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Gut Microbiota Predicts Treatment Response to Empagliflozin Among MASLD Patients Without Diabetes Mellitus

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ABSTRACT

Background and Aim: We aimed to investigate whether gut microbiota could predict the treatment response to pharmacological agents among metabolic dysfunction-associated steatotic liver disease (MASLD) patients without diabetes mellitus (DM), as data are lacking.

Methods: We prospectively followed up non-diabetic MASLD patients who used empagliflozin. Clinical, anthropometric, laboratory assessments and magnetic resonance imaging-proton density fat fraction (MRI-PDFF) were performed from baseline to week 52 (EOT). Baseline stool samples were collected, and shotgun DNA metagenomic sequencing was performed to profile microbiome. The primary outcome was treatment response to empagliflozin at EOT, defined as MRI-PDFF decline ≥30% at EOT from baseline. Linear discriminant analysis [LDA] effect size was used to identify putative bacterial species. Multivariable logistic regression was used to derive adjusted odds ratio (aOR) of outcome with bacterial species by adjusting for clinical factors.

Results: Twenty-two (48.9%) of 45 patients (median age: 56.9 years [IQR: 51.0–63.2]; male: 23 [51.1%]) achieved treatment response at EOT. There was difference in alpha diversity (Shannon index: $p < 0.001$; Simpson index: $p = 0.001$) and beta diversity ($p = 0.048$) in baseline microbiome between treatment response and non-response groups. *Faecalibacterium prausnitzii* ($\log_{10}$LDAscore = 4.27), *Lachnospira pectinoschiza* ($\log_{10}$LDAscore = 3.99), *Anaerostipes hadrus* ($\log_{10}$LDAscore = 3.98), *Roseburia faecis* ($\log_{10}$LDAscore = 3.97), *Roseburia inulinivorans* ($\log_{10}$LDAscore = 3.58) and *Agathobaculum butyriciproducens* ($\log_{10}$LDAscore = 2.77) were enriched in the treatment response group. *L. pectinoschiza* (aOR: 34.1; $p = 0.015$), *A. hadrus* (aOR:35.0;

Abbreviations: 95% CI, 95% confidence interval; ALP, alkaline phosphatase; ALT, alanine aminotransferase; aOR, adjusted odds ratio; AST, aspartate aminotransferase; AUROC, area under receiver operating curve; BMI, body mass index; DM, diabetes mellitus; EOT, end of treatment; FDR, false discovery rate; FXR, farnesoid X receptors; GGT, gamma-glutamyl transferase; GLP-1, glucagon-like peptide 1; IQR, interquartile range; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size; LPS, lipopolysaccharides; MASH, metabolic dysfunction-associated steatohepatitis; MASLD, metabolic dysfunction-associated steatotic liver disease; MRI-PDFF, magnetic resonance imaging-proton density fat fraction; NMDS, non-metric multidimensional scaling; OR, odds ratio; PERMANOVA, permutational multivariate analysis of variance; RCT, randomised controlled trial; rPDQS, rapid prime diet quality score; SCFAs, short-chain fatty acids; SGLT2, sodium glucose cotransporter-2; TGR5, transmembrane G-protein coupled receptor 5; WHO, World Healthy Organization.

Ho Yu Ng and Lina Zhang share co-first authorship.

$p = 0.032$) and *A. butyriciproducens* (aOR:22.3; $p = 0.023$) independently predicted treatment response but not clinical factors. These three species collectively predicted treatment response with AUROC of 0.89 (95% CI: 0.80–0.99).

Conclusions: Certain gut bacterial species, particularly the combination of *A. hadrus*, *L. pectinoschiza* and *A. butyriciproducens*, may predict treatment response to empagliflozin in MAFLD patients without DM.

1 | Introduction

The incidence of metabolic dysfunction-associated steatotic liver disease (MASLD) has been increasing across the globe, with an estimated prevalence as high as 32.4% [1]. MASLD can lead to severe consequences, including metabolic dysfunction-associated steatohepatitis (MASH), cirrhosis and hepatocellular carcinoma [2]. MASLD is one of the most common indications of liver transplantation in the United States [3]. In addition, hepatic steatosis also augments cardiovascular risk through its bidirectional relationship with some metabolic syndrome features, especially its association with a proatherogenic lipid profile [4]. Visceral adiposity and its associated chronic low-grade inflammation seen in MASLD are related to higher risk of cancer in MASLD patients [5].

While weight reduction and lifestyle modification (diet and physical activity) are the most effective ways to improve MASLD, it is difficult to achieve and sustain [6]. Therefore, pharmacological agents are important adjuncts in the treatment of MASLD. Sodium glucose cotransporter-2 (SGLT2) inhibitors have been shown to be effective in treating MASLD patients with DM in both experimental [7] and randomised controlled trials (RCTs), in which they were shown to reduce transaminase levels and liver fat content [8–10]. In some trials, the benefits of SGLT2 inhibitors persisted when compared to active controls (other antidiabetic agents), suggesting the benefits of SGLT2 inhibitors are independent of glycemic control [8]. SGLT2 inhibitors are suggested to improve MASLD via ameliorating systemic and tissue inflammation, as well as reducing oxidative stress through enhancing cellular antioxidative ability or diminishing free-radical generation [11]. However, it was observed that a significant proportion of patients with hepatic steatosis do not have DM [12]. A recent RCT showed that empagliflozin (an SGLT2 inhibitor) reduced hepatic steatosis to a greater extent than placebo in MASLD patients without DM (−2.49% vs. −1.43%; $p = 0.025$) [13].

Increasing evidence showed that the gut microbiota is implicated in MASLD. Gut microbiota dysbiosis is often observed as MASLD progresses, and there was increased abundance of Gram-negative bacteria such as *Proteobacteria* and decreased abundance of Gram-positive bacteria such as *Ruminococcaceae, Faecalibacterium, Coprococcus, Anaerosporobacter* and *Eubacterium* in MASID patients [14, 15]. Gut microbiota potentially influence the development of MASLD via production of various metabolites, including short-chain fatty acids (SCFAs), bile acids and amino acids. SCFAs such as acetate, propionate and butyrate can reduce fat deposition in liver by stimulating adipogenesis and inhibiting lipolysis in adipocytes [16], regulate the secretion of insulin and decrease insulin resistance via stimulating the secretion of glucagon-like peptide-1 (GLP-1) [17] and exert anti-inflammatory effect [18]. Bile acids can bind to farnesoid X receptors (FXR) and transmembrane G-protein coupled receptor 5 (TGR5) which are involved in glucose homeostasis [14]. Certain amino acids have also been shown to attenuate hepatic steatosis in aging mice models and exert hepatoprotective effects [19–21], and one study found that increased consumption of food which are rich in amino acids, especially lysine, threonine and valine, can help reduce the risk of hepatic steatosis [22].

However, there are currently no studies investigating whether gut microbiota influence the treatment response of pharmacological agents. Therefore, we aimed to investigate whether baseline gut microbiota composition can predict treatment response among MASLD patients without DM who have received SGLT2 inhibitors.

2 | Methods

2.1 | Study Design and Participants

This was a prospective cohort study opening recruiting adult MASLD patients without DM from the community [13]. The study protocol was approved by Institutional Review Board of Hospital Authority Hong Kong West Cluster and University of Hong Kong. Written informed consent was obtained from all study subjects.

They were confirmed to have hepatic steatosis (magnetic resonance imaging-proton density fat fraction [MRI-PDFF] ≥ 5%) [23] and fulfilled one of the five criteria of metabolic factors of MASLD [24]. Recruited subjects were then randomly allocated to receive either empagliflozin (Jardiance 10 mg; Boehringer Ingelheim, Ingelheim am Rhein, Germany) or placebo (i.e., control group) for 52 weeks (end of treatment, EOT).

A dietitian, who was blinded to allocation, educated subjects about MASLD and principles of healthy diet based on recommendations of American Dietetic Association [25], physical activity and weight control at baseline. We collected data on physical activity, including type, duration and frequency of exercises, as well as diet as per the questionnaire used for the rapid prime diet quality score (rPDQS), which is a validated diet quality screener that can reflect the quality of food intake, with scores ranging from 0 to 52 and a higher score indicating better diet quality [26]. Good lifestyle is defined as either level of exercises meeting World Health Organization (WHO) recommendation [27] or a high rPDQS score defined at the highest tertile.

Study subjects were followed until week 52 (EOT), with assessments at baseline, week 6, 12, 26, 40 and 52. We collected clinical data including subject's age, sex, medical history, drug history, diet, exercise, smoking and alcohol intake, as well as anthropometric measurements including body height, weight and body mass index (BMI). Blood samples were taken to assess ferritin level (a marker of hepatic necroinflammatory status) [28], liver function test (alanine aminotransferase [ALT],

Summary

- Sodium glucose cotransporter-2 (SGLT2) inhibitors have emerged as a potential treatment option for non-diabetic MASLD patients. While gut microbiota plays a role in MASLD progression, data on whether gut microbiota could predict treatment response to SGLT2 inhibitors in non-diabetic MASLD patients is lacking.
- We prospectively followed up non-diabetic MASLD patients who used empagliflozin for 1 year and found that certain gut bacterial species, especially the combination of *Anaerostipes hadrus*, *Lachnospira pectinoschiza* and *Agathobaculum butyriciproducens*, may predict treatment response to empagliflozin in MAFLD patients without DM.
- Further studies on animal models are needed to establish causality between gut bacterial markers and treatment response to empagliflozin.

aspartate aminotransferase [AST], alkaline phosphatase [ALP] and gamma-glutamyl transferase [GGT]) and metabolic parameters including fasting glucose and HbA1c.

At baseline and EOT, study subjects underwent MRI (1.5 T MRI scanner [Explorer Lift, General Electric Healthcare](A) to assess the liver fat content.

2.2 | Outcomes of Interest

The primary outcome was treatment response to empagliflozin at EOT, defined as MRI-PDFF decline ≥ 30%, among the empagliflozin users [29, 30]. MRI-PDFF decline ≥ 30% was shown to be associated with a higher odds of MASH resolution and histological response [29].

Secondary outcomes included (i) comparison of relative abundance of gut microbiota at EOT between the treatment response and non-response groups and (ii) dynamic changes of gut microbiota between baseline and EOT after intervention with empagliflozin.

2.3 | Exposure of Interest and Covariates

The primary exposures of interest are the baseline gut microbiota profile and the metabolic functional pathways. Covariates include the clinical data and blood parameters mentioned in the previous subsection.

2.4 | Stool Sample Collection, DNA Extraction and Sequencing

Stool samples at baseline before treatment and at EOT (week 52) were collected for the empagliflozin group but not the placebo group. Stool samples were collected in OMNIgene tube and stored at −80°C until extraction of total genomic DNA was performed using the Qiagen QIAamp DNA stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Library preparation of extracted genomic DNA for shotgun metagenomic sequencing was then performed using Nextera DNA Library Prep Kit (Illumina, California, USA). In brief, genomic DNA was first fragmented, and engineered transposome was used to tag genomic DNA fragments with adapter sequences. Limited cycle PCR was then used to add index adapter sequences to these tagged DNA. After amplification, PCR amplicons were purified using AMPure XP beads (Beckman-Coulter). The quality of the DNA library was first assessed by a Qubit fluorometer (Thermo Fisher Scientific), then by a Bioanalyzer (Agilent Technologies). After library preparation, we performed next-generation shotgun metagenomic sequencing using the Illumina NovaSeq 6000 platform (Illumina, San Diego, US) running at paired-end 150 bp, resulting in 10 Gb raw data per sample.

2.5 | Bioinformatics Analysis

We used fastp v0.20.1 [31] to process raw NGS reads to quality and perform adapter trimming to remove sequencing adapters and bases with poor quality. Trimmed reads were subjected to host sequence removal by Bowtie2 [32] to map reads against the human reference genome GRCh38.p13. MetaPhlAn (v3.0) [33] and HUMAnN (v3.0) [34] were used to infer the composition of microbial communities at species level and functional profile in each sample from the clean reads, respectively. Species coverage and relative abundance were then estimated.

2.6 | Statistical Analysis

All statistical analyses were performed using R statistical software (version 4.2.2, R Foundation for Statistical Computing, Vienna, Austria). Continuous variables were presented as median (interquartile range [IQR]), and categorical variables were presented as number of patients (percentage). Continuous variables of two groups were compared using the Mann–Whitney *U*-test, while categorical variables were compared using Chi-square test or Fisher's exact test. Alpha-diversity in terms of observed species and Shannon and Simpson index was calculated using *vegan* package in R Studio and compared using the Wilcoxon signed-rank test. Beta-diversity including Bray–Curtis compositional dissimilarity was compared using non-metric multidimensional scaling (NMDS). Permutational multivariate analysis of variance (PERMANOVA) was used to compare microbial communities of different samples. LEfSe (linear discriminant analysis effect size) was used to identify putative gut bacterial species and metabolic pathways with an absolute value of linear discriminant analysis (LDA) score ≥ 2. The highest quartile (top 75%) was used to define a high relative abundance of a particular bacterial species [35, 36].

We used a univariate logistic model to estimate the odds ratio (OR) of treatment response with different clinical factors as well as with a high relative abundance of putative gut bacterial species. Clinical factors and putative bacterial species of $p < 0.10$ on univariate logistic regression analysis were then incorporated into the multivariate regression model to estimate the adjusted OR (aOR) of treatment response. Spearman's correlation tests were used to analyse the correlation among continuous variables. False discovery rate (FDR) was used to correct for multiple comparisons in multiple hypothesis testing [37].

Additional analysis was performed by incorporating weight loss ≥ 5% at week 52 into the logistic regression model to investigate whether the effect of baseline gut microbiota profile on treatment response was independent of weight loss.

The receiver operating curve was generated by plotting 'sensitivity' against '1—specificity' at different values. The predictive performance of each putative bacterial species and their combination was expressed in terms of area under receiver operating curve (AUROC), with the 95% confidence interval (95% CI) being derived from bootstrapping by sampling with replacement from the original sample and repeating the process by 1000 times.

Subgroup analyses were performed according to good lifestyle in terms of exercises and diet as previously defined.

A two-sided *p*-value ≤0.05 was considered as statistically significant.

3 | Results

Between March 2021 and April 2022, we recruited 49 MASLD subjects without DM (median age: 56.9 years [IQR: 51.0–63.2]; male:23 [51.1%]); 45 had stool samples collected with sufficient DNA concentration to undergo shotgun metagenomics sequencing and were included into our present study.

Of these 45 subjects, 22 (48.9%) achieved treatment response to empagliflozin (i.e., MRI-PDFF decline ≥ 30%) at EOT, while 23 (51.1%) did not. Table 1 shows the baseline characteristics between the treatment response group and non-response group. There were 23 male subjects (51.1%), and the median age was 56.9 years (IQR:51.0–63.2). The median body weight was 72.2 kg (IQR:67.0–82.5), and 42 subjects (93.3%) were overweight or obese (defined as baseline BMI ≥ 23 kg/m^2). There was no significant difference in all baseline characteristics including MRI-PDFF (8.4% vs. 10.8%; $p = 0.382$), except for baseline AST (25.0 U/L vs. 29.0 U/L, $p = 0.042$).

TABLE 1 | Baseline characteristics between treatment response and non-response groups among empagliflozin users.

	Whole cohort ($n = 45$)	Treatment response[a] ($n = 22$)	Non-response[a] ($n = 23$)	*p*
Age	56.9 (51.0, 63.2)	57.4 (49.3, 66.5)	56.9 (52.6, 61.3)	0.629
Male sex	23 (51.1%)	11 (50.0%)	12 (52.2%)	0.884
Body weight (kg)	72.2 (67.0, 82.5)	70.7 (65.8, 75.1)	75.2 (67.3, 89.7)	0.095
Overweight or obese (baseline BMI ≥ 23 kg/m^2)	42 (93.3%)	21 (95.5%)	21 (91.3%)	> 0.999
Adequate level of exercises[b]	5 (11.1%)	4 (18.2%)	1 (4.3%)	0.187
Diet (rPDQS score tertile)				0.065
1st tertile	14 (31.1%)	4 (18.2%)	10 (43.5%)	
2nd tertile	12 (26.7%)	5 (22.7%)	7 (30.4%)	
3rd tertile	19 (42.2%)	13 (59.1%)	6 (26.1%)	
MRI-PDFF (%)	9.7 (5.9, 14.5)	8.4 (5.8, 13.2)	10.8 (6.3, 16.8)	0.382
Fasting glucose (mmol/L)	5.6 (5.1, 5.9)	5.8 (5.1, 6.1)	5.6 (5.2, 5.9)	0.593
HbA1c (%)	5.7 (5.5, 5.9)	5.7 (5.5, 5.9)	5.7 (5.4, 5.9)	0.873
ALT (U/L)	31.0 (21.0, 42.0)	26.0 (20.0, 35.0)	35.0 (24.5, 50.5)	0.056
ALT ≥ 40 U/L	14 (31.1%)	4 (18.2%)	10 (43.5%)	0.067
AST (U/L)	27.0 (21.0, 32.0)	25.0 (19.2, 29.0)	29.0 (24.5, 36.5)	0.042
AST > 40 U/L	5 (11.1%)	1 (4.5%)	4 (17.4%)	0.346
ALP (U/L)	74.0 (62.0, 86.0)	75.5 (71.0, 88.8)	74.0 (61.5, 78.5)	0.481
GGT (U/L)	33.0 (24.0, 47.0)	31.0 (21.2, 43.5)	33.0 (29.5, 48.5)	0.265
Ferritin (pmol/L)	733.0 (420.0, 1281.0)	738.0 (480.2, 1650.0)	733.0 (265.0, 1148.5)	0.350

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, gamma glutamyltransferase; HbA1c, haemoglobin A1c; MRI-PDFF, magnetic resonance imaging-proton density fat fraction; rPDQS, rapid prime diet quality score.
[a]Treatment response is defined as MRI-PDFF drop at week 52 from baseline ≥ 30%.
[b]Adequate level of exercises is defined as meeting World Health Organization recommendation (at least 150–300 min of moderate-intensity or 75–150 min of vigorous-intensity aerobic exercise per week).

3.1 | Baseline Gut Microbiota Composition Was Associated With Treatment Response to Empagliflozin

There was a significant difference in alpha diversity in terms of Shannon index ($p<0.001$) and Simpson index ($p=0.001$) (Figure 1A), as well as beta diversity by PERMANOVA analysis ($p=0.048$) (Figure 1B) between treatment response and non-response groups. Twelve baseline gut bacterial species were found to be enriched in the treatment response group compared to the non-response group by LEfSe analysis, six of which (*Faecalibacterium prausnitzii, Lachnospira pectinoschiza, Anaerostipes hadrus, Roseburia faecis, Roseburia inulinivorans*, and *Agathobaculum butyriciproducens*) were not zero-inflated (i.e., their median relative abundance was not equal to zero). They were associated with treatment response (*F. prausnitzii*: $\log_{10}$LDA score = 4.27, $p=0.039$; *L. pectinoschiza*: $\log_{10}$LDA score = 3.99, $p<0.001$; *A. hadrus*: $\log_{10}$LDA score = 3.98, $p=0.001$; *R. faecis*: $\log_{10}$LDA score = 3.97, $p=0.047$; *R. inulinivorans*: $\log_{10}$LDA score = 3.58, $p=0.011$; *A. butyriciproducens*: $\log_{10}$LDA score = 2.77, $p=0.002$) (Figure 2A) and were found to be significantly more abundant in the treatment response than the non-response group (*F. prausnitzii*: 7.70% vs. 4.80%, $p=0.040$; *L. pectinoschiza*: 2.05% vs. 0%, $p<0.001$; *A. hadrus*: 0.20% vs. 0.04%, $p=0.001$; *R. faecis*: 3.34% vs. 0.36%, $p=0.049$; *R. inulinivorans*: 0.59% vs. 0.10%, $p=0.011$; *A. butyriciproducens*: 0.13% vs. 0.04%, $p=0.002$) (Figure 2B).

In particular, the high baseline relative abundance of *L. pectinoschiza* (OR: 8.75, 95% CI: 1.91–63.47, $p=0.011$), *A. hadrus* (OR: 22.00, 95% CI: 3.60–429.20, $p=0.005$) and *A. butyriciproducens* (OR: 4.62, 95% CI: 1.14–23.87, $p=0.043$) was significantly associated with treatment response on univariate logistic regression. They remained significantly associated with treatment response on multivariable analysis (*L. pectinoschiza*–aOR: 34.05, 95% CI: 3.00–1172, $p=0.015$; *A. hadrus*–aOR:34.99, 95% CI: 2.21–1919, $p=0.032$; *A. butyriciproducens*–aOR:22.32, 95% CI: 2.164–632.6, $p=0.023$). On the other hand, routine clinical factors were not predictive factors of treatment response (all $p>0.05$) (Table 2).

Additional analysis incorporating weight loss ≥5% at week 52 (aOR:46.86, 95% CI: 3.04–2223, $p=0.017$) found that these three species remained significant (*L. pectinoschiza*–aOR: 79.91, 95% CI:4.47–6949, $p=0.013$; *A. hadrus*–aOR: 81.44, 95% CI:3.42–9849, $p=0.025$; *A. butyriciproducens*–aOR: 44.26, 95% CI:2.80–3055, $p=0.024$) (Table S1).

Table 3 and Figure 3 show that the AUROC of *L. pectinoschiza*, *A. hadrus and A. butyriciproducens* in predicting treatment response was 0.81 (95% CI: 0.69–0.94), 0.78 (95% CI: 0.64–0.91) and 0.77 (95% CI: 0.62–0.91), respectively. These three species collectively distinguished treatment response from no response with an AUROC of 0.89 (95% CI: 0.80–0.99), with a sensitivity of 0.82 (95% CI: 0.60, 0.95), specificity of 0.83 (95% CI: 0.61, 0.95), positive predictive value of 0.82 (95% CI: 0.60, 0.95), negative predictive value of 0.83 (95% CI: 0.61, 0.95), positive likelihood ratio of 4.70 (95% CI: 1.89, 11.71) and negative likelihood ratio of 0.22 (95% CI: 0.09, 0.54) (Table 3).

Moreover, among empagliflozin users with good lifestyle, these three species were found to have significantly higher relative abundance in the response group than in the non-response group (*L. pectinoschiza*: 2.77% vs. 0.00%, $p=0.003$; *A. hadrus*: 0.29% vs. 0.03%, $p=0.020$; *A. butyriciproducens*: 0.16% vs. 0.04%, $p=0.008$) (Fiure S1). Among empagliflozin users without good lifestyle, there was a significantly higher relative abundance in *L. pectinoschiza* (2.05% vs. 0.00%, p = 0.020), *A. hadrus* (0.13% vs. 0.07%, $p=0.029$) and *F. prausnitzii* (8.21% vs. 4.01%, $p=0.027$) in the response group than in the non-response group but not for *A. butyriproducens* (0.11% vs. 0.04%, $p=0.130$) (Figure S2).

3.2 | Baseline Metabolic Pathways Were Associated With Treatment Response to Empagliflozin

We identified 32 metabolic pathways that were enriched in the treatment response group, while three were depleted (Figure S3). Among these pathways, 18 belonged

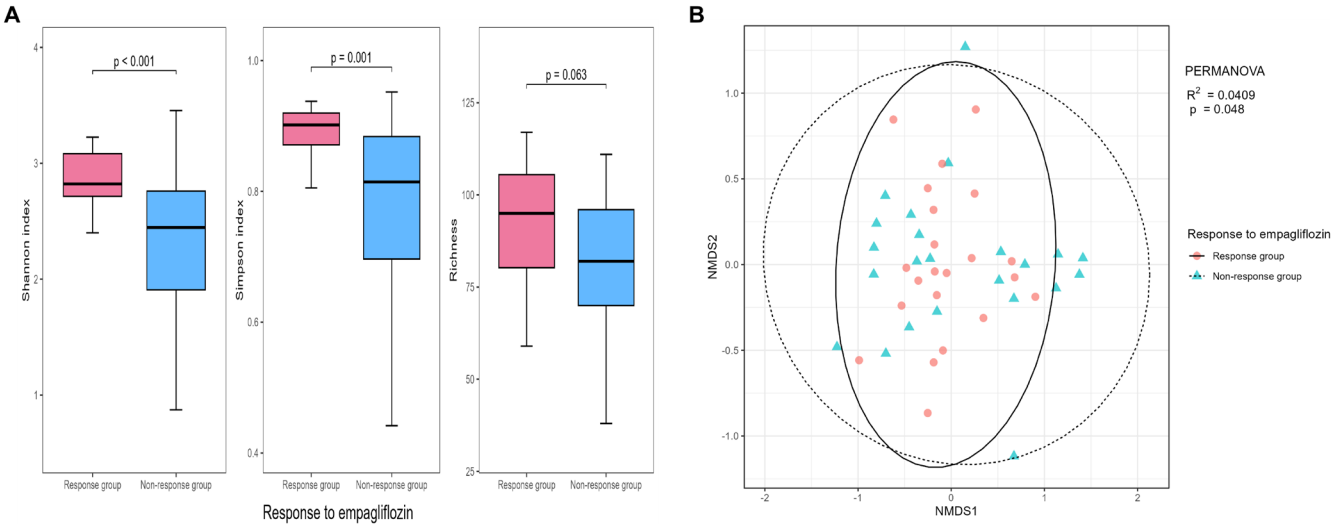

FIGURE 1 | (A) Alpha diversity of baseline gut microbiota in the treatment response group and non-response groups among empagliflozin users (B) Beta diversity of baseline gut microbiota in the treatment response group and non-response groups among empagliflozin users. NMDS, nonmetric multidimensional scaling.

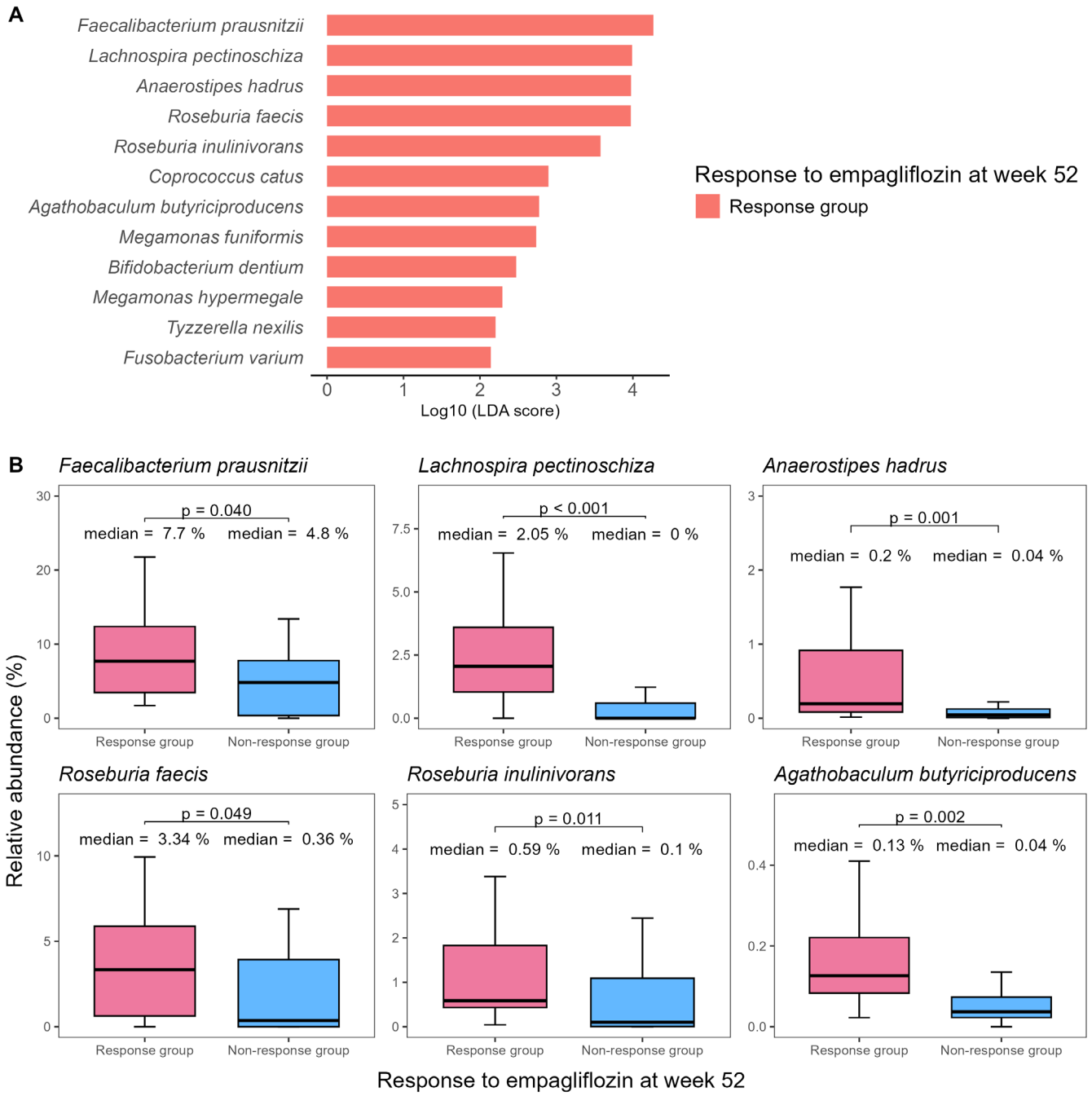

FIGURE 2 | (A) Baseline gut bacterial species enriched in the empagliflozin response group on LEfSe analysis (B) Comparison of relative abundances of putative baseline gut bacterial species identified on LEfSe analysis between treatment response group and non-response group. LEfSe, linear discriminant analysis effect size.

to 'Biosynthesis' category, 10 belonged to 'Degradation/Utilisation/Assimilation' category, six belonged to 'Generation of Precursor Metabolites and Energy' category and one belonged to 'Superpathways' category (Table S2). Of note, pathways enriched in the treatment response group included those that produced short-chain fatty acids (SCFAs), such as acetyl-CoA fermentation to butanoate II pathway ($\log_{10}$LDA score = 2.19, $p = 0.019$) and pyruvate fermentation to acetate and (S)-lactate I pathway ($\log_{10}$LDA score = 2.68, $p = 0.010$), as well as pathways that produced amino acids, such as L-ornithine biosynthesis I pathway ($\log_{10}$LDA score = 2.82, $p = 0.023$), L-arginine biosynthesis I ($\log_{10}$LDA score = 1.91, $p = 0.026$) and II ($\log_{10}$ LDA score = 2.93, $p = 0.025$) pathway, superpathway of L-cysteine biosynthesis (mammalian) ($\log_{10}$LDA score = 2.54, $p = 0.017$), as well as superpathway of L-lysine, L-threonine and L-methionine biosynthesis II ($\log_{10}$LDA score = 2.67, $p = 0.031$) (Table S2 and Figure S3).

3.3 | Correlation Between Baseline Gut Microbiota and Metabolic Pathways on Predicting Treatment Response to Empagliflozin

We performed Spearman's correlation between the baseline bacterial species and metabolic pathways identified on LefSe analysis (Figure 4). Notably, *A. hadrus* was positively correlated with

TABLE 2 | Univariate and multivariable logistic regression between treatment response and a combination of clinical factors and bacterial species.

	Univariate logistic regression			Multivariable logistic regression		
	OR	**95% CI**	***p***	**aOR**	**95% CI**	***p***
Age	1.022	0.951, 1.101	0.558			
Sex	0.917	0.282, 2.970	0.884			
Body weight (kg)	0.951	0.898, 0.999	0.063	0.988	0.913, 1.061	0.749
Overweight or obese (baseline BMI ≥ 23 kg/m^2)	2.000	0.178, 44.99	0.583			
Adequate level of exercises[a]	4.889	0.651, 100.3	0.172			
rPDQS score tertile at baseline						
1st tertile (reference)	—	—	—	—	—	—
2nd tertile	1.786	0.350, 9.699	0.486	16.34	1.071, 652.8	0.075
3rd tertile	5.417	1.271, 27.20	0.028	7.649	0.721, 130.4	0.113
MRI-PDFF (%)	0.951	0.854, 1.048	0.323			
Fasting glucose (mmol/L)	1.157	0.399, 3.427	0.787			
HbA1c (%)	1.048	0.149, 7.389	0.962			
ALT (U/L)	0.994	0.970, 1.013	0.572			
ALT ≥ 40 U/L	0.346	0.080, 1.299	0.128	0.591	0.048, 5.526	0.650
AST (U/L)	0.958	0.893, 1.012	0.166			
AST ≥ 40 (U/L)	0.226	0.011, 1.697	0.201			
Ferritin (pmol/L)	1.001	1.000, 1.002	0.227			
High relative abundance (defined as top 75%) of baseline gut bacterial species						
Faecalibacterium prausnitzii	2.714	0.707, 11.90	0.158			
Lachnospira pectinoschiza	8.750	1.914, 63.47	0.011	34.05	3.001, 1172	0.015
Anaerostipes hadrus	22.00	3.601, 429.2	0.005	34.99	2.209, 1919	0.032
Roseburia faecis	2.714	0.707, 11.90	0.158			
Roseburia inulinivorans	1.062	0.278, 4.068	0.928			
Agathobaculum butyriciproducens	4.615	1.138, 23.87	0.043	22.32	2.164, 632.6	0.023

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; aOR, adjusted odds ratio; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; GGT, gamma glutamyltransferase; HbA1c, haemoglobin A1c; MRI-PDFF, magenetic resonance imaging-proton density fat fraction; OR, odds ratio; rPDQS, rapid-prime diet quality score.

[a]Adequate level of exercises is defined as meeting the World Health Organization recommendation (at least 150–300 min of moderate-intensity or 75–150 min of vigorous-intensity aerobic exercise per week).

pathways that produced SCFAs, namely acetyl-CoA fermentation to butanoate II pathway ($r=0.47$, $p=0.024$) and pyruvate fermentation to acetate and (S)-lactate I pathway ($r=0.40$, $p=0.053$) with borderline significance. *A. hadrus* also had a significant positive correlation with the superpathway of L-lysine, L-threonine and L-methionine biosynthesis II ($r=0.44$, $p=0.035$). *F. prausnitzii* and *A. butyricidproducens* had significant positive correlation with L-arginine biosynthesis I pathway (*F. prausnitzii*: $r=0.71$, $p<0.001$; *A. butyriciproducens*: $r=0.45$, $p=0.027$), L-arginine biosynthesis II pathway (*F. prausnitzii*: $r=0.71$, $p<0.001$; *A. butyriciproducens*: r = 0.45, $p=0.031$) and superpathway of L-cysteine biosynthesis (mammalian) (*F. prausnitzii*: r = 0.86, $p<0.001$; *A. butyriciproducens*: $r=0.43$, $p=0.036$). *F. prausnitzii* and *A. butyriciproducens* were also positively correlated with L-ornithine biosynthesis I pathway with statistical significance ($r=0.66$, $p<0.001$) and with borderline significance ($r=0.39$, $p=0.060$), respectively. Additionally, *F. prausnitzii* had positive correlation with L-glutamate and L-glutamine biosynthesis pathway with borderline significance ($r=0.36$, $p=0.087$).

3.4 | Dynamic Changes of Gut Microbiota From Baseline to 1 Year

We found that there was no significant change in the relative abundance of the six putative gut bacterial species in both the treatment response (Figure S4) and non-response groups (Figure S5).

4 | Discussion

To our knowledge, our study was the first to investigate the association between gut microbiota composition and treatment response to empagliflozin in MASLD patients. We identified six potential baseline gut microbial markers, namely *F. prausnitzii, L. pectinoschiza, A. hadrus, R. faecis, R. inulinivorans* and *A. butyriciproducens*, as well as metabolic pathways that might predict treatment response to empagliflozin. Notably, *A. hadrus* remained enriched in the treatment response group regardless of lifestyle and remained predictive of treatment response on multivariable analysis including other clinical factors. *A. hadrus* was also positively correlated with pathways that produced SCFA and amino acids that might alleviate MASLD,

TABLE 3 | Performance of using high relative abundance of putative bacterial species in predicting empagliflozin treatment response.

	Lachnospira pectinoschiza	*Anaerostipes hadrus*	*Agathobaculum butyriciproducens*	Combination of three bacterial species
AUROC (95% CI)	0.814 (0.689–0.940)	0.779 (0.643–0.914)	0.765 (0.620–0.910)	0.893 (0.798–0.989)
Sensitivity	0.68 (0.45, 0.86)	0.32 (0.14, 0.55)	0.45 (0.24, 0.68)	0.82 (0.60, 0.95)
Specificity	0.83 (0.61, 0.95)	0.96 (0.78, 1.00)	0.87 (0.66, 0.97)	0.83 (0.61, 0.95)
PPV	0.79 (0.54, 0.94)	0.88 (0.47, 1.00)	0.77 (0.46, 0.95)	0.82 (0.60, 0.95)
NPV	0.73 (0.52, 0.88)	0.59 (0.42, 0.75)	0.62 (0.44, 0.79)	0.83 (0.61, 0.95)
PLR	3.92 (1.54, 9.99)	7.32 (0.98, 54.73)	3.48 (1.10, 11.01)	4.70 (1.89, 11.71)
NLR	0.39 (0.20, 0.73)	0.71 (0.53, 0.96)	0.63 (0.42, 0.95)	0.22 (0.09, 0.54)

Abbreviations: AUROC, area under receiver operating curve; CI, confidence interval; NLR, negative likelihood ratio; NPV, negative predictive value; PLR, positive likelihood ratio; PPV, positive predictive value.

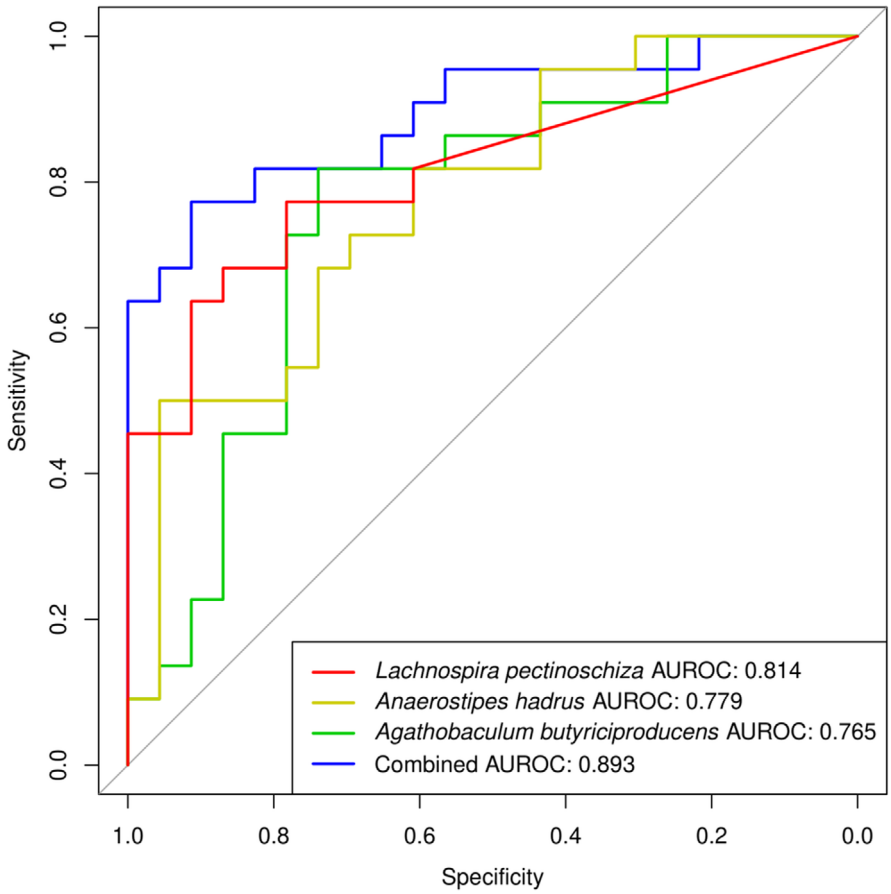

FIGURE 3 | Area under receiver operating characteristics curve of *Lachnospira pectinoschiza, Anaerostipes hadrus* and *Agathobaculuum butyriciproducens* and combination of these bacterial species in predicting treatment response to empagliflozin. AUROC, area under receiver operating curve.

highlighting it as a potential key bacterial species that might predict response to empagliflozin in treating MASLD. We summarise our study findings and prior literature in Figure 5.

Currently, weight reduction remains the most effective treatment for MASLD. Recommended pharmacological agents are mainly vitamin E and pioglitazone [38], but they are associated with various side effects, such as increased risk of prostate cancer and hemorrhagic stroke for vitamin E and weight gain, osteopenia/fracture, fluid retention, congestive heart failure and bladder cancer for pioglitazone. Newer agents like GLP-1 receptor agonists and resmetirom (a thyroid hormone receptor-beta agonist) also carry gastrointestinal side effects (nausea, vomiting, gastroesophageal reflux disease, constipation and diarrhoea) and are costly. In addition to its cardioprotective and renoprotective effects, SGLT-2 inhibitors showed promise as a potential treatment option for MASLD in patients with type 2 DM [9, 10] and without DM [13]. Furthermore, SGLT-2 inhibitors have been implicated in a lower risk of cancer incidence [39].

Gut microbiota has emerged as a potential factor that could influence the development and course of MASLD. Studies have found the presence of gut dysbiosis in MASLD patients and that certain gut microbiota signatures were consistently altered in MASLD patients compared with healthy individuals and mainly increased Gram-negative bacteria such as *Proteobacteria* and *Enterobacteriaceae* [14, 15]. On the genus level, consistent observations in MASLD patients included increased *Escherichia, Dorea and Peptoniphilus* and decreased *Anaerosporobacter, Coprococcus, Eubacterium, Faecalibacterium and Prevotella* [14, 15]. Potential mechanisms included the production of various metabolites by the gut microbiota, including SCFAs, bile acids and amino acids.

Our study demonstrated that there was significant difference in terms of baseline alpha- and beta-diversity between the treatment response and non-response groups, suggesting that gut dysbiosis might have hindered MASLD response to empagliflozin. On multivariable analysis, the high relative abundance of *L. pectinoschiza, A. hadrus* and *A. butyriciproducens* was significantly associated with treatment response, independent of other clinical factors. These three species collectively distinguished treatment response from no response with an AUROC of 0.89. Of note, baseline *L. pectinoschiza* and *A. hadrus* were enriched in the treatment response group regardless of lifestyle, making them more ideal as predictive markers. Notably, no other clinical factors could predict treatment response, except for weight loss > 5% at EOT. It is worth mentioning that these bacterial species remained predictive of treatment response independent of weight loss on sensitivity analysis, and weight

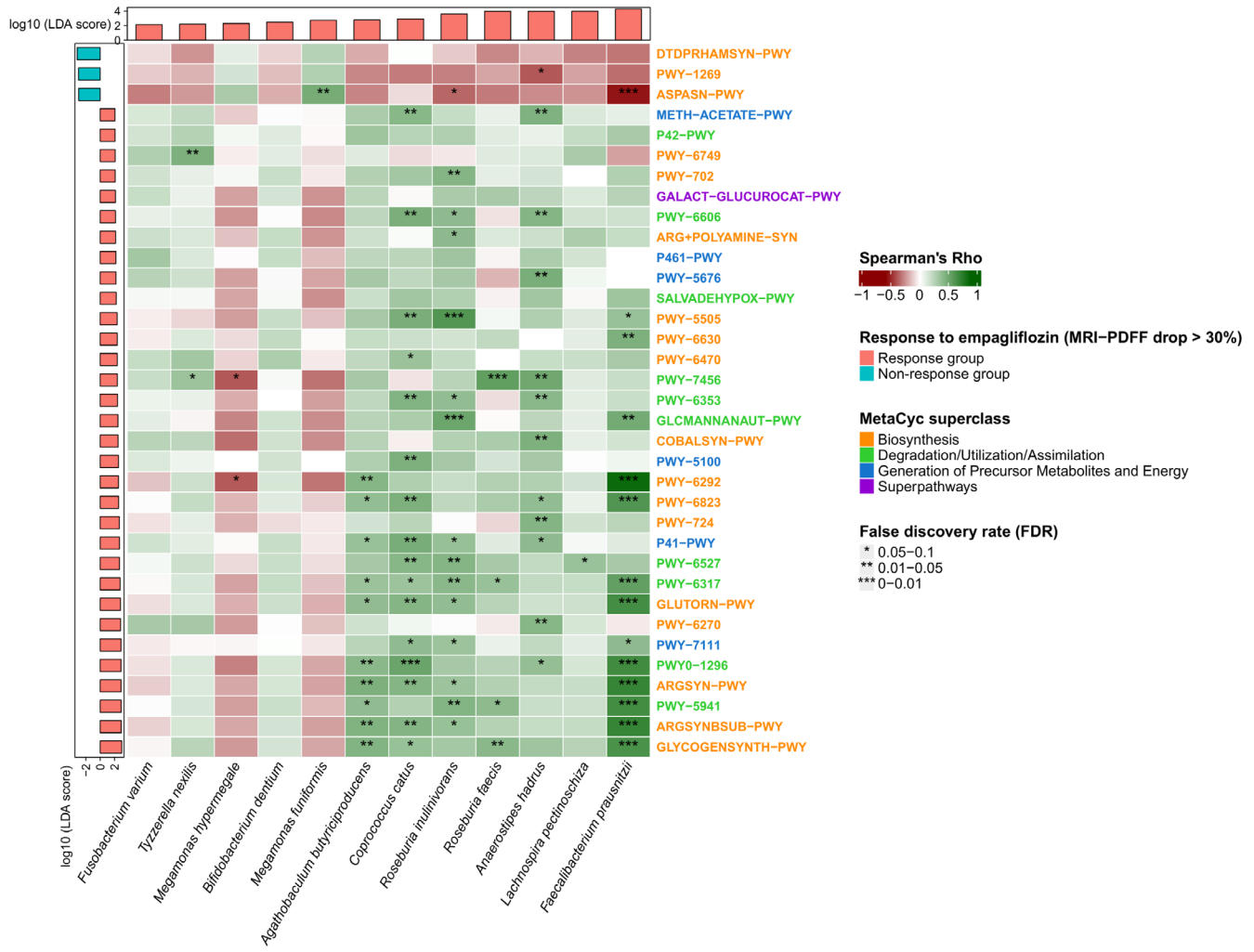

FIGURE 4 | Spearman correlation between baseline metabolic pathways and putative gut bacterial species.

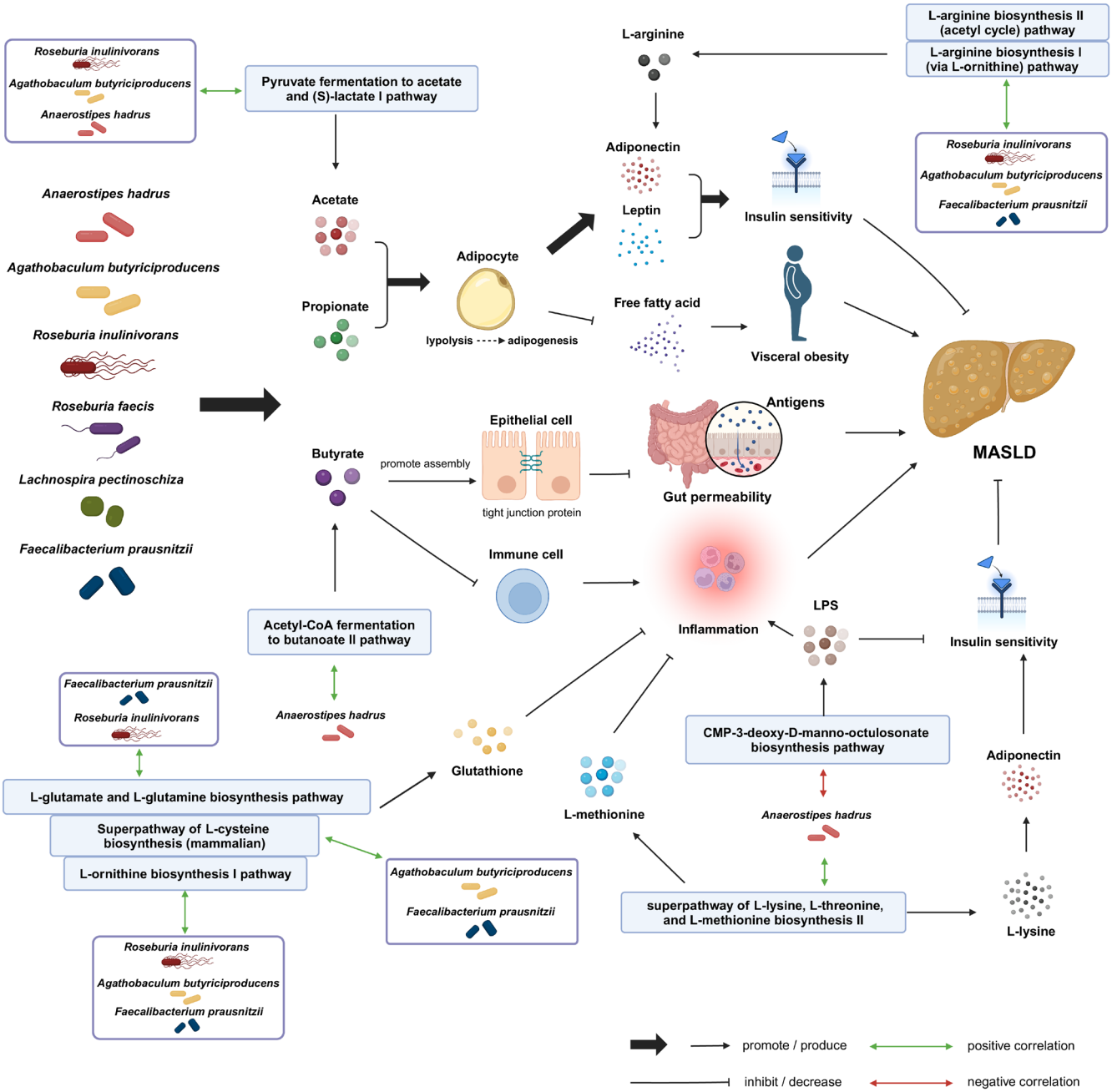

FIGURE 5 | Interaction between gut microbiota, metabolic pathways and metabolic dysfunction-associated steatotic liver disease based on current study findings and existing literature. *Anaerostipes hadrus, Agathobaculum butyriciproducens, Roseburia inulinivorans, Roseburia faecis, Lachnospira pectinoschiza* and *Faecalibacterium prausnitzii* produce at least one of the short-chain fatty acids (acetate, propionate and butyrate). Acetate and propionate facilitate adipocytes to produce adiponectin and leptin, which improve insulin sensitivity in cells and inhibit the production of free fatty acids to decrease visceral obesity. Butyrate can promote the assembly of tight junction proteins in gut epithelial cells, decrease gut permeability and inhibit immune cells to reduce inflammation. The pyruvate fermentation to acetate and (S)-lactate I pathway produces acetate to take function, while the acetyl-CoA fermentation to butanoate II pathway produces butyrate. The L-arginine biosynthesis II (acetyl cycle) pathway and L-arginine biosynthesis I (via L-ornithine) pathway produce L-arginine to increase the level of adiponectin. The superpathway of L-lysine, L-threonine, and L-methionine biosynthesis II produces L-lysine to increase adiponectin and L-methionine to inhibit inflammation. The superpathway of L-cysteine biosynthesis (mammalian), L-glutamate, and L-glutamine biosynthesis pathway, and L-ornithine biosynthesis I pathway produce glutathione to inhibit inflammation. The CMP-3-deoxy-D-manno-octulosonate biosynthesis pathway produces LPS, which promotes inflammation and dampens insulin sensitivity. LPS, lipopolysaccharides.

loss could not be used as a predictive marker of treatment response when commencement of empagliflozin for MASLD is being contemplated.

The potential beneficial role of these three species was also supported by human studies. A meta-analysis on publicly available sequencing data found that *A. hadrus* was depleted in patients

with progressive MASLD, supporting the possible beneficial role of *A. hadrus* in preventing MASLD progression [40]. An RCT which studied the effects of exercise and/or diet in treating MASLD also found that the family *Lachnospiraceae* and the genus *Ruminococcus* were increased in the intervention group [41], corroborating our study findings on the possible beneficial roles of *Anerostipes* and *Lachnospira* (members of the family *Lachnospiraceae*), as well as *Ruminococcus* in improving MASLD.

F. praustnitzii is also a well-known probiotic species. Administration of *F. prausnitzii* was shown to reduce hepatic fat content in high-fat-fed mice and improve hepatic transaminase levels [42]. Another study showed that a high hepatic fat content was correlated with a low abundance of *F. prausnitzii* [43]. Lipid metabolism-regulating adiponectin receptor (AdipoR) and lipid-oxidising citrate synthase (CS) were found to be overexpressed in *F. prausnitzii-treated* mice, which shifts fatty acid metabolism towards oxidation instead of synthesis [44]. *F. prausnitzii* treatment increased adiponectin expression (enhancing fat oxidation) and insulin sensitivity (increasing insulin receptor β (IRβ) expression and insulin-responsive hormone-sensitive lipase phosphorylation) [42]. *F. prausnitzii* may stimulate the expression of adiponectin through its cell wall, which is similar to that of Gram-positive bacteria [45]. These findings suggest that *F. prausnitzii* might aid in empagliflozin's effect on MASLD and thus can predict treatment response to empagliflozin.

Moreover, all six putative bacterial species enriched in the treatment response group produce SCFAs, including acetate, propionate and butyrate (Figure 5) [18, 46]. Acetate and propionate promote adipogenesis via GPCR43, inhibit lipolysis in adipocytes, reduce free fatty acids (FFAs) and alleviate liver fat deposition [16]. They also enhance leptin and adiponectin secretion, improving insulin sensitivity and glucose regulation [18]. Butyrate, meanwhile, has been shown to influence MASLD and MASH development by activating AMP-activated protein kinase (AMPK) and reducing inflammation [18].

Most of the pathways enriched in the response group are related to amino acid synthesis (Figure 5). The superpathway of L-lysine, L-threonine and L-methionine biosynthesis II positively correlated with *A. hadrus*, produces L-lysine and L-methionine, which enhance insulin sensitivity, reduce central obesity and attenuate hepatic injury [47]. Pathways for L-arginine, L-glutamate and L-glutamine synthesis, positively correlated with *F. prausnitzii* and *A. butyriciproducens*, were also enriched. L-arginine decreases fat deposition in the liver and intraperitoneal adipose tissue and downregulates concentrations of lipids and harmful lipoprotein in the serum [20]. The L-cysteine biosynthesis and L-ornithine biosynthesis pathways, which are positively correlated with *F. prausnitzii*, support glutathione production and are crucial for anti-inflammatory and metabolic functions in MASLD [19, 21, 48].

The pathways enriched in the non-response group were associated with antigen synthesis, such as lipopolysaccharides (LPS). 3-Deoxy-α-D-manno-octulosonate, produced by the CMP-3-deoxy-D-manno-octulosonate biosynthesis pathway, is a component of LPS. LPS induces liver inflammation by promoting NF-kB translocation through the LPS/TLR-4 pathway, leading to MASH [49]. Additionally, the LPS/TLR-4 pathway is also involved in insulin resistance [50].

We observed no significant changes in the relative abundances of the six putative bacterial species at 1 year from baseline. Other studies that have explored the effect of SGLT2 inhibitors on gut microbiome composition showed mixed results—one did not find significant effect on alpha diversity or microbial composition [51], while another found empagliflozin was associated with an increase in SCFA-producing bacteria and reduction in harmful bacteria such as *Escherichia–Shigella, Bilophila* and Hungatella [52].

Our study had some limitations. First, liver biopsy is the gold standard to diagnose and assess the histological severity of MASLD. However, it was not used due to its invasive nature. Instead, we used MRI-PDFF, which was considered the most accurate non-invasive test to quantify liver fat content [8]. Second, our cohort was composed of Chinese. As the gut microbiota composition varies greatly across different populations and geographic regions due to factors such as diet, lifestyle and socioeconomic status, our findings may not be generalizable to other populations. Third, validation of our study findings from other centers is lacking. Lastly, as our study only investigated the association of gut bacterial markers with treatment response to empagliflozin, it will be difficult to conclude if *A. hadrus* alone can serve as a substitute for empagliflozin. Further studies on animal models will be required to establish causal relationship, followed by randomised clinical trial to conclude the potential therapeutic role of *A. hadrus* in MASLD.

There are several clinical implications of our study findings. First, if further validated, the putative microbial markers could be used to assess whether a MASLD patient will respond to empagliflozin. It also lays the foundation for research into using stool microbial markers for predicting other approved medications for MASLD and may inspire further studies on animal models to characterise underlying mechanisms and causal relationships between the gut bacterial markers and treatment response to SGLT2 inhibitors in MASLD.

5 | Conclusions

Certain gut bacterial species could predict treatment response to empagliflozin in MASLD patients without DM. Further multi-center studies are warranted to confirm the study findings.

Author Contributions

Conceptualization: Ka Shing Cheung and Wai K. Leung. Methodology: Ka Shing Cheung and Rex Wan Hin Hui. Data collection: Jing Tong Tan and Rex Wan Hin Hui. Statistical analysis: Ho Yu Ng and Lina Zhang. Drafting of manuscript: Ho Yu Ng and Lina Zhang. Supervision and revision of the manuscript: Ka Shing Cheung, Wai Kay Seto, Man Fung Yuen and Wai K. Leung.

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Figure 5 was created with Biorender.com.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in Sequence Read Archive at https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1203401, reference number BioProject PRJNA1203401.

References

1. K. Riazi, H. Azhari, J. H. Charette, et al., "The Prevalence and Incidence of NAFLD Worldwide: A Systematic Review and meta-Analysis," *Lancet Gastroenterology & Hepatology* 7 (2022): 851–861.

2. R. Loomba, J. K. Lim, H. Patton, and H. B. el-Serag, "AGA Clinical Practice Update on Screening and Surveillance for Hepatocellular Carcinoma in Patients With Nonalcoholic Fatty Liver Disease: Expert Review," *Gastroenterology* 158 (2020): 1822–1830.

3. P. Burra, C. Becchetti, and G. Germani, "NAFLD and Liver Transplantation: Disease Burden, Current Management and Future Challenges," *JHEP Rep* 2 (2020): 100192.

4. M. S. Siddiqui, M. Fuchs, M. O. Idowu, et al., "Severity of Nonalcoholic Fatty Liver Disease and Progression to Cirrhosis Are Associated With Atherogenic Lipoprotein Profile," *Clinical Gastroenterology and Hepatology* 13 (2015): 1000–1008.e3.

5. A. Mantovani, A. Lonardo, N. Stefan, and G. Targher, "Metabolic Dysfunction-Associated Steatotic Liver Disease and Extrahepatic Gastrointestinal Cancers," *Metabolism* 160 (2024): 156014.

6. S. E. Keating, D. A. Hackett, J. George, and N. A. Johnson, "Exercise and Non-Alcoholic Fatty Liver Disease: A Systematic Review and Meta-Analysis," *Journal of Hepatology* 57 (2012): 157–166.

7. C. Komiya, K. Tsuchiya, K. Shiba, et al., "Ipragliflozin Improves Hepatic Steatosis in Obese Mice and Liver Dysfunction in Type 2 Diabetic Patients Irrespective of Body Weight Reduction," *PLoS One* 11 (2016): e0151511.

8. A. J. Scheen, "Beneficial Effects of SGLT2 Inhibitors on Fatty Liver in Type 2 Diabetes: A Common Comorbidity Associated With Severe Complications," *Diabetes & Metabolism* 45 (2019): 213–223.

9. M. S. Kuchay, S. Krishan, S. K. Mishra, et al., "Effect of Empagliflozin on Liver Fat in Patients With Type 2 Diabetes and Nonalcoholic Fatty Liver Disease: A Randomized Controlled Trial (E-LIFT Trial)," *Diabetes Care* 41 (2018): 1801–1808.

10. M. Shimizu, K. Suzuki, K. Kato, et al., "Evaluation of the Effects of Dapagliflozin, a Sodium-Glucose Co-Transporter-2 Inhibitor, on Hepatic Steatosis and Fibrosis Using Transient Elastography in Patients With Type 2 Diabetes and Non-Alcoholic Fatty Liver Disease," *Diabetes, Obesity & Metabolism* 21 (2019): 285–292.

11. F. Bonnet and A. J. Scheen, "Effects of SGLT2 Inhibitors on Systemic and Tissue Low-Grade Inflammation: The Potential Contribution to Diabetes Complications and Cardiovascular Disease," *Diabetes & Metabolism* 44 (2018): 457–464.

12. Z. M. Younossi, A. B. Koenig, D. Abdelatif, Y. Fazel, L. Henry, and M. Wymer, "Global Epidemiology of Nonalcoholic Fatty Liver Disease-Meta-Analytic Assessment of Prevalence, Incidence, and Outcomes," *Hepatology* 64 (2016): 73–84.

13. K. S. Cheung, H. Y. Ng, R. W. H. Hui, et al., "Effects of Empagliflozin on Liver Fat in Patients With Metabolic Dysfunction-Associated Steatotic Liver Disease Without Diabetes Mellitus: A Randomized, Double-Blind, Placebo-Controlled Trial," *Hepatology* 80 (2024): 916–927.

14. T. Hrncir, L. Hrncirova, M. Kverka, et al., "Gut Microbiota and NAFLD: Pathogenetic Mechanisms, Microbiota Signatures, and Therapeutic Interventions," *Microorganisms* 9, no. 5 (2021): 957, https://doi.org/10.3390/microorganisms9050957.

15. J. Aron-Wisnewsky, C. Vigliotti, J. Witjes, et al., "Gut Microbiota and Human NAFLD: Disentangling Microbial Signatures From Metabolic Disorders," *Nature Reviews. Gastroenterology & Hepatology* 17 (2020): 279–297.

16. Y.-H. Hong, Y. Nishimura, D. Hishikawa, et al., "Acetate and Propionate Short Chain Fatty Acids Stimulate Adipogenesis via GPCR43," *Endocrinology* 146 (2005): 5092–5099.

17. G. Tolhurst, H. Heffron, Y. S. Lam, et al., "Short-Chain Fatty Acids Stimulate Glucagon-Like Peptide-1 Secretion via the G-Protein–Coupled Receptor FFAR2," *Diabetes* 61 (2012): 364–371.

18. H. E. Leylabadlo, R. Ghotaslou, M. M. Feizabadi, et al., "The Critical Role of *Faecalibacterium prausnitzii* in Human Health: An Overview," *Microbial Pathogenesis* 149 (2020): 104344.

19. R. F. Butterworth and A. Canbay, "Hepatoprotection by L-Ornithine L-Aspartate in Non-Alcoholic Fatty Liver Disease," *Digestive Diseases* 37 (2019): 63–68.

20. C. Sellmann, C. Degen, C. J. Jin, et al., "Oral Arginine Supplementation Protects Female Mice From the Onset of Non-Alcoholic Steatohepatitis," *Amino Acids* 49, no. 7 (2017): 1215-1225.

21. M. Irie, T. Sohda, A. Anan, et al., "Reduced Glutathione Suppresses Oxidative Stress in Nonalcoholic Fatty Liver Disease," *Euroasian Journal of Hepato-Gastroenterology* 6, no. 1 (2016): 13–18, https://doi.org/10.5005/jp-journals-10018-1159.

22. X. Li, W. Ma, T. Yang, et al., "Higher Intakes of Lysine, Threonine and Valine Are Inversely Associated With Non-Alcoholic Fatty Liver Disease Risk: A Community-Based Case-Control Study in the Chinese Elderly," *Food Science and Human Wellness* 13 (2024): 191–197.

23. A. Tang, J. Tan, M. Sun, et al., "Nonalcoholic Fatty Liver Disease: MR Imaging of Liver Proton Density Fat Fraction to Assess Hepatic Steatosis," *Radiology* 267 (2013): 422–431.

24. M. E. Rinella, J. V. Lazarus, V. Ratziu, et al., "A Multi-Society Delphi Consensus Statement on New Fatty Liver Disease Nomenclature," *Hepatology* 78 (2023): 1966-1986.

25. M. L. Wheeler, M. Franz, P. Barrier, H. Holler, N. Cronmiller, and L. M. Delahanty, "Macronutrient and Energy Database for the 1995 Exchange Lists for Meal Planning: A Rationale for Clinical Practice Decisions," *Journal of the American Dietetic Association* 96 (1996): 1167–1171.

26. S. Kronsteiner-Gicevic, M. Tello, L. E. Lincoln, et al., "Validation of the Rapid Prime Diet Quality Score Screener (rPDQS), A Brief Dietary Assessment Tool With Simple Traffic Light Scoring," *Journal of the Academy of Nutrition and Dietetics* 123 (2023): 1541–1554 e7.

27. F. C. Bull, S. S. Al-Ansari, S. Biddle, et al., "World Health Organization 2020 Guidelines on Physical Activity and Sedentary Behaviour," *British Journal of Sports Medicine* 54 (2020): 1451–1462.

28. K. V. Kowdley, P. Belt, L. A. Wilson, et al., "Serum Ferritin Is an Independent Predictor of Histologic Severity and Advanced Fibrosis in Patients With Nonalcoholic Fatty Liver Disease," *Hepatology* 55 (2012): 77–85.

29. J. G. Stine, N. Munaganuru, A. Barnard, et al., "Change in MRI-PDFF and Histologic Response in Patients With Nonalcoholic Steatohepatitis: A Systematic Review and Meta-Analysis," *Clinical Gastroenterology and Hepatology* 19 (2021): 2274–2283.e5.

30. R. Loomba, B. A. Neuschwander-Tetri, A. Sanyal, et al., "Multicenter Validation of Association Between Decline in MRI-PDFF and Histologic Response in NASH," *Hepatology* 72 (2020): 1219–1229.

31. S. Chen, Y. Zhou, Y. Chen, and J. Gu, "fastp: an ultra-fast all-in-one FASTQ preprocessor," *Bioinformatics* 34 (2018): i884–i890.

32. B. Langmead and S. L. Salzberg, "Fast Gapped-Read Alignment With Bowtie 2," *Nature Methods* 9 (2012): 357–359.

33. D. T. Truong, E. A. Franzosa, T. L. Tickle, et al., "MetaPhlAn2 for Enhanced Metagenomic Taxonomic Profiling," *Nature Methods* 12 (2015): 902–903.

34. F. Beghini, L. J. McIver, A. Blanco-Miguez, et al., "Integrating Taxonomic, Functional, and Strain-Level Profiling of Diverse Microbial Communities With bioBakery 3," *eLife* 10 (2021): e65088.

35. H. Y. Ng, Y. Liao, R. Zhang, et al., "The Predictive Value of Gut Microbiota Composition for Sustained Immunogenicity Following Two Doses of CoronaVac," *International Journal of Molecular Sciences* 25 (2024): 2583.

36. L. N. Zhang, J. T. Tan, H. Y. Ng, et al., "Association Between Gut Microbiota Composition and Long-Term Vaccine Immunogenicity Following Three Doses of CoronaVac," *Vaccines (Basel)* 12, no. 4 (2024): 365, https://doi.org/10.3390/vaccines12040365.

37. Y. Benjamini and Y. Hochberg, "Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing," *Journal of the Royal Statistical Society. Series B (Methodological)* 57 (1995): 289–300.

38. M. E. Rinella, B. A. Neuschwander-Tetri, M. S. Siddiqui, et al., "AASLD Practice Guidance on the Clinical Assessment and Management of Nonalcoholic Fatty Liver Disease," *Hepatology* 77 (2023): 1797–1835.

39. R. Benedetti, G. Benincasa, K. Glass, et al., "Effects of Novel SGLT2 Inhibitors on cancer Incidence in Hyperglycemic Patients: A meta-Analysis of Randomized Clinical Trials," *Pharmacological Research* 175 (2022): 106039.

40. T. Wang, X. K. Guo, and H. Xu, "Disentangling the Progression of Non-Alcoholic Fatty Liver Disease in the Human Gut Microbiota," *Frontiers in Microbiology* 12 (2021): 728823.

41. R. Cheng, L. Wang, S. Le, et al., "A Randomized Controlled Trial for Response of Microbiome Network to Exercise and Diet Intervention in Patients With Nonalcoholic Fatty Liver Disease," *Nature Communications* 13 (2022): 2555.

42. E. Munukka, A. Rintala, R. Toivonen, et al., "*Faecalibacterium prausnitzii* Treatment Improves Hepatic Health and Reduces Adipose Tissue Inflammation in High-Fat Fed Mice," *ISME Journal* 11 (2017): 1667-1679.

43. E. Munukka, S. Pekkala, P. Wiklund, et al., "Gut-Adipose Tissue axis in Hepatic Fat Accumulation in Humans," *Journal of Hepatology* 61 (2014): 132–138.

44. J. Ha, S. Daniel, S. S. Broyles, and K. H. Kim, "Critical Phosphorylation Sites for Acetyl-CoA Carboxylase Activity," *Journal of Biological Chemistry* 269 (1994): 22162–22168.

45. M. Lopez-Siles, T. M. Khan, S. H. Duncan, H. J. M. Harmsen, L. J. Garcia-Gil, and H. J. Flint, "Cultured Representatives of Two Major Phylogroups of Human Colonic *Faecalibacterium prausnitzii* Can Utilize Pectin, Uronic Acids, and Host-Derived Substrates for Growth," *Applied and Environmental Microbiology* 78 (2012): 420–428.

46. W. Fusco, M. B. Lorenzo, M. Cintoni, et al., "Short-Chain Fatty-Acid-Producing Bacteria: Key Components of the Human Gut Microbiota," *Nutrients* 15 (2023): 2211.

47. K. Lian, Y. N. Feng, R. Li, et al., "Middle- and High-Molecular Weight Adiponectin Levels in Relation to Nonalcoholic Fatty Liver Disease," *Journal of Clinical Laboratory Analysis* 34 (2020): e23148.

48. S. K. Jain, D. Micinski, L. Huning, G. Kahlon, P. F. Bass, and S. N. Levine, "Vitamin D and L-Cysteine Levels Correlate Positively With GSH and Negatively With Insulin Resistance Levels in the Blood of Type 2 Diabetic Patients," *European Journal of Clinical Nutrition* 68, no. 10 (2014): 1148-1153.

49. J.-B. Soares, P. Pimentel-Nunes, R. Roncon-Albuquerque, and A. Leite-Moreira, "The Role of Lipopolysaccharide/Toll-Like Receptor 4 Signaling in Chronic Liver Diseases," *Hepatology International* 4 (2010): 659-672.

50. P. D. Cani, J. Amar, M. A. Iglesias, et al., "Metabolic Endotoxemia Initiates Obesity and Insulin Resistance," *Diabetes* 56 (2007): 1761–1772.

51. E. J. M. van Bommel, H. Herrema, M. Davids, M. H. H. Kramer, M. Nieuwdorp, and D. van Raalte, "Effects of 12-Week Treatment With Dapagliflozin and Gliclazide on Faecal Microbiome: Results of a Double-Blind Randomized Trial in Patients With Type 2 Diabetes," *Diabetes & Metabolism* 46 (2020): 164–168.

52. X. Deng, C. Zhang, P. Wang, et al., "Cardiovascular Benefits of Empagliflozin Are Associated With Gut Microbiota and Plasma Metabolites in Type 2 Diabetes," *Journal of Clinical Endocrinology & Metabolism* 107 (2022): 1888–1896.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.