THE EFFECT OF SHORT-TERM PROBIOTIC ADMINISTRATION IN NEWLY DIAGNOSED CHILDREN WITH TYPE 1 DIABETES MELLITUS ON GUT MICROBIOTA

By

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ABSTRACT

Background: *Type 1 diabetes mellitus (T1DM) is among the most well studied organ-specific autoimmune diseases, it is associated with a lot of life long complications and comorbidities. A number of studies revealed that adolescents with antibody positivity which later progressed to T1DM had reduced diversity of intestinal bacteria. Short chain fatty acids (SCFAs) producing bacteria such as Lactobacillus and bifidobacteria have a documented beneficial effect in autoimmune suppression.*

Purpose: *The aim of the study is to evaluate the alteration of gut microbiota between young patients with newly diagnosed T1DM, and to determine if modulation of gut microbiota could partly explain the aetiology of the disease and if this intervention could help in diabetic patients' management.*

Subjects and Methods: *This is a pilot study which involved 30 newly diagnosed patients with T1DM. The study was done during the period of April 2022 till September 2022. Patients were recruited from outpatient diabetes clinic, Ain shams University pediatric Hospitals, and they were selected by simple random method. Subjects were divided into 2 groups; Control group [A] and intervention group [B]. Patients in group [B] received probiotic enriched yogurt intake (200 g, 10 × 109 colony forming unit (CFU) of both Bifidobacteria spp. and Lactobacillus spp.) daily for 12 weeks duration. All patients were subjected for full history, clinical examination, and laboratory measurements; HbA1C, fasting C peptide, and fecal microbiota assessment by PCR analysis for Log count of Lactobacillus and Bifidobacteria at the onset of the study and after 12 weeks.*

Results: *Comparing both groups as regard the studied bacteria showed that the log count of Lactobacillus was significantly higher in group [B] than group [A] (p= 0.034). However, there was no significant difference between both groups as regards log count of Bifidobacteria. Both groups had significant improvement in glycemic control, lipid profile and BMI Z score. However, No significant difference was found between the two groups as regard data concerned with glycemic control and/or pancreatic function.*

Conclusion: *Probiotics use alone or in combination with other multi-strains of probiotics, are potentially the useful in improving the intestinal bacterial diversity of useful SCFAs producing bacteria in T1DM patients, further studies with larger number of patients, studying multi-strains probiotic effect and with longer follow up period is highly recommended to emphasize our current study results.*

Keywords: *gut microbiota, probiotic, autoimmunity, bifidobacteria, lactobacillus, Type 1 Diabetes mellitus.*

INTRODUCTION

The incidence of type 1 diabetes mellitus (T1DM) has increased rapidly worldwide. **(Hans H et al., 2018)** However, genetic susceptibility alone is not sufficient to explain why the prevalence of T1DM increases at a rate of 3% to 5% per year, when considering genetics in population to be relatively stable. **(Zheng P et al., 2018)** In recent years, the importance of environmental factors in T1DM, especially gut microbiome has been realized. T1DM develops as a result of an autoimmune process which results in destruction of the insulin secreting beta-cells of the pancreas. Several factors contribute to the development of T1DM; these factors are genetic susceptibility, viral infections and recently diet and intestinal microbiota. **(Pociot F and Lernmark A, 2016**; **Rewers M and Ludvigsson J, 2016)**. Several studies shows that altered gut bacterial microbiota (dysbiosis) is highly associated with the pathogenesis of T1DM, thus, targeting gut microbiota may serve as a therapeutic potential for patients with T1DM. One important cause for the rise of diabetes is dietary changes over the past years. Increased consumption of processed carbohydrates with low consumption of dietary fiber has been recognized as a major risk factor for development of diabetes and other auto-inflammatory diseases. Soluble and insoluble fibers are preferentially fermented by microbiota in the colon to generate SCFAs such as acetate (C2), propionate (C3) and butyrate (C4). SCFAs produced by the gut microbiota have beneficial anti-inflammatory and gut homeostasis

effects and prevent T1DM in mice. SCFAs may regulate lymphocytes and myeloid cells to facilitate the production of lymphocytes that promote immunity but prevent inflammatory diseases. SCFAs can activate G-protein-coupled receptor 43 (GPR43) which is the receptor for SCFAs on intestinal epithelial cells to enhance gut barrier function to prevent inflammatory diseases caused by invading bacteria. **(Chang H, 2017)** These functions are likely to contribute to suppression of autoimmune lymphocytes and T1DM. Reduced SCFA production indicates a loss of beneficial bacteria, commonly associated with chronic autoimmune and inflammatory diseases, including T1DM and type 2 diabetes. **(Bell KJ et al., 2022)** The abundance of SCFAs-producing bacteria as well as lactate-producing bacteria was reduced in patients with T1DM. The reduced number of Lactobacillus and Bifidobacterium could also be observed at the onset of T1DM **(Zheng P et al., 2018)**.

Sample size:

Using EPI INFO sample size calculator; with 0.05 alpha error, confidence interval of 0.95 and power of the study 0.80. The minimum sample size calculated to determine if modulation of gut microbiota could partly explain the aetiology of the disease and if this intervention could help in diabetic patients' management is 28 cases of newly diagnosed patients with T1DM.

Ethical consideration:

- This study was reviewed and approved according to Declaration of Helsinki **(as revised in Brazil, 2013)** by the Research Ethics Committee of Ain Shams University Hospitals, Faculty of Medicine.
- Informed written consent was taken from each subject before participation in the study.
- The aim and the steps of the study were explained to the caregivers of the patients before enrollment in the study.
- The patients had the right to withdraw from the study at any time.
- The data of the study is confidential and the patients have the right to keep it.

Conflict of interests:

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript and

there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication.

Funding:

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Inclusion criteria:

Diagnosis of T1DM was done based on International Society for Pediatric and Adolescent Diabetes (ISPAD) criteria of diabetes diagnosis in children, 2018. Children and adolescents with newly diagnosed T1DM were included with age ranging from 9 to 18 years old. They were recruited within 90 days from first diagnosis of T1DM.

Exclusion Criteria:

The following patients' groups were excluded from the study;

- Patients diagnosed with immunodeficiency diseases or significant cardiac, renal or hepatic diseases.
- Patients suffering from diseases which affect intestinal absorption such as celiac disease and thyroid disorders.
- Patients who were using probiotics supplements or gastrointestinal medicine earlier than conducting the study.
- Children with allergic reaction(s) to probiotics or prebiotics regimen.

Study design:

The study was carried out on 30 adolescents; all subjects were newly diagnosed patients with T1DM (recruited within 90 days of diagnosis of diabetes), they were recruited from the Paediatric and Adolescents Diabetes Clinic, Ain Shams University Hospital. Patients were selected by simple random method. Group [A] patients were the control group who did not receive probiotic enriched yogurt. Group [B] patients received 200 mg of probiotic enriched yoghurt daily for 12 weeks, compliance was followed up by regular meetings and telephone calls.

Patients' evaluation and laboratory investigations:

All participants were subjected to the following:

I. Full medical history taking with special emphasis on demographic data, family history of diabetes, duration of diabetes (in days), types of insulin therapy, dose of insulin therapy, frequency of blood glucose monitoring, history of

DKA and presence of diabetic complications.

II. Full clinical examination laying stress on: Vital data measurement including blood pressure, anthropometric measures were taken including weight in Kilograms (Kg) and height in centimeters (cm). Body mass index was calculated as Kg/m2 and plotted on the age and sex standard percentiles according to WHO growth charts for weight, height and BMI Z scores. Neurological examination was done to detect any signs of neuropathy. Finally, fundus examination was done by direct ophthalmoscope to detect retinopathy.

III. Laboratory Investigations:

a. Under complete aseptic conditions, 10mL of fasting venous blood were obtained by a clean venipuncture, two milliliters were placed in EDTA tube for subsequent assay of HbA1c, while the rest was evacuated in two plain test tubes. The serum was separated by centrifugation (1000x g for 15 minutes). Serum of one tube was immediately assayed for lipid profile, while the serum collected in the other tube was divided in three aliquots and stored at -20ºC for subsequent assay of fasting C-peptide, thyroid profile and celiac screening. Hemolysed samples were discarded. Repeated freezing and thawing was avoided.

b. Stool samples for bacterial PCR analysis were collected from all subjects at the onset of the study, and they were collected after 12 weeks for both groups; group [A] and group [B].

Preparation of the yogurt enriched with probiotic:

The probiotic component of the product used in the study contained a blend (one fermented milk cup contained 200 g, 10 × 109 CFU) of proprietary strains of Lactobacillus acidophilus CUL60, Lactobacillus acidophilus CUL21, Lactobacillus acidophilus NCFM, Bifidobacterium lactis HNO19, Bifidobacterium animalis supsp lactis CUL34, and Bifidobacterium bifidum CUL20. It was purchased from GNC Ultra Probiotic Complex (UK)

For probiotic yogurt preparation, pasteurized buffalo milk was inoculated with yogurt starter culture and activated probiotic bacteria mixture in 2/100 ratio at 37 °C. Then, the inoculated milk was divided into

200 mL volume in plastic cups covered with plastic lids and incubated for 6 h at 37 °C to form the curd. Finally, yogurt was transferred to refrigerator and stored at 4 °C till consumption. The Bifidobacterium sp. present in the fermented product was counted by adding 1 mL of the product to 9 mL saline (0.1 g/L); serial dilutions were done. Then, Bifidobacterium spp. was counted on LP- MRS agar after 48 h at 37 °C.

Bacterial quantification by real-time PCR:

Bifidobacterium and Lactobacillus count in stool assessment was done for Bifidobacterium and Lactobacillus strains in the collected stool samples before and after treatments by qRT-PCR technique (quantitative Real Time Polymerase Chain Reaction) which was performed as following:

DNA extraction briefly, 1 g of each stool sample was homogenized with 9 mL of phosphate buffer saline in a Stomacher 400 (Seward Ltd., London, United Kingdom) at full speed for 2 min. Then, 200 µL of the homogenate was used for the DNA extraction using the QIAamp DNA Stool Minikit (Qiagen, Germany) according to the manufacturer's instructions. Samples were kept refrigerated at −18 °C until being used for quantitative detection by RT-PCR.

Quantitative real-time PCR: 1 g of fecal material was homogenized with 9 ml of phosphate-buffered saline buffer in a Stomacher 400 (Seward Ltd., London, United Kingdom) at full speed for 2 min; 200 µl of the homogenate was used for the DNA extraction with the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. To test the different strains by real-time PCR, 200 µl of a culture (A600 1) of the microorganism was used.

The DNA extracts were frozen at 20°C until analysis. Samples (1 µl) were analyzed in 50-µl amplification reactions consisting of 1 PCR buffer II, 3.5 mM MgCl2, 0.2 µM each primer, 200 µM each deoxynucleoside triphosphate, 0.024 µM europium-labeled Bifidoprobe, 0.166 µM quencher probe, and 1.25 U of AmpliTaq Gold DNA polymerase. All reactions were performed on MicroAmp optical plates sealed with MicroAmp optical caps (Applied Biosystems).

Thermal cycling (iCycler) consisted of an initial cycle of 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 1 min, and

61°C for 45 s, and 35°C for 15 s. Europium fluorescence measurements were performed in real time at the end of each cycle with a Wallac Victor 1420 multilabel counter (Perkin Elmer, Turku, Finland) at 35°C to determine the threshold cycles (Ct) of individual reactions. The Ct was defined as the PCR cycle at which the europium signal-to-noise ratio crosses a threshold value of 1.5. The number of cells of bifidobacteria in the fecal samples was determined by comparing the Ct values obtained to the standard curve.

DNA extracts from the different samples were analyzed in duplicate in each PCR in two independent PCR runs. The 16S rDNA genes are mainly being used as target molecules, but as more bacterial sequences are becoming available, new specific primers and probes targeting other genes will be available in the near future to be used when the 16S ribosomal DNA is not an adequate target. It must be taken into account that bacterial quantification by real-time PCR can be influenced by differences in the number of rRNA operons between the quantified species or groups, sequence heterogeneity between different operons in the same species, and differential amplification of different DNA molecules.

The qPCR reactions were designed as follows: 95ºC for 10 min (1X), 95ºC for 10 sec (40X), 57ºC for 30 sec (1X), 72ºC for 30 sec (1X), and 95ºC for 10 sec (1X). The Ct values were averaged for each animal and primer set. Relative concentrations were calculated using the 2- ΔΔCT method.61 FastStart DNA Master SYBR Green (Roche, Indianapolis) was used for all qPCR reactions.

All universal primer pairs and specific TaqMan® probes developed during this study were tested with DNA samples (20 ng/µl) of the genera Lactobacillus, Bifidobacterium, Lactococcus and Streptococcus, which were used as positive and negative controls. Real-time PCR was performed using a Light Cycler® 480 (Roche, Mannheim, Germany) based on TaqMan® detection. Each singleplex real-time PCR sample contained 5 µl of DNA template, 10 µl Taq polymerase, 1 µl of each universal primer (10 µmol, Thermo Scientific, Dreieich, Germany), 1 µl of TaqMan® labeled specific primer (10 µmol, each 6-FAMTM/Dabcyl-labeled, TIB MOLBIOL, Berlin, Germany) and 2 µl of PCR grade water (Roche, Mannheim, Germany).

A single initial denaturation step of 10 min at 95° C was followed by 40 cycles of 95° C for 1 min (denaturation), 53° C for 30 s (annealing) and 73° C for 30 s (elongation). The fluorescence signal was measured at the end of each 73° C elongation step. Each real-time PCR included two technical repeats and the results were analyzed by using the Roche® Light Cycler 480® software.

Samples were kept refrigerated at -18 oC without been pasteurized. The samples were centrifuged at 3000 g for 30 min. Then, total DNA was isolated from the pellets. DNA was eluted in 100 μL and the purified DNA extracts were stored at 20 oC. Real Time PCR was used to characterize the bacterial DNA present in the breast milk samples. For this purpose, a series of genus-specific primer pairs were used. Real Time PCR for Bifidobacterium and Lactobacillus was performed according **Matsouki et al., 2002** and **Heilig et al., 2002**, respectively.

Calculation of bacterial colony counts by real time PCR:

The results were calculated by using linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis. Additionally, using the mean absorbance value for each sample was used to determine the corresponding concentration from the standard curve.

Statistical analysis:

The data has been updated, coded, and registered on a computer. The data analysis was done using the SPSS package (version 25). For categorical variables, descriptive statistics were provided as frequencies and percentages. Testing the normality of continuous data was done by using Kolmogorov-Smirnov and Shapiro-Wilk test, and normality plots as histogram and stem and leaf. The mean and standard deviation for continuous variables were used when normally distributed and when not normally distributed, as median and range. Age, duration of DM, and all lab investigations were not normally distributed, so the Mann Whitney test" U test" and Wilcoxon signed rank test were performed to compare these parameters between the two groups and within the same group, regarding the blood sugar parameters and bacteria counts they were normally distributed so, independent t-test and paired t-test were used to compare these parameters between

the two groups and within the same group. Chi-square was used to the gender distribution between the two groups. P-value ≤ 0.05 was considered significant.

RESULTS

The current study included 30 patients with T1DM; 14 females and 16 males with mean age 13 ± 2 years and mean diabetes duration 37 ± 27 days. Patients were divided into two groups; Group [A] patients [no. 15] were the control group who did not receive probiotic enriched yogurt. Group [B] patients [no. 15] received 200 mg of probiotic enriched yoghurt daily for 12 weeks and both groups were compared to each other at the onset of the study, and after 12 weeks. Our results will be demonstrated in the following tables and figures:

Table (1): Gender distribution among the studied group

		Subjects				Chi-squared test	P value
		Group A (N=15)		Group B (N=15)			
		N	%	N	%		
Gender	Male	6	40.0%	8	56.3%	0.819	0.336
	Female	9	60.0%	7	43.8%		

Table (2): Age, and duration of DM by days of the studied group at the onset of the study

	Subjects				p value
	Group A		Group B		
	Median	Range	Median	Range	
Age	14	10-18	12	10-17	0.299
Duration of diabetes in days	21	9 - 76	48	25-74	0.084

test of sig Mann Whitney "U test", sig p value<0.05

Table (1) and (2) are showing demographic data among the 2 groups of patients with T1DM. There was no statistically significant difference between the 2 groups as regards sex, age and duration of diabetes.

Table (3): BMI Z score and Lipid profile of the studied groups at the onset and after 12 weeks of the study

	Subjects				P_a
	Group A		Group B		
	Median	Range	Median	Range	
BMI_Z score [before]	-1.0	-2 - 0	-0.8	-2 - 0	0.831
BMI_Z score [after]	0.000	-1 – 0	0.0	-1.5 – 0	0.682
P_b	0.016*		0.004*		
Cholesterol mg/dl [before]	69	69 - 209	141	72 - 210	0.654
Cholesterol mg/dl [after]	87	71 - 159	121	73 - 207	0.232
P_b	0.001*		0.015*		
Triglycrides mg/dl [before]	99	52 - 250	83	32 - 189	0.264
Triglycrides mg/dl [after]	101	49 - 179	74	41 - 168	0.318
P_b	0.061*		0.351		
LDL [before]	54	23 - 100	53	41 – 98	0.741
LDL [after]	58	25 - 95	54	40 - 90	0.626
P_b	0.957		0.202		
HDL [before]	53	42- 64	48	34 - 58	0.264
HDL [after]	51	42 - 56	48	40 - 55	0.202
P_b	0.283		0.752		

"Pa = P value between two groups at baseline and post-intervention (Man Whitney for independent samples); Pb = P value within groups from baseline to 12 weeks (Wilcoxon signed-rank test)" * sig p value <0.05.

A comparative statistical analysis was conducted for each group separately for each studied covariant; both groups had statistically significant differences at the onset of the study and after 12 weeks.

Table (3) showing statistically significant differences at the onset of the study and after 12 weeks as regard the BMI Z score being higher after 12 weeks in both groups, lipid profile; cholesterol being lower after 12 weeks in both groups. However, there was no significant difference between group [A] and [B] as regard these studied parameters.

Table (4): HA1c % and fasting c- peptide of the studied groups at the onset and after 12 weeks of the study

	Subjects				
	Group A		Group B		
	Mean	±SD	Mean	±SD	P_a
HbA1c % [before]	11.00	1.85	11.38	1.57	0.549
HbA1c % [after]	8.75	1.31	8.65	1.70	0.681
P_b	<0.001*		<0.001*		
Fasting C peptide ng/ml [before]	0.20	0.16	0.22	0.15	0.547
af_Fasting C peptide ng/ml [after]	0.24	0.15	0.20	0.11	0.331
P_b	0.23		0.73		

"Pa = P value between two groups at baseline and post-intervention (independent samples t-test); Pb = P value within groups from baseline to post-intervention (paired t- test)" * sig p value <0.05.

Table (4) showing HBA1c level decreased after 12 weeks in both groups. No significant difference was found at the onset and after the intervention as regards fasting c-peptide. However, there was no significant difference between group [A] and [B] as regard these studied parameters.

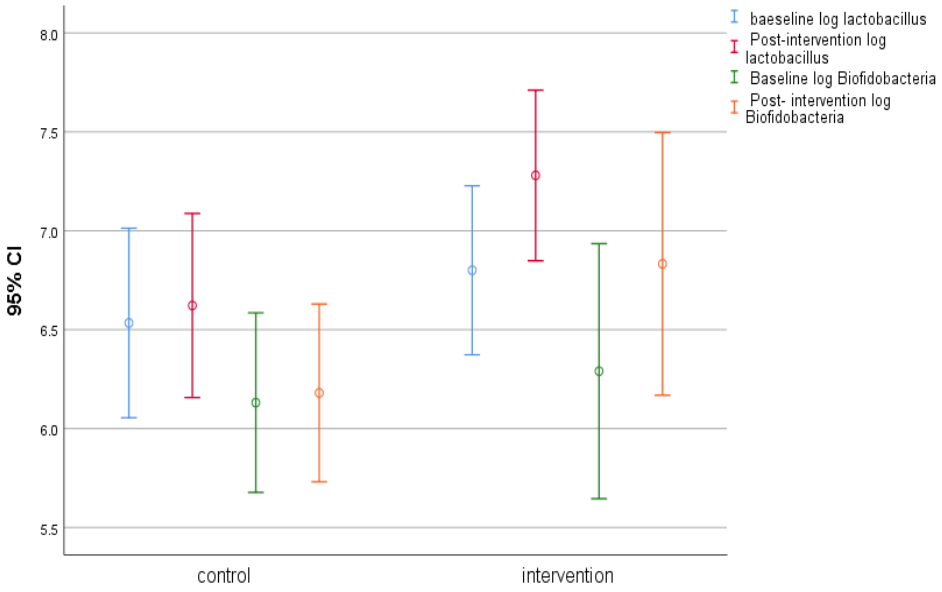

Figure (1): Comparison between control group and intervention group in patients with newly diagnosed diabetes as regards Log count of Lactobacillus and log count of Bifidobacteria

As shown in **Figure (1)**; Log count of Lactobacillus and log count of Bifidobacteria was evaluated for both groups separately at baseline and after 12 weeks; both groups showed a statistically significant difference in the log counts of bacteria at baseline and after 12 weeks; ($p = 0.03$ for log count of Lactobacillus in Group A, $p<0.001$ for log count of Lactobacillus in Group B), ($p<0.001$ for log count of Bifidobacteria in Group A, $p<0.001$ for log count of Bifidobacteria in Group B). Comparing both groups as regard the studied bacteria showed that the log count of Lactobacillus was significantly higher in the intervention group who received probiotic enriched yoghurt (Group [B]) than the control group (Group [A]) ($p= 0.034$). However, there was no significant difference between both groups as regards log count of Bifidobacteria.

DISCUSSION

Gut microbiota in human intestinal tract comprises a total genome that is near 150 times more than the human genome. **(Lepage P et al., 2013)** Recently, it has been proven that gut microbiota plays an important role in regulating metabolic functions and is associated with many diseases' aetiology such as obesity, insulin resistance, autoimmune diseases, and tumour **(El Samahy MH et al., 2018)**.

In our study, there was a highly significant difference at baseline and after 12 weeks as regard BMI Z score, HbA1c % and fasting lipid profile in both the control group and the intervention group. This is attributed to the natural course of the disease and the start of insulin therapy; all subjects were newly diagnosed young patients with T1DM at the start of the study, they suffered from diabetic ketoacidosis with different grades; they had weight loss due to hyperosmolar state; water loss and lack of insulin. Also, dyslipidemia is well documented at the start of diabetes diagnosis due to chronic hyperglycemia **(Soliman H and Ibrahim A, 2021)** 12 weeks after the diagnosis all our patients had already started insulin therapy with improvement of all the previously mentioned parameters in both groups. However, on comparing both groups [A] and [B] together there was no statistically significant difference between these studied parameters.

The log count of Lactobacillus was significantly higher in the intervention group [B] ($p<0.001$) than the control group [A] ($p= 0.034$). However, there was no significant difference between the two groups as regards log count of Bifidobacteria. These results are in line with **Laitinen et al.** who suggested the beneficial role of probiotic administration in newly diagnosed T1DM children. **(Laitinen k et al., 208) Kumar et al., 2021** reported that supplementation of probiotic regularly results in significant reduction of the lipid profile and HbA1C% in young patients with T1DM **(Kumar S et al., 2021)**.

Along with **Groele et al.** who reported that it remains unclear which probiotics, alone or in combination, and at which doses, are potentially the most beneficial for young patients with T1DM. **(Groele L et al., 2017**, **Gomes AC et al., 2014)**. So, we recommend further studies to explore the effects of various gut microbiota modifications on islet cell autoimmunity and to include other probiotics, as their effects are largely strain specific with

investigating different doses effect on patients with T1DM.

CONCLUSION

By giving adolescents with newly diagnosed T1DM probiotic supplement for 3 months duration, a significant higher values of Lactobacillus log count was documented. According to our study, the administration of specific strains of probiotic could be of beneficial role upon its clinical implementation. However, it remains unclear which probiotics, alone or in combination, are potentially useful for management of T1DM patients, further studies with larger number of patients, studying multi-strains probiotic effect and with longer follow up period is highly recommended to emphasize our study results.

LIMITATIONS OF STUDY

Limitations of our study must be addressed, firstly; short duration of probiotic yogurt supplementation for 3 months duration was one of the study limitations, but it was a matter of difficulty to give probiotic regularly for longer periods under the study purposes to adolescents with newly diagnosed T1DM. Secondary; only two types of probiotic bacteria were evaluated. Howver, both Lactobacillus spp. and Bifidobacteria spp were proofed internationally to be the most essential and beneficial to be studied.

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