The Effect of Short-Term Probiotic Administration in Newly Diagnosed Children with Type 1 Diabetes Mellitus on Gut Microbiota

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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 main objective of the study is to assess the alterations of gut microbiota among young patients with newly diagnosed T1DM, and to identify 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 the two samples' log counts of the examined bacteria revealed that sample [B] had a substantially greater log count of Lactobacillus than sample [A] ($p=0.034$). The log count of Bifidobacteria did not vary significantly between the two groups, though. Both groups had significant improvement in glycemic control, lipid profile and BMI Z score. However, No significant variation was detected 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

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Introduction

Globally, the prevalence of type 1 diabetes mellitus (T1DM) has increased remarkably. (Hans H et al., 2018) Therefore, even if genetics in the community were remarkably constant, genetic vulnerability alone would not be enough to describe why the frequency of T1DM rises by 3% to 5% annually. (Zheng P et al 2018) The significance of environmental variables, particularly the gut microbiome, in T1DM has recently come to light. T1DM occurs from an autoimmune response that causes the pancreatic beta cells that secrete insulin to be destroyed. T1DM is caused by a number of variables, including genetic predisposition, viral infections, and more lately, nutrition and intestinal microbiome. (Pociot F and Lernmark A, 2016; Rewers M and Ludvigsson J, 2016). Targeting the gut microbiota might well have therapeutic effects for T1DM patients as evidence from numerous research indicates that dysbiosis, or abnormal gut bacterial flora, is strongly related to the pathophysiology of the disease. Dietary modifications in the last few years are a significant factor in the growth of diabetes. The progression of diabetes and other auto-inflammatory illnesses has been linked to a higher intake of refined carbohydrates and poor intake of dietary fibre. Microbes in the colon selectively ferment soluble and insoluble fibres to produce SCFAs such acetate (C2), propionates (C3), and butyrates (C4). Mice without T1DM benefit from the anti-inflammatory and gut homeostasis activities of SCFAs generated by the gut microbiota. SCFAs can control myeloid and lymphoid cell development to facilitate the formation of lymphocytes that not only support immunity but also guard against inflammatory disorders. Intestinal epithelial cells' G-protein-coupled receptors 43 (GPR43), which serve as the receptors for SCFAs, could be activated by SCFAs to improve gut barrier performance and ward against inflammatory disorders brought on by invasive microorganisms. (Chang H, 2017) These processes most likely lead to the reduction of T1DM and autoimmune cells. Decreased SCFA synthesis suggests a loss of good bacteria, which is frequently linked to chronic inflammatory and autoimmune illnesses, particularly T1DM and type 2 diabetes. (Bell KJ et al, 2022) In T1DM patients, there was a decrease in the number of bacteria that produce lactate and SCFAs. At the beginning of T1DM, there were fewer Lactobacillus and Bifidobacterium bacteria present. (Zheng P et al., 2018)

Sample size

Using the EPI INFO sample size calculator, the sample size was calculated using a 0.05 alpha error, a 0.95 confidence interval, and a 0.80 power. The minimum sample size needed to assess whether altering gut microbiota could assist elucidate some of the genesis of the disease and whether this treatment may aid in the treatment of diabetic individuals is 28 cases of recently diagnosed T1DM patients.

Ethical consideration

- The Research Ethics Committee of Ain Shams University Hospitals, Faculty of Medicine, examined and authorized this analysis in accordance with the Declaration of Helsinki (as amended in Brazil, 2013).

- 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 writers have no apparent conflicts of interest. All co-authors have examined and authorized the manuscript's content, and none of the authors have any conflicting financial interests to mention. We confirm that the submission is original and hasn't already been accepted by another publication.

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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. comprehensive medical history gathering with a focus on demographic information, family history of diabetes, and the duration of the disease (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 includes height in centimetres and bodyweight in kilogrammes (Kg) (cm). Based on WHO growth reference for bodyweight, height, and BMI Z scoring, body mass index was computed as Kg/m2 and displayed on the age and sex normal percentiles. 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. A sterile venipuncture was used to extract 10mL of fasting venous blood. Two millilitres of this blood were deposited in an EDTA tubes for later HbA1c testing, and the remaining blood was emptied into two conventional test tubes. Centrifugation was used to extract the serum (1000x g for 15 minutes). Whereas the serum obtained in the other container was separated into three aliquots and kept at -20oC for later assays of fasting C-peptide, thyroids profile, and celiac screening, the serum from the first tube was promptly analysed for lipid profile. Specimen that were hemolyzed were discarded. We prevented repeatedly freezing and thawing.

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].

processing of the probiotic-enriched yoghurt

A proprietary blend of the probiotic strains L. acidophilus CUL60, Lactobacillus acidophilus CUL21, Lactobacillus acidophilus NCFM, Bifidobacterium lactis HNO19, Bifidobacterium animalis supsp lactis CUL34, and Bifidobacterium bifidum CUL20 was included in the product utilized in the research (one fermented milk bowl comprised The seller was GNC Ultra Probiotic Complex (UK).

Pasteurized buffalo milk was combined in a 2/100 ratio with activating probiotic bacteria combination to create probiotic yoghurt. This process was carried out at 37 °C. The inoculated milk was then split into 200 mL portions and placed in plastic containers with plastic covers, where it was cultured for 6 hours at 37 °C to create the curd. The yoghurt was then placed in the refrigerator and kept there until consumption at 4 °C. By combining 1 mL of the fermentation process with 9 mL of saline (0.1 g/L), serial dilutions were performed in order to quantify the Bifidobacterium sp. contained in the product. After 48 hours at 37 °C, Bifidobacterium spp. were enumerated on LP-MRS agar.

Bacterial quantification by real-time PCR

Quantitative Real Time Polymerase Chains Reaction (qRT-PCR) was used to determine the number of Bifidobacterium and Lactobacillus strains in the stool specimens that were obtained before and after therapy. The procedure was carried out as follows:

Isolation of DNA Essentially, a Stomacher 400 (Seward Ltd., London, United Kingdom) was used to homogenise 1 g of every stool specimen with 9 mL of phosphates buffer saline at high speed for 2 min. The manufacturer's protocols were followed to obtain 200 µL of the homogenate's DNA using the QIAamp DNA Stool Minikit (Qiagen, Germany). Specimens were employed for identification and quantification by RT-PCR after being stored chilled at -18 °C.

Quantitative real-time PCR: 200 µl of the homogenate was utilized for DNA isolation with the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. 1 g of faecal material was homogenised with 9 ml of phosphates-buffered saline buffers in a Stomacher 400 (Seward Ltd., London, United Kingdom) at full speed for 2 min. 200 µl of the microorganism's cultures (A600 1) were used to perform real-time PCR tests on the various strains.

Before testing, the DNA isolates were frozen at 20°C. 1 PCR buffers II, 3.5 mM $MgCl_2$, 0.2 mM per primer, 200 mM every deoxynucleosides triphosphates, 0.024 µM europium-labeled Bifidoprobe, 0.166 mM quencher probes, and 1.25 U of AmpliTaq Gold DNA polymerase were used in 50 µl

amplifying processes to evaluate the samples (1 µl). On MicroAmp optical plates with MicroAmp optical caps in place (Applied Biosystems), all processes were carried out.

An introductory cycling of 95°C for 10 min was followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, 61°C for 45 s, and 35°C for 15 s. Thermal cycling (iCycler) was used to test the effectiveness of the device. At the conclusion of every cycle, real-time observations of the fluorescence of europium were made using a Wallac Victor 1420 multilabel counting (Perkin Elmer, Turku, Finland) the threshold cycles (Ct) of actively responding at 35 °C. The PCR cycles at which the europium signals-to-noise ratio reaches a threshold limit of 1.5 is known as the Ct. By contrasting the Ct results to the standard curve, the amount of bifidobacteria cells in the faecal samples was calculated.

Within every PCR, duplicate DNA extraction from the various samples were examined throughout the course of two separate PCR cycles. The majority of target molecules employed are 16S rDNA genes, However, when more bacterial sequencing become accessible, new targeted promoters and probes for other genes will soon be made accessible and can be utilised in situations where the 16S ribosomal DNA is not a suitable target. It is important to remember that distinctions in the amount of rRNA operons between the quantitative organisms or groups, sequential heterogeneity between various operons within the same organisms, and discrepancy amplification of various DNA molecules can all have an impact on bacterial quantification by real-time PCR.

The temperature settings for the qPCR processes were 95°C for 15 minutes (1X), 95°C for 10 sec (40X), 57°C for 30 sec (1X), 72°C for 30 sec (1X), and 95°C for 10 sec (1X). Every animal's and priming set's Ct scores were averaged. The 2-ΔΔCT technique was employed to calculate comparative values. All qPCR experiments used Roche's FastStart DNA Masters SYBR Green (Indy).

All universal primers combinations and specific TaqMan® probes developed during this inquiry were tested using DNA species (20 ng/l) of the genera Lactobacillus, Bifidobacterium, Lactococcus, and Streptococcus as either positively or negatively controls. Real-time PCR was performed using a Light Cycler® 480 (Roche, Mannheim, Germany) with TaqMan® monitoring. 5 µl of DNA template, 10 µl Taq polymerases, 1 µl of TaqMan® marked specific primers (10 mol, each 6-FAMTM/Dabcyls-labeled, TIB MOLBIOL, Berlin, Germany), 1 µl of global primer sets (10 mol, Thermo Scientific, Dreieich, Germany), and 2 µl of PCR grading water were all included in each individual plex real-time (Roche, Mannheim, Germany).

40 cycles of 95° C for 1 minute (denaturation), 53° C for 30 s (annealing), and 73° C for 30 s were performed after a single initial denaturation phase lasting 10 minutes at 95° C. (elongation). At the conclusion of each phase of 73° C elongation, the fluorescence signal was evaluated. Every real-time PCR contained two technical replicates, and Roche® Light Cycler 480® software was used to analyse the outcomes.

The specimens were not pasteurised and were kept chilled at -18 oC. For 30 minutes, the specimens were centrifuged at 3000 g. The pellets were then used to extract total DNA. Purified DNA isolates were kept at 20 oC after being processed DNA was eluted in 100 µL. The bacterial DNA in the specimens of breast milk was identified using Real Time PCR. A number of genus-specific primers pairs

were utilised for this. According to Matsouki et al. (2002) and Heilig et al. (2002), real-time PCR was carried out for Bifidobacterium and Lactobacillus, correspondingly.

Calculation of bacterial colony counts by real time PCR

The average absorbance of each control was plotted against its dosing on a sheet of linear graph paper, with the absorbance value on the vertical (Y) axis and the density on the horizontal (X) axis. This allowed for the calculation of the findings. Furthermore, the relevant dosage from the standard curve was calculated using the average absorption coefficient for each specimen.

Statistical analysis

Using a computer, the dataset has been changed, processed, and recorded. The SPSS programme was used for the data processing (version 25). Descriptive statistics were presented as frequencies and percentages of categorical variables. Using the Shapiro-Wilk and Kolmogorov-Smirnov tests, as well as normality plots like the histogram and stem and leaf diagram, it was determined whether continuous data were normally distributed. Since continuous data were not normally distributed, the medians and ranges were utilised instead of the mean and standard deviation. The Mann Whitney analysis, often known as the "U test," and the Wilcoxon signed rank assessment were used to assess the variables between the two groups and within a single category because age, the period of DM, and all lab tests were not normally distributed. In order to contrast the blood sugar levels and bacterial counting between the two groups and within a single group, independent t-test and paired t-test were utilised because they were normally distributed variables. To compare the sex ratio of the two groups, chi-square was utilised. P-values lower than or equal to 0.05 were deemed 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%		
	Female	9	60.0%	7	43.8%	0.819	0.336

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 statistical variation 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		

"P_a = P value between two groups at baseline and post-intervention (independent samples t-test); P_b = 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 variation 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.

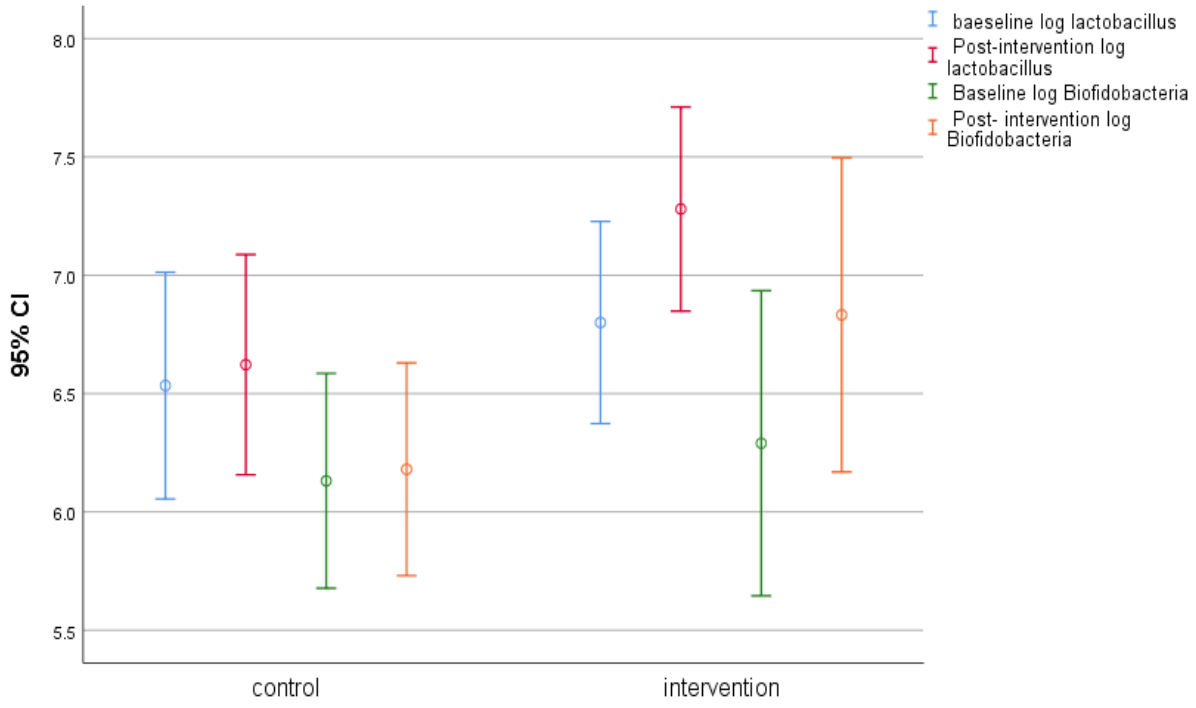

Figure (1): Comparison between control group and intervention group in patients with newly diagnosed diabetes as regards Log count of Lactobacillus and log count of Bifidobacteria

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

The human digestive tract's gut microbiota has a genome that is almost 150 times larger than the human genome. (2013) (Lepage P et al.) Lately, it has been established that the gut microbiota

regulates metabolic processes and is linked to the aetiology of many disorders, including obesity, insulin resistance, autoimmune disorders, and tumours. (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 stated that it's yet unknown which probiotics, individually or in combination, and at what dosages, may be the most helpful for younger T1DM patients. (Groele L et al., 2017, Gomes AC et al., 2014). Therefore, as probiotics' activities are usually strain-specific, we advise future research into how diverse gut microbiota alterations affect islet cell autoimmunity as well as other probiotics' impacts on T1DM patients.

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, Whether probiotics, individually or in combination, may be helpful for treating T1DM sufferers is still unknown, 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 the 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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