT CGA CTT3) that amplify a 1500 bp fragment.

The genous specific primers of identification of *Lactobacilli* were designed on 16SrRNA as described by Dubernet et al.[11].

The genus specific primers, (Sigma, USA) for *Lactobacilli* amplifies a 123 bp fragment. LbL MA1-rev (5-CTC AAA ACT AAA CAA AGT TTC-3) and R16-1 (5′-CTT GTA CAC ACC GCC CGT CA-3).

1.2.3.3. PCR conditions and procedure. PCR reaction mixture (25 µl) was composed of 25 pmol of each primer, 0.2 mM of each dNTP, 1 × PCR buffer 50 mM Tris–HCl, PH 8.8, 2.5 mM MgCl2, 15 Mm (NH4)2SO4 0.45% Triton X-100, 10 ng of bacterial DNA and 1U of Taq DNA Polymerase (Biotech International, Australia).

The PCR was carried out in a Touchdown Thermal Cycler (Hybaid Middlesex, UK).

The amplification program is as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles consisting of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and a 7 min final extension step at 72 °C. Amplification products were subjected to electrophoresis in 1% agarose gels (Electrophoresis grade, Invitrogen) in TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.2) followed by ethidium bromide staining (5 µg ml) and visualized under UV light as shown in Images 2 and 3.

The strain *L. acidophilus* ATCC 4356 was used as positive control.

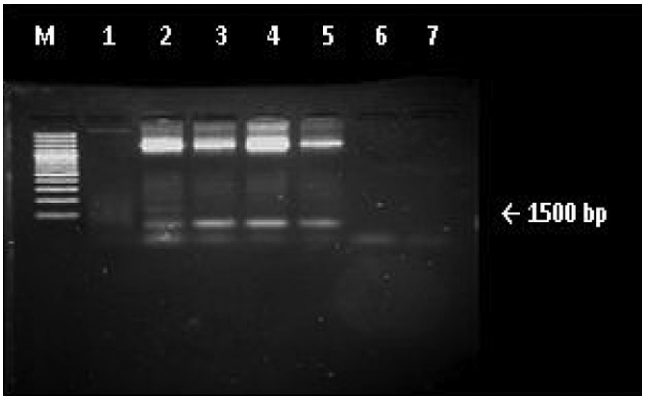

Image 2 Gel electrophoretic separation of PCR product of 16 S universal bacterial gene 1500 bp fragment.

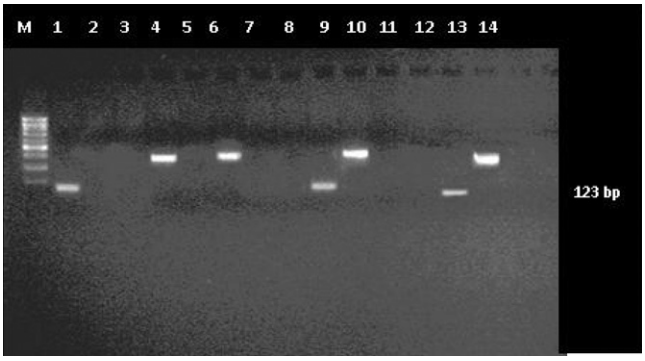

Image 3 Gel electrophoresis of *Lactobacillus acidophilus* gene amplification product at 123 pb fragments (at well 1, 9, 13).

2. Statistical analysis

Data were analyzed using SPSS (Statistical Program for Social Sciences) version 12. Data were expressed as mean ± standard deviation (SD). Data were analyzed using Student's *t*-test and one-way analysis of variance (ANOVA) with post Hoc analysis (Tukey's test) for the comparison of two and three groups, respectively. Chi-square test was used to compare categorical data. Pearson correlation was used for measuring the correlation between the different variables. A *p*-value of less than 0.05 was considered significant.

3. Results

Demographic and clinical characteristics of the studied groups are shown in Table 1. There were no significant differences between the three groups as regards age and gender. Compared

Table 1 Demographic, clinical characteristics and biochemical parameters of the study groups.

	Group A (controlled diabetic patients) $N = 20$	Group B (uncontrolled diabetics patients) $N = 20$	Controls $N = 20$	P-value
Age (years)	52.70 ± 6.79	52.95 ± 7.96	53 ± 1	> 0.05
Gender no (%)				> 0.05
Male	8 (40%)	10 (50%)	10 (50%)	
Female	12 (60%)	10 (50%)	10 (50%)	
BMI[a]	34.18 ± 5.87	32.26 ± 1.67	27.01 ± 2.3	0.001**
Total leukocytic count (10^3/cmm)	6.455 ± 1.318	5.672 ± 1.956	5.625 ± 1.245	> 0.05
PMNL[a]	55 ± 3	56 ± 4	54 ± 2	> 0.05
Fasting blood glucose (mg/dl)	122.15 ± 10.86	200.35 ± 34.961	81.23 ± 8.273	< 0.001**
Postprandial blood sugar (mg/dl)	162.15 ± 9.3	303.3 ± 43.552	122.81 ± 12.139	< 0.001**

All values are expressed as mean ± SD except gender which is expressed as no. (%).
[a] BMI, body mass index; PMNL, polymorph nuclear leukocytosis.
** $P < 0.05$ significant.

Table 2 Phagocytosis index % among the study groups.

	Group A (controlled diabetic patients)	Group B (uncontrolled diabetic patients)	Healthy controls	P-value
Phagocytosis index	33.65 ± 6.235	20.90 ± 2.174	38.25 ± 5.398	$P < 0.001$*
Tukey's test				
Controlled diabetics vs. uncontrolled diabetics $P < 0.001$*	Controlled diabetics vs. healthy controls $P < 0.05$*		Uncontrolled diabetics vs. healthy controls $P < 0.001$*	

* $P < 0.05$ significant.

Table 3 *Lactobacillus acidophilus* count among the study groups.

Characteristic	Group A (controlled diabetic patients)	Group B (uncontrolled diabetic patients)	Healthy controls	P-value
[a]*Lactobacillus a.* Count (CFU/ML)	430 ± 212.999	560 ± 153.554	163.75 ± 85.878	$P < 0.001$**
Tukey's test				
Controlled diabetics vs. uncontrolled diabetics $P < 0.001$**	Controlled diabetics vs. healthy controls $P < 0.001$**		Uncontrolled diabetics vs. healthy controls $P < 0.05$**	

[a] CFU, colony forming unit.
** $P < 0.05$ significant.

to the control group, diabetic patients showed significantly higher BMI, fasting blood glucose and postprandial blood glucose levels. Total leukocytic count and PMNL did not differ significantly between the study groups. Among diabetic patients, HbA1c was 6 ± 0.44 among those with good glycemic control and was 7.3 ± 0.32 among those with poor glycemic control.

On comparing the phagocytosis index between the three groups, we observed a significant reduction of their values among uncontrolled diabetic patients (20.90 ± 2.174) than the controlled diabetic patients and the control group (33.65 ± 6.235 and 38.25 ± 5.398 respectively) as shown in Table 2.

We observed on culturing of the stool of the subjects for microflora that the count of *L. acidophilus* in diabetics was significantly higher than in controls ($P = < 0.001$; means of 495.00 ± 194.738 CFU/ml vs. 163.75 ± 85.878 CFU/ml, respectively). Further analysis revealed that *L. acidophilus* count was higher among uncontrolled diabetics than controlled diabetics and healthy controls as shown in Table 3.

Correlations between *L. acidophilus* count in stool among controlled and uncontrolled diabetic patients and the various studied parameters are shown in Table 4. There was a positive correlation between *L. acidophilus* count in stool and plasma glucose levels (FBS, PPBS, HBA1c), which was observed among both groups, being more significant among the uncontrolled group. In addition, *L. acidophilus* count was negatively correlated with the phagocytosis index % among both groups with significant correlation among the uncontrolled group. Regarding the BMI, no correlation was found between the

Table 4 Correlation of *Lactobacillus acidophilus* count and the various studied parameters among controlled and uncontrolled diabetics.

	Controlled diabetic patients		Uncontrolled diabetic patients	
	r	*P*-value	*r*	*P*-value
Age	−0.288	0.218	0.291	0.213
BMI[a]	0.437	0.054	0.282	0.228
FBS[a]	0.22	0.34	0.595	0.006**
PPBS[a]	0.07	0.75	0.621	0.003**
HBA1c[a]	0.268	0.254	0.681	0.001**
TLC[a]	0.110	0.644	−0.378	0.100
PMNL[a]	0.167	0.482	−0.315	0.176
Phagocytosis index	−0.031	0.896	−0.643	0.002**

[a] BMI, body mass index; FBS, fasting blood sugar; PPBS, postprandial blood sugar; TLC, total leukocytic count; PMNL, polymorph nuclear leukocytosis; HbA1C, glycosylated hemoglobin.
** $P < 0.05$ significant.

Table 5 Correlation of phagocytosis index % and the various studied parameters among controlled and uncontrolled diabetics.

	Controlled diabetic patients		Uncontrolled diabetic patients	
	r	*P*-value	*r*	*P*-value
Age	0.202	0.392	−0.481	0.032
BMI[a]	−0.063	0.792	0.089	0.710
FBS[a]	−0.214	0.36	−0.653	0.002**
PPBS[a]	−0.12	0.65	−0.517	0.02**
HBA1c[a]	−0.314	0.178	−0.804	0.000**
TLC[a]	−0.197	0.406	0.028	0.907
PMNL[a]	0.220	0.351	0.175	0.459
Lactobacillus acidophilus count	−0.031	0.896	−0.643	0.002**

[a] BMI, body mass index; FBS, fasting blood sugar; PPBS, postprandial blood sugar; TLC, total leukocytic count; PMNL, polymorph nuclear leukocytosis; HbA1C, glycosylated hemoglobin.
** $P < 0.05$ significant.

quantification of *L. acidophilus* and the diabetic state of the patients.

The phagocytosis index % correlated negatively and significantly with the values of plasma glucose as determined by FBS, PPBS and HbA1c among uncontrolled diabetic patients and negatively, though not significantly, with plasma glucose values among controlled diabetic patients as shown in Table 5.

4. Discussion

Evidence that the gut microbiota composition can be different between healthy and obese and/or type 2 diabetic patients has led to the investigation of this environmental element as a key factor in the pathophysiology of metabolic diseases [12].

The results of this study showed that the significantly higher levels of the *Lactobacillus* group in diabetic subjects compared to controls, have also been reported in relation to type 2 diabetes in mouse models [13] and to obesity in human adults [14].

It has been proposed that some of the gut microbiota directed increased monosaccharide uptake from the gut and instructed the host to increase hepatic production of triglycerides associated with the development of insulin resistance [15]. This is in line with our study which revealed a significant increase in *L. acidophilus* count in uncontrolled diabetics in comparison to controlled diabetics as well as significant positive correlation between *L. acidophilus* counts and HbA1c, fasting blood glucose and 2 h postprandial blood glucose in uncontrolled diabetics and a significant positive correlation between *L. acidophilus* counts and HbA1c in all diabetics. Further explanation for the above finding was that several studies have described biomechanical remodeling as well as morphological remodeling of the gastrointestinal wall in experimental diabetic rats [16]. Frokjaer et al. [17] have shown that both the neuronal function of the contractile system as well as the structural apparatus of the gastrointestinal tract may be affected in patients with longstanding diabetes mellitus and diabetic autonomic neuropathy (DAN).

On the contrary, Yadav and his colleagues [18] found that following the administration of *L. acidophilus* and *Lactobacillus casei* to high fructose induced type 2 diabetes in rats there was a decrease in glycogen accumulation in the liver, significantly delayed onset of glucose intolerance, hyperglycemia, hyperinsulinemia, dyslipidemia, and oxidative stress indicating a lower risk of diabetes and its complications.

Several reports have demonstrated that the gut microbiota participates in the development of obesity by several mechanisms, i.e., by harvesting energy from the diet, by modulating the synthesis of gut peptides involved in energy homeostasis, and/or by regulating fat storage [19].

This study revealed no significant correlation between *L. acidophilus* counts and body mass index (BMI) in all the diabetics either controlled or uncontrolled. On the contrary, Khan et al. [20] reported that in his animal experimental

study, the administration of a single dose of *Lactobacillus* spp. was associated with a significant weight gain. In another study, obese patients presented significantly higher concentrations of *Lactobacillus* sp. in their feces than lean controls [21]. Larsen et al. [5] reported that type 2 diabetes mellitus was associated with compositional changes in the intestinal microbiota, as obese diabetics presented significantly higher levels of *Lactobacillus* sp. Moreover, Thuny and his associates [22] found that treatment with vancomycin in humans resulted in a major and significant weight gain as they speculated that the weight gain was induced by the growth promoting effect of *Lactobacillus* sp. in patients who had been treated by vancomycin, as these bacteria are known to be resistant to glycopeptide.

Chronic hyperglycemia can impair a wide range of functions in neutrophils including chemotaxis, adherence, phagocytosis, intracellular killing, superoxide anion O_2, hydrogen peroxide H_2O_2 and sorbital production. This is consistent with many studies which confirmed that clinical investigations of diabetic and experimental studies of diabetic rats and mice have clearly demonstrated consistent defects in PMN chemotactic, phagocytic, and antimicrobial activities [23].

Regarding the association of phagocytic index with HbA1c, fasting blood glucose and postprandial blood glucose, we found that there is a highly significant negative correlation of phagocytosis index with HBA1c and a significant negative correlation with fasting and postprandial blood sugar in uncontrolled diabetics. This is in agreement with Alba-Loureiro et al. [24] who stated that the reduction in leukocyte phagocytosis and bactericidal activity showed a significant correlation with increases in blood glucose levels. The reduction of blood glucose levels by insulin treatment of diabetic patients has been reported to be significantly correlated with improvement of neutrophil phagocytosis capacity [2].

This finding confirmed that chronic hyperglycemia impairs phagocytic function of neutrophils and this contributes to the high susceptibility to and severity of infections in diabetes mellitus.

This study is the first and the only available one to correlate between phagocytic activity and *L. Acidophilus* counts in diabetic patients. In uncontrolled diabetics, we found a significant negative correlation. While in controlled diabetics, there was no significant correlation.

In conclusion, no one can judge the true link between diabetes mellitus and gut microbiota, if it is a cause or an outcome. It was known that high fat feeding changed the gut microbiota toward decreasing the number of beneficial bacteria together with an increase in the number of hazardous bacteria resulting in a state of metabolic endotoxaemia which might trigger an inflammatory response and play a role in the development of diabetes. The exact mechanism by which gut microbiota lowers systemic inflammatory tone is poorly understood and needs further investigations.

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