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Fermented beetroot modulates gut microbial carbohydrate metabolism in prediabetes and prevents high-fat diet induced hyperglycemia in a prediabetic model

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ABSTRACT

The global increase in prevalence of (pre-)diabetes demands immediate intervention strategies. In our earlier work, we demonstrated *in vitro* antidiabetic potential of a fermented beetroot product (PN39). Here, we examined the impact of PN39 on glucose tolerance and gut microbiota in C57BL/6J male mice and on prediabetic (PD) subjects' stool microbiota. In mice, high-fat diet (HFD) consumption for 9 weeks resulted in hyperglycemia and impaired glucose tolerance (GT) while concomitant consumption of PN39 and HFD (PN39+HFD) prevented GT impairment. Meanwhile, feeding the mice with HFD for 5 weeks to induce PD and later administering them with PN39 for 4 weeks (PD + PN39) neither improved fasting blood glucose nor GT. Relative to control groups, the gut microbiota of both PD mice and humans were characterized by decreased *Clostridia UCG-014* and Lactobacilli as well as significantly altered gut microbial carbohydrate metabolism. Feeding PN39 together with HFD preserved *Clostridia UCG-014* and Lactobacilli, increased short chain fatty acid production relative to mice fed with HFD only. Treating gut microbiota of PD subjects with PN39 however increased *Clostridia UCG-014* and Lactobacilli populations and increased short chain fatty acids concentrations in the stools. In both mice and humans, PN39 treatment rectified the altered microbial carbohydrate metabolism observed in their PD counterparts. This suggests that the gut microbial modulatory effects of PN39 coupled with its capacity to regulate gut microbial glucose metabolism, likely played a role in preventing PD in mice receiving PN39+HFD. Taken together, our results indicate that PN39 could act as a potent antidiabetic functional food for preventing diabetes and its associated dysbiosis.

1. Introduction

Prediabetes indicates a stage of dysglycemia between normoglycemia and diabetes (American Diabetes Association, 2020) and is characteristic of individuals who are at risk of future diabetes. The World Health Organization defines prediabetes as impaired glucose tolerance characterized by a 2-h plasma glucose concentration between 7.8 and 11.0 mmol/L during an oral glucose tolerance test or an impaired fasting plasma glucose concentration between 6.1 and 6.9 mmol/L (World Health Organization, 2006). The global prevalence of prediabetes among individuals aged 19–99 years is about 8 % (Echouffo-Tcheugui and Selvin, 2021) and still growing. This underscores the need for prediabetes surveillance for effective diabetes prevention policy implementation. It is estimated that up to 5 % of prediabetics develop type 2

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diabetes mellitus (T2DM) annually (Gerstein et al., 2007), while up to 70 % of them will develop T2DM at some point in their lifetime (Tabák et al., 2012). Risk factors of prediabetes include genetics (Zeng et al., 2023), increased Western diet consumption (Demaria et al., 2023), inactive lifestyle, sleep deprivation (Wang et al., 2023), and high levels of stress (Ingrosso et al., 2023). Prediabetes is strongly linked to serious pathologies such as decreased heart-rate change (Gerritsen et al., 2000; Wu et al., 2007), erectile dysfunction (Tucker et al., 2023), retinopathy (Sune et al., 2023) and macrovascular disease (Raffaele et al., 2020). Although the current strategy for managing prediabetes is lifestyle modification (exercise, weight loss and dietary modification), this approach may be inadequate owing partly to poor understanding of the pathophysiology of the condition (Wu et al., 2020).

Recent studies have shown the potential of the gut microbiota to affect host glucose metabolism and hence alterations in the gut microbial structure and function may result in hyperglycemia (Takeuchi et al., 2023; Zhou et al., 2019). In fact, it is known that gut microbial dipeptidyl peptidase 4 (DPP4), just like host DPP4 (an important target for T2DM management), can hydrolyze host's glucagon-like peptide-1 to compromise host glucose homeostasis. Thus, inhibiting gut microbial DPP4 activity can improve host glucose metabolism drastically (Wang et al., 2023). Studies indicate that certain gut bacteria help regulate host blood sugar levels by breaking down fecal carbohydrates and so a decrease in these bacteria may contribute to prediabetes (Takeuchi et al., 2023; Wu et al., 2020). More so, short chain fatty acids (SCFAs) producing bacteria are severely diminished during prediabetes (Karlsson et al., 2013). Modulation of the gut microbiota can result in a significant microbial functional shift to increase SCFAs production (Forslund et al., 2015) which stimulates insulin secretion (Pingitore et al., 2017).

Given that the gut microbiota represents an essential modifiable factor worth considering for preventing and/or managing T2DM, several antidiabetic functional foods have been developed (Ofosu et al., 2023; Tong et al., 2024). Among them, fermented foods such as fermented beetroot have attracted attention due to their beneficial health effects and gut microbiota modulatory potentials (Daliri et al., 2023) as well as being sources of novel probiotics (Megur et al., 2023). Yet, while some functional foods have shown promise to prevent progression from prediabetes to T2DM, their mechanisms and impact on the gut microbiota are poorly studied (Ofosu et al., 2023). For this reason, this study was intended to investigate the ability of an antidiabetic beetroot product we developed earlier (here-in referred to as PN39) (Daliri et al., 2023) to ameliorate prediabetes in C57BL/6J male mice and modulate their gut microbiota structure and physiology. In our previous study, PN39 demonstrated strong antioxidant potential and inhibited both carbohydrate digestion and DPP4 activity *in vitro*. Meanwhile, the composition of mice and human microbiotas are distinct which limits the translation of microbiota research findings between these species (Beresford-Jones et al., 2022). Thus, we also examined the impact of PN39 on prediabetic human gut microbiota samples using an *in vitro* pH-controlled colonic model to better understand the perturbations of the microbiota metabolome interface in prediabetes and identify possible potential therapeutic targets.

2. Materials and methods

2.1. Animals

For this study, 40 male C57BL/6J mice of 4-month-old (Janvier Laboratories, France) were used. Permission was granted by the Lithuanian State Food and Veterinary Service to perform the experiment (No. B1-866), and the husbandry and experiments complied with the requirements of Directive (2010)/63/EU. The animals were kept under controlled conditions (22 °C ± 1, 40 % humidity, 12-h light/dark cycle (lights off at 20:00 h), food and water supply *ad libidum*) under veterinary supervision.

2.2. Diet and animal feeding

The animals were divided into 4 groups (n = 10 per group) and were fed with different diet for 9 weeks. The groups were as follows: High-fat diet (HFD); control diet (healthy mice); PN39+High-fat diet (PN39+HFD); High-fat diet feeding for 5 weeks + PN39 for 4 weeks (HFD + PN39). The control diet (Altromin C 1090–10) was composed of 10 % energy from fat while the HFD (Altromin C 1090–60) consisted of 60 % energy from fat (Altromin, Soest, Germany). PN39 was prepared by inoculating blended red beetroot with 8 LogCfu/mL of *Latilactobacillus curvatus* PN39 for 72 h at 45 °C (Daliri et al., 2023). The fermented beetroot drink (PN39), containing 20 % (v/v) fermented beetroot juice, was freshly prepared and placed in housing bottles that were changed twice per week.

2.3. Animal weight and glucose tolerance test

The animals were weighed twice a week. At the end of the final time point, a glucose tolerance test was performed to check insulin resistance as previously described (Burokas et al., 2018). During the glucose tolerance test, glucose was measured at different time points up to 2 h. The animals were not fed for 12 h before the experiment but were provided with water. The dose of 2 g/kg glucose was injected intraperitoneally and 10 μL of blood was collected from the snipped tail to measure glucose levels using a CONTOUR®NEXT GEN glucose meter (Ascensia Diabetes Care, NJ, USA). The animals were culled by cervical dislocation after the study. Cecal contents from each mice group (n = 10) were pooled together to obtain 3 samples per group and used for microbiota and short chain fatty acids analysis.

2.4. Human stool collection and in-vitro-gut fermentation

Informed consent was provided by each volunteer prior to their participation in the study. The study was conducted according to the guidelines of the Declaration of Helsinki, and the University of Roehampton (London, UK) Research Ethics Committee (LS 22/374) approved all procedures involving human subjects. Six fecal samples were obtained from three prediabetic subjects (Hb1ac > 6.0; age = 59.0 ± 6.4 years; 2 females) and three nondiabetic subjects (Hb1ac < 6.0; age = 38.7 ± 4.9 years, 2 male). None of the donors had received antibiotics or probiotics for at least 3 months before sampling, nor steroids or other drugs with a proven impact on gut microbiota composition over the preceding 3 months. Fecal samples were collected by participants using stool collection bags (Longniddry, UK) and DNase/RNase-free tubes. Specimens were stored at −20 °C immediately after sampling and transported in freezer bags to the University of Roehampton, for storage at −80 °C. Fecal samples from each individual in each group (prediabetic and non-diabetic donors) were pooled together to allow for intra-individual consistency regarding relative abundance of the main phyla including specific genera associated with clinical benefits. Fecal samples were defrosted and diluted (1:10 *w/v*) in phosphate-buffered saline (0.1 mol/L phosphate-buffered solution, pH 7.4) and homogenized (Stomacher 400, Seward, UK) for 2 min at 240 paddle beats per min. *In vitro* colonic fermentation was performed in an anaerobic pH-controlled batch culture system following a previously reported method (Obayiuwana et al., 2023) to assess the impact of PN39 on fecal microbiota. Inulin (Sigma-Aldrich Sp, Pozna'n, Poland) was used as control and samples were collected for SCFA and metagenomics analysis after 0, 4, 8 and 24 h of fermentation.

2.5. DNA extraction and 16S rRNA sequencing

The microbial genomic DNA of fecal and cecum samples (*ca.* 200 mg) was extracted using QIAamp PowerFecal Pro DNA Kit which included the stages of DNA isolation and purification following the manufacturer's instruction. The purity and quantity of total genomic DNA were

monitored via NanoQuant plate (Tecan Spark 10 M plate reader) and fragment analyzer system (5400 Agilent). Depending on individual concentration (300–560 ng μL^{-1}), the DNA was diluted to 1 ng μL^{-1} for further analytical procedures. Next generation sequencing was carried out by Macrogen Europe (Amsterdam, The Netherlands). Briefly, DNA that qualified quality control measures were used for library construction. The DNA samples were randomly fragmented and ligated by a 5′ and 3′ adapter before being used for the library construction. The fragments were amplified by polymerase chain reaction followed by purification by gel electrophoresis. After the fragments were captured on a lawn of surface-bound oligos complementary to the library adapters, they were amplified into unique clonal clusters through bridge amplification. Illumina SBS technology was used to generate base-by-base sequencing. The base calls binary was converted into FASTQ utilizing the Illumina package bcl2fastq.

2.6. Sample processing for SCFA analysis

Cold isopropanol (1 mL) was added to a vial containing the stool or cecal content and homogenized in a bead beater (Precellys, Bertin Technologies, Montigny Le Bretonneux, France) applying three cycles at 6000 rpm for 30 s per cycle. The homogenized sample in solvent (approximately 700 μL) was centrifuged at 16,000 g for 15 min at 4 °C (Eppendorf 5430 R Centrifuge, Hamburg Germany). For SCFAs analysis, we employed a procedure modified from Valdivia-Garcia et al. (2022). Samples (20 μL) were mixed with 20 μL of an internal standard solution (10 mM $^{13}C_2$ acetic, 10 mM $^{13}C_1$ propionic and 10 mM $^{13}C_2$ butyric acid) and 20 μL LC-MS grade water. Derivatization was achieved by addition of 20 μL of 200 mM 3-nitrophenylhydrazine hydrochloride (3-NPH; Sigma Aldrich, Steinheim, Germany; prepared in 50 % v/v acetonitrile in water) and 20 μL of 120 mM N-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) prepared in 6 % (v/v) pyridine in ethanol solution (Sigma Aldrich, Steinheim, Germany). The plate was covered with an aluminum mat and agitated at 1400 rpm for 10 s before heating at 40 °C for 30 min. The derivatization reaction was quenched by adding 150 μL 0.1 % formic acid solution in LC-MS grade water, again agitating for 10 s at 1400 rpm. A total of 100 μL of the supernatant was transferred to a high-recovery LC-MS plate for UHPLC-MS/MS analysis.

2.7. Ultra-high-performance liquid chromatography with tandem mass spectrometry

Chromatographic separation was achieved on a Waters Acquity UHPLC system using a binary gradient on a Waters CSH C18 Column (100 mm × 2.1 mm, 1.7 μm particle size, 130 Å pore size). Mobile phase A consisted of 0.1 % formic acid solution in LC-MS grade water, mobile phase B consisted of 0.1 % formic acid in LC-MS grade acetonitrile. The gradient started with a flow rate of 0.35 mL/min at 20 % B for 2 min, followed by a linear increase to 55 % at 8 min, a switch to 99 % B at 8.1 min, held for 1.4 min, followed by re-equilibration at 20 % from 9.5 min to 10.5 min. The flow rate increased to 0.4 mL/min at 8.1 min and returned to the initial rate after 1 min. Column temperature was kept constant at 40 °C. SCFAs detection was performed on a Waters Xevo TQ-Sμ in negative electrospray ionisation (ESI) mode with multiple reaction monitoring (MRM) transitions taken from Valdivia-Garcia et al. (2022). The MS capillary voltage was set at 2.4 kV; cone temperature, 150 °C; desolvation gas flow, 550 L/h; and desolvation temperature, 350 °C. Data were exported using MSConvertGUI (Chamata et al., 2020) and analyzed using an in-house workflow we developed earlier (Behrends et al., 2011).

2.8. Data statistical analysis

For analysis of annotated and quantified SCFA concentrations, GraphPad Prim 9.0 (GraphPad Prism version 9.0.0 for Windows, GraphPad Software, California, USA) was used for analysis of means (2 sample t-tests) and Wilcoxon tests performed with 95 % confidence limits. Linear regression equations from standard calibration curves, r-square, accuracy and precision values with coefficient of variation percentages (relative standard deviation) and standard errors of the mean were calculated in Excel 365 (Microsoft 365, Microsoft Corporation, Version 16.0). For data normal distribution and homogeneous variance, ANOVA followed by Tukey comparison test was used to determine differences between groups. Repeated measures of two-way ANOVA was applied for assessing differences in body weight and in glucose tolerance test results. Differences were considered significant for $p < 0.05$. p values were consistently corrected for multiple comparisons using Bonferroni correction and correlations plots were adjusted for False discovery rate using Benjamini-Hochberg. GraphPad Prism was used for ANOVA statistical analysis (version 9.3.1; USA). Spearman correlation matrix analysis was performed using JASP 0.17.2.1 software (Amsterdam, Netherlands). Based on the relative abundance of each genus, MicrobiomeAnalyst 2.0 (Lu et al., 2023) was used to generate principal coordinate analysis (PCoA) plots for beta diversity analysis as well as to calculate observed OTUs, Abundance-based Coverage Estimator (ACE), Simpson's and Shannon's alpha diversity in the stool samples from each group. Linear discriminant analysis Effect Size (LEfSe) plots were generated using the method described by earlier (Segata et al., 2011). KEGG pathways were generated by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) and statistically significant differences between the microbial profiles based on Welch's *t*-test was assessed using the Statistical analysis of taxonomic and functional profiles (STAMP) software (Parks et al., 2014).

3. Results

3.1. Effects of HFD and PN39 consumption on body weight, dysglycemia and blood glucose tolerance

Relative to healthy mice, feeding mice with HFD increased weight gain with or without PN39 consumption (Fig. 1A). The HFD group had significant increase in blood glucose levels ($p = 0.001$), and PD + PN39 mice did not reduce blood glucose levels (HFD vs PD + PN39, $p = 0.09$). Also, both HFD and PD + PN39 mice showed significant impairment in glucose tolerance (Fig. 1C). However, PN39+HFD mice neither demonstrated a spike in blood glucose levels ($p = 0.001$) nor an altered glucose tolerance when compared with the healthy group. No significant change was observed in the serum insulin levels of all the experimental groups (Fig. 1C) though HFD consumption increased the homeostasis model assessment of insulin resistance (HOMA-IR) index.

3.2. Effects of HFD and PN39 consumption on gut microbial ecology and function

Gut microbial community richness measured by observed OTU and ACE indices indicated significantly reduced richness ($p = 0.031$) in the HFD group relative to the healthy group. Meanwhile, the microbial richness of PN39+HFD and PD + PN39 were not different ($p > 0.05$) from the healthy control group. Simpson's and Shannon's diversity indices showed no significant differences in the microbial community diversity among the mice groups (Fig. 2).

3.3. Beta-diversity was examined by a principal coordinate analysis (PCoA) performed on the unweighed UniFrac distances computed among samples to compare the composition of microbiota (Fig. 3)

The gut microbiota of HFD fed mice clearly separated on PC2 explaining 14.7 % of the variation, and variation obtained for the PC1 was 53.8 %. PN39 treated mice seem to separate from both healthy mice and HFD fed control mice on PC1 accounting for the highest variation. PERMANOVA analysis of the 16S dataset showed that there was a

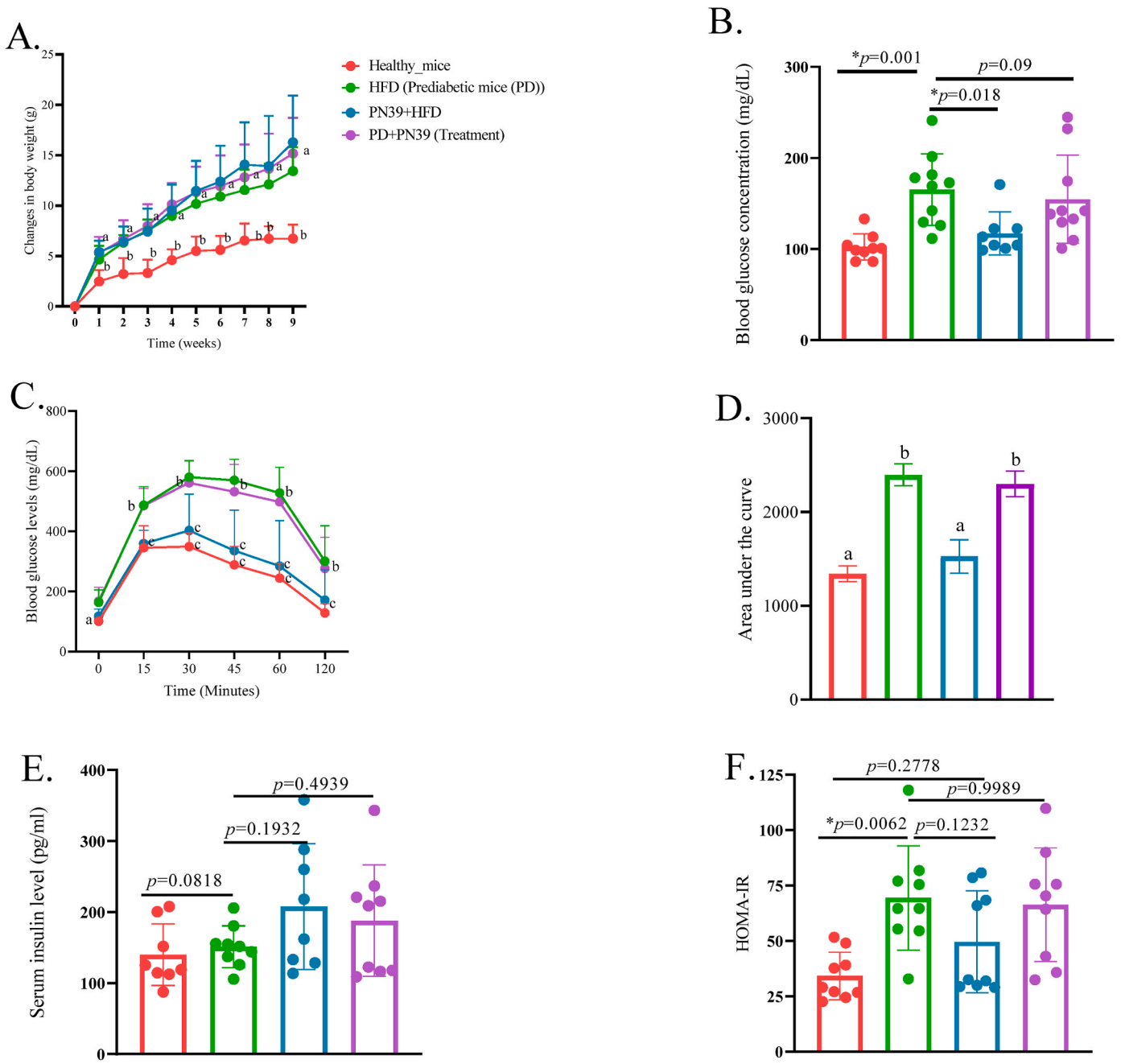

Fig. 1. Effects of PN39 consumption on A. body weight, B. fasting blood glucose on week nine after mice were fasted for 12 h, C. Intraperitoneal Glucose Tolerance Test measured for 120 min on the 9th week, D. area under the curve for blood glucose tolerance test on the 9th week, E. serum insulin levels on the 9th week of study, F. HOMA-IR (Homeostatic Model Assessment for Insulin Resistance) determined after the 9th week of study. Columns or time points with different alphabets (a, b) are significantly different (one-way ANOVA, Tukey's multiple comparisons tests, * = $p < 0.05$). N = 8–10 mice and each data point in A. and C. represents mean ± SEM.

significant effect of HFD and PN39 on the gut microbiota (Table 1).

The microbiota in the mice groups were further compared using the LEfSe method to perform high-dimensional class comparisons with a particular focus on metataxonomic analysis. LEfSe provides biological class explanations to establish statistical significance, biological consistency and effect size estimation of predicted biomarkers. A total of 14 taxa showed significantly higher relative abundances in healthy mice compared to the PD group, with an LDA score higher than 2.0. Relative to PD mice gut microbiota, *Lactobacillus* and *Clostridia UCG_014* genera were significantly more abundant in PN39+HFD group, while PD + PN39 mice were enriched in *Clostridia UCG_014* and *Muribaculaceae* genera. Microbial functions predicted by PICRUSt (based on marker gene sequences) KEGG pathways indicated a significant elevation in carbohydrate, energy, nucleotide and xenobiotic metabolism but a reduced lipid metabolism in PD gut microbiota relative to healthy mice. Meanwhile, for PN39+HFD and PD + PN39 groups, significantly reduced carbohydrate and nucleotide metabolism and increased lipid metabolism were predicted (Fig. 4).

3.4. Effects of HFD and PN39 consumption on gut microbial SCFAs and glucose tolerance

Compared to control mice, HFD feeding decreased the levels of all the measured SCFAs except valerate (Fig. 5A). Cecal content of

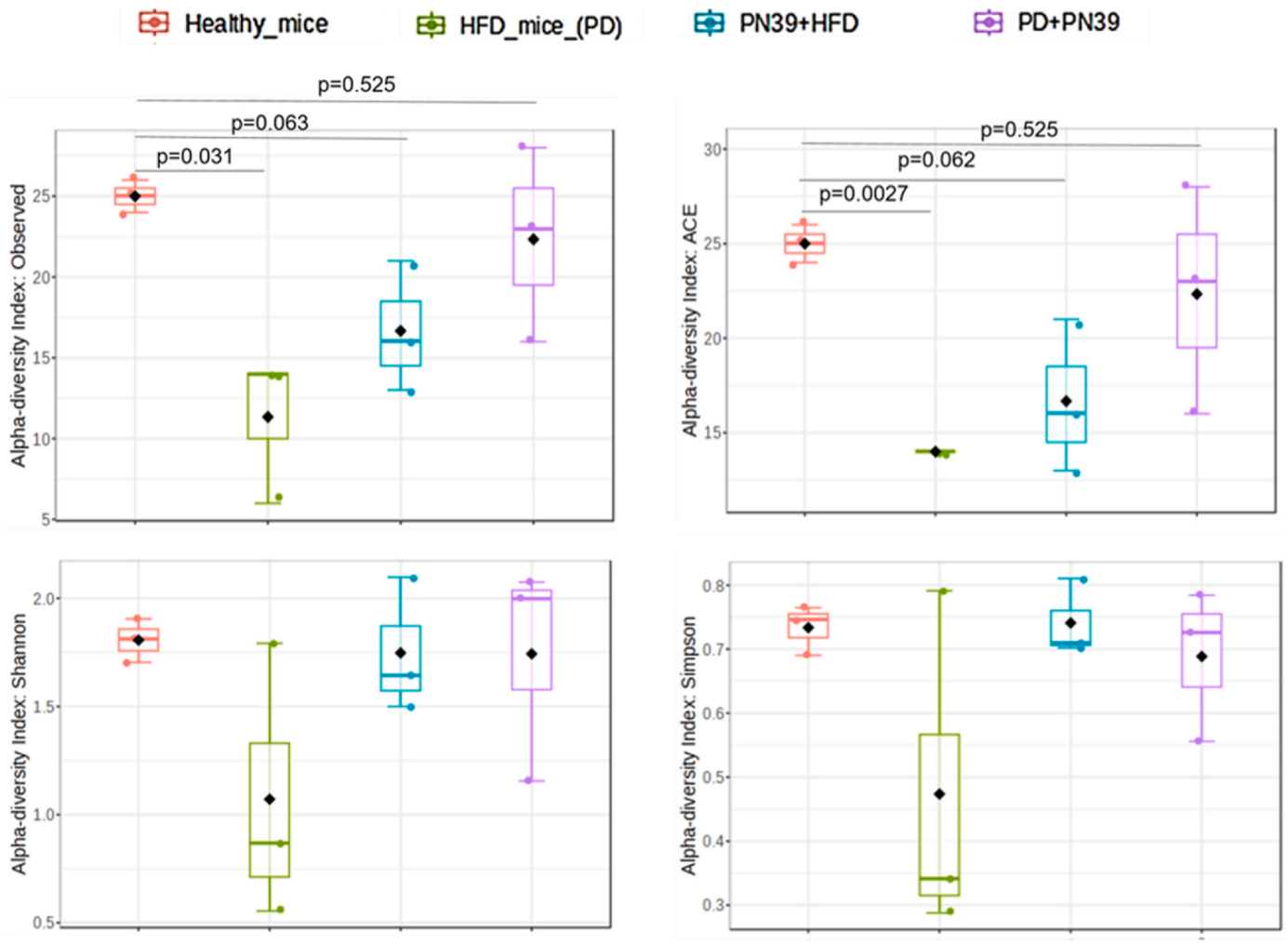

Fig. 2. Measures of alpha diversity for healthy, HFD (prediabetic/PD), PN39+ HFD and PD + PN39 mice. The number of observed OTUs, ACE index, Shannon index and Simpson's index were used to measure/analyze the alpha diversity of the mice cecal microbiota $p < 0.05$ indicates statistical significance using ANOVA. All these indices (ACE, Shannon, Simpson) are calculated based on the OTU data (equivalent to "genera detected" in ecology) not on taxa data. $N = 10$ mice randomly pooled into 3 groups.

PN39+HFD mice showed higher levels of butyrate, propionate, lactate and valerate, compared to HFD. Meanwhile, only cecal caproate was higher ($p = 0.041$) in PD + PN39 mice relative to HFD. Spearman's correlation showed that *Lactobacillus* was significantly and positively correlated with lactate levels but negatively correlated with blood glucose levels (Fig. 5A). Conversely, *Clostridia UCG_014* was significantly and positively correlated with propionate, butyrate, valerate and caproate (Fig. 5B).

3.5. Effects of PN39 on prediabetic human gut microbiota composition and physiology

Based on the top 20 most abundant bacteria in fecal samples, prediabetic subjects were characterized by significantly higher relative abundances of *Stenotrophomonas* and *Escherichia–Shigella* compared to non-diabetic subjects. Fermenting PD fecal samples with inulin for 24 h did not restore *Lactobacillus* levels to those observed in non-diabetic controls but significantly decreased levels of *Stenotrophomonas* genus. However, culture of PD stools with PN39 for 24 h restored the levels of lactobacilli and reduced the abundance of *Stenotrophomonas* and *Escherichia–Shigella* (Fig. 6). Microbial functional prediction indicated a significant alteration in carbohydrate, amino acid and energy metabolism but reduced lipid metabolism in PD gut microbiota relative to non-diabetic subjects. Meanwhile, culturing PD fecal microbiota with PN39 rectified microbial carbohydrate metabolism while inulin rectified both carbohydrate and lipid metabolism (Fig. 6B).

3.6. Effects of PN39 on prediabetic human gut microbiota, SCFAs production and lactate

Among the samples tested, stool of prediabetic subjects contained the lowest amounts of SCFAs. Co-incubation of PD stools with PN39 increased the production of all the measured SCFAs except isobutyrate. Similarly, PD stool fermentation with inulin increased the production of all the SCFAs measured in the study (Fig. 7).

4. Discussion and conclusion

It is well established that consumption of Western diet (*high-fat, high-fructose, and high-cholesterol*) can cause insulin impairment and that dietary modification can mitigate the condition (Hsu et al., 2014). Indeed, functional foods are rich in bioactive compounds and their consumption have been shown to prevent, treat and/manage such metabolic diseases while modulating the gut microbiota (Ofosu et al., 2023; Tong et al., 2024). Therefore, in our quest to examine the ability of PN39 to prevent prediabetes, we studied the effect of concomitant consumption of PN39 and HFD on glucose tolerance in C57BL/6J mice. Our results showed that nine weeks concomitant consumption of HFD and PN39 did not

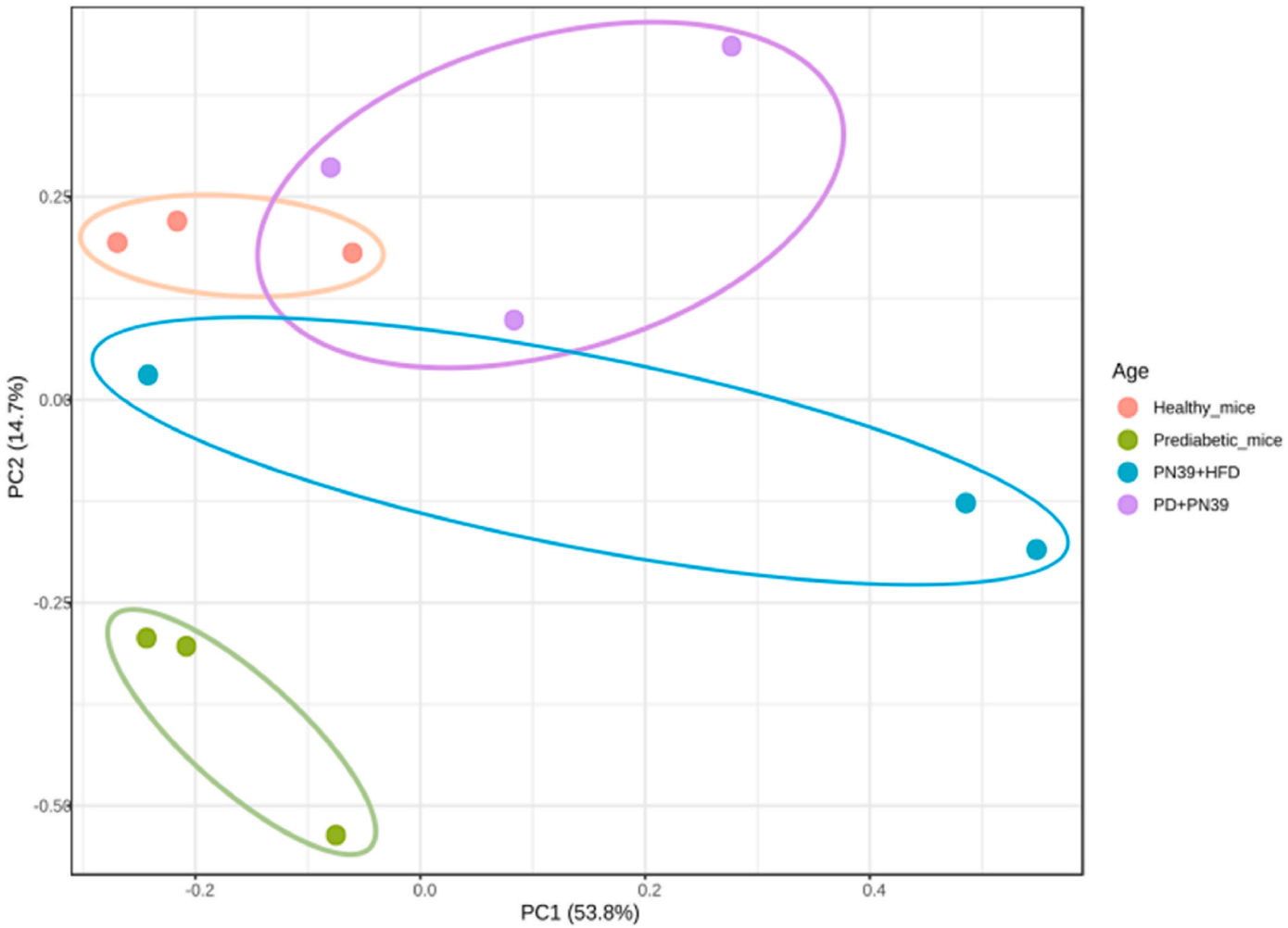

Fig. 3. Representation of principal component analysis (PCoA) performed for healthy, prediabetic, PN39+ HFD and PD + PN39 mice based on unweighted UniFrac metric. The cecum contents of mice (n = 10) were randomly pooled into 3 groups (to make up for inter-individual variations).

Table 1
Permutational multivariate analysis of variance (adonis) and multivariate homogeneity of groups dispersions analysis (betadisper) results for gut microbial communities associated with healthy, prediabetic, PN39+ HFD and PD + PN39 mice.

	Df	Mean Squares	F-value	R^2	*p*-value
Group	12	0.966132	1.724154	0.340911	*0.016
Residuals	40	0.560352	NA	0.659089	NA
Total	52				

*Significant *p*-value at $p < 0.05$.

disrupt mice glucose tolerance indicating that PN39 prevented blood glucose spike (hyperglycemia) although it did not prevent weight gain (Fig. 1). Meanwhile, consumption of HFD alone for 9 weeks drastically impaired blood glucose clearance as the mice demonstrated a significantly high HOMA-IR index relative to the control group although they showed similar serum insulin levels (Fig. 1E and F). A four-week administration of PN39 to mice that had already been fed HFD for 5 weeks did not improve the condition (Fig. 1B and C). This is probably because the prediabetic state might have been established by the 5-week of HFD consumption which was irreversible by PN39 intervention. Previous studies have shown that HFD consumption increases the expression of adipocyte NADPH oxidase complex and liver genes associated with fatty acid oxidation which elevates reactive oxygen species production and oxidative stress, leading to impaired glucose tolerance (Matsuzawa-Nagata et al., 2008). It is therefore likely that the strong antioxidant capacity of PN39 (Daliri et al., 2023) contributed to its ability to prevent prediabetes when simultaneously administered with HFD.

Considering the involvement of the gut microbiota in the onset of prediabetes (Megur et al., 2022; Wu et al., 2020), the influence of HFD and PN39 consumption on the gut microbial ecology was studied. Our results showed that HFD consumption decreased gut microbial richness but not diversity (Fig. 2). The gut microbial communities of mice consuming PN39 were more similar to each other and differed from that of the HFD group (Fig. 3, Table 1). Interestingly, the gut microbiota of both prediabetic mice and prediabetic humans were characterized by a diminished relative abundance of *Lactobacillus* genus and an increased relative abundance of known prediabetes risk factors such as *Helicobacter* (in mice) (Bajaj et al., 2014; Chen et al., 2015; Draz et al., 2018) and *Escherichia-Shigella* (in humans) (Diener et al., 2021; Mei et al., 2024; Zhong et al., 2019) compared to non-diabetic groups (Figs. 4D and 6C). Although PN39 supplementation in prediabetic mice did not raise *Lactobacillus* levels to those seen in control mice, it increased *Clostridia UCG_014* levels and inhibited *Helicobacter* populations. In prediabetic human microbiota however, PN39 restored *Lactobacillus* and *Clostridia UCG_014* abundance to that observed in non-diabetic subjects and suppressed both *Escherichia-Shigella* and *Stenotrophomonas* (Fig. 6B). Meanwhile, PN39 effectively prevented HFD associated *Lactobacillus* and *Clostridia UCG_014* reduction and suppressed pathobiont growth in the cecal microbiota of prediabetic mice. This suppression effect was

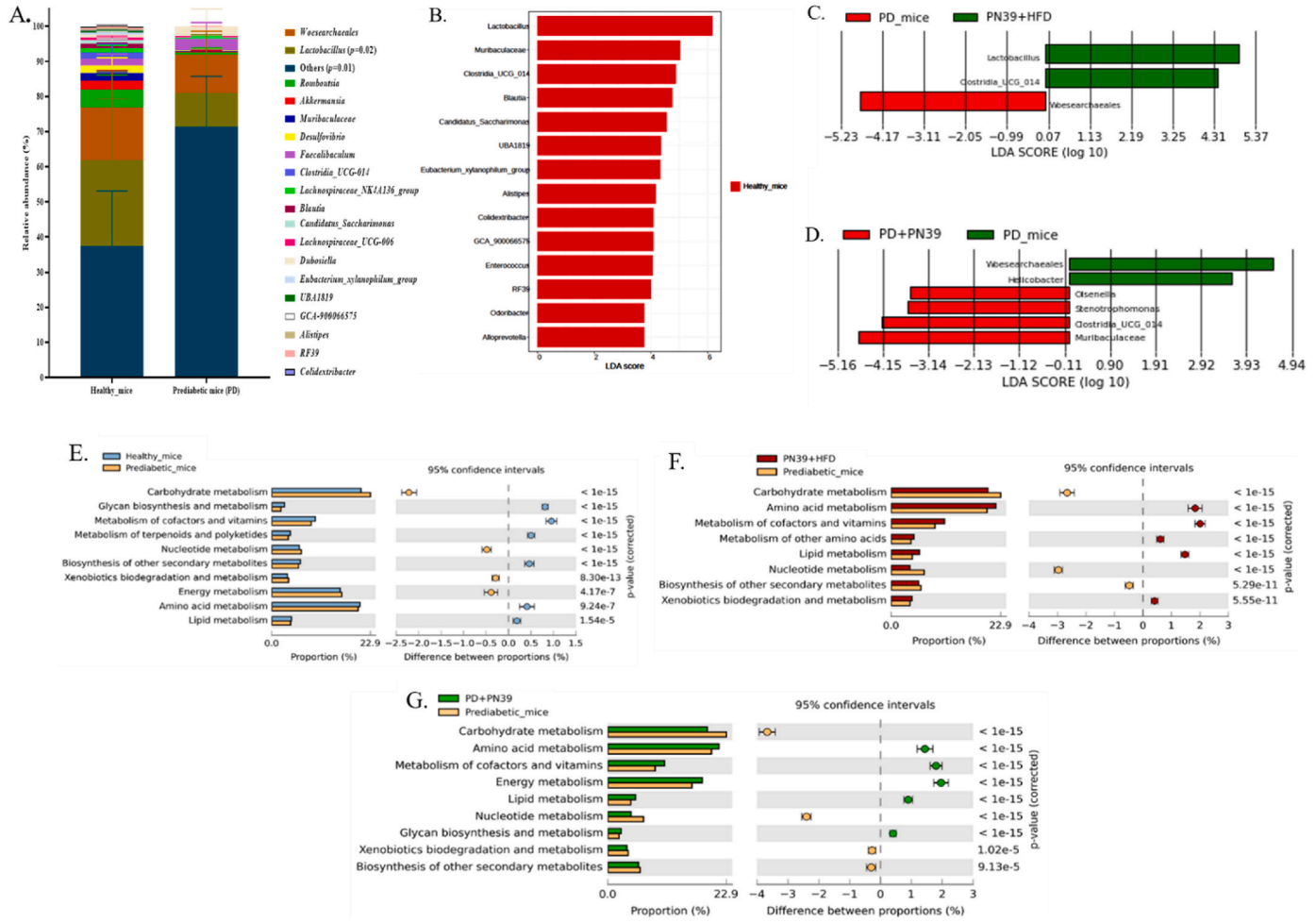

Fig. 4. A. Top 20 most abundant genera in healthy and PD mice, B. Differential gut microbial composition in healthy, PN39+HFD and PD + HFD mice groups. LEfSe determines the biological relevance of significant enrichment of taxa and ranks them by effect size. LDA score indicates the magnitude of the effect size. Taxa enriched in the healthy mice, PN39+HFD and PD + HFD groups relative to
PD group are described with a positive LDA score, C. Differential PICRUSt prediction based on KEGG pathways for healthy vs PD, PN39+HFD vs PD and PD + HFD vs PD mice groups by STAMP software. Blue bars represent healthy mice, yellow bars represent PD mice, red bars represent PN39+HFD and green bars represent PD + HFD group. An extended error bar plot indicates differences in the predicted functional profiles of gut microbiota between groups. Bar plots on the left side present the mean proportion of each KEGG pathway. Dot plots on the right display the differences in mean proportions between the two groups using *p*-values.

likely due to the elevated lactate concentration produced by lactobacilli to lower gut pH as detected in PN39+HFD cecum (Fig. 5). Additionally, the significant correlation between lactobacilli and lactate further reinforces this assertion. Meanwhile, the pathobiont genera suppressed by PN39 in prediabetic mice and humans differed, reflecting variations in their gut microbiota compositions, despite similarities in function (Beresford-Jones et al., 2022). Functional prediction based on the composition of the studied communities indicated potential alterations in gut microbial carbohydrate, lipid and energy metabolism in prediabetic mice and humans relative to their non-diabetic counterparts. Meanwhile, consuming PN39 alongside HFD prevented alterations in the aforementioned microbial metabolic pathways (Fig. 4G and 6E) which are strongly associated with host insulin resistance (Pedersen et al., 2016; Takeuchi et al., 2023). Similarly, based on functional prediction, PN39 could significantly modulate gut microbial carbohydrate metabolism in prediabetic mice and humans to levels comparable to control groups. To ascertain which microbial metabolites may be involved in mitigating host hyperglycemia, we performed stool and cecal SCFAs analysis. It was found that PN39 enhanced microbial butyrate, propionate, caproate and valerate production (Figs. 5 and 7) which are known to improve host insulin signaling and energy homeostasis (Kimura et al., 2013). A previous study (Liu et al., 2020) has shown that lactate produced by lactobacilli can be converted to butyrate, valerate, propionate and caproate by *Clostridia* which agrees with the current study (Fig. 5B). It is therefore probable that PN39 acted by promoting lactobacilli and *Clostridia UCG_014* growth, stimulating lactate production by lactobacilli and its conversion into antidiabetic SCFAs by *Clostridia UCG_014* thereby mitigating prediabetes. Also, considering that many lactobacilli and *Clostridia* produce bile salt hydrolase (Lv et al., 2024; Guzior et al., 2024) that regulate bile acids metabolism, which also tightly linked to glucose and lipid metabolism, it is likely that the elevation of these microbes could contribute to glucose homeostasis in the host.

Overall, our study demonstrates that high-fat diet consumption reduces gut lactobacilli and *Clostridia UCG_014* populations, disrupts microbial carbohydrate metabolism and decreases SCFA production thereby contributing to hyperglycemia. Meanwhile, consuming HFD together with PN39 prevented prediabetes and mitigated gut dysbiosis as well as microbial carbohydrate metabolism while promoting antidiabetic SCFA production. Coupled with its potential to modulate host carbohydrate digestion and DPP-IV activity in our previous studies, PN39 could serve as a functional food for mitigating diabetes and its associated gut dysbiosis. Clinical trials are however needed to validate our findings.

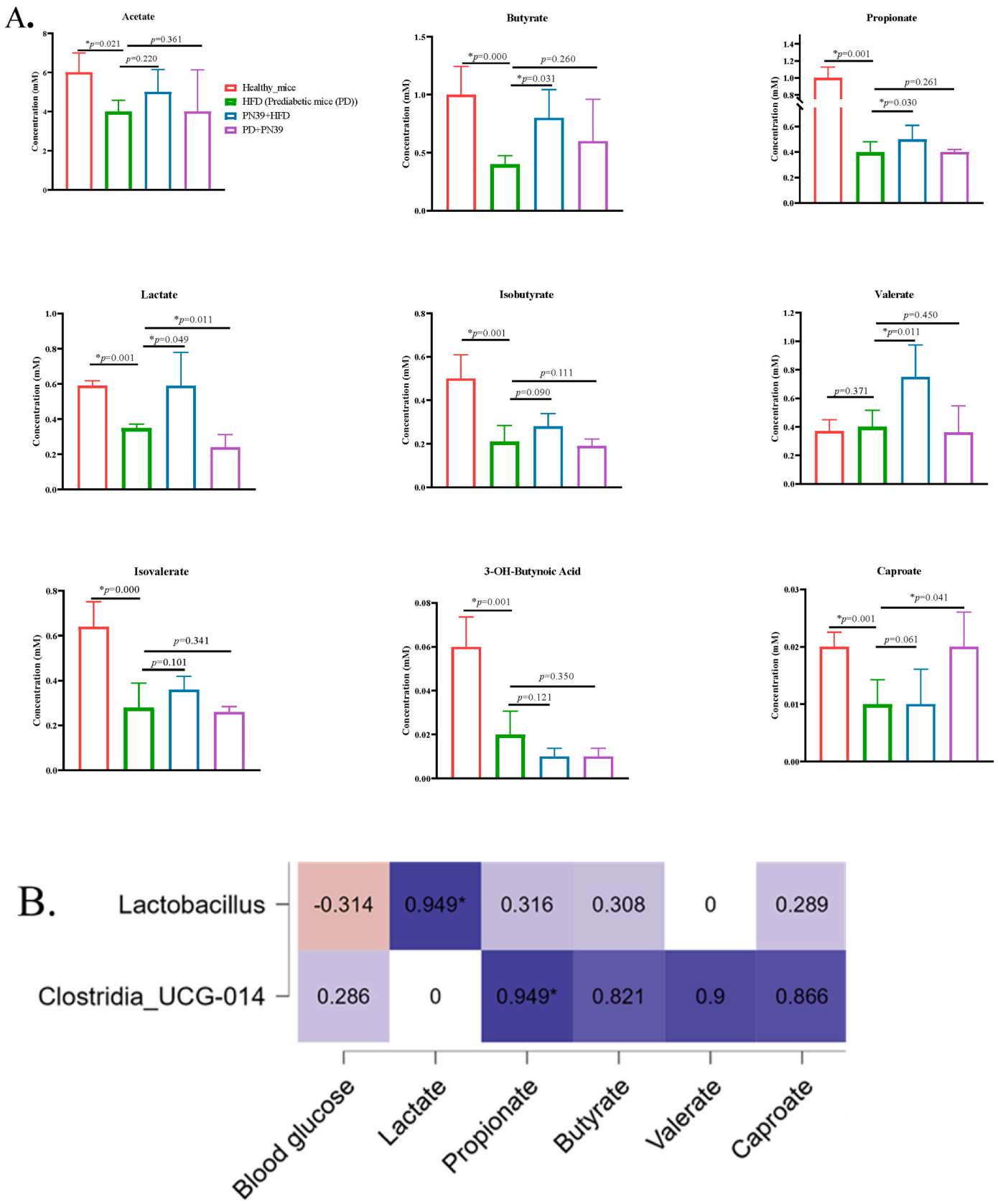

Fig. 5. A. Impact of PN39 consumption on mice cecal SCFAs. Data are expressed as Mean ± SEM for each group (n = 10). B. Heatmap showing Spearman's correlation coefficients between *Lactobacillus* and *Clostridia UCG_014* and glucose tolerance as well as SCFAs levels in the cecum of PN39+HFD mice. Blue squares indicate positive linear correlation while white squares indicate no linear relationship. Significant correlations are indicated inside the heatmap with * ($p < 0.01$).

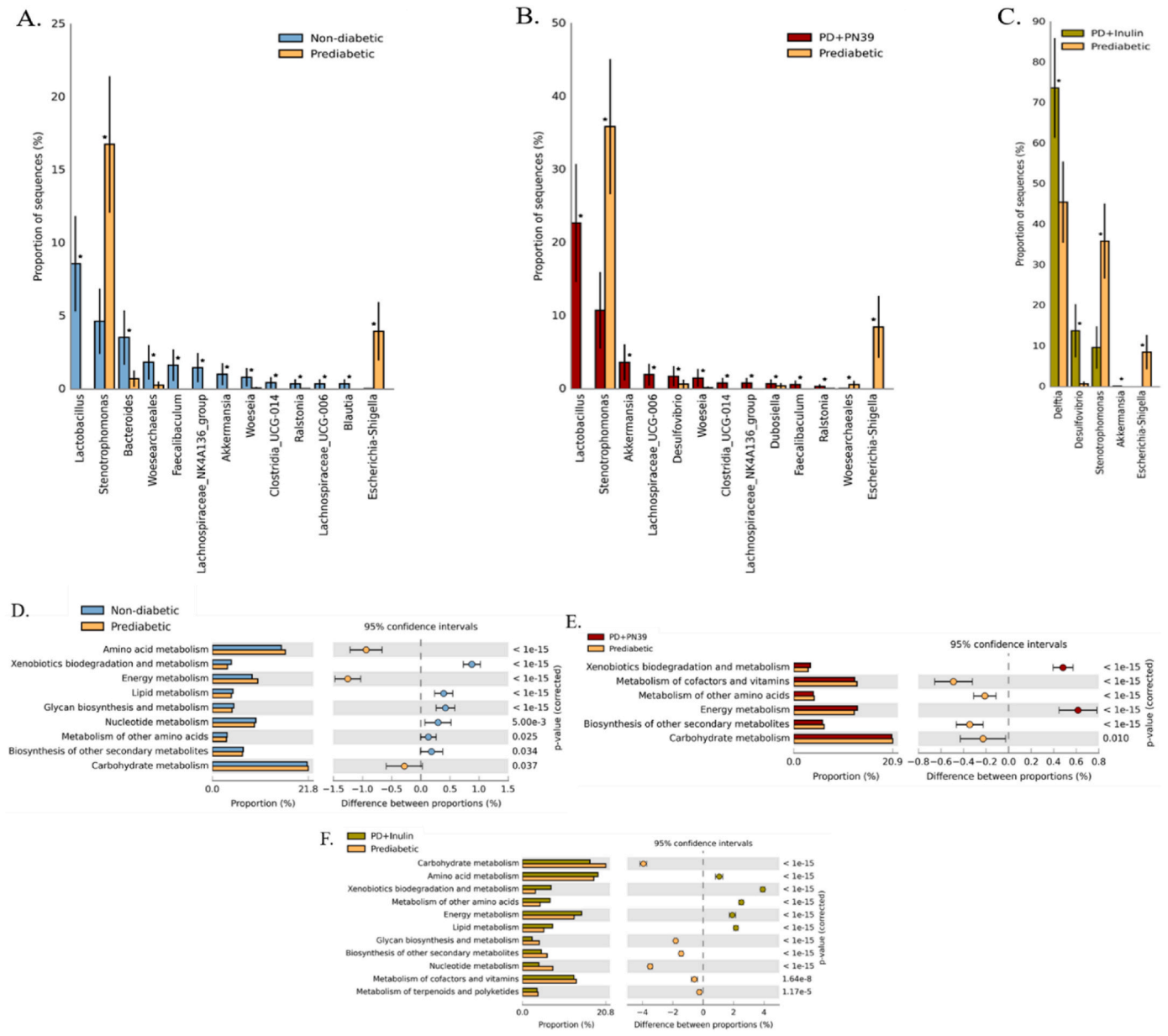

Fig. 6. Impact of PN39 and inulin on *in vitro* fermentation of prediabetic human fecal microbiota. A. Relative abundance of top 20 genera in non-diabetic donors versus prediabetic donors, B. PD + PN39 versus prediabetic donors and C. PD + Inulin versus prediabetic donors. Differential PICRUSt was used to predict KEGG pathways that were significantly different among the sample groups after *in vitro* fermentation of PD stool samples with either PN39 or inulin. D. Differential KEGG pathways between non-diabetic volunteer gut microbiota and prediabetic volunteer gut microbiota, E. differential KEGG pathways PD + PN39 gut microbiota and prediabetic volunteer gut microbiota, F. differential KEGG pathways between PD + Inulin gut microbiota and prediabetic volunteer gut microbiota. For each comparison, the mean proportion of predicted KEGG pathways (left) and difference in mean proportions (right) were illustrated.

CRediT authorship contribution statement

Eric Banan-Mwine Daliri: conceived and designed the study, drafted the manuscript. **Ashwinipriyadarshini Megur:** was involved in animal handling and data collection. **Jonas Mingaila:** were involved in animal data analysis and interpretation. **Akshay Kumar Vijaya:** were involved in animal data analysis and interpretation. **Toma Balnionytė:** were involved in animal data analysis and interpretation. **Debalina Sakar:** performed HPLC and SCFA data analysis, All authors reviewed the results and approved the final version of the manuscript. **Yaiza Carnicero-Mayo:** performed HPLC and SCFA data analysis, All authors reviewed the results and approved the final version of the manuscript. **Volker Behrends:** performed HPLC and SCFA data analysis, All authors reviewed the results and approved the final version of the manuscript. **Adele Costabile:** conceived, designed the study and revised the final manuscript. **Aurelijus Burokas:** conceived, designed the study and revised the final manuscript.

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Declaration of competing interest

All authors declare no financial or non-financial competing interests.

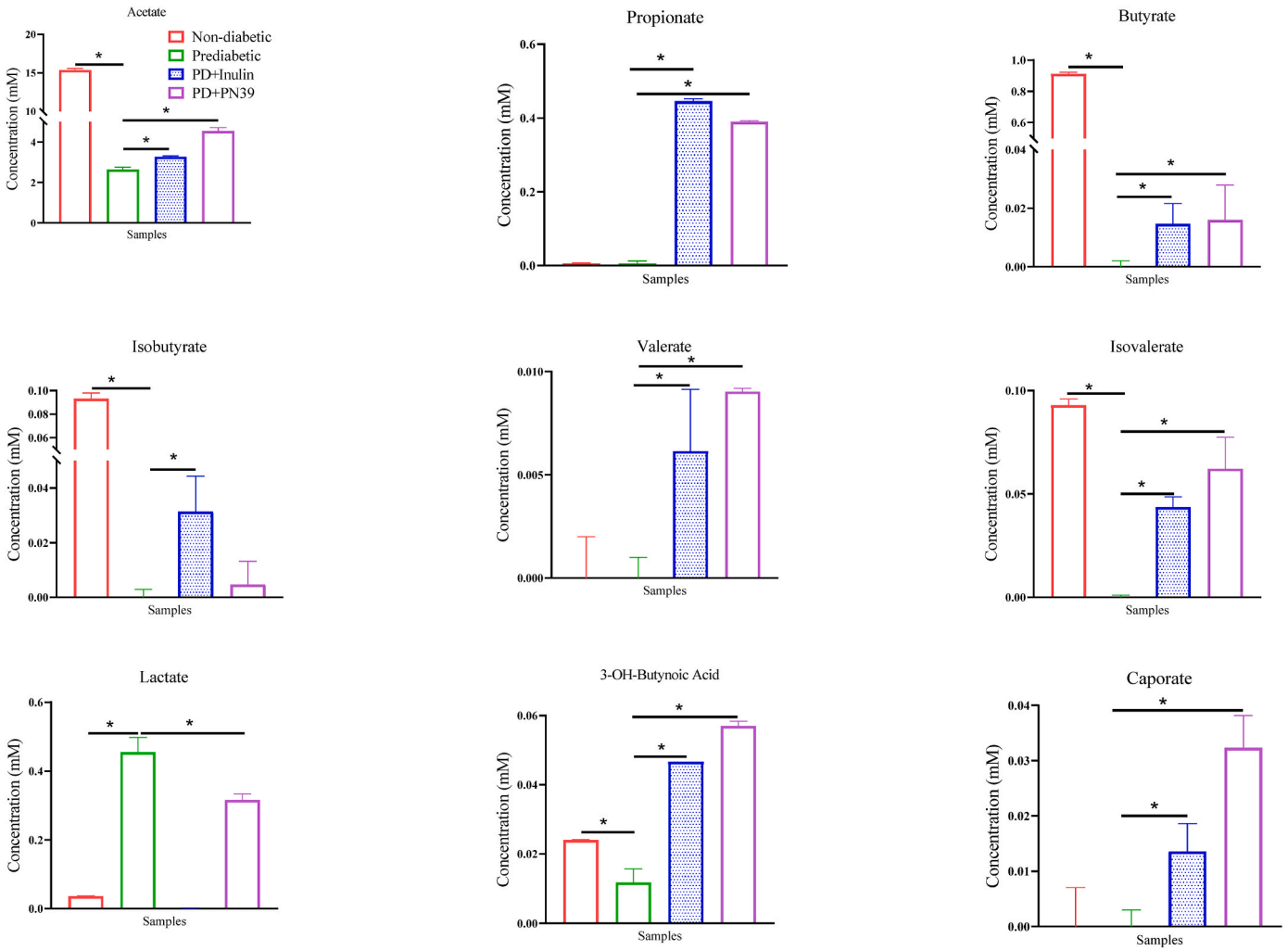

Fig. 7. Quantification of stool SCFAs and lactate in non-diabetic and prediabetic subjects as well as prediabetic stool samples co-incubated with PN39 or inulin for 24 h. For each group (n = 3), mean ± SEM is represented (one-way ANOVA, Tukey's multiple comparisons tests, * = $p < 0.05$).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2025.101052.

Data availability

Data will be made available on request.

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