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Article

Changes in Composition and Diversity of the Intestinal Microbiota, Associated with Carbohydrate Consumption in Type 2 Diabetes *Mellitus* Patients

Beatriz Elina Martínez-Carrillo ^{1,*}, Amapola De Sales-Millán ¹, José Félix Aguirre-Garrido ², Roxana Valdés-Ramos ¹, Flor de María Cruz-Estrada ¹ and José Arturo Castillo-Cardiel ³

¹ Laboratorio de Investigación en Nutrición, Facultad de Medicina, Universidad Autónoma del Estado de México, Toluca, México

² Departamento de Ciencias Ambientales, Universidad Autónoma Metropolitana, Lerma, México

³ Universidad Autónoma de Durango, Durango, México

* Correspondence: martinez_elina9@hotmail.com; Tel.: +52 7222672188

Abstract: Type 2 diabetes *mellitus* (T2DM) is a multifactorial disease, influenced by dietary and environmental factors that can modify the intestinal microbiota. The aim was to evaluate changes in composition and diversity of the intestinal microbiota, associated with carbohydrate (CH) consumption in T2DM patients. Forty patients participated, with and without T2DM. Feces samples were collected for the characterization of microbial diversity from the massive sequencing of the 16S rRNA gene. Carbohydrate consumption was quantified from the FCF, the groups were categorized according to BMI and BMI + CH consumption. The group without T2DM showed normal biochemical and anthropometric parameters, although it had high carbohydrate consumption compared to the group with T2DM. At the *Phylum* level, there were differences in relative abundance, Control overweight group (CL-OW>CH) and T2DM-Normal Weight > CH patients, had increased *Bacteroides* and decreased *Firmicutes*. In contrast, CL-OW>CH and T2DM-OW<CH, showed reduced *Bacteroidetes* and elevated *Firmicutes* amount. At the genus level, the differences were in the relative abundance of *Roseburia*, *Clostridium_IV*, *Prevotella*, and *Sporobacter*, associated with the consumption of carbohydrate. The groups that consumed high amounts of carbohydrates, regardless of whether they had diabetes *mellitus* and overweight, had a significantly reduced proportion of *Faecalibacterium*, an altered proportion of *Bacteroides*. The high consumption of carbohydrate showed considerable modifications in the composition and diversity of the bacterial communities.

Keywords: Carbohydrate; Body Mass Index; Type 2 Diabetes *Mellitus*; Microbiota

1. Introduction

Type 2 diabetes *mellitus* (T2DM) occurs when the pancreas does not produce enough insulin or the body does not use it effectively, which causes glucose intolerance [1]. It has been estimated that in 2015 there were 415 million people with diabetes *mellitus*; by 2040, the number will be 642 million [2]. In Mexico, diabetes *mellitus* is the main cause of morbidity and mortality in those aged between 45 and 64 years [3]. It is a multifactorial disease involving genetic, dietary, lifestyle (such as sedentarism) [4], and environmental factors (such as the intestinal microbiota) [5]. The intestinal microbial composition is considered a factor of environmental origin because it differs between patients according to their environments [6]. In this sense, the type, quantity, and quality of the diet modifies patients' microbiota, which causes dysbiosis that influences the pathophysiology of T2DM [7]. The intestinal microbiota is defined as a complex and diverse community dominated by bacteria living in the intestine [8]. In adults, the most abundant bacterial phyla are *Bacteroidetes* (Gram-negative bacteria) and *Firmicutes* (Gram-positive bacteria) [9]; *Actinobacteria*, *Fusobacterium*, and *Verrucomicrobia* are variable between individuals [10]. Each nutrient consumed in the diet alters the

composition of the intestinal microbiota, promoting the expansion of microorganisms in a particular way [11]. Studies describe the effect of high-fat diets [12], but no studies involve the influence of consumption of different types of carbohydrates on the intestinal microbiota. For example, most individuals, include sucrose in different proportions as a dietary additive [13]. Hydrolyzed sucrose is retained in the distal intestine, exposing the microbiota to significant amounts of fructose and glucose for a longer period [14]. Sucrose has been found to decrease the abundance of *Bacteroides thetaiotaomicron*, which are important regulators of intestinal colonization [15]. For this reason, the objective of this work was to evaluate the changes in the composition and diversity of the intestinal microbiota associated with carbohydrate consumption in type 2 diabetes *mellitus* patients.

2. Results

2.1. Socio-Demographic Data

The median age in the T2DM group was higher (49 years) than the median age in the control group (42 years). In the T2DM subgroups, the T2DM-OW subgroup was younger (median 47 years), compared to the T2DM-NW subgroup (median 50 years). In the CL-OW and CL-NW subgroups, the median ages were 36 and 45 years, respectively. All the patients were healthy, without pathologies at the time of the study for the control group and the T2DM patients didn't have aggregate pathologies recorded. In each group we found 18 women and 2 men. In the diabetes mellitus group, a total of 12 patients had a 6-10-year evolution of T2DM, while only 8 had less than 5 years of diagnosis. The treatment most frequently taken by patients was metformin (40%), followed by insulin (25%), the combination of metformin plus glibenclamide (20%), the combination of insulin plus metformin (10%), and the combination of sitagliptin plus metformin (5%).

2.2. Anthropometric Evaluation

The BMI was of 24.75 kg / m² for the control group and a BMI of 24.8 kg / m² for the T2DM group. The BMI was categorized, with the following findings in the control group: 12 patients with normal weight and 8 with overweight. In the T2DM group, 12 had normal weight, and 8 were overweight.

2.3. Biochemical Evaluation

The biochemical parameters of glycaemia (144 ±9.1 mg / dL) and HbA1c (8.95 ±2.2 mg / dL), were found to be significantly elevated (*P* < 0.001, in both cases) in the T2DM group, compared to the control group (glycaemia: 82 ±3.9 mg / dL and HbA1c: 5.25±0.356 mg / dL). The glucose and HbA1c results, the control group patients of CL-OW subgroup showed elevation in both parameters compared with the patients of CL-NW subgroup. In contrast, in the T2DM group, the T2DM-NW subgroup of patients showed elevated glycaemia and HbA1c, compared to the T2DM-OW subgroup (Table 1). Regarding triglycerides, a difference was found between the control and the T2DM group (*P* <0.024); the differences between subgroups was observed in T2DM-OW subgroup showed high triglycerides, compared the T2DM-NW, CL-OW, and CL-NW subgroups (Table 1). On the other hand, total cholesterol was lower in the control group compared to the T2DM group (*P*<0.029), however, there are no significant differences between the subgroups (*P*<0.519), as shown in Table 1.

Table 1. Biochemical data of patients with and without Diabetes *mellitus*.

	CL-NW n = 12	CL-OW n = 8	T2DM-NW n = 12	T2DM-OW n = 8	
	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	<i>P</i> value
Serum glycaemia	81.1 ±1.7	83.3 ±5.9	150 ± 7.1**	135 ±4.3**	0.001*
Hb1Ac	5.1 ±0.206	5.4 ±0.471	9.2 ±1.5**	8.4 ±3.3**	0.001*
TG	128 ±3.6	155 ±8.3	208 ± 1.5	251 ±1.5	0.119
CT	175.2 ±2.3	174 ±2.4	195 ±6	192 ±3.9	0.519

2.4. Dietary Evaluation

The dietary evaluation between the groups showed significant differences ($P < 0.009$). The group with T2DM consumed less energy (1,266 Kcal / day) with an adequate proportion of carbohydrates (113 g / day), fiber (13 g / day), protein (60 g / day), and lipids (34 g / day); in comparison with the patients without diabetes, who consumed higher energy (2,284 Kcal / day), carbohydrates (249 g / day), fiber (25 g / day), proteins (86 g / day), and lipids (66 g / day). The control group consumed more CH such as sucrose (22 g / day), starch (18 g / day), fructose (8 g / day) except glucose (3.5 g / day); compared to the T2DM group who consumed 30 g / day of starch, 25 g / day of sucrose, the same proportion of fructose (8.6 g / day), with a higher amount of glucose (6 g / day), they consumed more CH than the T2DM group.

2.5. Microbiota Analysis

The richness and diversity of OTUs were estimated using the Chao 1 index as an indicator of richness, the Shannon and Inversa indices of Simpson as indicators of diversity, and the Pielou index as an indicator of equity (Tables 2a and 2b). Under these approaches, the fecal bacterial communities of the T2DM-NW subgroup showed lower values compared to those obtained in the T2DM-OW subgroup. This was contrary to what was observed in the CL-OW subgroup, which had richness and diversity greater than that of the T2DM-OW subgroup. The percentage of coverage was very similar in all samples of the different subgroups (Tables 2a and 2b). The results of the massive sequencing directed to the V4-V5 region of the 16S rRNA gene of the fecal samples were used for taxonomic characterization at different levels. For the analysis of relative abundance, the levels of *Phylum* and *genus* were considered in the subgroups categorized by BMI and BMI + CH. The analysis of the relative abundance at the *Phylum* level showed that the taxonomic distribution of the subgroups BMI and BMI + CH had a higher abundance of *Firmicutes* and *Bacteroidetes*, followed by *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia*. In groups categorized according to BMI, *Firmicutes* were represented mostly in the CL-OW subgroup (70.62%) and *Bacteroidetes* in the T2DM-OW subgroup (54%). *Proteobacteria* had a higher representation in the CL-NW subgroup (0.95%), while *Actinobacteria* did not reach a representativeness of greater than 0.5% in all subgroups. However, the subgroups with the highest representation were T2DM-NW (0.27%) and CL-NW (0.26%); *Verrucomicrobia* (1.20%) was represented mostly in the T2DM-NW subgroup (Figure S2). In the BMI + CH subgroups, the *Firmicutes* were represented mostly in the CL-OW < CH subgroup (75.30%). This was contrary to *Bacteroidetes*, which had a higher representation in the T2DM-OW < CH subgroup (54%), followed by T2DM-NW > CH (49%). *Proteobacteria* showed greater representation in the T2DM-NW > CH (1.16%) and CL-NW < CH (1.04%) subgroups. *Actinobacteria* had a majority in the CL-NW > CH subgroup (0.65%) and was so low in the other subgroups, it did not reach 0.5%, while *Verrucomicrobia* (1.43%) was represented mostly in the T2DM-NW < CH subgroup. The results of the evaluation of relative abundance at the *genus* level showed that the taxonomic distribution according to BMI and BMI + CH had a greater abundance in 25 taxa, with the results of the CL-NW subgroup taken as a reference.

Table 2. a. Number of qualified readings, estimators of richness, species, diversity and uniformity of the bacterial communities present in samples of patients without T2DM categorized by BMI.

Control Group	Nseqs ^a	Coverage ^b (%)	OTU ^c	Chao1 (lci ^d -hci ^e)	Inv-Simpson (lci ^d -hci ^e)	Shannon (lci ^d -hci ^e)	Pielou
CL-NW (n = 12)							
M15	25575	99.75	278	412 (357-520)	6 (5.94-6.12)	2.81 (2.8-2.83)	0.993
M45	55419	99.82	437	594 (572-638)	6.27 (6.21-6.34)	4.75 (4.72-4.79)	0.991
M13	58542	99.86	612	857 (795-97)	4.74 (4.70-4.78)	3.45 (3.44-3.47)	0.994
M23	57984	99.86	1029	1297 (1245-1391)	6.44 (6.41-6.47)	4.44 (4.43-4.45)	0.998
M29	98637	99.86	906	1264 (1162-1374)	5.29 (5.26-5.31)	4.12 (4.11-4.13)	0.997
M32	56648	99.86	1125	1468 (1436-1666)	4.39 (4.35-4.44)	3.10 (3.09-3.11)	0.998

M33	45032	99.88	832	1160 (1059-1363)	5.73 (5.71-5.75)	4.60 (4.59-4.61)	0.997
M34	64036	99.83	1081	1396 (1344-1508)	6.55 (6.5-6.6)	4.99 (4.98-5)	0.996
M37	50042	99.86	458	597 (505-641)	4.66 (4.62-4.71)	2.46 (2.45-2.47)	0.996
M38	5021	99.66	1191	1490 (1488-1688)	6.52 (6.38-6.67)	4.86 (4.82-4.87)	0.998
M43	46370	99.87	883	1239 (1217-1288)	6.03 (5.98-6.09)	4.60 (4.58-4.61)	0.997
M46	39738	99.83	950	1290 (1188-1334)	6.32 (6.23-6.40)	4.55 (4.53-4.56)	0.998
CL-OW (n = 8)							
M48	6333	99.82	1146	1503 (1471-1704)	5.59 (5.51-5.68)	4.75 (4.73-4.76)	0.997
M49	14430	99.83	998	1330 (1318-1434)	5.41 (5.36-5.45)	4.85 (4.84-4.88)	0.994
M6	8343	99.75	1037	1345 (1253-1410)	5.71 (5.63-5.8)	4.84 (4.82-4.86)	0.997
M16	59311	99.85	681	908 (849-1009)	5.99 (5.95-6)	4.9 (4.8-5)	0.979
M17	52056	99.84	1015	1332 (1267-1450)	4.87 (4.82-4.92)	3.43 (3.42-3.44)	0.997
M19	40529	99.81	861	1128 (1072-1229)	6.14 (6-6.2)	4.75 (4.74-4.76)	0.999
M25	64872	99.88	822	1091 (1080-1202)	5.43 (5.41-5.45)	4.59 (4.58-4.61)	0.778
M30	21765	99.74	625	884 (872-919)	4.94 (4.86-5)	2.14 (2.13-2.16)	0.992

The parameters were estimated using MOTHUR. Samples from patients without T2DM (M). ^aNseqs = Sequence numbers were obtained after removing low quality sequences (N ≥ 2; homopolymers ≥8) and short sequences (<200 bp). ^b The smallest library (5021 sequences) was used for normalization of the data. The results presented are the average of 1000 repetitions. ^cOTU = number of operational taxonomic units defined over the maximum distance of 3%. ^dlci = lower confidence interval. ^ehci = upper confidence interval.

Table 2. b. Number of qualified readings, estimators of richness, species, diversity and uniformity of the bacterial communities present in stool samples of patients with T2DM categorized by BMI.

T2DM Group	Nseqs ^a	Coverage ^b (%)	OTU ^c	Chao1 (lci ^d -hci ^e)	Inv-Simpson (lci ^d -hci ^e)	Shannon (lci ^d -hci ^e)	Pielou
T2DM-NW (n = 12)							
DM5	44662	99.86	715	944 (897-1037)	5.65 (5.6-5.7)	4.3 (4.29-4.32)	0.995
DM38	28103	99.84	1130	1471 (1431-1559)	5.62 (5.54-5.7)	4 (4.05-4.08)	0.995
DM1	42462	99.81	703	890 (830-998)	6.24 (6.17-6.32)	3.47 (3.46-3.48)	0.997
DM2	63930	99.84	1047	1346 (1276-1454)	5.98 (5.92-6)	3.53 (3.52-3.54)	0.997
DM6	51875	99.86	879	1172 (1125-1257)	3.77 (3.74-3.8)	3 (3-3.03)	0.995
DM8	53503	99.85	790	1057 (993-1175)	4.27 (4.22-4.31)	3.89 (3.88-3.9)	0.997
DM9	63090	99.82	908	1168 (1090-1299)	4.62 (4.56-4.68)	4.53 (4.52-4.54)	0.999
DM17	37353	99.83	1187	1517 (1460-1629)	6.53 (6.46-6.61)	4.87 (4.86-4.88)	0.998
DM19	10255	99.74	738	1097 (1073-1161)	4.72 (4.6-4.86)	3.95 (3.92-3.97)	0.994

DM27	33597	99.79	680	943 (866-1093)	7.1 (7-7.19)	5 (5.01-5.03)	0.998
DM50	40129	99.82	931	1278 (1203-1425)	6.95 (6.88-7)	4 (4.06-4.08)	0.998
DM51	38123	99.81	799	1056 (981-1198)	6.64 (6.59-6.7)	4.48 (4.47-5)	0.894
T2DM-OW (n = 8)							
DM16	46862	99.84	1009	1328 (1275-1425)	5.37 (5.32-5.43)	4.82 (4.81-4.83)	0.999
DM20	67321	99.86	799	1125 (1052-1253)	4.87 (4.84-4.9)	3.33 (3.32-3.34)	0.996
DM21	58523	99.85	1250	1609 (1531-1749)	6.23 (6.18-6.28)	5.23 (5.22-5.24)	0.997
DM22	58044	99.87	1011	1390 (1333-1495)	4.85 (4.81-4.9)	4 (4.06-4.08)	0.997
DM23	39135	99.84	981	1270 (1220-1368)	5.57 (5.52-5.63)	4.75 (4.74-4.77)	0.996
DM28	37674	99.85	922	1366 (1327-1443)	4.75 (4.69-4.82)	2.92 (2.9-2.93)	0.996
DM42	18130	99.74	933	1228 (1166-1367)	5.14 (5-5.25)	3.84 (3.82-3.86)	0.994
DM42	71836	99.86	646	879 (809-1000)	5.96 (5.92-6)	4 (4.04-4.06)	0.998

The parameters were estimated using Mothur. Samples from patients with T2DM (DM). ^a Nseqs = Sequence numbers were obtained after removing low quality sequences (N ≥ 2; homopolymers ≥8) and short sequences (<200 bp). ^b The smallest library (5021 sequences) was used for normalization of the data. The results presented are the average of 1000 repetitions. ^c OTU = number of operational taxonomic units defined over the maximum distance of 3%. ^d lci = lower confidence interval. ^e hci = upper confidence interval.

In the categorization based on BMI (Figure S2) the following were found: CL-NW subgroup: *Faecalibacterium* and *Bacteroides*; CL-OW subgroup: *Megamonas* and *Catenibacterium*; T2DM-NW subgroup: *Lachnospiraceae_incertae_sedis* and *Ruminococcus*; and T2DM-OW subgroup: *Prevotella* and *Clostridium_IV*. On the other hand, in the subgroups categorized according to BMI + CH, the following were found: CL-NW > CH: *Faecalibacterium* and *Roseburia*; CL-NW < CH: *Streptococcus* and *Dorea*; CL-OW > CH: *Blautia* and *Ruminococcus*; CL-OW < CH: *Megamonas* and *Catenibacterium*; T2DM-NW > CH: *Bacteroides* and *Coprococcus*; T2DM-NW < CH: *Lachnospiraceae_incertae_sedis* and *Lactobacillus*; and T2DM-OW < CH: *Prevotella* and *Clostridium_IV*, as seen in Table 3.

Table 3. Average relative abundance at the gender level, categorized for consumption of Carbohydrates per group.

CLNW			CLOW		T2DMNW		T2DMOW	
n = 12			n = 8		n = 12		n = 8	
>200 g HCO	<i>Faecalibacteriu</i>	23.92	<i>Faecalibacteriu</i>	23.3	<i>Bacteroides</i>	29.75	No patient	
	<i>m</i>	18.48	<i>m</i>	21.23	<i>Prevotella</i>	14.19	consumed	
	<i>Prevotella</i>	8.47	<i>Bacteroides</i>	15.23	<i>Faecalibacteriu</i>	10.13	more than	
	<i>Roseburia</i>	8.11	<i>Prevotella</i>	11.09	<i>m</i>	8.47	200 g of CHO	
	<i>Blautia</i>	8.47	<i>Blautia</i>	7.5	<i>Roseburia</i>	6.82	per day, then	
	<i>Roseburia</i>	5.85	<i>Ruminococcus</i>	4.83	<i>Blautia</i>	30.64	we don't	
	<i>Bacteroides</i>	26.7	<i>Parabacteroides</i>	16.82	Less than 5%		have samples	
	Less than 5%		Less than 5%				in this group.	
n = 4			n = 4		n = 4		n = 0	
< 200 g HCO	<i>Faecalibacteriu</i>	20.49	<i>Faecalibacteriu</i>	16.3	<i>Faecalibacteriu</i>	16.63	<i>Prevotella</i>	52.13
	<i>m</i>	16.79	<i>m</i>	15.52	<i>m</i>	12.15	<i>Faecalobacteriu</i>	13.57
	<i>Bacteroides</i>	10.87	<i>Roseburia</i>	11.41	<i>Prevotella</i>	12.1	<i>m</i>	5.94
	<i>Prevotella</i>	9.32	<i>Prevotella</i>	10.89	<i>Roseburia</i>	9.85	<i>Blautia</i>	5.03

<i>Blautia</i>	8.36	<i>Megamonas</i>	10.3	<i>Bacteroides</i> ,	8.22	<i>Roseburia</i>	23.33
<i>Roseburia</i>	4.10	<i>Bacteroides</i>	10.28	<i>Blautia</i>	5.71	Less than 5%	
<i>Streptococcus</i>	15.67	<i>Blautia</i>	25.3	<i>Lachnospirace</i>			
Less than 5%		Less than 5%		<i>a_</i>	35.34		
				<i>incertae_sedis</i>			
				Less than 5%			
n = 8		n = 4		n = 8		n = 8	

Different taxonomic groups were identified at the gender level with statistical significance according to the condition of BMI + CH. The results showed 4 taxa with statistical significance: a) *Roseburia* ($P = 0.025$), b) *Clostridium_IV* ($P = 0.017$), c) *Prevotella* ($P = 0.017$), and d) *Sporobacter* ($P = 0.020$), these correlations by level between the BMI + CH. Furthermore, to test for differences in bacterial composition between samples, a principal component analysis (PCA) was performed for all genus-level 16S rRNA gene reads in STAMP. In this analysis, the difference that exists between the dominances of the bacterial communities and the characteristics of each subgroup based on BMI and BMI + CH was observed. In the BMI group, the T2DM-OW subgroup showed a position clearly differentiated from that of the other subgroups. In contrast, in the BMI + CH group, the T2DM-OW < CH subgroup is separated from the other subgroups.

The data represent the Means $\pm$ Standard Deviations of the biochemical values of patients with and without diabetes mellitus. One-way ANOVA* was performed to analyze differences between groups, with Tukey’s post hoc** test to compare intragroup differences. Significance was considered with a $p > 0.05$. Control Normal Weight (CL-NW), Control Overweight (CL-OW), Type 2 Diabetes mellitus Normal Weight (T2DM-NW), Type 2 Diabetes mellitus Overweight (T2DM-OW).

The table shows the percentage and type of bacteria at the genus level classified by group of patients who consumed more than 200 g of CH or less than this amount. The percentage of less than 5% represents genera of bacteria with little representation in the bacterial microenvironment.

Figure S1.
Study groups: Control and Type 2 Diabetes Mellitus, classified by Body Mass Index (BMI) and subcategorized by BMI + HCO consumption.

Figure S2a and S2b.
Structure bacterial communities of the faeces samples of the CL and T2DM groups and their respective subgroups according to BMI. Average relative abundance at the *Phylum* level of the BMI subgroups. Only taxa with mean relative abundances $> 0.05\%$ were graphed. Control Normal Weight (CL-NW), Control Overweight (CL-OW), Type 2 Diabetes Mellitus Normal Weight (T2DM-NW), Type 2 Diabetes Mellitus Overweight (T2DM-OW).

Figures S3a and S3b.
Structure of the bacterial communities of faeces samples for CL and T2DM groups, with the subgroups categorized by BMI. Average relative abundance at the gender level of the BMI subgroups. Taxa with mean relative abundance $P > 0.05\%$ were plotted. Control Normal Weight (CL-NW), Control Overweight (CL-OW), Type 2 Diabetes Mellitus Normal Weight (T2DM-NW), Type 2 Diabetes Mellitus Overweight (T2DM-OW).

3. Discussion

In this study, the dietary evaluation showed higher carbohydrate consumption in the healthy control group than in the patients with T2DM. This phenomenon acts as a risk factor for the healthy group but is protective for the diabetic group. For example, the dietary recommendation in patients with T2DM establishes a carbohydrate consumption less than 45%, and sugar consumption less than 10% / day of the total energy consumed. [22]; a sucrose consumption below 5% has been proposed [23]. In this study, both groups consumed more than 50% carbohydrates and 10% sucrose in their diet, situation that can be associated with the presence of overweight in these patients [24]. The richness and diversity of the bacterial communities of the feces samples of the subgroups categorized by BMI and BMI + CH (Tables 2a and 2b, and Figure S2) allowed us to observe the modifications in

the structure of the bacterial communities of these subjects. In the T2DM-NW > CH subgroup, richness was much lower compared to that of the CL-NW > CH subgroup, while in the CL-OW < CH subgroup, there was greater richness than in the T2DM-OW < CH subgroup (Tables 2a and 2b). These results show the changes in the diversity of the intestinal microbiota in patients with overweight, T2DM, and high carbohydrate consumption. Similar behavior has been reported in overweight patients, who tend toward obesity and who present changes in the diversity of the intestinal microbiota, which leads to low-grade inflammation [25] and, consequently, in the long term, may be the cause of T2DM [26]. Currently, that information is unclear. It is known that the type of intestinal microbiota will depend on the quality and quantity of the nutrients consumed, particularly carbohydrates. Consumption of metformin in patients with T2DM for glycemic control has been reported to cause changes in the intestinal microbiota [27]. With the results that this study obtained, it can be sustained that the consumption of carbohydrates and the status of being overweight alter the structure of the intestinal microbiota of patients with T2DM [28].

From the characterization of bacterial communities of feces, it was possible to detect that the relative abundance of *Firmicutes* in the T2DM-OW subgroup decreased by almost 50% of the total (*Firmicutes* / *Bacteroidetes* ratio) compared to in the CL-OW group, where the percentage of *Firmicutes* reached 70.62% (Figure S2). These findings contrast with the results of the study by Chávez-Carbajal et al. [18], who found that the group with T2DM + metformin presented an increase in *Bacteroidetes* and a decrease in *Firmicutes*, allowing for the association of metformin as a modifier of the microbiota. In addition, an interesting datum is that the abundance percentages of the T2DM-OW group (*Bacteroidetes* 54% and *Firmicutes* 45.23%) of this study (Figure S2) are like those of the T2DM group without metformin (*Bacteroidetes* 53.18% / *Firmicutes* 43.11%) from the work of Chávez-Carbajal et al. [18]. The foregoing makes it possible to clarify that there is a close relationship between overweight, T2DM, metformin consumption, and changes in the intestinal microbiota. Similarly, other studies show a clear relationship between overweight/obesity, T2DM [29], and the diversity of *Bacteroidetes* vs *Firmicutes* [7], where patients with T2DM and obesity have a lower percentage of *Bacteroidetes* (19.5%) and a higher percentage of *Firmicutes* (55.7%) compared to patients without T2DM (*Bacteroidetes*: 32.1% / *Firmicutes*: 36.9%) [30]. The importance of maintaining a normal weight in patients suffering from T2DM is clear, as being overweight/obese could affect the therapeutic effects of metformin [29,30]. When the subgroups of BMI + CH are combined, it can be inferred that suffering from T2DM combined with high consumption of carbohydrate could be the main cause of the changes in the abundance of *Firmicutes*. This was observed in the T2DM-NW > CH subgroup (49.72%), in which there was less *Firmicutes* in comparison to the CL-OW > CH subgroup (56.59%). On the other hand, this same *Phylum* was higher in the CL-OW < CH subgroup (75.30%), a combination of overweight and low carbohydrate consumption without DMT2, was lower in the T2DM-NW < CH subgroup (67.25%), in which patients are suffering from T2DM, with low carbohydrate consumption and normal weight; overweight plus T2DM and low carbohydrate consumption (T2DM-OW < CH, 45.23%) also showed a lower percentage of *Firmicutes* (Figure S2). This is consistent with published data regarding dysbiosis attributed to *Firmicutes* of the intestinal microbiota in women [31]; 90% of patients in this study were women. Additionally, it was reported that *Firmicutes* could contribute to the development of obesity [32], which agrees with the results of this study, as there is an abundance of *Firmicutes*, associated with overweight/obesity, in Mexican women. On the other hand, the prevalence of *Bacteroidetes* was more represented in the T2DM-OW < CH subgroup (54%), with a lower proportion of *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia* found (Figure S2). In relation to *Bacteroidetes* and *Actinobacteria*, it is believed that they could be of importance to the growth of *Firmicutes*, as they could be inhibiting their growth [33]. The present study found that the *Bacteroides* (29.75%) had a higher relative abundance than the other genera of the T2DM-NW > CH subgroup. It has been reported that changes in the abundance of *Bacteroides* could be attributed to the amount consumed vs. absorption; for example, sucrose, which is not absorbed in the small intestine, is absorbed in the colon, where it also interacts with the colonic microbiota [34]. This has also been reported in Japanese patients with T2DM [50]. These results are

controversial because the ingestion of sucrose produces a reduction in *Bacteroides* by inhibiting the expression of the BT3172 gene, which is essential for its survival [15,35,36].

Another genus associated with a high prevalence of prediabetes [37] and childhood obesity [38] is the *Megamonas*, which, in this study, showed a high prevalence in the CL-OW < CH subgroup. In addition, *Megamonas* was found to be highly enriched in a group with normal glucose tolerance [39], revealing that the different species of *Megamonas* have some particular functions; some are capable of fermenting glucose into acetate and propionate, short-chain fatty acids (SCFA), which are beneficial to health [40].

The genus *Prevotella* appeared with higher relative abundance in the T2DM-OW < CH subgroup. *Prevotella* is a bacterium associated with chronic intestinal inflammation that has been found to be not very abundant in the Chinese population with T2DM [41]. In this study, when the abundance analysis was performed between the subgroups, *Prevotella* was identified within the group of most abundant intestinal bacteria. The abundance of these bacteria increases in subgroups with T2DM, making dysbiosis evident in these patients [6,7,42]. In addition, the inflammation of the intestinal mucosa mediated by this bacterium promotes systemic inflammation, with increased intestinal permeability and translocation of bacterial products [43,44]. The genus *Faecalibacterium* presented a higher relative prevalence in the CL-NW > CH subgroup and a lower relative prevalence in the T2DM-NW > CH subgroup. *Faecalibacterium* is one of the most abundant bacterial species in the healthy human intestine [45]. Currently, *F. prausnitzii* is the only known species of the genus *Faecalibacterium* that, in recent years, has been of great interest as a biomarker of intestinal health [46]. The decrease in the abundance of this bacterial genus is associated with inflammatory bowel syndrome (IBS), inflammatory bowel disease (IBD), colorectal cancer, and T2DM [47]. However, the effects derived from the interaction between *F. prausnitzii* and the consumption of carbohydrate in patients with T2DM are not yet known. There are variations in the microbial composition of the subgroups categorized by BMI and BMI + CH. At the genus level, a clear bacterial profile was observed within the T2DM-OW and T2DM-OW < CH subgroups. This finding would help to confirm the existence of a microbial signature in T2DM, reported in other studies [34]. Therefore, this study could serve as a basis for identifying the microbial molecular fingerprint of the colon in patients with T2DM, overweight, and carbohydrate consumption. The fact that the intestinal microbiota is a dynamic ecosystem that changes with the type of diet [11] could explain the modifications found with the consumption of carbohydrate in this study. The carbohydrate consumption significantly marked the abundance of certain bacteria in the different subgroups of BMI + CH. At the gender level, there was a significant increase in *Roseburia* and *Clostridium IV* (CL-NW > CH), as well as *Prevotella* (T2DM-OW < CH) and *Sporobacter* (T2DM-NW > CH). These findings provide insight into the effect and interaction between sucrose consumption and the changes it causes in the host's intestinal microbiota [10, 14, and 48]. In the colon, the interaction between the microbiota and the products of sucrose metabolism [34] can influence the inflammatory state, the function of the intestinal barrier, and, therefore, the development of complications of DM2. [49]. With this study, new nutritional education strategies can be proposed, which favor the reduction of carbohydrate in the diet and, with this, modulate various bacterial groups associated with the development of TDM2.

4. Materials and Methods

4.1. Ethics Statement

The present study protocol was reviewed and approved by the ethics committee of the hospital and the Faculty of Medicine (project 015/2018) of the Universidad Autónoma del Estado de México (UAEMéx). All participants included in the study gave their informed consent in accordance with the Declaration of Helsinki revised in 2013 [16].

4.2. Study Design

This was a prospective, cross-sectional, and comparative study. One hundred subjects with and without a diagnosis of T2DM were invited to attend the hospital's outpatient clinic an informative

talk. From this, forty subjects of both sexes attended the outpatient clinic of the General Hospital “Dr. Nicolás San Juan” in Toluca, State of Mexico. A brief medical history was given to assess whether the patients met the inclusion criteria.

4.3. Study Subjects

Of the 100 subjects invited to participate, 40 met the inclusion criteria. These were divided into 2 groups: 1) Type 2 Diabetes *mellitus* group, T2DM (n = 20); and 2) Control group of healthy subjects without Diabetes *mellitus*, CL (n = 20). The inclusion criteria for both groups were 25 to 65 years old, without any other pathology at the time of the study and less than 10 years of suffering the disease, regardless of chronological age. The following exclusion criteria were used in both cases: pregnancy; chronic alcohol, drug, or tobacco use; and acute or chronic autoimmune, bacterial, or viral diseases.

4.4. Anthropometric Evaluation

To calculate the body mass index (BMI), each participant's body weight in kilograms was determined using a Tanita-brand scale model Bf-682 (Monterrey, Mexico) after an 8-hour fast. The participants were placed standing on the scale, with the soles of their feet on the scale's surface. Height was measured while standing, without shoes, with a stadiometer (Seca® Model 240; Accuracy ± 2 mm, Seca GmbH & Co., Hamburg, Germany). Once these data were obtained, BMI was calculated using the following formula: weight (Kg) / height (m^2). Once the BMI was obtained, it was categorized as follows: a) Normal weight (NW = 18.5 - 24.99 Kg / m^2), b) Overweight (OW = 25.0 - 29.99 Kg / m^2), and c) Obese > 30 Kg / m^2 .

4.5. Determination of Biochemical Profile

To obtain the blood samples, the patients were asked to fast for 8 hours prior to the collection of the sample. Whole venous blood was collected in tubes with heparin (BD, Vacutainer, Franklin Lakes, NJ, USA). Glycaemia, glycosylated haemoglobin, total cholesterol and triglycerides were determined from whole blood, using immunoturbidimetric tests with reinforcing particles (Innovastar, Diasys Diagnostic Systems, Holzheim, Germany).

4.6. Dietary Evaluation and Patient Categorization

A Food Consumption Frequency (FCF) questionnaire was used to evaluate the food consumed during the last month. From this information, the energy intake of the macronutrients (carbohydrates, lipids, and proteins) was calculated for each patient, with the help of two instruments: The Mexican List of Composition of Food Equivalents “Mexican System of Equivalents” [17] and the FoodData Central, U.S. Department of Agriculture (<https://fdc.nal.usda.gov/>) [18]. From the intake in grams per day of CH, a consumption classification was made in more than 200 g / day (> 200) or less than 200 g / day (< 200), considering the proportion of sucrose, fructose, glucose and maltose. With these data, the groups were categorized by BMI and sub-categorized according to BMI + carbohydrate consumption as shown in Figure 1.

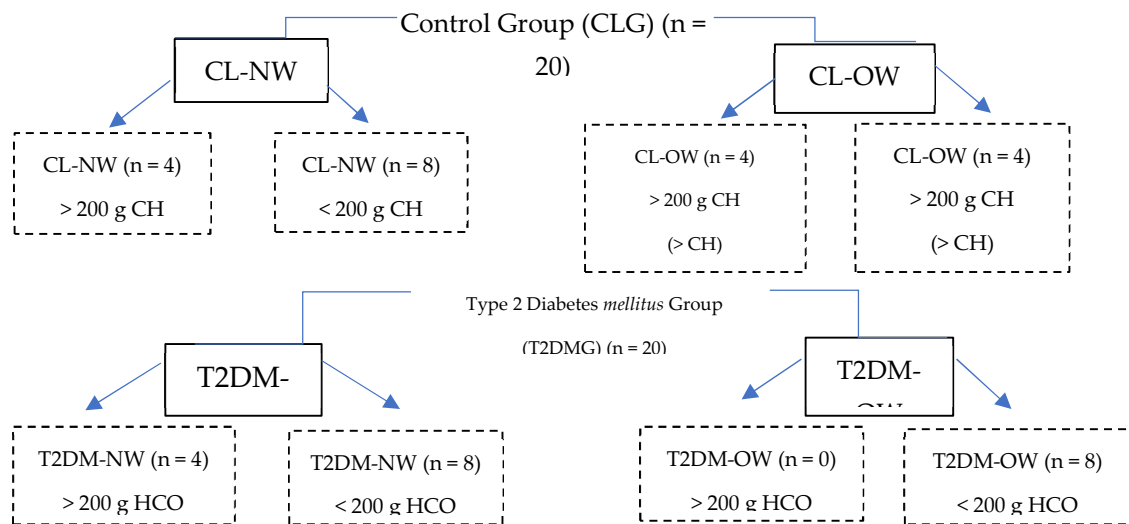


Figure 1. Classification of groups by Body Mass Index and subgroups categorized by Body Mass Index plus carbohydrate consumption. Carbohydrate (CH), Control Normal Weight (CL-NW), Control Overweight (CL-OW), Type 2 Diabetes mellitus Normal Weight (T2DM-NW), Type 2 Diabetes mellitus Overweight (T2DM-OW).

4.7. Collection of Feces

To collect feces samples, a sterile bottle was provided with instructions for correct collection and storage. Once the samples were collected, they were processed in a sterile microbiological hood. Four aliquots with 1 g of feces were stored in sterile 1.5 mL micro tubes at -70°C for subsequent batch analysis [19].

4.8. Analysis of the Fecal Microbiota

1). DNA extraction from faeces

The 40 samples from the study participants were processed, using 150 mg of feces from each patient, for DNA extraction, following the instructions provided in the protocol of the Quick-DNA™ Fecal / Soil Microbe Miniprep Kit from the Zymo Research brand. The times of mechanical lysis with disruptor were modified from 10 to 15 minutes; cooling cycles were at the same times.

1). Gel electrophoresis of DNA extraction and Metagenomics' Material Amplification Test (PCR)

Electrophoresis was performed at 110 Volts for 35 minutes on samples of 3 µL of DNA loaded on 1% agarose gels (100 mL of 1X Buffer TAE per 1 g of agarose). A total of 1 mL of DNA from each sample was used to amplify by PCR targeting the 16S rRNA gene, to verify that they were capable of being amplified [20].

3). Massive sequencing

4.10. Illumina Sequencing by Amplicons of the V4-V5 Region of the 16s rRNA Gene

The 40 samples resulted in metagenomics DNA that met the quality of purity and concentration (260/280 = A1.8-2.0). They were sent to the sequencing service of the Center for Comparative Genomics and Evolutionary Bioinformatics (CGBE) of Dalhousie University, Canada. Massive sequencing of the Illumina MiSeq type (300 + 300 bp PE) was requested [21].

4.11. Bioinformatic Analysis of the Sequences

The 16S rRNA data were processed with Mothur software version 1.39.5, following the pipeline recommended by the developer of the MiSeq SOP. The selected reads met the following criteria: no ambiguous bases, a minimum length of 200 bp, and homopolymers with a maximum length of 8 bp. Similarly, removal of the chimeric sequences and the lineages of eukaryotes, mitochondria, and

chloroplasts, unknown and unclassified, was carried out. Subsequently, the diversity of the sequences by operational taxonomic units (OTU) was examined considering quality readings with a dissimilarity of 3%, while the rarefaction curves were calculated in a similarity of 97% with the Mothur alpha diversity flow. Different metrics were calculated to evaluate the bacterial communities, including the number of OTUs observed, the Shannon diversity indices and Pielou uniformity, and the Chao 1 and Inv-Simpson estimators for species richness. The calculation of these parameters was carried out by normalizing all the libraries. Finally, the composition of the bacterial communities was determined by beta diversity. The abundance was expressed as a percentage with respect to the total number of sequences in each sample (relative abundance) [20,21]. Subsequently, the file generated by Mothur was analyzed using the Statistical Analysis of metagenomics Profiles Test (STAMP) software, and the results were represented by graphs. The obtained sequences were registered in the NCBI BioSample database, at the following link: <https://www.ncbi.nlm.nih.gov/sra/PRJNA807457>, with the number to access and cite these SRA data: PRJNA807457.

4.12. Statistical analysis

The anthropometric, biochemical, and diet profiles were analyzed using SPSS v22 software, employing the non-parametric Kruskal-Wallis test. To identify the differences between the subgroups of the intestinal microbiota in the taxonomic levels of *Phylum*, Class, Order, and Genus, the relative abundance (%) of bacterial communities and the principal component analysis (PCA) we used the ANOVA test and the Kruskal-Wallis test to calculate p values < 0.05 in STAMP [18,21].

5. Conclusions

The composition and diversity of the gut microbiota in patients with and without T2DM were significantly different. The habitual consumption of carbohydrate is closely related to differences in the taxonomic levels of *Phylum* and genus. Although more studies are necessary, it can be concluded that, with a reduction in the consumption of 10% *per day*, it is possible to modulate the bacterial communities in healthy patients and, through this, perhaps help to prevent dysbiosis and the development of T2DM. But, with an increase in the consumption (more than 15%), the modification in the proportion and diversity of the microbiota was altered. This was observed in the groups that consumed high amounts of carbohydrates (CL-NW $>$ CH, CL OW $>$ CH, T2DM-NW $>$ CH), regardless of whether they had diabetes mellitus and overweight or not, which significantly reduced the proportion of *Faecalobacterium*, altered the proportion of *Bacteroides*, with important modifications in other bacterial genera. The high consumption of carbohydrate considerably modified the composition and diversity of the bacterial communities. If comorbidities such as T2DM and overweight are added to this, the changes in the intestinal microbiota will be more evident. In this study, carbohydrate consumption showed, at the genus level, an abundance of bacteria, such as *Faecalobacterium*, *Bacteroides*, *Blautia*, *Roseburia*, *Prevotella*, and *Sporobacter*.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used “Conceptualization, BEMC and ADSM; methodology, JFAG and BEMC; validation, JACC and RVR; FDMCE; ADSM and BEMC writing—original draft preparation, and editing. All authors have read and agreed to the published version of the manuscript.

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