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Research article

Gut microbiota composition and type 2 diabetes: Are these subjects linked Together?

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ABSTRACT

Purpose: Evidence suggests that changes in the composition of gut microbiota may be linked to metabolic disorders including type 2 diabetes (T2D). The present study aims to evaluate the compositional changes of the intestinal microbiota in patients with T2D as compared to healthy individuals.

Methods: In this case-control study, there were 18 T2D patients and 18 healthy individuals who served as controls. To profile the gut microbiota in both groups, bacterial DNA was extracted from fecal samples and analyzed using quantitative real-time polymerase chain reaction (qPCR).

Results: The study discovered that diabetics had significantly greater frequencies of the genus *Bacteroides* and the phylum *Bacteroidetes* than did controls ($P = 0.03$ and $P < 0.001$, respectively). Conversely, the *Actinobacteria* and *Firmicutes* phyla were significantly more abundant in the controls ($P=0.01$ for both). No significant differences were observed in the fecal populations of the genus *Enterococcus*, *Clostridium clusters IV* and *XIVa*, phylum *Proteobacteria*, and all bacteria between the studied groups ($P=0.88$, $P=0.56$, $P=0.8$, $P=0.99$, and $P=0.7$, respectively).

Conclusions: Our findings confirm that T2D may be associated with the gut microbiota fluctuations. These findings may be valuable for developing strategies to control or treatment T2D by restoring the intestinal microbiota through the strategic administration of specific probiotics/prebiotics and lifestyle and dietary modifications.

1. Introduction

The human body maintains a complex and dynamic symbiosis with a diverse array of microorganisms, collectively known as the microbiome, which plays a significant role in modulating human health [1,2]. Predominantly composed of bacteria, the human

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microbiome is estimated to harbor over 10^{14} bacterial cells within the intestinal tract, encoding a genetic repertoire that surpasses the diversity of the human genome [3,4]. Taxonomic surveys at the phylum level have revealed that the majority of the gut microbiota is represented by *Bacteroidetes* and *Firmicutes*, with lesser but notable contributions from *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, *Verrucomicrobia*, and *Spirochaetes* [5–7]. The *Bacteroidetes* phylum encompasses three classes, with the class *Bacteroidia* being the most extensively studied, largely due to its well-known genera of *Prevotella* and *Bacteroides*. *Firmicutes*, the most prevalent bacterial phylum in the human gut, comprises over 200 genera, with the majority of its intestinal representatives falling into two critical clusters: *Clostridium cluster IV* and *Clostridium cluster XIVa* [5,8]. The *Enterococcus* genus, a member of *Firmicutes*, has been the subject of considerable research, more so than other *Firmicutes* genera [9]. These clusters include taxonomically diverse members from the genera of *Clostridium*, *Ruminococcus*, and *Eubacterium* [10]. The gut microbiota is a crucial component of a complex ecosystem of microorganisms inhabiting the intestinal tracts of humans and animals, interacting with the intestinal barrier's physical and chemical aspects and the immune, neuromotor, and enteroendocrine systems [11,12]. Intestinal microorganisms engage in a symbiotic relationship with the host, affecting nutrition, immunity, health, and disease. The human intestinal mucosa, a frequent portal of entry for pathogens, houses a significant proportion of immune system cells that can be modulated by gut microorganisms [13,14].

According to available data, the gut bacteria play a major role in regulating energy homeostasis. Moreover, disruptions in the gut bacterial community, known as dysbiosis, have been associated to different chronic diseases, including rheumatoid arthritis, non-alcoholic fatty liver, obesity, type 2 diabetes (T2D), cancer, inflammatory bowel disease, autism, and cardiovascular diseases [15–17]. Diabetes mellitus or T2D is a metabolic condition marked by elevated blood sugar levels due to issues with insulin production, insulin effectiveness, or a combination thereof [18]. The occurrence of diabetes is swiftly rising across both developing and developed nations [19]. The count of individuals with diabetes has climbed from 153 million in 1980 to 382 million in 2013, with estimates forecasting a surge to over 590 million by 2035 globally [19,20]. T2D is the predominant form of diabetes, representing about 90 % of all diabetes cases. Contemporary investigations have shown that T2D emerges from a complex interplay between genetic predispositions and environmental influences [21,22]. The rising incidence of diabetes over the last several decades has been linked to socioeconomic changes and lifestyle factors, such as decreased physical activity and a diet high in calories [23]. Emerging studies indicate that compositional changes (dysbiosis) within the human gut microbiota, leading to an imbalance in the production of metabolites, may be a contributing factor in the pathogenesis of T2D. This dysbiosis is believed to play a crucial role in the onset of prediabetic states such as insulin resistance and chronic inflammation, which are implicated in the development of T2D [24]. The results indicate that certain bacterial species exhibit a positive correlation with fasting glucose levels and glycosylated hemoglobin (HbA1c), whereas other strains show a negative correlation with HbA1c, fasting glucose, and plasma triglycerides. This recommends that these bacterial strains may play a role in the association with T2D [25]. Consequently, it is crucial to elucidate the intricate interaction between the host and its gut microbiota, particularly in the situation of abnormal metabolism of glucose. Under pathological states, the disruption of host molecules regulation can induce alterations in the composition of the intestinal microbiota. Conversely, the microbiota plays a regulatory role that can influence the progression of T2D [25].

The alterations in gut microbiota composition are thought to play a direct role in instigating a low-grade inflammatory condition that may serve as a catalyst in the onset of T2D [22,26,27]. Research has demonstrated that the imbalances between *Bacteroidetes* and *Firmicutes* phylum subspecies is associated with fasting blood sugar levels and T2D [28]. Indeed, they found that the *Firmicutes*-to-*Bacteroidetes* ratio is lower in subjects with T2D than in non-diabetics. Evidence indicates that the gut microbiota of patients with T2D tends to have a higher abundance of Gram-negative bacteria from the *Bacteroidetes* and *Proteobacteria* phyla, which can induce endotoxemia through elevated levels of circulating lipopolysaccharides (LPS). This state may trigger chronic, low-grade inflammation, irregular expression and secretion of various inflammatory cytokines like interleukins, activation of macrophages, oxidative stress, and ultimately contribute to insulin resistance and the development of T2D [29–34]. The presence of *Bacteroidetes* correlates with higher LPS levels, while a decrease in *Bacteroidetes* is associated with reduced metabolic endotoxemia and a lower inflammatory state. Conversely, *Proteobacteria* are known to be highly pro-inflammatory. It is well-established that this subclinical, pro-inflammatory state, driven by LPS-dependent production of inflammatory cytokines such as Tumor Necrosis Factor-α, Interleukin-1, and Interleukin-6, promotes the progress of insulin resistance and subsequently type 2 diabetes [35].

Gut bacteria may facilitate enhanced absorption of monosaccharides from the intestine and prompt the host to elevate liver triglyceride production, potentially exacerbating insulin resistance [36]. Additionally, dysbiosis within the gut bacteria is recognized to impact the production of short-chain fatty acids (SCFAs), modify bile acid profiles, and influence the endocannabinoid system. This can result in decreased levels of glucagon-like peptide 1 and 2 and peptide YY, which are crucial regulators of glucose metabolism and insulin secretion by activating gut hormone receptors. These changes leading to a compromised gut barrier and setting off a cascade of events that culminate in reduced insulin sensitivity, heightened inflammation, increased oxidative stress, elevated steatosis, and an increase in fat mass. These factors influence the epigenetic regulation of genes involved in inflammation and insulin resistance in T2D [37,38]. The concentrations of SCFAs are altered in individuals with type 2 diabetes. Specific studies have indicated that gut dysbiosis significantly affects SCFA concentrations in people with T2D [39]. An increase in SCFA production has been observed to enhance energy extraction, which is attributed to a higher intake of dietary calories. SCFAs have been shown to have various beneficial effects on gut metabolism in obesity, which frequently accompanies diabetes [38]. It is also important to note that diabetes is linked to an increased presence of opportunistic pathogens and a decreased abundance of butyrate producing bacteria [28]. An elevation in the levels of pathogenic bacteria, including *Enterobacteriaceae*, *Escherichia coli* various *Clostridiales*, *Prevotella copri*, *Bacteroides vulgatus*, *Bacteroides caccae*, and *Lactobacilli*, has been identified in the gut microbiota of individuals with T2D [35]. Various investigations from around the globe have revealed relative or substantial variations in fecal bacterial composition—including differences at the phylum, cluster, genus, and species levels—between individuals with T2D and those without the condition [16,29,31,40–42].

Recent research specifies that the risk of developing diabetes is largely associated with shifts in the equilibrium of the gut

microbiota, rather than the activity of a solitary microbe. However, the precise mechanisms connecting the gut microbiota to metabolic diseases, including T2D, remain to be fully understood. It is not yet determined whether specific alterations in microbiota composition directly lead to T2D in humans or if microbial dysbiosis is merely a manifestation of underlying metabolic disorders [24,43]. Nonetheless, scientists have pinpointed potential impacts of the microbiome on human metabolic processes, including its involvement in metabolic conditions such as obesity and T2D. To explore the evidence of gut bacterial modifications in T2D patients, a case-control study was designed to assess the composition of the gut microbiota, encompassing dominant bacterial phyla, clusters, and genera, in individuals with T2D versus non-diabetic, healthy controls, utilizing a molecular technique tailored for this investigation.

2. Materials and methods

2.1. Study participants, samples collection and DNA extraction

The DNA used in this research was extracted from fecal samples collected from 18 adult patients diagnosed with type 2 diabetes and 18 non-diabetic adults. This material was obtained from a study previously conducted by our team at the Microbiology Department of the Medical Sciences Faculty, Iran University of Medical Sciences, located in Tehran, Iran [41]. As detailed in our earlier work, participants in both the diabetic and non-diabetic groups were carefully matched based on several demographic characteristics and health-related criteria, including age, gender, race, and geographical location. All diabetic patients had a glycated hemoglobin (HbA1c) level of less than 10 % and a known diabetes duration of less than five years as inclusion criteria. To maintain the integrity and consistency of our results, we conducted thorough interviews with patients to ascertain their medical histories, with a particular focus on any gastrointestinal diseases and the potential use of antibiotics, prebiotics, and probiotics. In accordance with the study's stringent inclusion and exclusion criteria, individuals who had used antibiotics, probiotics, prebiotic products, or any other medical interventions that could influence the digestive system (such as drugs that disrupt the gastrointestinal tracts) within the two months preceding sample collection were not included in the study. Similarly, participants who experienced gastrointestinal disorders during this two-month period were also excluded from the research. In order to ensure the stability of the microbiota composition over time in the content of the gastrointestinal system and capture the dynamic nature of the gut bacteria, fecal specimens were collected from participants in both the diabetic and control groups on three occasions with a time interval of two weeks between the first two sampling times and ten days between the second and third sampling times. Each participant's samples were evaluated separately. The data from the three samples collected from each participant were then aggregated, and the average (mean threshold cycle [CT] values obtained from real-time qPCR method) were utilized for subsequent data analysis.

The dietary habits of participants were evaluated using a Food Frequency Questionnaire (FFQ) developed and validated for Iranian adults. The physical activity levels of participants in both the diabetic and control groups were assessed using the short version of the International Physical Activity Questionnaire (IPAQ).

Table 1
Specific primers and TaqMan probes targeting bacterial 16S rDNA coding regions.

Target Bacteria	Primer/Probe	Oligonucleotide sequence (5' – 3′)	Size (bp)	Product size (bp)	Ref.
Phylum *Proteobacteria*	primer F	CAAAKACTGACGCTSAGGTG	20	96	[44]
	primer R	GGCACAACCTBCAARTCG	18		
	Probe	AATCCTGTTTGCTCCCCACGCTTTC	25		
Phylum *Firmicutes*	primer F	CGAACGGGATTAGATACC	18	186	[44]
	primer R	CGAATTAAACCACATACTCC	20		
	Probe	CCCCGTCAATTCCTTTGAGTTT	22		
Phylum *Bacteroidetes*	primer F	GTGGTTTAATTCGATGATACGC	22	154	[44]
	primer R	CGCTCGTTATGGGACTTAAG	20		
	Probe	CCTCACGGCACGAGCTGACG	20		
Phylum *Actinobacteria*	primer F	CCGTTACTGACGCTGAGGAG	20	141	[44]
	primer R	GCGGGATGCTTAACGCG	17		
	Probe	TAGATACCCTGGTAGTCCACGCCGTA	26		
Genus *Enterococcus*	primer F	TAGAGAAGAACAAGGABGAKAGT	23	171	This study
	primer R	GGGCTTTCACATCAGACTTA	20		
	Probe	CGGTATCTAACCAGAAAGCCACGG	24		
Genus *Bacteroides*	primer F	GCAGTGAGGAATATTGGTCAA	21	97	This study
	primer R	TCCYNTATAAAAGAAGTTTRCAAYC	25		
	Probe	ATCCTTCACGCTACTTGGCTG	21		
Clostridium cluster IV	primer F	GCACAAGCAGTGGAGT	16	538	This study
	primer R	CTTCCTCCGTTTTGTCAA	18		
	Probe	AGGGTTGCGCTCGTT	15		
Clostridium cluster XIVa	primer F	GCAGTGGGGAATATTGCA	18	245	This study
	primer R	CTTTGAGTTTCATTCTTGCGAA	22		
	Probe	AAATGACGGTACCTGACTAA	20		
All bacteria	primer F	ACTCCTACGGGAGGCAG	17	161	This study
	primer R	GACTACCAGGGTATCTAATCC	21		
	Probe	TGCCAGCAGCCGCGGTAATAC	21		

* Primers F: forward, R: reverse, bp: base pair, Ref.: References.

2.2. Primers and probes designing

Five sets of primers and TaqMan probes applied in the present study were designed for the first time, while the other sets were obtained from the study conducted by Mohammadzadeh et al. (2021) [44]. The gradient-PCR approach was employed to assess the quality and specificity of the primers designed for conducting Real-Time PCR and to determine the optimal annealing temperature for these primers. The primers and probes targeted the bacterial 16S rDNA gene. Bacterial 16S rRNA sequences were obtained from the SILVA High-Quality Ribosomal RNA database [45] and converted to 16S rDNA sequences. Various websites, such as NCBI [46], Silva [47], Probebase [48], IDT [49], and EMBL-EBI [50], as well as AlleleID software (version 7.5), were used at different stages to design specific primer and probe sequences targeting the bacterial 16S rDNA gene. All sequences were aligned using the NCBI [46] and EMBL-EBI [51] databases accessible through the relevant websites and the AlleleID software. The specific sequences of primers and TaqMan probes are listed in Table 1.

2.3. Real-time qPCR and microbial quantification

Quantities of the fecal microbiota composition were measured by TaqMan quantitative PCR (qPCR) using 16S rDNA specific primers and probes in a Rotor-Gene Q real-time PCR cycler (Qiagen Corbett, Hilden, Germany) system. The 5′ end of the target probe was labeled with 6-carboxyfluorescein (FAM) dye as a reporter, and the 3′ end was tagged with Black Hole Quencher (BHQ) as a quencher. Every qPCR test was run in triplicate, and analysis and computation were done using the mean values. Each reaction mixture with a total volume of 20 μl contained 12 μl of TaqMan qPCR Master Mix (Takara Bio Inc., Shiga, Japan), 0.5 μl of each forward and reverse primer, 0.5 μl of TaqMan probe, 1 μl of template DNA and 5.5 μl of sterilized distilled water. All the steps were done on ice with minimal contamination and maximum accuracy in sampling. As shown in Table 2, Real-time qPCR was performed using cycle conditions specific to each primer pair and TaqMan probe. Negative controls, which included all elements of the reaction mixture except for template DNA, were performed in every analysis, and no amplified DNA product was ever detected. The amplified product at the end of each cycle was identified via the fluorescent signal curve, which signifies the reaction and successful amplification. The CT (Threshold Cycle) value was recorded using the threshold line within the exponential phase of the amplification curves, which runs parallel to the x-axis. The data presented are the mean values obtained from triplicate real-time PCR reaction analyses for each individual sample, ensuring the minimization of test errors.

Standard curves were constructed to determine the abundance of bacteria in each sample, including the *Proteobacteria* phylum, the *Firmicutes* phylum, the *Bacteroidetes* phylum, the *Actinobacteria* phylum, the *Enterococcus* genus, the *Bacteroides* genus, the *Clostridium cluster IV*, the *Clostridium cluster XIVa*, and all bacteria present. The standard curves were created using 9-fold serial dilutions of genomic DNA from pure bacterial cultures of known concentration, ranging from 10^1 to 10^{10} copies/gram of stool. For each dilution, the Real-Time PCR assay was conducted in duplicate. The serial dilutions were loaded sequentially into the instrument to execute the qPCR reaction in a single run. The mean Ct values derived at the end of the reaction, along with the corresponding concentrations for each DNA dilution, were documented in the device's software for the purpose of generating a standard curve. The standard curves were created following the tutorials provided by Applied Biosystems [52] and normalized to the frequency of the 16S rRNA gene for each group of bacteria. The efficiency of Real-Time qPCR method, which is based on the line slope, was calculated using the standard curve and by the formula Efficiency = $10^{(-1/\text{slope})}$-1. The acceptable range for the slope of the line was between −3.1 and −3.6, which corresponds to a reaction efficiency of 90 %–110 %, indicating appropriate qPCR efficiency. A correlation coefficient (R^2) of 0.99 or higher was confirmed. The copy numbers of bacteria whose 16S rRNA operon copy numbers had not been published were determined by averaging the operon numbers of the closest bacterial species from the ribosomal RNA database, or rrnDB, as described previously [41]. By utilizing the Ct value obtained from the samples and positioning it within the standard curve, we were able to ascertain the quantity of DNA present in the unknown sample, which expressed as the bacterial load per gram of stool.

Table 2
Time and temperature conditions of real-time qPCR cycles for all investigated bacteria in the present study.

Stage		Temperature	Time	Cycles No.
Initial holding		95 °C	30 s	
Denaturation		95 °C	5 s	
Annealing/Extension	Phylum *Proteobacteria*	55 °C	30 s	35
	Phylum *Firmicutes*	56 °C		
	Phylum *Bacteroidetes*	59 °C		
	Phylum *Actinobacteria*	58 °C		
	Genus *Enterococcus*	53 °C		
	Genus *Bacteroides*	52 °C		
	Clostridium cluster IV	57 °C		
	Clostridium cluster XIVa	56 °C		
	All bacteria	58 °C		

2.4. Bacterial standard strains

Bacterial standard strains used in this study were obtained from the American Type Culture Collection (ATCC) (*Bifidobacterium bifidum* ATCC 29521, *Prevotella intermedia* ATCC 25611D-5, *Bacteroides fragilis* ATCC 25285, *Lactobacillus acidophilus* ATCC 4356) and Persian Type Culture Collection (PTCC) (*Enterococcus faecalis* PTCC 1237, *Escherichia coli* PTCC 1270, *Enterobacter cloacae* PTCC 1003, *Clostridium acetobutylicum* PTCC 1492, *Clostridium perfringenes* PTCC 1765).

2.5. Ethical approval

This study's methods were all carried out strictly in compliance with the Helsinki criteria and ethical standards. The Iran University Human Ethics committee accepted the research protocol and experimental design, guaranteeing the safety of human subjects and conformity to moral standards. All participants gave their informed consent before beginning the study, and their privacy and confidentiality were scrupulously protected during the whole process of gathering and analyzing data.

2.6. Statistical analysis

We utilized Minitab software version 16.2.0 and SPSS software version 20.0 (SPSS Inc., Chicago, IL, USA) for statistical analysis. The sample size was determined to be able to identify a difference in the mean bacterial quantity between the T2D patients and the healthy individuals of at least 2×10^5 copies per gram of feces. Six participants were required for each group based on a power of 80 %, an alpha error of 0.05, and an anticipated standard deviation of 1.13×10^5 copies per gram of feces [40]. However, we expanded the sample size to 18 diabetic patients and 18 healthy subjects to increase the study power. To compare the means of the various variables among the research groups, an independent sample *t*-test was employed. Using Spearman Rank, the linear correlation between the variables was calculated. A *p-value* of less than 0.05 was considered statistically significant. All data were expressed as mean ± standard deviation (mean ± SD). Box and Whisker charts were used to visually represent the qPCR data.

3. Results

3.1. Study participants

The study involved 18 individuals with T2D and 18 non-diabetic controls, all within the age range of 35–65 years and exhibiting body mass indices (BMI) between 18.5 and 35 kg/m^2. As anticipated, participants with T2D displayed higher levels of fasting blood glucose (FBS: 125.10 ± 29.58 versus 79.50 ± 6.37 mg/dl) and HbA1c (6.68 ± 1.03 % versus 4.96 ± 0.54 %) compared to the non-diabetic group. Table 3 provides a detailed overview of the characteristics of the participants in both the diabetic and healthy groups.

3.2. Food frequency questionnaire analysis

The evaluation of the food frequency questionnaire (FFQ) indicated notable variations in the consumption of total sugar, glucose, monounsaturated fatty acids, cholesterol, total fat, vitamin B12, and thiamin between participants in the diabetic and healthy groups. Conversely, no significant differences were observed between the two groups regarding their dietary intake of total fiber, carbohydrates, protein, polyunsaturated fatty acids, vitamin B6, riboflavin, biotin, iron, and selenium. Furthermore, despite a significant difference in total daily energy intake, the macronutrient compositions (protein, carbohydrates, and fat) were found to be similar in both study groups (Fig. 1).

Table 3
Characteristics of the participants in both case and control groups in the present study.

Characteristics	Diabetic group	Control group	*P-value*
Number	18	18	–
Male/female	7/11	7/11	–
Age (year)	54.3 ± 7.63	52.1 ± 7.56	0.62
Height (cm)	166.22 ± 8.15	164.39 ± 7.42	0.48
Weight (kg)	72.28 ± 10.32	65.72 ± 6.57	0.03
BMI (kg/m2)	26.3 ± 3.12	24.43 ± 2.98	0.07
Diabetes duration (years)	–	3 ± 1.88	–
Glucose (mg/dL)	149 ± 9.68	93 ± 3.25	<0.001
FBS (mg/dl)	125.1 ± 29.58	79.5 ± 6.37	<0.001
HbA1C (%)	6.68 ± 1.03	4.96 ± 0.54	<0.001

Values are presented as mean ± SD. Interactions between both study groups were analyzed using the Mann-Whitney *U* test. Values are significantly different for $P < 0.05$.

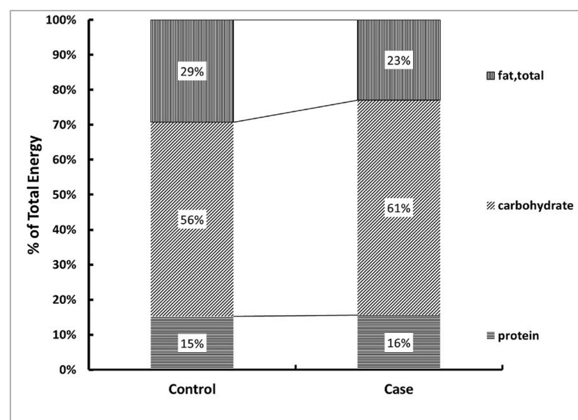

Fig. 1. Nutritional macronutrient (protein, carbohydrate and fat) compositions in the diabetic and non-diabetic study groups (in total daily calorie intake).

3.3. International Physical Activity Questionnaire analysis

The analysis of the physical activity questionnaire revealed no significant difference in the average physical activity scores between the healthy and diabetic groups (1494 ± 1030 MET-min/week versus 1305 ± 975 MET-min/week; *P value* = 0.59). Nonetheless, distinctions in activity patterns were observed between the two groups. Notably, only 22.0 % of diabetic patients reported engaging in vigorous physical activity 1 to 3 times per week (mean: 2393 MET-min/week), whereas 49.0 % indicated low or moderate physical activity 2–5 times per week (mean: 1277 MET-min/week). In comparison, 89.0 % of healthy individuals reported low or moderate physical activity 3 to 5 times per week (mean: 1342 MET-min/week), and 58.0 % participated in vigorous physical activity 2 to 4 times per week (mean: 2640 MET-min/week).

3.4. Quantitative PCR analysis of gut bacterial compositions

In this study, qPCR was employed to assess variations in fecal microbiota composition between T2D patients and healthy controls, focusing on specific taxa: phyla *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, genera *Enterococcus* and *Bacteroides*, and *Clostridium clusters IV* and *Clostridium cluster XIVa*, as well as total bacterial content. Following the exclusion of outliers, intra-group analysis indicated significant differences in the abundance of three phyla and one genus of bacteria between participants in the healthy and diabetic groups. The results of gut bacterial quantification were as follows.

- *Bacteroidetes* Phylum: The prevalence of *Bacteroidetes* was significantly greater in the diabetic individuals compared to the healthy group (*P value* = 0.03).
- *Firmicutes* Phylum: The frequency of *Firmicutes* was significantly higher in healthy individuals than in diabetic subjects (*P value* = 0.01).
- *Actinobacteria* Phylum: The abundance of *Actinobacteria* was significantly elevated in non-diabetic individuals relative to the diabetic group *(P value* = 0.01).
- *Proteobacteria* Phylum: The copy number of *Proteobacteria* was similar between diabetic and healthy participants (*P value* = 0.99).

Table 4
Statistical results and quantity of bacteria per gram of fecal samples quantified by Real-time qPCR in both case and control groups.

Bacterial species	Copies/gr of fecal		Levene's Test		*t*-test for Equality of Means	
	Case (N = 18)	Control (N = 18)	F	Sig.	T	Sig. (2-tailed)
Phylum *Proteobacteria*	3.43E8±3.13E8	3.44E8±3.03E8	0.1	0.74	−0.08	0.99
Phylum *Firmicutes*	7.47E8±0.3.31E8	2.30E9±2.28E9	51.79	<0.001	−2.6	0.01
Phylum *Bacteroidetes*	3.10E9±3.45E9	2.37E9±2.47E9	5.27	0.02	0.72	0.03
Phylum *Actinobacteria*	4.94E8±3.19E8	9.26E8±6.48E8	5.6	0.02	−2.49	0.01
Genus *Enterococcus*	5.58E6±3.36E6	5.75E6±3.79E6	0.8	0.37	−0.149	0.88
Genus *Bacteroides*	8.23E6±1.01E6	6.7E5±2.7E5	35.29	<0.001	28.89	<0.001
Clostridium cluster IV	7.51E7±8.03E6	7.67E7±8.88E6	0.05	0.82	−0.58	0.56
Clostridium cluster XIVa	8.36E7±1.14E7	8.45E7±1.05E7	0.82	0.37	−0.25	0.81
All bacteria	7.91E9±9.48E8	7.88E9±8.77E8	0.15	0.7	0.38	0.94

Values are presented as means ± SD and expressed as copy numbers per gram of stool. Relationships between both groups were analyzed using the T test and Levene's Test. Values are significantly different for $P < 0.05$.

- *Bacteroides* Genus: The prevalence of *Bacteroides* was significantly higher in diabetic patients compared to controls (P *value* < 0.001).
- *Enterococcus* Genus: The quantity of *Enterococcus* in the gut microbiota of healthy individuals was marginally higher than in the patient group, but the difference was not statistically significant (P *value* = 0.88).
- *Clostridium cluster IV*: No significant difference was observed in the copy number of *Clostridium cluster IV* between healthy individuals and T2D patients (P *value* = 0.56).
- *Clostridium cluster XIVa*: The abundance of *Clostridium cluster XIVa* was slightly higher in the gut microbiota of the control group compared to the patient group, although the difference was not statistically significant (P *value* = 0.8).
- All Bacteria: There was no significant difference in overall bacterial abundance between the diabetic and control groups, with counts being very similar (P *value* = 0.94).

The differences in intestinal bacterial compositions between the two study groups are illustrated in Table 4, Fig. 2 and 3.

3.5. Correlation between body mass indices and quantity of targeted bacterial compositions

Spearman rank correlation analysis showed a significant positive correlation (r = 0.39, *P-value* = 0.02) between BMI and the abundance of the genus *Bacteroides*, as determined by qPCR. In contrast, no significant correlations were found between BMI and the levels of other bacterial groups (all *P-values* > 0.05). The correlations between intestinal microbiota counts and BMI are depicted in Fig. 4.

4. Discussion

In the present study, we hypothesized that T2D in humans could be linked to specific gut microbiota and that intestinal microbiota in patients with T2D differ from those in non-diabetic people. Therefore, we focused on the characteristics of the gut microbiota

	Case	Control
Phylum *Bacteroidetes*	↑ (Sig)	
Phylum *Firmicutes*		↑ (Sig)
Phylum *Actinobacteria*		↑ (Sig)
Phylum *Proteobacteria*		↑ (No Sig.)
Genus *Enterococcus*		↑ (No Sig.)
Genus *Bacteroides*	↑ (Sig)	
Clostridium cluster IV		↑ (No Sig.)
Clostridium cluster XIVa		↑ (No Sig.)
All Bacteria	↑ (No Sig.)	

Fig. 2. Differentiation of intestinal bacterial frequency between T2D patients and non-diabetic controls. Values are significantly (Sig.) different with $P < 0.05$ and no significantly (No Sig.) different with $P \geq 0.05$.

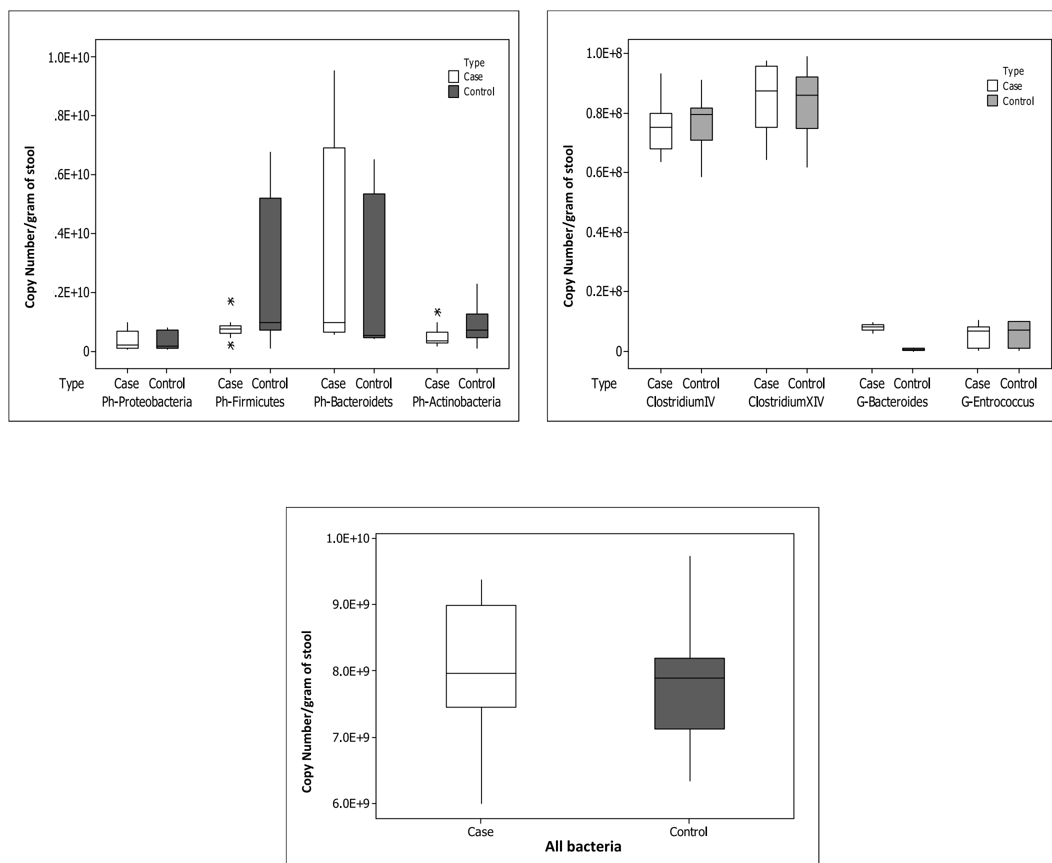

Fig. 3. Quantification of intestinal bacteria by TaqMan-real time qPCR and expression as copy number of bacteria per gram stool in human adults with T2D (white boxes; N = 18) and healthy controls (grey boxes; N = 18). Boxes show the upper (75 %) and the lower (25 %) percentiles of the data. Whiskers indicate the highest and the lowest values. Outlier Points were shown by *. Ph: Phylum, G: Genus.

population in patients with T2D compared to those in a group of healthy participants. Emerging evidence from large-scale 16S rRNA gene sequencing, quantitative real-time PCR (qPCR), and fluorescent in situ hybridization (FISH) studies has indicated a potential relationship between the gut microbiota composition and metabolic conditions including T2D and obesity [15,32,53,54]. In alignment with these observations, our findings reveal that while there were no substantial variations in the overall bacterial load between the T2D patients and the non-diabetic controls, there were significant differences in the composition of the fecal microbiota between the healthy and diabetic patients.

Our research has elucidated that the link between T2D and alterations in the composition of the intestinal microbiota is predominantly observed at the phylum and genus taxonomic levels. Notably, the relative abundance of the *Bacteroidetes* phylum was markedly elevated in individuals with T2D when compared to their non-diabetic counterparts. Conversely, the abundances of the *Actinobacteria* and *Firmicutes* phyla were significantly greater in the healthy people than in those with diabetes. As a result, the *Bacteroidetes* to *Firmicutes* ratio was significantly elevated in T2D patients relative to healthy controls. These findings concur with recent research by Larsen et al. [31], Wu et al. [40], and Le Chatelier et al. [55], which also reported a significantly higher *Bacteroidetes* to *Firmicutes* ratio in T2D patients compared to healthy individuals. In contrast, Remely et al. [29] observed a higher prevalence of the *Firmicutes* phylum in T2D group, leading to a higher *Firmicutes* to *Bacteroidetes* ratio in the diabetic subjects compared to healthy participants. Interestingly, our study found that the proportions of the *Proteobacteria* phylum were comparable between healthy individuals and those with diabetes, a finding that is consistent with studies by Remely et al. [29], Wu et al. [40], Le Chatelier et al. [55], and Larsen et al. [31], which showed no significant differences in the abundance of *Proteobacteria* between diabetic and non-diabetic groups. To our knowledge, there has been only one study [16] indicating a significantly higher abundance of *Proteobacteria* in T2D patients compared to the healthy group. Furthermore, our research revealed a significantly greater abundance of *Actinobacteria* in the healthy group compared to those with T2D, a result that differs from the findings of Larsen et al. [31], who reported no significant difference in the concentration of *Actinobacteria* between diabetic and control subjects.

In our study, the abundances *of Clostridium Cluster IV* and *Clostridium Cluster XIVa* were not significantly different between the patients and controls. Similarly, Remely et al. [29] show no meaningful differences in frequencies of these two clusters between the study groups. In the current research, the concentration of genus *Bacteroides* was significantly higher in diabetic patients compared to the controls. In contrast to this finding, Larsen et al. [31], Wu et al. [40], and Qin et al. [56] showed that despite a higher level of

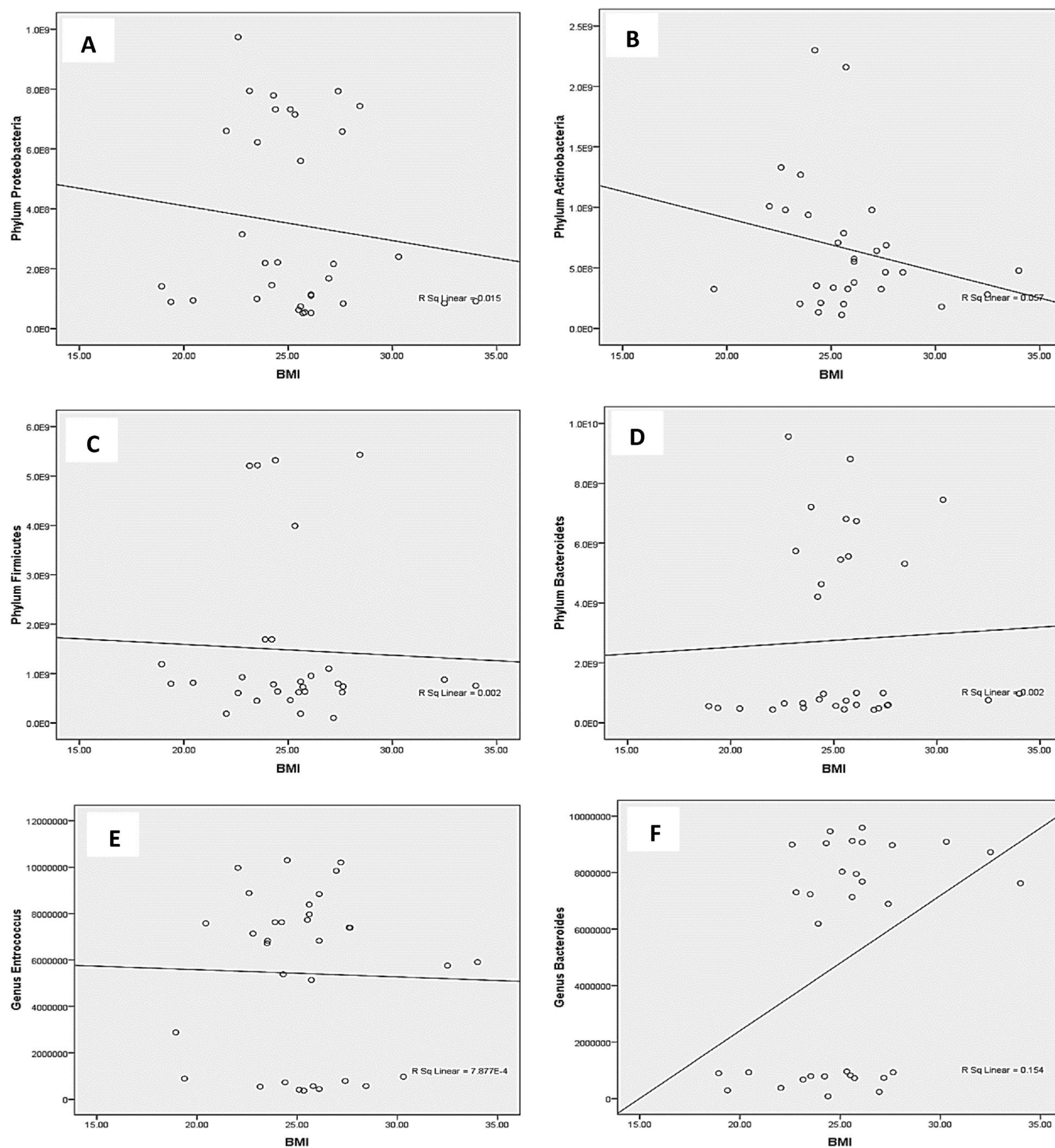

Fig. 4. Correlation of Body Mass Indices with (A) Phylum *Proteobacteria* (r = −0.12, *P-value* = 0.5), (B) Phylum *Actinobacteria* (r = −0.23, *P-value* = 0.2), (C) Phylum *Firmicutes* (r = −0.04, *P-value* = 0.82), (D) Phylum *Bacteroidetes* (r = 0.04, *P-value* = 0.79), (E) Genus *Enterococcus* (r = −0.02, *P-value* = 0.87), (F) Genus *Bacteroides* (r = 0.39, *P-value* = 0.02), (G) *Clostridium cluster IV* (r = −0.24, *P-value* = 0.17), (H) *Clostridium cluster XIVa* (r = −0.06, *P-value* = 0.73) and (I) All bacteria (r = −0.26, *P-value* = 0.14).

Bacteroides in patients with T2D compared to healthy subjects, there were no significant differences between the two groups in the abundance of this bacteria. Furthermore, in the current study, the fecal composition of genus *Enterococcus* was not significantly different between the two groups studied. This result is in agreement with the findings of Remely et al. [29] showing no meaningful changes in the abundance of *Enterococcus* between diabetic and non-diabetic subjects. In the genus and species level, we have previously shown significant differences in the quantity of some genera (*Lactobacillus* and *Bifidobacterium*) and species (*Faecalibacterium*

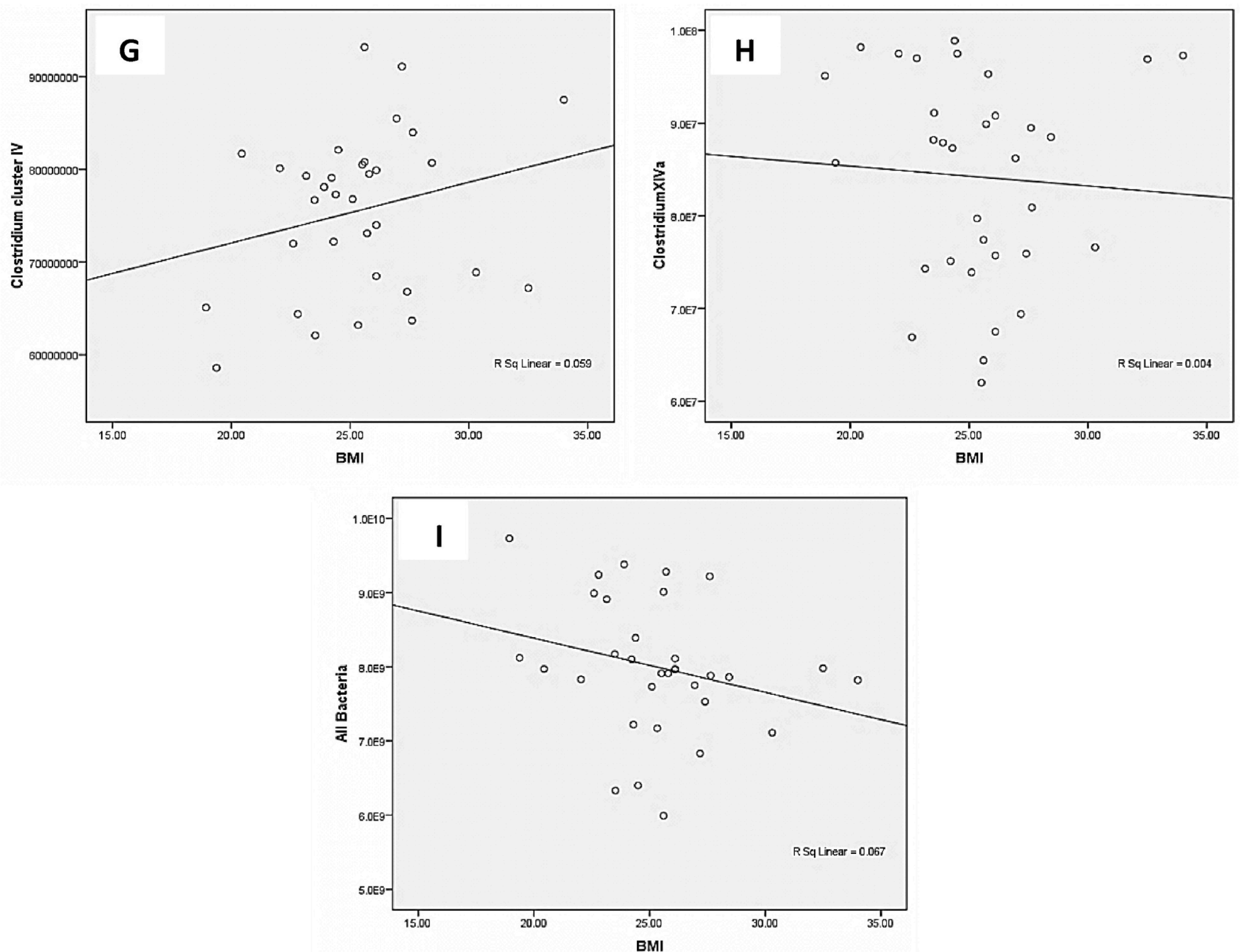

Fig. 4. (*continued*).

prausnitzii) between T2D patients and healthy individuals, supporting a link between alternation in the gut bacterial composition and diabetes [41,42]. Our findings indicate that all bacteria load, encompassing the entire microbial community, does not significantly differ between individuals with T2D and those without the condition. This observation aligns with the conclusions drawn by Larsen et al. [31] and Remely et al. [29], who also reported a lack of significant differences in the total number of intestinal bacteria between T2D patients and healthy counterparts. These collective results imply that the association between T2D and the gut microbiota is more likely to be related to alterations in the microbial community structure and balance of gut microbiota rather than the influence of a particular microorganism or changes in the overall bacterial abundance. So, gut microbiota dysbiosis may not uniformly affect all bacterial populations. This highlights the complexity of the gut microbiota's role in T2D and suggests that the disease may be associated with specific microbial signatures rather than global changes in the microbiota [57]. It's possible that the lack of significant differences in certain bacterial populations between studied groups reflects the natural variability of the gut microbiota rather than a lack of association with T2D. A core set of essential bacterial species, crucial for vital functions of the gastrointestinal tract, is consistently found across individuals living in diverse conditions and is common to all human populations [58]. The lack of difference in some bacteria could suggest their resilience to the metabolic changes associated with T2D. In some cases, the gut microbiota may exhibit compensatory mechanisms where changes in certain bacterial populations are offset by changes in others, maintaining an overall balance that does not significantly differ between healthy and diabetic individuals. Similarly, The ability to detect significant differences in bacterial populations can be influenced by the study design limitations, including the selection of participants and the sample size, disease duration, and the dynamic nature of the host-microbiota interaction. The gut microbiome is naturally dynamic and can fluctuate over time. The study's time frame might not have captured significant changes in specific bacterial populations. While it may seem counterintuitive to the hypothesis linking T2D to gut microbiota changes, it actually highlights the specificity and intricacy of these associations, warranting further research to unravel the detailed mechanisms at play.

A number of case-control studies have examined the profile of bacterial diversity in T2D patients relative to non-diabetic controls, and they have consistently found no significant differences in the bacterial diversity of the gut microbiota between these two groups. Nonetheless, these studies have identified fluctuations or imbalances in the abundance of specific bacterial phyla, clusters, genera, and

species ([31,40,41]), suggesting that the composition of the dominant fecal microbiota is significantly different between T2D patients and healthy individuals.

Considering the well-known association between T2D and obesity, it was anticipated that there would be notable correlations between BMI and various bacteria that are more abundant in individuals with diabetes. Contrary to this expectation, the analysis revealed a single significant correlation: that between BMI and the abundance of genus *Bacteroides*. Our analysis did not identify any statistically significant correlation between the participants' BMI and the frequency of *Actinobacteria*, *Bacteroidets*, *Firmicutes*, *Proteobacteria*, *Enterococcus*, *Clostridium cluster IV*, *Clostridium cluster XIVa* or total bacterial load. These findings are congruent with the results reported by Remely et al. [29] and Larsen et al. [31], indicating no significant correlation between the bacterial composition of gut microbiota and BMI in both lean and obese subjects, although the genus *Bacteroides* was not specifically evaluated in their studies. Moreover, our previous research [41,42] did not reveal significant correlations between BMI and other fecal bacterial genera, such as *Lactobacillus*, *Fusobacterium*, *Bifidobacterium*, and *Prevotella*, or specific species including *Bifidobacterium longum, Bacteroides fragilis*, and *Faecalibacterium prausnitzii*. In contrast, Million et al. [59] demonstrated a significant negative correlation between the abundance of the phylum *Bacteroidetes* and BMI in obese individuals. Koliada et al. [60] reported a significant positive correlation between BMI and the counts of *Firmicutes* phylum, but found no significant difference between BMI and the *Bacteroidetes* phylum in their study groups. Interestingly, our current study identified a significant positive correlation between BMI and the quantity of the *Bacteroides* genus, which differs from the findings of Koliada et al. [60], who observed a significant negative correlation between the concentration of the *Bacteroides* genus and BMI levels.

The variations and consistencies observed in the results of the studies comparing the bacterial composition of gut microbiota in T2D patients and healthy individuals, as well as the differing correlations between bacterial counts and BMI, may be partly attributed to a range of factors including genetic predisposition, ethnicity, geographical location, environmental and occupational exposures, medical history, physical activity levels, lifestyle, and dietary habits among study participants. In our study, we endeavored to mitigate the influence of confounding variables such as physiological factors by ensuring that healthy and patient groups were matched for age, gender, race, living environment, and the avoidance of medications or foods that could potentially impact the outcomes, such as antibiotics and probiotic products and other drugs affecting the human gastrointestinal system. Furthermore, it is important to recognize that the technical design of a study, including appropriate sampling techniques, precise design of bacterial-specific primers and probes for real-time quantitative PCR, specificity and efficiency of molecular methods used to detect and measure the number of bacteria and meticulous setup of all work steps, plays a critical role in determining the final outcomes. Consequently, some of the observed heterogeneity across studies may stem from differences in research methodologies. In summary, while it is well-established that metabolic disorders such as T2D are associated with alterations in gut microbiota composition, the causality remains ambiguous. It's unclear whether specific changes in gut microbiota community contribute to the development of T2D or if microbial dysbiosis is merely a consequence of the disease [24,61]. However, several mechanisms suggest how microbial dysbiosis could impact the pathophysiology of metabolic disorders like T2D: First, increased gut permeability due to gut microbiota dysbiosis allows bacterial endotoxins like LPS into the bloodstream. These endotoxins can trigger low-grade inflammation, which is a hallmark of T2D. The inflammation can lead to insulin resistance, a key feature of T2D, by affecting the signaling pathways in muscle, liver, and adipose tissue [35,62]. Second, dysbiosis can reduce the production of SCFAs, such as butyrate, propionate, and acetate, which have anti-inflammatory properties and improve insulin sensitivity, contributing to T2D development [63]. Third, Certain gut bacteria can influence the production of incretin hormones, such as GLP-1, which are released in response to food intake and help regulate blood glucose levels by stimulating insulin secretion and inhibiting glucagon release. Dysbiosis can disrupt the balance of these hormones, affecting glucose homeostasis and contributing to T2D [64]. Forth, The gut microbiota also plays a role in the breakdown and absorption of nutrients, influencing how the body harvests and stores energy. Changes in the microbiota composition can lead to increased energy harvest from the diet, contributing to obesity, which is a significant risk factor for T2D [35,63]. To achieve a deeper insight into the role of gut microbiota in the pathogenesis of metabolic disorders, including T2D, and to elucidate the interplay between these conditions, further long-time studies employing standardized methodologies are required.

Understanding the link between gut microbiota composition and T2D offers several broader implications and significant insights for the understanding and management of the disease. This knowledge paves the way for dietary and lifestyle interventions aimed at modulating the microbiota composition to improve metabolic health. For example, diets rich in fiber can promote the growth of beneficial gut bacteria that produce SCFAs. Additionally, the tailored use of probiotics and prebiotics emerges as a novel therapeutic approach to target dysbiosis associated with T2D. The gut microbiota's role in T2D suggests that personalized medicine approaches could be developed, taking into account an individual's unique microbiota composition to tailor treatments more effectively. Furthermore, monitoring changes in the gut microbiota could serve as a biomarker for early T2D diagnosis, enabling timely interventions and prevention strategies. In conclusion, the interplay between gut microbiota composition and T2D pathophysiology is complex but offers exciting opportunities for new therapeutic strategies and a deeper understanding of the disease.

The most important limitations of this study are as follows: First, The study included a relatively small sample size of 18 participants in each group, which may limit the generalizability of the findings. Second, The study's cross-sectional case-control design does not allow for the determination of causality. Whether the observed variations in gut microbiota are a cause or an effect of type 2 diabetes remains unclear. To determine the temporal link between changes in gut microbiota and the onset of type 2 diabetes, longitudinal studies are required. Third, Although the study attempted to control for confounding variables such as age, gender, living geographical location, and race, other factors such as diet, taking some medications with possible effects on the gut microbiota, and physical activity levels could influence the gut microbiota composition and were not fully accounted for. Forth, the study population may not be representative of other populations due to regional and ethnic differences in diet and lifestyle, which can affect gut microbiota composition. The findings may not be generalizable to populations with different backgrounds. Fifth, the use of qPCR for microbial

quantification relies on the specificity and sensitivity of the primers and probes used. There is a potential for bias if these are not optimized for all relevant bacterial taxa or if there are variations in the copy number of the 16S rRNA gene among different species. Sixth, our study relies on self-reported data for factors such as diet and physical activity, which can be subject to recall bias and may not accurately reflect participants' true behaviors. Seventh, since case-control studies are observational and retrospective in nature, they are prone to several types of bias, including: recall bias, selection bias, confounding bias, temporal ambiguity, and misclassification bias. Finally, The study focuses on the composition of the gut microbiota but does not address the functional aspects of the microbiome, which could be crucial in understanding how microbial changes relate to the pathophysiology of T2D. Addressing these limitations in future research could enhance the understanding of the relationship between gut microbiota and T2D, potentially leading to new strategies for disease prevention and treatment. Despite the aforementioned limitations, these findings contribute to our comprehension of how alterations in the gut microbiota may related to the development of diabetes and the potential connection between the microbiota and T2D. Further investigation is warranted to delve into the biological plausibility and the dynamic interplay between the intestinal bacteria and diabetes.

5. Conclusion

The results of the current research enhance our basic knowledge about the fecal bacterial communities in patients with T2D**.** Our research has identified significant variations in the copy numbers of certain gut bacteria between diabetic and healthy groups, as determined by quantitative PCR (qPCR) analysis. Therefore, it can be concluded that T2D is associated with changes in the composition of the gut microbiota. This specificity can help in understanding the role of gut microbiota in the pathogenesis of T2D. Additional research is warranted to investigate changes in other bacterial populations across various taxonomic levels in T2D subjects. The insights gained from this study could be instrumental in devising strategies to manage T2D by modulating the intestinal microbiota. Such strategies may include lifestyle modifications such as dietary adjustments, weight management, increased physical activity, and the avoidance of harmful environmental and occupational exposures. Furthermore, the strategic administration of specific probiotics and prebiotics, or other effective interventions designed to restore the beneficial and commensal microbiota within the gastrointestinal tract, could offer a viable therapeutic option.

CRediT authorship contribution statement

Shabnam Razavi: Conceptualization, Data curation, Supervision, Writing – review & editing. **Nour Amirmozafari:** Conceptualization, Methodology, Resources, Software. **Abed Zahedi bialvaei:** Validation, Visualization, Writing – review & editing. **Fatemeh Navab-Moghadam:** Investigation, Methodology, Project administration. **Mohammad E. Khamseh:** Investigation, Validation. **Fariba Alaei-Shahmiri:** Data curation, Investigation, Supervision. **Mansour Sedighi:** Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Writing – original draft.

Data availability

All data generated or analyzed during this study are included in this published article [and its tables and figures].

Ethical approval

This project was approved by the Iran University Human Ethics committee (Ethical code: IR.IUMS.REC 1395.9221133206). We received a signed form of consent from all study participants for using samples and data obtained from Food Frequency Questionnaire (FFQ) and International Physical Activity Questionnaire (IPAQ) for the analyses presented here.

Consent to participate

The DNA extracted from fecal samples and demographic data were obtained from a previous study conducted in the Microbiology Department of Medical Sciences Faculty, Iran University of Medical Sciences in Tehran, Iran. All patients provided written informed consent for studies.

Consent for publication

The participant has consented to the submission of this article to the journal. We confirm that the manuscript, or part of it, has neither been published nor is currently under consideration for publication. This work and the manuscript were approved by all coauthors.

Code availability

The code is available from the corresponding author upon request.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

T2D	Type 2 Diabetes
BMI	Body Mass Indices
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
Ct	Threshold Cycle
LPS	Lipopolysaccharide
rDNA	Ribosomal Deoxyribonucleic acid
FBS	Fasting Blood Sugar
SPSS	Statistical Package for the Social Sciences

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