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Research Article

Metagenomics analysis of the gut bacterial microbiome in D2T patients in Misan Governorate, Iraq

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Abstract: The microbiome is the genetic material of all microbes, viz. bacteria, fungi, parasites, and viruses that live within the human digestive tract; the human gut microbiome co-evolves with its host for thousands of years. Thus, these microorganisms can evaluate any host's health status, including metabolic diseases such as DM2. In this study, thirty-six stool specimens were collected from participant patients (20) and control (16) who attended Alemara laboratory in Misan governorate. The investigation period was from September 2021 to February 2022. The results showed that many types of bacteria in the human intestine belong to the phyla of *Firmicutes*, *Bacteroidetes, Verrucomicrobia, Proteobacter, Lentisphaerae, Elusimicrobia*, and *Tenericutes* species. Our findings also showed no significant differences in the microbiome between diabetes mellitus type 2 and controls (P=0.099) using different bioinformatics approaches. The *Verrucomicrobia* (2.9%), *Proteobacteria* (12.70%) and *Fusobacteria* (0.47%) display the highest percentages in diabetes mellitus type 2 compared to the control group (0.5, 9.06 and 0%), respectively. The *Firmicutes* (36.78%), *Bacteroidetes* (44.89%), *Tenericutes* (0.195%), and *Actinobacteria* (0.34%) revealed the lowest percentages in diabetes mellitus type 2 compared with the control group (39.9, 47.6, 1.7 and 0.48%), respectively.

Keywords: 16SrRNA, Microbiome, Monoplex PCR, Alpha diversity, T2D.

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Introduction

Communities of microorganisms often form relationships with more complex hosts, like plants and humans, that can range from symbiotic to parasitic and pathogenic (Lederberg & McCray 2001). This community of microorganisms is commonly referred to as a microbiome. The microbiome comprises microorganisms and their DNA (Resell et al. 2012). The human microbiome is everywhere from the skin to the intestines (Prowls et al. 2014).

Type 2 diabetes mellitus (T2D) is a chronic, multifactorial metabolic disease. It is characterized by the body's inability to absorb glucose (hyperglycemia) due to a decrease in insulin secretion, insulin action (insulin resistance), or a combination of both. T2D patients have insulin resistance or relative insulin deficiency. Environmental and genetic predispositions are the essential causes of T2D, i.e. a sedentary lifestyle and excessive feeding of an unhealthy diet may be causes of T2D. The incidence of T2D is rising exponentially because the pathogenesis of T2D is multifactorial, and the alternation of different gene products may be considered (Stumvoll et al. 2005).

The gut microbiome plays the main role in regulating glucose and energy balance. It also plays a main role in obesity and controlling blood sugar, viz. type 2 diabetes mellitus (Harris et al. 2012). Alpha diversity describes the variety in a sample, such as a faecal sample (Gilbert & Lynch 2019). Many indices show the diversity of species in a sample. The three

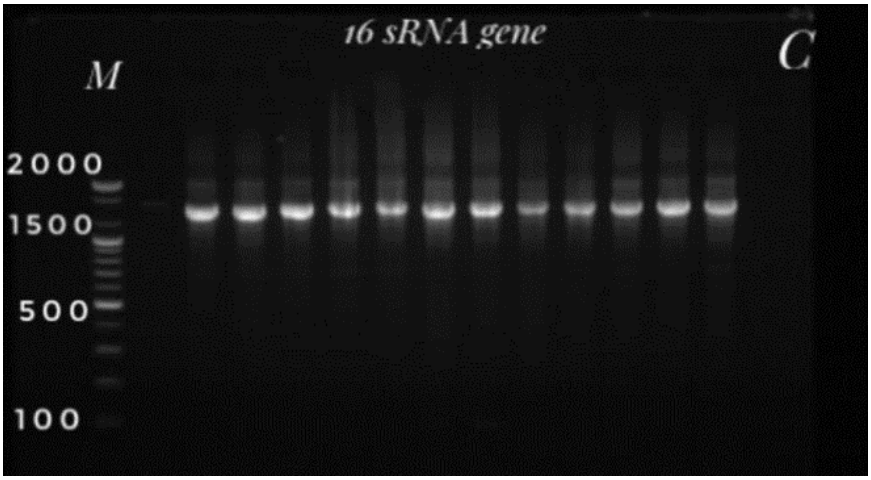

Fig.1. *Agarose gel electrophoresis of PCR product 16sRNA gene, (1500bp), where M: ladder (100bp), all sample positive results, C:* Control (only primer+ Master Mix), the gel stained by ethidium bromide (0.5 µg/ml) the electrophoresis was running in 70 volt*s for one hour.*

factors considered in diversity indices, which are the number of species, the number of singleton taxa, and the number of doubleton taxa. Since recording the microbiota's abundance is impossible. Therefore, the diversity index is used since it shows both richness and evenness (Borcard et al. 2018). This index gives rare species additional consideration (Xia & Chen 2018). Some debate on the fact that the gut bacterial microbiome has an impact on the development of DM2. Hence, this work aimed to study the differences in the gut bacterial microbiome between DM type 2 patients with those of the healthy group.

Materials and methods

Sample collection: The participants were from Misan Governorate, and their ages ranged 30-40 and 40-50 years. In six months, from September 2021 to February 2022, the samples were collected and immediately transferred under aseptic conditions to the laboratory. Thirty-six stool samples were collected, including 16 healthy and 20 DM2 patients who attended Alemara laboratory in the Misan governorate. Stool specimens were collected according to strict guidelines designed to minimize the influence of outside variables. We did not include in our study any patients who meet the following criteria: Pregnant and lactating women and patients with blood pressure, duodenal ulcer, cancer, autoimmune diseases, atherosclerosis, pneumonia, and colitis. Following the steps outlined in the QIMamp DNA stool mini kit (Qiagen, Germany) manual, DNAs were extracted from a 0.2-gram stool specimen.

Molecular identification.

PCR amplification of 16S rRNA gene of bacteria isolated from stool was performed by Polymerase Chain Reaction (PCR) assay, and the PCR conditions were performed as described by Miyoshi et al. (2005) with some modifications. The 16S rRNA primers prepared by Bioneer (South Korea) were used in this study (Miyoshi et al. 2005) following 27F 5´-AGAGTTTGATCCTGGCTCAG 3´ and 1492R 5´-GGTTACCTTGTTACGACTT 3´. All components of PCR were assembled in a PCR tube and mixed by cooling microcentrifuge for 10 secs at 850rpm. A PCR reaction mix was prepared using the AccuPower® PCR Master Mix kit, manufactured by the Bioneer Korean Company. All PCR reactions were performed in a final volume of 25µL using 4µL of extracted DNA as template, 5µL of master mix, forward primer one µL, reverse primer one µL, and molecular grade water 14µL. Then PCR amplification was carried out in a thermocycler (Prime, UK) with the following thermal conditions: an initial denaturation at 94°C for one min, followed by 30 cycles each of one min denaturation at 94°C,

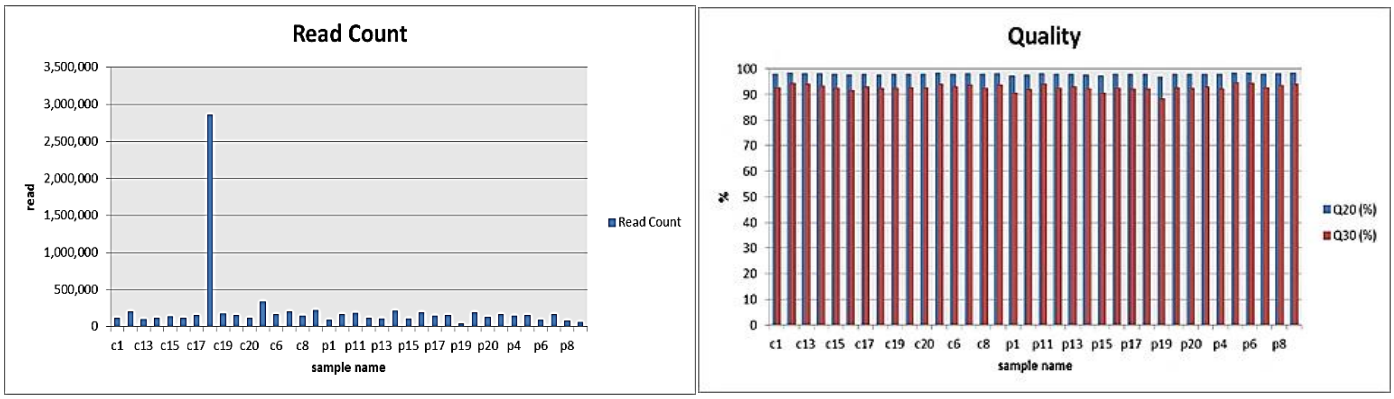

Fig.2. *Assembly of microbiome in diabetes mellitus type 2 and control group.*

35 s annealing at 52°C, one min extension at 72°C, and final extension at 72°C for seven min.

Finally, the PCR product was held at 4°C. The amplified PCR products were separated by electrophoresis (Consort, Belgium) in 1.5% agarose gel stained with ethidium bromide and visualized with a UV transilluminator (Electrofor, Italy). Next Generation Sequencing was performed by Psomagen (USA), including library preparation and quality control, and microbiome assembly, pre-processing, and clustering by CD-HIT-OUT. Analysis of abundance and diversity indices was done using the Alpha indices of Chao, Shannon, and Simpson.

Statistical analysis: The abundance of the bacterial populations in the healthy and patient groups was assessed by the independent samples PERMANOVA and PCO. The R software package was used for cluster analysis. $P>0.05$ was considered a statistically significant difference.

Results and discussion

It has been hypothesized that people with type 2 diabetes have a different gut microbiota composition than healthy people (Masella et al. 2012). In this work, pyrosequencing of the V3-V4 region of the 16S rRNA gene was used to test this hypothesis in a group of Iraqi adults with a wide age range. The 16S rRNA gene has been a mainstay of sequence-based bacterial analysis for decades. However, sequencing of the full gene has only recently become a realistic prospect. The 16S gene can provide taxonomic resolution at species and strain levels in bacteria (Johnson et al. 2019).

16S r RNA gene amplification: Subunit ribosomal gene of 16S rRNA are used to evaluate bacterial phylogeny (Woese & Fox 1977; Fox et al. 1997), and it is a conserved element of the transcriptional machinery found in all DNA-based lifeforms (Moffatt & Cookson 2017). In this gene, nine regions are stable, and nine (V1-V9) hypervariable (Chakraborty et al. 2007). Distinguishing bacteria is done by evaluating these hypervariable regions; typically, one or two are used. We can quickly analyze the bacterial makeup of several samples using NGS of 16S rRNA using gene amplicon sequencing. Quantitative estimations of the number of bacteria present in a sample can then be done by mapping the amplified sequences to a database. In this study, V3-V4 region of the 16s rRNA gene was amplified to diagnose the microbiome and its relationship to diabetes mellitus type II disease. All bacterial isolates were successfully identified using the universal bacterial pair primers used for the 16SrRNA gene fragment (Fig. 1). A positive result was recorded for all bacterial selections with an amplification band corresponding to 1500 bp. The next-generation sequencing (NGS) technologies yield useful data for describing microbial compositions in an ecosystem (Lin & Peddada 2020).

Assembly of the microbiome: The results showed many types of bacteria in the human intestine, belong to the phyla of Firmicutes, Bacteroidetes,

Table 1. The Results of pre-processing and clustering of OUT picking method (denovo) of diabetes mellitus type 2 and control group.

Results of pre-processing	
Sample count	36
Read count	1,433,100
Alpha diversity	1,042
Counts/Sample summary	
Min	3,371.0
Max	419,760.0
Median	21,595,5
Mean	39,808,333
Filtered read count	
Ambiguous	0
Wrong prefix or primer	383,206
Sequence of prefix or primer	CCTACGGG(ACGT)GGC(AT)GCAG
Low-quality	40.105
Chimera	229,673
Other	5,907,140

Sample count: The total number of samples; **Read count:** The total number of sequence reads; **Alpha diversity** corresponds to species diversity in sites habitats at a local scale; **Min:** Minimum number of sequence per sample; **Max:** Maximum number of sequences per samples; **Median:** The number separating the higher half of a data samples; **Mean:** The average number of the sequence of samples; **Ambiguous:** Filtered seqs with ambiguous bases calls; **Low-Quality**: Filtered seqs with low-quality bases (Quality score offset 33); **Chimera**: Filtered seqs with chimeric reads, Denoising: Filtered seqs with all other noise.

Verrucomicrobia, Proteobacter, Lentisphaerae, Elusimicrobia, and Tenericutes species. Our findings also showed no significant differences in the microbiome between diabetes mellitus type 2 and controls (P=0.099) using different bioinformatics approaches. The results of microbiome assembly are shown in Figure 2 elucidated the total bases, read count, Nucleotide (N)%, GC content%, and phred quality score (Q20% and Q30%), which are used to indicate the measure of base quality in DNA sequencing where high consistency of a sequenced base indicated by greater values of phred. A phred score of 20 indicates the likelihood of finding an incorrect base call among 100 bases (Goswami & Sanan-Mishra 2022). The score of 30 means that the error probability is 1 (1000 or 99.9% accuracy for bases in the assembled sequence) (Lapidus 2009). Rojo (2021) mentioned that the community assembly (CA) is a topic of growing interest in ecology due to global change. Jones et al. (2022) stated that microbiome assembly gives rise to an individual's microbiome.

Pre-processing and clustering by CD-HIT-OUT: Our results of pre-processing and clustering of OUT picking method (denovo) of diabetes mellitus type 2 and control group are shown in Table 1. Fu et al. (2012) clarified that CD-HIT is widely used for clustering biological sequences to reduce sequence redundancy and improve the other sequence analyses in response to the rapid increase in sequencing data produced by next-generation sequencing technologies. It is the latest developments in technology that affect medical next-generation sequencing research, which has the potential to enhance clinicians' diagnostic and treatment methods targeted treatments (Metzker, 2010; Casey et al., 2013).

Andermann et al. (2022) pointed out that Alpha diversity refers to diversity on a local scale, describing the species diversity (richness) within a functional community i.e. it describes the observed species diversity within a defined plot or within a defined ecological unit, such as a pond, a field, or a patch of forest. Alpha diversity provides an overview

Table 2. Community richness and diversity of microbiome in patient and control samples.

	Total		Control		Patient	
	Mean	Median	Mean	Median	Mean	Median
Variables						
OTUs	224.9166667	220.5	243.8125	202	209.8	245.5
Chao1	262.0682361	249.9981618	279.9626646	227.25	247.7526934	280.2875
Shannon	4.74858159	4.877944974	4.780537027	4.877944974	4.72301724	4.918846901
Gini-simpson	0.903188263	0.929802378	0.910449757	0.926498466	0.897379069	0.933713965
Good's coverage	0.998124033	0.998309745	0.998515959	0.998457029	0.997810492	0.998309745

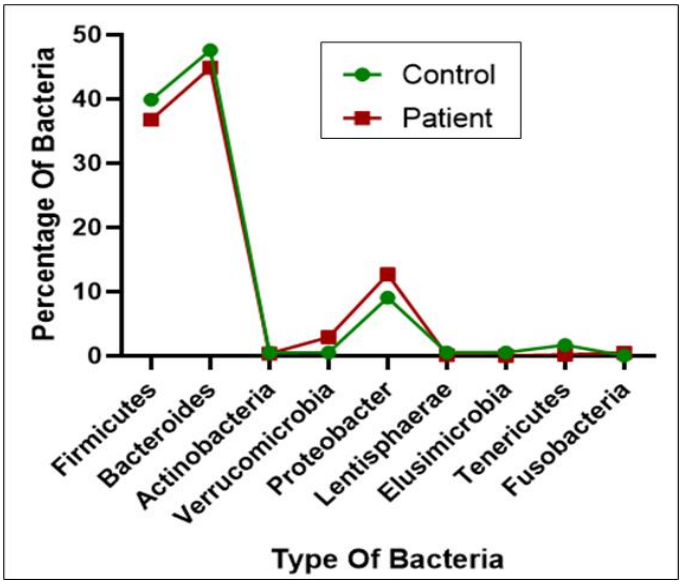

Fig.3. Percentages of bacteria in diabetes mellitus type 2 patients and control group.

of the richness (number of taxonomic categories), evenness (distribution of group abundances), or both of an ecological community's structure (Table 2). A typical first step in microbial ecology is to analyse the alpha diversity of the data from amplicon sequencing to identify differences between environments (Willis 2019).

Our results showed a high percentage of *Bacillota* (*Firmicutes*) in 39.9% of the control group compared to 36.78% of the patient group (Table 2, Fig. 3). A similar result was reported by Larsen et al. (2010), that *Firmicutes* reduced in the T2D patients. The results of the current work disagree with Zhang et al. (2013), who showed an increase in the abundance of *Bacillota* in T2D patients of China. The differences in results between Iraq and other countries may be

Table 3. Percentages of bacteria in diabetes mellitus type 2 patients and control group.

Type of bacteria	Patient%	Control%
Bacillota (Firmicutes)	36.78	39.9
Bacteroides	44.89	47.6
Actinobacteria	0.34	0.48
Verrucomicrobia	2.9	0.5
Proteobacter	12.7	9.06
Lentisphaerae	0.1	0.5
Elusimicrobia	0	0.5
Tenericutes	0.195	1.7
Fusobacteria	0.47	0

due to lifestyle and geographic region. Han & Lin (2014) reported that the change in the abundance of *Bacillota* in relation to diabetes had been attributed to differences in ancestry, geographic regions, eating habits, and research methods. A study conducted by Karlsson et al. (2013) on European female T2D patients revealed a decrease in the abundance of butyrate-producing bacteria, including *F. prausnitzii* were deduced to be highly discriminant for T2D by the Mammalian Gene Collection (MGC) model analysis, and this is in agreement with our results. Our results also revealed a high percentage of *Bacteroides* as 47.6% in the control group compared to 44.89% in patients.

The percentages of *Firmicutes* and *Bacteroidetes* increased in the control group compared to DM2 patients (Table 3, Fig. 3). Schwiertz et al. (2010) pointed out that the predominant bacterial types in healthy and DM2 patients are *Firmicutes* and *Bacteroidetes*. Qin et al. (2012) found a positive

correlation between the ratio of *Bacteroidetes* to *Firmicutes* and lower glucose tolerance. Nookaew (2013) mentioned that the pathogens that seize opportunities and enrichment of other sulfate reduction and oxidative stress-reducing microbial activities resist stress in the larger European population.

Based on the results, other bacteria such as *Verrucomicrobia*, *Proteobacteria*, and *Fusobacteria*, unlike *Firmicutes* had a higher percentage in DM2 patients than healthy people. Ley et al. (2005) and Turnbaugh et al. (2006) showed that an increase in *Firmicutes* taxa might be associated with increased energy absorption from food and low-grade inflammation. Also, our results showed a large abundance of *Verrucomicrobia* bacteria that agrees with the result of Fujio-Vejar et al. (2017) in Chilean, where a high abundance of *Verrucomicrobia* was reported. This phylum is a member of the superphylum Verrucomicrobia, which includes relatively related bacteria with unusual properties such as having a complex and dynamic inner membrane system, in some respects closer to eukaryotic cells. It includes a few genera isolates from freshwater and soil animal feces. Previous studies show that *Verrucomicrobia* may be a potential marker of type 2 diabetes (Barlow et al. 2015). Some researchers suggest that people with type 2 diabetes have a decreased number of bacteria that produce short-chain fatty acids (e.g., acetate, propionate, and butyrate) (Lv et al. 2018). As stated by Zhang et al. (2021) that there were significant differences in the number of bacteria among patients with PreDM and T2DM and the control group. Compared with the control group, Proteobacteria bacteria were significantly higher in the PreDM group (P=0.006). Fusobacterium, were positively associated with T2D as described by study of Gurung et al. (2020).

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