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Improved Glucose Homeostasis in Obese Mice Treated With Resveratrol Is Associated With Alterations in the Gut Microbiome



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Oral administration of resveratrol is able to improve glucose homeostasis in obese individuals. Herein we show that resveratrol ingestion produces taxonomic and predicted functional changes in the gut microbiome of obese mice. In particular, changes in the gut microbiome were characterized by a decreased relative abundance of Turicibacteraceae, Moryella, Lachnospiraceae, and Akkermansia and an increased relative abundance of Bacteroides and Parabacteroides. Moreover, fecal transplantation from healthy resveratrol-fed donor mice is sufficient to improve glucose homeostasis in obese mice, suggesting that the resveratrol-mediated changes in the gut microbiome may play an important role in the mechanism of action of resveratrol.

The polyphenol, resveratrol, has shown promising results in the management of clinical symptoms associated with early type 2 diabetes (T2D) (1,2). To date, resveratrol has been shown to exert antidiabetic effects via multiple mechanisms, including anti-inflammation and antioxidant effects (3), as well as by increasing incretin secretion (4). In addition, it is debated whether resveratrol has direct insulin-sensitizing effects on peripheral tissues (5) or whether intraorgan signaling is the primary mechanism of action (6). However, given the fact that resveratrol has low bioavailability when administered orally and largely arrives unmetabolized in the colon, it is likely that resveratrol can interact with the gut

microbiota (7), and this may contribute to the antidiabetic effects of resveratrol.

Here we confirm that resveratrol administration leads to marked changes in the composition of the gut microbiota in obese mice (8,9), which is associated with improved insulin sensitivity. We also expand these findings to include a more detailed analysis of the involvement of the gut microbiota in the observed improvement in glucose homeostasis. To do this, we transplanted fecal matter from chow-fed or resveratrol-fed donor mice to conventional mice fed an obesogenic high-fat/high-sugar (HFHS) diet. Transplantation of the fecal matter from resveratrol-fed mice, but not chow-fed mice, was sufficient to recapitulate the improvement in glucose homeostasis observed with oral resveratrol treatment. Together, these findings indicate that alterations in the gut microbiota may play a pivotal role in mediating the beneficial metabolic effects of resveratrol. Thus, our findings have significant clinical implications because dysbiosis may help to explain the mixed results seen in human resveratrol trials with some studies showing a metabolic benefit although others do not (10).

RESEARCH DESIGN AND METHODS

Mouse Model of Diet-Induced Obesity

This investigation conforms with the guidelines of the Canadian Council on Animal Care and the University

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of Alberta Animal Policy and Welfare Committee. Male C57BL/6N mice (8 weeks of age, $n = 40$) were obtained from Charles River Laboratories and single housed on a 12-h light/dark cycle (0600 to 1800 h light) with ad libitum access to food and water. In brief, mice were randomly assigned into four groups and fed 1) chow, 2) chow plus 0.4% resveratrol, 3) HFHS (45 kcal% fat, 17 kcal% sucrose), or 4) HFHS plus 0.4% resveratrol ($n = 10$ /group). Mice were fed their respective diets ad libitum for 8 weeks and single housed to eliminate the confounding effect of cohousing on the microbiota.

Glucose Tolerance Tests

Mice were fasted for 5–6 h and then injected intraperitoneally with 2 g/kg body wt glucose dissolved in sterile 0.9% saline. Glucose levels were detected in blood collected from the tail tip prior to and at 10, 20, 30, 60, 90, and 120 min after the injection using an ACCU-CHEK Advantage Glucometer (Roche Diagnostics, Laval, QC, Canada) as described previously (11).

Fecal Microbiota Transplantation

Fresh fecal matter from chow and resveratrol-fed donor mice that were fed their respective diets for 8 weeks were collected as described previously (12), and aliquots of pooled fecal slurry from 10 mice were frozen and stored at -80°C until used for fecal microbiota transplantations (FMTs). A separate cohort of single-housed, non-germ-free C57BL/6N ($n = 20$) mice were fed an HFHS diet for 5 weeks, fasted overnight prior to receiving their first FMT of fecal slurry (200 μL ; each FMT dose was prepared from an average weight of 40 mg of fecal matter, equivalent to two fresh fecal pellets from the donor) from chow-fed or resveratrol-fed donor mice via oral gavage. Mice then received two additional FMTs (in the absence of fasting), which were administered every second day for a total of three FMTs. During this time, all mice continued to be fed their original HFHS diet. Glucose tolerance tests (GTTs) were then performed to assess glucose clearance, as described above. In a separate experiment, fresh fecal matter from HFHS-fed and HFHS plus resveratrol-fed donor mice that were fed their respective diets for 8 weeks were collected as described previously (12), and aliquots of pooled fecal slurry from 10 mice were frozen and stored at -80°C until used for FMT. The remainder of the experiment remained the same as described above.

Gut Microbial Profiling

Cecal samples from mice fed chow, resveratrol, HFHS, and HFHS plus resveratrol diets were collected after a fast for 5–6 h in sterile autoclaved DNase- and RNase-free Eppendorf tubes. Genomic DNA was extracted from cecum samples followed by Illumina-compatible multiplex PCR amplification of the variable 3 region of the 16S rRNA gene and sequencing using the MiSeq platform. A custom in-house pipeline was used to process the FASTQ files (McMaster Genome Facility, McMaster University, Hamilton, ON, Canada), as described previously (13). Sequences were trimmed and aligned with Cutadapt and PANDAseq (14,15) and then grouped into operational taxonomic units (OTUs) that were

based on 97% similarity with AbundantOTU+ (15,16). QIIME (Quantitative Insights Into Microbial Ecology) (17) was used to assign OTUs against the 2011 version of the Greengenes reference database (18) and to calculate α - and β -diversity, as previously described (13,17). OTUs were assigned to the closest root of the phylogenetic tree, which can result in different OTUs being assigned to the same classification. The prediction of metagenome functional content from 16S recombinant DNA library was developed using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) software, and PICRUSt predictions were categorized as levels 1–3 into KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways (19). To identify pathways with differentiating abundance in the different groups, the linear discriminant analysis (LDA) effect size (LEfSe) algorithm was used with the online interface Galaxy (<http://huttenhower.sph.harvard.edu/galaxy/root>).

Combined Direct Flow Injection and Liquid Chromatography–Tandem Mass Spectrometry Compound Identification and Quantification

We applied a targeted quantitative metabolomic approach to analyze the serum samples using a commercially available metabolomics system (AbsoluteIDQ p180 Kit; BIOCRATES Life Sciences, Innsbruck, Austria) as described previously (20). This kit, in combination with an ABI 4000 QTRAP System (Applied Biosystems/MDS Sciex) mass spectrometer equipped with a reverse-phase high-performance liquid chromatography column, can be used for the targeted identification and quantification of up to 181 different endogenous metabolites, including amino acids, acylcarnitines, biogenic amines, glycerophospholipids, sphingolipids, and sugars. Isotope-labeled internal standards and other internal standards are integrated in the kit plate filter to permit absolute metabolite quantification. Fecal slurries from chow- and resveratrol-fed mice that were used for FMTs of obese mice were also analyzed for levels of short-chain fatty acid (SCFA) acylcarnitines as described above.

Statistical Analysis

Results are expressed as the mean \pm SEM or box-and-whisker plots. Blinding was not possible for these experiments. The variance was similar between all groups being tested, and the Shapiro-Wilk normality test was applied to test for normality. Statistical methods were not used to predetermine sample size. Statistical analyses were performed using GraphPad Prism software. Pairwise comparisons were performed using an unpaired two-tailed Student t test. Multiple groups were compared by one-way ANOVA or two-way ANOVA and Tukey post hoc test when appropriate because of the small sample size. P values <0.05 were considered to be significant. The Benjamini-Hochberg multiple-testing adjustment procedure was conducted in R in order to account for the false discovery rate (FDR), where FDR-corrected P values were estimated for all taxonomic data. Results from PICRUSt analysis were evaluated for significance using the LEfSe tool with P values set at 0.05 and an LDA cutoff score of 3.0 (21). The most significant

subset of metabolites was identified by using univariate analysis for $P < 0.1$ for principal component analysis and partial least squares discriminant analysis. Data normalization consisted of log transformation and Pareto scaling. Univariate analysis of serum metabolites was performed by unpaired two-tailed Student t test and Wilcoxon Mann-Whitney test (P value with W value). FDR q values were calculated to consider the corrections needed for multiple comparisons. Heat-map and clustering analyses were performed using MetaboAnalyst. Clusters were formed based on the Euclidian distance metric, and the associated analysis was performed using a correlation test.

RESULTS

To investigate the mechanisms underlying resveratrol's effects on glucose homeostasis, we fed 8 week old male C57BL/6N mice either a chow, a chow plus 0.4% resveratrol, an HFHS (45 kcal% fat, 17 kcal% sucrose) or a HFHS plus 0.4% resveratrol diet. This dose of resveratrol was intentionally chosen so as to align with previous studies that showed improved glucose homeostasis in obese mice (22,23). Compared to chow-fed mice, consuming an HFHS diet for 8 weeks impaired glucose clearance during a GTT (Fig. 1A and B). Consistent with previous reports (8,24), obese mice administered resveratrol had significantly improved glucose tolerance (Fig. 1A and B). Resveratrol did not alter body mass in HFHS-fed mice (Fig. 1C), despite a significant decrease in fat mass in resveratrol-fed obese mice (Fig. 1D). In addition, resveratrol significantly altered acylcarnitine and phosphatidylcholine metabolism, as evidenced by a significant separation in serum metabolomic profiles of HFHS-fed and HFHS plus resveratrol-fed mice (Fig. 1E, Supplementary Table 1, and Supplementary Fig. 1).

Since changes in the composition and function of the gut microbiome are strongly implicated in the development of obesity and T2D (25), we performed 16S RNA-based bacterial profiling of cecum samples. Confirming some recent reports (3,9,26), we show that resveratrol administration altered the commensal gut microbial community in the cecum of obese mice (Fig. 1F and G). Phylum-level changes showed a higher ratio of Bacteroidetes to Firmicutes in resveratrol-fed obese mice compared with vehicle-treated obese mice (Fig. 1F and G), and the cecal contents of resveratrol-fed obese mice were characterized by a decreased relative abundance of Turicibacteraceae, *Moryella*, Lachnospiraceae, and *Akkermansia* and an increased relative abundance of *Bacteroides* and *Parabacteroides* (Fig. 1H). These findings were also confirmed by LEfSe analysis of the bacterial taxons (Fig. 1I). Linear regression using a Spearman correlation analysis between bacterial taxons and the area under the curve (AUC) from the GTT indicated that no significant correlations exist between any single bacterial group shown in Fig. 1H and the GTT AUC (data not shown). Metagenomic predictions using PICRUSt (19) and LEfSe analysis of the cecal microbiota showed distinct microbial functional profiles of the resveratrol- and vehicle-fed obese mice (Fig. 1J and Supplementary Tables 2 and 3). The

top discriminative microbial pathways in resveratrol-fed obese mice included several metabolic pathways, including carbohydrate, amino acid, and energy metabolism, along with replication and repair pathways (Fig. 1J). In contrast, the top discriminative microbial pathways in obese mice included pathways related to bacterial chemotaxis, flagella assembly, motility pathways, and environmental information processing (Fig. 1J). Overall, these data suggest that the glucose-lowering effects of orally administered resveratrol were associated with significant modification of gut microbial composition and predicted functional pathways in obese mice (27), although shotgun metagenomics would be required to directly measure the functional pathways involved.

In order to ascertain whether resveratrol-induced changes in the gut microbiota may be involved in the improvement in glucose homeostasis, we fed a separate cohort of 8-week-old mice an HFHS diet for 5 weeks and then by oral gavage administered three FMTs collected from donor mice fed either a chow diet or a resveratrol diet (Fig. 2A). Interestingly, 1 week after the final FMT dose, obese mice that had received a resveratrol-FMT displayed robust improvements in glucose clearance, whereas control-FMTs had no effect in obese mice (Fig. 2B and C). An improvement in glucose clearance was also observed in obese mice receiving FMT from donor mice maintained on an HFHS plus resveratrol diet (Supplementary Fig. 2). Importantly, this improvement in glucose homeostasis occurred in the absence of a reduction in body weight (Fig. 2D). For these FMT experiments, we also performed bacterial sequencing of cecum samples and show, using the Bray-Curtis similarity index, that the bacterial community in the cecum from obese mice receiving control-FMT was different from obese mice receiving resveratrol-FMT (Fig. 2E). Obese mice receiving resveratrol-FMT had higher levels of *Parabacteroides* and lower relative abundance of *Moryella* and *Akkermansia* (Fig. 2F), which is similar to our results obtained with oral resveratrol feeding. Metagenomic predictions using PICRUSt and LEfSe analysis (19) of the cecal microbiota again showed distinct microbial functional profiles between the pre-FMT obese mice and mice that received either a control-FMT or resveratrol-FMT (Fig. 2G and Supplementary Table 4). As was seen in the resveratrol-fed obese mice, the top discriminative microbial pathways in the pre-FMT obese mice included several metabolic pathways related to bacterial motility, membrane transport, and environmental information processing, whereas mice receiving a control-FMT had higher carbohydrate, energy, and amino acid metabolism levels, and mice receiving a resveratrol-FMT had higher levels of glycan biosynthesis, genetic information processing, and replication and repair pathways.

Four clusters of metabolites were identified that were associated with changes in gut bacterial composition of obese mice after resveratrol-FMT or chow-FMT. These were identified using a Spearman correlation test (Supplementary Table 5). A significant correlation was found between Proteobacteria and four of the five metabolites in cluster 2 [PC ae C40:1, PC ae C42:1, PC ae C42:2, and SM (OH) C24:1],

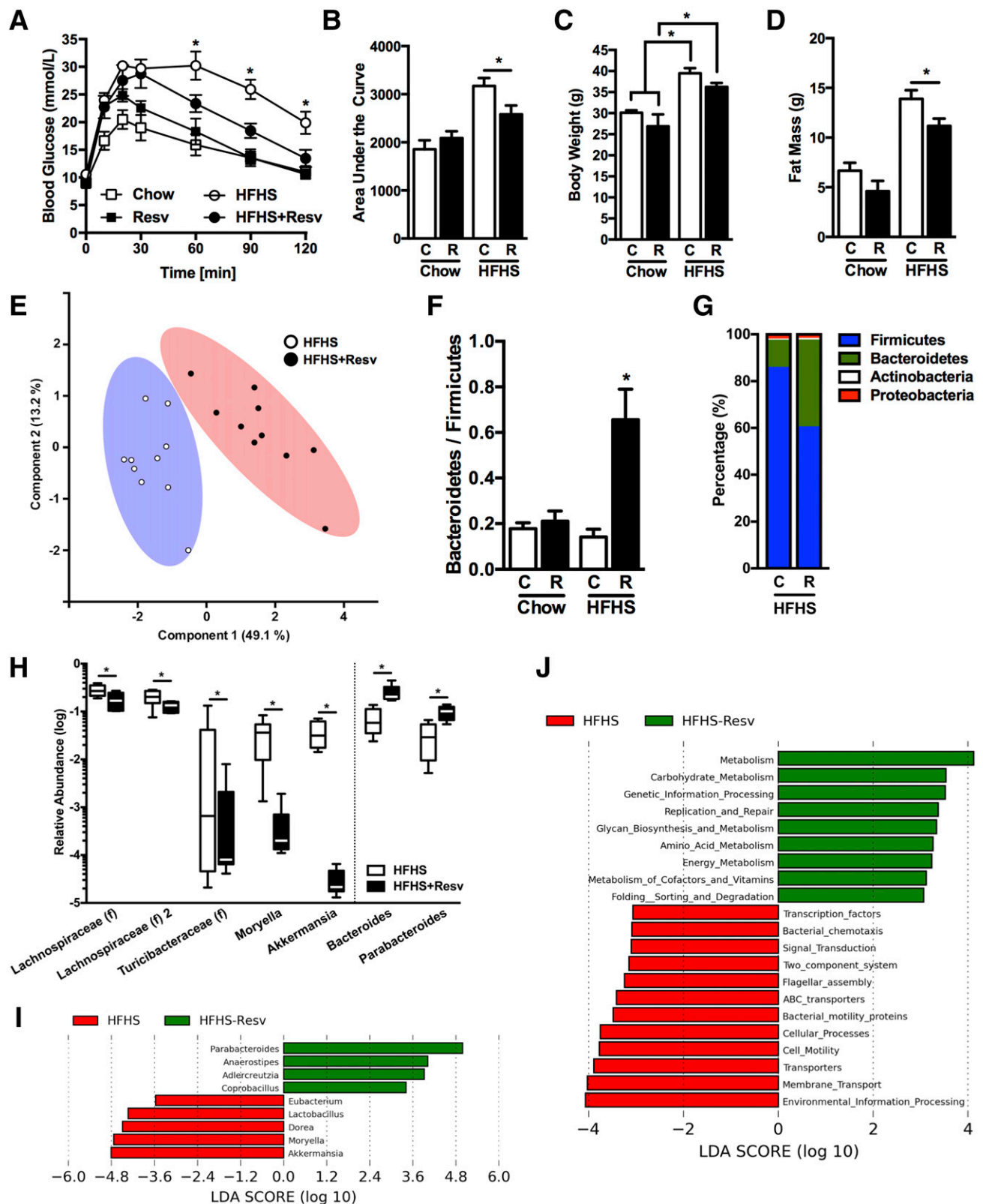


Figure 1—Resveratrol feeding improves glucose homeostasis, serum metabolomic profiles, and composition and predicted function of the gut microbiome in obese mice. **A**: Glucose tolerance tests of mice maintained on a chow diet (Chow), a chow diet supplemented with resveratrol (Resv), an HFHS diet, or an HFHS diet supplemented with resveratrol (HFHS+Resv) ($n = 7$ /group) for 8 weeks. **B**: Glucose clearance represented by the AUC of the glucose tolerance tests in either chow-fed mice (Chow) or HFHS-fed mice (HFHS) supplemented without resveratrol (C) or with resveratrol (R). Body weight ($n = 9$ – 10 /group) (**C**) and fat mass (**D**) in all groups of mice ($n = 5$ /group). **E**: Serum metabolomic profiles of mice fed HFHS or HFHS+Resv diets ($n = 10$ /group). **F**: Ratio of Bacteroidetes to Firmicutes in the cecum of mice from all four groups (Chow, $n = 5$, and other groups, $n = 6$). **G**: Representative commensal gut microbial community in HFHS-fed mice without

consisting of phosphatidylcholines and a sphingomyelin. Our analyses show a decrease in Proteobacteria in obese mice after resveratrol-FMT compared with a chow-FMT. Furthermore, the presence of these metabolites was also decreased in mice maintained on a diet supplemented with resveratrol. Cluster 4, consisting of acylcarnitines, was associated with changes in Actinobacteria and Verrucomicrobia (Supplementary Table 5). In particular, our findings indicate that butyrylcarnitine (C4) was decreased in mice maintained on a diet supplemented with resveratrol.

Previous evidence (28) has shown that changes in the gut microbiota can strongly impact energy harvesting from the diet. Further, gut fermentation produces metabolites, including SCFAs, that have been proposed to have critical roles in the maintenance of energy homeostasis (i.e., energy expenditure and adiposity) and insulin sensitivity of the host (29). Therefore, we used metabolomics to screen fecal samples used for FMT for all SCFA acylcarnitines, and we observed either no differences between groups or that several SCFA acylcarnitines were below the limit of detection (Supplementary Table 6). These data confirm that improved glucose homeostasis in these obese mice receiving FMT from resveratrol-fed donor mice is independent of a direct supplementation of SCFAs produced in donor mice. However, the effect observed in the mice receiving resveratrol-FMT could have involved an increased production of luminal SCFA by transplanted microbes.

DISCUSSION

Our data suggest that resveratrol-induced alterations in the gut microbiota are associated with improved glucose homeostasis in obese mice and that these changes may be an important mechanism by which resveratrol mediates its beneficial metabolic effects. Although this association is consistent with previous studies (3,9), we did not test whether altered microbiota is essential for the ability of resveratrol to improve glucose homeostasis in obese mice and, thus, cannot definitively state that this is the case. Indeed, antibiotics could be tested in this model in order to confirm that antibiotics can prevent the effects of resveratrol (13,30,31). However, despite the limitations of the current study, previous studies (3,32) have shown that resveratrol is able to reduce tissue inflammation and endotoxemia, suggesting that correcting obesity-related alterations in gut microbiota, low-grade inflammation, and metabolic endotoxemia (33) may be involved in this effect. In this context, our findings show that decreases in Proteobacteria with resveratrol-FMT are associated with changes in phosphatidylcholines and

sphingomyelins. Proteobacteria are associated with inflammation and can be indicative of imbalances in the microbiome (34). Indeed, the inability to control levels of Proteobacteria has been shown to underlie cecal inflammation (35), which can lead to local and systemic inflammation, ultimately contributing to metabolic dysfunction (34). Similarly, phosphatidylcholines and sphingomyelins have been implicated in inflammation and the inflammatory pathway (36). Thus, it is possible that resveratrol-FMT lowers the inflammatory state of obese mice, contributing to the improvement in their glucose homeostasis.

Earlier reports emphasized the antimicrobial effects of resveratrol (3), which showed a decrease in the relative abundance of the following three species of bacteria: *Parabacteroides johnsonii*, *Alistipes putredinis*, and *Bacteroides vulgatus*. In agreement with that study, we show that the relative abundance of certain microbial families (Lachnospiraceae and Turicibacteraceae) and genera (*Moryella* and *Akkermansia*) were also decreased with resveratrol treatment. However, we show that the relative abundance of certain genera and families of bacteria are also increased as a result of resveratrol supplementation (*Bacteroides* and *Parabacteroides*) or after FMT from donor mice fed a resveratrol-supplemented diet (*Lactococcus*, *Parabacteroides*, and Lachnospiraceae) (2). Furthermore, our findings were in agreement with those of previous studies showing that resveratrol increases the ratio of Bacteroidetes to Firmicutes (9). Thus, although our findings are in general agreement with those of previous studies demonstrating that resveratrol can alter the gut microbiota, there are some differences in specific microbial changes. It is possible that these observed differences could be attributed to housing environments (isolated vs. conventional), diets (high-fat vs. HFHS diet), and/or model species (mouse vs. rat) used in the different studies.

Previous findings also showed that C4 is increased in the plasma of patients with T2D (37). Thus, increases in C4 may be associated with an insulin-resistant/diabetic phenotype that may be decreased by resveratrol supplementation and resveratrol-FMT through the alteration of a specific bacterial phylum. In addition, since resveratrol supplementation improves glucose homeostasis in obese mice via increased portal vein concentrations and intestinal content of glucagon-like peptide-1 (3), the effects we observed herein may also involve this mechanism. Moreover, we report reduced abundance of *Akkermansia* in the resveratrol-treated obese mice, which corresponds with improved glucose tolerance. Although it is tempting to speculate that a reduced

resveratrol (C) or with resveratrol (R). *H*: Relative abundance of Lachnospiraceae, Turicibacteraceae, *Moryella*, *Akkermansia*, *Bacteroides*, and *Parabacteroides* in the cecum of mice fed HFHS or HFHS+Resv diets ($n = 6$ /group). LEfSe analysis of bacterial taxons (*I*) and metagenomic predictions (*J*) using PICRUST and LEfSe analysis of the cecal microbial functional profiles between mice fed HFHS ($n = 6$) or HFHS+Resv diets (Log LDA >3.0 ; $n = 6$). Values in *A–D* and *F* are shown as the mean \pm SEM, and those in *H* are box-and-whisker plots. * $P < 0.05$; analyzed by two-way ANOVA with Tukey post hoc test in *A*, and analyzed by one-way ANOVA with Tukey post hoc test in *C* and *F*. Data in *B*, *D*, and *H* were analyzed vs. HFHS-resveratrol determined by unpaired two-tailed Student *t* test, and results in *H* were corrected for FDR. Only functional categories meeting a log LDA significant threshold value of >3 are shown in *I* and *J*.

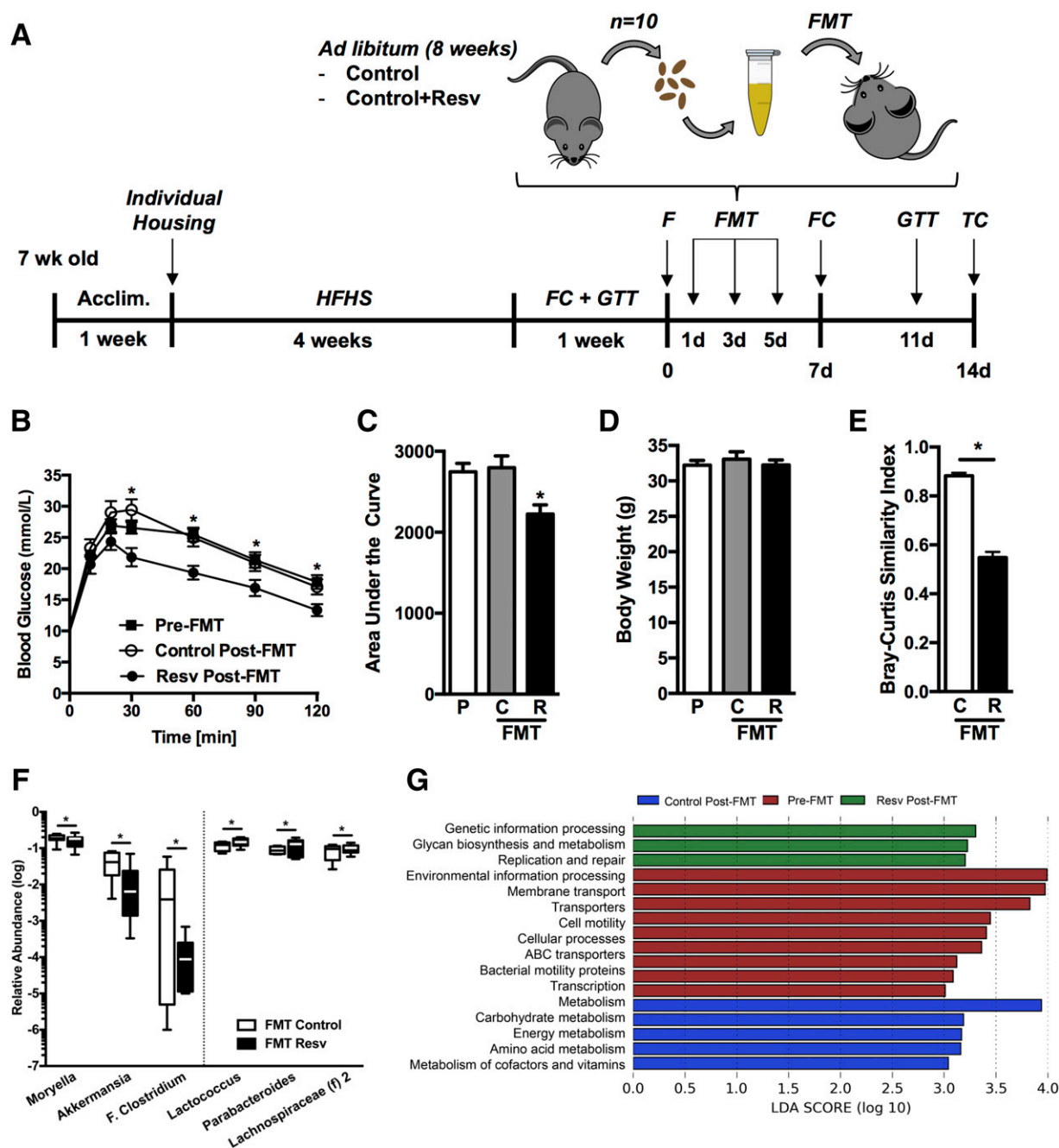


Figure 2—FMTs from donor mice fed a resveratrol diet are sufficient to improve glucose homeostasis and alter the gut microbiota in obese mice. **A:** Male C57BL/6N mice (8 weeks old) were fed an HFHS diet for 5 weeks. Fecal samples were collected (FC) and baseline GTTs were completed during the week before receiving FMTs. Following an overnight fast on day 0 (F), mice received FMTs (FMT) every other day for a total of three FMT doses on days 1, 3, and 5 (1d, 3d, and 5d). Mice were fasted overnight only prior to the first FMT dose and subsequently were fed their original HFHS diet ad libitum during the last two FMT doses and throughout the remainder of the study. Fecal samples were collected (FC) again on day 7 (7d), and post-FMT GTTs (GTT) were completed on day 11 (11d). Tissues were collected (TC) after a 5–6 h fast on day 14 (14d). **B:** GTTs of HFHS-fed mice at baseline prior to receiving FMTs (Pre-FMT; $n = 20$) and after randomization to receiving an FMT from chow-fed mice (Control Post-FMT; $n = 9$) or from chow plus resveratrol-fed donor mice (Resv Post-FMT; $n = 11$). **C:** Glucose clearance represented by the AUC of the glucose tolerance tests from HFHS-fed mice prior to FMT (P) and after FMT from control-FMT (C) and Resv-FMT (R). **D:** Body weight in all groups of mice (P, $n = 20$; R, $n = 11$; and C, $n = 9$). **E:** Bray-Curtis similarity index comparing the bacterial community in the cecum from obese mice receiving either control-FMT (C, $n = 8$) and Resv-FMT (R, $n = 9$). **F:** Relative abundance of *Moryella*, *Akkermansia*, *Bacteroides*, *F. Clostridium*, *Lactococcus*, *Parabacteroides*, and *Lachnospiraceae* in the ceca of HFHS-fed mice following control-FMT ($n = 8$) or Resv-FMT ($n = 9$). **G:** Metagenomic predictions using PICRUSt and LEfSe analysis of the cecal microbial functional profiles between the pre-FMT, control-FMT ($n = 9$ /group), and Resv-FMT mice ($n = 11$ /group). Values in B–E are shown as the mean \pm SEM, and those in F are box-and-whisker plots. * $P < 0.05$; analyzed by two-way ANOVA with Tukey post hoc test in B and by one-way ANOVA with Tukey's post hoc test in C and D. Data in F were analyzed by unpaired two-tailed Student t test corrected for FDR. Only functional categories meeting a log LDA significant threshold value of >3 are shown in G.

level of *Akkermansia* contributes to the beneficial effects of resveratrol, this is in contrast to other findings showing that *Akkermansia muciniphila* abundance inversely correlates with body weight and glucose tolerance (38,39), so additional work in this area is necessary before any conclusions can be drawn.

On the basis of the rapid and dramatic improvement in glucose homeostasis in obese mice receiving FMTs from resveratrol-fed donor mice, our data appear to rule out direct effects of circulating resveratrol on peripheral target tissues. However, it is possible that changes in the gut microbial community work in conjunction with resveratrol to induce these beneficial effects. In fact, on the basis of the FMT studies, it is quite possible that a metabolite of resveratrol and/or bacterial-derived metabolites induced by resveratrol in donor mice is responsible for producing the beneficial effects observed in obese mice receiving FMTs. In addition, future studies using heat-inactivated FMT from resveratrol-fed donor mice could help address whether or not live gut microbiota transplanted during the FMT are necessary for improving glucose homeostasis in obese mice. Thus, although a better understanding of the contents of the FMT that may influence glucose homeostasis in obesity is clearly warranted, our findings not only highlight a previously underappreciated site of action for resveratrol in the gut but may also assist in the eventual identification of the resveratrol-mediated mechanisms responsible for improved glucose homeostasis in obesity and aid in the discovery of new treatment modalities.

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Author Contributions. M.M.S. and T.T.K. contributed to the study design and data analysis and interpretation; performed the experiments; and drafted and reviewed the manuscript. E.D. contributed to the study design and data analysis and interpretation and performed the experiments. C.-L.M.S., S.M.H., N.J.B., and G.M. performed the experiments. H.P. contributed to the study design and data analysis and interpretation and performed the experiments. D.S.W., K.L.M., J.D.S., and J.R.B.D. contributed to the study design and data analysis and interpretation

and drafted and reviewed the manuscript. J.R.B.D. 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.

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