Association of metformin administration with gut microbiome dysbiosis in healthy volunteers

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Abstract

Background

Metformin is a widely used first-line drug for treatment of type 2 diabetes. Despite its advantages, metformin has variable therapeutic effects, contraindications, and side effects. Here, for the very first time, we investigate the short-term effect of metformin on the composition of healthy human gut microbiota.

Methods

We used an exploratory longitudinal study design in which the first sample from an individual was the control for further samples. Eighteen healthy individuals were treated with metformin (2 × 850 mg) for 7 days. Stool samples were collected at three time points: prior to administration, 24 hours and 7 days after metformin administration. Taxonomic composition of the gut microbiome was analyzed by massive parallel sequencing of *16S rRNA* gene (V3 region).

Results

There was a significant reduction of inner diversity of gut microbiota observed already 24 hours after metformin administration. We observed an association between the severity of gastrointestinal side effects and the increase in relative abundance of common gut opportunistic pathogen *Escherichia-Shigella* spp. One week long treatment with metformin was associated with a significant decrease in the families *Peptostreptococcaceae* and *Clostridiaceae*_1 and four genera within these families.

Conclusions

Our results are in line with previous findings on the capability of metformin to influence gut microbiota. However, for the first time we provide evidence that metformin has an immediate effect on the gut microbiome in humans. It is likely that this effect results from the increase in

abundance of opportunistic pathogens and further triggers the occurrence of side effects associated with the observed dysbiosis. An additional randomized controlled trial would be required in order to reach definitive conclusions, as this is an exploratory study without a placebo control arm. Our findings may be further used to create approaches that improve the tolerability of metformin.

Introduction

Metformin is a biguanide agent that is widely used as a first-line treatment of type 2 diabetes (T2D) [1]. Metformin has several advantages, including high safety indicators, high efficacy, neutral or lowering effect on body mass, and cardioprotective effects [2–4], resulting in broad indications for use over the 60 years it has been on the market. Nevertheless, metformin also has variable therapeutic effects, contraindications, and side effects which indicate the urgent need for a personalized approach when choosing treatment strategies [5].

It has been shown that intravenously administered metformin is less effective than its orally administered form [6]. Furthermore, metformin reaches a 30–300 times higher concentration in mucosa of small intestine compared to plasma, and up to 30% of the drug is eliminated through the feces [7, 8]. In addition, a delayed-release formulation of metformin improves glycemic control to the same extent as the immediate-release form despite lower systemic exposure [9]. These findings have led to the hypothesis that the effects of metformin are partially explained by its interaction with the gut microbiome. The connection between the effects of metformin and the gut microbiome has been supported by several recent studies [10–17]. These studies suggest that the gut microbiome is involved in both the therapeutic and side effects of the drug, yet details of this interaction remain obscure.

Current knowledge regarding the interaction between metformin and the gut microbiome highlights that metformin reduces inner diversity of the gut microbiome in mice fed a high-fat diet [13] and its administration increases relative abundance of *Akkermansia muciniphila* [10–14]. There is also evidence that metformin increases the abundance of some other mucin degrading and short-chain fatty acids producing genera [10], as well as opportunistic pathogens such as *Escherichia* spp. [11, 12]. Modulation of the gut microbiome is also hypothesized to be responsible for the anti-obesity action of metformin, not only in T2D patients but in prediabetic populations as well [18].

However, as pointed out previously, many of the earlier studies of the gut microbiome did not control for treatment regimens in T2D patients, subsequently leading to divergent conclusions [11]. It appears plausible that some of the potential clinical effects, e.g., metabolic control of longevity [19], anticancer properties [20], and testosterone lowering in patients with polycystic ovary syndrome [21] occur through alterations in the microbiome. Therefore, in this exploratory longitudinal study we evaluated the short-term effect of oral metformin administration on the human gut microbiome composition and diversity in healthy individuals, and the possible connection between these changes and metformin-related gastrointestinal (GI) side effects.

Materials and methods

Study design

Eighteen healthy volunteers of Caucasian origin were included in this exploratory study through the Genome Database of Latvian Population [22] as a part of an ongoing clinical trial

(50 individuals to be included in total), by assessing the 25 individuals available at the time. Baseline characteristics and registered clinical parameters are shown in Table 1. Major exclusion criteria were as follows: (1) use (during the past two months) of antibiotics, immunosuppressive drugs, corticosteroids, proton pump inhibitors, or pharmaceutical-grade probiotics; (2) oncological, autoimmune, or chronic gastrointestinal tract diseases, or T2D; (3) diarrhea in the past week; and (4) use of any other medications that are not compatible with metformin. A full list of inclusion/exclusion criteria can be found in the S1 Text. All participants, after full explanation of the purpose and nature of all procedures used, gave signed informed consent containing detailed information on the project (Fig 1). The study was carried out in accordance with the Declaration of Helsinki, and approved by the Central Medical Ethics Committee (1/16-05-12) and State Agency of Medicines of the Republic of Latvia (17–1723), clinical trial registration number: 2016-001092-74 (www.clinicaltrialsregister.eu).

Participants took metformin (850 mg tablets; Berlin-Chemie AG, Germany) twice daily during meals with a glass of water for a period of 7 days. Diet, physical activities, and side effects were registered daily in special questionnaires during the whole study period. Dietary data were registered using a 7-day food record during the week of metformin use, and an additional 2-day food record was filled before starting the use of metformin. We consulted a certified nutritionist and data from the dietary registry were divided into 11 food groups and labeled as follows: (1) milk and dairy products, (2) vegetables, (3) fruits, (4) meat and its products, (5) fish, (6) croppers, (7) nuts and seeds, (8) fat, (9) snacks, (10) sweetened drinks, and (11) alcohol. The cumulative summary characterizing the 7-day food records for each food group was expressed as a percentage from the combined amount of food consumption during the metformin treatment (S2 Table).

The primary endpoint of this study was the detection of significant changes in taxonomical composition of the gut microbiome. The secondary endpoint was the possible correlation between specific taxonomic units and the development of GI side effects. Compliance with the study was ensured by thorough explanation and detailed written instructions of the study protocol. Unused tablets were returned to the principal investigator.

All individuals were concurrently involved in an ongoing methylation profile analysis in leukocytes from whole blood samples taken at three specific time points during the study (unpublished data).

Sample collection

Blood samples for hematological and biochemical analyses were collected in the fasting state 1–3 days before starting metformin administration. Data were used to evaluate significant health indicators for kidney and liver function, as well as other criteria characterizing the

Table 1. Characteristics of the study group.

Characteristic	Value
Females/Males, n (%)	11 (61.1%)/ 7 (38.9%)
Age (years), median [IQR]	25.5 [7.5]
BMI, median [IQR]	24.2 [3.5]
ALAT (U/l), median [IQR]	20.5 [10.8]
Creatinine (μmol/l), median [IQR]	71.5 [13.5]
Fasting plasma glucose (mmol/l), median [IQR]	5.1 [0.5]

ALAT–alanine aminotransferase, BMI–body mass index, IQR–interquartile range

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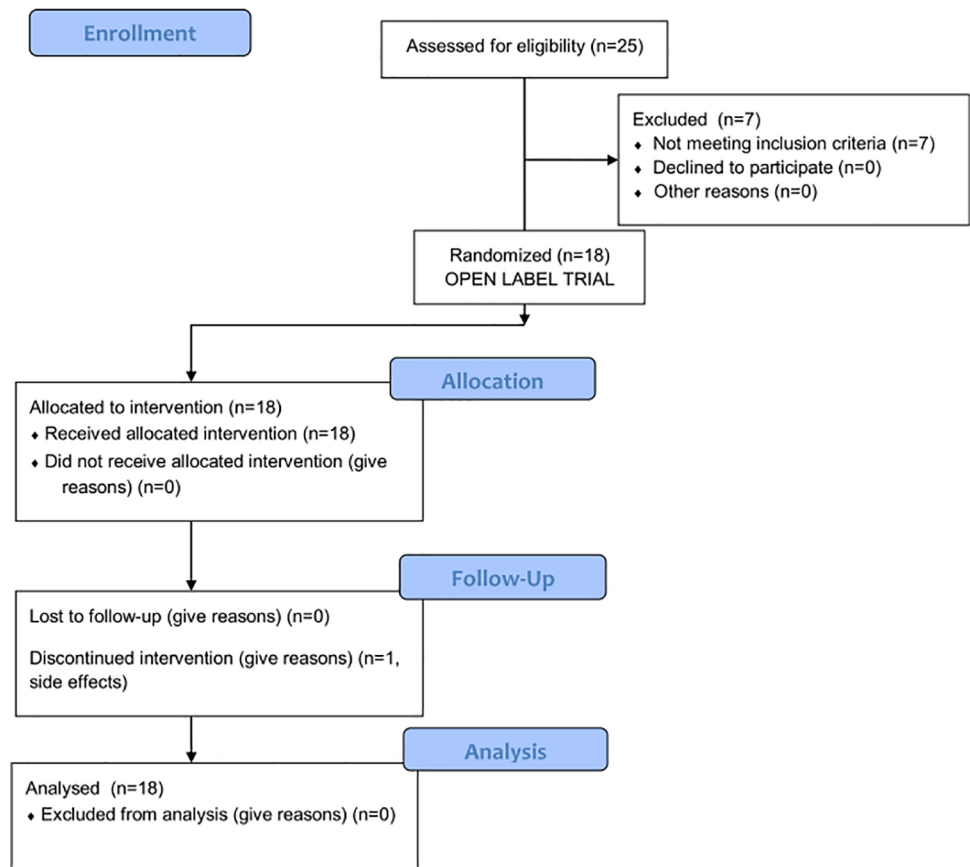
CONSORT 2010 Flow Diagram

Fig 1. CONSORT flowchart of the open-label trial.

suitability of individuals for medicament therapy. All hematological and biochemical analyses were conducted in the same certified clinical laboratory.

Stool samples in two aliquots were collected at three time points: before starting metformin treatment (M0) and 24 hours (M24h) and 7 days (M7d) after the first intake of metformin. After collection, fecal samples were stored at room temperature until delivery to the laboratory, and frozen at −80˚C as soon as possible but not later than within 24 hours of collection [23, 24]. Sample collection, storage and handling were done by following our developed standard operation procedures with the aim to minimize unnecessary freezing and thawing cycles and to reduce the possibility of artefacts caused by temporary storage at room temperature.

Bacterial DNA preparation and sequencing analysis

Microbial DNA was extracted from frozen stool samples using FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) and FastPrep Instrument according to the instructions of the manufacturer. DNA concentrations of the extracted samples were evaluated using Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and the integrity of the extracted microbial DNA was validated by agarose gel electrophoresis.

For each sample, the V3 region of the *16S rRNA* gene was amplified using the Probio_Uni/Probio_Rev primer set [25]. Each primer contained IonXpress adapter sequence and a unique barcode sequence. The amplified PCR products were purified using NucleoMag magnetic beads (Macherey-Nagel, Düren, Germany), and their quantity and quality were evaluated with the Agilent 2100 Bioanalyzer DNA High Sensitivity chip (Agilent Technologies, Santa Clara, CA, USA). Sequencing of the amplicon libraries was performed with Ion Torrent Personal Genome Machine (PGM) System (Thermo Fisher Scientific; Ion 318 Chip Kit v2, Ion PGM Hi-Q Sequencing Kit, minimal sequencing depth per sample– 250 000 reads) according to the instructions of the manufacturer.

Preprocessing and statistical methods

Raw sequence data were processed using mothur software v.1.39.1 [26]. Analyses were done using a modified version of the publicly accessible MiSeq SOP. In the sequence filtering, step reads were removed if they were 75 bp or shorter, or contained ambiguous bases or homopolymers longer than eight bases. A representative sequence from each cluster was chosen and used to identify taxonomic groups from the SILVA database v.123 [27]; the flip parameter was set as true. Chimeric sequences and sequences containing potential sequencing errors were removed using UCHIME [28] or pre-clustering (threshold = 2), respectively. Operational taxonomic units were defined at ≥99% sequence identity, using the OptiClust algorithm. Reads were classified using the naïve Bayesian classifier [29].

The correlation between gut microbiome taxa and the defined food groups was evaluated with Spearman's correlation analysis and the results were adjusted for multiple testing using the Benjamini–Hochberg method.

Statistical analyses were performed on taxonomic units found in at least 50% of samples with R program v.3.2.2 packages edgeR, limma, phyloseq, DESeq, vegan (adjustment for multiple testing by Benjamini–Hochberg method), and graphics were created with package ggplot2. Sample normalization was done as implemented in edgeR (calcNormFactors function) or the relative abundances were used if necessary. Additional analysis to detect differential abundance was performed using the Linear discriminant analysis Effect Size (LEfSe) method [30] integrated in the Galaxy framework. In particular, the non-parametric Kruskal–Wallis sum-rank test was used to detect differentially abundant taxa, and Linear Discriminant Analysis (LDA) was used to estimate the effect size. The genus level alpha diversity of each sample was calculated by the Shannon index [31], beta diversity across samples was evaluated with non-metric multidimensional scaling (NMDS) using Bray–Curtis distances. Permutational multivariate analysis of variance (PERMANOVA) was used (permutations = 9999) for comparing the analyzed groups of ordinations. Statistical significance for changes of Shannon index and for taxonomic units between specific sample groups was evaluated by Wilcoxon signed-rank test.

Results

Main characteristics of the samples

In total 53 stool samples were obtained from 18 healthy individuals. All characteristics depicted in Table 1, except for age and ALAT, corresponded to the Gaussian distribution. One participant withdrew from the trial at the fifth day of metformin administration due to severe GI side effects. The stool sample from this individual was collected after five days long metformin administration, and during the analysis it showed high similarity to all other M7d samples, so it was further analyzed together with this group.
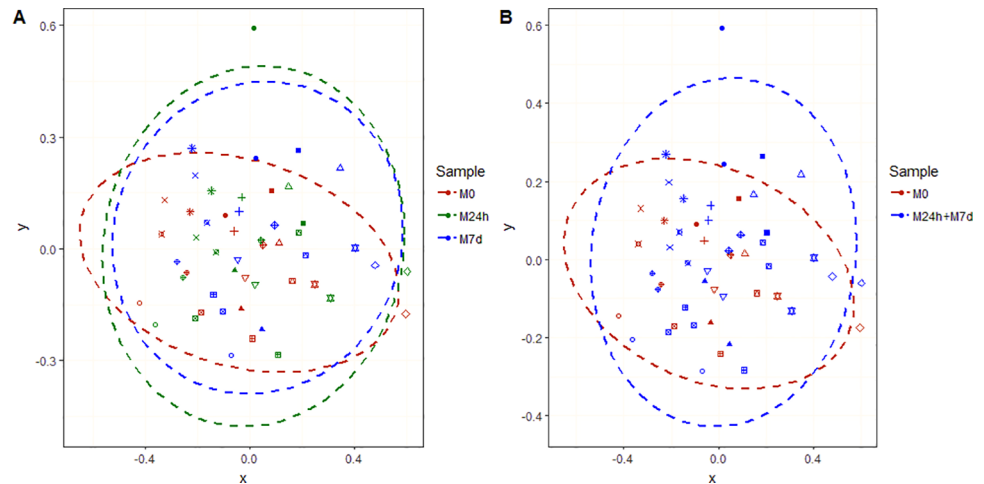
Fig 2. NMDS plots representing diversity between samples at genus level based on Bray–Curtis distances. (A) Comparison between all sample groups. (B) Comparison between M0 sample and samples during metformin administration (M24h + M7d). Ellipses represent the 95% confidence interval surrounding each group of samples. Different symbols represent participants of the study.

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After evaluation of registered side effects, we divided individuals into three groups according to the severity of GI side effects observed during the metformin administration: (1) no side effects (n = 3); (2) mild side effects defined by meteorism, stomach ache, nausea, and loss of appetite (n = 6); and (3) severe side effects defined by loose stools 1–3 times a day, diarrhea, and vomiting (n = 9). Only four individuals had loose stools (1–2 times per day) on day 1 of the study. The average time of occurrence for severe side effects was the day 3 of treatment. Full description on the registered adverse events can be found in S1 Table.

To evaluate the general differences in gut microbiota between the control sample and the samples taken after metformin administration we performed ordination analysis (Fig 2A and 2B) based on Bray–Curtis distances. As expected, gut microbiome communities were specific to each individual (PERMANOVA: R^2 = 0.74, p = 0.001) (S1 Fig). Thus, for further comparison of ordinations we used each individual as a nested factor. The analysis did not show any significant difference between the three groups of samples as defined by time points (M0, M24h, and M7d) (PERMANOVA: R^2 = 0.028, p = 0.078). Merging together both of the sample groups collected during and after metformin administration (M24h and M7d) and comparison with the control sample (M0) revealed a significant difference (PERMANOVA: R^2 = 0.019, p = 0.036).

Metformin reduces inner diversity of the gut microbiome

Comparing the Shannon index between the groups (Fig 3) we found that metformin therapy significantly reduces inner diversity of the gut microbiome immediately after the first two or three doses of metformin. After 7 days of metformin administration the inner diversity of the gut microbiome in study participants slightly increased, but was still significantly lower than before the use of metformin.

Changes in abundance of opportunistic pathogens in groups with different severity of GI side effects

To determine if the reduced inner diversity of the microbiome was associated with further gut microbiome dysbiosis, we analyzed changes in the abundance of common gut opportunistic
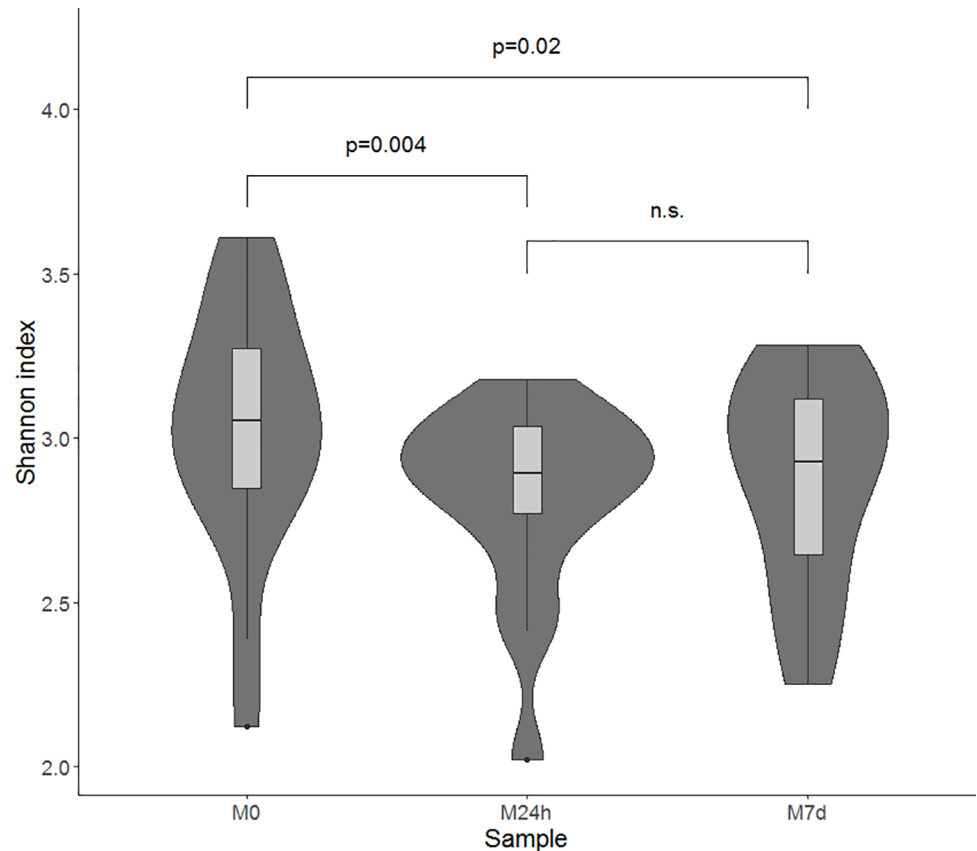
Fig 3. Alpha diversity changes during metformin therapy, evaluated at different time points. Samples marked as follows: M0—before starting metformin treatment; M24h - 24 hours after first intake of metformin; M7d - after 7 days treatment with metformin. Violin plot characterizing Shannon indexes combines boxplots, representing the median value and interquartile ranges, with kernel density plots.

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pathogen *Escherichia-Shigella* spp. We used the Wilcoxon-rank test for targeted analysis of possible changes in the relative abundance of *Escherichia-Shigella* genus comparing the three time points. There was no significant changes observed between the M0 (MED = 0.03%; IQR = 0.37%) and M24h ((MED = 0.05%; IQR = 0.14%) or M7d (MED = 0.46%; IQR = 1.04%). The relative abundance of these opportunistic pathogens was increased in the M7d sample when compared to M24h sample.

In order to test the possible relation of these changes with observed side effects we compared the changes in relative abundance of *Escherichia-Shigella* spp., as well as overall alpha diversity in different GI side effect categories (Fig 4). The inner diversity in the M7d sample compared to M24h sample increased only in groups with side effects. Thus in the group with mild side effects the median Shannon index for M7d sample was 3.03 (IQR = 0.21) compared to 2.97 (IQR = 0.15) in M24h sample, while in the group with severe side effects median was 2.88 (IQR = 0.66) for M7d sample compared to 2.72 (IQR = 0.42) for the M24h sample. We also observed increased presence of *Escherichia-Shigella* spp. in the samples taken before metformin administration from the participants later experiencing mild or severe side effects with the following median values of 0.21% (IQR = 1.57%) and 0.13% (IQR = 0.33%) respectively. The presence of *Escherichia-Shigella* spp. in the group with no side effects was beyond detectable limits.
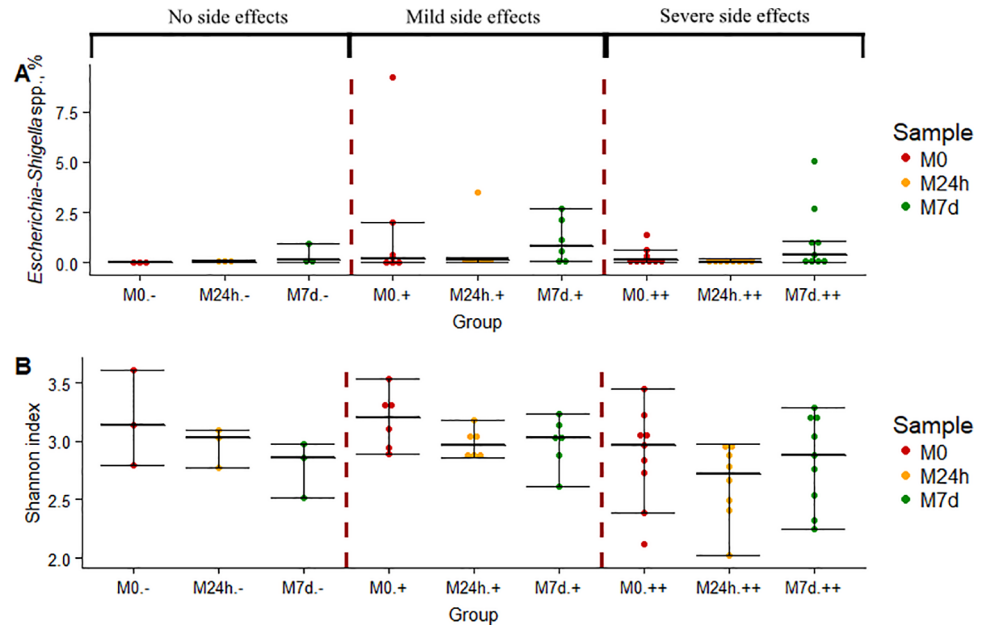
Fig 4. Changes in gut microbiome alpha diversity and abundance of opportunistic pathogen *Escherichia-Shigella* spp. at different time points within groups defined according to severity of GI side effects. (A) Changes in the relative abundance of *Escherichia-Shigella* spp. (B) Inner diversity changes, characterized by Shannon index. Samples marked as follows: M0—before starting metformin treatment; M24h - 24 hours after first intake of metformin; M7d - after 7 days treatment with metformin. Groups defined by observed side effects: "–"no side effects (n = 3), "+" mild side effects (n = 6), "++" severe side effects (n = 9). Dot plots depict median, 25th percentile and 75th percentile of data in each group. Dots beyond the bounds of the whiskers represent outliers.

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Differential abundance of taxonomic groups

To observe in-depth changes in the composition of the gut microbiome we used edgeR and evaluated the statistical significance of differential abundance of taxonomic groups between time points at every taxonomical level (phylum, class, order, family and genus). In total, 220 taxonomic groups presented in at least 50% of samples were tested. The main results are summarized in Table 2. There were no significant changes in representation of taxonomic groups at the phylum level at any of the contrasts between the M0, M24h, and M7d samples. One week treatment with metformin was associated with significant decreases in the families *Peptostreptococcaceae* and *Clostridiaceae_1* and four genera within these families: *Peptostreptococcaceae_*unclassified (family *Peptostreptococcaceae*), *Clostridiaceae_1_*unclassified (family *Clostridiaceae_1*), *Asaccharospora* (family *Peptostreptococcaceae*), and *Romboutsia* (family *Peptostreptococcaceae*). Comparison of the M24h and M7d samples showed significantly increased abundance of the order *Enterobacteriales,* including the only family in this order–*Enterobacteriaceae* with the genus, *Escherichia-Shigella*.

In addition, for graphic representation of differentially abundant taxa as well as their effect sizes and phylogenetic relationship, the LEfSe method was performed (Fig 5). This method detected 17 differentially abundant taxonomic clades, which mainly matched with those found with edgeR analysis.

In order to verify the findings from previous publications reporting that metformin increased abundance of *Akkermansia* spp., we performed a targeted Wilcoxon–rank test. Comparisons between two pairs were significant: M0 vs. M7d (p = 0.03) and M24 vs. M7d (p = 0.01) but the significance disappeared after performing the correction for multiple testing.

Table 2. Main significant changes in taxonomic units at all taxonomic levels.

Taxonomic level	Taxonomic group	Average abundance in sample groups,%			P–value [FDR*]		
		M0	M24h	M7d	M0 vs. M24h	M24h vs. M7d	M0 vs. 7d
Class	*Proteobacteria* unclassified	0.019	0.008	0.02	0.03 [0.62]		
	Gammaproteobacteria	1.16	0.50	1.71		0.002 [0.05]	0.008 [0.13]
	Verrucomicrobiae	0.45	0.30	1.14		0.03 [0.20]	
	Bacilli	1.02	0.83	1.31		0.03 [0.20]	0.04 [0.17]
	Epsilonproteobacteria	0.003	0.007	0.01			0.01 [0.13]
	Negativicutes	2.38	1.90	1.34			0.02 [0.15]
	*Proteobacteria*_unclassified	0.02	0.008	0.02	0.02 [0.68]		
	Enterobacteriales	0.99	0.41	1.55		**0.002 [0.04]**	0.005 [0.12]
Order	*Verrucomicrobiales*	0.45	0.30	1.14		0.03 [0.26]	
	Lactobacillales	1.00	0.81	1.29		0.03 [0.26]	0.04 [0.36]
	Selenomonadales	2.38	1.90	1.34			0.02 [0.26]
	Peptostreptococcaceae	1.17	0.93	0.23		**0.001 [0.02]**	**4.24E-06 [0.0002]**
	Clostridiaceae_1	0.70	0.51	0.13		**0.008 [0.12]**	**3.41E-05 [0.0007]**
Family	*Enterobacteriaceae*	0.99	0.41	1.55		**0.001 [0.02]**	0.004 [0.05]
	Streptococcaceae	0.58	0.41	0.68		0.01 [0.14]	
	Verrucomicrobiaceae	0.45	0.30	1.13		0.03 [0.21]	
	*Peptostreptococcaceae*_unclassified	0.91	0.72	0.18	0.04 [0.97]	**0.0006 [0.04]**	**1.86E-06 [0.0002]**
	*Clostridiaceae_1*_unclassified	0.63	0.49	0.10		0.032 [0.08]	**8.40E-06 [0.0005]**
	Asaccharospora	0.17	0.15	0.03		0.003 [0.08]	**1.64E-05 [0.0006]**
	Romboutsia	0.09	0.06	0.02		0.002 [0.07]	**2.92E-05 [0.0009]**
	Escherichia-Shigella	0.80	0.27	1.00		**0.0006 [0.04]**	0.008 [0.14]
Genus	*Streptococcus*	0.45	0.35	0.61		0.007 [0.16]	0.02 [0.31]
	*Enterobacteriaceae*_unclassified	0.19	0.13	0.48		0.01 [0.19]	0.004 [0.11]
	Ruminiclostridium_6	0.45	0.35	0.08		0.03 [0.45]	0.006 [0.13]
	Akkermansia	0.44	0.30	1.13		0.03 [0.48]	
	Ruminococcaceae_UCG-008	0.02	0.03	0.04			0.01 [0.16]
	Blautia	1.45	2.04	2.02			0.04 [0.52]

* Tendencies that maintained significance after false discovery rate (FDR) correction are marked in bold.

This genus was present in 37 of 53 samples, but the tendency and direction of changes in abundance were not consistent in all individuals for this taxa.

In conclusion, to evaluate the possible confounding effect of diet, Spearman correlation analysis was carried out. We did not find any significant association between the changed taxa and our defined food groups after performing the correction for multiple testing.

Discussion

In order to observe unbiased short-term effects of metformin on the gut microbiome we used an exploratory longitudinal study design and included healthy individuals. We believe that this design should have minimized false associations and conclusions arising from unaccounted treatment status by metformin or other medications in T2D patients, including the unknown true duration of T2D before diagnosis and the high interindividual variation of the gut microbiome. It has been recognized that, in similar time series studies, individuals can be treated as their own controls before and during treatment [32]. In addition, the strong effect size in previously described metformin studies [12] allowed us to consider the longitudinal study design

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B
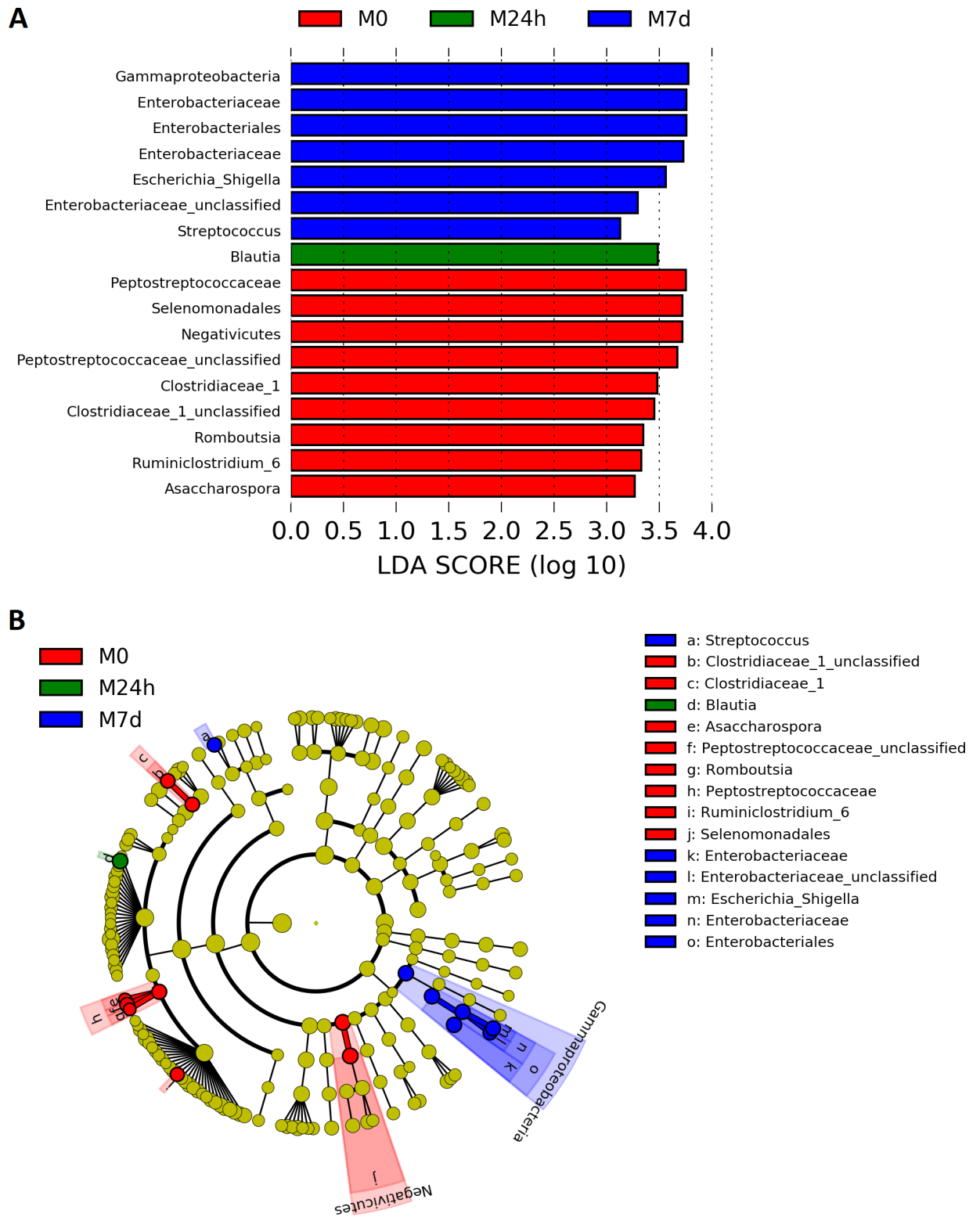
Fig 5. Comparison of LDA effect size of the significantly differentiating microbial taxa deduced using LefSe analysis. (A) Differences in abundance of taxonomic groups among all three sample. LDA cutoff = 2. Differentiating feature analysis was carried out with Kruskal–Wallis test raw p-value cutoff = 0.05. (B) Cladogram illustrating the phylogenetic relationship among the significantly differentiating gut microbiome taxonomic groups among the M0, M24h, and M7d samples.

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as sufficiently powerful to achieve the goal of our study. Taking in account our results, this study design has as well prevented any confounding effects induced by the known high inter-individual variety of diet [33], as we did not find any significant association between the changed taxonomic composition and data from the 7-day food record. Furthermore, there has been an increase in the use of metformin beyond diabetes, so this research may give additional insights into general features of the interaction between metformin and the gut microbiome, which may be further applicable to its use across a broad range of diseases, such as Alzheimer's disease [34], polycystic ovary syndrome [21], various types of cancers [20], and prevention of diabetes in individuals with prediabetic symptoms [35]. We also chose to include the first sampling point 24 hours after metformin administration in order to observe the effects as soon as possible and to avoid the potentially strong influence of diarrhea and other side effects known to occur after metformin administration. The generally accepted incidence of metformin-induced GI side effects is 20 – 30% [36, 37]. However, our data agreed with recent reports [38, 39], as we observed a high rate of side effects in our study (50% of study group experienced strong and 33% experienced mild side effects). This could be explained by the rather high initial dose of metformin, or the possibility that the design of the recent studies was more feasible for patients, which ensured higher treatment adherence and higher rate of reporting side effects.

Our findings that show the reduction in inner diversity of the gut microbiome during metformin treatment was in line with the previously observed effects of metformin effects in mice and rat models [13, 17]. In addition, a recent study using metagenome sequencing showed that metformin improves microbial gene richness among T2D patients, while metformin users generally have lower gene richness than healthy controls have [11]. It should be noted that, in our case, the reduction of diversity was observed at the markedly short time period of 24 hours, in the absence of diarrhea (only four participants experienced loose stools on the day 1). The small increase in inner diversity when comparing the M24h and M7d samples indicates the tendency of the gut microbiome to regain its ecological equilibrium even in participants experiencing diarrhea, as seen in the group of participants with severe side effects, in which nine people experienced loose stools or diarrhea. Likewise, this explains the growth of opportunistic pathogens including members from genus *Escherichia-Shigella* spp., which in previous studies has been associated with metformin treatment in T2D patients [11, 12]. Although, we cannot attribute the rapid increase of this genus between 24-hour and 7-day time points as a direct effect of metformin, this effect could be ensured by the trait of persistence of this genus [40] and high abilities to adapt [41]. Therefore, it can occupy the space open due to unfavorable conditions created by yet fully unknown effect of metformin. In other words, the reduced diversity in the gut presents *Escherichia-Shigella* spp. the free niche needed to emerge in larger numbers compared to the concurrent bacterial species. The connection between reduced alpha diversity and the further increase in the representation of opportunistic pathogens has been described before in the context of antibiotic treatment, various diseases and aging [11, 42–45].

The characteristic GI side effects in most cases manifest at the beginning of metformin therapy and usually disappear after several weeks [46, 47]. Several species from *Escherichia-Shigella* spp. have been identified as pathogens [48]. Assuming that the reason for adverse effects may be an increase of such opportunistic pathogens from *Escherichia-Shigella* spp., later reduction of adverse reactions could be associated with specific characteristics of these taxonomic groups. *Escherichia* and *Shigella* are two closely related genera that share bioenergetic mechanisms that allow them to fill a specific niche in the gut microbiome ecosystem [49]. Despite a competitive advantage as a facultative anaerobe, the population of *Escherichia coli* is known to be dependent on substrates provided by polysaccharide-degrading anaerobes [50]. Thus, the rapid initial growth might be terminated by the lack of mono- and disaccharides caused by

reduced abundance of anaerobic mucus-associated taxonomic groups and increased competition for the limited amount of energy substrates within the taxa. Also, T2D therapy accompanying a specific diet with reduced amount of simple carbohydrates [51] may play a role in limiting the amount of substrate. That could lead to further stabilization of the microbial ecosystem and recovery of metformin tolerance. Nevertheless, the initial side effects are the main reason for metformin discontinuation in 5% of patients [37]. Our results show an increased initial presence of *Escherichia-Shigella* spp. in the samples taken before metformin administration from the participants later experiencing side effects versus those without side effects (*Escherichia-Shigella* spp. below detectable limits). Development and implementation of a test for the presence of pathogens prior to metformin administration may allow stratification of treatment strategies (e.g. dose reduction or use of slow release forms) in high-risk patients.

A limitation of the present approach is the fact that analysis of *16S rRNA* sequencing results merge together various *Escherichia-Shigella* spp. species and strains with a wide spectrum of functions, effects, and ways of interaction [52]. Therefore, further metagenomic analysis in a longitudinal study providing information on gene richness, composition, and metabolic pathways could give deeper taxonomic and functional insight into the specificity of metformin-induced changes.

In addition, the sample collection procedure that involved temporary storage at room temperature prior to freezing can be seen as a possible limitation of the study. However, it has been shown in various studies that such approach does not significantly alter the microbiome composition if the storage is up to 24 hours [23, 24].

Despite the fact that it is still hard to distinguish whether dysbiosis of the gut microbiome is the cause or consequence of T2D and a specter of various other diseases, many therapeutic effects of gut microbiome modulation have been proven already [14, 53, 54]. It has been suggested that, despite induction of GI associated side effects, metformin may also exert its positive effects through its capability to modulate the gut microbiome. The strongest observable and specific effect of metformin in our study was the reduction in abundance of the family *Peptostreptococca-ceae* and three genera within it. Members of this family, in principle, have been associated with compromised health—one of the most convincing examples being *Clostridium difficile*. Increased abundance of *Peptostreptococcaceae* has also been associated with such conditions as non-alcoholic fatty liver disease [55], ulcerative colitis [56], and colorectal cancer [57], as well as with reduced lifespan [58]. In addition, reduced abundance of this family has been found in mice fed with a low-fat diet [59] or with calorie restrictions [58]. Interestingly, both families, significantly decreased by metformin, have been described to show similar response tendencies in various studies. Both *Peptostreptococcaceae* and *Clostridiaceae_1* possibly mediate the effect of eugenol treatment on mucus production in mice [60] and may be associated with dietary protein restriction induced improvement of ileal barrier function in pigs [61].

In the context of T2D or metformin therapy, the family *Peptostreptococcaceae* in general has not been described before, but previous studies have found significantly reduced abundance of one genus within it–*Intestinibacter* spp.–associated with metformin treatment [11, 12]. The functional role of this genus is still unclear, as it has been defined only recently [62]. We did not observe any statistically significant changes in the abundance of this genus that might be explained by analysis of healthy individuals in our study group.

The possibly controversial role of these taxa could be explained by potential differences in genera and species composition within these families between human and animal gut microbiomes. Overall, these changes in taxonomic units show that metformin may have beneficial effects through modification of possibly unfavorable human gut microbiome composition.

Unlike previous studies, we did not observe a significant increase in abundance of *Akkermansia* spp. after correction. One of the reasons may be the low prevalence of this genus in our

study group that can be explained by population, age, or disease status based differences when comparing to other studies.

Another intriguing question is the mechanism of how metformin modifies the gut microbiome. Recently, it has been shown that metformin has a direct effect on some, but not all of the gut microbiome bacteria, that was demonstrated by decreased growth in the presence of metformin *in vitro* [12]. It is not yet clear whether these direct effects of metformin are sufficient to explain the broad range of taxa affected in gut. Alternatively, the microbiome changes at least in part can be the result of systemic effects of metformin on the host (e.g. altered enterohepatic circulation of bile acids and salts) as suggested in McCreight et al. (2016) [7]. Our data, however, show rapid metformin-induced effects, and thus are in favor of the direct action of metformin, although this has to be proven using additional *in vitro* studies.

In conclusion, we were able to present direct evidence of effects of metformin on the gut microbiome in humans using prospective study, and associate these changes with metformin side effects. As this is an exploratory study without a placebo control arm, it would require additional randomized controlled trial in order to reach definitive conclusions. Nevertheless, our results indicate the possibility of developing a personalized approach in metformin therapy by pre-screening gut microbiota for abundance of opportunistic pathogens, followed by adjusted therapeutic strategies in patients with higher risk of developing side effects.

Supporting information

S1 Text. List of inclusion/exclusion criteria.
(DOCX)

S2 Text. CONSORT checklist.
(DOCX)

S3 Text. Clinical trial information.
(PDF)

S1 Table. Summary of the registered side effects during the metformin use.
(DOCX)

S2 Table. Summary of 7-day food record during the metformin treatment.
(DOCX)

S1 Fig. Taxonomic composition in all samples at family level. Plot visualizes the high interindividual diversity represented by most abundant taxonomic groups at family level. Each individual is marked with a personal identification code.
(PDF)

Acknowledgments

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