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Metformin and DPP-4 Inhibitor Differentially Modulate the Intestinal Microbiota and Plasma Metabolome of Metabolically Dysfunctional Mice

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Running Title: Metformin, DPP-4i and the Gut Microbiota

Key Messages: [1] Several large scale cross-sectional clinical studies have recently demonstrated the propensity of common medication to alter the composition and diversity of the gut microbiota. [2]

Metformin, a relatively old drug for which the mechanism of action is incompletely understood, has a far greater effect on the gut microbiota than a novel DPP-4 inhibitor. [3] Metformin therapy reduced α -diversity, while increasing relative abundances of several genera associated with metabolic function - *Akkermansia*, *Parabacteroides* and *Christensenella*.

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Data statement: The data generated by Illumina sequencing were deposited in the NCBI Sequence Read Archive (SRA) and are available under Ac. No. PRJNA414526 (HFC) and PRJNA497977 (MET and PKF).

ABSTRACT

Background | Recent evidence indicates that the gut microbiota is altered considerably by a variety of commonly prescribed medications. This study assessed the impact of two antidiabetic therapeutics on the gut microbiota and markers of cardiometabolic disease in metabolically dysfunctional mice.

Materials & Methods | C57BL/6 mice were fed a high-fat diet for 24-weeks while receiving one of two antidiabetic therapeutics - metformin or DPP-4 inhibitor, PKF-275-055 - for the final 12 weeks. Mice were assessed for weight gain, glucose and cholesterol metabolism, and adiposity. In addition, caecal microbiota was analysed by 16S compositional sequencing and plasma metabolome was analysed by LC-MS/MS.

Results | Both therapeutics had similar metabolic effects, attenuating mesenteric adiposity, improving cholesterol metabolism and insulin sensitivity. However, multivariate analyses of microbiota and metabolomics data revealed clear divergence of the therapeutic groups. While both metformin and PKF-275-055 mice displayed significantly decreased Firmicutes/Bacteroidetes ratios, only metformin harboured metabolic health-associated *Akkermansia*, *Parabacteroides* and *Christensenella*. Paradoxically, metformin also reduced α -diversity, a metric frequently associated with host metabolic fitness. PKF-275-055 mice displayed elevated levels of butyrate-producing *Ruminococcus* and acetogen *Dorea*, with reduced levels of certain plasma sphingomyelin, phosphatidylcholine and lysophosphatidylcholine entities. In turn, metformin reduced levels of acylcarnitines, a functional group associated with systemic metabolic dysfunction. Finally, several associations were identified between metabolites and altered taxa.

Conclusions | This study represents the first direct comparison of the microbiota-modifying effects of metformin and a DPP-4 inhibitor, and proposes several putative microbial targets both in terms of novel therapeutic development and adverse effect prevention.

INTRODUCTION

Current projections forecast that insulin resistance and the associated metabolic sequelae of the metabolic syndrome are set to become one of the greatest global threats to human health [1, 2]. While primary concern must be placed with upstream stemming of this epidemic, detailed exploration of current antidiabetic therapies is also of particular importance. We currently lack clear mechanistic detail for a number of such therapies, and complete comprehension may contribute to the development of novel targets or personalised therapeutics. One factor which has only recently garnered considerable attention in this regard is the gut microbiome. The adult human gastrointestinal tract is host to a staggering diversity of bacteria, fungi and viruses, the genetic material of which vastly eclipses that of our own genome [3]. Due to this same genetic and metabolic potential, the gut microbiome has long been suspected as implicit in human extra-intestinal health, including factors of metabolic regulation [4]. Relatively large-scale clinical cross-sectional observational studies have demonstrated a contributory role for the gut microbiome in host metabolic health. Indeed, a robust correlation was previously identified between increased microbiota diversity, or gene richness, and host metabolic fitness [5]. Moreover, the propensity of the gut microbiota to produce metabolites such as branched chain amino acids has been implicated in the pathogenesis of insulin resistance in a large Danish cohort [6].

In addition to contributing to host health and disease, research indicates that the gut microbiome often alters or is altered by certain pharmacological interventions [7]. A large study combining clinical datasets from two independent Belgian and Dutch studies revealed that medications -such as antibiotics, proton pump inhibitors, osmotic laxatives, progesterone, antidepressants and TNF- α inhibitors- explained the greatest degree of variance between the microbiota composition of faecal samples [8]. Moreover, a recent analysis of the >2700 participant UKTwins Study cohort uncovered microbiome composition associations for dozens of commonly prescribed oral and even topical medications [9]. Indeed, certain drugs are now recognised to be metabolised by gut microbiota members in reactions such as glucuronidation, reduction, oxidation and hydrolysis [10]. In addition, bile-modifying microbes, with which the gut is highly populated [11],

can further alter drug kinetics by increasing or decreasing solubility, absorption and excretion [12, 13]. In line with this, the composition of one's gut microbiota, which displays degrees of inter-individual variation, may explain a degree of the variance in the personalised response observed with certain oral pharmaceutical therapies. One such drug which has been shown to exhibit microbiota-mediated action is metformin. Old knowledge indicating that certain antibiotics affect metformin efficacy gave the first clue that the microbiota may play a role in the drugs mechanism. Preclinical studies identified *Akkermansia muciniphila* as a potential key microbe mediating the mechanistic effects of metformin, since the Verrucomicrobia species was consistently found to be enhanced in the presence of the drug [14]. This effect has also been reproduced and confirmed in randomised, controlled and cross-sectional clinical trials [15-17], and the corrective effects of *Akkermansia muciniphila* in models of metabolic dysfunction have previously been demonstrated in a preclinical setting by Everard *et al.* [18].

Under the current National Institute for Health and Care Excellence guidelines [19], the management of type-2 diabetes mellitus (T2DM) follows an order of attempted dietary-control, subsequently in combination with biguanide therapy (*i.e.* metformin), followed by combination with SGLT2 inhibitor, sulfonylurea, thiazolidinedione or dipeptidase peptidyl (DPP)-4 inhibitor. The first therapeutic assessed in this study, metformin, is one of the most widely prescribed antidiabetic drugs globally; while the second therapeutic, PKF-275-055, is a member of the relatively novel DPP-4 inhibitor class of drugs. Although the effects of metformin on the microbiota have been well characterised, detailed metabolomics has not been applied to such studies. In addition, the microbiota-modifying effects of DPP-4 inhibitors, and in particular PKF-275-055, is currently unknown. Identification and comparison of the distinct microbial signatures associated with both interventions (metformin and PKF-275-055) may reveal novel targets for the management of diabetes and systemic metabolic dysfunction. Herein, we explore and compare the effects of these functionally-distinct antidiabetic therapies on the microbiota composition and plasma metabolome of metabolically dysfunctional mice.

METHODS

Animals, Diet & Therapies

C57BL/6 male mice (3 weeks old; Envigo UK) were used in this diet-induced obese mouse model. All mice were acclimatised for 1 week in the housing facility prior to trial commencement. Animals were group-housed (3-4/cage) at room temperature (21°C) in a 12-h light–dark cycle (light cycle 07:00-19:00). The study was performed in APC Microbiome Ireland, University College Cork and experimental procedures were carried out as per the protocols approved by the University College Cork Ethics Committee, with a license acquired through the Health Products Regulatory Authority. Mice were randomised into the three groups and maintained on a high-fat diet (Open Source Diets [D12492 – 60% kcal from fat; Research Diets Inc.]) *ad libitum* for 24 weeks, while receiving one of two antidiabetic therapies for the final 12 weeks. Prior to commencement of interventions, high-fat fed mice were compared against mice receiving normal chow for their glucose handling and insulin sensitivity, in order to confirm the validity of the diet-induced metabolic dysfunctional model (Supplementary Figure S1). At this point, high-fat diet fed mice were randomised into three groups: high-fat control (HFC; *n* 14), metformin (MET; *n* 14) and PKF-275-055 (PKF; *n* 14). The interventions were delivered in the drinking water with the following doses: metformin 300 mg/kg/d (Sigma Aldrich Ireland) and PKF-275-055 15 mg/kg/d (Novartis Ireland). The dosage for both therapies were calculated and adjusted each day from the daily water intake and weekly animal body weights.

Metabolic Test Battery

Intraperitoneal Glucose Tolerance Test. At week 11 of intervention, mice were fasted from the start of light-cycle for 6 hours prior to the Intraperitoneal Glucose Tolerance Test (IPGTT). At baseline, duplicate blood glucose readings were recorded from blood collected from the tail vein (Accu-Chek glucometers and strips; Roche) and 1 g D-glucose/kg of bodyweight was delivered by injection into the intraperitoneum of the mouse. Subsequently, blood glucose was recorded again in duplicate at 15,

45, 90 and 120 min following glucose challenge. In addition, bloods were drawn at 0 and 15 min for quantification of plasma insulin (Mouse Insulin ELISA, Mercodia).

Insulin Tolerance Test. At week 11 of intervention, mice were fasted in the same manner for the insulin tolerance test (ITT) as for the IPGTT. Following duplicate baseline blood glucose readings, intraperitoneal injection of 0.75 IU insulin/kg of bodyweight was administered. Blood glucose was then monitored in the same way at 15, 45, 90 and 120 min following insulin challenge.

Mixed Meal Gavage. At week 11 of intervention, mice were fasted in the same manner as the previously described tests prior to the mixed meal gavage (MMG). Each mouse received a gastric oral gavage of 200 μ l Ensure Plus (Abbott Nutrition, Ireland) and blood glucose readings were recorded in duplicate at 0, 2, 4 and 18 h post gavage. Plasma samples were stored and cholesterol levels were later quantified (EnzyChrom colorimetric assay; Cambridge Biosciences, UK).

Plasma Metabolomics

Plasma was obtained from mice at cull and frozen at -80°C prior to shipment under regularly replenished dry-ice to University of Alberta, Canada. The Biocrates AbsoluteIDQ p180 Kit (BIOCRATES Life Sciences AG, Austria) was utilised to detect and quantify ~140 metabolites within each plasma sample. Following initial extraction and derivatization steps, a range of specific amino acids, biogenic amines, acylcarnitines, lysophosphatidylcholine, phosphatidylcholines, sphingomyelins and hexoses present in the samples were detected and quantified on an ABI 4000 Q-Trap mass spectrometer (MDS Sciex) run in conjunction with a reverse-phase HPLC-column.

Compositional Microbiota Sequencing

Microbial DNA extraction, 16S rRNA amplification and sequencing. Caecal contents were collected from individual mice following 12 weeks of drug intervention. Total metagenomic DNA was extracted from caecal contents with the QIamp® PowerFecal® DNA Kit (Qiagen, Milano, Italy)

where an additional bead beating step was incorporated into the protocol. Extracted DNA was quantified using the NanoDrop™ 8000 Spectrophotometer (Thermo Fisher Scientific). Total genomic DNA was then subjected to PCR amplification by targeting a 464-bp fragment of the 16S rRNA variable region V3-V4 using the specific bacterial primer set 341F (5'- CCTACGGGNGGCWGCAG -3') and 806R (5'- GACTACNVGGGTWTCTAATCC -3') with overhang Illumina adapters [20, 21]. Unique barcodes were attached to the forward primer for facilitating the differentiation of samples. Amplicons were cleaned with the Agencourt AMPure kit (Beckman Coulter) following the manufacturer's instructions, and DNA was quantified using the Quant-iT PicoGreen dsDNA kit (Invitrogen). Amplicons were mixed and combined in equimolar ratios, and the quality and purity of the library was checked with the High Sensitivity DNA Kit (Agilent, Palo Alto, CA, USA) by the Bioanalyzer 2100 (Agilent). The library was sequenced on an Illumina MiSeq platform at CIBIO (Center of Integrative Biology) – University of Trento, Italy.

Bioinformatic analysis. Sequences obtained from Illumina sequencing were processed using Quantitative Insights Into Microbial Ecology (QIIME) software package version 1.9 [22]. The paired-end reads were associated to the corresponding sample through the unique barcode and joined by using the fastq-join method [23]. Quality filtering of Illumina data was performed as part of split_libraries_fastq.py, using the per-nucleotide Phred quality score ($q > 20$), which works on a per-nucleotide basis, truncating reads at the position where their quality begins to drop [22, 24]. Uclust was then used for clustering the reads left into operational taxonomic units (OTUs) at 97% identity [25]. PyNAST was used to align OTUs with a minimum alignment of 150 bp and 80% of minimum identity, and taxonomy was assigned by using Ribosomal Database Project (RDP) classifier 2.0.1 [26, 27] QIIME was used to generate alpha (Chao1, PD whole tree, Simpson, Shannon, observed OTUs) and beta diversities (Bray Curtis) distance matrices, and principal coordinate analysis (PCoA) plots were generated based on the beta diversity distance matrices. Finally, the Galaxy Lab platform for analysing linear discriminant analysis (LDA) effect size (LEfSe) was applied to sequencing data in order to identify taxa most associated with each group [28].

Statistical Analysis

All metabolic datasets were analysed by one-way analysis of variance (ANOVA) in Prism 5 (GraphPad Software Inc.). Metabolomic data was log normalised prior to ANOVA, multivariate and correlation analyses in the MetaboAnalyst metabolomics analysis suite [29]. Sparse partial least square discriminant analysis (sPLS-DA) was also performed on metabolomic data [30]. For metabolomic data, a false discovery rate (FDR) q value less than 0.05 was considered statistically significant.

RESULTS

Metabolic Parameters

All animals gained significant weight over the study period (Fig. 1B; $p < 0.0001$). MET mice presented with significantly lower weights when compared to both HFC and PKF at several weekly timepoints throughout the study. However, both MET and HFC groups had similar weight gain at the end of the study. Despite this, MET mice were significantly lighter than PKF mice at week 24 ($p < 0.05$). In addition, at sacrifice both therapeutic groups displayed a reduced quantity of mesenteric adipose tissue when compared to the HFC (Fig. 1C; $p < 0.01$).

Complete MMG challenge at week 23 (*i.e.* week 11 of intervention period) demonstrated that both therapeutics improved mouse cholesterol metabolism, as both groups had returned to a significantly lower level of plasma cholesterol compared to the HFC group by 18 h (Fig. 1D; $p < 0.01$). Although neither therapy significantly improved glucose handling during IPGTT, MET treated mice demonstrated a reduced level of insulin 15 min post glucose loading (Fig. 1E; $p < 0.01$). In addition, during the IPITT, MET treated mice displayed a more rapid and sustained blood glucose depletion response to insulin challenge when compared to HFC and PKF, respectively (Fig. 1F; $p > 0.05$).

Plasma Metabolomics

sPLS-DA of plasma metabolomics data displays neat clustering of MET specimen away from those of both HFC and PKF groups (Fig. 2A). The component metabolites of greatest importance in this sPLS-DA plot are PC ae C34:3, C16:1, C18:1, C14, SM OH C16:1, C18:2, phenylalanine, PC aa C40:6, C16:2, SM OH C14:1, in order of effect (Fig. 2B). With respect to amine entities, PKF mice displayed increased levels of phenylalanine and reduced levels of glutamate when compared to both other groups (Fig. 2C; $p < 0.05$). Both therapeutic groups presented with reduced threonine, while solely MET mice had reduced levels of methionine sulfoxide (Fig. 2C; $p < 0.05$). With regards sphingomyelins, MET mice displayed increased levels of SM OH C14:1, SM OH C16:1 and SM C18:0; while, conversely, PKF mice showed reduced levels of the latter two metabolites (Fig. 2D; $p < 0.05$). Analysis of phosphatidylcholine metabolites demonstrated a multitude of PKF-mediated reductions including PC ae C34:3, PC ae C36:5, PC ae C42:3, PC aa C36:0, PC aa C40:1, PC aa C42:4 and lysoPC a C18:0 (Fig. 2E; $p < 0.05$). MET mice were found to have increased levels of PC ae C34:3 and decreased PC ae C42:3 (Fig. 2E; $p < 0.05$). Finally, the two therapeutics acted conversely with regards acylcarnitine entities. PKF mice displayed increased levels of hexadecenoylcarnitine (C16:1), octadecenoylcarnitine (C18:1) and octadecadienylcarnitine (C18:2; Fig. 2F; $p < 0.05$); while MET mice presented with reduced levels of tetradecanoylcarnitine (C14) and hexadecenoylcarnitine (C16:1; Fig. 2F; $p < 0.05$).

Microbiota Composition

PCoA of 16S compositional sequencing data demonstrates clear and consistent clustering of the MET mice microbiota as distinct from both HFC and PKF (Fig. 3A). The PCoA-related scatterplot demonstrates the microbial taxa most associated with each vector (Fig. 3B). The MET microbiota cluster most neatly associated with *Mucispirillum*, *Bacteroides*, *Parabacteroides*, *Clostridiales*, *Christensenella* and *Akkermansia* (Fig. 3B). Interestingly, the MET microbiota displayed reduced levels of several α -diversity metrics, including Shannon diversity index, when compared to both HFC ($p < 0.0001$) and PKF (Fig. 3C; $p < 0.001$).

In broad terms, LEfSe analysis revealed that the HFC microbiota was associated with Firmicutes, while the MET microbiota was defined by Bacteroidetes, *Parabacteroides* and *Akkermansia* (Fig. 4A/B). In turn, the PKF microbiota appeared to associate most closely with the family *Ruminococcaceae* and a number of low abundance taxa (Fig. 4A). With regards to phylum level alterations, PKF mice presented with reduced levels of Firmicutes compared to HFC (46.4 vs. 52.4 %; $p = 0.05$), while MET mice displayed lower levels of the taxon compared to both other groups (38.7 %; $p < 0.01$). Conversely, the MET microbiota showed increased levels of Bacteroidetes compared to HFC (49.5 vs. 40.8 %; $p < 0.01$), and Verrucomicrobia compared to both PKF and HFC (3.4 vs. 0.1 and 0.01 %; $p < 0.001$). Both MET ($p < 0.001$) and PKF ($p < 0.05$) mice had a reduced Firmicutes:Bacteroidetes ratio (Fig. 4C). When compared to HFC, PKF mice displayed increased levels of the genera *Bacteroides* (19.2 vs. 11.2 %; $p < 0.01$), *Dorea* (1.0 vs. 0.2 %; $p < 0.00001$; Fig. 4D) and *Ruminococcus* (2.0 vs. 1.2 %; $p < 0.01$; Fig. 3D); while displaying reduced levels of *Odoribacter* (1.5 vs. 3.5 %; $p < 0.05$) and *Lachnoclostridium* (17.4 vs. 24.2 %; $p < 0.01$; Fig. 3D). In turn, when compared to the HFC, MET mice presented with significantly increased levels of *Bacteroides* (20.6 vs. 11.2 %; $p < 0.001$), *Akkermansia* (3.4 vs. 0.02 %; $p < 0.001$; Fig. 4E), *Parabacteroides* (6.2 vs. 1.0 %; $p < 0.001$; Fig. 4F), *Christensenella* (0.1 vs. 0.01 %; $p < 0.00001$; Fig. 4G) and *Clostridiales_other* (4.8 vs. 1.0 %; $p < 0.00001$); while displaying decreased levels of AF-12 (0.3 vs. 0.9 %; $p < 0.01$), *Muribaculum* (10.3 vs. 14.5 %; $p < 0.001$), *Clostridiaceae_other* (0.1 vs. 0.6 %; $p < 0.00001$), *Lachnoclostridium* (16.8 vs. 24.2 %; $p < 0.01$), *Coprococcus* (1.1 vs. 3.5 %; $p < 0.05$), *Dorea* (0.04 vs. 0.2 %; $p < 0.00001$), *Papillibacter* (4.6 vs. 6.4 %; $p < 0.05$), *Oscillospira* (8.0 vs. 12.5 %; $p < 0.01$), *Ruminococcus* (0.7 vs. 1.4 %; $p < 0.00001$), *Desulfovibrio* (0.1 vs. 1.1 %; $p < 0.00001$) and *Desulfovibrionaceae* (0.0 vs. 0.2 %; $p < 0.00001$; Fig. 3D).

Microbiota-Metabolite Correlation

When conducting correlation analysis between the key taxonomic microbiota alterations observed and plasma metabolome, a range of positive and negative associations with *Dorea* and *Christensenella* relative abundance were identified (Supplementary Figure S2). *Dorea* relative abundance was

positively correlated with several acylcarnitines (C2, C161, C182, C141, C14, C142, C162, C181 and C141-OH; $q < 0.05$) and phenylalanine ($q < 0.05$), and negatively correlated with a range of phosphatidylcholines (PC ae C343, PC aa C406, PC aa C424, PC ae C424, PC aa C361 and PC aa C362; $q < 0.05$) and a single sphingomyelin (SM OH C161; $q < 0.05$). Conversely, *Christensenella* relative abundance was positively correlated with phosphatidylcholines (PC ae C343, PC aa C361 PC aa C424, PC aa C362, PC ae C342, PC ae C365 and PC ae C321; $q < 0.05$) and two sphingomyelins (SM OH C141, SM OH C161; $q < 0.05$), as well as ornithine and alanine ($q < 0.05$). In turn, *Christensenella* relative abundance was negatively correlated with several acylcarnitines (C14, C2, C161, C182, C141-OH, C142 and C162; $q < 0.05$), phenylalanine and kynurenine ($q < 0.05$).

DISCUSSION

The intestinal microbiome has garnered significant attention and scrutiny over the past two decades as an influencer of host health and disease. While we acknowledge the major impact that diet imposes on our gut microbiome [31], until recently, little consideration had been given to the potential impact that the thousands of regularly prescribed oral medications may have on this plastic enteric ecosystem. While metformin has been widely publicised for its microbiota-modulating and *Akkermansia*-enhancing attributes, the effects of more novel classes of antidiabetic therapeutics have not been extensively researched. In line with this, we set out to assess the effect of a novel DPP-4 inhibitor on the gut microbiota composition of metabolically dysfunctional mice. In addition, our objectives extended to directly compare these effects with those of metformin, in order to assess whether such alterations may be the result of metabolism correction, or rather if they are therapy specific. Finally, we examined the downstream effects of both therapeutics on several classes of potentially metabolically important host plasma metabolites.

Metformin remains a first-line therapeutic in the management of T2DM, acting in part through the suppression of hepatic gluconeogenesis and promotion of insulin sensitivity [32], while also contributing to a small degree of weight loss [33]. In line with this, MET mice in the present study displayed increased insulin sensitivity and improved cholesterol handling, with reduced weight

gain and mesenteric adiposity. The vildagliptin analogue PKF is a DPP-4 inhibitor and therefore confers its beneficial effects by extending the half-life of endogenous GLP-1. Consistent with this, PKF animals demonstrated improved cholesterol metabolism, a trend towards improved glucose handling and reduced mesenteric adipose tissue. These physiological changes are, for the most part, in line with the expected outcomes for each metabolic parameter [34-36], suggesting that the therapeutics functioned reasonably well in this animal model of metabolic dysfunction.

Already mentioned, the administration of metformin to both animal and man has been shown to alter the composition of the gut microbiota with potency [17], and is rivalled in the consistency of these effects only by antibiotic regimes. In the current study and animal model of metabolic dysfunction, metformin produced many of the same effects which have been demonstrated previously, thereby reiterating this consistency. The primary identified enteric member of the Verrucomicrobia phylum, *Akkermansia* is a genus which has received a great deal of attention for its association with metabolic health [37]. This effect appears to be due to metformin-mediated increases in goblet cells, which in turn secrete mucin, a key energy source of *Akkermansia* [38]. The Delzenne and Cani group of the Catholic University of Louvain have made inroads in elucidating the putative mechanisms through which *Akkermansia* may contribute to host health in rodent models [39], focusing primarily on the increased levels of inflammation-attenuating endocannabinoids and promotion of the gut barrier integrity observed in animals exposed to the live microbe [40]. In fact, the same group has conducted the first ever human clinical trial involving *Akkermansia* as an intervention in subjects with obesity and metabolic dysfunction (ClinicalTrials.gov ID: NCT02637115). The results of this small-scale phase I trial are eagerly awaited.

In addition to the increased relative abundance of *Akkermansia*, MET mice displayed increases in fibre-fermenting genera *Parabacteroides*, a taxon which has been repeatedly associated with metabolic health [41-43], as well as metformin therapy, independently of metabolic status [44]. A recent preclinical intervention study in which obese mice were gavaged with *Parabacteroides distasonis* demonstrated that the microbe generated succinate and secondary bile acids in the intestinal

lumen, thereby instigating intestinal gluconeogenesis and activating farnesoid X receptor, which in turn improved insulin sensitivity and produced a leaner phenotype [45]. Finally, the increase in *Christensenella* relative abundance following metformin intervention may be of particular interest, as this genera was previously associated with a lean BMI in a clinical study examining nearly 1000 individuals from twinsets [46]. In the same study, the transfer of the obese phenotype into mice through microbiota transplantation from an obese donor was attenuated by the addition of a member of the *Christensenella* genus. However, it is important to note that the metformin-mediated augmentation of *Christensenella* in the present study was, albeit interindividually consistent, quite modest and therefore may not be clinically relevant.

Curiously, metformin appeared to drastically reduce the gut microbiota α -diversity of obese mice, an effect which has been reported previously in rodents [47]. This result initially appears paradoxical in light of current knowledge, as microbiota diversity and gene richness have been found to associate tightly with metabolic fitness in human cohorts [5]. Although this is most likely the direct result of mucin production-mediated *Akkermansia* population inflation, it may be worth considering whether metformin inhibits the growth of certain microbial taxa, thereby facilitating the expansion of other metabolically beneficial micro-organisms. In fact, the antimicrobial attributes of metformin are now a topic of discussion having been demonstrated against several common pathogens *in vitro* [48, 49] and also in a clinical context against tuberculosis infection [50-52]. In addition, metformin therapy is often associated with adverse gastrointestinal tract symptoms, with up to a quarter of patients experiencing bloating, nausea or diarrhoea [53]. It may be of interest to stratify clinical patients on metformin monotherapy that are symptomatic and non-symptomatic of gastrointestinal symptoms and to examine the composition of the gut microbiota to assess whether this may be microbially-mediated.

In this rodent model of metabolic dysfunction, DPP-4 inhibitor intervention significantly increased the relative abundances of *Dorea*, *Ruminococcus* and *Bacteroides*, all of which are characterised as containing SCFA-producing species. While *Dorea* spp. have the propensity to

generate acetate [54], *Ruminococcus* spp. are known to produce acetate and butyrate, and *Bacteroides* spp. contribute to the intestinal levels of both acetate and propionate [55]. Indeed, the DPP-4 inhibitor sitagliptin has been shown to enhance the abundances of other SCFA-producing genera, namely *Roseburia* and *Blautia* [56]. If these bacterial abundance increases relate to augmented SCFA production, then this would likely have a significant impact on metabolic function in the host. For example, acetate is known to participate in cholesterol synthesis pathways and lipogenesis, while also modulating appetite [57, 58]. In addition, propionate has been demonstrated to impact upon hepatic gluconeogenesis and cholesterol metabolism [59]. Finally, butyrate acts as an energy source for colonic enterocytes and, in turn, contributes to tight junction integrity [60]. In line with all this, a recent study examining the effects of vildagliptin on the rodent innate immune system and glucose homeostasis revealed that the caecum of these animals contained substantially greater levels of propionate [61], suggesting that the compositional microbiota effects translate into functional attributes and may contribute to the therapeutic phenotype.

Metabolomics analysis of the mouse plasma revealed a mixed story in which the therapeutics produced substantially divergent profiles. Although both antidiabetic groups displayed reduced threonine levels -a free amino acid which has been shown to correlate with fasting plasma glucose in T2DM patients- this was the extent of their overlap with respect to amine entities [62]. Interestingly, glutamate was found to be depleted in the plasma of PKF mice and this metabolite was also found to be associated with degree of insulin resistance in the previously mentioned T2DM cohort [62]. In addition, metformin treated mice displayed reduced concentrations of methionine sulfoxide, a proposed putative marker of oxidative stress related in diseases such as diabetes [63].

Several sphingolipids were also found to be altered following antidiabetic therapy. While PKF mice demonstrated decreased levels of certain sphingomyelin species, MET appeared to have the counter effect. Intriguingly, while MET mice displayed increased plasma levels of several sphingomyelin entities, a previous PICRUST analysis (*i.e.* Phylogenetic Investigation of Communities by Reconstruction of Unobserved States - a bioinformatic tool which uses phylogenetic data to infer

functional capacity) of the metformin-induced rodent microbiota displayed an increase in the levels of microbes associated with sphingolipid metabolism pathways [47]. Abundances of such entities are often associated with insulin resistance [64] and have been found to antagonise the activity of adipocyte peroxisome proliferator-activated receptor- γ in obese women [65], thereby promoting the diabetic phenotype [66]. Indeed, this makes the increased abundance following metformin therapy a curious, but interesting result.

Significant attention has recently been given to microbial phosphatidylcholine metabolism due to the downstream cardiovascular effects of this pathway and its key metabolites trimethylamine-*N*-oxide. However, the levels of circulating phosphatidylcholine entities may also offer insight into the metabolic state of the subject. While little alteration in phosphatidylcholine metabolism was noted in MET mice, those treated with the DPP-4 inhibitor displayed reduced levels of multiple metabolites of this class. Importantly, many closely related metabolites have been proposed as early predictive biomarkers of T2DM progression in humans [67, 68], due to their association with insulin resistance. Finally, the reduced levels of two acylcarnitines in the MET mice may also be of biological importance. Increased levels of these entities have been reported in the plasma of individuals with insulin insensitivity, with the running hypothesis that fatty acid oxidation rate may be reacting more rapidly than the tricarboxylic acid cycle in obese and metabolically dysfunctional individuals [69, 70], leading to the accumulation of intermediates such as acylcarnitines [71, 72].

As previously eluded to, T2DM and its associated comorbidities already represent one of the largest contributors to morbidity and mortality in the current global population. Beyond the vastly augmented risk of heart disease associated with insulin resistance and the metabolic syndrome [73], patients and health services must persist with the downstream life-changing sequelae of the disease, such as distal neuropathies and retinopathies. With this in mind, it is of paramount importance that novel efficacious and tolerable therapeutics are developed to combat this life-threatening disease and the taxing economic ramifications which it poses. We must first gain a complete understanding of the mechanisms and effects of older and current antidiabetic formulations in order to develop such novel

therapeutic regimes. In line with this, exploration of the intestinal microbiota modifying effects may be of central importance. Finally, as diabetic management is often not straightforward, increasing numbers of patients are receiving multiple or combination oral therapeutics, such as a combined sitagliptin-metformin formulation. The interactive effects of such medications should be considered in future preclinical and clinical studies, in order to understand the ramifications to the gut microbiota.

Herein we have explored for the first time the microbiota and metabolome effects of a novel DPP-4 inhibitor, PKF-275-055, and compared them directly against the well-characterized biguanide, metformin. The data presented indicates that, while metformin had a greater overall effect on the composition of the intestinal microbiota, the subtler alterations observed in the PKF treated mice may indeed be of biological significance due to the metabolic capabilities of the taxa involved. Future directions should include the isolation and application of these potentially metabolically important microbes to models of metabolic disease. In addition, the microbiota of clinical patients on metformin monotherapy should be examined and stratified by their gastrointestinal symptoms in order to explore any potential role of this microbial ecosystem in the variability of this phenotype. Finally, we have identified specific plasma metabolites which may be of importance to the therapeutic course of these antidiabetic compounds, either as markers or disease modifying compounds, and explored the associations of these metabolites with altered taxa. In conclusion, the intestinal microbiota composition of metabolically dysfunctional mice is altered in a treatment-specific manner, which may point to a role of the gut microbiota in their mechanisms of action.

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FIGURE LEGEND**Figure 1 | Study Design, Body Profiling and Metabolic Assessment Following Antidiabetic**

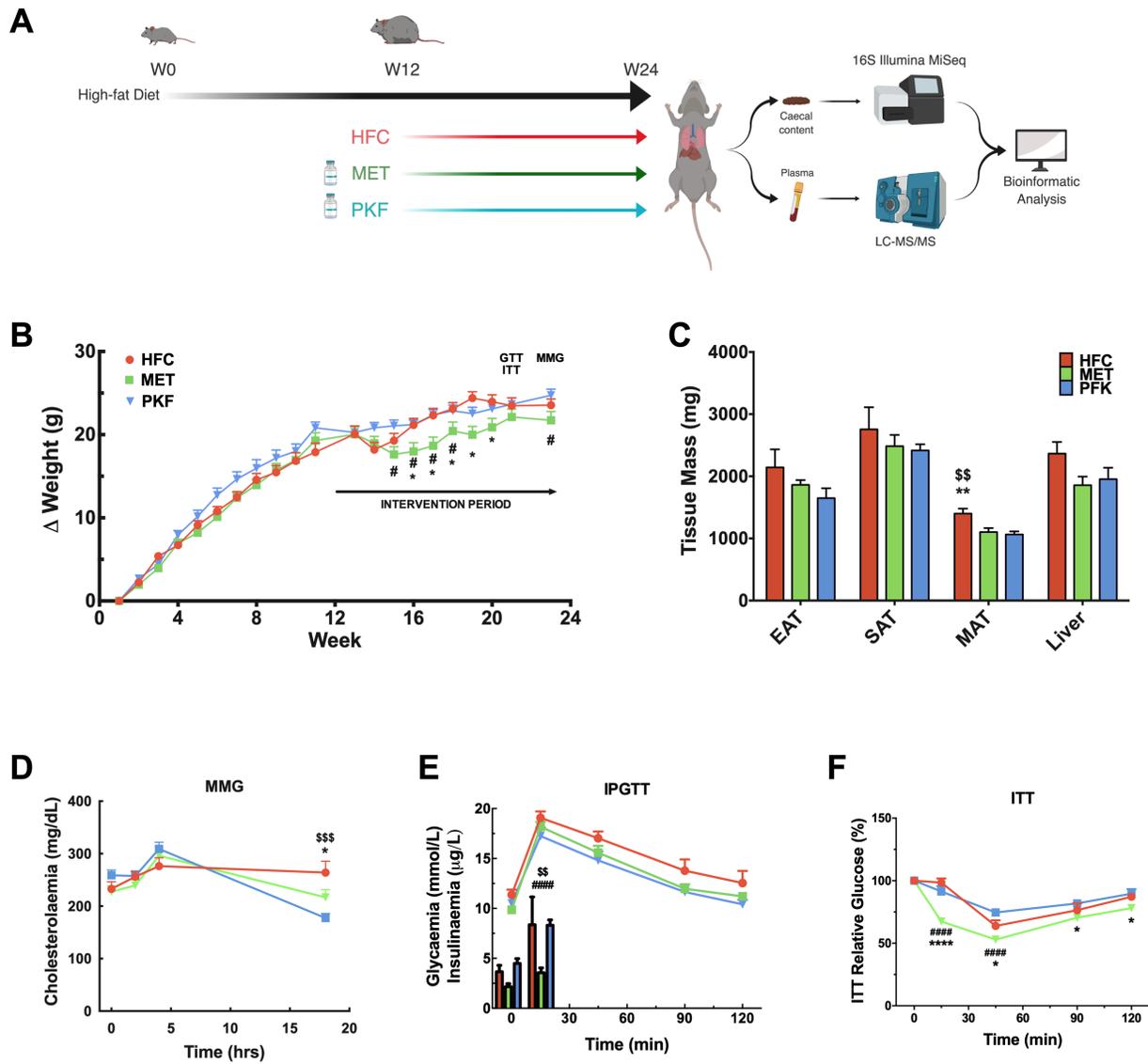
Therapy. (A) Mouse trial design and procedures detailed above, with high-fat diet represented by yellow bar and intervention by broken grey bar. (B) Delta change in body weights over the pre-feeding (white background) and intervention (red background) periods for HFC [red; $n = 13$], PFK [blue; $n = 14$] and MET [green; $n = 14$]. Tall dark red boxes represent metabolic test weeks (GTT/ITT and MMG, respectively). (C) Epididymal (EAT), subcutaneous (SAT) and mesenteric adipose tissue (MAT), as well as liver tissue weights are depicted. (D) Post-prandial serum cholesterol levels during the 18 hours following MMG. (E) Serum glucose (lines) and insulin (bars) levels during GTT. (F) Serum glucose levels during ITT. Data was analysed by one-way ANOVA with Bonferroni correction. Significant differences between groups are represented as follows: HFC-MET $^*(p < 0.05)$, $^{**}(p < 0.01)$, $^{***}(p < 0.001)$, $^{****}(p < 0.0001)$; HFC-PFK $^{\$}(p < 0.05)$, $^{\$\$}(p < 0.01)$, $^{\$\$\$}(p < 0.001)$, $^{\$\$\$\$}(p < 0.0001)$; MET-PFK $^{\#}(p < 0.05)$, $^{\#\#}(p < 0.01)$, $^{\#\#\#}(p < 0.001)$, $^{\#\#\#\#}(p < 0.0001)$. Plots depict replicates with mean and SEM.

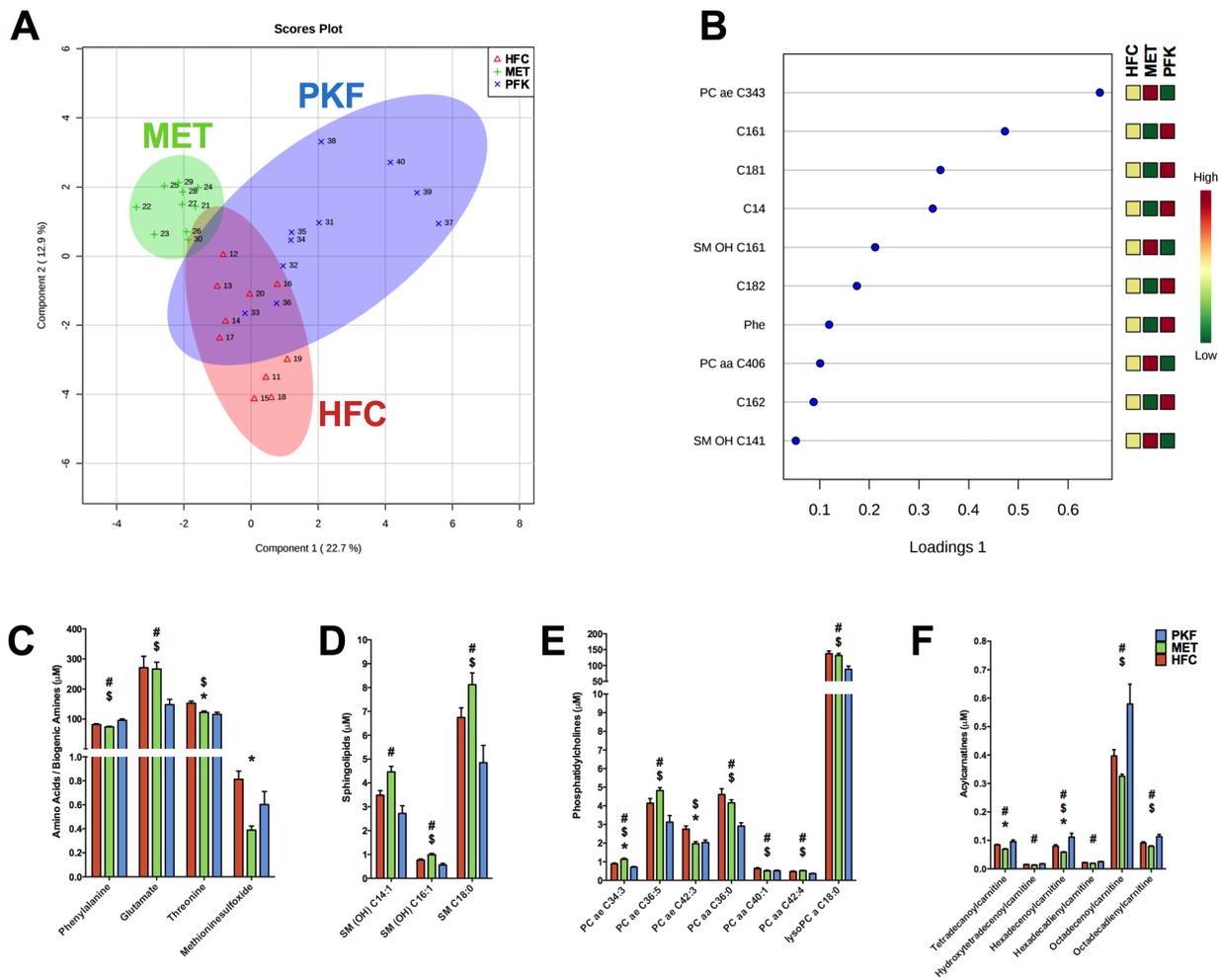
Figure 2 | Plasma Metabolomics Reveals Contrasting Effects of Metformin and DPP-4 Inhibitor

Therapy. (A) Partial least squares discriminant analysis plot displays PFK [blue], MET [green] and HFC [red] samples. (B) Plot loadings graph demonstrating the variables of importance to the partial least squares discriminant analysis and the group with which each variable is positively (green box) or negatively (red box) related. Quantitative data is displayed for the metabolites which were significantly altered by PFK or MET; these includes amino acids and biogenic amines (C), sphingolipids (D), diacyl-phosphatidylcholine (PC aa) and acyl-alkyl-phosphatidylcholine (PC ae; E) and acylcarnitines (F). Data was analysed by one-way ANOVA and a false discovery rate was applied. Significant differences between groups are represented as follows: HFC-MET $^*(q < 0.05)$, $^{**}(q < 0.01)$, $^{***}(q < 0.001)$, $^{****}(q < 0.0001)$; HFC-PFK $^{\$}(q < 0.05)$, $^{\$\$}(q < 0.01)$, $^{\$\$\$}(q < 0.001)$, $^{\$\$\$\$}(q < 0.0001)$; MET-PFK $^{\#}(q < 0.05)$, $^{\#\#}(q < 0.01)$, $^{\#\#\#}(q < 0.001)$, $^{\#\#\#\#}(q < 0.0001)$. Plots depict replicates with mean and SEM.

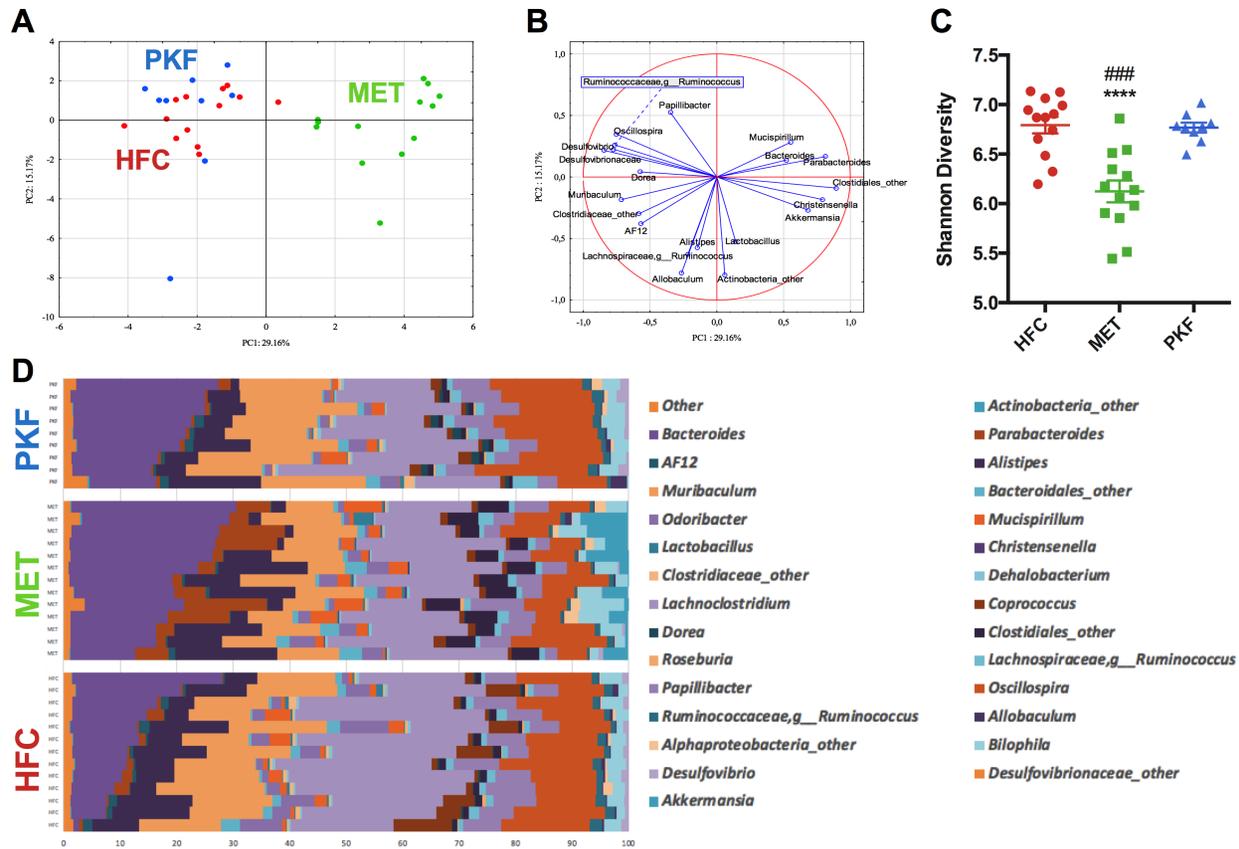
Figure 3 | Gut Microbiota Composition is Substantially Altered by Metformin and, to a Lesser Extent, DPP-4 Inhibitor Therapy. (A) Principle coordinate analysis (PCoA) of compositional sequencing data. (B) Scatterplot associated with PCoA plot. (C) Shannon diversity index of microbiota sequencing data. (D) Genera relative abundances of each sample displayed in horizontal and divided into PFK [blue], MET [green] and HFC [red]. Data was analysed by one-way ANOVA with a Bonferroni correction. Significant differences between groups are represented as follows: HFC-MET ****($p < 0.0001$); MET-PFK ####($p < 0.001$). Plots depict replicates with mean and SEM.

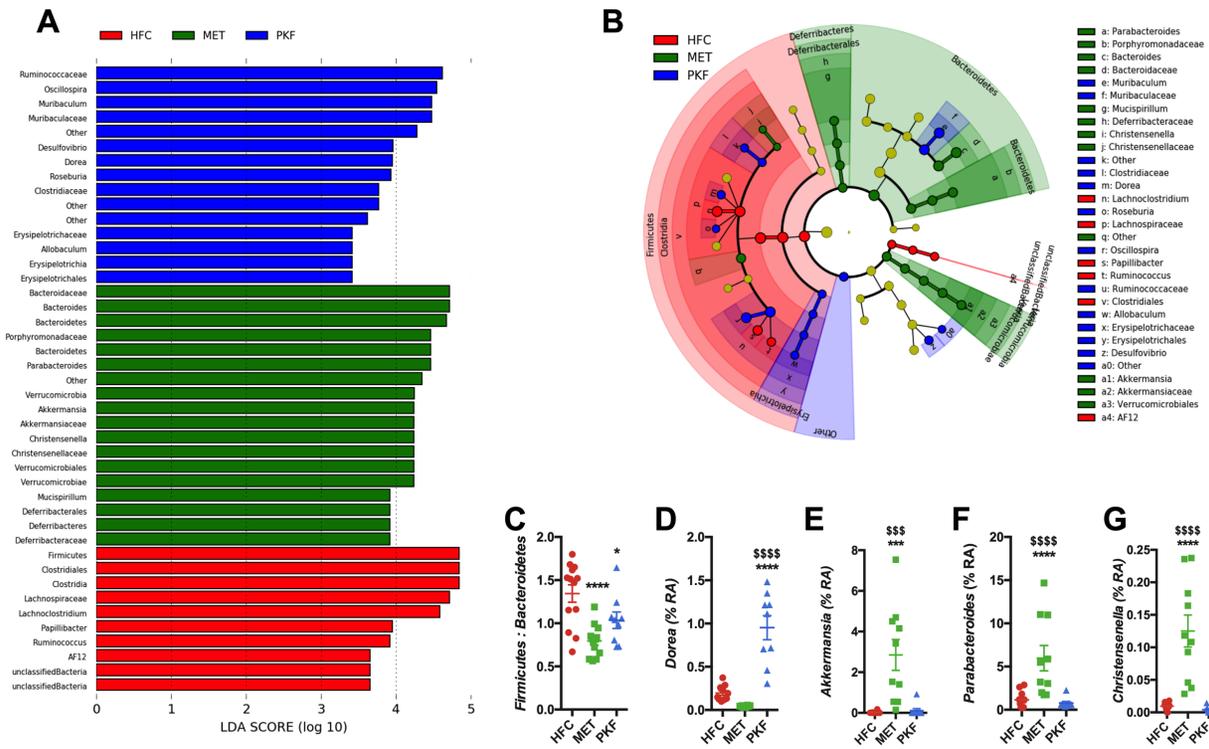
Figure 4 | Both Metformin and DPP-4 Inhibitor Therapy Associate with Unique Taxonomic Markers and Enhance the Relative Abundances of Potentially Metabolically Important Microbes. Linear discriminant analysis (LDA) effect size (LEfSe) (A) barchat and (B) cladogram representation of taxa associated with HFC and antidiabetic therapies. (C) Ratio of relative abundances of Firmicutes to Bacteroidetes. (D) Relative abundances of the genus *Dorea*. (E) Relative abundances of the genus *Akkermansia*. (F) Relative abundances of the genus *Parabacteroides*. (G) Relative abundances of the genus *Christensenella*. PFK [blue], MET [green] and HFC [red]. Significant differences between groups are represented as follows: HFC-MET *($q < 0.05$), **($q < 0.01$), ***($q < 0.001$), ****($q < 0.0001$); HFC-PFK $^{\$}$ ($q < 0.05$), $^{\$\$}$ ($q < 0.01$), $^{\$ \$ \$}$ ($q < 0.001$), $^{\$ \$ \$ \$}$ ($q < 0.0001$); MET-PFK $^{\#}$ ($q < 0.05$), $^{\#\#}$ ($q < 0.01$), $^{\#\#\#}$ ($q < 0.001$), $^{\#\#\#\#}$ ($q < 0.0001$). Plots depict replicates with mean and SEM.





ACCEPTED





SUPPLEMENTARY DATA

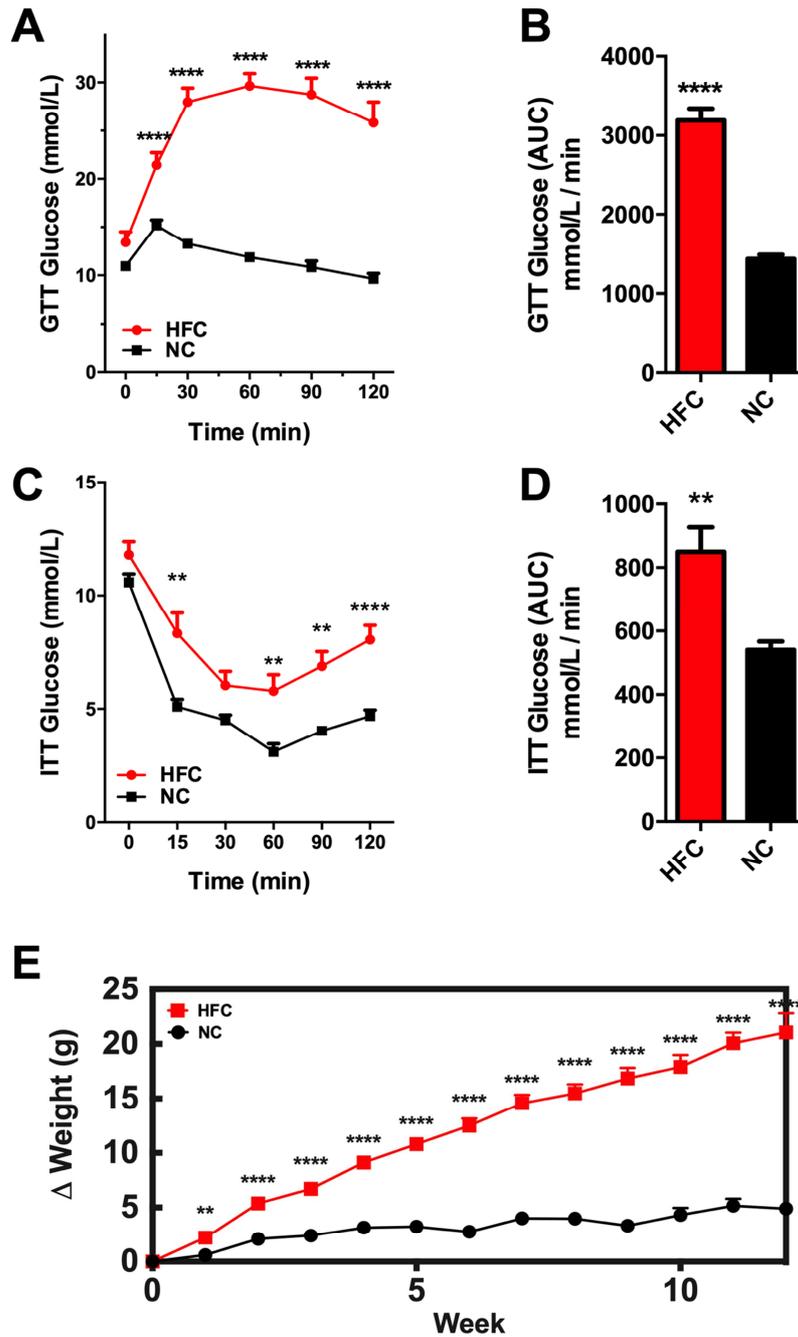


Figure S1. Glucose and Insulin Tolerance Tests for Confirmation of Metabolic Dysfunction in Diet Induced Obesity Model. At week 12, prior to commencement of antidiabetic interventions, high-fat diet fed animals (HFC) were assessed against animals receiving only normal chow (NC) for their glucose handling by intraperitoneal glucose tolerance test [A/B] and by insulin tolerance test [C/D]. In addition, mouse weight was monitored over the initial 12 weeks to demonstrate and validate the diet-induced obesity forecasted in the HFC animals [E].

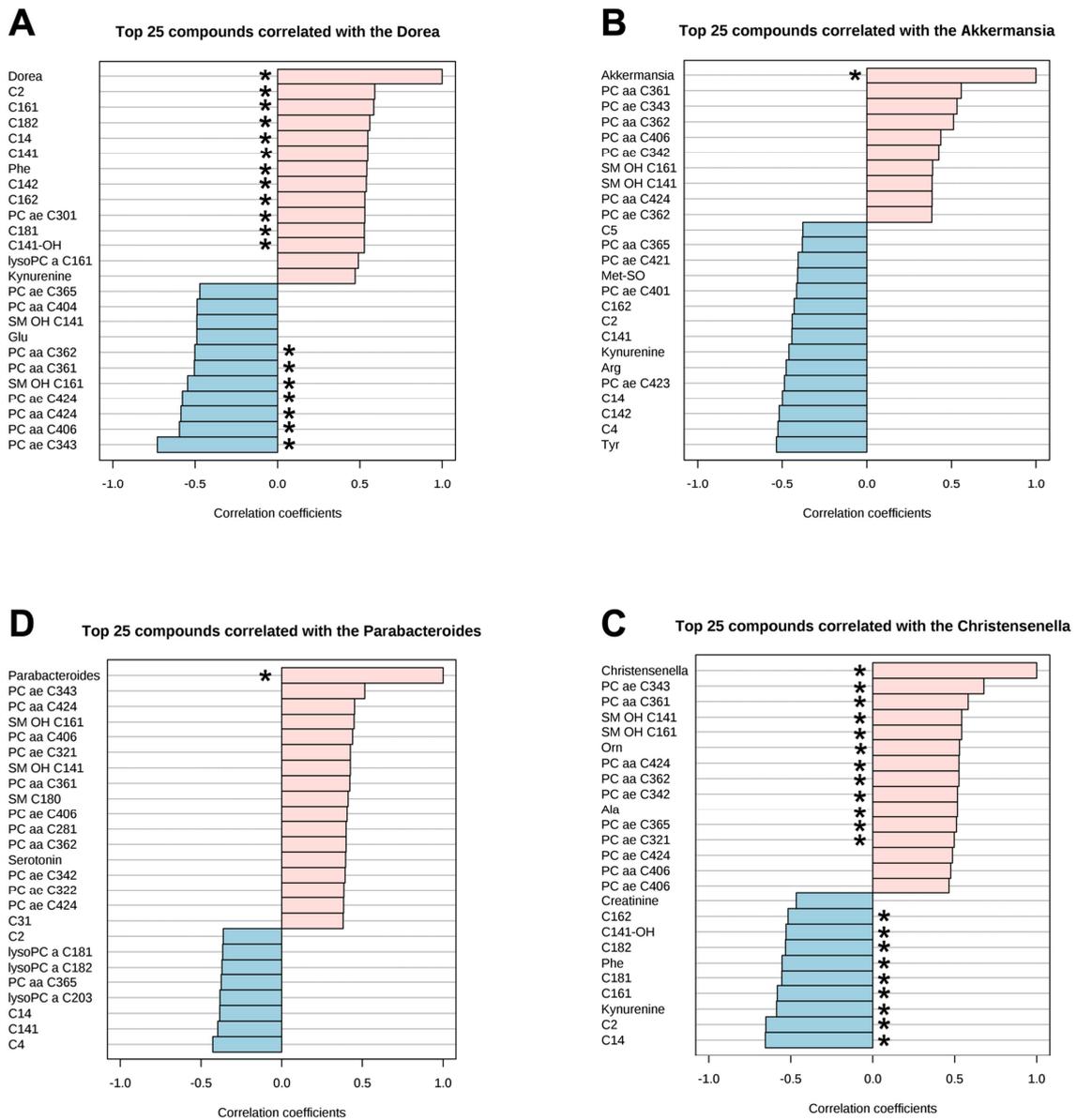


Figure S2. Correlation Analysis Between Key Taxonomic Alterations and Plasma Metabolomics. Pearson correlation analysis was carried out between key taxonomic alterations highlighted in Figure 4 and plasma metabolomics data for each animal. The above plots depict the top 25 compounds correlated with [A] *Dorea*, [B] *Akkermansia*, [C] *Parabacteroides* and [D] *Christensenella* genera relative abundances, with the correlation co-efficient displayed on the x-axis. Statistically significant interactions are annotated with *, which indicates an FDR q value of < 0.05.