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PARTICULARITIES OF THE INTESTINAL MICROBIOME COMPOSITION IN ROMANIAN PATIENTS WITH METABOLIC SYNDROME AND TYPE 2 DIABETES

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Abstract

Introduction. The composition of gut microbiota can be affected by type 2 diabetes mellitus (T2DM) and metabolic syndrome (MS), its improvement is frequently ignored in metabolic disorders.
The objective of the study was a comparative evaluation of the faecal microbiota of healthy controls and of T2DM and MS patients (n = 150).
Materials and methods. A retrospective cross-sectional study was performed, between July 2019-December 2020 in the Bio-standard laboratory Oradea, Romania. A group of 75 patients with non-insulin dependent T2DM and MS (study group = SG) and 75 healthy subjects (control group = CG) has been included in the study. The composition of the microbiome and the influence of oral antidiabetic treatment on the microbiome in SG patients were compared.
Results. 8 out of 19 species of the analysed bacteria had a different relative abundance in the SG, compared to CG; *Enterococcus* spp. were significantly higher (3.18x106 vs 1.94x106, p=0.004), while *Akkermansia*

Résumé

Particularités de la composition du microbiome intestinal chez les patients roumains atteints de syndrome métabolique et de diabète de type 2

Introduction. La composition du microbiote intestinal peut être affectée par le diabète de type 2 (T2DM) et le syndrome métabolique (SM), son amélioration est fréquemment ignorée dans les troubles métaboliques.
L'objectif de l'étude était une évaluation comparative du microbiote fécal de témoins sains et de patients DT2 et SM (n = 150).
Matériaux et méthodes. Une étude transversale rétrospective a été réalisée entre juillet 2019 et décembre 2020 dans le laboratoire Bio-standard Oradea, Roumanie. Un nombre de 75 patients atteints de DT2 non insulinodépendant et de SM (groupe d'étude = GE) et 75 sujets sains (groupe témoin = GT) ont été inclus dans l'étude. La composition du microbiome et l'influence du traitement antidiabétique oral sur le microbiome chez les patients SG ont été comparées.

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muciniphila (2.55x109 vs. 4.66x109, p<0.001) and Eubacterium spp. (3.51x108 vs 4.16x108, p=0.047) had significantly lower values in the SG vs. CG.
Conclusions. The results indicated differences in the relative abundance of microbial species between the two groups, with significant changes in gut microbiome in the SG.

Keywords: metabolic syndrome, type 2 diabetes, weight status, glucidic profile, lipidic profile.

List of abbreviations:
BMI – body mass index
CG – Control group
DBP – diastolic blood pressure
HbA1c – glycosylated haemoglobin A1c
HDLc – high-density lipoprotein cholesterol
LDLc – low-density lipoprotein cholesterol
MS – metabolic syndrome
NAFLD – non-alcoholic fatty liver disease
SCFA– short-chain fatty acid
SG – study group
SBP – systolic blood pressure
T2DM – type 2 diabetes

Résultats. 8 des 19 espèces de bactéries analysées avaient une abondance relative différente dans le GE, par rapport au GT; *Enterococcus spp.* étaient significativement plus élevés (3,18x106 contre 1,94x106, p=0,004), tandis qu'*Akkermansia muciniphila* (2,55x109 contre 4,66x109, p<0,001) et *Eubacterium* spp. (3,51x108 vs 4,16x108, p=0,047) avaient des valeurs significativement plus faibles dans le SG vs. CG.
Conclusion. Les résultats ont indiqué des différences dans l'abondance relative des espèces microbiennes entre les deux groupes, avec des changements significatifs dans le microbiome intestinal dans le GE.

Mots-clés: syndrome métabolique, diabète de type 2, statut pondéral, profil glucidique, profil lipidique.

Introduction

The most common metabolic disorder worldwide is type 2 diabetes mellitus (T2DM), its specific features including peripheral insulin resistance in the liver, skeletal muscle and adipose tissue, and deficiencies in the insulin secretion[1]. In several developed countries of North America and Europe, the prevalence of T2DM grew alarmingly over the last decades[2]. An increased incidence of obesity and metabolic syndrome (MS) (two factors strongly associated with the risk of diabetes) was also observed[3]. Obesity is linked to hypertension, dyslipidaemia, insulin resistance and hyperglycaemia, these together being known as "metabolic syndrome"[4].

MS has a multifactorial aetiology, with diverse associations between factors like genetic predisposition, behaviours, diet, and the environment[5]. Physiologic risk factors (including lipo-toxicity, cortisol, systemic inflammation, oxidation) are linked to the pathogenesis and appearance of metabolic disorders (i.e. T2DM, obesity, hypertension, non-alcoholic fatty liver disease (NAFLD), etc)[6]. The risk of occurrence for T2DM, as well as of cardiovascular disorders, is enhanced by these metabolic disorders, which also determine increased rates of mortality and morbidity[4].

Besides genetic background which determines T2DM, other factors like physical activity and diet are also important in the occurrence and gravity of T2DM and MS. The advanced knowledge revealed microbiota to be an important factor for human health, presenting new pathways for fundamental and clinical studies of T2DM[7].

Microbiota consists of various living microorganisms like archaea, bacteria and fungi found in a specific environment[8]. It can regulate health, nutrition and diseases of the host and can be found on or within the host[9]. Although the exact composition of intestinal microbiota is not known, the improvement in metagenomic techniques recently began to reveal the variety of our microbial partners (human microbiome). Each human race contains at least 160 of these species, from a consortium of 1000 to 1150 prevalent bacterial species. 90% of the bacterial phylotype belongs to the two phyla viz bacteria, followed by *Actinobacteria* and *Proteobacterium*, *Bacteroidetes* and *Firmicutes*[10].

The diversity and composition of the gut bacteria have been intensely studied, as well as their impact on health and diseases[11], including obesity[12], inflammation[13], and T2DM[14]. Various studies revealed that decreased ratio of *Bacteroidetes* and increased ratio of *Firmicutes* were linked to insulin resistance and obesity[15,16]. Contradictory results were revealed by Larsen et al., suggesting that the proportion of *Bacteroidetes* to *Firmicutes* was positively and considerably associated with plasma glucose levels, and the *Betaproteobacteria* class was greatly enhanced in the

gut microbiome of diabetic subjects[17]. Further research on the connection between metabolic diseases and gut microbiota may determine better therapy schemes, precise monitorization of the disease, as well as new medicines production. In this study, the faecal microbiota of patients with T2DM and MS vs. healthy controls were compared, totalling the information of 150 subjects.

The objective of the study was to characterize the quantitative composition of intestinal microbial communities in adults with T2DM and MS. Furthermore, the hypothesis that intestinal microbial communities in patients with diabetes are different in individuals treated with oral antidiabetics and hypoglycaemic diet compared to those on a hypoglycaemic diet only was examined. This approach allows the evaluation of the microbiota response in humans, correlated with both drug and food interventions.

Materials and methods

A retrospective cross-sectional study was performed on the database of "Biostandard SRL" laboratories, Oradea, Romania, in the interval July 2019 – December 2020 (18 months), the only medical analysis centre that performed the microbiome analysis in Romania, at that time. The study group (SG) included 75 patients with non-insulin dependent T2DM and with MS. From the same database as well, 75 healthy patients (control group, CG) have been selected. The composition of the microbiome in the two groups was comparatively analysed, and in the SG, the influence of the treatment with oral antidiabetics on the microbiome has been also analysed.

Bacterial groups in faecal samples were quantified by polymerase chain reaction (qPCR), using the RealTimePCR Equipment (DNA Technology, Research and Production" LLC, JSC 2017, Russia), Multiplex kits (Immunodiagnostik Gmbh Germany), MutaPLEX AKM/FEAB PCR and MutaPLEX EU/BAC/BIF PCR tests, applied after a prior extraction of the genetic material (using ZymoBIOMICS DNA extraction Kit) and spotlighting the an-aerobic groups [18]. Aerobics group and fungus were cultured on convectional culture medias Hektoen, Mackonkey, Columbia Blood Agar, Sabouraud, and quantified by colonies counting technique.

The research was conducted in accordance with the WMA Ethical Declaration of Helsinki[19], and was approved by the Ethics Commission of the Council of Medicine and Pharmacy Faculty, University of Oradea, Romania (11/27.03.2021). Each patient included in this study signed an informed consent.

The software packages Jasp, SPSS v17 and Microsoft Excel have been used for the statistical analysis. The first step was to obtain a complete descriptive statistic which was performed by calculating the central tendency and descriptive indicators, and by plotting the most important results. After that, the Shapiro – Wilk test was applied to identify data distribution. To see if the observed differences can be considered statistically significant, a Mann – Whitney test (between the two studied groups) was applied. In the end of the study, a risk analysis was run to see if not going under treatment can be considered a risk factor; in this regard, the odds ratio (OR) parameter has been calculated, the 95% confidence interval was considered, and the chi square test was applied. For all results, α=0.05 has been considered.

Results

Demographic data and clinical characteristics

Because in most cases it was revealed that the data are not normally distributed ($p<0.05$), non-parametrical tests have been chosen. Tests were performed to identify statistical differences between the mean values of age, body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP), glycated haemoglobin (HbA1c), glucose of the two groups (with and without disease). Mann – Whitney test has been applied in this purpose and significant differences ($p<0.05$) resulted in the case of BMI, HbA1c and glucose (Table 1).

Comparison of the intestinal microbiota

At the level of intestinal microbiota, 11 bacterial species have been found at identical or very similar levels in both the SG and CG. For *Proteus* spp., *Serratia* spp., *Morganella morganii*, and *Pseudomonas* spp., the value $1x10^3$ was recorded in all cases; for Staphylococcus aureus and *Geotrichum* spp. $1x10^2$, and *Candida Albicans*, *Candida Nonalbicans*, the value 1x10 was recorded in all cases. *Enterobacter* spp., *Citrobacter spp.*, and *Firmicutes* spp./*Bacteroides* spp. were slightly different in the two groups (Table 2).

The Mann – Whitney test was applied to see if there are significant differences in the number of bacterial species in patients with or without the disease. We obtained significant difference: in *Enterococcus* spp. ($p=0.004$), in *Akkermansia muciniphila* $p<0.001$, and in *Eubacterium* spp. (Table 3). In the SG, *Escherichia coli* values, *Bacteroides* and *Enterococcus* spp., were higher than in the CG, while the rest of the analysed bacteria had lower values in the SG, as Figure 1 reveals.

In the SG, over 50% of patients did not follow antidiabetic treatment (54.70%), following only a hypoglycaemic diet. However, the most used oral

Table 1. The central tendency and dispersion indicators for age, BMI, glucose level, HbA1c, SBP, DBP

Descriptive statistics	*Age*		*BMI*		*Glucose*		*HbA1c*		*SBP*		*DBP*	
	CG	*SG*	*CG*	*SG*	*CG*	*SG*	*CG*	*SG*	*CG*	*SG*	*CG*	*SG*
Valid	75	75	75	75	75	75	75	75	75	75	75	75
Missing	0	0	0	0	0	0	0	0	0	0	0	0
Mean	47.533	54.68	29.615	30.349	117.96	123.267	6.917	7.207	138.867	139.333	79.96	79.253
Std. Error of Mean	0.634	1.207	0.317	0.319	2.036	2.514	0.071	0.069	0.786	0.813	0.678	0.687
Median	48	54	29	29.69	115	123	6.8	7.2	140	140	80	80
Mode	45	45	27.09	29.3	132	155	6.5	6.8	140	140	80	80
Std. Deviation	5.49	10.45	2.749	2.766	17.636	21.775	0.618	0.595	6.807	7.039	5.874	5.953
Variance	30.144	109.194	7.557	7.653	311.039	474.171	0.382	0.354	46.333	49.55	34.498	35.435
Skewness	-0.38	0.65	1.683	1.694	0.204	0.248	0.381	0.429	0.127	0.362	-0.352	-0.226
Kurtosis	0.415	0.129	3.434	3.604	-1.192	-1.185	-0.074	-0.145	-0.926	-0.428	-1.04	-1.095
Shapiro-Wilk (SW)	0.968	0.964	0.846	0.838	0.946	0.937	0.979	0.968	0.898	0.909	0.899	0.901
p-value of SW	0.056	0.032	<0.001	<0.001	0.003	0.001	0.26	0.051	<0.001	<0.001	<0.001	<0.001
Range	30	51	13.13	13.13	62	77	3.1	2.6	25	25	20	20
Minimum	29	36	25.42	26.12	88	88	5.5	6.2	130	130	70	70
Maximum	59	87	38.55	39.25	150	165	8.6	8.8	155	155	90	90

Note: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycated haemoglobin.

Table 2. Microbiome analysis

Intestinal Microbiota	*Control group*	*Study group*
Eschericia coli	$7.391\pm2.790 \times 10^{6}$	$7.468\pm2.753 \times 10^{6}$
Proteus spp.	$1.000\pm0.000 \times 10^{3}$	$1.000\pm0.000 \times 10^{3}$
Klebsiella spp.	$1.173\pm0.978 \times 10^{3}$	$1.533\pm1.833 \times 10^{3}$
Enterobacter spp.	$1.297\pm1.021 \times 10^{3}$	$1.293\pm0.161 \times 10^{3}$
Serratia spp.	$1.000\pm0.000 \times 10^{3}$	$1.00\pm0.00 \times 10^{3}$
Morganella morganii	$1.000\pm0.000 \times 10^{3}$	$1.000\pm0.000 \times 10^{3}$
Citrobactr spp.	$1.000\pm0.000 \times 10^{3}$	$1.081\pm0.69 \times 10^{3}$
Pseudomonas spp.	$1.000\pm0.000 \times 10^{3}$	$1.000\pm0.000 \times 10^{3}$
Enteroccocus spp.	$2.217\pm2.320 \times 10^{6}$	$3.377\pm2.790 \times 10^{6}$
Staphylococcus aureus	$1.000\pm0.000 \times 10^{2}$	$1.000\pm0.000 \times 10^{2}$
Akkermansia munciniphila	$4.475\pm2.156 \times 10^{9}$	$2.545\pm1.841 \times 10^{9}$
Faecalibacterium prausnitzii	$4.088\pm2.302 \times 10^{10}$	$4.070\pm2.388 \times 10^{10}$
Eubacterium spp.	$4.156\pm2.358 \times 10^{8}$	$3.505\pm2.298 \times 10^{8}$
Bifidobacterium spp.	$4.251\pm2.500 \times 10^{9}$	$4.452\pm2.490 \times 10^{9}$
Bacteroides spp.	$4.324\pm2.227 \times 10^{8}$	$4.529\pm2.372 \times 10^{8}$
Raport Firmicutes spp./*Bacteroides* spp.	$1.128\pm1.071 \times 10^{0}$	$1.124\pm1.669 \times 10^{0}$
Candida Albicans	$1.000\pm0.00 \times 10^{1}$	$1.00\pm0.000 \times 10^{1}$
Candida Nonalbicans	$1.000\pm0.000 \times 10^{1}$	$1.00\pm0.000 \times 10^{1}$
Geotrichum spp.	$1.000\pm0.000 \times 10^{2}$	$1.000\pm0.000 \times 10^{2}$

antidiabetics were metformin + glibenclamide combinations (26.70%), and metformin (12.00%). The therapeutical administered schemes are presented in Table 5. Also, it was analysed the bacterial species level in the 75 patients from the SG, in the case of treatment presence/ absence. The descriptive analysis is presented in Table 4. For the SG, a Mann – Whitney test was applied to find out if patients with treatment have a different gut microbiome structure. Significant differences have been obtained in the case

Table 3. Bacterial abundance in control group (CG) *vs* study group (SG)

Intestinal Microbiota	*CG*	*SG*	*P**
Escherichia coli	7.391±2.790 x10^6	7.468±2.753 x10^6	0.822
Klebsiella spp.	1.173±0.978 x10^3	1.533±1.833 x10^3	0.186
Enteroccocus spp.	2.217±2.320 x10^6	3.377±2.790 x10^6	0.004
Akkermansia munciniphila	4.475±2.156 x10^9	2.545±1.841 x10^9	< .001
Faecalibacterium prausnitzii	4.088±2.302x10^{10}	4.070±2.388x10^{10}	0.819
Eubacterium spp.	4.156±2.358 x10^8	3.505±2.298 x10^8	0.047
Bifidobacterium spp.	4.251±2.500 x10^9	4.452±2.490 x10^9	0.584
Bacteroides spp.	4.324±2.227 x10^8	4.529±2.372 x10^8	0.560

*Independent Samples Mann-Whitney test

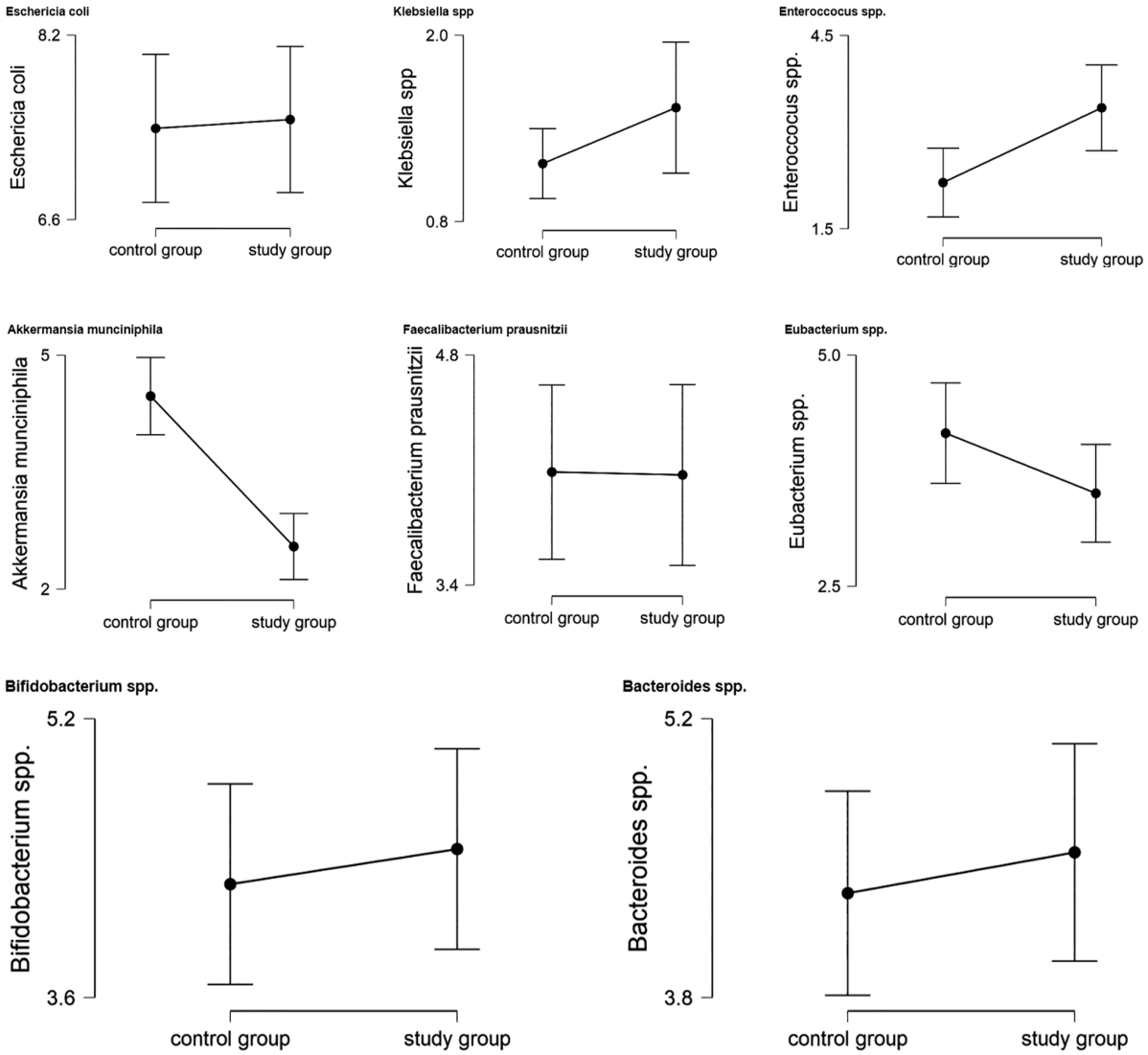

Figure 1. Differences in the number of bacterial species

of *A. muciniphila*, p=0.002 (Table 5); the patients that followed a treatment have significantly higher values. In all the tested cases, except *Faecalibacterium prausnitzii*, a growth was registered in the group of patients who had followed a treatment. The entire analysis is detailed in Figure 2.

In the end of the study, a risk analysis has been performed. Considering this cross-sectional study, it

Table 4. Frequencies of oral antidiabetic treatment

Antidiabetic treatment	*Frequency*	%
Metformin + glibenclamide	20	26.667
Metformin	9	12.000
Gliclazide	5	6.667
Hypoglucidic diet	41	54.667
Total	75	100

Table 5. Bacterial abundance in patients with or without antidiabetic treatment

Intestinal Microbiota	*Sample 1*	*Sample 2*	*P**
Escherichia coli	$7.253\pm2.893 \times 10^6$	$7.661\pm2.652 \times 10^6$	0.517
Klebsiella spp.	$1.000 \pm0.000 \times 10^3$	$1.976 \pm2.403 \times 10^3$	-
Enteroccocus spp.	$3.377\pm2.790 \times 10^6$	$3.259 \pm2.320 \times 10^6$	0.624
Akkermansia munciniphila	$2.545\pm1.841 \times 10^9$	$4.475\pm2.156 \times 10^9$	0.002
Faecalibacterium prausnitzii	$4.070\pm2.388 \times 10^{10}$	$4.088\pm2.302 \times 10^{10}$	0.360
Eubacterium spp.	$3.505\pm2.298 \times 10^8$	$4.156\pm2.358 \times 10^8$	0.979
Bifidobacterium spp.	$4.452\pm2.490 \times 10^9$	$4.251\pm2.500 \times 10^9$	0.624
Bacteroides spp.	$4.529\pm2.372 \times 10^8$	$4.324\pm2.227 \times 10^8$	0.624

*Mann-Whitney U test. [a]Variance in *Klebsiella* spp. is equal to 0 after grouping on Sample, 1 = without treatment, 2 = with treatment

Table 6. The risk analysis

Contingency table				*Results*
Akkermansia munciniphila	$\leq 5 \times 10^9$	$> 5 \times 10^9$	*Total*	
Without treatment	38	3	41	p = 0.027 OR = 5.227 OR ∈ (1.31; 21.14)
With treatment	24	10	34	
Total	62	13	75	

was verified if the lack of treatment can be considered a risk factor of having a lower *A. muciniphila* quantity. After applying the risk analysis, it resulted that the patients from the group with the disease, who were not under treatment, were significantly more likely to have a lower *A. muciniphila* quantity (OR=5.277,95% ∈ (1.31;21.14), p=0.027) (Table 6).

Discussion

More and more data indicate that the development, susceptibility, progression, and severity of T2DM are affected by gut microbiota. Insulin resistance, T2DM, low-grade inflammation and obesity are linked with dysbiosis, altered gut microbiota, that probably indicates a causal role connecting these disorders[20]. Experimental studies and various human trials have shown particular gut bacteria decreased or enriched in T2DM in contrast to healthy controls[20], and support the connection between gut microbiome and other various MS components[21].

As the aim of this study was to determine some peculiarities of the composition of the intestinal microbiome in patients with T2DM and MS, our research identified differences in the relative abundance of bacteria in the SG with T2DM and MS. *Escherichia coli*, *Citrobacter* spp., and Bacteroides spp. values resulted in being insignificantly higher than in the CG, while the values of *Enterococcus* spp. were significantly higher; moreover, for *Enterobacter* spp. and *Firmicutes* spp./ *Bacteroides*, the values were insignificantly lower in the SG vs. CG. *A. munciniphila* and *Eubacterium spp.* had significantly lower values in the SG compared to the CG.

Generally, compared to healthy subjects (controls), T2DM patients presented increased quantities of branch chain amino acid synthesizing bacteria (*Bacteroides vulgatus* and *Prevotella copri*), reduced quantities of opportunistic pathogens (*Bacteroides caccae* and *Clostridium hathewayi*) and sulfate-metabolizing bacteria (*Desulfovibrio, Lactobacillus gasseri, and Lactobacillus reuteum*), and reduced

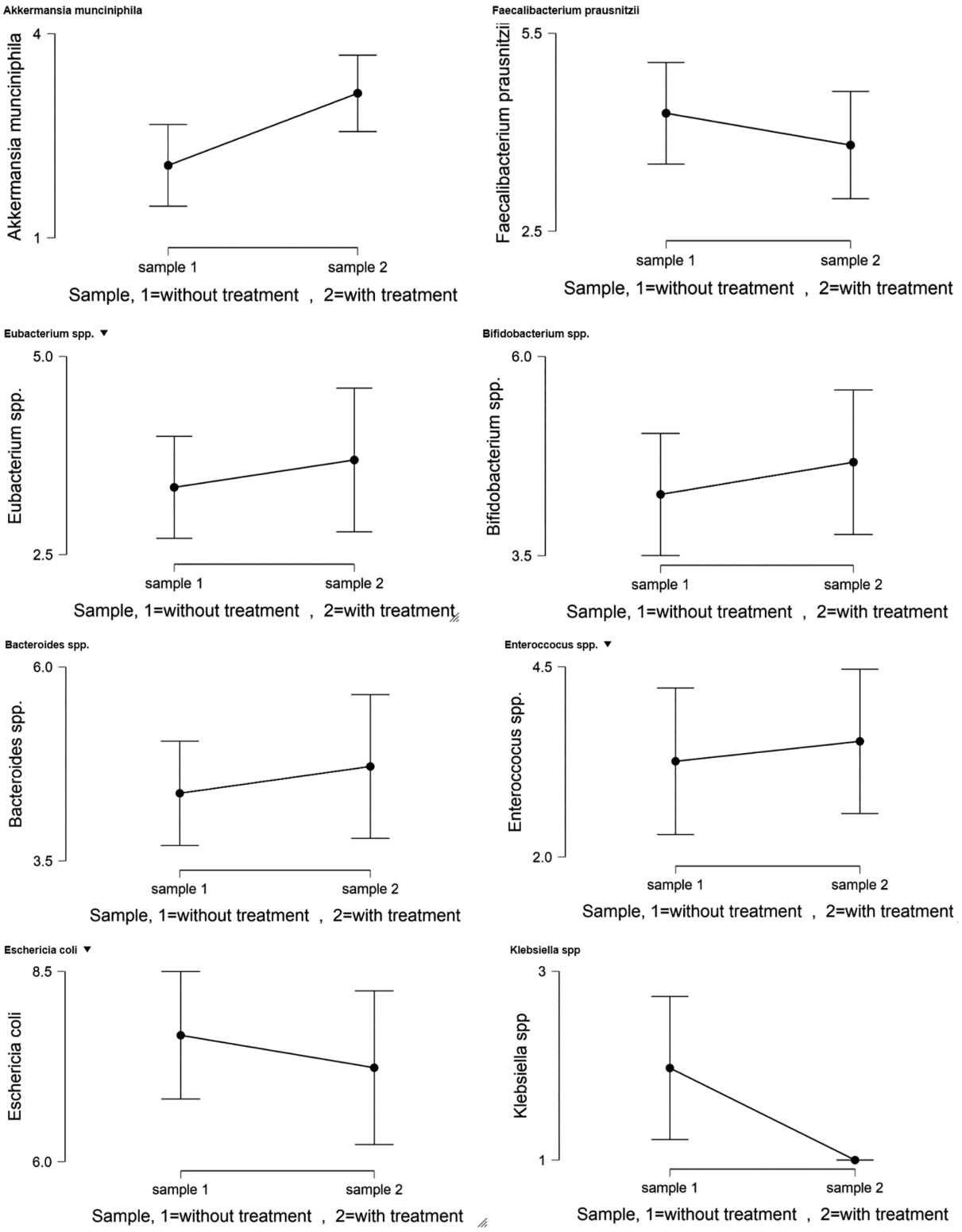

Figure 2. Data distribution of the bacterial abundance in patients with or without treatment.

quantities of tryptophan metabolite producing bacteria (Bacteroides, Bifidobacterium) and short-chain fatty acid (SCFA) producing bacteria (*Eubacterium rectale*, *Faecalibacterium prausnitzii*, *Akkermansia*, and *Bifidobacterium*)[15,22,23].

The most indicated beneficial genera in T2DM trials are *Bacteroides* and *Bifidobacterium*. *Bifidobacterium*, a genus comprising microbes that probably offer protection against T2DM, is systematically supported by the literature. Almost all studies indicate a negative

link between T2DM and this genus[24-28], though a study indicates opposite outcomes[29]. No significant differences in *Bacteroides* and *Bifidobacterium* were reported in the present study between the SG (they resulted in higher amounts in patients treated with oral antidiabetics) and CG.

The host metabolic functions can be influenced by drugs that can alter the gut microbiome (drug – microbiome – metabolism axis). Between biguanides, metformin has various effects, it improves glycaemic control and reduces cardiovascular mortality in T2DM patients who are overweight; in newly diagnosed T2DM patients, it is used as first-line treatment and can also prevent T2DM[30]. It is also suggested by various evidences that metformin modulates gut microbiota[31] in high-fat diet (HFD)-fed rodents and humans; additionally, together with high quantities of *A. muciniphila*, a mucus-degrading gut bacteria, ameliorates glycemia[31].

The structure of gut microbiota is changed by metformin in both humans and mice, bringing it to a form comparable to that of a healthy host[32]. A decreased quantity of *Intestinibacter* spp. and *Clostridium* spp. and a high quantity of *Bifidobacterium bifidum*, *A. muciniphila, Lactobacillus, Escherichia, Shigella* spp. are determined by metformin[33].

As T2DM is a progressive condition, in which higher degrees of hyperglycaemia are depicted, and there is a necessity to increase progressively the dose to preserve the glycemia in normal ranges, combined therapy was applied to target multiple mechanisms[34]. The combinations of metformin and DPP-4 inhibitors, metformin-thiazolidinediones and metformin-sulfonylureas are the most frequently used[35]. Currently, there is limited data on the influence of various combination of therapies on gut microbiome.

In the present study, most patients were administered metformin, alone or in combination with glibenclamide, and the variations in the number of bacteria in the gut microbiome have been determined. *Eubacterium, Bifidobacterium, Enterococcus* and *Bacterioides* spp. had an insignificantly higher abundance in the treated group, while the other determined species were insignificantly lower. *A. muciniphila* was significantly increased in patients with antidiabetic therapy. In recent years, there has been an increase in attention paid to *A. muciniphila* because of benefits found and proven in reducing body weight, data published in the literature emphasizing the need for colonization with *Akkermansia* spp. of patients describing metabolic disorders[30]. Both *A. muciniphila* and mucosal pathology have been modified; moreover, the incidence of inflammatory bowel disease and appendicitis was inversely associated with it[36], demonstrating the negative association of intestinal *A. muciniphila* with obesity, diabetes, and other MS[36,37]. The results of our study suggest that antidiabetic drug treatment combined with diet may significantly influence the intestinal microbiome in patients with diabetes and MS. Several studies to evaluate the response of intestinal microbiota, both to drug and dietary interventions, are needed to pave the way for effective therapeutic approaches in the treatment of T2DM with MS.

Conclusions

The simultaneous presence of T2DM and MS produces changes in the composition of the intestinal microbiome in the studied patients, leading to a significant increase in the values of *Enterococcus* spp., and a significant decrease in *A. muciniphila* and *Eubacterium* spp. Antidiabetic treatment combined with hypoglycaemic diet positively influence the composition of the intestinal microbiome in patients with both disorders, registering a significant increase in *A. muciniphila*.

Authors Contribution:

Conceptualization, R.A.C.A., D.M.T., and C.M.V.; methodology, R.A.C.A. and S.B.; software, R.M.; validation, D.M.T. and S.G.B.; formal analysis, R.M. and S.B.; investigation, R.A.C.A., S.B., and A.G.T; resources, R.A.C.A.; data curation, R.A.C.A. and A.G.T.; writing–original draft preparation, R.A.C.A., D.M.T., C.M.V. and S.B.; writing–review and editing, R.A.C.A., D.M.T., R.M, C.M.V. and S.B.; visualization, D.M.T. and S.B.; supervision, S.B.; project administration, S.B. All authors have read and agreed with the final version of this article.

Compliance with Ethics Requirements:

"The authors declare no conflict of interest regarding this article"

"The authors declare that all the procedures and experiments of this study respect the ethical standards in the Helsinki Declaration of 1975, as revised in 2008(5), as well as the national law. Informed consent was obtained from all the patients included in the study"

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