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ORIGINAL ARTICLE

Human gut microbiota and its possible relationship with obesity and diabetes

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Abstract

Background Obesity and diabetes are public health problems that are leading causes of death in the world. Recent surveys suggest that there is a relationship between diabetes and bacterial residents of the gastrointestinal tract.

Objective This case-control study was designed to evaluate the composition of the gut microbiota in patients with type 2 diabetes (T2DM) and obesity compared to the healthy people.

Methods A total of 91 adult subjects (25 patients diagnosed with T2DM, 48 obese patients, and 18 healthy individuals) were included in the study. The gut microbiota composition was investigated by quantitative real-time polymerase chain reaction (qPCR) method using bacterial 16S rRNA gene.

Results The frequency of all bacterial species in the obese group compared to the control group have significantly changed ($p < 0.05$) except *Bacteroides fragilis*, whereas the level of bacterial composition was not changed significantly ($p > 0.05$) in the diabetic patients versus the control ones, except for *Bacteroides* phylum and *Lactobacillus* spp. Moreover, the mean body mass index (BMI) in control, T2DM, and obese groups were 24.28 ± 3.00, 26.83 ± 3.29, and 44.65 ± 3.73, respectively. Our analysis showed a positive correlation between diabetic patients plus obese ones and the number of bacteria ($p < 0.05$).

Conclusions To sum up, these findings show that specific changes in microbial community composition are associated with T2DM and obesity. More extensive, our survey suggests that modulation of the microbiome warrants further investigation as a potential therapeutic strategy for metabolic diseases.

Keywords Gut microbiota · Obesity · Type 2 diabetes mellitus · Real-time PCR

Introduction

Over 10–100 trillion microbes colonize in each part of human body, including the skin, vagina, oronasopharyngeal cavity, and of course gastrointestinal tract [1, 2]. The gastrointestinal microbiota generally refers to the microbial composition in the gut, which contains various types of microorganisms such as bacteria, viruses, archaea, fungi, as well as phages [3]. Among all different bacterial species, which colonized in human guts, five phyla are most abundant, including *Firmicutes*, *Bacteroidetes*, *Verrucomicrobia*, *Actinobacteria*, and *Proteobacteria* [3]. These microorganisms perform variety

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of functions that the human body has no ability to do them by itself. Some studies have suggested that human gut microbiota could play key role in the host immune system, permeability of the intestine and time of pass, increasing the secretion of the metabolic endotoxin (LPS), modulation of neurohormonal function, epithelial cell proliferation, and gut barrier function. Moreover, gut microbes also perform a wide range of bile acid modifications and interfere in cholesterol reduction and in the biosynthesis of some B vitamins and vitamin K, isoprenoids, and amino acids such as lysine and threonine [4, 5]. Therefore, human microbiome can also affect metabolism and may readily lead to the obesity and its related disorders in the host.

Dysbacteriosis, which is known as dysbiosis, is a term for a microbial maladaptation or imbalance on or inside the body. This imbalance has been reported to be associated with diseases, such as malnutrition, inflammatory bowel disease, neurological disorders, rheumatoid arthritis, autism, allergies, and cancer, as well as obesity and diabetes [6–9].

Obesity and type 2 diabetes (T2DM) are global health challenges [3]. Obesity represents the intensity of body fat that results in a higher body mass index (BMI), which may negatively contribute to morbidity and mortality. According to the World Health Organization (WHO) mean BMI guideline [10], generally accepted BMI ranges are underweight, under 18.5 kg/m^2; normal weight, 18.5–25 kg/m^2; overweight, 25–30 kg/m^2; and obese, over 30 kg/m^2. BMI is a major indicator of health, so the high levels of it can increase the risk of various diseases such as cardiovascular disease, diabetes, chronic kidney disease, retinopathy, and several cancers. Additional studies identified that alteration in the equilibrium of the bacterial phyla, especially the low levels of *Bacteroides* and high *Firmicutes* abundances, conduce increased weight gain and obesity [11]. Diabetes is a chronic disease that is correlated with the failure of the pancreas to produce enough insulin relative to body needs, and the body is unable to effectively utilize the insulin it produces [12, 13]. T2DM is also a main cause of renal failure, retinopathy and blindness, and limb amputations that transpire in a situation with decreased blood flow and neuropathy in the feet. Insulin resistance is a critical factor linking obesity that promotes the risk of diabetes, as increasing adipose tissue mass related to insulin resistance [14].

Danish et al. in 2010 showed that the healthy individuals significantly had higher amounts of phylum *Firmicutes* and *Prevotella* spp. compared to T2DM patients [15]. In other studies, Gram-negative bacteria in T2DM patients have developed quite more in intestinal microbiota, principally those parts of the *Proteobacteria* and *Bacteroidetes* phyla [16, 17]. But the question is, how we can link these results about T2DM patients to their BMI? Or better say, how we can explain the association between obesity and T2DM with the imbalance of gut microbial populations? Hence, to achieve a correct answer, we designed this study in our country.

Materials and methods

Study enrollment and collection of specimens

In this study, we collected 91 stool samples from the Institute of Endocrinology and Metabolism Research and Training Center in Tehran, Iran. Among these 91 patients, 25, 48, and 18 of them were T2DM, obesity, and non-diabetic individual patients, respectively, and the mean age of all volunteers was 56 ± 8 years. We matched age, gender, and their current living environment for all 91 participates.

The participants were included using the following criteria: (1) Obese patients with BMI > 40 who have not had diabetes; (2) T2DM patients with glycated hemoglobin (HbA1c) < 10% whose T2DM was diagnosed less than 5 years; and (3) no subjects had taken antibiotic, probiotic/prebiotic products, or any other medical treatment influencing gut microbiota for 2 months before the beginning of the samples collection. In addition, patients were interviewed for their history of gastrointestinal diseases, dietary habits, and physical activity levels in both case and control groups. Participants who had suffered from gastrointestinal disorders during this period were also excluded from the study.

All participants' stool samples were collected on three occasions by sterile cups instantly after defecation and brought them to the laboratory in 2 h. Fecal samples were immediately stored at 70 °C upon arrival in microbiology laboratory.

Extraction and purification of DNA from stool samples

Total microbial DNAs were extracted from all stool specimens using QIAamp® DNA Stool mini kit (Qiagen Retsch GmbH, Hannover, Germany) according to the manufacturer's protocol. DNA quality and concentrations were determined by Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and agarose gel electrophoresis. Whole extracted DNAs were immediately stored at 20 °C.

Design of the oligonucleotide primers and probes

The specific sequences of primers and TaqMan probes are shown in Table 1. Desired specificity of the primer pairs was verified by submitting the sequences to the FASTA database search program provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and Probe Match program provided by the Ribosomal Database Project (rdp.cme.msu.edu/ html/). The primers and probes against selected species or group of specific target sequences were synthesized commercially by Pishgam Biotech Co., Iran.

Table 1 16S rDNA gene-targeted specific primers and TaqMan probes used in this study

Target bacteria	Primer Probe[a]	Oligonucleotide sequence	Product size (bp)	Tm	Ref.
F. prausnitzii	Primer F	ATAATGACGGTACTCAACAAGGA	171[b]	59 °C	21
	Primer R	ACAGTTTTGAAAGCAGTTTATGG			
	Probe	ACTTCCAACTTGTCTTCCCGCCTG			
B. fragilis	Primer F	CGAGGGGCATCAGGAAGAA	136	59 °C	21
	Primer R	CGGAATCATTATGCTATCGGGTA			
	Probe	CTTGCTTTCTTTGCTGGCGACCG			
B. longum	Primer F	GTGGCTTCGACGGGTAG	200	59 °C	21
	Primer R	ACGGGTAAACTCACTCTCG			
	Probe	TTGCTCCCCGATAAAAGAGGTTTACA			
G Lactobacillus	Primer F	GTCTGATGTGAAAGCCYTCG CCAGGG	204[c]	60 °C	16
	Primer R	TATCTAATCCTGTTYG YCACCGCTA			
	Probe	CACATGRAGTTCCACT			
G Bifidobacterium	Primer F	GGTTAACTCGGAGGAAGG GTACCGG	85	60 °C	16
	Primer R	CCATTGTAGCA CGTCAGATCATCA			
	Probe	TGCCCCTTACG			
G Fusobacterium	Primer F	GTATGTCRCAAGCGTTATCC AACGCA	100	60 °C	16
	Primer R	ATACRGAGTTGAGC CCTAGACGCG			
	Probe	CTTTACGCCCAAT			
Ph Firmicutes	Primer F	CGAACGGGATTAGATACC	186	60 °C	This
	Primer R	CGAATTAAACCACATACTCC			
	Probe	CCCCGTCAATTCCTTTGAGTTT			
Ph Bacteroidetes	Primer F	GTGGTTTAATTCGATGATACGC CGCT	154	60 °C	This
	Primer R	CGTTATGGGACTTAAG CCTCACGG			
	Probe	CACGAGCTGACG			
Ph Proteobacteria	Primer F	CAAAKACTGACGCTSAGGTG	96	60 °C	This
	Primer R	GGCACAACCTBCAARTCG			
	Probe	AATCCTGTTTGCTCCCCACGCTTTC			
Ph Actinobacteria	Primer F	CCGTTACTGACGCTGAGGAG GCGGGA	141	60 °C	This
	Primer R	TGCTTAACGCG TAGATACCCTGGTA			
	Probe	GTCCACGCCGTA			

[a] Primers F (forward), R (reverse), and probes targeting the 16S rDNA gene

Real-time PCR conditions and optimization

Real-time TaqMan qPCR in Rotor-Gene 6000 real-time PCR cycler (Qiagen Corbett, Hilden, Germany) was used to characterize the bacterial DNA present in the stool samples. Triplicate samples were routinely used for the determination of DNA by real-time PCR, and the mean values were calculated. The real-time qPCR reaction was performed in a total volume of 20 ml including 0.5 ml of forward primer, 0.5 ml of reverse primer, 0.5 ml of TaqMan probe, 12 ml of Probe Ex Taq (Probe qPCR) Master Mix (Takara Bio, Shiga, Japan), 1 ml of template DNA, and 5.5 ml sterilized ultrapure water. The real-time qPCR reaction conditions for amplification of DNA were 95 °C for 30s, followed by 40 cycles of denaturation at 95 °C for 5 s, and annealing/extension at different temperatures associated with any bacteria for 30 s. Negative controls including all the elements of the reaction mixture except template DNA were performed in every analysis. According to our previous study [18], positive control strains used in this study were obtained from the American Type Culture Collection (ATCC).

To construct standard curves for the real-time PCRs, all bacterial standard strains were cultured on BHI agar (Merck, Germany). A suspension was made in BHI broth (Merck, Germany) and DNA was extracted. The DNA concentration was determined 3 times by using the Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, USA), and the mean value was used for further calculations. Standard curves were created according to Applied Biosystems tutorials [19] and normalized to the copy number of the 16S rRNA gene for each species.

Statistical analysis

Statistical analysis was performed with SPSS for Windows, version 18.0 (SPSS, Inc., Chicago, Ill.) and Minitab version 16.2.0. An independent sample *t* test was used to compare the means of different variables between the study groups. Linear correlation between the variables was estimated by Pearson correlation. Ninety-five percent confidence intervals (CI) for sensitivity and specificity were calculated. All data were expressed as mean ± standard deviation, and the real-time

Table 2 Weight and BMI variables in control, diabetic, and obese groups

	Normal	Diabetic			Obese			Diabetic/Obese	
	Mean ± SD	Mean ± SD	Mann-Whitney test		Mean ± SD	Mann-Whitney test		Mann-Whitney test	
			T	Sig.		T	Sig.	T	Sig.
Weight	64.38 ± 6.80	74.27 ± 10.67	− 3.314	0.002	120.5 ± 16.58	16.88	< 0.001	− 10.89	< 0.001
BMI	24.28 ± 3.00	26.83 ± 3.29	− 2.425	0.021	44.6 ± 3.73	19.44	< 0.001	− 17.66	< 0.001

PCR results were presented by Box and Whisker charts, graphically.

Results

Table 2 summarizes the weight and the BMI variations in all the three groups. Pursuant to this table, the means of weight in control, diabetic, and obese groups were 64.38 ± 6.80, 74.27 ± 10.67, and 120.58 ± 16.58, respectively. Furthermore, the means of BMI in these three groups were 24.28 ± 3.00, 26.83 ± 3.29, and 44.65 ± 3.73, respectively.

In this case-control study, real-time PCR analysis was performed to evaluate the differences in composition of fecal microbiota in diabetic, obese, and healthy individuals for *F. prausnitzii*, *B. fragilis*, *B. longum*, *Lactobacillus* spp., *Bifidobacterium* spp., *Fusobacterium* spp., *Firmicutes* phylum, *Bacteroidetes* phylum, *Proteobacteria* phylum, and *Actinobacteria* phylum. Observations related to quantification of bacterial groups are represented in Table 3 and illustrated in Figs. 1 and 2.

According to Table 3, frequency of bacterial species in obese group in comparison with control group are changed significantly ($p < 0.05$) with one exception, *Bacteroides fragilis*. On the other hand, none of bacteria in diabetic group were not changed significantly ($p > 0.05$) versus control group, except *Bacteroides* phylum and *Lactobacillus* spp. Figures 1 and 2 can show us patterns of distribution of fecal microbiota composition in different weight and different groups, respectively. Impressive observations related to quantification of bacterial genera were as follows:

- *Lactobacillus* and *Bifidobacterium* spp: Weight gain significantly reduced *Lactobacillus* spp. copy number between the control and the diabetic vs the obese subjects ($p < 0.001$).
- *Fusobacterium* spp: The quantity of *Fusobacterium* spp. did not have correlation with the weight in the T2DM group and healthy ones ($p < 0.001$), though there was significant changes in *Fusobacterium* spp. frequency between the obese group vs other ones ($p < 0.001$).
- *Bacteroidetes* phylum: Surprisingly, *Bacteroidetes* phylum was significantly more frequent in the diabetic patients compared with the control and obese subjects ($p < 0.001$).
- *Firmicutes*, *Proteobacteria*, and *Clostridium cluster iv* phyla: No significant difference was observed in copy number of *Firmicutes*, *Proteobacteria*, and

Table 3 Different types of bacteria in the control, diabetic, and obese patients

Type of bacteria	Control ($n = 18$)	Diabetic ($n = 25$)			Obese ($n = 48$)		
	Copies/g of fecal	Copies/g of fecal	Levene's test		Copies/g of fecal	Levene's test	
			z	Sig.		*z*	Sig.
B fragilis	7.7E+15 ± 3.3E+16	3.1E+08 ± 8.4E+08	− 0.86	0.384	5.8E+07 ± 1.3E+08	− 1.010	.313
B longum	2.3E+10 ± 2.9E+10	4.2+10 ± 8.5E+10	− 0.032	0.975	2.3E08 ± 5.1E+08	− 6.175	< 0.001
F prausnitzii	7.7E+15 ± 3.3E+16	4.1E+07 ± 1.5E+08	− 0.987	0.319	2.8E+08 ± 4.3E+08	− 3.398	.001
Ph Bacteroidetes	1.8E+10 ± 2.3#+10	8.5E+11 ± 2.9E+11	− 5.330	< 0.001	8.9E+09 ± 1E+10	− 2.441	.015
Ph Firmicutes	5.2E+10 ± 2.8E+10	2.9E+10 ± 2.2E+10	− 0.333	0.739	3.4E+08 ± 1.1E+09	− 6.201	< 0.001
G Bifidobacterium	2.2E+10 ± 2.8E+10	2.3E+10 ± 1.7E+10	− 0.333	0.739	3.4E+08 ± 1.1E+09	− 6.201	< 0.001
Ph Proteobacteria	5.2E+10 ± 2.8E+10	6E+10 ± 4.7E+10	− 0.032	0.975	6.9E+08 ± 1.9E+09	− 6.223	< 0.001
C cluster iv	1.7E+11 ± 1.2E+11	1.3E+11 ± 1.1E+11	− 1.382	0.167	8.6E+08 ± 1.3E+09	− 6.223	< 0.001
G Fusobacterium	4.7E+10 ± 7.9E+10	3.8E+10 ± 4E+10	− 0.934	0.35	1.3E+07 ± 4.6E+07	− 6.235	< 0.001
G Lactobacillus	4.2E+09 ± 3.1E+09	2.5E+10 ± 2.1E+10	− 4.591	< 0.001	2.4E+08 ± 7.7E+08	− 5.770	< 0.001

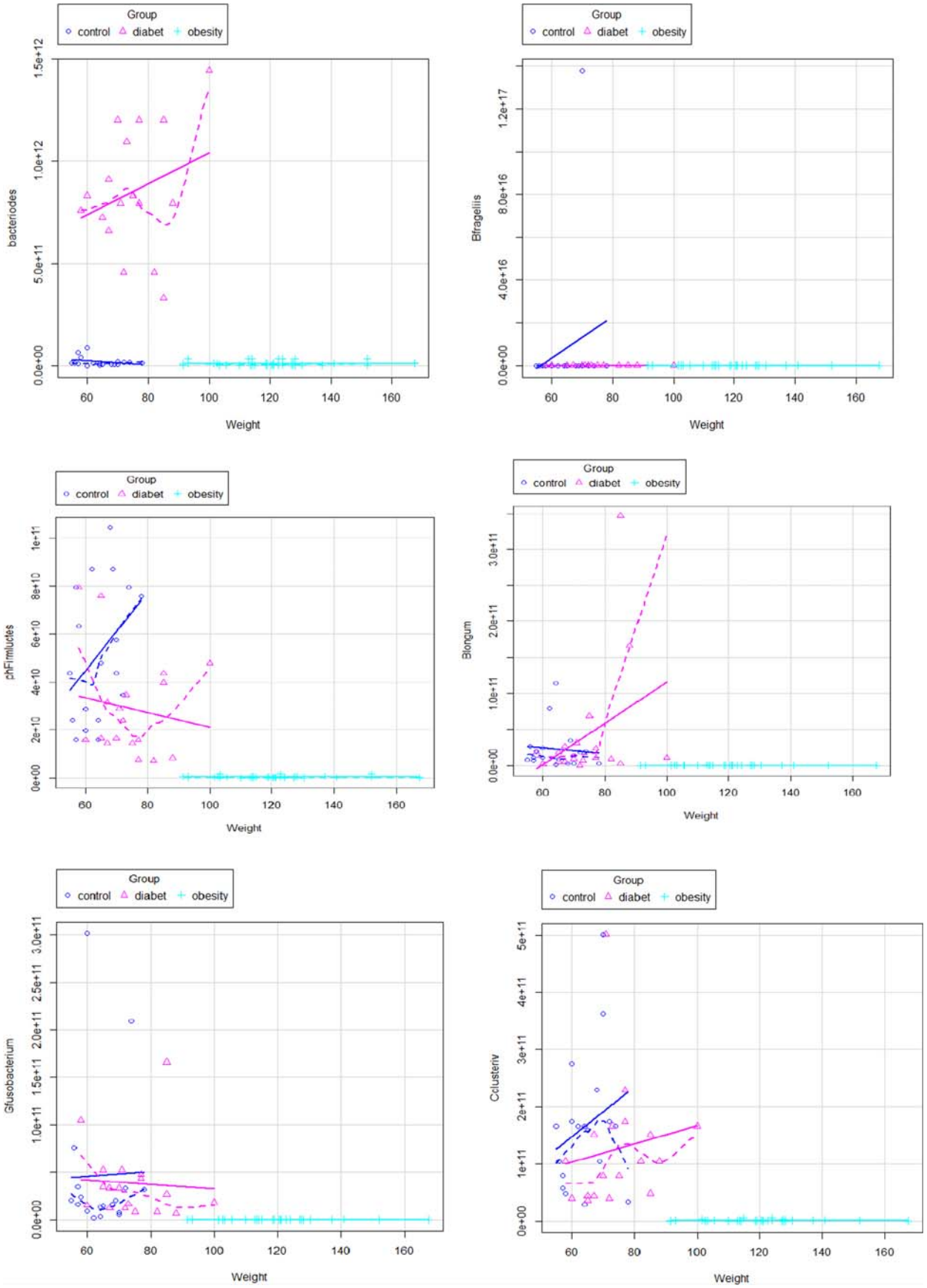

Fig. 1 The scatter plots of various types of microbiota in control, obese, and diabetic patients for different weights

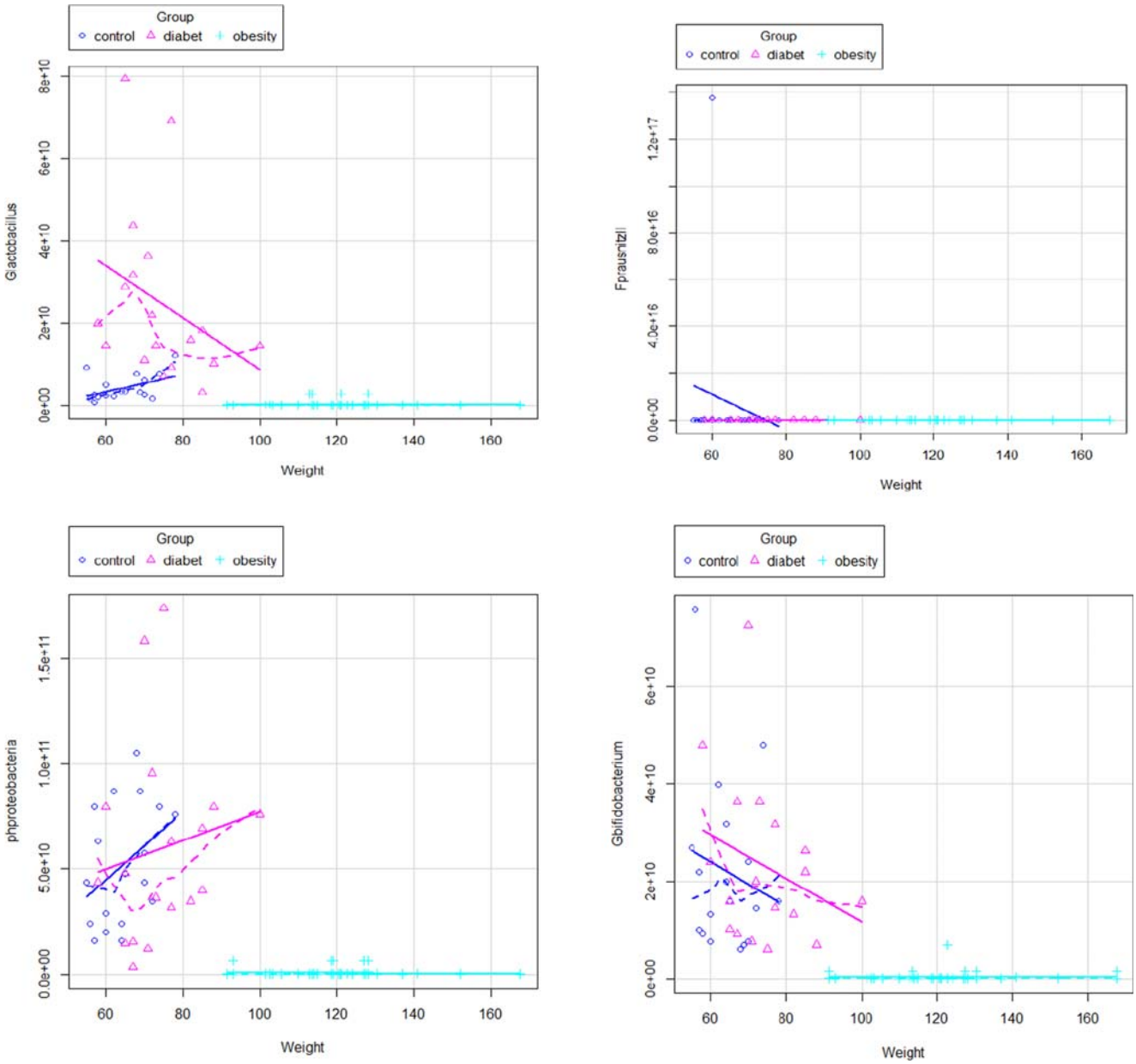

Fig. 1 (continued)

Actinobacteria phyla between the T2DM and the control group, although weight gain significantly reduced copy number of these bacterial phyla in the obese subjects ($p < 0.001$).

Discussion

The current research showed that the intestinal microbiota composition of healthy is heterologous as compared with the diabetic and obese patients. Various studies have shown that changes in compounds of specific genera and intestinal bacterial species in human or animal models may lead to prolonged diseases like diabetes, obesity, IBD, cancer, and autism [20, 21]. The study of Murri et al. on the T1D patients displayed a noteworthy distinction in the *Lactobacillus*, *Bifidobacterium*, *Prevotella*, *Clostridium*, and *Bacteroides* genera levels as well as *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* phyla levels among diabetic and control groups [22]. In the present study, we discovered that the level of *Firmicutes* phylum in healthy subjects was considerably higher than diabetic patients in contrast with the findings of Remely et al. and Larsen et al. [23, 24]. Wu et al. [25] showed a significant higher concentration of *Bifidobacterium* in control groups in comparison with T2DM patients; however, in our study it

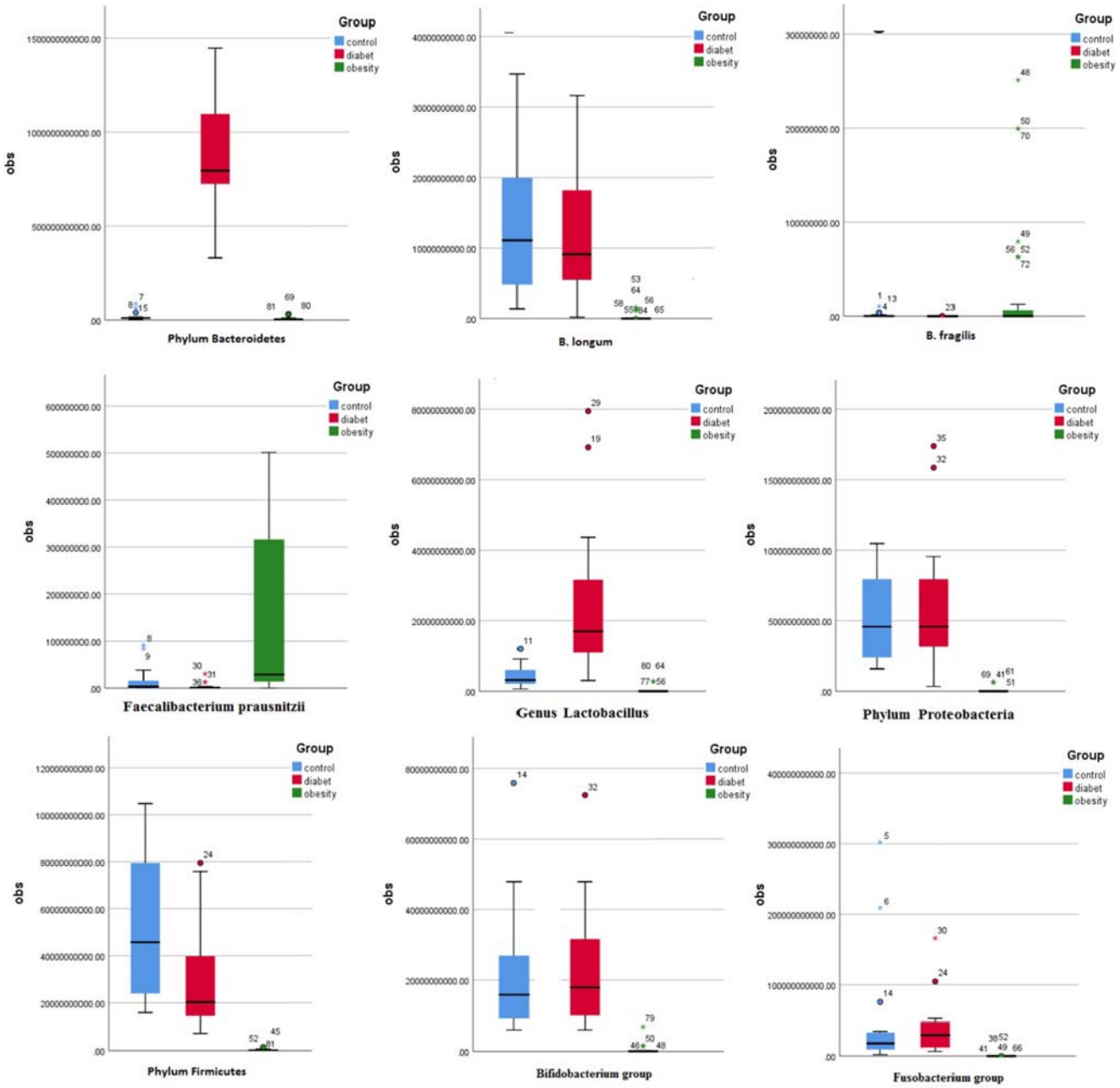

Fig. 2 Number of different types of microbiota in control, diabetic, and obese groups

was higher than T2DM. Also, Remely et al. did not show important dissimilarity in the number of copies of *Bifidobacterium* genus among the control and case individuals [24]. It should be noted that in our previous research, the *Bifidobacterium longum* species concentration belonging to the genus *Bifidobacterium* was not significantly different between diabetic and non-diabetic individuals [26]. We also discovered that the frequency of the *Prevotella* and *Fusobacterium* groups in the diabetic group were reasonably higher than the healthy ones. However, the alterations were not statistically noteworthy. Constantly, Remely et al. [24] and Larsen et al. [23] did not show a significant difference in *Prevotella* level between T2DM patients and healthy subjects. Correspondingly, according to Field et al. [27], *Fusobacterium* was not expressively different in stool specimens collected from T2DM patients related to the controls. Nevertheless, Casarin et al. [28] reported that *Fusobacterium* genus was much lower in the control group than in T2DM patients.

Most likely, T2DM is associated with the substitution in the balance of intestinal microbiota, neither the achievement of a microbe nor a simple change in diversity. For example, Wu et al. [25] investigated the levels of bacterial assortment in

T2DM and non-diabetic patients and showed that there was not any substantial difference between bacterial species in the two groups. Though, they pointed to a meaningful change in the number of bacterial phyla, genera, and species [25]. They believed that the combination of dominant bacteria in the intestinal microbiota of T2DM individuals was not similar to healthy subjects. Regarding previous studies that indicated an association between T2DM and overweight/obesity, we also showed significant correlation between BMI and all the microbial groups, *Bacteroides fragilis*.

Remely et al. [24] and Larsen et al [26] and our results are consistent in overweight individuals, but it contrasts with some studies on the relationship between BMI and this group of bacteria [29, 30]. On the other hand, Million et al. [30], Ignacio et al. [29] showed a positive and noteworthy connection between *Lactobacillus* concentration and BMI in obese subjects, and Saber et al. [31] exhibited a meaningful negative correlation between *Lactobacilli* count and BMI. As with our findings, Million et al. [30], Collado et al. [32] and Remely et al. did not correlate results between *Bifidobacterium* and BMI. In contrast, Ignacio et al. [29] found a negative correlation between BMI and *Bifidobacterium*. Andoh et al. [33] showed a significant positive correlation between *Fusobacterium* and BMI; nevertheless, in the present study, there was no association between this bacterial frequency and BMI in the studied groups. Si et al [34] reported that *Prevotella* spp. have a significant correlation with BMI, but it is not in line with our results. These controversial results about alterations in the composition of intestinal bacteria in T2DM patients and also ambiguous findings in relation between different groups of bacteria and BMI can be explicated by heterogeneity in assorted factors like genetic background, ethnicity, geographical location, environment and occupation, disclosures, medical history, potential underlying diseases/disorders, lifestyle habits, and dietary routines of individuals during studies.

In the present study, we tried to limit the maximum perplexing variables where the members in the T2DM and control group were coordinated by age, sex, race, living environment, and non-interventional medications and food that might affect the outcome (such as various antibiotics, probiotics, and prebiotics). It should be kept in mind that the design of a technical study (i.e., a specific bacterial primer and probe design for a real-time qPCR) is of significant importance in the final output of the study. Thus, some of these heterogeneities may return to the study design. In general, in the present study, we exhibited that T2DM and obese are associated with changes in the gut microbes. Conversely, due to the scheme of this study, we remained restricted to creating a causative connection concerning the changes in the intestinal microbial composition and the disease. Moreover, it is uncertain whether T2DM and obese are created by microbial changes or that it is just a replication of the condition of the disease. This matter should be taken into consideration in longitudinal research.

Conclusion

The outcomes of this study enhance basic knowledge about the gut microbiome in T2DM and obese patients. This survey shows the abundance of different types of bacteria in the gut, which in turn can affect health. In addition, eating habits and probiotics supplements may induce change in the gut microbiota and stabilize microbial communities, which help to prevent or delay T2DM and obese. Nowadays, there is a clear need to continue to explore the roles of the microbiome in obesity and T2DM to facilitate the development of effective therapeutic strategies.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This project was done based on the ethical guidelines as previously approved by the Iran University of Medical Science, Tehran, Iran (project no: IR.IUMS.REC.1397.1072).

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