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ORIGINAL ARTICLE

The Diversity of Gut Microbiota among Type 1 and Type 2 Egyptian Diabetic Patients

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ABSTRACT

Background: *Gut microbiome could have central role in development of type 1 and type 2 diabetes mellitus (T1D) & (T2D).* ***Objectives:*** *The aim of the present study is to detect the composition of gut microbiota among T1D and T2D patients compared to healthy individuals.* ***Methodology****: The intestinal microbial composition of diabetic and heathy groups was investigated by both traditional culture techniques and polymerase chain reactions with amplification of the 16-23S rRNA intergenic spacer (IS) region.* ***Results****: By applying culture, diabetic groups showed a non-significant higher Firmicutes/ Bacteroidetes ratio (4.7% in T1D and 9% in T2D) compared to control group (4%) P value is 0.2058. While, PCR method showed that the diabetic group had a non-significant higher percentage of Lactobacillus spp., Faecalibacterium prausnitzii, Alistipes spp., Bacteroides thetaiotaomicron and Akkermansia muciniphila compared to healthy group (P value=0.407).* ***Conclusion:*** *Both diabetic groups showed a non-significant Firmicutes/Bacteroidetes ratio compared to control group by both culture and PCR techniques.*

INTRODUCTION

Diabetes mellitus (DM) is a chronic disease wherein patients have higher blood glucose level associated with low grade inflammation and cytokines production[1,2]. Type 1 diabetes mellitus is an autoimmune disease that elicited by genetic factors. On the other hand, type 2 diabetes is the most common form of diabetes represented with high proportion in developing countries and caused by deficiency in pancreatic insulin production or insulin action unresponsiveness or both[3,4]. DM is the ninth major cause of death globally [5]. World health organization states that diabetes is the second most common cause of death after cardiovascular disease in Egypt [4]. The prevalence of diabetes among adults in Egypt represents about 15.56%[6].

In recent years, many evidences revealed that environmental factors like diet, physical activities and gut microbiome are considered as essential modulator of DM [7]. The gut microbiome is usually considered as a functional and measurable organ [8]. The composition of gut microbiota varies along the gut, and differs from one to another according to nutritional status and dietary lifestyle.[9] Mammalian gut microbiota mainly involves four main phyla: Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria which are essential for the host metabolic activity and physiology upregulation [10,11]. Gut microbiome diversity is very essential for immune system development and consequently protection from several diseases [12]. Currently, little is known about the relationships between T1D and the gut microbiota. One study in Finland reported that children with T1D have lower percentage of Firmicutes in comparison to Bacteroidetes [13]. Intestinal permeability was significantly increased in the T1D patients, indicating poor intestinal barrier function and increased gut permeability to exogenous antigens with consequent production of autoantibodies leading to pancreatic β-cell damage[14]. Additionally positive correlations were reported between plasma glucose levels and the ratios of Bacteroidetes to Firmicutes in T2D patients [15,16]. Recently, it was registered that type 2 diabetes in humans was correlated to a low percentage of butyrate producing microbiota along with high abundance of *Lactobacillus* spp. [15,17]. Furthermore, Betaproteobacteria were more abundant in type 2 diabetes patients than the healthy controls. These observations may be attributed to the endotoxin induced inflammatory response of Gram-negative Bacteroidetes and Proteobacteria [18].

The aim of the current study was to compare different gut microbiota spp. that predominate in type 1 and type 2 diabetic patients and healthy individuals by applying both culture dependent and culture independent molecular techniques. Culture method was carried to assess the gut microbiota profile, with special consideration to *Lactobacilli*, *Clostridium*, *Bacteroides*, *Bifidobacterium* spp. and *E. coli*. Molecular techniques are based on PCR with amplification of the 16-23S rRNA IS with different specific primer pairs. T*o the best of our knowledge*; this is the first study to compare the gut microbiota of T1D and T2D among Egyptian diabetic patients.

METHODOLOGY

Ethical considerations

The study protocol was approved by the ethics committee of the Faculty of Pharmacy, Girls Al-Azhar University. All participants provided an informed consent before collection of stool samples.

Study design and patients criteria:

This is a Case-Control study. It was conducted on 99 adults; 80 diabetic patients (40 T1D and 40 T2D). The patients were randomly selected from Diabetes Outpatient Clinic of Al-Azhar University Hospitals and included different classes of diabetic groups (diabetic only, diabetic with hypertension and diabetic with fatty liver). Nineteen healthy volunteers group matched for age and gender were also included as control over a study period from January 2016 to February 2017 (table1). Patients taking antibiotic within previous three months, or suffering from inflammatory bowel disease, acute or chronic diarrhea, smokers, alcohol abusers, pregnant or lactating women and those with history of mental illness were excluded from the study. Clinical history was reported and patients were classified into two different groups according to blood glucose level and presence of other medical diseases (table 2).

Table 1: Characterization of study participants.

Patients	T1D	T2D	Healthy volunteers
Males	16	19	13
Female	24	21	6
Age (years)	20-70	20-70	25-65
Total	40	40	19

T1D: Type 1 diabetes mellitus T2D: type 2 diabetes mellitus

Table 2: Different classes of diabetic patients included in the study.

Patients	T1D. No (%)	T2D No (%)
Patients with controlled blood glucose level:		
1.Diabetic only	12 (75%)	13(81.25%)
2. Diabetic with hypertension	2 (12.5%)	2 (12.5%)
3. Diabetic with liver disease	2 (12.5%)	1(6.25%)
	16	**16**
Patients with uncontrolled blood glucose level:		
1.Diabetic only	14 58.33%)	16 (66.66)
2. Diabetic with hypertension	7 (29.16%)	6 (25%)
3. Diabetic with fatty liver disease	3 (12.5%)	2(83.33)
Total	**24** **40**	**24** **40**

T1D: Type 1 diabetes mellitus T2D: type 2 diabetes mellitus

Stool samples collection and processing

Fresh stool samples were collected in sterile screw caped cups and were processed as soon as possible at Research Laboratory of Microbiology & Immunology Department, Collage of Pharmacy, Al-Azhar University. Part of the stool was inoculated in cooked meat media (Oxoid® Limited, Basingstoke, UK) and mixed thoroughly for aerobic and anaerobic cultivation and the other part was placed on normal saline and stored at -70°C for molecular assay (PCR).

Culture techniques:

One drop of homogenized stool sample was transferred with micropipette to blood agar (Difico, Detroit, USA) plate for incubation under aerobic condition and Columbia blood agar (Oxoid® Limited, Basingstoke, UK) plate supplemented with vitamin K and hemin (Sigma Aldrich, USA) for incubation under anaerobic condition. Diverse media and biochemical tests were used for isolation and identification of bacterial spp. For Gram-negative aerobic bacteria; MacConkey agar, eosin methylene blue (EMB) agar, triple sugar iron agar (Lab M, UK), oxidase, indole production, citrate utilization, urease, methyl red, Voges Proskaure and phenyl alanine deaminase tests were used according to identification scheme of MacFaddin, [19]. For aerobic Gram-positive bacteria; blood agar, nutrient agar (Lab M, UK), catalase, coagulase, DNAase, and mannitol fermentation tests were used for isolation and identification of *Staphylococcus* spp. Streptococci and Enterococci were identified by blood haemolysis and bile esculin hydrolysis tests. *Bacillus* spp. were identified using starch hydrolysis, motility on semisolid agar and catalase test according identification scheme of Koneman [20].

For isolation of anaerobic bacteria, processing of primary anaerobic plates was performed as soon as possible and incubated in anaerobic jar (Oxoid® Limited, Basingstoke, UK) in presence of anaerogen Gas Pack and resazurine indicator strip (Oxoid® Limited, Basingstoke, Hampshire, England) immediately at 35-37 °C and examined after 3-7 days.

All colonies detected were confirmed to be pure and are ensured to be anaerobic by performing aerotolerance test according to Engelkirk, [21]. Pure anaerobic colonies were immediately Gram stained and cultured on special culture media selective for different anaerobic bacteria. For identification of anaerobic Gram-positive bacteria, neomycin blood agar, egg yolk agar, and thioglycolate gelatin medium were used. *Clostridium* spp. were identified using several biochemical tests as carbohydrate fermentation using protease peptone yeast extracts media, indole, urease**,** esculin hydrolysis and gelatin liquefaction tests according to scheme described by Summanen *et al.* and Morton, [22,23]**.** For isolation of *Lactobacillus* spp., De Man, Rogosa and Sharpe (MRS) agar (Mast group limited, Merseyside, UK) medium was used. On the other hand, for isolation and

identification of Gram-negative anaerobic bacilli as *Bacteroides* and *Fusobacterium* spp,; Bacteroides bile esculin agar and kanamycin-vancomycin lacked blood agar supplemented with vitamin K and hemin, bile esculin hydrolysis, catalase and indole tests were used according Sutter *et al*. and Mangels, [24,25].

DNA extraction

Total microbial DNA of each fecal sample (180 to 220 mg) were extracted using QIAamp® DNA stool mini kit (Qiagen, Hilden, Germany) following the manufacturer´s protocol instruction. DNA quality and concentrations were determined by Nano drop spectrophotometer (Implen, Germany) and agarose gel-electrophoresis (Apelex, France) and were stored at -20°C.

PCR primers and conditions

PCR was carried out using three universal primer pair (table 3) targeting 16S-23S IS region according to Remley *et al.* [26].

Table 3: Primers used in the study

Primers For PCR targeting 16S-23S IS region	
Primer	**Sequence**
FirISf	5´CTGGATCACCTCCTTTCTAWG-3´
BacISf	5 -CTGGAACACCTCCTTTCTGGA-3´
DUISr1	5´ -AGGCATCCACCGTGCGCCCT-3´
DUISr2	5´ -AGGCATTCACCRTG-CGCCCT-3´
DUISr3	5´ -AGGCATCCRCCATGCGCCCT-3´

For each sample two PCR reactions were done. One reaction was composed of one forward primer specific for *Firmicutes* and *Actinobacteria* (FirISf) with each of three reverse primers (DUISr1, DUISr2 and DUISr3) and the other reaction was composed of one forward primer specific for *Bacteroidetes* (BacISf) to beaded with the same reverse primers, so 6 PCR amplification reactions were carried out for each samples.

PCR reaction mixture (100µl) was composed of 5µl of DNA, 50 µl of a Mastermix (Thermo Scientific, Waltham, USA) 2 µl of bovine serum albumin, 1µl of a forward primer, 0.34µl of each reverse primer and 41µl of nuclease free water. The PCR was carried out in thermal Cycler (Bio Cycler TC-S, BOECO, Germany) The amplification cycles were; initial heating step at 72°C for 2 minutes followed by 35 cycles consisting denaturation at 94°C for 30 seconds then, annealing at 56°C for 45 seconds, extension at 72°C for 1 minute, and 5 minutes final extension at 72°C. Amplification products of PCR were visualized and seprated in 1.5% highly pure molecular biology grade agarose (Bioline, London, UK) using tris acetate EDTA (TAE) as buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.2) (Thermo Scientific, Waltham, USA) with ethidium bromide stain (5µg/ml) (Suvchem, Mumbai, India) and visualized under UV transilluminator (Vilber Lourmant, France). For accurate sizing of the separated DNA fragments, two Gene Rulers (100bp and 50bp) DNA molecular weight markers (Thermo Scientific, Waltham, USA) were used.

Statistical analysis:

Data were analyzed using chi-square test by *SPSS software* program (version 14.0, Chicago, IL, USA), *p* value < 0.05 was considered as statistically significant. Data were expressed as mean ± standard deviation (SD) and were analyzed using one-way analysis of variance (ANOVA) for the comparison between different groups.

RESULTS

Distribution of gut flora among the studied groups by culture techniques

Firmicutes/ Bacteroidetes ratio was higher in both diabetic groups (4.7% in T1D and 9% in TD) compared to healthy group (4%). The main detected Firmicutes were *Clostridium* spp., *Bacillus* spp., *enterococci* and *Lactobacilli* (Table 4).

Table 4: Distribution of fecal gut flora among T1D, T2D groups (both patients with and without controlled blood glucose level) and healthy group.

Phylum	Type of bacteria	T1D			T2D			
		T1D with uncontrolled blood glucose level(24) No. (%)	T1D with controlled blood glucose level (16) No. (%)		T2D with uncontrolled blood glucose level(24) No. (%)	T2D with controlled blood glucose level (16) No. (%)		Healthy group (20) No. (%)
Aerobic bacteria			**Total No. (%)**			**Total No. (%)**		
Proteobacteria	***Escherichia coli***	8 (19.5)	12 (18.4)	**20(18.9)**	6 (14.2)	11 (18)	**17(16.5)**	**13 (18)**
	***Klebsiella* spp.**	0	5 (7.6)	**5(4.7)**	0	3 (4.9)	**3(3)**	**4 (5.5)**
	***Proteus* spp.**	3 (7.3)	2 (3)	**5(4.7)**	4 (9.5)	1 (1.6)	**5(4.8)**	**0**
	Unidentified Gram negative	1 (2.3%)	3 (4.6%)	**4**	3 (7.1)	2 (3.2)	**5(4.8)**	**7 (9.7)**
Firmicutes	***Enterococci* spp.**	3 (7.3)	9 (13.8)	**12(11.3)**	6 (14.2)	5 (8.1)	**11(10.7)**	**6 (8.3)**
	***Bacillus* spp.**	6 (14.6)	9 (13.8)	**15(14.1)**	5 (11.9)	12 (19.6)	**17(16.5)**	**5 (6.9)**
	Coagulase negative staphylococci (CoNS)	2 (4.8)	3 (4.6)	**5(4.7)**	3 (7.1)	6 (9.8)	**9(8.7)**	**5 (6.9)**
	***Streptococcus* spp.**	0	2 (3)	**2(1.8)**	2 (4.7)	0	**2(2)**	**3 (4.1)**
	S. aureus.	1 (2.3)	1 (1.5)	**2(1.8)**	1 (2.3)	0	**1(1)**	**0**
Total		24 (58.5)	46 (70.7)	**70(66)**	30 (71.4)	40 (65.5)	**70(68)**	**43(59.7)**
Anaerobic bacteria								
Firmicutes	***C. sporogense***	4	2	**6**	2	3	**5**	**3**
	C.ramosum	0	2	**2**	0	0	**0**	**0**
	C. perfringen.	1	1	**2**	0	0	**0**	**0**
	C. novyi.	0	0	**0**	1	1	**2**	**1**
	Unidentified spp.	2	3	**5**	4	3	**7**	**3**
	Total	7 (17)	8 (12.3)	**15(14.2)**	7 (16.6)	7 (11.4)	**14(13.5)**	**7 (9.7)**
	***Peptostreptococcus* spp.**	0	0		0	2 (3.2)	**2(2)**	**0**
	***Lactobacillus* spp.**	2 (4.8)	3(4.6)	**5(4.7)**	2 (4.7)	5 (8.1)	**7(6.8)**	**7 (9.7)**
Bacteroidetes	***Bacteriodes* spp.**	7 (17)	5 (7.6)	**12(11.3)**	3 (7.1)	4 (6.5)	**7(6.8)**	**8 (11.1)**
Actinobacteria	***Bifidobacterium* spp.**	1 (2.3)	0	**1(1)**	0	0		**3 (4.1)**
Fusobacteria	***Fusobacterium* spp.**	0	3 (4.6)	**3(2.8)**	0	3 (4.9)	**3(3)**	**4 (5.5)**
Total		17 (41.5%)	19(29.3%)	**36(34)**	12(28.6%)	21 (34.5)	**33(32)**	**29**
Firmicutes/ Bacteroidetes ratio (F/B)		**56/12 (4.7%)**			**63/7 (9%)**			**33/8 (4%)**
	S.D	**2.8**	**3.6**		**2.4**	**3.7**	**3.6**	
	Total	41	65	**106**	42	61	**103**	**72**

T1D: Type 1 diabetes mellitus T2D: type 2 diabetes mellitus *P value* is 0.2058 considered not significant.

In type 1 diabetic group the most isolated aerobic bacteria were *E. coli* (18.9 %), *Bacillus* spp. (14.1%) and *Enterococci* spp. (11.3%) while the most prevalent isolated anaerobic bacteria were *Clostridium* spp. (14.2%), *Bacteroides* spp. (11.3%) and *Lactobacillus* spp. (4.7 %). In type 2 diabetic groups, *Escherichia coli* (16.5%), *Bacillus* spp. (16.5%) and *Enterococci* spp. (10.7%) were the most commonly isolated aerobic bacteria while the most isolated anaerobic bacteria were *Clostridium* spp. (13.5%), *Bacteroides* spp. (6.8%) and *Lactobacillus* spp. (6.8%). In healthy group the most isolated aerobic bacteria were (*Escherichia coli* (18%), *Enterococcus* (8.3%) and *Bacillus* spp. (9.7%) while the most prevalent anaerobic bacteria were *Bacteroides* spp. (11.1%), *Lactobacillus* spp. (9.7%) and *Clostridium* spp. (9.7%) (Table 4). No significant difference was found between the three diabetic groups and isolated species *P value* is 0.2058**.**

PCR analysis of 16S-23S rRNA intergenic spacer region

The microbiome diversity of twenty five fecal DNA specimens from patients with T1D, T2D and healthy individuals was examined, they include 6 samples of T1D with controlled blood glucose level with no other medical diseases (T1CN), 6 samples of T1D with uncontrolled blood glucose level and no other medical diseases,(T1NN), 4 samples of T2D with controlled blood glucose level and no other medical diseases (T2CN), 2 samples of T2D with un controlled blood glucose level and hypertension (T2NH), 3 samples of T2D with uncontrolled blood glucose level and no other medical diseases (T2NN) and 4 samples from healthy

group. Products of PCR were separated by gel electrophoresis and were visualized under UV transilluminator (figure 1,2) and analyzed according to IS of Deutsche Stammsammlung (DSM) German strain mentioned by Remley *et al.*[26]

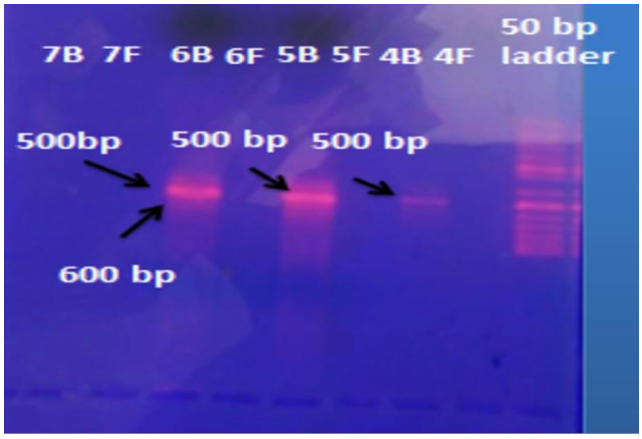

Fig. 1: PCR amplification products of T2D group**.** showed three bands at 500 bp for *L. acidophilus or L. reuteri)* at well 4B, 5B and 6B. Also, there is one band at 600bp for *Akkermansia muciniphila* at well 6B. *(F: Firmicutes and B: Bacteroidetes*).

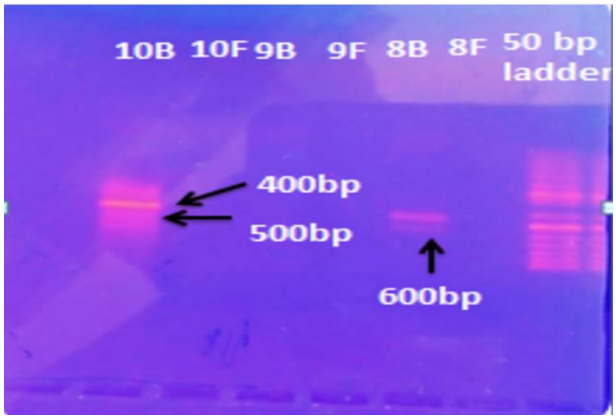

Fig. 2: PCR amplification products of T1D group. show one band at 500 bp for *L. acidophilus or L. reuteri)* at well 10B and one band at 400 bp at well 10B for *Alistipes fingoldi* . Also, there is one band at 600 bp at well 8B for *Akkermansia muciniphila.* (F: Firmicutes and B: Bacteroidetes).

The results indicated that both T1D and T2D groups had higher percentage (indicated by No of bands visualized in the gel) of *Lactobacillus* spp., *Faecalibacterium prausnitzii, Alistipes* spp., *Bacteroides thetaiotaomicron* and *Akkermansia muciniphila* compared to healthy group, though there was no significant difference (*P value* = 0.407) (figure 3) .

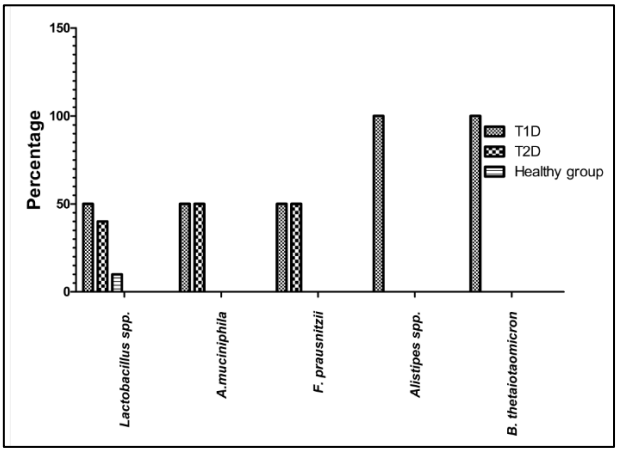

Fig. 3: Frequency of bacterial spp. detected by PCR among T1D, T2D and healthy group.
T1D: Type 1 diabetes mellitus T2D: type 2 diabetes mellitus

Lactobacillus spp. and *A. muciniphila* represented the most abundant bands among the bacterial species. Higher level of *Lactobacillus* spp. was observed in T1CN and T2CN groups than others. Higher level of *A. muciniphila* was observed in T2D groups (T2CN and T2NH) followed by T1D groups (T1CN and T1NN) (*P value* = 0.619).

DISCUSSION

An altered configuration of the gut microbial community has emerged as a new factor that aid in development of type 2 diabetes. The misuse of antibiotics and utilization of low fiber highly processed diet and other prebiotics had altered our natural flora [27]. In the present work we attempted to minimize variables, as much as possible, where diabetic and healthy groups were with similar age, dietary habit and living in the same environment. The current study revealed that the fecal microbial composition was different in T1D group and T2D group compared to healthy group by applying both traditional culture techniques and PCR analysis. This evidence that gut microbiota is a key factor in the pathophysiology of metabolic diseases [28].

Higher Firmicutes/Bacteroidetes ratio was observed in diabetic groups compared to control group by applying culture technique. This was in agreement with Remely *et al.* [26]. Bacteroidetes mainly involved in assembly of enzyme linked to lipid and carbohydrate metabolism while, Firmicutes possess significantly more resulting in increased fermented end products, including nutrient transportation and SCFAs [29]. Increased evidence has revealed that T2D patients exhibited an altered intestinal microbiota which was characterized by higher Firmicutes/Bacteroidetes ratio with an increase of some endotoxins producing Gram-negative bacteria and various opportunistic

pathogens[30,31]. In contrast, other study did not confirm this high ratio [32].

By using culture techniques it was observed that there was a slightly higher percentage of *Lactobacillus* spp. in diabetic patients (4.7% in T1D and 6.8% in T2D) compared to healthy individuals (9.7%) This result was found to be in agreement with Remely *et al*[26]., Larsen *et al*[31]., and Sedighi *et al*.[33] who reported higher abundance of *Lactobacillus* spp. in diabetic group compared to control group. Lactobacillus genus has well documented immunomodulating properties and might possibly contribute to chronic inflammation in diabetic patients [34].

On the other side it was noticed that *E. coli* have similar percentage in healthy and diabetic groups (about 18%). Our results were consistent with study in Egypt carried by Saber *et al*., [34] who showed that the most abundant genera of gut microbiota were *E. coli* and *Lactobacilli* spp. Enterobacteria are a major cause of mortality in T2D patients that may be attributed to the fact that *Bacteroidetes* and *Proteobacteria* might enhance the production of lipopolysaccharide and endotoxin [35]. *Bifidobacterium* spp. was higher in healthy group than diabetic patients by applying culture techniques. This was in agreement with Wu *et al*.,[30] and Sedighi *et al*.[33] who reported the presence of lower concentration of *Bifidobacterium* in T2D patients compared to the controls. Also, de Goffau *et al* [36] reveled that Bifidobacterium were less abundant in T1D. In contrast, Remely *et al.* [32] found no significant differences in copy number of genus Bifidobacterium between the diabetic and control groups. Lower percentage of *Bifidobacterium* spp. in the colon have been associated with a number of disorders like diabetes *as they* decrease butyrate concentrations and decrease numbers of bacterial butyrate producers in the human colon [37].

In the present study, by using culture techniques a slightly higher percentage of *Bacteroides* spp. in diabetic patients (11.3% in T1D and 6.8 % in T2D) compared to healthy group (11.1%) were detected. This was in contrast to Chiu *et al.*[38] who found greater *Bacteroides* spp. richness in normal weight healthy individuals than obese individuals.

A. muciniphila, belonging to the phylum *Verrucomicrobia*. It is a mucus degrading bacterium that obtains its energy sources independently from mucus coating the intestinal tract and constitute about 3-5% of gut microbiota biomass [26]. In the present study, PCR results revealed that *A. muciniphila* were more prevalent in both T1D and T2D compared to control group. This result was found to be in agreement with Qin *et al.*[15] and Remely *et al.*[26] who showed that *A. muciniphila* was more abundant in T2D. Additionally PCR technique in this study revealed that *F. prausnitzii* was highly present in T1D and T2D while it wasn't detected in control group. This result was consistent with Remely *et al.*[26] who showed that a significant increase in abundance of *F. prausnitzii* among T2D diabetes groups compared to healthy controls. In contrast to de Goffau *et al* [36] who found that *F. prausnitzii* was decreased among T1D patients compared to healthy control. *F. prausnitzii* represents in average about 5-15% of the total fecal microbiota in healthy individuals, growing evidence is emphasizing the importance of *F. prausnitzii* in human's gastrointestinal performance and for anti-inflammatory properties [39]. Furthermore it was observed that *Alistipes* spp. was only detected in T1D, this was in agreement with Gavin *et al.*[40] *Alistipes* may promote mucous production and a healthy epithelial barrier in autoantibody negative T1D patients, as it is positively correlated with production of different mucous layer proteins, adhesion molecule and some pancreatic proteins and negatively correlated with fibrillin-1 and a cluster of heavy and light chain antibody variable regions [40].

Conclusion and Recommendation

This study declared the existence of diversity in gut microbiota between T1D and T2D patients compared to healthy group by applying both culture and PCR techniques. It is unclear if gut microbiota dysbiosis is a cause or outcome of diabetes as so; further studies should be done on large number of patients to compare gut microbiota between poorly controlled and well controlled diabetic patients by more sensitive techniques as 16S rRNA sequencing.

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Conflict of interest: The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

REFERENCES

1. Dandona P, Aljada A, Bandyopadhyay A. Inflammation: the link between insulin resistance, obesity and diabetes. Trends in immunology. 2004; 25(1): 4-7.

2. Devaraj S, Dasu MR, Jialal I. Diabetes is a proinflammatory state: a translational perspective. Expert review of endocrinology & metabolism. 2010; 5(1): 19-28.
3. Essue B, Mirzaei M, Leeder, SR, Colagiuri R. Epidemiology of diabetes. The serious and continuing illness policy and practice study (SCIPPS). The Menzies Centre for Health Policy. 2007.
4. Chatenoud L. World Diabetes Day: perspectives on immunotherapy of type 1 diabetes. European journal of immunology. 2015; 45(11): 2968-2970.
5. Zheng Y, Ley SH, Hu FB. Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. Nature Reviews Endocrinology. 2018; 14(2): 88.
6. Hegazi R, El-Gamal M, Abdel-Hady N, Hamdy O. Epidemiology of and risk factors for type 2 diabetes in Egypt. Annals of global health. 2015; 81(6): 814-820.
7. Atkinson MA, Eisenbarth GS, Michels AW. Type 1 diabetes. The Lancet 2014; *383*(9911): 69-82.
8. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Mende DR. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 2010; 464(7285): 59.
9. Conlon M, Bird A. The impact of diet and lifestyle on gut microbiota and human health. Nutrients 2015; 7(1): 17-44.
10. Giongo A, Gano KA, Crabb DB, Mukherjee N, Novelo LL, Casella G, Veijola R. Toward defining the autoimmune microbiome for type 1 diabetes. The ISME journal 2011; *5*(1): 82.
11. Rowland I, Gibson G, Heinken A, Scott K, Swann J, Thiele I, Tuohy K. Gut microbiota functions: metabolism of nutrients and other food components. European journal of nutrition 2018; 57(1): 1-24.
12. Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. Cell 2014; 157(1): 121-141.
13. Davis-Richardson AG, Ardissone A N, Dias R, Simell V, Leonard M T, Kemppainen K M, Ilonen J. Bacteroides dorei dominates gut microbiome prior to autoimmunity in Finnish children at high risk for type 1 diabetes. *Frontiers in microbiology* 2014; *5*: 678.
14. Vehik K, Dabelea D. The changing epidemiology of type 1 diabetes: why is it going through the roof?. Diabetes/metabolism research and reviews 2011; 27(1): 3-13.
15. Qin J, Li Y, Cai Z, Li S, Zhu J, Zhang F, Peng Y. A Metagenome-wide association study of gut microbiota in type 2 diabetes. Nature 2012; 490 (7418): 55.
16. Larsen N, Vogensen FK, Van Den Berg FW, Nielsen DS, Andreasen AS, Pedersen BK, Jakobsen M. Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. PloS one 2010; 5(2): e9085.
17. Karlsson FH, Tremaroli V, Nookaew I, Bergström G, Behre CJ, Fagerberg B, Bäckhed F. Gut metagenome in European women with normal, impaired and diabetic glucose control. Nature 2013; 498(7452): 99.
18. Aw W, Fukuda S. Understanding the role of the gut ecosystem in diabetes mellitus. Journal of diabetes investigation 2018; 9(1): 5-12.
19. MacFaddin JF. Biochemical testes for identification of medical bacteria, 3rd edition. Lippincott William & Wilkins 2000.
20. Koneman EW, Allen SD, Janda WM, Schreckenberger PC, and Winn, WC. Color atlas and textbook of diagnostic microbiology, 5th edition. Lippincott-Raven Publishers, U.S.A 1997.
21. Engelkirk PG and Duben-Engelkirk JL. Laboratory diagnosis of infectious diseases: Essentials of diagnostic microbiology. Lippincott Williams & Wilkins 2008.
22. Summanen P, Baron EJ, Citron DM, Strong CA, Wexler HM and Finegold M. Wadsworth Anaerobic Bacteriology Manual, 5th edition. Star Publishing Co., Belmont, Calif 1993.
23. Morton A. Anaerobic bacteriology In: Clinical microbiology procedures handbook Ch: 4. Garcia, L.S and Isenberg, H.D. eds. 2nd edition. ASM press 2007.
24. Sutter VL, Citron DM and Finegold SM. Wadsworth Anaerobic Bacteriology Manual. 3rd edition. C.V. Mosby Company system 1980.
25. Mangels JI. Anaerobic bacteriology In: Clinical microbiology procedures handbook Ch: 4. Garcia, L.S and Isenberg, H.D. eds. 2nd edition. ASM press 2007.
26. Remely M, Hippe B, Zanner J, Aumueller E, Brath HG, Haslberger A. Gut microbiota of obese, type 2 diabetic individuals is enriched in *Faecalibacterium prausnitzii*, *Akkermansia muciniphila* and *Peptostreptococcus anaerobius* after weight loss. Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders) 2016; 16(2): 99-106.
27. Allin H, Nielsen T, Pedersen O. Mechanisms in endocrinology: gut microbiota in patients with type 2 diabetes mellitus. *European Journal of endocrinology*, 2015; *172*(4), 167-177.

28. Dantas N, G. The effects of antibiotics on the microbiome throughout development and alternative approaches for therapeutic modulation. Genome medicine 2016; 8(1): 39.
29. Duncan SH, Hold G L, Barcenilla A, Stewart C S, Flint H J. *Roseburia intestinalis* sp. nov., a novel saccharolytic, butyrate-producing bacterium from human faeces. International journal of systematic and evolutionary microbiology 2002; 52(5):1615-1620.
30. Wu X, Ma C, Han L, Nawaz M, Gao F, Zhang X, Wang J Molecular characterisation of the faecal microbiota in patients with type II diabetes. Current microbiology 2010; 61(1): 69-78.
31. Larsen N, Vogensen FK, van den Berg FW, Nielsen DS, Andreasen AS, Pedersen BK, Jakobsen M. Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. PloS one 2010; 5(2): 9085.
32. Remely M, Dworzak S, Hippe B, Zwielehner J, Aumüller E, Brath H, Haslberger A. Abundance and diversity of microbiota in type 2 diabetes and obesity. J Diabetes Metabolism 2013; 4(253): 2.
33. Sedighi M, Razavi S, Navab-Moghadam F, Khamseh ME, Alaei-Shahmiri F, Mehrtash A. Amirmozafari N. Comparison of gut microbiota in adult patients with type 2 diabetes and healthy individuals. Microbial pathogenesis 2017; 111: 362-369.
34. Saber SM, Othman HB, ElMasry S, Magdy A, Zidan HF. Correlation between body mass index and gut microbiota in adults. Int. J. Curr. Microbiol. App. Sci 2017; 6(2): 778-787.
35. Wanping A, Shinji F. Understanding the role of the gut ecosystem in diabetes mellitus. J Diabetes Investig 2018; 9: 5–12.
36. De Goffau MC, Fuentes S, van den Bogert B, Honkanen H, de Vos WM, Welling GW, Harmsen HJ. Aberrant gut microbiota composition at the onset of type 1 diabetes in young children. Diabetologia 2014; 57(8): 1569-1577.
37. Rivière A, Selak M, Lantin D, Leroy F, De Vuyst L. Bifidobacteria and butyrate-producing colon bacteria: importance and strategies for their stimulation in the human gut. Frontiers in microbiology 2016; 7: 979.
38. Chiu CM, Huang WC, Weng SL, Tseng HC, Liang C, Wang WC, Huang HD. Systematic analysis of the association between gut flora and obesity through high-throughput sequencing and bioinformatics approaches. BioMed research international 2014.
39. Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermúdez-Humarán LG, Gratadoux JJ Grangette C. *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proceedings of the National Academy of Sciences 2008; 105(43): 16731-16736.
40. Gavin P G, Mullaney JA, LooD, Lê Cao, K A, Gottlieb P A, Hill M M, Hamilton-WilliamsE E. Intestinal metaproteomics reveals host-microbiota interactions in subjects at risk for type 1 diabetes. *Diabetes care*, 2018; *41*(10), 2178-2186.