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Exploitation of Absolute qPCR to Estimate *Lactobacillus* and *Bifidobacterium* Count in Human Gut as Indicator of Diabetic Mellitus Complication

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Abstract

Diabetes mellitus is a form of metabolic disorder where patients are incapable to organize glucose metabolism. The most common types are Type I and Type II, constituting about 10% and 90% of cases, respectively. The cause of type I diabetes, which usually spreads in children and adolescents, is the disability of the endocrine system to produce insulin. On the other hand, The most common type of diabetes, type II diabetes, is often presented in adults. It is usually presented as a collection of insulin deficiency and insulin resistance. This work was done to estimate the count of microbiota in diabetics to find an approach for detection and follow-up treatment. The count of two types of bacteria *Lactobacillus* and *Bifidobacterium* was determined using qPCR based on the standard curve that was created from the serial decimal dilution of samples containing an unknown number of bacteria taken from probiotic capsules. The main results of this study show that the *Lactobacillus* count was affected by diabetes types, where a decrease was observed in the mean value in the case of diabetes type I group (32978.13) compared with the control group (610680.26). The mean value in diabetes type II was close to that of the control group (682199.27). While, the count of the *Bifidobacterium* showed a significant reduction in the mean value in both type I and type II diabetes groups (7521.70, 51880.82, respectively), compared with the control group (63405999.00).

Keywords: Diabetes mellitus, microbiota, *Lactobacillus*, *Bifidobacterium*, absolute qPCR.

استثمار تقنية تفاعل البلمرة المتسلسل الكمي المطلق لتقدير أعداد بكتريا *Lactobacillus* و *Bifidobacterium* في امعاء الانسان كمؤشر للتدخلات المرضية لداء السكري

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الخلاصة

داء السكري هو شكل من أشكال الاضطراب الابيضحي حيث يكون المرضى غير قادرين على تنظيم عملية أيض الجلوكوز. أكثر انواع مرض السكري شيوعاً هما النوع الأول والنوع الثاني، حيث يظهران حوالي 10% و 90% من الحالات، على التوالي. ان سبب مرض السكري من النوع الأول، الذي ينتشر عادة في الأطفال والمراهقين، هو عدم قدرة نظام الغدد الصماء لإنتاج الأنسولين. اما النوع الثاني من مرض السكري،

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غالبًا ما يكون عند البالغين وعادة يحدث بسبب نقص الأنسولين و كذلك مقاومة الأنسولين. ان هدف هذا البحث هو لتقدير عدد الميكروبات في مرضى السكر والتي يمكن استخدامها كمؤشر للمرض وكذلك متابعة العلاج. تم تحديد عدد بكتيريا *Lactobacillus* و *Bifidobacterium* باستخدام qPCR بناءً على المنحنى القياسي الذي تم إنشاؤه من سلسلة التخفيف العشرية للعينات التي تحتوي على عدد معلوم من البكتيريا المأخوذة من كبسولات المكملات الغذائية (probiotic). أظهرت النتائج الرئيسية لهذه الدراسة أن عدد بكتيريا *Lactobacillus* تأثرت بمرض السكري ، حيث لوحظ انخفاض في قيمة المتوسط الحسابي في حالة مجموعة السكري من النوع الأول (32978.13) مع مجموعة التحكم (610680.26). أما بالنسبة للقيمة المتوسط الحسابي لمرض السكري من النوع الثاني كان قريبًا من متوسط قيم المجموعة الضابطة (682199.27). بينما أظهر عدد بكتيريا *Bifidobacterium* انخفاضًا كبيرًا في قيمة المتوسط الحسابي في كلتا الحالتين من داء السكري ، النوع الأول والنوع الثاني (7521.70 ، 51880.82) على التوالي مع المجموعة الضابطة (63405999.00).

Introduction

Diabetes mellitus is a form of metabolic disorder where patients are incapable to organize glucose metabolism. The most common types are Type I and Type II, constituting about 10% and 90% of cases, respectively [1]. The cause of type I diabetes, which usually spreads in children and adolescents, is the disability of the endocrine system to produce insulin due to immune-mediated destruction of β cells. On the other hand, the most common type, type II diabetes, is often presented in adults. It is usually presented as a collection of insulin deficiency and insulin resistance [2]. "Microbiota" is a term used to characterize microorganisms which normally inhabit the human skin, gut, vagina, upper respiratory tract, and the throat. Their wide collection of genes is called "microbiome". Around 100 trillion microorganisms inhabit the human intestine, that represents 10 times the number of eukaryotic cells in the human body [3]. Gut bacteria and the host live in a commensal manner. Gut bacteria play a remarkable role in human health, such as aiding in the digestion of cellulose, synthesizing vitamin K, promoting angiogenesis and enteric nerve function, and supplying essential nutrients [2]. However, they can also be harmful because of their composition change when the intestinal ecosystem is exposed to abnormal changes such as in cases of the use of antibiotics, stress, illness, bad dietary habits, aging, and lifestyle. Dysbiosis in the gut bacteria communities is able to cause numerous chronic diseases, such as obesity, cancer, hypertension, diabetes, inflammatory bowel disease, and autism. Further, lack of balance in the composition of gut bacteria was linked with intestinal symptoms, such as abdominal pain, diarrhea, and bloating [4]. Lactic acid bacteria (LAB) include numerous genera within the order Lactobacilliales, one of which is *Enterococcus*, that are acid tolerant. *Streptococcus* and *Lactobacillus* species are within the most well characterized. *Lactobacillus* is a genus of anaerobic or small-scale aerophilic, gram-positive, catalase-negative, nonsporulating organisms that are found in various habitats [5]. Generally, they do not synthesize porphyrinoids and have no heme-dependent action. They have outgrowth temperature that ranges from 2 to 53 °C and they can develop in a pH in the range of 3 and 8. Typical growth temperature and pH in general are 30–40 °C and 5.5–6.2, respectively [6]. The major metabolic final result of lactobacilli is lactic acid during glucose fermentation. Lactic and succinic acids are produced too, however, just in small amounts [7]. It has been proposed that lactobacilli can also be useful for controlling autoimmune diseases such as inflammatory bowel disease (IBD), celiac disease, and type 1 diabetes [8,9]. *Bifidobacterium* is a pleomorphic rod, Gram-positive, non-spore forming, anaerobic, and used to be firstly named *Bacillus Bifidus communis* [10,11]. It has been shown that Bifidobacteria had different effects that promote health, that include the abstraction of procarcinogens, immunomodulation, banning of diarrhea and intestinal infections, and the synthesis of vitamins. It also contributes to the production of the antimicrobial agents against severe intestinal bacteria as well as the integrity of the epithelium through the prevention of the invasion of the pathogenic bacteria [12].

The goal of this study is the assessment of the effects of diabetes on the normal flora count, especially *Lactobacillus* and *Bifidobacterium*.

Materials and Methods

Collection of Samples:- In this experimental study, 50 samples of the stool were collected and distributed 25 samples from Diabetes patients and 25 samples from apparently healthy people. The stool was collected from persons aged between 9- 67 years during the period from July 2017 to May 2018. They were diagnosed at the Ramadi teaching Hospital for Diabetes.

Extracting of DNA:-Total stool DNA was extracted from stool samples of patients and control by AccuPrep Stool DNA Extraction Kit from Bioneer (cat no. K-3036) as described by the instruction manual. The extracted samples were checked for purity and concentration by nanodrop Dihan (Korea).

Primers:-The primers for the detection of normal flora *Lactobacillus* and *Bifidobacterium* (Table-1) were designed according to the sequence of specific genes obtained from NCBI (<https://www.ncbi.nlm.nih.gov/>) using primer3plus program available online (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/>).

Table 1-The primers used in this study.

Bacteria	Primer	Sequence 5—3	Annealing Temperature	Reference
<i>Lactobacillus</i>	Lac F	TGGAAACAGGTGCTAATACCG	58	This study
	Lac R	CCATTGTGGAAGATTCCC		
<i>Bifidobacterium</i>	Bif F	CCACCGTTACACCGGGAA	62	This study
	Bif R	GGGTGGTAATGCCGGATG		

Construction of Standard Curves for Bacteria Copy Number Determination

The standard curve method is generally established on the threshold cycle Ct values of each an input set of known DNA concentrations or a dilution series of a reference DNA sample. The standard curves was designed for *Lactobacillus* and *Bifidobacterium* bacteria through DNA extracted from probiotics capsules from Protexen Pharmaceuticals, where each capsule contained 200 million bacterial cells. A 10-fold serial dilution series of the extracted DNA, ranging from 1×10^5 to 1×10^9 , was used to construct the standard curves for both *Lactobacillus* and *Bifidobacterium*. CT values in all dilution were measured by using a real-time qPCR with the *Lactobacillus* and *Bifidobacterium* sets to generate the standard curves for these bacteria. The logarithm of their initial template copy numbers was plotted against the CT values. Each standard curve was created through linear regression of the plotted points.

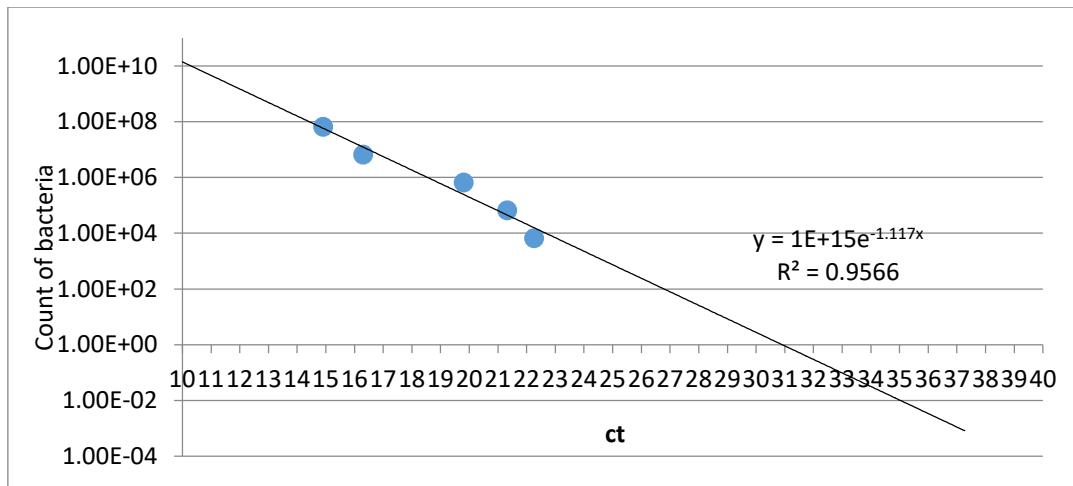
Estimation of Bacterial Numbers:- The bacterial numbers for both *Lactobacillus* and *Bifidobacterium* in patients and control stool samples were determined by qPCR depending on the standard curve and employing a ready to use sybr green qPCR kit in 20 ul reaction sample. Predenaturation was performed at 95°C for 5 min, then denaturation was achieved at 95°C for 20 sec, followed by annealing\extension at 55-60°C for 40-45 sec.

Statistical Analysis:- Data of the current study were analyzed by using SPSS v.22 program. Nominal data were described by number and percentage and compared by using (X^2). Numeric data were described by (Mean \pm SD). T-test was used to compare between two numeric variables, while the F test (ANOVA) was used to compare three numeric variables or more. A level of significance of $\alpha=0.05$ was applied to the tests.

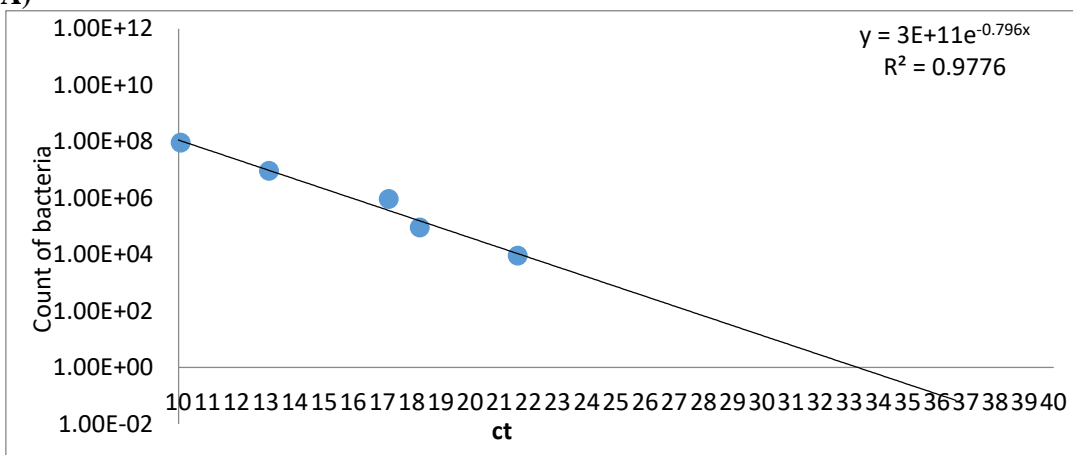
Results

A real-time PCR experiment was performed and the standard curve is shown Figures-(1a and b). The CT values for *Lactobacillus* were, sequentially from the highest concentration to the lowest concentration, as follows: 10.09, 13.11, 17.22, 18.28, and 21.64. In addition, the CT values for *Bifidobacterium* were, sequentially from the highest concentration to the lowest concentration, as follows: 14.92, 16.31, 19.82, 21.33, and 22.27.

All the results and the numbers mentioned in the standard curve experiment were calculated on the basis that the sample taken in DNA extraction is 200 mg of the stool as well as of probiotics.



(A)



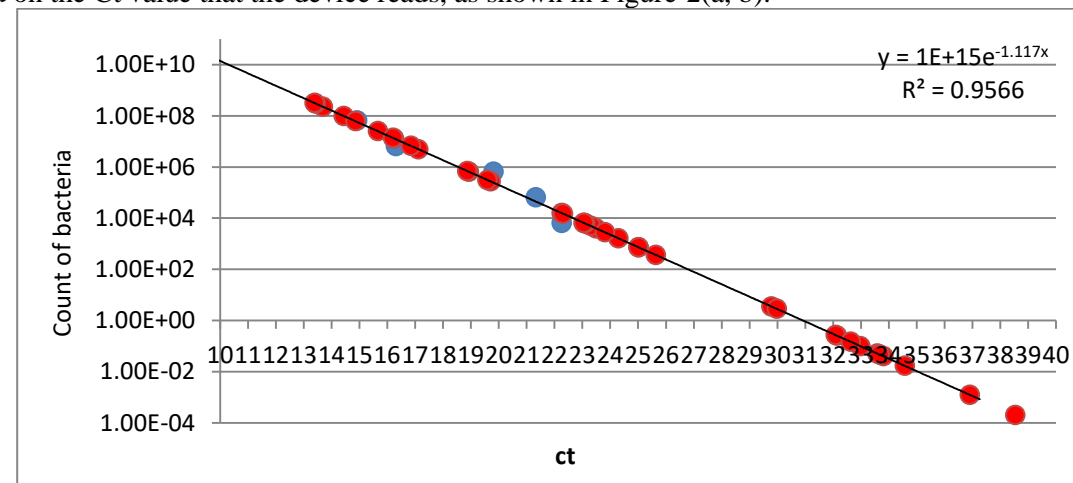
(B)

* The blue dots represent samples of a series of dilutions from the probiotics.

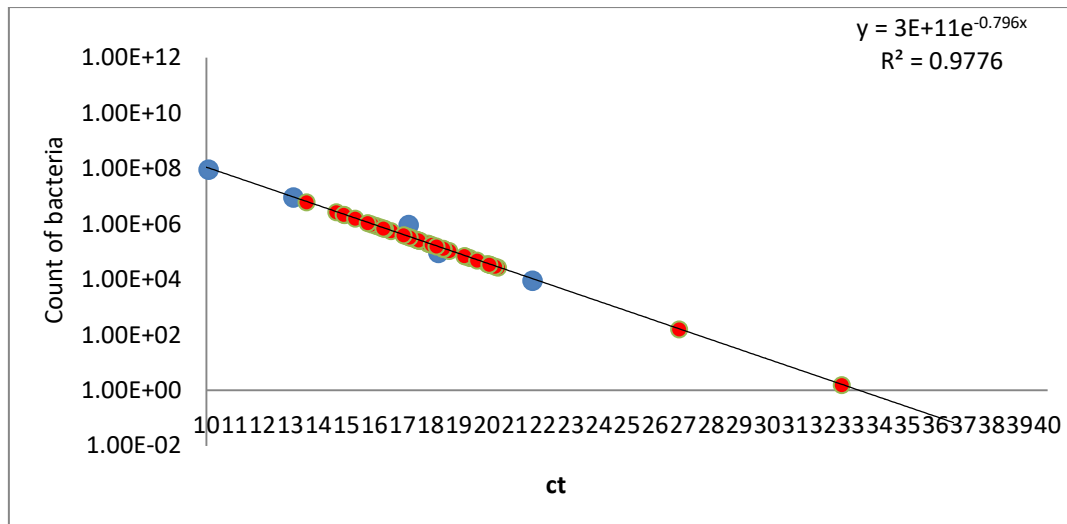
Figure 1-Construction of the real-time PCR standard curves for Lactobacillus. For each set, determined CT values were plotted against the logarithm of their known initial copy number (n). (A) Bifidobacterium (B) Lactobacillus.

Estimation of the Numbers of Bacteria Through the Standard Curve.

The real-time PCR device calculates the number of bacteria automatically based on the standard curve generated by the standard samples, whose numbers are already known, where the process is based on the Ct value that the device reads, as shown in Figure-2(a, b).



(A)



(B)

* The blue dots represent samples of a series of dilutions from the probiotics

*Red dots represent study samples

Figure 2-Estimation of cell numbers through the standard curve. Blue dots represent standard samples, red dots represent samples of the trial under study (a) for *Bifidobacterium* (b) for *Lactobacillus*.

The results of this study showed that there was a difference in the mean values of the number of *Lactobacillus* bacteria between the two groups (normal and diabetic) of samples. The mean values of *Lactobacillus* from normal and diabetic specimens were 610680.26 and 623179.17, respectively. Likewise, it is shown that there was a difference in the mean value of the number of *Bifidobacterium* bacteria between the two groups of samples. The mean values of *Bifidobacterium* from normal and diabetic specimens were 63405999.00 and 47848.17, respectively, as shown in Table-2.

Table 2-The number of bacteria in the sample groups (normal and diabetes patients).

Types	N	Concentrations of bacteria			
		<i>Bifidobacterium spp.</i>		<i>Lactobacillus spp.</i>	
		Mean	SD	Mean	SD
Normal	17	63405999.00 ^a A	110063588.90	610680.26 ^a B	718968.86
Diabetic	22	47848.17 ^c B	158531.98	623179.17 ^a A	1327333.68

*Small letters compare vertically between groups.

*Capital letters compare horizontal between two types of bacteria for each disease.

The current study showed that the number of *Lactobacillus* bacteria was affected by types of diabetes, as shown in Table-3.

Table 3-Concentration of *Lactobacillus* in diabetes type I and diabetes type II.

Diabetic types	<i>Lactobacillus spp.</i>		
	N	Mean	SD
Type I	2	32978.13 ^b	371.23
Type II	20	682199.27 ^a	1380918.21
Total	22	623179.17	1327333.68

*Small letters compare vertically between groups.

The results showed a difference in the mean value of the *Lactobacillus* bacteria number between the two groups of diabetes (type I, type II). The mean values were 32978.13 and 682199.27, respectively (Table-3). Comparing the mean values of *Lactobacillus* in diabetes types (Table-3) and the control group (Table-2), there was an observed decrease in the mean value in the case of a diabetes type I group. The mean value of diabetes type II was close to that of the control group.

The current study showed that the number of *Bifidobacterium* bacteria was affected by types of diabetes, as shown in Table-4.

Table 4-The concentration of Bifidobacterium bacteria in diabetes type I and diabetes type II

Diabetic types	<i>Bifidobacterium spp.</i>		
	N	Mean	SD
Type I	2	7521.70 ^b	10633.29
Type II	20	51880.82 ^a	166083.27
Total	22	47848.17	158531.98

*Small letters compare vertically between groups.

A difference in the mean value of the *Bifidobacterium* bacteria number was shown between the two groups of diabetes, type I and type II (7521.70 and 51880.82, respectively) (Table-4). There were significant differences in the numbers of *Bifidobacterium* bacteria in type I diabetes and those in type II diabetes, as shown in Table-4. When comparing the mean values of *Bifidobacterium* in diabetes types (Table-4) with the control group (Table-2), a significant reduction was observed in the mean value in both cases of diabetes, type I and type II groups. The results of this study demonstrated that the number of *Bifidobacterium* and *Lactobacillus* bacteria was affected by diabetes, with the counts being related to the diabetic type, as shown in the Table-5.

Table 5-The concentration of Bifidobacterium and Lactobacillus bacteria in the diabetic type (diabetic type I and diabetic type II)

Diabetic types	Concentrations of bacteria				
	<i>Bifidobacterium spp</i>			<i>Lactobacillus spp</i>	
	N	Mean	SD	Mean	SD
Type I	2	7521.70 ^b B	10633.29	32978.13 ^b A	371.23
Type II	20	51880.82 ^a B	166083.27	682199.27 ^a A	1380918.21
Total	22	47848.17	158531.98	623179.17	1327333.68

* Small letters compare vertically between groups.

* Capital letters compare horizontal between two types of bacteria for each disease.

The results of this study revealed that the mean value of *Lactobacillus* bacteria was much higher than that of *Bifidobacterium* bacteria in diabetic type I. The mean values for *Bifidobacterium* bacteria and *Lactobacillus* bacteria were 7521.70 and 32978.13, respectively (Table-5). There were significant differences in the numbers of *Bifidobacterium* and *Lactobacillus* bacteria in diabetic type I.

Moreover, in the case of diabetic type II, the mean value of *Lactobacillus* bacteria was much higher than that of *Bifidobacterium* bacteria. The mean values for *Bifidobacterium* bacteria and *Lactobacillus* bacteria were 51880.82 and 682199.27, respectively (Table-5). There were significant differences in the numbers of *Bifidobacterium* and *Lactobacillus* bacteria in diabetic type II.

Discussion

The standard curve is the basis for the absolute quantification application, which is prepared from samples with recognized concentrations. For an unknown sample, the concentration could then be determined by easy interpolation of its PCR sign (cycle quantification value Cq) through this standard curve [13]. This method is faster and less expensive compared with DNA hybridization and has no

safety-related problems. Furthermore, the method is simple to implement and can be applied to observe the copy number of a plasmid in the study of time-course or in a recombinant bioprocess [14]. The standard curve method is one of the most accurate ways to determine the number of bacterial cells in the sample. This corresponds to the method we used to determine the number of bacteria.

The richness and composition of intestinal microbiota within the host rely on the symbiotic relationship. They are modified by diet, age, host health, ethnicity and genetics and thus are highly variable and unique between persons [15]. Microorganisms develop in the gut with their host and adapt to the environment in which they live [16]. Because intestinal bacteria have major effects on human health and disease, there is an increasing trend to test the ability use them as a new goal to block and treat many chronic diseases and to ensure additional research to target them in different ways to combat resistance to diseases associated with intestinal bacteria [4].

Many studies have confirmed that compositional modifications in specific species and genera patterns of intestinal microorganism in human or animal may also cause many chronic diseases such as cancer, obesity, diabetes, and autism. It was suggested that the composition of gut microbiota in patients with type 2 diabetes is different from that of healthy individuals [17]. A study at the level of human metagenome showed significant association with bacterial genes, metabolic pathways, and specific gut microbes in T2D patients. These patients showed higher levels of *Lactobacillus spp.* than non-diabetic patients [18]. It was detected that the *Lactobacillus* levels were significantly rising in patients with diabetes than in the healthy group. This remark was pointing a significantly higher massiveness of the *Lactobacillus* group in stool samples of type 2 diabetics [17, 19]. Acarbose intake in T2D or hyperlipidemic patients was moreover shown to increase the levels of *Bifidobacterium* and *Lactobacillus* [20]. It was also revealed that probiotics such as *Bifidobacterium* and *Lactobacillus* are depleted in the diabetic rats' stools [16]. Individuals with diabetes have fewer *Faecalibacterium prausnitzii* and *Bifidobacterium*, both Gram + with anti-inflammatory properties [21].

Children with T1D showed greater counts of *Veillonella*, *Bacteroides*, and *Clostridium*, accompanied by decreased counts of *Lactobacillus* and *Bifidobacterium* than healthy children [21].

In Diabetes Type II, the current study results were consistent with the results of other researchers that are reported above. We observed that *Lactobacillus* levels were higher in diabetics. But the rise was so slight that it was not statistically significant. The results of *Bifidobacterium* bacteria were consistent with other previous results, where they showed lower counts but the decline was significant. As for diabetes type I, the current study is also in line with the previous studies, where we observed a clear reduction in the numbers of *Lactobacillus* and *Bifidobacterium*.

It has been observed that diabetes treatments can cause shifts in gut microbiome, as in metformin therapy. It also changes the microbiota composition, increasing the abundance of *Escherichia spp.*, *Lactobacillus*, and *A. muciniphila*. and reducing the profusion of some pathogens [22]. Whilst sitagliptin was able to block the reduction of *Bifidobacterium* and appeared to exacerbate *Lactobacillus* deficiency [16].

Conclusion

In this study, we observed that the numbers of *Lactobacillus spp.* did not change in T2D, while they were low in T1D. As for *Bifidobacterium spp.*, the numbers were reduced in the both cases of diabetes.

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Conflict of Interest The is no conflict of interest.

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