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Original article

Changes in *Akkermansia muciniphila* and its relationship with dietary habits in type 2 diabetic obese patients

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Summary. *Background:* Recent studies have suggested that gut bacteria play a crucial role in obesity and diabetes. *Akkermansia muciniphila (A.muciniphila)*, which is a mucin-degrading bacterium and resident of the mucus layer, could affect gut barrier function through its composition and interaction with the host. This study was aimed to investigate *Akkermansia muciniphila* changes and its relationship with dietary habits in type 2 diabetic obese patients. *Methods:* In this case-control study, diabetic (n=36) and non-diabetic obese subjects (n=32) were recruited. Fasting blood sample and fecal samples were collected from all participants. Food frequency questionnaire (FFQ) was used to evaluate dietary habits of participants. Additionally, DNA extraction and qPCR were done for *A.muciniphila.*Data was analyzed by SPSS (Ver.13). *Results:* Mean *A.muciniphila* colony forming unit (CFU) was significantly different between two groups (p=0.022). Results showed an inverse correlation between fasting blood sugar (FBS) and *A.muciniphila* CFU in healthy group (Beta=-0.395, p=0.017). There was a positive correlation between *A.muciniphila* and dairy consumption in the diabetic group (Beta=0.355, p=0.034). *Conclusion: A.muciniphila* counting was different among diabetic and non-diabetic subjects and had a strong relationship with dietary habits. Nevertheless, further studies are required.

Keywords: *Akkermansia muciniphila*, Type 2 diabetes , Dietary habits

Introduction

Over the last decades, the prevalence of obesity and associated disorders such as the metabolic syndrome and type 2 diabetes (T2D) has increased dramatically worldwide. In addition, the risk of developing other diseases namely atherosclerosis, nonalcoholic fatty liver disease, and certain cancers increase with obesity. Based on the results obtained from several studies, the gut microbiota majorly contributes to obesity and related disorders (1). The world prevalence of diabetes among adults (aged 20–79 years) was 6.4% in 2010, and probably it would increase up to 7.7% by 2030. It is expected that between 2010 and 2030, the number of adults with diabetes in developing and developed countries would increase up to 69% and 20%, respectively (2). Obesity (abdominal or central) and insulin resistance associated with obesity are the main factors

of T2D (3). Moreover, size of adipocyte, type of food intake, and physical activity are other factors related to obesity. For this purpose, human intestinal microbiota has been evaluated to determine their influence on human metabolism and physiology. The human intestine plays an important role in balancing and maintaining the critical reactions and protecting the human cells. Studies indicated that intestinal bacteria are different in number in lean and obese individuals (1, 4-6). Several studies have been accomplished on mouse models with a genetic tendency for obesity in order to survey the impact of the gut microbiota composition on body weight gain and adiposity (4). Studies have revealed that gut microbiota is associated with the development of adipose tissue, insulin resistance, and mild inflammation which are precursors of obesity. Since 2010, the relationship between gut bacteria and obesity (microbesity) has been stressed by numerous studies (7). *Akkermansia muciniphila* (*A. muciniphila*) is one of the main bacteria which is found in the intestinal mucosal layer of humans and has a protective role against external disturbing factors. *A.muciniphila* also has a positive effect on obesity and its comorbidities(8). Researchers claimed that the number of this bacterium was much less in obese and diabetic mice. Additionally, non-digestible fibers such as oligofructose represent a positive effect on the gut microbiota by restoring the normal bacterial population. The intestinal barrier is protected by the presence of these bacteria and on the contrary, their absence leads to weight gain, fat storage, and inflammatory reactions in the adipose tissue, and eventually insulin resistance. Scientists have come to this understanding that adding *A.muciniphila* to the diet of mice that became obese by consuming a high fat diet revealed significant results, however the same results were not observed in normal mice. In the obese mice, fat accumulation and obesity-related metabolic defects decreased but it had no effect on their appetite. Interestingly, after adding the bacteria to the diet of mice, the concentration of the bacteria increased (7). Increasing the bacteria maintains the rate of blood sugar at an appropriate level; normalizes the adipose tissue, adipogenesis, and fatty acid oxidation; and improves the intestinal barrier function (9). Studies indicated that healthy and live bacteria can make such results, since heat-killed bacteria were not efficient (8, 9). The development of obesity and metabolic syndrome are complex processes involving genetic and environmental factors. Diet is one of the main factors affecting the composition of the intestinal bacteria. Intestinal flora has a complex metabolic activity and is different between lean and obese individuals. Losing weight in obese people can lead to the improvement of the normal intestinal flora. A diet rich in fiber improves the intestinal flora by reducing the levels of pathogenic bacteria(1). Due to the lack of human studies determining the relationship between *A.muciniphila* and metabolic factors and also eating habits, the object of this study was to compare the *A. muciniphila* CFU count in the stool of T2D patients and non-diabetic patients, and asses its relationship with dietary habits.

Material and methods

Participants

This case-control study included 36 diabetics and 32 non-diabetics (overweight and obese) with a body mass index (BMI) ranged 25-35 kg/m^2. Patients were selected from medical centers of Tabriz University of medical sciences by simple sampling. Controls were selected among non-diabetic subjects who were matched according to BMI, age (18-50 years), sex and habitat. Additionally, medical and family history was collected from all participants. Inclusion criteria were having T2D, being in the BMI range of 25-35 kg/m^2, 18-50 years, and not receiving antibiotic or probiotic treatment. Exclusion criteria were using insulin, vitamin, minerals, oils rich in omega 3 fatty acids, receiving antibiotic or probiotic treatment in the last 6 months, and diagnosed with other disorders.

Anthropometric measures

Anthropometric measurements were done with light clothing without shoes. Weight was measured to the nearest 0.1 kg using a standardized analogue scale (Seca, Hamburg, Germany), and height was measured to the nearest 0.1 cm using a portable stadiometer (Seca). Moreover, BMI (weight in kilograms divided by height in meters squared) was calculated for each individual. All of the measurements were carried out by an expert and in a constant condition to reduce measurement error.

Nutritional information

Food frequency questionnaire (FFQ) was used to evaluate dietary habits of participants (10). The questionnaire consisted of 132 items regarding dairy, bread and cereal, meat, vegetable, fruit, fat, and sugar consumption. The FFQ was filled out for each individual according to daily, weekly, monthly, or yearly consumption and eventually calculated based on weekly usage.

Serological tests

Fasting blood sample were obtained from all participants in order to assess fasting blood sugar (FBS), cholesterol and triglycerides and sent immediately to the laboratory. Blood samples were centrifuged and stored at -70˚C and then measured using enzymatic-colorimetric techniques.

DNA extraction and qPCR

Fecal samples from healthy and diabetic participants were collected. One gram of fresh fecal samples was used and suspended in 10 milliliter of phosphate-buffered saline (PBS). The suspension was vortexed for 10 minutes and centrifuged at 1000 *g* for 1 minutes to remove debris (Intestinal epithelial cells, plant cells, etc.). One milliliter of the supernatant was transferred to a new tube and then centrifuged at 10000 *g* for 10 minutes to plate all the bacteria. After discarding the supernatant, the plate of bacteria was used for extracting DNA by using a QIAamp DNA stool mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions.

After aligning the 16s ribosomal RNA sequences of human intestinal flora and *A.muciniphila* (Clustal w software), the specific primers were designed by Oligo 7 software from the variable regions of the 16S rRNA gene sequence of *A.muciniphila*. The GenBank program from NCBI (BLASTn) was used to verify that both primers were specific to *A.muciniphila* only. The sequences of primers were:

AKmF:5'-TTACTCTAGTCTCGCAGTATCATG-3' and

AKmR:5'-AAGATAGTACCACAAGAGGAAGAG-3'.

The size of the PCR product for using in Real Time PCR was 181 bp (base pair). To check the specificity of the designed primer, PCR amplification was performed using the template DNA from several intestinal bacteria (data not shown). Quantitative PCR amplification was performed with a BioRAD system (IQ5, USA). Each 20 µL reaction mixture was composed of Power SYBR green PCR master mix (Applied Biosystems, United Kingdom), 0.4µM of the specific primers, and 50ng of template DNA. The Q-PCR conditions were as follows: 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 s, 57˚C for 30 s, and 72˚C for 15 s, and a final extension at 72˚C for 5 min. The melting curve was drawn after amplification to distinguish the specificity of the PCR product.

Standard curves were created using serial 10-fold dilutions of Genomic DNA from *A.muciniphila* strain Muc (ATCC® BAA-835™). The bacterial concentration of each sample was calculated by comparing the cycle threshold (CT) values obtained from the standard curve. All samples were analyzed in triplicates.

This experiment was repeated ten times for different ranges of standard *A.muciniphila*. Mean *A.muciniphila* CT values are presented in table 1. The relationship between mean CT and *A.muciniphila* CFU is logarithmic.

Table 1. mean standard *A.muciniphila* CT values and CFU counts.

A.muciniphila	Standard *A.muciniphila* CFU	Mean CT of standard *A.muciniphila*	Ln(Standard *A.muciniphila* CFU)
STANDARD1	1000000	18.54	13.81551
STANDARD2	100000	21.69	11.51293
STANDARD3	10000	25.14	9.21034
STANDARD4	1000	28.51	6.907755

CFU: Colony Forming Unit, CT: Cycle Threshold, Ln: Logarithm

Figure 1 shows the relationship between log(CFU) with different ranges of CT. According to figure 1 there is a linear relation between CT and log(CFU). Based on the curve fitting of log(CFU) according to CT, the following data are achieved:

Ln(CFU) = -0.69 (CT) + 26.556

CFU = exp (-0.69 (CT) + 26.556)

Statistical methods

The Statistical Package for Social Science (SPSS) version 13 was used for the statistical analysis. The influential variables were adjusted between two groups (Diabetic and Non-Diabetic) at the beginning of the study. The normality of variables were tested by the Kolmogorov–Smirnov test. Mean differences between two groups were tested using independent sample T-test and for variables that normality was not evaluated Mann-Whitney U Test was applied. An Error Bar of 95% confident interval was used for *A.muciniphila* CFU between two groups. Additionally, linear regression with Stepwise method was used for comparing each independent variable with CFU between two groups; R and Adjusted R Square was reported. The level of significance was set at 0.05 and all results were expressed as Mean±SEM (standard error of mean).

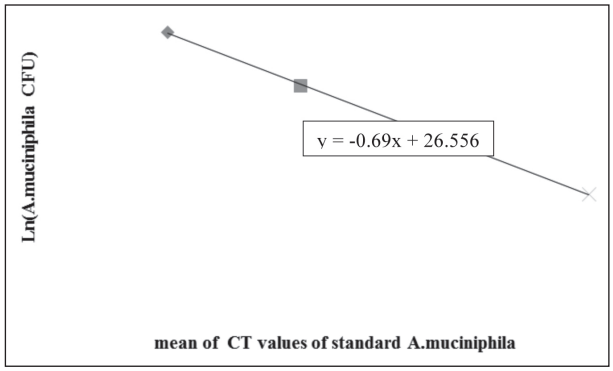

Figure 1. log(*A.muciniphila* CFU) with different range of CT of *A.muciniphila*.

Results

In this study, 36 diabetic and 32 non-diabetic subjects were studied. Age of participants was 18-50 years. Table 2 shows BMI, FBS, Cholestrol, triglycerides, *Akkermansia muciniphila* Ct value and *Akkermansia muciniphila* CFU/100mg stool in two groups. Mean *A.muciniphila* CFU was significantly different between diabetic and non-diabetic subjects (391294.43 vs 843427.87, p=0.022)

Figure 2 shows the comparison of *A.muciniphila* CFU between two groups with a 95% confidence interval (CI) corresponding to a p-value of 0.022.

Results from FFQ forms for dietary habits assessment indicated that mean weekly consumption of four food groups (dairy, vegetables, meat and sugar) were significantly different between groups (table 3).

Table 4 indicates the correlation between *A.muciniphila* CFU/100mg stool and food groups and FBS in both groups. The correlation of *A.muciniphia* CFU counts with food groups were significant only in regard to dairy (Beta=0.355, p=0.034) and fruit

Table 2. Comparison of serological variables and *Akkermansia muciniphila* CFU count between two groups (n=68).

Variable	T2D group (n=36) mean±SEM	Healthy group (n=32) mean±SEM	p value
BMI (kg/m2)	30.2±0.56	29.9±0.54	0.698#
FBS (mmol/l)	152.17±2.89	95.03±1.39	<0.001#
Cholesterol (mg/dl)	189.06±6.91	184.84±5.83	0.773$
Triglycerides (mg/dl)	209.81±13.26	150.53±11.45	0.001#
Akkermansia muciniphila Ct value[1]	22.39±0.65	20.34±0.8	0.052$
Akkermansia muciniphila CFU/100mg stool[2]	391294.43±83352.77	843427.87±171828.55	0.022#

#: Independent T-test $: Mann-Whitney U Test; 1: Cycle threshold (in stool samples), 2: Colony Forming Unit was measured in 100 milligrams of stool.; BMI: Body Mass Index, FBS: Fasting Blood Sugar

(Beta=-0.328, p=0.049) in the diabetic group. Additionally, in the non-diabetic group the correlation of *A.muciniphia* CFU count was significant in regard to meat (Beta=0.363, p=0.027) and FBS (Beta=-0395, p=0.017). There was an inverse correlation between FBS and *A.muciniphila* CFU; however it was only significant in the non-diabetic group.

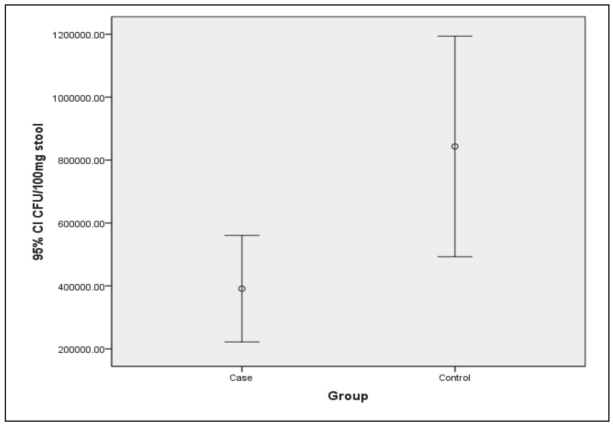

Figure 2. Comparison of *Akkermansia muciniphila* CFU between two groups.

Discussion

This study demonstrated a significant difference of *A.muciniphila* CFU between diabetic and non-diabetic subjects. Previous studies have shown similar results. Marlene et al. (11), investigated the gut microbiota in obese, lean and T2D individuals. Obese and T2D patients were on a nutrition counseling intervention. The T2D patients received an additional therapy with GLP-1-Agonist. They observed that *A.muciniphila* increased throughout the study period in the diabetic patients. Everard et al (8) assessed the role of *A.* in gut microbiota, gut barrier, physiological and hemostatic functions in obese and T2D subjects. Inevitably, they

Table 3. Comparison of dietary items between two groups (n=68).

Variable	T2D group (n=36) mean±SEM	Healthy group (n=32) mean±SEM	*p* value
Dairy	9.12±0.65	13.85±0.99	<0.001$
Bread & cereals	28.69±0.79	29.64±0.67	0.369#
Meat	23.21±1.04	26.43±1.09	0.026#
Vegetable	38.7±1.27	33.04±0.75	<0.001#
Fruit	28.41±1.24	28.08±0.84	0.844$
Fat	19.85±0.9	17.99±0.46	0.136$
Sugar	9.18±0.91	22.85±1.04	<0.001$

#: Independent T-test $: Mann-Whitney U Test; Variables are based on consumption per week

Table 4. Correlaion between *A.muciniphila* CFU and variables in diabetic and healthy groups.

	Groups	Variables	B±SE	Standardized Beta	P Value*	R	Adjusted R Square
CFU	T2D(n=36)	Dairy	45247.87±20441.33	0.355	0.034	0.432	0.137
		Fruit	-22156.27±10818.36	-0.328	0.049		
	Healthy(n=32)	Meat	57196.99±24478.65	0.363	0.027	0.548	0.252
		FBS	-35780.74±14101.44	-0.395	0.017		

**Linear Regression With backward method between CFU with Some important Variables.; FBS: Fasting blood sugar*

observed that the abundance of the bacteria had decreased in obese and diabetic mice. Prebiotic treatment restored *A.muciniphila* to basal levels and also reversed metabolic endotoxemia and related disorders. Multiple evidences indicate that endotoxaemia in obesity and type 2 diabetes could be due to gut barrier disruption which is associated with an increase in paracellular gut permeability (12-15). Tight-junction proteins linking epithelial cells together such as claudin, occludin, and zonula occludens1 regulate the paracellular permeability (16). Eventually, any gut barrier disruptions may increase the absorption of bacterial parent compounds such as gut microbiota-derived lipopolysaccharide (LPS), leading to metabolic endotoxaemia, inflammation and metabolic diseases (9, 12, 17-20). Indeed, LPS is a powerful proinflammatory molecule produced from the cell wall of Gram-negative bacteria (20). Larsen et al (21) assessed the differences between the composition of the intestinal microbiota in humans with T2D and non-diabetic individuals as control. This study indicated that T2D is associated with compositional changes in intestinal microbiota of humans. In another study (22), *A.muciniphila* was investigated in preschool children with and without overweight and obesity. They observed that the level of *A.muciniphila* was significantly lower in the obese/overweight children(22).

Our study demonstrated significant differences in FBS, triglyceride and *A.muciniphila* CFU/100mg stool between two groups. Yassour and et al (23) analyzed metagenomes from 36 fecal samples drawn from healthy monozygotic Korean twins over time; the investigators studied the association between T2D-related biomarkers (for example, BMI, FBS, triglycerides, cholesterol) and microbial clades and functions. They found that the abundance of *A.muciniphila* was negatively correlated with BMI, FBS, and insulin levels (23).

In our study results of FFQ analyzes showed significant differences between two groups in the consumption of diary, meet, vegetables and sugar. Marlene et al detected the differences of gut microbiota in obese and type 2 diabetics in relation to FFQ and showed that there were no significant differences in the consumption of grain, vegetables, fruits, meets, dairy products and fish between diabetic and obese subjects(11). We also investigated the correlation between the abundance of *A.muciniphila* and frequency of consuming different food groups. Results showed that in the diabetic group, there was a positive correlation between *A.muciniphila* and dairy products, while this correlation was reverse for fruits.

Dao et al study showed A.muciniphila in overweight and obese adults is correlated with an improved metabolic status and better suitable clinical outcomes after Calorie restriction(24). A line of studies showed *A.muciniphila* controls fat mass storage and glucose homeostasis via two mechanisms. Firstly by restoring the mucosal layer and controlling the host mucus turnover (25). Second, the abundance of *A.muciniphila* is associated with higher L-cell activity such as GLP-1 and GLP-2 secretion that regulate epithelial cell proliferation and gut barrier function (15, 20).

Our study had the following limitations: Firstly, the small sample size clearly presents the most significant limitation to this study. Second, it would have been precise if we had measured other markers of diabetes and inflammatory markers. Third, assessing lean and normal weight diabetics and also non-diabetic lean and normal weight individuals alongside our study population would have brought stronger evidence to clarify the results obtained from this study. However, the present study has been performed for the first time in Iran and may be the most significant brightness of this study.

Conclusion

Our results indicate that the abundance of *A.muciniphila* was significantly lower in T2D patients in comparison to healthy subjects. The significant correlation was seen between *A.muciniphila* abundance and dairy/fruit groups in T2D patients. Also this correlation was seen between *A.muciniphila* abundance and meat group consumption and FBS level in healthy subjects. Thus, it seems vital to concentrate on gut microbiota and host interaction to better understand the involvement of *A.muciniphila* in the development of T2D. Additionally, searching for other bacteria that could perform similar results is another aspect that must be taken into account.

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