



Metformin Affects Gut Microbiome Composition and Function and Circulating Short-Chain Fatty Acids: A Randomized Trial

Noel T. Mueller,^{1,2} Moira K. Differding,^{1,2} Mingyu Zhang,^{1,2} Nisa M. Maruthur,² Stephen P. Juraschek,³ Edgar R. Miller III,² Lawrence J. Appel,² and Hsin-Chieh Yeh²

Diabetes Care 2021;44:1462–1471 | <https://doi.org/10.2337/dc20-2257>

OBJECTIVE

To determine the longer-term effects of metformin treatment and behavioral weight loss on gut microbiota and short-chain fatty acids (SCFAs).

RESEARCH DESIGN AND METHODS

We conducted a 3-parallel-arm, randomized trial. We enrolled overweight/obese adults who had been treated for solid tumors but had no ongoing cancer treatment and randomized them ($n = 121$) to either 1) metformin (up to 2,000 mg), 2) coach-directed behavioral weight loss, or 3) self-directed care (control) for 12 months. We collected stool and serum at baseline ($n = 114$), 6 months ($n = 109$), and 12 months ($n = 105$). From stool, we extracted microbial DNA and conducted amplicon and metagenomic sequencing. We measured SCFAs and other biochemical parameters from fasting serum.

RESULTS

Of the 121 participants, 79% were female and 46% were Black, and the mean age was 60 years. Only metformin treatment significantly altered microbiota composition. Compared with control, metformin treatment increased amplicon sequence variants for *Escherichia* (confirmed as *Escherichia coli* by metagenomic sequencing) and *Ruminococcus torques* and decreased *Intestinibacter bartlettii* at both 6 and 12 months and decreased the genus *Roseburia*, including *R. faecis* and *R. intestinalis*, at 12 months. Effects were similar in comparison of the metformin group with the behavioral weight loss group. Metformin versus control also increased butyrate, acetate, and valerate at 6 months (but not at 12 months). Behavioral weight loss versus control did not significantly alter microbiota composition but did increase acetate at 6 months (but not at 12 months). Increases in acetate were associated with decreases in fasting insulin. Additional whole-genome metagenomic sequencing of a subset of the metformin group showed that metformin altered 62 metagenomic functional pathways, including an acetate-producing pathway and three pathways in glucose metabolism.

CONCLUSIONS

Metformin, but not behavioral weight loss, impacted gut microbiota composition at 6 months and 12 months. Both metformin and behavioral weight loss altered circulating SCFAs at 6 months, including increasing acetate, which correlated with lower fasting insulin. Future research is needed to elucidate whether the gut microbiome mediates or modifies metformin's health effects.

¹Department of Epidemiology, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD

²Welch Center for Prevention, Epidemiology, and Clinical Research, Johns Hopkins University, Baltimore, MD

³Division of General Medicine and Primary Care, Beth Israel Deaconess Medical Center, Boston, MA

Corresponding author: Noel T. Mueller, noelmueller@jhu.edu

Received 11 September 2020 and accepted 24 March 2021

Clinical trial reg. no. NCT02431676, clinicaltrials.gov

This article contains supplementary material online at <https://doi.org/10.2337/figshare.14340419>.

© 2021 by the American Diabetes Association. Readers may use this article as long as the work is properly cited, the use is educational and not for profit, and the work is not altered. More information is available at <https://www.diabetesjournals.org/content/license>.

Emerging evidence indicates that the biological effects of metformin, the most commonly used glucose-lowering drug worldwide (1), originate in the intestine. The intestine is the primary reservoir for metformin, with accumulation in the jejunum 30–300 times that of the plasma (2). When metformin is administered intravenously, bypassing the intestine, it does not lower glucose (3). Moreover, a delayed-release oral metformin, that delivers the drug to the lower bowel with lower plasma exposure, has a greater glucose-lowering effect than extended-release metformin, providing further evidence for gut-mediated mechanisms of metformin (4).

The gut-mediated health effects of metformin may specifically stem from alterations in the gut microbiota. Following a seminal study by Cabreiro et al. (5), which demonstrated that metformin extends the life span of *Caenorhabditis elegans* by altering folate and methionine production by *Escherichia coli*, cross-sectional studies (6–8), single-arm studies (9–11), and a short-term trial (12) have provided evidence that metformin impacts gut microbiota composition. In particular, human studies have shown that metformin consistently increases the abundance of *E. coli* and decreases *Intestinibacter bartlettii* (7,9–13). In mice, broad-spectrum antibiotics abrogate metformin's microbial effect (14). There is also evidence that metformin increases short-chain fatty acid (SCFA) production in humans (12). Yet, an outstanding and salient question for research and clinical applications is whether metformin's effects on the microbiome and SCFAs are transient or persist long-term and whether they play a role in metformin's health effects.

We conducted a randomized trial to examine the effects of metformin treatment and the effects of behavioral weight loss on gut microbiota composition and SCFAs over 12 months in overweight and obese adults. We hypothesized that consistent with findings of shorter-term trials, long-term metformin treatment alters gut microbiota composition by increasing the abundance of *E. coli* and decreasing *I. bartlettii*, resulting in increased circulating SCFAs. We compared participants randomized to metformin with 1) participants randomized to a self-directed care arm (control) and 2)

participants randomized to a behavioral weight loss arm. The latter comparison allowed us to examine whether metformin's effects differed from the effects of behavioral weight loss, which we hypothesized would also alter microbiome composition and circulating SCFAs, consistent with results of similar prior research (15).

RESEARCH DESIGN AND METHODS

Participants

The Survivorship Promotion In Reducing IGF-1 Trial (SPIRIT) is a single-center, 3-parallel-arm randomized trial that was originally designed to evaluate the effect of metformin and behavioral weight loss on insulin-like growth factor and its binding protein (16,17). From August 2015 through December 2016, we recruited, from the Baltimore metropolitan area, overweight or obese adults (BMI ≥ 25 kg/m²) who had a history of a malignant solid tumor diagnosis and completed cancer treatment >3 months prior to enrollment. The major exclusion criteria were medication-treated diabetes, nonfasting blood glucose ≥ 11.1 mmol/L or HbA_{1c} $\geq 7\%$, current or prior regular use of metformin within the past 3 months, and significant renal disease or dysfunction, defined as estimated glomerular filtration rate <45 mL/min. Eligibility was determined by Web, phone, and in-person contacts (16).

We enrolled 121 participants and randomized them to one of 3 arms: 1) metformin treatment ($n = 42$), 2) coach-directed behavioral weight loss ($n = 39$), and 3) self-directed care (control group) ($n = 40$). The study protocol was approved by the institutional review board at Johns Hopkins University. All participants provided written informed consent.

Description of Randomized Groups

Metformin

Participants were randomized to receive up to 2,000 mg/day of metformin for 12 months. All metformin was provided by the study. Dose was increased, as tolerated, to a goal of 2,000 mg/day by a study physician. Those not tolerating advancement of dosing were left at lower doses. Participants also received metformin-related education and counseling from a study staff member immediately following randomization. An investigational new drug waiver was granted for SPIRIT for the use of

metformin outside of type 2 diabetes management.

Coach-Directed Behavioral Weight Loss

Participants received a coach-directed behavioral intervention that promoted calorie reduction, exercise, and the Dietary Approaches to Stop Hypertension (DASH) diet. The intervention was based on the call center-directed intervention from the Practice-based Opportunities for Weight Reduction (POWER) trial (18). The weight loss goal was 5% of baseline weight within 6 months and then weight maintenance until 12 months.

Self-directed Care (Control)

Participants had an informational visit where they were given the National Institutes of Health booklets *Aim for a Healthy Weight* and *What I Need to Know about Physical Activity and Diabetes* and a link to the Centers for Disease Control and Prevention website: "Healthy Weight." No further instructions were given to the participants.

Study Visits and Data Collection

We collected baseline and follow-up data by telephone and through in-person visits. The enrollment process involved a telephone contact, an in-person visit during which we collected baseline data, and a second in-person visit at which we notified participants of their assigned group. We asked participants to make in-person follow-up visits 3, 6, and 12 months after randomization. At each of these visits, we measured weight on a high-quality, calibrated digital scale, with the participant wearing light, indoor clothes and no shoes. We measured height once at study entry. Trained research staff performed the standardized measurements.

Collection and Storage of Biospecimens

We collected stool and fasting serum at baseline, 6 months, and 12 months. Aliquots of serum were stored at -80°C . Participants collected their own stool at home fresh with the OMNIgene kit (19) and refrigerated it and within 1 day brought it to ProHealth, where it was stored at -80°C until analysis.

Gastrointestinal Symptoms

Participants were asked at 3, 6, and 12 months to complete a questionnaire about

gastrointestinal symptoms during the past month, including diarrhea/loose stools, constipation, bloating, nausea/upset stomach, and heartburn. The four ordinal options for each symptom were 1) did not occur, 2) mild, 3) moderate, and 4) severe.

Laboratory Methods

16S rRNA Sequencing

A KingFisher robot deposited ~0.5 g stool in each bead plate well (MagAttract PowerSoil DNA KingFisher Kit; QIAGEN) and extracted microbial DNA per the manufacturer's instructions. Extracted DNA was quantified with the Quant-iT High-Sensitivity dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA). PCR amplification was performed with the Thermo Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific) with 1–10 ng extracted DNA. Samples were denatured for 2 min at 98°C, after which were 30 cycles of the following: 98°C for 20 s, 55°C for 15 s, 72°C for 30 s, and 72°C for 10 min. After PCR, libraries were prepared with use of the standard operating procedures described by Kozich et al. (20). Ten μ L PCR product was used to normalize the SequelPrep Normalization Prep Plate Kit (cat. no. A1051001; Thermo Fisher Scientific) to 1–2 ng/ μ L, and 5 μ L was pooled from each normalized sample into a single library per 96-well plate. The DNA Clean & Concentrator Kit (cat. no. D4013; Zymo Research) was used to concentrate library pools. For quality control steps, a dilution series for each pooled library was first performed and the Agilent bioanalyzer and high-sensitivity dsDNA assay (cat. no. 5047-4626) was used to analyze approximate fragment size and verify library integrity. The QIAquick Gel Extraction Kit (QIAGEN; cat. no. 28706) was used to purify pools with unintended amplicons. The KAPA Library Quantification Kit for Illumina (cat. no. KK4824) was used to determine pooled library concentrations. Library pools were diluted to 4 nmol/L and denatured with 2.0 N NaOH. Pools were loaded at 8 pmol/L with a PhiX spike-in of 20%. The 16S rRNA V4 region of purified libraries was then paired-end sequenced with the 500-cycle MiSeq Reagent Kit (cat. no. MS-102-2003; Illumina, San Diego, CA) with use of an Illumina MiSeq and primers and sample-

specific barcode demultiplexing as described by Kozich et al. (20).

We used the DADA2 R package (version 1.7.5) to complete read quality control and resolution of amplicon sequence variants (ASVs) (21). Following inspection of aggregate positional sequence quality (Supplementary Fig. 1), we trimmed forward and reverse reads before position 15 and after position 220 (5' and 3' ends, respectively) to omit severe drops in quality due to end effects. We applied filters to remove individual reads from the data set if they contained any Ns, mapped to any part of the PhiX genome, or were predicted based on quality values to have a maximum expected error >2 (filterAndTrim function).

We calculated error model estimation on a randomly selected subset of ~1 million bases from the run using the learnErrors function. We applied this aggregate model to every sample during final pooled amplicon sequence denoising via the dada function with other parameters set to default values. We performed pooled chimera detection and removal via the removeBimeraDe novo function (5153525 nonchimeric sequences out of 7108481 merged denoised sequences [~72.5%]). We classified the taxonomy of each ASV independently via SINTAX using the Ribosomal Database Project (RDP), version 16, of the 16S rRNA gene sequence database (22) as distributed on the USEARCH website. We aligned ASVs using the MSA package (23) and computed a maximum likelihood phylogenetic tree with the phangorn package (24), as previously described (25). We then combined the tree and the ASV-count table with other participant data and loaded it into the phyloseq package for statistical analysis (26).

Whole-Genome Shotgun Metagenomic Sequencing

To further characterize metformin effects on microbiome species and function, we selected a subsample of eight participants in the metformin arm who were able to tolerate at least 500 mg/day metformin at the 6-month visit and were matched on sex (four male, four female), and we performed whole-genome shotgun metagenomic sequencing on their stool samples collected at baseline and the 6-month

visit. Shotgun sequencing was done on the same samples with Illumina NextSeq technology. Approximately 18.64 gigabases (Gbases) were generated with 150 \times 2 paired-end reads. Each sample yielded a median of 1.03 Gbases. After sequencing, reads were separated according to the barcode used in the library preparation. Initial quality evaluation was done with FastQC, version 0.11.5. Processing took place in three steps: paired-end read joining, removing of contaminants, and trimming. Paired-end reads were joined with use of FLASH, version 1.2.11 (27). Reads that mapped to the human genome were removed. Finally, sequences were trimmed according to their quality values with Trimmomatic, version 0.36, using custom parameters (28). Joining the paired reads reduced the library size by 24.5%. Approximately 0.18% of the stitched reads mapped to the human genome and were removed. Read trimming using quality filters removed 6.03% of the screened reads. At the end of quality control, the median number of quality-filtered reads per sample was 563,328. Spearman correlations between microbiome signatures identified by shotgun and 16S rRNA sequencing were high: genera, range 0.52–0.99; species, 0.72–0.91; and α -diversity metrics, 0.63–0.66.

SCFA Quantification

Fasting serum samples were spiked with a solution of eight stable labeled internal standards and subjected to protein precipitation. After centrifugation, an aliquot of the supernatant was derivatized. The reaction mixture was analyzed by liquid chromatography–tandem mass spectrometry on an Agilent 1290/AB SCIEX QTRAP 5500 system. The peak areas of the respective analyte product ions were measured against the peak area of the corresponding internal standard product ions. Quantitation was performed with a weighted least squares regression analysis generated from fortified calibration standards prepared immediately prior to each run. SCFA data below or above the limit of quantitation were extrapolated beyond the lower or upper limit of quantitation.

Coefficients of variation were all below <7%.

Statistical Analysis

Primary outcomes were 6-month change in stool microbiota and serum SCFAs. We chose to assess primary outcomes at 6 months to maximize data collection at the time of the greatest anticipated treatment effect and to minimize a decrease in sample size due to censoring. Moreover, the 6- and 12-month end points allowed us to determine whether the impacts of metformin extended beyond the time points reported in shorter-term trials. The primary contrasts were the metformin arm versus control arm and the behavioral weight loss arm versus control arm. Informed by prior studies (9–12), we hypothesized that metformin but not behavioral weight loss increases abundance of *E. coli* and decreases *I. bartlettii* and that both metformin and behavioral weight loss increase serum SCFAs.

Change in Gut Microbiome Diversity

We used the phyloseq estimate_richness function to compute the Shannon diversity index and distance function to compute the pairwise weighted UniFrac distance matrix. We used generalized linear regression to examine the change in Shannon diversity and change in the first principle coordinate of weighted UniFrac distances between baseline and 6 months and 12 months within intervention arm.

Change in Gut Microbiome Composition and Function

We first compared the differential abundance of ASVs within intervention arm using paired Wilcoxon ranked sum tests on the centered log-ratio transformed values of a set of Monte Carlo Dirichlet resampling instances, as implemented in the ALDEx2 Bioconductor/R package (29). After identifying ASVs that were differentially abundant between baseline and 6 months and 12 months, we examined the difference in ASV relative abundance change across treatment arms using ANCOVA. We used Wilcoxon tests to determine differences in microbiome function.

Change in Serum SCFA Concentrations

After normalizing serum SCFA concentrations through log transformation, we used ANCOVA to compare between-group differences in SCFA change from baseline to 6 months and baseline to 12 months.

Correlations of Gut Microbiome, SCFAs, and Clinical Parameters

We examined correlations of select bacterial ASVs and taxa with serum SCFAs, metabolic parameters, and other clinical parameters in the metformin arm using Kendall rank correlation coefficients.

In addition to our primary intention-to-treat analyses, we conducted additional microbiota-outcome analyses among participants randomized to metformin and able to tolerate at least 500 mg/day. We considered a two-sided $P < 0.05$ to be statistically significant for a priori hypotheses, including the effects of metformin on *E. coli* and *I. bartlettii* and on SCFAs. For agnostic testing (no a priori hypotheses), including effects on microbiome function and correlations of taxa with clinical parameters, we considered a false discovery rate (FDR)-corrected $P < 0.05$ as significant.

RESULTS

Baseline Characteristics

Of the 121 randomized participants, 79% were female, 46% were Black, and the mean age was 60 years. Average BMI was 35 kg/m², and breast cancer was the most commonly reported cancer. Distributions of sociodemographic factors and clinical factors (Table 1), as well as gut microbiome diversity (permutational multivariate ANOVA by intervention arm for weighted UniFrac, $P = 0.92$; ANOVA by intervention arm for Shannon diversity, $P = 0.84$) and composition (Supplementary Fig. 2), were balanced across intervention arms at baseline.

Participant Follow-up

All participants completed the study, and >90% returned stool at the 6-month or 12-month visit. Noncompliance was evenly distributed across intervention arms. The CONSORT diagram is provided in Supplementary Fig. 3.

Change in Microbiome Diversity

No intervention changed Shannon diversity or weighted UniFrac at 6 or 12 months (Supplementary Fig. 4). However, among the subsample selected for shotgun metagenomics ($n = 8$), metformin increased metagenomic functional pathway richness (Supplementary Table 1) and altered the metagenomic functional variation from baseline to 6 months (Supplementary Table 2).

Change in Microbiome Composition

Metformin increased relative abundance of *Escherichia* (confirmed as *E. coli* by shotgun metagenomics), while decreasing *I. bartlettii* and *Roseburia intestinalis* at 6 and 12 months (Fig. 1) (non-FDR corrected). Metformin also decreased *Roseburia faecis* at 6 months and increased *Ruminococcus torques* at 12 months. Findings were similar if not stronger in microbiome analyses restricted to the 27 (of 38) and 23 (of 32) participants able to tolerate at least 500 mg/day metformin at 6 and 12 months, respectively (Supplementary Fig. 5). Moreover, the effect of metformin on *E. coli* was dose dependent (Supplementary Fig. 6). There were no significant changes in microbial ASVs within the coach-directed behavioral weight loss arm or the self-directed arm (control) at 6 months or 12 months (Fig. 1). Accounting for intake of fruits and vegetables, fiber, or fat did not change the results.

Compared with the self-directed arm (control) or the coach-directed arm, the metformin arm had increased relative abundance of *E. coli* and decreased relative abundance of *I. bartlettii* at the 6- and 12-month visits. The metformin arm (vs. self-directed) also had decreased abundance of *Roseburia* (genus), *R. faecis*, and *R. intestinalis* at 12 months; metformin (vs. coach-directed) had decreased abundance of *Roseburia* (genus) at 12 months and *R. intestinalis* at 6 months and 12 months, and metformin (vs. self-directed) had increased *R. torques* at 6 and 12 months (Fig. 2 and Supplementary Table 3).

Metformin Effects on Microbiome Species and Functional Pathways

For the eight metformin participants selected for whole-genome shotgun metagenomic sequencing, metformin affected 6 of the 156 detected species before FDR correction (Supplementary Table 4). Consistent with 16S models, *E. coli* (increased by metformin) and

Table 1—Characteristics of participants in SPIRIT (N = 121)

	Coach-directed	Metformin	Self-directed
Participants in each arm (unless otherwise indicated), n	39	42	40
Female sex, n (%)	32 (82.1)	32 (76.2)	32 (80.0)
Age at baseline, years, mean (SD)	60.9 (9.7)	59.6 (9.0)	58.8 (8.5)
Race/ethnicity, n (%)			
Black	17 (43.6)	20 (47.6)	18 (45.0)
White	21 (53.8)	22 (52.4)	22 (55.0)
American Indian	1 (2.6)	0 (0.0)	0 (0.0)
Education, n (%)			
High school or less	8 (20.5)	8 (19.0)	4 (10.0)
Some college	5 (12.8)	9 (21.4)	13 (32.5)
College graduate	26 (66.7)	25 (59.5)	23 (57.5)
Cancer type, n (%)			
Breast	19 (48.7)	27 (64.3)	22 (55.0)
Colon	3 (7.7)	3 (7.1)	4 (10.0)
Prostate	3 (7.7)	4 (9.5)	4 (10.0)
Number of cancers, n (%)			
1	34 (87.2)	37 (88.1)	37 (92.5)
>1	5 (12.8)	5 (11.9)	3 (7.5)
BMI, kg/m ² , mean (SD)			
Baseline	35.3 (5.0)	35.0 (6.3)	34.7 (4.9)
6 months	33.8 (5.4), n = 38	33.9 (6.6), n = 40	34.4 (5.3)
12 months	34.1 (5.6), n = 38	33.5 (6.8), n = 37	34.5 (5.3)
Weight, kg, mean (SD)			
Baseline	93.8 (13.9)	97.7 (20.7)	95.7 (14.8)
Change from baseline to 6 months	−3.5 (4.9), n = 38	−2.6 (4.1), n = 40	−0.8 (3.0)
Change from baseline to 12 months	−2.7 (5.0), n = 38	−3.5 (5.7), n = 37	−0.4 (3.9)
Fasting insulin, mU/L, mean (SD)			
Baseline	14.2 (6.9)	16.4 (10.8)	16.9 (8.5)
Change from baseline to 6 months	−3.7 (5.2), n = 38	−1.4 (14.5), n = 40	−2.2 (4.8)
Change from baseline to 12 months	−2.3 (6.2), n = 37	−5.5 (11.0), n = 37	−2.3 (9.0), n = 39
hs-CRP, mg/L, mean (SD)			
Baseline	5.2 (7.3)	4.6 (4.1)	6.0 (6.8)
Change from baseline to 6 months	0.5 (5.9), n = 38	0.6 (2.8), n = 40	−1.2 (4.0)
Change from baseline to 12 months	−0.5 (3.4), n = 37	0.4 (3.8), n = 37	−1.3 (4.1), n = 39
HbA _{1c} , %, mean (SD)			
Baseline	5.7 (0.6), n = 38	5.8 (0.5)	5.7 (0.5), n = 39
Change from baseline to 6 months	−0.1 (0.4), n = 35	−0.1 (0.4), n = 39	−0.0 (0.2), n = 39
Change from baseline to 12 months	−0.1 (0.4), n = 36	−0.0 (0.8), n = 37	−0.0 (0.3), n = 38
Fasting glucose, mg/dL, mean (SD)			
Baseline	98.9 (13.6)	102.9 (15.3)	99.8 (13.5)
Change from baseline to 6 months	−1.5 (9.6), n = 38	−2.9 (14.8), n = 40	−1.0 (9.0)
Change from baseline to 12 months	−0.8 (10.7), n = 37	−2.0 (34.7), n = 37	0.1 (10.0), n = 39
Log-transformed serum SCFAs, baseline, mean (SD)			
Acetate	7.19 (0.48)	7.22 (0.50)	7.24 (0.49)
Propionate	4.94 (0.37)	5.04 (0.45)	4.98 (0.38)
Isobutyrate	6.26 (0.25)	6.26 (0.26)	6.31 (0.27)
Butyrate	4.16 (0.32)	4.12 (0.37)	4.24 (0.44)
Methylbutyrate	4.39 (0.40)	4.40 (0.36)	4.37 (0.47)
Isovalerate	4.26 (0.47)	4.28 (0.45)	4.17 (0.50)
Valerate	2.98 (0.36)	2.94 (0.43)	2.97 (0.34)
Hexanoate	4.25 (0.21)	4.17 (0.20)	4.23 (0.20)

Abbreviations: SD, standard deviation; BMI, body mass index; HbA_{1c}, hemoglobin A_{1c}; hs-CRP, high sensitivity C-reactive protein; SCFA, short chain fatty acid.

R. intestinalis (decreased by metformin) were significant (before FDR correction) in the shotgun metagenomic models. In addition, *Eubacterium rectale*, *Bifidobac-*

terium longum, *Bacteroides uniformis*, and *Faecalibacterium prausnitzii* were significant (before FDR correction) in metagenomic models. In agnostic

functional-pathway testing, we found that metformin significantly altered 62 functional pathways after FDR correction (Supplementary Table 5), including

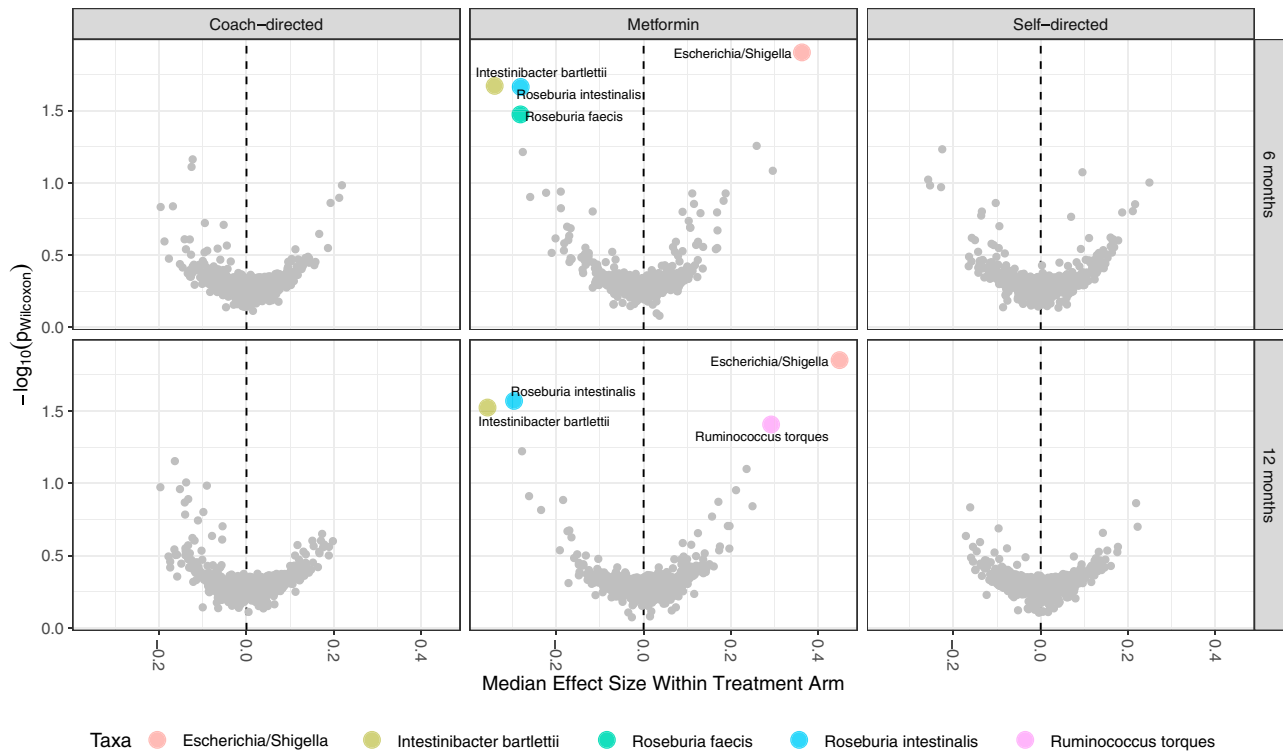


Figure 1—Change in relative abundance of gut microbiota ASVs between baseline and 6 months (top row) and baseline and 12 months (bottom row) in coach-directed weight loss arm, metformin arm, and self-directed weight loss arm. Colored ASVs are statistically significant at $P < 0.05$ before FDR correction. Colored dots to the left are higher in relative abundance at baseline, and colored dots to the right are higher at either 6 months or 12 months. Sample sizes by treatment arm: coach-directed baseline $n = 36$, 6 months $n = 35$, and 12 months $n = 35$; metformin baseline $n = 40$, 6 months $n = 38$, and 12 months $n = 32$; and self-directed baseline $n = 38$, 6 months $n = 36$, and 12 months $n = 38$.

an acetate-producing pathway, and three pathways in glucose metabolism: glucose and glucose-1-phosphate degradation, sucrose degradation IV, and starch degradation.

Change in SCFAs

Compared with self-directed care (control), metformin and coach-directed behavioral interventions increased acetate at 6 months but not at 12 months (Fig. 3 and Supplementary Table 6). Metformin (vs. self-directed) also increased butyrate and valerate at 6 months, but not at 12 months, and coach-directed behavioral weight loss (vs. self-directed) decreased methylbutyrate at 12 months (Fig. 3 and Supplementary Table 6).

Correlations of Bacteria With SCFAs and Clinical Factors

Overall, change in bacterial taxa was weakly correlated with change in serum SCFAs, while increases in acetate were significantly correlated with decreases in fasting insulin, and increases in *E. coli* were nonsignificantly correlated with decreases in fasting insulin after FDR correction (Supplementary Fig. 7).

Furthermore, while those in the metformin arm had diarrhea more than the other intervention arms (Supplementary Fig. 8), diarrhea was not significantly correlated with SCFAs or the microbial features affected by metformin (Supplementary Fig. 7), suggesting that the diarrheal effects of metformin did not influence our results.

CONCLUSIONS

In our randomized trial of racially diverse overweight and obese cancer survivors, metformin affected long-term gut microbiota composition, increasing *E. coli* and *R. torques* and decreasing *I. bartlettii* and *R. intestinalis* at 6 and 12 months postrandomization. The effects of metformin on *E. coli*, *I. bartlettii*, and *R. intestinalis* were consistent in comparisons with effects among subjects in the self-directed control arm (who did not lose weight) and the behavioral weight loss arm (who lost an amount of weight similar to that of the metformin arm), suggesting that the metformin effects are independent of weight loss. Metformin also altered 62 functional

pathways, including acetate production and glucose metabolism, and increased serum SCFAs butyrate, acetate, and valerate at 6 months, but these SCFA effects did not persist at 12 months. Coach-directed behavioral weight loss, which resulted in a mean loss of 3.5 and 2.7 kg at 6 and 12 months, respectively, had negligible effects on the gut microbiome and increased acetate at 6 months but not 12 months.

Our results add to multiple lines of evidence (7,9–14,30,31) suggesting that the provenance of metformin's physiologic effects may derive from changes in gut microbiota. Notably, our trial shows that metformin affects microbiota composition over 12 months of treatment. Our finding that metformin advantages *E. coli* growth and disadvantages *I. bartlettii* is consistent with findings of cross-sectional studies (6,7), single-armed trials (9–11), and one short-term randomized trial (12). Functional analysis of *I. bartlettii*, formerly known as *Clostridium bartlettii* (32), shows that it is able to degrade fucose, hinting at

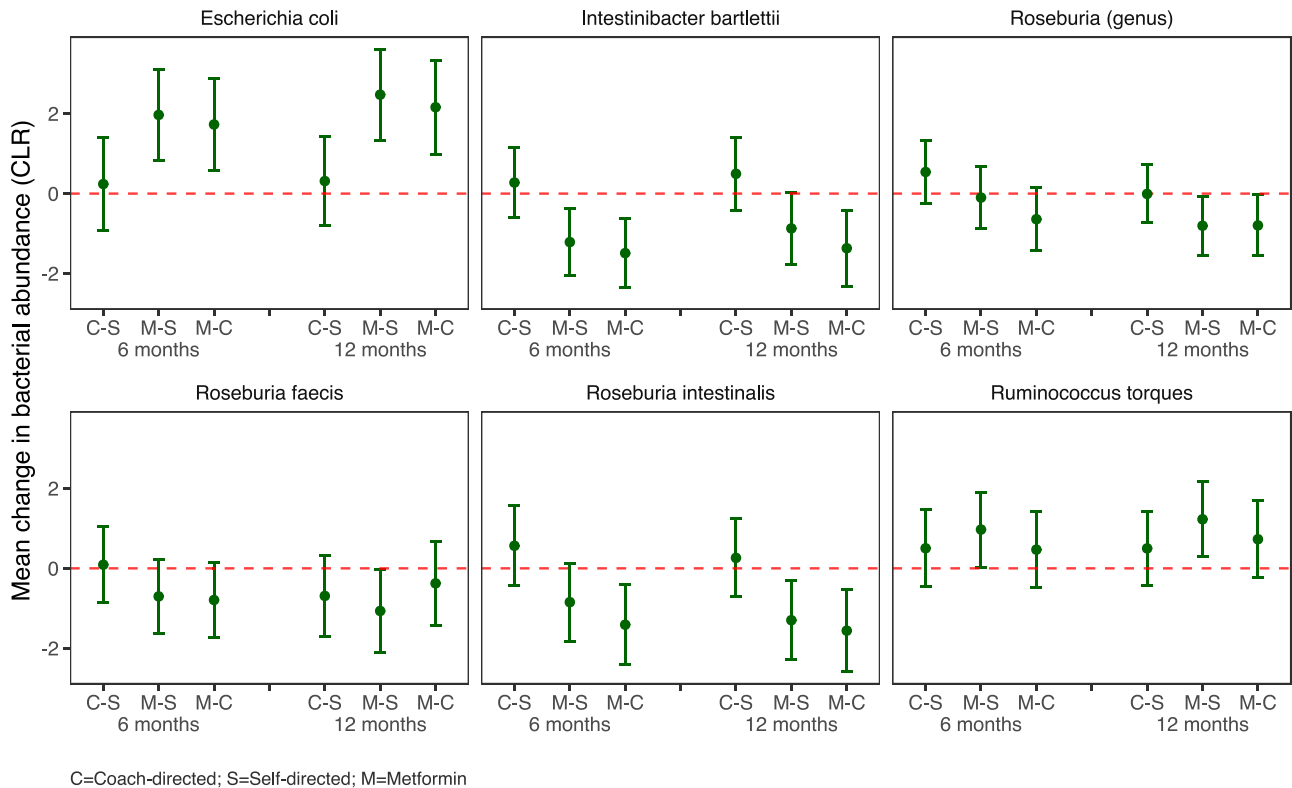


Figure 2—Comparison of mean change in center log ratio (CLR) transformed bacterial abundances from baseline to 6 months and 12 months between treatment arms. Point estimates and 95% CIs are provided in Supplementary Table 3. Sample sizes by treatment arm: coach-directed (C) baseline $n = 35$, 6 months $n = 35$, and 12 months $n = 35$; metformin (M) baseline $n = 38$, 6 months $n = 38$, and 12 months $n = 32$; and self-directed (S) baseline $n = 38$, 6 months $n = 36$, and 12 months $n = 38$.

indirect involvement in mucus degradation. Interestingly, consistent with the pattern seen in our study, pigs susceptible to colonization by enterotoxigenic *Escherichia* spp. had lower abundance of *I. bartlettii* (33). In a recent flux balance analysis, *I. bartlettii* was associated with substantial (>50%) alterations in propionate metabolism, whereas in the same study *E. coli* was associated with substantial (>50%) alterations in butyrate and vitamin B2 metabolism. *Escherichia* spp. have also been linked with pathway changes in lipopolysaccharide production among other pathways (34). Most recently, using shotgun metagenomic sequencing in participants from three separate cohort studies, Vila et al. (7) found that *E. coli* was the major contributor to the functional changes associated with metformin use.

Pertinent to our findings on metformin and SCFAs, Forslund et al. (6) demonstrated enhanced butyrate and propionate production potential in

metformin-treated adults with type 2 diabetes. This finding was corroborated by a trial by Wu et al. (12), who found that metformin increased fecal concentrations of butyrate and propionate in men over 6 weeks. Zhernakova et al. (35) also observed higher fecal SCFAs, namely, propionate, in 15 metformin users compared with 9 nonusers. Our findings extend this work, showing that metformin increases SCFA-producing *R. torques*, increases SCFA-production pathways, and impacts serum acetate and butyrate at 6 months. However, the effect of metformin on these SCFAs was diminished at 12 months. The cause of the waning effect on SCFAs, and its relevance to health, requires more research. We postulate that it may be due to compensatory use of SCFAs as alternative fuel during prolonged negative energy balance (36).

Of note, we observed that increases in serum acetate correlated with reductions in fasting insulin. This finding is consistent with prior research (37),

including ours (38), and the pathways through which acetate influences insulin sensitivity and glucose metabolism are a topic of active investigation (39). In our study, we also found that metformin-induced increases in *E. coli* correlated with increases in serum acetate along with decreases in fasting insulin, raising the possibility that specific *E. coli* strains may increase acetate production and, in turn, improve insulin sensitivity. On the other hand, acetate was increased and insulin was decreased at 6 months in the behavioral weight loss arm, suggesting that these concomitant changes may not be unique to the microbial effects of metformin.

Unlike in other studies (10,40), baseline abundance of *E. coli* or change in abundance of *E. coli* was not associated with diarrhea in our study. Forslund et al. (6) found that metformin enriched virulence factors and gas metabolism genes attributable to increased *Escherichia*. We did,

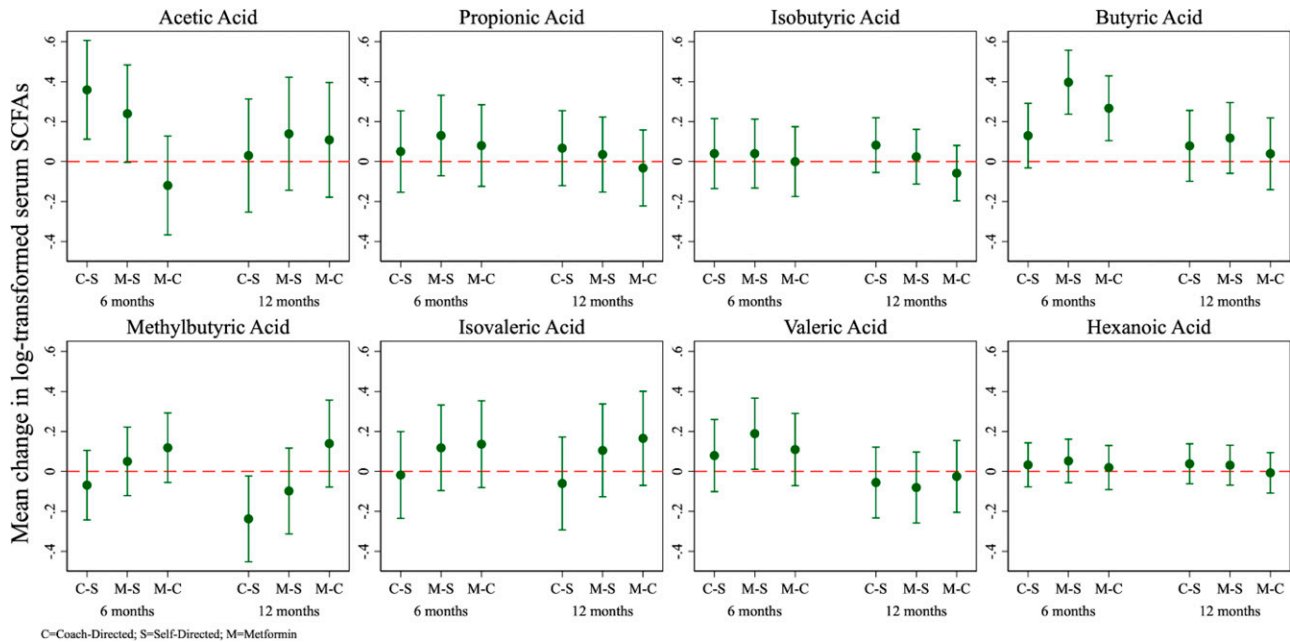


Figure 3—Comparison of mean change in log-transformed serum SCFAs from baseline to 6 months and 12 months between treatment arms ($n = 118$ at 6 months; $n = 113$ at 12 months). Point estimates and 95% CIs are provided in Supplementary Table 6. C, coach-directed; M, metformin; S, self-directed.

however, find that metformin decreased *I. bartlettii*, which is folate spore forming and resistant to stomach acid. Future trials are needed to determine whether giving *I. bartlettii* as a probiotic in conjunction with metformin could reduce gastrointestinal side effects (41).

There are numerous strengths of our trial. Our randomized trial is the largest and longest randomized trial of metformin on the gut microbiome, and it is the first trial to examine the effects of metformin on the microbiome and SCFAs beyond 6 months. Other strengths are that we enrolled a diverse population, with 46% Black participants, and we had a high retention rate. Our study also has limitations. Approximately 30% of participants randomized to metformin reported that they were not able to tolerate the drug at the follow-up visits. The primary reason why participants were not able to tolerate metformin was gastrointestinal side effects, which is consistent with the literature: that up to 30% of treatment-naïve individuals experience have digestive disorders (42–45). Including these participants in our primary analysis likely attenuated the effects, as evidenced by analyses restricted to participants on met-

formin who took at least 500 mg/day. Furthermore, we cannot rule out the possibility that the results may not generalize to a noncancer population, although the mean interval between end of cancer treatment and trial start date for participants enrolled was ~9 years and thus the influence of cancer treatment on gut microbiota is likely minimal—if there is any at all. Also, our population had a high average BMI and did not have type 2 diabetes; research is needed to determine whether the results generalize to participants with lower body weight and dysregulated insulin. Finally, the negligible effects of our behavioral weight loss intervention on the gut microbiota may be due to lack of extreme diet modification or to a long-term resilience of the microbiota despite changes in diet and weight, as recently observed (46). We also cannot rule out that smaller effects were missed because of limited statistical power.

In conclusion, results from our randomized trial extend evidence about the longer-term effects of metformin and modest behavioral weight loss on gut microbiota and serum SCFAs. Consistent with prior shorter-term trials, metformin altered gut microbiota

composition—increasing *E. coli* and decreasing *I. bartlettii*—at 6 and 12 months of follow-up and these effects were distinct from behavioral weight loss, which had negligible effects on microbiota in our trial. Moreover, the increases in *E. coli* were correlated with increases in serum acetate, which correlated with decreases in fasting insulin, suggesting that microbiota changes may play a role in metformin's effect on insulin regulation. Whether heterogeneity in microbial effects corresponds to long-term clinical outcomes is still unknown and should be investigated in larger studies. Nevertheless, our study helps to elucidate the biologic mechanisms by which metformin affects health outcomes and identifies microbes that could be future targets for the prevention of metabolic disorders or gastrointestinal symptoms known to affect drug tolerability.

Funding. SPIRIT was funded by the Maryland Cigarette Restitution Fund. N.T.M. was supported by the National Heart, Lung, and Blood Institute of the National Institutes of Health (K01HL141589). H.-C.Y. was supported in part by the National Cancer Institute's Cancer Centers Support Grant to the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins (5P30CA006973). M.Z. is supported by the American

Heart Association (Award Number: 827990). M.K.D. is supported by the National Heart, Lung, and Blood Institute, NIH Grant Number T32 HL007024.

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. N.T.M. contributed to acquisition, analysis, and interpretation of the data and conception, design, drafting, and revision of the manuscript. M.K.D. contributed to analysis and interpretation of the data and drafting and revision of the manuscript. M.Z. contributed to analysis and interpretation of the data and drafting and revision of the manuscript. N.M.M. contributed to interpretation of the data and revision of the manuscript. S.P.J. contributed to interpretation of the data and revision of the manuscript. E.R.M. contributed to interpretation of the data and revision of the manuscript. L.J.A. contributed to acquisition and interpretation of the data and revision of the manuscript. H.-C.Y. contributed to acquisition and interpretation of the data and conception, design, and revision of the manuscript. All authors approved the final draft submitted. N.T.M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References

- Inzucchi SE, Bergenstal RM, Buse JB, et al.; American Diabetes Association (ADA); European Association for the Study of Diabetes (EASD). Management of hyperglycemia in type 2 diabetes: a patient-centered approach: position statement of the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD). *Diabetes Care* 2012;35:1364–1379
- Bailey CJ, Wilcock C, Scarpello JH. Metformin and the intestine. *Diabetologia* 2008;51:1552–1553
- Bonora E, Cigolini M, Bosello O, et al. Lack of effect of intravenous metformin on plasma concentrations of glucose, insulin, C-peptide, glucagon and growth hormone in non-diabetic subjects. *Curr Med Res Opin* 1984;9:47–51
- Buse JB, DeFronzo RA, Rosenstock J, et al. The primary glucose-lowering effect of metformin resides in the gut, not the circulation: results from short-term pharmacokinetic and 12-week dose-ranging studies. *Diabetes Care* 2016;39:198–205
- Cabreiro F, Au C, Leung KY, et al. Metformin retards aging in *C. elegans* by altering microbial folate and methionine metabolism. *Cell* 2013;153:228–239
- Forslund K, Hildebrand F, Nielsen T, et al.; MetaHIT consortium. Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. *Nature* 2015;528:262–266
- Vich Vila A, Colliv V, Sanna S, et al. Impact of commonly used drugs on the composition and metabolic function of the gut microbiota. *Nat Commun* 2020;11:362
- de la Cuesta-Zuluaga J, Mueller NT, Corrales-Agudelo V, et al. Metformin is associated with higher relative abundance of mucin-degrading *Akkermansia muciniphila* and several short-chain fatty acid-producing microbiota in the gut. *Diabetes Care* 2017;40:54–62
- Bryrup T, Thomsen CW, Kern T, et al. Metformin-induced changes of the gut microbiota in healthy young men: results of a non-blinded, one-armed intervention study. *Diabetologia* 2019;62:1024–1035
- Elbere I, Kalnina I, Silamikelis I, et al. Association of metformin administration with gut microbiome dysbiosis in healthy volunteers. *PLoS One* 2018;13:e0204317
- Sun L, Xie C, Wang G, et al. Gut microbiota and intestinal FXR mediate the clinical benefits of metformin. *Nat Med* 2018;24:1919–1929
- Wu H, Esteve E, Tremaroli V, et al. Metformin alters the gut microbiome of individuals with treatment-naïve type 2 diabetes, contributing to the therapeutic effects of the drug. *Nat Med* 2017;23:850–858
- Forslund K, Hildebrand F, Nielsen T, et al. Disentangling the effects of type 2 diabetes and metformin on the human gut microbiota. *Nature* 2015;528:262–266
- Shin NR, Lee JC, Lee HY, et al. An increase in the *Akkermansia* spp. population induced by metformin treatment improves glucose homeostasis in diet-induced obese mice. *Gut* 2014;63:727–735
- Dao MC, Everard A, Aron-Wisniewsky J, et al.; MICRO-Obes Consortium. *Akkermansia muciniphila* and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology. *Gut* 2016;65:426–436
- Juraschek SP, Plante TB, Charleston J, et al. Use of online recruitment strategies in a randomized trial of cancer survivors. *Clin Trials* 2018;15:130–138
- Yeh HC, Maruthur NM, Wang NY, et al. Effects of behavioral weight loss and metformin on insulin-like growth factors in cancer survivors: a randomized trial. *J Clin Endocrinol Metab*. 22 April 2021 [Epub ahead of print]. DOI: 10.1210/clinem/dgab266
- Appel LJ, Clark JM, Yeh HC, et al. Comparative effectiveness of weight-loss interventions in clinical practice. *N Engl J Med* 2011;365:1959–1968
- Choo JM, Leong LE, Rogers GB. Sample storage conditions significantly influence faecal microbiome profiles. *Sci Rep* 2015;5:16350
- Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ Microbiol* 2013;79:5112–5120
- Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods* 2016;13:581–583
- Cole JR, Wang Q, Fish JA, et al. Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res* 2014;42:D633–D642
- Bodenhofer U, Bonatesta E, Horejš-Kainrath C, Hochreiter S. msa: an R package for multiple sequence alignment. *Bioinformatics* 2015;31:3997–3999
- Schliep KP. phangorn: phylogenetic analysis in R. *Bioinformatics* 2011;27:592–593
- Callahan BJ, Sankaran K, Fukuyama JA, McMurdie PJ, Holmes SP. Bioconductor workflow for microbiome data analysis: from raw reads to community analyses. *F1000 Res* 2016;5:1492
- McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 2013;8:e61217
- Magoč T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 2011;27:2957–2963
- Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014;30:2114–2120
- Fernandes AD, Reid JN, Macklaim JM, McMurrough TA, Edgell DR, Gloor GB. Unifying the analysis of high-throughput sequencing datasets: characterizing RNA-seq, 16S rRNA gene sequencing and selective growth experiments by compositional data analysis. *Microbiome* 2014;2:15
- Lee H, Ko G. Effect of metformin on metabolic improvement and gut microbiota. *Appl Environ Microbiol* 2014;80:5935–5943
- Zhang X, Zhao Y, Xu J, et al. Modulation of gut microbiota by berberine and metformin during the treatment of high-fat diet-induced obesity in rats. *Sci Rep* 2015;5:14405
- Song YL, Liu CX, McTeague M, Summanen P, Finegold SM. *Clostridium bartlettii* sp. nov., isolated from human faeces. *Anaerobe* 2004;10:179–184
- Messori S, Trevisi P, Simongiovanni A, Priori D, Bosi P. Effect of susceptibility to enterotoxigenic *Escherichia coli* F4 and of dietary tryptophan on gut microbiota diversity observed in healthy young pigs. *Vet Microbiol* 2013;162:173–179
- Rosario D, Benfeitas R, Bidkhorji G, et al. Understanding the representative gut microbiota dysbiosis in metformin-treated type 2 diabetes patients using genome-scale metabolic modeling. *Front Physiol* 2018;9:775
- Zhernakova A, Kurilshikov A, Bonder MJ, et al.; LifeLines cohort study. Population-based metagenomics analysis reveals markers for gut microbiome composition and diversity. *Science* 2016;352:565–569
- Wong JM, de Souza R, Kendall CW, Emam A, Jenkins DJ. Colonic health: fermentation and short chain fatty acids. *J Clin Gastroenterol* 2006;40:235–243
- Layden BT, Yalamanchi SK, Wolever TM, Dunaif A, Lowe WL Jr. Negative association of acetate with visceral adipose tissue and insulin levels. *Diabetes Metab Syndr Obes* 2012;5:49–55
- Mueller NT, Zhang M, Juraschek SP, Miller ER, Appel LJ. Effects of high-fiber diets enriched with carbohydrate, protein, or unsaturated fat on circulating short chain fatty acids: results from

the OmniHeart randomized trial. *Am J Clin Nutr* 2020;111:545–554

39. Hernández MAG, Canfora EE, Jocken JWE, Blaak EE. The short-chain fatty acid acetate in body weight control and insulin sensitivity. *Nutrients* 2019;11:1943
40. Florez H, Luo J, Castillo-Florez S, et al. Impact of metformin-induced gastrointestinal symptoms on quality of life and adherence in patients with type 2 diabetes. *Postgrad Med* 2010;122:112–120
41. Olgun A. “Metformin-resistant” folic acid producing probiotics or folic acid against metformin’s adverse effects like diarrhea. *Med Hypotheses* 2017;106:33–34
42. Bouchoucha M, Uzzan B, Cohen R. Metformin and digestive disorders. *Diabetes Metab* 2011;37:90–96
43. Dandona P, Fonseca V, Mier A, Beckett AG. Diarrhea and metformin in a diabetic clinic. *Diabetes Care* 1983;6:472–474
44. Bailey CJ. Biguanides and NIDDM. *Diabetes Care* 1992;15:755–772
45. Krentz AJ, Ferner RE, Bailey CJ. Comparative tolerability profiles of oral antidiabetic agents. *Drug Saf* 1994;11:223–241
46. Fragiadakis GK, Wastyk HC, Robinson JL, Sonnenburg ED, Sonnenburg JL, Gardner CD. Long-term dietary intervention reveals resilience of the gut microbiota despite changes in diet and weight. *Am J Clin Nutr* 2020;111:1127–1136