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ORIGINAL RESEARCH

Are There Differences in Gut Microbiome in Patients with Type 2 Diabetes Treated by Metformin or Metformin and Insulin?

Sylwia Dzięgielewska-Gęsiak[1], Edyta Fatyga[1], Magdalena Piłot[1], Aleksander Wierzgoń[2], Małgorzata Muc-Wierzgoń[1]

[1]Department of Internal Medicine Prevention, Medical University of Silesia in Katowice, Katowice, Poland; [2]Department of Environmental Biotechnology, Faculty of Energy and Environmental Engineering, Silesian University of Technology, Gliwice, Poland

Correspondence: Sylwia Dzięgielewska-Gęsiak, Department of Internal Preventive Medicine, Medical University of Silesia in Katowice, 41-902 Bytom, Piekarska 18 st, Katowice, Poland, Tel/Fax +48/32/39-76-527, Email sgesiak@sum.edu.pl

Introduction: Recently, gut microbiota has been described as being involved in the health and diseases of the host, and together with diet and drugs may influence metabolic health. Yet, there is still no answer which type of treatment plays the most important role in the interplay of gut microbiota and type of treatment for type 2 diabetes (T2DM). An attempt was made to answer the question of which factors have the most significant impact on the intestinal microbiome in the context of metformin or metformin+insulin use in treatment of the patients with T2DM. Thus the aim of the study was to compare the gut microbiome profiles of patients with T2DM and two of the most traditional treatment methods.

Methods: T2DM patients treated by metformin (Met) and metformin+insulin (Met+Ins), with the treatment duration of 5–10 years were enrolled. Biochemically blood glucose and glycated hemoglobin (HbA1c), lipids and kidney function were investigated and the quantitative and qualitative examination of the fecal intestinal flora were performed through the next-generation sequencing.

Results: There were no significant differences in the study of the gut microbiome: the dominant bacterial phyla were *Firmicutes* and *Verrucomicrobia*, while *Bacteroidetes* and *Proteobacteria* shared smaller proportions in both groups. However, the group Met+Ins had worse metabolic control in terms of blood glucose and HbA1c in comparison with the Met group.

Conclusion: As there are no differences in gut microbiome in T2DM patients treated with metformin only or metformin plus insulin, adding insulin in the treatment of T2DM may delay late diabetic complications development.

Keywords: gut microbiome, type 2 diabetes, metformin, metformin+insulin

Introduction

The last decades have been characterized by remarkable advances in our understanding of type 2 diabetes (T2DM) mechanisms which are explained not only by reduced insulin secretion from β cells but also increased: glucagon secretion from α cells, hepatic glucose production, renal glucose reabsorption and lipolysis; reduced: glucose uptake in peripheral tissues, the incretin effect in the small intestine; and neurotransmitter dysfunction and insulin resistance in the brain.[1] Recently, a gut microbiota has been described, which is involved in the health and diseases of the host, and together with diet and drugs have an influence on metabolism.[2–5] It was found that the level of *Bifidobacterium* was significantly higher in healthy individuals in comparison with T2DM individuals, while *Lactobacillus* was more frequent in T2DM patients.[6] Moreover, in adults with T2DM a reduction in *Clostridia* class, which belongs to *Firmicutes* phyla was found. What is more, a significant, positive correlation was found for plasma glucose concentration and the ratios of *Bacteroidetes* to *Firmicutes* as well as the ratios of *Bacteroides-Prevotella* group to *C. coccoides-E. rectale* group.[7] The data suggests that the composition changes of gut-microbiome are dynamic and depend on diet, exposure to drugs including antibiotics and, in particular, in response to disease.[8,9] In relation to T2DM, changes in microbiota composition are associated with impaired β-cell function, insulin resistance development, increased intestinal permeability which

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promotes a pro-inflammation status and endotoxemia.[10] Thus, by influencing gut microbiome, we may treat T2DM and its complications.

Pharmacomicrobiomics, a rapidly growing branch of medicine, is the study of the interactions between microbiome and drugs.[11] The remodeling microbiota induced by drugs depends on the drugs' pharmacodynamics and pharmacokinetics.[12] In T2DM metformin improves glucose homeostasis and exerts hypoglycemic effects by affecting the gut microbiota (significantly increasing the abundance of the phylum *Verrucomicrobia*, genus *Akkermansia*, and species *Akkermansia muciniphila*), through which it maintains the intestinal barrier function, increases the production of short-chain fatty acids, regulates bile acid metabolism, and affects glucose homeostasis.[13] While intensive insulin therapy recovers diabetes-associated gut structural abnormalities and restores the microbiome landscape.[14]

Yet, there is still no answer as to which type of treatment plays the most important role in the interplay of gut microbiota and type of treatment for T2DM subjects. In this study, an attempt was made to answer the question of which factors have the most significant impact on the intestinal microbiome in the context of metformin or metformin+insulin use as the most traditional treatment for patients with T2DM with the treatment duration 5–10 years.

Materials and Methods

Ethics

The current study protocol was registered with the Bioethical Committee of the Medical University of Silesia in Katowice. The Committee wrote that "the project does not meet the criteria of a medical experiment in the context of law and does not require assessment by the bioethical committee; However, failure to obtain the consent requirement does not release the applicant from compliance with generally applicable laws and standards" (Letter KNW/0022/KB1/39/19). Based on this decision, written informed consent was not required of our study nor was separate patient consent required for our statistical analysis or research. Still, according to the Declaration of Helsinki 2013 the participation in the study was voluntary and informed. Moreover, participants were informed in detail about the study. Thus, patient data has been encoded in accordance with the pseudo-anonymization procedure, which means that personal data is processed in such a way that it cannot be assigned to a specific data subject, without the use of an additional "key."

Patients Inclusion Criteria

The study included patients with type 2 diabetes with the duration treatment 5–10 years. A total of 358 patients with type 2 diabetes, using *Metformin* or *Metformin* plus *Insulin* treatment were invited to the study. The mean dose of metformin for both patient groups was 2000 mg/day. Among those with insulin treatment 55% of them used to use human insulin (short acting/intermediate or mixed) and 45% of them had analogue insulin (rapid acting, long acting or mixed).

Patients Exclusion Criteria

If T2DM patients were on medication other than metformin or insulin as a hypoglycemic agents they were excluded from the study.

According to Lee et al[15] current smokers had an increased proportion of the phylum B*acteroidetes* with decreased *Firmicutes* and *Proteobacteria* compared with never smokers, whereas there were no differences between former and never smokers. So current smokers and alcohol consumers were excluded from the study. In addition, individuals were also excluded if they used to use medication that increase microbiome phylla: pro-, pre-, or symbiotics (including over the counter [OTC] and supplements) as well as those medications that may change microbiome profiles: inhibitor proton pumps, medications that change stool frequency or intestines movement, systemic anti-inflammatory drugs, antibiotics, including antifungal treatment.

Participants Previously Diagnosed

- Malignant tumors,
- Bowel-inflammatory diseases,
- Heart failure,

- End stage renal disease,
- Liver cirrhosis were excluded from the present study.

During participant selection, the presence of deviations including pathology in the results of a laboratory examination panel, served as the basis for excluding the patient from the study. The laboratory examination panel was carried out in the morning, on an empty stomach, and included a complete blood count; liver panel: bilirubin, ALAT, AST; C-reactive protein, and a cancer-markers panel.

Clinical Assessment

Following recruitment, patients underwent a complete clinical assessment and biochemical investigation.

Biochemical Investigations

Classical Self-Monitoring of Glycaemia

A seven-day daily glycemic control was analyzed, which consisted of determining the concentration of glucose in capillary blood using dry strip tests with a glucometer. The test consisted of puncturing the previously washed side surface of the fingertip and smearing a blood drop sample of the appropriate size on the test field. Patients used Accu-Chek Active blood glucose meters by Roche Diagnostics. The respondents kept self-control notebooks in which they wrote down the time and the measurement results, the dose of anti-diabetic drugs, the size and composition of meals, and additional information on physical exertion and stressful situations. Then the mean glucose (mean Glu) was calculated.

Determination of Plasma Glucose and Glycated Hemoglobin

Fasting plasma glucose (G0') concentration was determined by an enzymatic method with hexokinase and glucose-6-phosphate dehydrogenase, on dimension Xpand Plus Systems (Siemens Healthcare Diagnostics). The analytical measurement range of the test was 0–27.8 mmol/l.

Glycated hemoglobin (HbA1c) was measured in whole blood by a method according the National Glycohemoglobin Standardization Program (NGSP), anchored to the IFCC reference method and traceable to the Diabetes Control and Complications Trial (DCCT) reference. The D-10 equipment of Bio-Rad (Hercules, CA, USA) was used. The HbA1c was expressed either as the percentage of HbA1c fraction (IFCC) in total hemoglobin or mmol/mol (NGSP).

Lipids Measurements

Concentration of total plasma cholesterol (TC), high-density lipoproteins cholesterol (HDL-C), and triglycerides (TAG) were assessed by enzymatic methods on the Dimension Xpand Plus Systems (Siemens Healthcare Diagnostics) equipment.

Total cholesterol (TC): The concentration of total cholesterol in the serum was determined by the enzymatic method with cholesterol esterase/cholesterol oxidase. The analytical measurement range of the test was 1.3–15.5 mmol/l.

HDL-cholesterol (HDL-C): The high density lipoproteins fraction was obtained from the plasma blood after precipitation of chylomicrons, very low density lipoproteins and low-density lipoproteins with dextran sulfate in the presence of magnesium sulphate; after centrifugation in a supernatant (containing HDL lipoproteins), the cholesterol concentration was determined through a direct enzymatic method with cholesterol esterase/cholesterol oxidase. The analytical measurement range of the test was 0.08–3,89 mmol/l.

Triglycerides (TAG): Serum triglycerides concentration was determined by an enzymatic method (lipase/glycerol kinase/glycerol-3-phosphate oxidase/peroxidase). The analytical measurement range of the test was 0.17–11.3 mmol/l.

Plasma low-density lipoproteins cholesterol (LDL-C) concentration was calculated according to Friedewald formula: [LDL-C] = {[TC] (mmol/L) – [HDL-C] (mmol/L)} – [0.45xTAG (mmol/L)], if TAG <4.56 mmol/L. Those patients with TAG >4.56 were excluded from the study.

Plasma cholesterol of non-HDL (non-HDL-C) fraction was calculated from the formula: [non-HDL-C] (mmol/L) = [TC] (mmol/L) – [HDL-C] (mmol/L).

Kidney Function

Kidney function was assessed by fasting creatinine (cre) concentration (reference range 44–88 umol/l) automatically on COBAS Integra system (Roche Diagnostics, Basel, Switzerland). Moreover, an estimated Glomerular Filtration Rate (eGFR) was calculated by the Modified Diet in Renal Disease formula (MDRD).

Analysis of Stool Samples

During the months before stool collection took place, the investigated participants were not treated with antibiotics, NSAIDs, metamizole, paracetamol, steroids, iron preparations, or other drugs used in gastrointestinal diseases (including proton pump inhibitors). They also did not take probiotics, prebiotics and/or symbiotics.

Ultimately, after all clinical and biochemical inclusion/exclusion criteria, 60 persons were entitled to fecal microbiome profiling.

Stool samples (2 grams) were collected by individuals in sterile containers and were delivered within two hours to the laboratory, where they were frozen at −80 degrees Celsius. Then, the samples were transferred in a subjected manner to the molecular laboratory.

Qualitative and Quantitative Analysis of the Intestinal Microbiome

The quantitative and qualitative examination of the fecal intestinal flora was performed by the next-generation sequencing (NGS) method of A&A Biotechnology (Gdynia, Poland) in cooperation with Macrogene Inc. (Korea) based on studies by Klindworth A et al.[16] The analysis was carried out in the following stages:

1. DNA isolation from frozen human stool samples. Isolation using mechanical and enzymatic lysis and purification on ion-exchange membranes. DNA eluate parameters: a minimum concentration 0.1ng/μL in a minimum volume of 20 μL per sample.
2. Illumina SBS DNA sequencing technology. The Amplicon library was prepared from supplied DNA by amplifying the V3–V4 region using a pair of primers and incorporating illumina adapters with indexes. Due to the expected repeatability of the tests and the need for comparison with other results, the required DNA sequencing procedure is based on the studies of Klindworth A et al.[16]
3. Testing the input material in terms of quality (electrophoresis) and quantity with the aid of Victor 3 fluorometry using picrogreen, as well as checking the quality of the library itself.
4. Sequencing by means of the original Illumina kits (Herculase II Fusion DNA Polymerase Nextera XT Index Kit V2) on the MISec platform in 2×300 bp paired reading mode (v3 chemistry = 600 cycle), with up to 100 K readings per sample.
5. The open-source Statistical Analysis of Metagenomic Profiles (STAMP (v 2.1.3)) according to Klindworth A et al.[16]

Statistical Analysis

Statistical analysis was calculated by Statistica (version 13.3) for Windows. The sample size calculation was based on an α value of 0.01 to allow comparisons between investigated groups. The Shapiro–Wilk test was used to check the normality of distributions of variables in the 60 elderly individuals (all investigated patients) and treated with metformin or metformin plus insulin groups. Because most of the data had a non-normal distribution a nonparametric Mann–Whitney *U*-test was used to assess the differences between the Met and Met+Ins groups. A p-value < 0.05 or lower was considered statistically significant.

Results

Table 1 summarizes the characteristics of the investigated T2D patients (the results are expressed as median and interquartile range) in whole T2DM individuals and T2DM treated by metformin (Met) and metformin+insulin (Met +Ins) groups. The analyzed Met (n=27) and Met+Ins (n=33) groups differed in terms of metabolic control: fasting plasma

Table 1 The Characteristics of the Investigated T2D Patients (the Results are Expressed as Median and Interquartile Range)

	All T2DM N=60	Met N=27	Met+Ins N=33	p
Age [years]	64.0 (59.5–72.0)	70.0 (61.0–78.0)	63.0 (58.0–69.9)	0.03
Diabetes duration [years]	8.2 (5.9–8.9)	7.9 (5.8–9.0)	8.4 (6.0–9.2)	>0.05
Glu 0' [mmol/l]	7.3 (6.2–9.7)	6.8 (5.9–7.6)	8.9 (6.7–11.4)	0.02
HbA1c [%]	7.2 (6.2–8.7)	6.2 (5.9–6.6)	8.2 (7.3–9.3)	0.000001
HbA1c [mmol/mol]	55.0 (44.0–72.0)	44.0 (41.0–49.0)	66.0 (56.0–78.0)	0.00001
Glu mean [mmol/l]	8.9 (7.3–11.3)	7.3 (6.9–7.9)	10.5 (9.0–12.2)	0.00001
TC [mmol/l]	4.65 (3.81–5.17)	4.66 (4.09–5.32)	4.47 (3.81–5.14)	>0.05
HDL-C [mmol/l]	1.25 (0.99–1.50)	1.34 (1.05–1.82)	1.15 (0.91–1.4)	>0.05
LDL-C [mmol/l]	2.47 (1.74–3.12)	2.38 (1.76–3.07)	2.49 (1.71–3.18)	>0.05
Non-HDL-C [mmol/l]	3.25 (2.43–3.83)	3.15 (2.18–3.86)	3.36 (2.5–3.83)	>0.05
TAG [mmol/l]	1.60 (1.32–2.08)	1.46 (1.18–1.71)	1.97 (1.45–2.31)	0.01
Crea [umol/l]	68.0 (58.8–89.9)	73.5 (59.8–85.7)	65.1 (57.7–93.1)	>0.05
eGFR [mL/min/1.73m^2]	60.0 (57.8–60.0)	60.0 (59.0–60.0)	59.0 (56.8–60.0)	>0.05

glucose concentration (p=0.02), the HbA1c value (p<0.000001) and mean glucose concentration (p=0.001), as well as triglyceride concentration (p = 0.01).

In the investigated T2DM patients the relative abundance composition (Figure 1) of the microbial population at the phylum level showed that the most common were *Firmicutes*.

Although the group Met+Ins had worse metabolic control in terms of blood glucose and HbA1c levels and a tendency for weaker kidney function (p not statistically significant) in comparison with the Met group, the analysis of microbiome biodiversity did not show statistically significant differences. The dominant bacterial phyla were *Firmicutes* and *Verrucomicrobia*, while *Bacteroidetes* and *Proteobacteria* shared smaller proportions in both investigated groups (Met vs Met+Ins). The predominant order in each group was *Clostridiales* with abundances of 46.0% in Metformin group (Figure 2) and 49.0% in Metformin+Insulin group (Figure 3). The *Verrucomicrobiales* order represents 18.0% and 14.0%, respectively. Both analyzed groups had the same abundances of *Bacteroidales* order (9.0%). *Proteobacteria* were represented by *Enterobacteriales*: 7.0% in Metformin group and 9.0% of Metformin+Insulin group. The abundances of the other orders which represents 1% of microbiome for investigated groups describe Figures respectively 4 for Metformin group and 5 for the Metformin+Insulin group (Figures 4 and 5).

It was found that the use of metformin or metformin+insulin is not associated with significant changes in Shannon's diversity (Figure 6).

Discussion

Thanks to next-generation sequencing (NGS) we could anticipate pathogen-host interactions.[17] The technic provides a new perspective in understanding the pathomechanisms of various diseases, including diabetes, and elucidated the role of medications on gut microbiota. Some research has added to our knowledge of crosstalk between gut microbiota and antidiabetic drug action.[18,19] By the use of NGS in this study we investigated the pharmacomicrobiomics in terms of intestinal microbiome composition and two different types of the most used treatments of T2DM: by metformin or metformin+insulin.

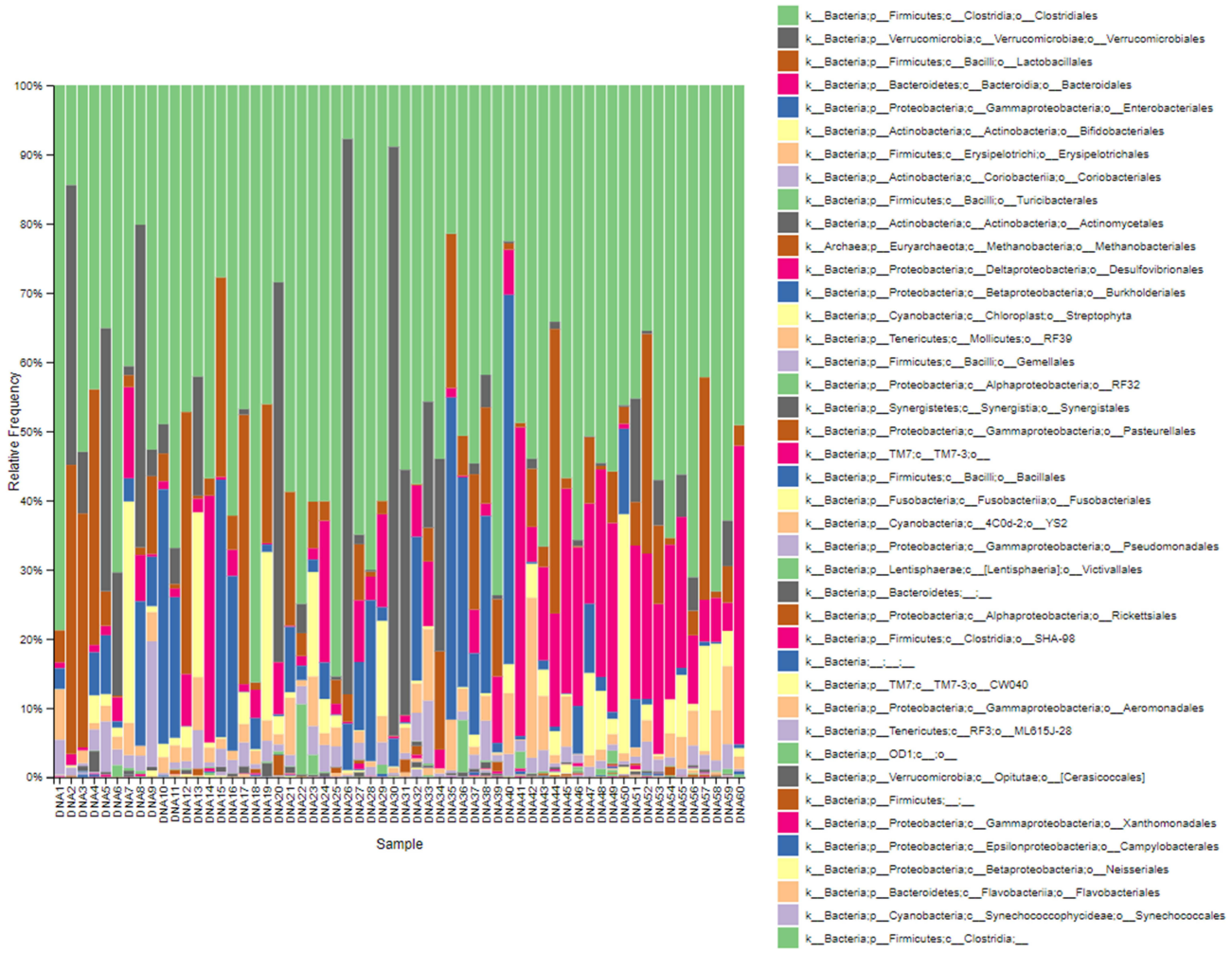

Figure 1 Relative frequency of the microbial population at the orders level in all investigated T2DM patients.

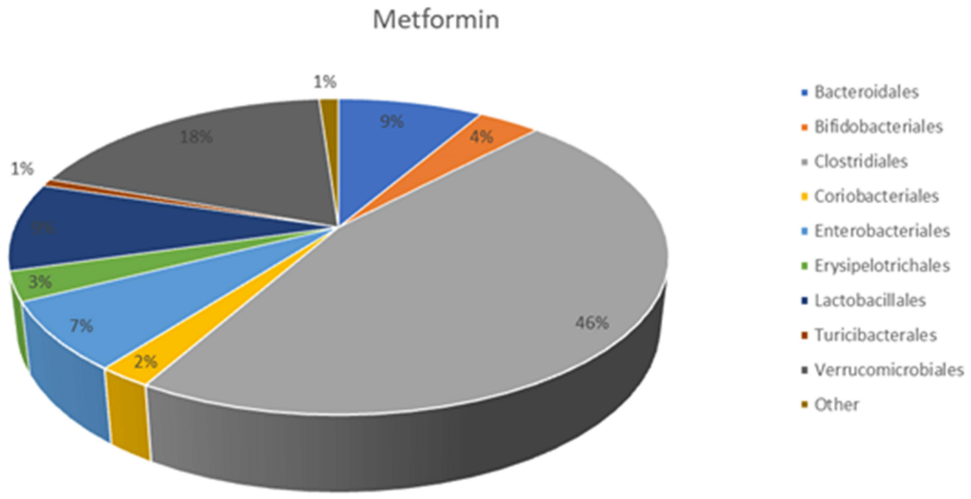

Figure 2 Percentages of the microbial population at the orders level of Metfromin group of T2DM patients.

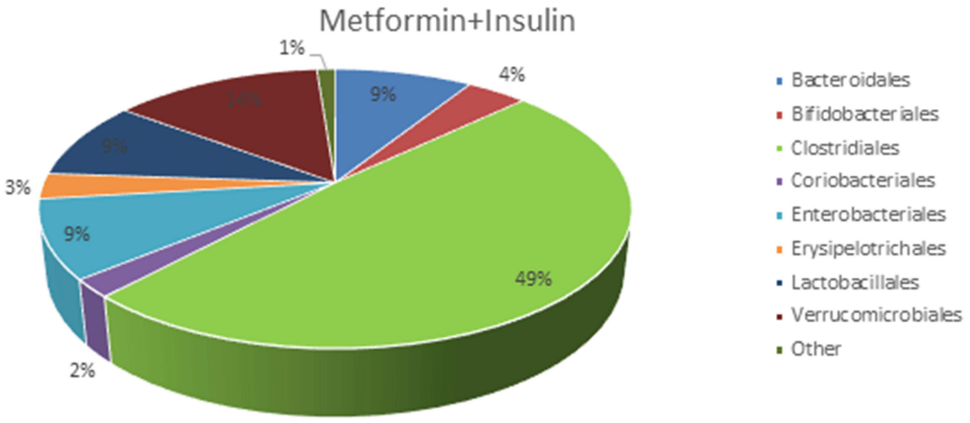

Figure 3 Percentages of the microbial population at the orders level of Metformin+Insulin group of T2DM patients.

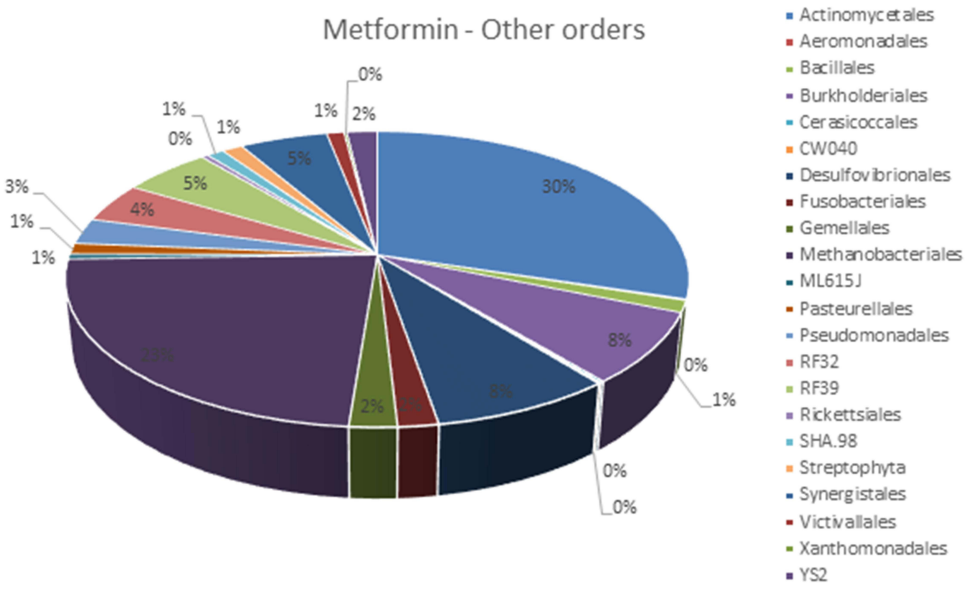

Figure 4 Percentages of the microbial population at the orders level of Metformin group of T2DM patients – The 1% from the other bacterial orders.

Despite the array of drugs, metformin is still the most frequently and widely used drug in the treatment of T2DM.[20] For almost 70 years the biguanide is the first line agent in T2DM treatment and exerts various effects through different actions. Despite a known glucose-lowering effect and insulin sensitizing potential it has a beneficial effect on the growth of metabolically beneficial bacteria, such as *Akkermansia muciniphila, Lactobacillus spp. or Escherichia spp*. and reduce the number of other potentially unfavorable bacteria such as *Intestinibacter*.[21] It should be remembered that the use of metformin may also influence short-chain fatty acid production and increase butyrate-producing taxa, thus influencing gut microbiome.[22,23] In particular the treatment by metformin is sub-served by an increase of *Akkermansia muciniphila* which is known as a mucin-degrading bacteria and belongs to the *Verrucomicrobiales* order.[13] In our study, the abundances of *Verrucomicrobiales* was 18.0% in the Metformin group and 14.0% in the Metformin+Insulin group. As was stated in the results, the most common order in both investigated groups was *Clostridiales*, which belongs to *Firmicutes* phylum, however, the Shannon diversity for all bacteria was not significant. It is in contrast with the work of Chávez-Carbajal et al who found the Shannon index showed a richness of diversity in those T2DM patients treated with polypharmacy+insulin in gut bacterial communities in comparison with only metformin treated patients.[24] However, in the available literature, there are few reports regarding the effect of insulin therapy on the intestinal microbiome. Wang

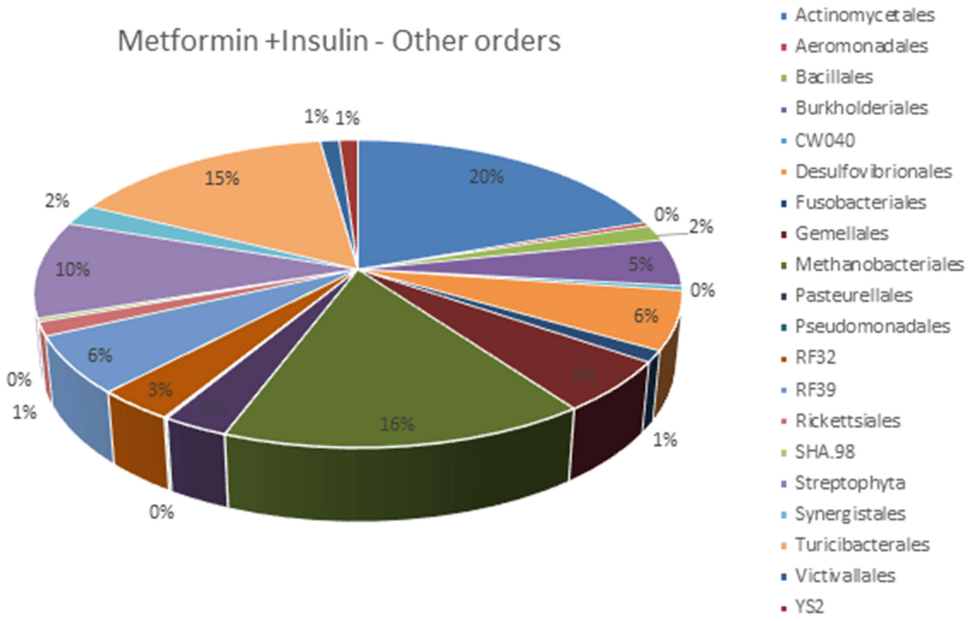

Figure 5 Percentages of the microbial population at the orders level of Metformin+Insulin group of T2DM patients – The 1% from the other bacterial orders.

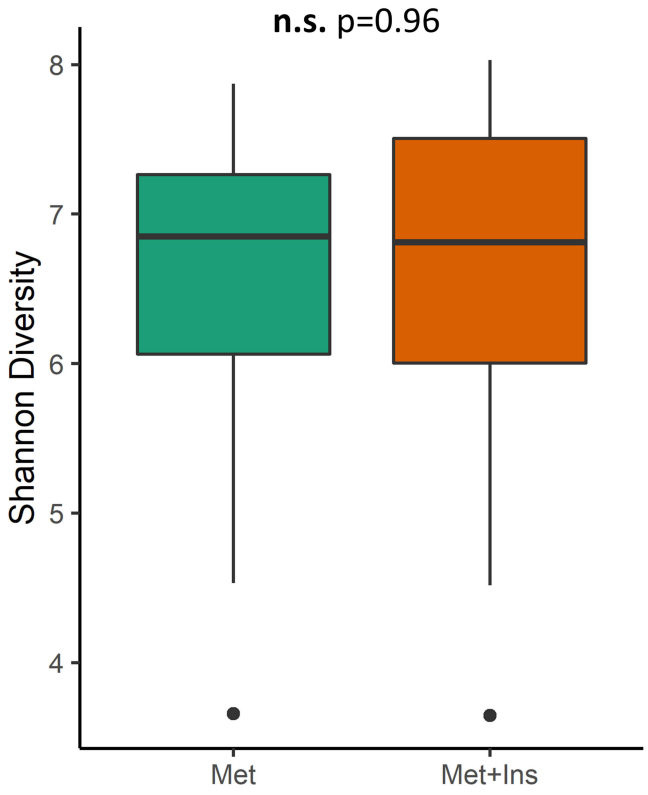

Figure 6 Shannon diversity of microbiome in investigated groups.

et al indicate that intensive insulin therapy recovers diabetes-associated gut structural abnormalities and restores the human microbiome.[14]

On the other hand, dysbiosis may play a role in the development of diabetes complications, or their appearance during the course of the disease.[25] Yet, it was found that *Firmicutes*, particularly *Lachnospiraceae* overgrowth were found to be

in a close relationship between impaired glucose metabolism and kidney failure progression.[26,27] Nevertheless, in our investigated groups the Met+Ins had higher fasting and mean glucose concentrations as well as HbA1c levels and a tendency towards weaker kidney function in comparison to the Met group but there were no significant diversity in the gut microbiome. Moreover, phylum *Actinobacteria* is involved in lipid metabolism.[28]

In our investigated patients the Met+Ins group had higher triglyceride concentrations but the *Bifidobacteriales* abundances between both groups were the same.

The polypharmacy in diabetes is intended to improve metabolic outcomes. Weaker metabolic outcomes should result in treatment intensification adding other oral antidiabetic drugs or insulin, which is administered parenterally.[29] It is known, however, that therapeutic inertia may occur – which means slower intensification of treatment than results of HbA1c are recommended in clinical guidelines.[30,31] Moreover, the inertia may be due to the medical staff or patients. The therapeutic inertia may be an explanation of the weaker metabolic outcome in our clinical cohort treated with metformin+insulin. In our two clinical cohorts, no differences in gut microbiome is the strength of our results, which could avoid therapeutic inertia by adding insulin to treat diabetes. Thus it may prevent late diabetic complications but not restore all metabolic outcomes.

Limitations of the Study

This study is limited by some factors and should be interpreted with caution. The main limitation is the small number of participants in both groups (27/33) and the heterogeneity in the Met+Ins group in regards to the types of insulin and insulin therapy models used. However, and this is strength of the study, we investigated only the most traditional type of treatment (metformin or metformin+insulin) with long duration of treatment. We did not investigate the influence of other factors on gut microbiome neither novel therapies nor diet. Moreover, diet is well known to influence the gut microbiome in T2DM and obesity.[2,3] Simultaneously, due to the small number of participants in both groups, microbiome profile was not analyzed depending on their BMI, fasting/postprandial glycemia, and HOMA value.

Conclusion

Patients with diabetes need different types of treatments. There are no differences in gut microbiome in patients treated with metformin or metformin plus insulin. Thus, adding insulin in the treatment of diabetes may delay late diabetic complications development.

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Ethics Approval and Consent to Participate

The current study protocol was registered with the Bioethical Committee of the Medical University of Silesia in Katowice. The Committee wrote that the project does not meet the criteria of a medical experiment in the context of law and does not require assessment by the bioethical committee; However, failure to obtain the consent requirement does not release the applicant from compliance with generally applicable laws and standards. (Letter KNW/0022/KB1/39/19). The decision of the Committee was final. Based on this decision, written informed consent was not required of our study nor was separate patient consent required for our statistical analysis or research. Still, according the Declaration of Helsinki 2013 the participation in the study was voluntary and informed. Moreover, participants were informed in detail about the study. Thus, patients data has been encoded in accordance with the pseudo-anonymization procedure, which means that personal data is processed in such a way that it cannot be assigned to a specific data subject, without the use of an additional "key."

Disclosure

The authors report no conflicts of interest in this work.

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