Gut Microbiota Patterns in Obese and Type 2 Diabetes (T2D) Patients from Romanian Black Sea Coast Region

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Gut microbiota plays a major role in the process of food absorption and low grade inflammation, two key steps in obesity and diabetes mellitus occurrence. Gut microbiota metabolites, such as short chain fatty acids (SCFA), have an important impact over the metabolic pathways like insulin signalling, incretin production and inflammation. [1-3] We aimed to study the microbiota patterns in obese and T2D patients from Black Sea Coast region, considering the ethnic mixture, environmental and geographical particularities, involving diet or various habits in this area. 100 patients and 100 controls matched by age, gender and ethnicity were studied regarding feaces predominance of Lactobacillus and Bifidobacterium species. We compared the results of microbiota patterns from patients to those obtained in a similar control group of healthy subjects. The standard pour plate 0.05% L-cystine enriched method was used to obtain the bacterial cultures and anaerobic conditions. Morphological and biochemical tests were used to identify the Lactobacillus and Bifidobacterium spp. Fecal organic acid concentrations were explored in frozen samples. The association between bacterial counts/organic acid concentrations and independent variables, including age, diet, ethnicity and other risk factors were calculated using multivariable linear regression analysis. Pearson's correlation coefficients were calculated to detect associations between fecal bacteria counts/organic acid concentrations and laboratory variables (serum biomarkers, body mass index, age, and severity of obesity/ T2D according to international scales). Junk and sweet diets, lack of physical activity and familial aggregation of hypercholesterolemia and diabetes were significantly more often present in our T2D/obese patients than in controls. The bacterial counts of the L. acidophilus, L plantarum and L. reuteri subgroups of Lactobacillus sp were significantly lower among patients with T2D and obesity than in controls. The counting of Bifidobacterium spp revealed a higher presence of B. bifidum in controls than in obese or T2D patients. Diet type (junk food and sweets), BMI (>25) and personal history of metabolic disorders were associated with decreased counts of L acidophilus and increased counts of L. fermentum and B. adolescentis in T2D patients. Ethnicity, metabolic disorders history and junk and sweet diet were associated with low counts of L. acidophilus and L. reuteri and low counts of B. longum. Junk and sweet diet was associated with low counts of B. bifidum. Romanian ethnicity and metabolic disorders were associated with low counts of B. choerinum at obese patients, independent of age or previous antidiabetic treatments. The concentrations of acetic and butyric acids were significantly lower in all patients groups, while the concentrations of valeric acid were significantly higher in patients with untreated T2D and obese patients compared to the controls. Low counts of L. acidophilus and L. reuteri were positively correlated with the increased levels of HbA1c, LDL cholesterol, TG and inflammatory markers such as CRP, ESR and IL-6, no matter of diet, age, ethnicity or metabolic disorders history. Also, low counts of B. bifidum and B. infantis were positively correlated with high levels of CRP, IL-6 and TG. In obese patients, statistic analysis results showed that low counts of L. acidophilus, L. plantarum, L. johnsonii and L. reuteri were positively associated with increased levels of CPR, IL-6 and TG, while low counts of B. bifidum, B infantis and B. breve were positively correlated with higher counts of CPR, LDL cholesterol and TG. Low counts of B. bifidum and B choerinum were positively correlated with low counts of HDL cholesterol in Romanian ethnicity patients and in those with previous metabolic disorders. Low bacterial counts of some particular strains of Lactobacillus spp and Bifidobacterium spp were positively correlated with diet type, BMI, Romanian ethnicity and personal history of metabolic disorders obese and T2D patients from Romanian Black Sea Coast Region.

Keywords: microbiota, patterns, diabetes type 2, obesity, risk factors

Metabolic disorders have reached an alarming stage over the world. Epidemiologic studies have shown that obesity and T2D gained an exponential increase in the last 2 decades [4- 11]. Sedentary lifestyle and increased food consumption, especially highly processed meat, sweets and fats have been attributed as main causes for obesity and T2D occurrence [3-7]. Meanwhile, researchers demonstrated that environmental and genetic factors play also a role in metabolic disorders development, by influencing the gut microbiota patterns [3]. Along with other factors, gut microbiota plays an important role in digestion, vitamin synthesis and metabolism. The exact mechanism linking gut microbiota and obesity is not yet completely understood, still, it was postulated that gut microbiota contribute to low-grade inflammation and regulate fatty acid tissue composition [3]. It was demonstrated that high-fats diets influence the gut microbiota patterns by increasing the gram negative bacilli containing lipopolysaccharides in their capsule, which can activate the inflammatory pathways [12, 13]. In contrast, short-chain fatty acids (e.g. butyrate, acetate, propionate) produced by bacterial fermentation of insoluble dietary fiber in the bowel can bind to GPR41 and GPR43 [14-16] leading to reduced inflammation and increased glucagon-like peptide (GLP)-

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1 and Peptide YY (PYY) secretion improving the insulin sensitivity [17, 18]. The short chain fatty acid butyrate is a substrate of energy for the mucosal cells lining the colon, whereas acetate and propionate enhance hepatic gluconeogenesis and lipogenesis. Secondary bile acids resulted from bacteria metabolism of bile acids bind to the GPCR TGR5, increasing GLP-1 production from L-cells and energy consumption in muscles providing further improvements in insulin sensitivity [19]. Still, the huge variety of gut microbes, the ethnic variation of populations studied and large variations between individuals make difficult to fix the exact contribution of gut microbiota to the development of obesity and diabetes [20]. The hypothesis of modulating the gut microbiota to control the spread of obesity and diabetes holds a tremendous therapeutic potential for treating these two metabolic endemic disorders [1]. Based on numerous reports, it seems that the geographical differences in microbiota composition result from the different dietary habits in a region or a country. A recent study [21] showed a geographical gradient in the infant microbiota across Europe where northern infants showed higher levels of *Bifidobacteria*, *Atopobium, C. perfringens, C. difficile* while Southern infants presented higher proportion of *Bacteroides*, *Eubacteria* and *Lactobacillus*. [22] Lactobacilli like eubacteria and enterococci have been described in a study made on Estonian infants while Swedish infants showed clostridia and bacteroides in much higher quantity [23, 24]. There were also studies focused on microbiota in adults from different areas of Europe reporting higher levels of bifidobacteria in Italian adult subjects compared to Swedish, French or German adults [25]. Based on this background, we aimed to study the microbiota patterns of obese and T2D patients from Black Sea Coast region, considering the ethnic mixture and geographical particularities involving genetics, diet and various personal habits in this geographical area.

Experimental part

Material and method

We studied the microbiota spectrum of 100 patients diagnosed according to national and international guidelines with obesity and diabetes type 2 (T2D) in Internal Medicine Ist Clinic of Emergency St. Apostle Andrew Hospital of Constanta County and we compared results to those obtained in a similar control group of healthy subjects. The exclusion criteria were applied during enrollment stage. Previous antibiotic therapy, severe gastrointestinal disorders, already diagnosed or treated gastroenteritis one month prior to enrollment, viral or other types of hepatitis, including alcoholic or autoimmune type, cardiac, renal or vascular severe disorders, inflammatory bowel diseases were documented and excluded prior to study onset. All patients were informed and provided their verbal consent in order to participate to this study, according to ethic regional requirements. The research was conducted in accordance with Good Clinical Practice Guidelines and the Declaration of Helsinki, and the Institutional Review Board approved the study protocol.

Stool analysis

Stool samples were collected in sterile containers from all patients and control groups. Specimens were stored at -20 degrees and transferred in a coupled manner subject/control to the microbiology lab. Bacteria strains were investigated after dilution of samples with NaCl (0.85% m/v) enriched with 0.05% L-cysteine. We studied 6 species of *Lactobacillus*: *L. acidophilus, L reuteri, L. fermentum, L. plantarum, L. salivaricus and L. johnsonii* and 6 strains of *Bifidobacterium specie*: *B. Longum, B. adolescentis, B bifidum, B infantis, B. breve and B choerinum.*

The standard pour plate method was used to obtain the bacterial cultures. 25 mL of MRS (De Man, Rogosa and Sharpe) agar was used for each plate. 0.05% of L - cystine enriched plates to provide anaerobic conditions (0% = O2, 10% = CO2, 10% = H2). We placed plates in anaerobic gas pak jar (provided by Merck). Then, samples were incubated at 37°C for 72 hours. Morphological and biochemical tests including sugar fermentation, catalase and methyl red tests were used to identify the *Lactobacillus species*. Quantitative fluorometric assay was used to identify the *Bifidobacterium genus*. Biochemical methods using fructose-6-phosphate phosphoketolase enzyme, sugar fermentation tests and the susceptibility to mupirocin tests were done to identify the *Bifidobacterium species*.

Measurement of organic acid concentrations and pH

Fecal organic acid concentrations were explored in frozen samples, homogenized in a volume of 20 mL 0.15 mol/L perchloric acid and then centrifuged for 10 min, prior being maintained at 4°C for 12 h. We passed the supernatants obtained through a thin filter and measured the organic acid concentration using chromatography method.

Biochemical assays from blood samples

Blood samples were obtained from patients and control subjects at the time of enrollment. Serum levels of glycated hemoglobin (HbA1c), high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and triglycerides (TG) were measured using standard techniques. The plasma levels of C-reactive protein (CRP) and interleukin (IL)-6 were done using ELISA analysis.

Statistics

SPSS Student Version 18.0 (Valuepack - 6th Edition) was used to make the statistic analysis. t-test, Fisher's test and the two-tailed ÷2 test were used to compare different results. $P < 0.05$ was considered as being statistically significant. The association between bacterial counts/organic acid concentrations and independent variables, including age, diet, ethnicity and other risk factors were calculated using multivariable linear regression analysis. Pearson's correlation coefficients were calculated to detect associations between fecal bacteria counts/organic acid concentrations and laboratory variables (serum biomarkers, body mass index, age, and severity of obesity/T2D according to international scales).

Results and discussions

The risk factors analysis revealed that diet rich in junk and sweet (p=0.010, respectively p=0.009), lack of physical activity (p=0.039, respectively 0.008) and familial aggregation of hypercholesterolemia and diabetes (p=0.031, respectively 0.042) were significantly more present in our T2D/obese patients than in controls. The levels of HbA1c (p = 0.006, respectively p=0.001), LDL cholesterol (p = 0.003, respectively p=0.004), inflammatory markers, CRP (p = 0.018, respectively 0.034) and IL-6 (p = 0.023 respectively 0.032), had also significantly higher levels in patients with T2D/obesity than in the controls (table1).

Fecal bacteria composition

Total fecal bacteria counts did not differ statistically between patients (12.24± 0.4 $\log_{10}$ cells/g feaces (12.6±

	T2D patients N=50	obesity patients N=50	Controls N=100	P values
Gender				
Males	24[48]	26[52]	50[50]	0.23;0.41
Females	26[52]	24[48]	50[50]	0.33;0.47
Age, mean	63.61±10.34	62.45±9.27	65.33±8.11	0.45; 0.67
Diet type				
Vegetarian	6[12]	1[2]	14[28]	0.021*; 0.001*
Mediterranean	12[24]	8[16]	19[38]	0.044*; 0.028*
Junk food	19[38]	24[48]	8[16]	0.036*; 0.031*
Sweets	13[26]	17[34]	9[8]	0.025*; 0.030*
Ethnicity				
Romanian ethnicity	42	39	44	
Other	8	11	6	
Physical activity				
Absent	30	38	16	0.039*; 0.008*
Mild/Moderate	14	18	19	0.071 ; 0.091
Intense	6	6	15	0.058 ; 0.070*
Familial history of T2D	27	24	14	0.031*; 0.042*
Familial history of obesity	29	35	10	0.052 ; 0.057
HbA1c (%)	7.1	6.4	5.4	0.006*; 0.001*
LDL(mg/dl)	180[120-215]	193[167-290]	110[89-137]	0.003*; 0.004*
HDL (mg/dl)	38[35-41]	34[28-39}	47[39-68]	0.066 ; 0.062
TG				0.055 ; 0.068
CRP (mg/dl)	1.2[0.56-2.11]	1.9[0.88-3.04]	0.03[0.02-0.05]	0.018*; 0.034*
Il-6 (pg/ml)	2.8[1.6-3.1]	2.4[1.3-2.8]	1.5[1.2-2.0]	0.023*; 0.032*

HDL=high density lipoprotein; LDL= low density lipoprotein; TG = triglicerides; HbA1c = glycated hemoglobin A1c; CRP = C-reactive protein; Variables are represented by numbers, percent [%] means ± standard deviations or as medians (interquartile range); * = statistically significant;

Table 1
RISK FACTORS FOR T2D/ OBESITY IN STUDY GROUPS

Studied species	Bacterial counts (log_{10}cells/gram)					Detection rate (%) *			
	T2D**	Obesity**	Controls	*p*-value	*q****	T2D	Obesity	Controls	*p*-value****
Lactobacillus sp.									
L. acidophilus	11.25±0.7	10.97±1.5	7.22±0.4	**0.003**	0.14	50	50	50	1.00
L. reuteri	10.97±1.5	10.23±0.8	8.23±0.7	**0.029**	0.18	50	50	50	1.00
L. fermentum	8.87	8.83	8.21±1.1	0.522	0.33	50	49	50	0.84
L. plantarum	±0.54	±0.91	7.92±0.7	**0.047**	0.29	50	50	50	1.00
L. salivaricus	11.11±1.0	9.49±1.1	8.88 ±0.9	0.222	0.18	47	50	50	0.88
L. johnsonii	8.23±0.51	8.57±0.82	9.99±1.1	0.098	0.36	49	48	50	0.64
L. ruminis	9.97±0.2	9.95±0.84	9.41±1.2	0.410	0.29	50	50	49	0.84
	9.22±3.0	9.41±1.29							
Bifidobacterium sp.	8.05±0.5	7.99±0.52	9.45±0.82	0.451	-0.28	50	50	50	1.00
B. Longum,	9.04±1.60	9.04±1.60	9.94±1.31	0.077	-0.31	50	50	50	1.00
B. adolescentis,	8.90	7.91	11.01±0.1	**0.041**	- 0.23	50	50	50	1.00
B bifidum,	±0.39	±0.39	11.93±1.8	0.064	-0.12	50	50	50	1.00
B infantis,	10.83±0.9	10.83±0.9	10.88±1.2	0.109	-0.45	47	47	48	0.92
B. breve	9.13±0.38	9.13±0.38	9.03±1.29	0.098	-0.41	50	50	50	1.00
B. choerinum	8.88±0.49	7.88±0.49							

* the percentage of fecal samples containing the specific specie above the detection threshold ; ** means and standard deviations; *** q value calculated according to Benjamini and Hochberg method; **** Fisher's t test.

Table 2
FECAL BACTERIA COUNTS IN PATIENTS AND CONTROLS.

0.9 log_{10} cells/g feaces) and controls (12.3 ± 0.5 log_{10} cells/g feaces) (p = 0.67). However, the bacterial counts of the *L. acidophilus, L plantarum* and *L. reuteri* subgroups of *Lactobacillus spp* were significantly lower among patients with T2D and obesity than in controls (p = 0.003, q = 0.14; 0.047, q=0.29 respectively p=0.029, q=0.18). The counting of *Bifidobacterium spp* revealed a higher presence of *B. bifidum* in controls than in obese or T2D patients (p=0.041, q =- 0.23) (table 2).

Comparisons of fecal bacteria count between T2D/obesity patients and controls

Multivariate linear regression analysis was done in order to detect correlations between bacterial counts and independent variables such as age, ethnicity, BMI, diet, familial history of metabolic disorders and antidiabetic treatments. Diet type (junk food and sweets), BMI (>25) and personal history of metabolic disorders were associated with decreased counts of *L acidophilus* (p=0.048, β =-0.341; p=0.033, β =-0.118; p=0.022, β =-0.058), L plantarum (p=0.039, β =-0.207, p=0.041, β =-0.231, p=0.036, β =-0.115) and L reuteri (p=0.032 β =-0.187, p=0.041, β =-0.142 p=0.019, β =-0.166) and increased counts of L. fermentum (p=0.022 β =0.232, p=0.041, β =0.142, p=0.019, β =0.115) L. salivaricus (p=0.028 β = 0.563, p=0.047 β =0.621, p=0.033 v =0.198) and also, decreased counts of B. bifidum (p=0.012 β =-0.123, p=0.037 β =-0.199, p=0.044 β =-0.201) and B. adolescentis (p=0.028 β =-0.144, p=0.040 β =-0.176, p=0.041 β =-0.210) in T2D patients, while Romanian ethnicity, metabolic disorders history and junk and sweet diet were associated with low counts of L. acidophilus (p=0.045, β =-0.199, p=0.033, β =-0.167, p=0.042, β =-0.381) and L reuteri (p=0.008

Table 3

MULTIVARIABLE LINEAR REGRESSION ANALYSIS TO IDENTIFY THE PERSONAL RF FOR BACTERIAL COUNTS IN T2D.

	Bacterial count											
	Lactobacillus sp.							*Bifidobacterium sp.*				
	L. acidophilus p val (β)	*L. reuteri* p val (β)	*L. fermentum* p val (β)	*L. plantarum* p val (β)	*L. salivarius* p val (β)	*L. johnsonii* p val (β)	*L. ruminis* p val (β)	*B. longum* p val (β)	*B. adolescentis* p val (β)	*B bifidum* p val (β)	*B infanis* p val (β)	*B. breve* p val (β)	*B. choerinum* p val (β)
age	**0.12(-0.07)**	**0.07(-0.42)**	**00.87(0.255)**	**0.08(-0.244)**	**0.07(-0.34)**	**0.06(0.14)**	**0.54(0.64)**	**0.06(-0.92)**	**0.09(-0.62)**	**0.22(-0.24)**	**0.1 (0.21)**	**0.4(0.18)**	**0.49(-0.13)**
ethnicity	**0.36(-0.34)**	**0.10(-0.28)**	**0.077(0.349)**	**0.07(-0.298)**	**0.66(-0.28)**	**0.09(0.18)**	**0.34(0.44)**	**0.05(-0.69)**	**0.07(-0.44)**	**0.13(-0.26)**	**0.9(0.21**	**0.1(0.14)**	**0.41(-0.33)**
BMI	**0.03(-0.11)**	**0.04(-0.14)**	**0.041(0.142)**	**0.04(-0.231)**	**0.02(-0.56)**	**0.03(0.21)**	**0.87(0.71)**	**0.13(-0.26)**	**0.04(-0.17)**	**0.03(-0.19)**	**0.4(0.18)**	**0.8(0.68)**	**0.13(-0.26)**
diet	**0.04,(-0.34)**	**0.03(-0.18)**	**0.022(0.232)**	**0.03(-0.207)**	**0.04(-0.62)**	**0.11(0.63)**	**0.71(0.49)**	**0.18(-0.33)**	**0.02(-0.14)**	**0.01-(0.12)**	**0.4(0.34)**	**0.8(0.37)**	**0.08(-0.19)**
History of MD	**0.02(-0.05)**	**0.01(-0.16)**	**0.019(0.166)**	**0.03(-0.115)**	**0.03(-0.19)**	**0.09(0.27)**	**0.56(0.23)**	**0.31(-0.47)**	**0.04(-0.21)**	**0.04(-0.20)**	**0.6(0.28)**	**0.6-0.22)**	**0.34(-0.29)**
anti T2D therapy	**0.28(-0.51)**		**0.062(0.339)**	**0.05(0.388)**	**0.06(-0.45)**	**0.07(0.57)**	**0.83(0.49)**	**0.09(-0.55)**	**0.18(-0.32)**	**0.41(-0.33)**	**0.7(0.28)**	**0.3(0.22)**	**0.09(-0.13)**

Table 4

MULTIVARIABLE LINEAR REGRESSION ANALYSIS TO IDENTIFY THE PERSONAL RF FOR BACTERIAL COUNTS IN OBESITY

	Bacterial count											
	Lactobacillus sp.							*Bifidobacterium sp.*				
	L. acidophilus p val (β)	*L. reuteri* p val (β)	*L. fermentum* p val (β)	*L. plantarum* p val (β)	*L. salivarius* p val (β)	*L. johnsonii* p val (β)	*L. ruminis* p val (β)	*B. longum* p val (β)	*B. adolescentis* p val (β)	*B bifidum* p val (β)	*B infanis* p val (β)	*B. breve* p val (β)	*B. choerinum* p val (β)
age	0.11(-0.44)	0.061(0.43)	0.51(-0.28)	0.09(0.27)	0.05(-0.27)	0.07(0.17)	0.35(0.39)	0.06(-0.92)	0.25(-0.51)	0.09(-0.19)	0.6 (-0.44)	0.32(0.48)	0.23(-0.31)
ethnicity	**0.04(-0.19)**	**0.008(0.10)**	0.06(-0.25)	0.11(019)	0.62(-0.36)	0.05(0.13)	0.32(0.37)	**0.03(-0.11)**	0.09(-0.25)	0.28 (-0.29)	0.9(-0.51)	0.18(0.37)	**0.04(-0.29)**
diet	**0.04 (-0.34)**	**0.014(0.16)**	0.072(-.33)	0.08(0.27)	0.09(-0.39)	0.10(0.38)	0.55(0.41)	**0.00(-0.12)**	0.07(-0.17)	**0.04 (-0.27)**	0.2(-0.27)	0.31(0.45)	0.09(-0.34)
History of MD	**0.03(-016)**	**0.010(0.15)**	0.057(-.38)	0.09(0.19)	0.07(-0.26)	0.09(0.21)	0.41(0.49)	**0.02(-0.10)**	0.07(-0.33)	0.06(-0.31)	0.08 (-0.21)	0.25 (-0.26)	**0.03(-0.16)**
anti T2D therapy	0.25(-0.30)	0.082(0.34)	0.069(-.42)	0.05(0.36)	0.08(-0.28)	0.14(0.56)	0.48(0.51)	0.09(-0.55)	0.17(-0.40)	0.33(-0.18)	0.07(-0.28)	0.41 (-0.29)	0.28(-0.34)

β =-0.100 p=0.010 β =-0.159 p=0.014 β =-0.106) and low counts of B. longum (p=0.037 β =- 0.111, p=0.025 β =- 0.106, p=0.009 β =- 0.129), junk and sweet diet was associated with low counts of B. bifidum (p=0.044, β =- 0.276) and Romanian ethnicity and history of metabolic disorders were associated with low counts of B choerinum (p=0.042 β =- 0.298, p=0.032 β =- 0.161,) in obese patients, independent of age or previous treatments. No other associations were found (table 3 ad 4).

Correlation between obesity and T2D and fecal organic acid concentration

The statistic analysis was accomplished in order to evaluate correlations between patients and control groups regarding the fecal organic acid concentration. According to the history of previous hypoglycemic treatments, patients with T2D were split in 2 groups, in order to exclude the possible interactions of anti-diabetes drugs with the fecal acid concentration. Also, patients taking lipid- lowering drugs were advised to stop the medication 2 weeks prior to enrollment. The total organic acid concentration was significantly lower (p =0.036, p =-0.129 respectively p =0.027, p =-0.115) in untreated diabetes and obese patients (75.23 ± 17.1 mmol/g feces, respectively 77.18 ± 9.22 mmol/g feces) than in controls (111.8 ± 21.5mmol/g feces). The contents of total organic acid concentration was also lower in T2D patients taking anti-diabetes drugs, but without statistic significance (p=0.081, *ns*) compared to controls. The concentrations of acetic and butyric acids were significantly lower (p = 0.025, q = -0.018, p = 0.040, q =-0.183 p = 0.022, q = -0.191 respectively, p = 0.038, q = -0.133, p = 0.033, q = -0.210 p = 0.041, q = -0.184) in all patient groups, while the

Fecal organic acid concentration	Patients		
	Untreated T2D	T2D + hypoglycemic treatment	Obesity
	q (p val)	*q* (p val)	*q* (p val)
Total	**-0.129(0.036)**	0.123(0.091)	**-0.115(0.027)**
Butyric acid	**-0.133(0.038)**	**- 0.210(0.033)**	**-0.184(p=0.041)**
Valeric acid	**0.212(0.031)**	0.177(0.071)	**0.165(0.022)**
Acetic acid	**-0.018(0.025)**	**-0.183(0.040)**	**-0.191(0.022)**

Table 5
MULTIVARIABLE LINEAR REGRESSION ANALYSIS TO IDENTIFY THE CORRELATION BETWEEN T2D/ DIABETES AND FECAL ORGANIC ACID CONCENTRATION

** q= standardized regression coefficient*

Table 6
MULTIVARIABLE LINEAR REGRESSION ANALYSIS TO IDENTIFY THE CORRELATION BETWEEN SERUM VARIABLES AND FECAL BACTERIAL COUNTS OF *Lactobacilus AND Bifidobacterium sp* IN T2D

Lactobacillus species	**Serum variables**						
	CPR p val (β)	**ESR p val (β)**	**IL-6 p val (β)**	**HbA1c p val (β)**	**LDL cholesterol p val (β)**	**HDL cholesterol p val (β)**	**TG p val (β)**
L. acidophilus	**0.044(-0.199)**	**0.022(-0.186)**	**0.031(-0.179)**	**0.034 (-0.129)**	**0.011(-0.184)**	0.090(-0.293)	**0.003(-0.091)**
L. ruminis	0.234(-0.451)	0.076 (-0.206)	0.092(-0.222)	0.088(-0.199)	0.173(-0.187)	0.075(-0.234)	0.058(-0.224)
L. fermentum	0.106(-0.188)	0.078(-0.211)	0.105(-0.189)	0.106(-0.190)	0.234(-0.451)	0.107(-0.194)	0.134(-0.251)
L. plantarum	0.169(-0.201)	0.098(-0.258)	0.234(-0.451)	0.075(-0.234)	0.222(-0.201)	0.061(-0.177)	0.105(-0.178)
L. salivaricus	0.166(-0.199)	0.206(-0.388)	0.301(-0.301)	0.133(-0.240)	0.055(-0.202)	0.098(-0.258)	0.169(-0.199)
L. johnsonii	0.121(-0.219)	0.067(-0.144)	0.199(-0.199)	0.107(-0.178)	0.099(-0.258)	0.106(-0.188)	0.173(-0.187)
L.rueuteri	**0.034(-0.177)**	**0.038(-0.176**	**0.022(-0.149)**	**0.022 (-0.107)**	**0.031(-0.156)**	0.093(-0.301)	**0.016(-0.105)**
Bifidobacterium species.							
B. longum	0.129(-0.207)	0.108(-0.197)	0.104(-0.188)	0.222(-0.206)	0.238(-0.404)	0.098(-0.258)	0.122(-0.145)
B. adolescentis	0.111(-0.177)	0.173(-0.187)	0.093(-0.376)	0.116(-0.185)	0.104(-0.188)	0.109(-0.194)	0.173(-0.187)
B. bifidum	**0.017(-0.085)**	0.102(-0.182)	**0.042(-0.177)**	0.234(-0.333)	0.209(-0.256)	0.134(-0.210)	**0.027(-0.148)**
B. infantis	**0.028(-0.095)**	0.128(-0.198)	**0.037(-0.181)**	0.077(-0.238)	0.098(-0.258)	0.155(-0.167)	**0.044(-0.172)**
B. breve	0.111(-0.186)	0.201(-0.191)	0.234(-0.451)	0.111(-0.177)	0.173(-0.187)	0.188(-0.165)	0.177(-0.190)
B. choerinum	0.145(-0.167)	0.078(-0.211)	0.176(-0.177)	0.173(-0.187)	0.093(-0.376)	0.159(-0.155)	0.063(-0.198)

concentrations of valeric acid were significantly higher (p = 0.031, q = 0.212, respectively p = 0.022, q = 0.165) in patients with untreated T2D and obese patients compared to the controls. Regarding the fecal *p*H examination, results did not reveal any difference in *p*H between studied patients (6.77± 0.49) and controls (6.60 ± 0.83), (p = 0.22) (table 5).

Correlation between different species of Lactobacilus and Bifidobacterium and serum variables

The correlations between different species of *Lactobacilus* and *Bifidobacterium spp* with the serum variables revealed that in T2D patients with/without hypoglycemic treatments in the history, low counts of *L. acidophilus* and *L. reuteri* were positively correlated with the increased levels of HbA1c, LDL cholesterol, TG and inflammatory markers such as CRP, ESR and IL-6, no matter of diet, age, ethnicity or metabolic disorders history (p=0.034, q=-0.129; p=0.011, q=-0.184; p=0.003, q=-0.091, p=0.044, q=-0.199; p=0.022, q=-0.186; p=0.031, q=-0.179 respectively p=0.022, q=-0.107; p=0.031, q=-0.156; p=0.016, q=-0.105, p=0.034, q=-0.177; p=0.038, q=-0.176; p=0.022, q=-0.149); also, low counts of B. bifidum and B. infantis were positively correlated with high levels of CRP, IL-6 and TG (p=0.017, q=-0.085; p=0.042, q=-0.177; p=0.027, q=-0.148 respectively p=0.028, q=-0.095; p=0.037, q=-0.181; p=0.044, q=-0.172) (table 6). In obese patients, statistic analysis results showed that low counts of L. acidophilus, L. plantarum, L. johnsonii and L. reuteri were positively associated with increased levels of CPR, IL-6 and TG (p=0.030, q=-0.111; p=0.027, q=-0.193; p=0.043, q=-0.115; p=0.007, q=-0.079, p=0.005, q=-0.091; p=0.020, q=-0.117; p=0.042, q=-0.122, p=0.009, q=-0.173, p=0.006, q=-0.110 respectively p=0.046, q=-0.105; p=0.007, q=-0.106, p=0.011, q=-0.126) while lower counts of B. bifidum, B infantis and B. breve were positively correlated with higher counts of CPR, LDL cholesterol and TG (p=0.012, q=-0.181; p=0.027, q=0.185; p=0.040, q=-0.095; p=0.034, q=-0.116, p=0.047, q=-0.210; p=0.028, q=-0.157; p=0.041, q=-0.175; p=0.037, q=-0.183, p=0.041, q=-0.133) ; Also, lower counts of B. bifidum and B choerinum were positively correlated with lower counts of HDL cholesterol (p=0.033, q=0.141 respectively p=0.007, q=0.099) in Romanian ethnicity and patients with previous metabolic disorders (table 7).

Literature focused on the study of human gut microbiota suggests that from all factors, the main contributor to the diversity and equilibrium of intestinal microbiota seems to be the diet [26]. During pregnancy, the infant's gut is sterile, the first contact of digestive tract with bacteria being the delivery moment, when the infant goes in contact with the maternal vaginal of skin microbiota. Immediately after the first population of child's gut, changes in the diet can be responsible for 57 % variations in microbiota [27]. This feature can be proved by the study of breast-fed infants

Table 7

MULTIVARIABLE LINEAR REGRESSION ANALYSIS TO IDENTIFY THE CORRELATION BETWEEN SERUM VARIABLES AND FECAL BACTERIAL COUNTS OF *Lactobacilus* AND *Bifidobacterium sp* IN OBESITY

Lactobacillus species	**Serum variables**						
	CPR p val (β)	**ESR p val (β)**	**IL-6 p val (β)**	**HbA1c p val (β)**	**LDL cholesterol p val (β)**	**HDL cholesterol p val (β)**	**TG p val (β)**
L. acidophilus	**0.030(-0.111)**	0.083(-0.227)	**0.027(-0.193)**	0.105(-0.189)	0.224(-0.451)	0.063(-0.198)	**0.043(-0.115)**
L. ruminis	0.111(-0.176)	0.078(-0.228)	0.112(-0.173)	0.219(-0.251)	0.211(-0.300)	0.077(-0.220)	0.145(-0.247)
L. fermentum	0.085(-0.239)	0.116(-0.197)	0.081(-0.209)	0.077(-0.238)	0.176(-0.199)	0.105(-0.189)	0.139(-0.231)
L. plantarum	**0.007(-0.079)**	0.085(-0.239)	**0.005(-0.091)**	0.133(-0.241)	0.138(-0.230)	0.287(-0.351)	**0.020(-0.117)**
L. salivaricus	0.126(-0.201)	0.133(-0.240)	0.099(-0.197)	0.167(-0.200)	0.116(-0.197)	0.085(-0.216)	0.123(-0.200)
L. johnsonii	0.085(-0.239)	0.173(-0.208)	**0.009(-0.173)**	0.099(-0.196)	0.085(-0.239)	0.078(-0.228)	**0.006(-0.110)**
L.reuteri	**0.046(-0.105)**	0.106(-0.190)	**0.007(-0.106)**	0.234(-0.451)	0.138(-0.209)	0.169(-0.231)	**0.011(-0.126)**
Bifidobacterium species.							
B. longum	0.221(-0.351)	0.155(-0.199)	0.078(-0.189)	0.195(-0.289)	0.130(-0.190)	0.144(-0.182)	0.178(-0.207)
B. adolescentis	0.207(-0.211)	0.234(-0.451)	0.117(-0.187)	0.178(-0.201)	0.165(-0.199)	0.071(-0.211)	0.092(-0.189)
B. bifidum	**0.012(-0.181)**	0.201(-0.300)	0.088(-0.211)	0.234(-0.351)	**0.027(-0.185)**	**0.033(-0.141)**	**0.040(-0.095)**
B. infantis	**0.034(-0.116)**	0.199(-0.199)	0.106(-0.197)	0.222(-0.201)	**0.047(-0.210)**	0.145(-0.201)	**0.028(-0.157)**
B. breve	**0.041(-0.175)**	0.116(-0.197)	0.097(-0.219)	0.196 (-0.343)	**0.037(-0.183)**	0.109(-0.188)	**0.041(-0.133)**
B. choerinum	0.188(-0.220)	0.139(-0.211)	0.115(-0.196)	0.173(-0.260)	0.112(-0.177)	**0.007(-0.099)**	0.115(-0.194)

who shows more *Bifidobacteria spp.* compared to formula fed babies [28-29] which have a more diverse microbiota, with higher levels of *Bacteroids spp.* and *Lactobacillus spp.* [30]. Consecutive to these study results, the type of feeding immediately after child's birth is supposed to be the second element involved in microbiota changes and health evolution. The diversification of diet is another important step in child's microbiota changes, the moment and the quantities of the new elements introduced in child's diet having an important influence on host's future gut microbiota balance and personal well-being. [31] The use of antibiotics is, on the other hand, a decisive factor that influences the gut microbiota despite the diet. The early administration of antibiotics during childhood demonstrated to influence the microbiota populations. The intake of antibiotics in the first 6 months of life have a crucial effect on the microbiota during adult period, the imbalance of microorganisms ratio and the obesity being much more commonly present in this category of patients [32]. Advances in molecular biology techniques, such as next-generation sequencing has been contributed to better explore and understand the human gut microbiota spectrum [33-36]. New saprophyte or potential pathogen bacteria are still studied and new strategies for maintaining microbiota equilibrium are proposed. The dietary changes using the prebiotics and probiotics were first recommended to restore the physiological microbiota contents and to control the metabolic activity of the gut microorganisms. Lactulose and inulin, carbohydrate-like compounds, were two chemical formulas added to diet of patients with gut microbiota disturbances and metabolic secondary disorders from whom specialists expected good results, targeting especially the *Lactobacillus* and *Bifidobacterium spp.* It was also stated that prebiotics could restore the count of *L. reuteri* strain, involved in dyslipidemia occurrence. The restoration of normal *L. reuteri* strain count can reduce the fat gut absorption, decreasing the high level of LDL-cholesterol (low-density lipoprotein cholesterol). Our study confirmed the presence of low counts of *L. reuteri spp* in our patients groups, this feature being found to be correlated with junk food and sweets, BMI (>25) and personal history of metabolic disorders (p=0.032 β =-0.187, p=0.041, β =-0.142 p=0.019, β =-0.166) in patients with T2D and with Romanian ethnicity, metabolic disorders history and junk and sweet diet in patients with obesity (p=0.008 β =-0.100 p=0.010 β =-0.159 p=0.014 β =-0.106). There are chemical formulas combining the two compounds called synbiotics, in order to have the maximum additive benefit. Along with the diet and antibiotic intake history, other personal and environmental factors alter the gut microbiota. The spectrum of microorganisms populating the human gut varies according to geographical area, to ethnicity or diet habits and to the genetic features of a specific population. Physicians are still debating around the subject and are trying to merge data regarding microbiota patterns in different geographical areas, in order to prevent disorders related to microbiota gut disturbances, globally. Following this general concern on the topic, we studied the microbiota spectrum in two metabolic disorders as T2D and obesity over the geographical area of Romanian Black-Sea Coast, which contains a mixture of populations, ethnic groups and different alimentary habits. We studied the *Bifidobacterium spp*, along with *Lactobacillus spp* which are the most well-known inhabitants of our digestive tract, being part of the normal gut flora producing vitamins, bacteriocins (antibacterial chemicals) and antibiotic-like substances. *Lactobacillus species*, which constitute a significant component of the human gut microbiota, are

Gram-positive, facultativ anaerobic or microaerophilic, rod-shaped, non-spore-forming bacteria converting sugars to lactic acid. For example, in women of European ancestry, they are a major part of the vaginal microbiota which invade and populate the infants gut during delivery [37]. Our study results showed that the low bacterial counts of the *L. acidophilus, L plantarum and L. reuteri* subgroups of *Lactobacillus spp* were significantly more present among patients with T2D and obesity than controls (p = 0.003, q = 0.14 respectively p=0.029, q=0.18). On the other hand, *Bifidobacterium spp*, found in fermented dairy foods, especially yogurt, are Gram-positive, anaerobic, branched rod-shaped bacterium which also ferment sugars to produce lactic acid [38]. For example, *B. longum* genome codes for many proteins specialized for the catabolism of oligosaccharides.[39] In our study, Romanian ethnicity, metabolic disorders history and junk and sweet diets were associated with low counts of *B. Longum spp* in patients with obesity (p=0.037 β =- 0.111, p=0.025 β =- 0.106, p=0.009 β =- 0.129) while Romanian ethnicity and previous metabolic disorders were associated with low counts of *B. choerinum spp.* (p=0.042 β =- 0.298, p=0.032 β =- 0.161,) at obese patients, independent of age or previous antidiabetic treatments.

Research data demonstrated that gut bacterial products regulate important T2D and obesity pathways such as insulin signaling, inflammation and glucose homeostasis [1, 40-48]. The effect of microbiota on T2D and obesity incidence seems to be related mostly to SCFA titers. SCAFA, bacterial products resulted from colonic fermentation of the gut microbes, contribute to energy metabolism and balance the redox state in the gut. This mechanism includes the anaerobic breakdown of dietary fiber, protein and peptides. The most important SCFA bacterial products are acetate, propionate, and butyrate. Acetate and propionate are mostly produced by *Bacteroidetes phylum*, while butyrate is produced by the *Firmicutes phylum* and *Bifidobacterium spp.* They have been shown to exert beneficial effects on body weight, glucose homeostasis and insulin sensitivity. It was demonstrated that the dietary supplementation with butyrate can reduce the insulin resistance through increasing energy expenditure and mitochondria function [49]. In addition, oral administration of butyrate and acetate were proved to have a protective action against diet-induced obesity [50] and improve glucose tolerance [44, 51]. Also, gut microbiota is involved in the production of key insulin signaling molecules such as GLP-1 and PYY through SCFA and its binding to FFAR2 [52]. GLP-1 and PYY have favorable effects by decreasing insulin resistance and the functionality of β-cells [52]. *Bifidobacterium spp.* demonstrated to have anti-inflammatory effect in mice through the production of GLP2 and reducing intestinal permeability [43]. Along with these few examples on the potential impact of gut microbiota on the development of T2D and obesity, our study results revealed that the concentrations of acetic and butyric acids were significantly lower in patients compared to controls (p = 0.025, q = -0.018, p = 0.040, q =-0.183 p = 0.022, q = -0.191 respectively, p = 0.038, q = -0.133, p = 0.033, q = -0.210 p = 0.041, q = -0.184), while the concentrations of valeric acid were significantly higher (p = 0.031, q = 0.212, respectively p = 0.022, q = 0.165) in patients with untreated T2D and obese patients. We also founded that the total organic fecal acids concentration was significantly lower (p =0.036, p =-0.129 respectively p =0.027, p =-0.115) in untreated diabetes and obese patients (75.23 ± 17.1 μmol/g feces, respectively 77.18 ± 9.22 μmol/g feces) than in the controls (111.8 ± 21.5 μmol/g feces).

The multivariable linear regression analyze studied the correlation between fecal counts of *Lactobacillus spp.* and *Bifidobacterium spp* and different serum abnormal variables discovered in our patient groups.

Data on the interaction of microbiota with food and obesity/T2D brought new hypothesis for the obesity/fat diet relationships with inflammation. The inflammatory state that accompanies the metabolic syndrome shows no signs of infection or autoimmunity and no massive tissue injury seems to have taken place [53].

The severity of the inflammatory activation is not important as severity being often called l*ow-grade* chronic inflammation. Furthermore, some researchers named this inflammatory state as *metaflammation*, meaning metabolically triggered inflammation [1, 54] or *parainflammation* as a term to define an intermediate state between basal and inflammatory states [1, 53,55]. Whatever the term used, the inflammatory process that characterizes the metabolic syndromes seems to have its own unique features, its mechanisms being far from fully discovery. [1, 53, 56]. Recent studies confirmed the positive association between obesity and inflammatory markers, mainly CRP protein in women [1, 53, 57, 58], but also with other inflammatory markers, both in women and men [59, 60]

Our study results proved that no matter of personal variable features, low counts of different strains of *Lactobacillus spp* and *Bifidobacterium spp* are always correlated with high levels of inflammatory tests and metabolic tests disturbances. High levels of ESR, IL-6, respectively CPR and increased levels of HbA1c, LDL cholesterol and TG were correlated with low counts of L acidophilus and L reuteri (p=0.034, q=-0.129; p=0.011, q=-0.184; p=0.003, q=-0.091, p=0.044, q=-0.199; p=0.022, q=-0.186; p=0.031, q=-0.179; p=0.022, q=-0.107; p=0.031, q=-0.156; p=0.016, q=-0.105, p=0.034, q=-0.177; p=0.038, q=-0.176; p=0.022, q=-0.149) and high levels of CRP, IL-6 and TG were positively correlated with low counts of B. bifidum and B. infantis (p=0.017, q=-0.085; p=0.042, q=-0.177; p=0.027, q=-0.148 respectively p=0.028, q=-0.095; p=0.037, q=-0.181; p=0.044, q=-0.172) in T2D patients with/without hypoglycemic treatments in the history, no matter of diet, age, ethnicity or metabolic disorders history. In obese patients, results were a bit different, increased levels of CPR, IL-6 and TG were positively correlated with low counts of L. acidophilus, L. plantarum, L. johnsonii and L. reuteri (p=0.030, q=-0.111; p=0.027, q=-0.193; p=0.043, q=-0.115; p=0.007, q=-0.079, p=0.005, q=-0.091; p=0.020, q=-0.117; p=0.042, q=-0.122, p=0.009, q=-0.173, p=0.006, q=-0.110 respectively p=0.046, q=-0.105; p=0.007, q=-0.106, p=0.011, q=-0.126) while higher counts of CPR, LDL cholesterol and TG were positively correlated with lower counts of B. bifidum, B infantis and B. breve (p=0.012, q=-0.181; p=0.027, q=0.185; p=0.040, q=-0.095; p=0.034, q=-0.116, p=0.047, q=-0.210; p=0.028, q=-0.157; p=0.041, q=-0.175; p=0.037, q=-0.183, p=0.041, q=-0.133). In addition, low counts of HDL cholesterol were correlated with low counts of B. bifidum and B choerinum (p=0.033, q=0.141 respectively p=0.007, q=0.099) but only in Romanian ethnicity patients and in those with previous metabolic disorders history.

Conclusions

Gut microbiota imbalance seems to be related with two key metabolic diseases impacting pathways like energy homeostasis and inflammation in patients diagnosed with T2D and obesity from south Romanian Black Sea Coast Region, in concordance with literature data. Low bacterial

counts of some strains of *Lactobacillus spp* and *Bifidobacterium spp* were positively correlated with food type, BMI, Romanian ethnicity and personal history of metabolic disorders in our patients groups. Pre/probiotic treatments with supplementation of lacking elements (*L acidophilus, L plantarum,L reuteri, B bifidum, B infantis, B. breve and B choerinum*) could have additive efficacy in our T2D and obese medical care.

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