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# Steatotic Liver Disease in Younger Adults is Associated With Altered Gut Microbiology

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## ABSTRACT

**Background and Aims:** Steatotic liver disease (SLD) is a leading cause of chronic liver disease worldwide. As SLD pathogenesis has been linked to gut microbiome alterations, we aimed to identify SLD-associated gut microbiome features early in SLD development by utilising a highly characterised cohort of community-dwelling younger adults.

**Methods and Results:** At age 27 years, 588 participants of the Raine Study Generation 2 underwent cross-sectional assessment. Hepatic steatosis was quantified using a validated magnetic resonance imaging (MRI) volumetric liver fat fraction (VLFF) equation (HepaFat). Of the 588 participants, 488 (83%) were classified as having 'no SLD' (VLFF ≤ 3.55%), 76 (12.9%) with 'mild–moderate' SLD (VLFF: 3.56%–13.4%) and 24 (4.10%) with 'severe' SLD (VLFF > 13.4%). Stool microbiome profiling identified an association between severe SLD and lower microbiota alpha diversity (observed features [$p = 0.015$], Pielou evenness [$p = 0.001$] and Shannon diversity [$p = 0.002$]) compared to no SLD. Faecal microbiota composition differed significantly between no SLD and both mild–moderate ($p = 0.004$) and severe SLD groups ($p = 0.001$). There was no significant difference in microbiota dispersion between SLD groups. Reduced relative abundance of short-chain fatty acid producing bacteria, and higher levels of proinflammatory bacterial taxa, were both significantly associated with severe SLD ($q < 0.05$).

**Abbreviations:** ALD, alcohol-associated liver disease; AMPK, AMP-activated protein kinase; ASVs, amplicon sequence variants; BMI, body mass index; BP, blood pressure; DQES v2, Cancer Council Victoria Dietary Questionnaire for Epidemiological Studies; FDR, False-Discovery Rate; Gen2, generation 2; HOMA-IR, homeostasis model of insulin resistance; IPAQ, International Physical Activity Questionnaire; IQR, interquartile range; IR, insulin resistance; LinDA, linear models for differential abundance; LPS, lipopolysaccharide; MASH, metabolic dysfunction-associated steatohepatitis; MASLD, metabolic dysfunction-associated steatotic liver disease; Met-ALD, MASLD and increased alcohol intake; MRI, magnetic resonance imaging; NMDS, Non-Metric Multi-Dimensional Scaling; PAMPs, pathogen-associated molecular patterns; PERMANOVA, permutational analysis of variance; PPI, proton pump Inhibitor; QIIME2, Quantitative Insights Into Microbial Ecology 2; SCFA, short-chain fatty acids; SLD, steatotic liver disease; VLFF, volumetric liver fat fraction.

Geraint B. Rogers and Leon A. Adams share senior authorship.

**Conclusions:** SLD in younger adults is associated with reduced intestinal microbial diversity and a pattern of bacterial taxa depletion that is consistent with other chronic inflammatory conditions. Our characterisation of gut microbiome characteristics in early SLD development provides a potential basis for risk identification and reduction.
**Trial Registration:** The Raine Study is registered in the Australian New Zealand Clinical Trials Registry (ACTRN12617001599369)

## 1 | Introduction

Steatotic liver disease (SLD) is a leading cause of chronic liver disease worldwide [1, 2]. The most common aetiology of SLD, metabolic dysfunction-associated steatotic liver disease (MASLD), has a global prevalence of 30% in adults [2] with a predicted prevalence of 56% by 2040 [3]. MASLD is defined by an excessive amount of fat in the liver (steatosis) that is unaccounted for by factors such as alcohol use, and which is associated with cardiometabolic risk factors [1]. A subset (approximately 14%) develop metabolic dysfunction-associated steatohepatitis (MASH) [4], characterised by progressive inflammation, hepatocyte damage and pericellular fibrosis, which may progress to advanced fibrosis and cirrhosis.

Multiple components of the gut-liver axis are believed to influence the development of MASLD, including changes in intestinal permeability, immune homeostasis, systemic inflammation and insulin resistance (IR) [5]. IR is a major contributor to intracellular lipid accumulation in the liver, which in turn impairs insulin signalling and worsens IR [6]. As MASLD progresses, the resultant increase in IR exacerbates liver steatosis through an influx of free fatty acids into hepatocytes, which act as substrates for *de novo* hepatic lipogenesis [6]. The metabolic load from the free fatty acid influx contributes to endoplasmic reticulum stress and hepatic lipotoxicity, further contributing to disease progression [6].

Increased gut permeability results in greater transmission of bacteria and pathogen-associated molecular patterns (PAMPs) to the liver via the portal circulation. For example, animal studies have demonstrated that lipopolysaccharide (LPS), derived from the outer membrane of gram-negative bacteria, triggers systemic and hepatic inflammation and an inflammatory cascade that gives rise to IR, thereby further promoting the accumulation of hepatic lipid [7]. Furthermore, as MASLD progresses to advanced fibrosis, gram-negative bacteria become more prevalent within the gut microbiota, and corresponding increases in circulating LPS are likely to further augment hepatic inflammation, contributing to the development of advanced fibrosis [8].

Gut microbes can also produce factors that are protective against steatotic liver disease. These include short-chain fatty acids (SCFA), principally butyrate, acetate and propionate, that are synthesised through saccharolytic fermentation of carbohydrates in the colon. Transport of SCFAs to the liver via the portal circulation are integral to liver health, regulating hepatic metabolism and FFA flux from adipocytes [9]. SCFAs also act as substrates for hepatic gluconeogenesis and *de novo* lipogenesis [9, 10], and contribute to the integrity of the gut epithelial barrier [7, 9].

The majority of clinical studies investigating the gut microbiome in SLD have involved small case–control cohorts and have focused on aging populations, where confounding factors, such as diabetes and medications, are more prevalent [11]. These investigations have also typically relied on less accurate approaches to SLD characterisation, such as ultrasound [11]. A meta-analysis seeking to identify a gut microbiome signature in SLD found significant heterogeneity between studies [11]. Whilst some studies reported MASLD to be associated with lower microbial diversity and altered stool microbiota composition [8, 12–16] others described an increase in diversity or no association [11]. Broadly, those with SLD exhibited a lower microbial diversity, an increased prevalence of proinflammatory bacterial genera, such as *Escherichia* and *Fusobacterium*, and a lower relative abundance of taxa such as *Coprococcus* and *Ruminococcaceae*, which are known to produce the anti-inflammatory metabolite butyrate [11].

Whilst the contribution of the gut microbiome to SLD pathogenesis may provide an opportunity to predict or prevent disease development, such strategies require a deeper understanding of microbiological changes that occur in younger individuals, prior to the development of advanced liver disease. To address this knowledge gap, we investigated the relationship between intestinal microbiome characteristics with markers of liver steatosis in a well-characterised population of community-dwelling younger adults from the Raine Study.

## 2 | Methods

### 2.1 | Study Population

Participants and metadata were drawn from the Raine Study, a prospective longitudinal multigenerational cohort study based in Western Australia [17]. The Raine Study was conducted in accordance with the Declaration of Helsinki and was approved by the University of Western Australia Human Research Ethics committee (reference number: 2022/ET000237). All participants provided written, informed consent. The Raine Study is registered in the Australian New Zealand Clinical Trials Registry (ACTRN12617001599369).

Our assessment focused on the Generation 2–27 year follow-up undertaken between 2016 and 2018, which is broadly representative of the wider Australian population at this age [17]. Follow-up included a comprehensive general medical history and lifestyle questionnaire, anthropometric measurements by trained assessors, magnetic resonance imaging (MRI) of the liver and faecal sample collection for microbiome analysis. Blood samples were taken after an overnight fast and analysed at the PathWest Laboratory, Royal Perth Hospital, for liver function tests, glucose, lipids, high sensitivity C-reactive protein and insulin.

To be included, participants were required to have undergone an MRI, completed the study general questionnaire and provided a

**Summary**

- Steatotic/fatty liver disease (SLD) is a leading cause of chronic liver disease worldwide.
- SLD development has been linked to the types of microbes, which live within the lower intestinal tract.
- We have found that younger adults with SLD have fewer types of bacteria in their gut and lower levels of specific bacteria, as has been reported in certain other chronic inflammatory conditions.

faecal sample for microbiome analysis. Exclusion criteria were potentially other causes of liver disease or hepatic steatosis, such as viral hepatitis, autoimmune hepatitis and conditions which may impact the gut microbiome including: inflammatory bowel disease, coeliac disease, diagnosed malignancy (excluding non-melanoma skin cancer), immunosuppressant medications (methotrexate and azathioprine) and previous bariatric surgery (including gastric lap band surgery).

### 2.2 | Liver Imaging

Liver MRI studies were conducted with a Siemens Magnetom Aera 1.5T (Siemens AG, Erlangen, Germany) [18]. Hepatic steatosis (dependent variable) was quantified using a validated volumetric liver fat fraction (VLFF) equation (HepaFat) as previously described by St Pierre et al. and approved by the Food and Drug Administration [19]. An MRI volumetric liver fat fraction cut-off of greater than 3.55% has been validated with comparison to liver histology to confirm the presence and severity of steatosis [19, 20]. Participants were initially divided into those with and without SLD for analysis using the cut-off of $>3.55\%$. Following the exclusion of those without SLD (VLFF $\leq 3.55\%$, $n=488$), VLFF values for those with SLD (VLFF $>3.55\%$, $n=100$) were sub-divided into quartiles. Participants were then divided into three groups for analysis; no SLD (VLFF $\leq 3.55\%$), mild–moderate SLD (VLFF 3.56%–13.4%) and severe SLD (VLFF $>13.4\%$). Severe SLD was defined by membership of the upper quartile (VLFF $>13.4\%$, $n=24$).

### 2.3 | Faecal Samples, DNA Extraction and 16S rRNA Gene Amplicon Sequencing

Stool samples were collected from participants using Omnigene GUT collection kits (DNA Genotek, Ottawa, Canada) in accordance with the manufacturer's instructions. Approximately 0.5 mL of stool was aliquoted into a 2 mL screw cap and stored at −80°C until analysis. Bacterial DNA was extracted from all faecal samples using QIAamp PowerFecal Pro DNA Kit (QIAGEN, Straße, Hilden, Germany) as per the manufacturer's instructions after homogenising samples using FastPrep (MP Biomedicals, Santa Ana, USA) for bead-beating (4 m/s for 30s + 30s cooling +30s). DNA concentration was then quantified using Quant-iT.

The Illumina 16S Metagenomic Sequencing Library Preparation protocol (Illumina Inc., San Diego, USA) was used with modifications to perform 16S rRNA gene sequencing, as previously described [21]. Modified universal primers 27F and 519R were used to generate amplicons of the V4 hypervariable region. In order to enable multiplexing, the Nextera XT Index kit (Illumina) was used to conduct dual-indexing of amplicons. Quantification of barcoded libraries was performed using Quant-iT dsDNA HS Assay Kit (Thermo Fisher Scientific, USA) and samples were pooled at equal concentration. Total bacterial abundance was determined using a validated 16S rRNA gene qPCR assay [22]. Total bacterial abundance data were used to convert the relative abundance of bacterial taxa that were identified as discriminant based on SLD parameters to obtain absolute abundance equivalents.

Paired-end sequencing of the final library using a Miseq Reagent kit v3 ($2\times300$ bp) was performed on a Miseq Illumina platform by the South Australian Genomics Centre (Adelaide, Australia). Paired-end 16S rRNA sequence reads were then merged, demultiplexed and amplicon sequence variants (ASVs) assigned using Quantitative Insights Into Microbial Ecology 2 (QIIME2) (release 2023.9) and the DADA2 plugin. Taxonomic assignment was performed against the SILVA 16S ribosomal database (version 132) (clustered at 97% sequence identity). Once spurious sequence variants were removed, samples were subsampled to a read depth of 8525 (lowest read depth) for microbiome analysis. Taxa relative abundances were calculated at phylum and genus level. Metrics for determining alpha diversity and Bray–Curtis dissimilarity were determined using QIIME2. Raw sequencing data is publicly accessible from the NCBI SRA database (Bioproject ID PRJNA1182921).

### 2.4 | Metadata

Metadata were collected from the comprehensive self-reported general questionnaires, MRI, blood tests, clinical and anthropometric measures [18]. Sex was included as a binary (female/male) variable, as reflected by biological status at birth. Comorbidities and medication use were self-reported and coded as a yes/no from the general questionnaire. Exposure to antibiotics was related to a 6-month period prior to stool sample collection. As the veracity of antibiotic use data could not be independently verified, the data was not sufficiently robust to merit inclusion. Proton pump inhibitor (PPI) use in participants was low overall ($<1\%$), lacking sufficient statistical power for inclusion in sub-analyses. Body mass index (BMI) was calculated using anthropometric measurements. Self-reported alcohol intake was included as a covariate due to known associations between steatosis and alcohol intake. Alcohol consumption was quantified using a 7-day recall questionnaire with pictorial representation of the type and serves of alcohol consumed each day recorded and converted to grams per day. Physical activity was assessed using the International Physical Activity Questionnaire [23]. Daily caloric intake was calculated from the Cancer Council Victoria Dietary Questionnaire for Epidemiological Studies (DQES v2) [24].

### 2.5 | Statistical Analysis

Characteristics of the study population across SLD status were assessed using Mann-Whitney and Kruskal–Wallis tests for

continuous non-normally distributed variables and chi-squared test for categorical variables. All data were checked for normality using Kolmogorov–Smirnov test.

Microbiota analysis was performed based on both SLD status and by SLD severity (no SLD, mild–moderate and severe SLD). Alpha diversity metrics (Shannon diversity, observed features and Pielou's evenness) were compared using Mann–Whitney and Kruskal–Wallis tests for nonparametric data, and visualised using box and whisker plots in GraphPad Prism (version 10.0.2). Beta-diversity (Bray–Curtis dissimilarity) were compared by permutational analysis of variance (PERMANOVA) using the function 'adonis2' from the vegan R package (v2.6-4), adjusting for potential covariates for steatosis (sex, BMI and alcohol intake) [25]. Beta-diversity dispersion was also compared using the PERMDISP PRIMER 7 (version 7.0.23) from PRIMER-e with the PERMANOVA+ package [26]. Beta-diversity differences were visualised by Non-metric Multi-Dimensional Scaling

**TABLE 1** | The Raine Study Gen2-27 year follow-up population characteristics by steatotic liver disease status.

| | SLD, $n = 100$ (17%) | No SLD, $n = 488$ (83%) | $p$ |
|---|---|---|---|
| Female (%) | 39 (39%) | 266 (54.5%) | 0.005 |
| Current smoker (%)[a] | 15 (15%) | 69 (14%) | 0.836 |
| Alcohol intake (g/day)[b] | 5.91 (22.7) | 9.43 (22.5) | 0.176 |
| ≥ 20 g per day in females ($n$, %) | 4 (10.3%) | 54 (20.3%) | 0.170 |
| ≥ 30 g per day in males ($n$, %) | 17 (27.9%) | 56 (25.2%) | 0.480 |
| Energy intake (including fibre) (kcalories/day)[c] | 1880 (552) | 1844 (724) | 0.862 |
| IPAQ: Total MET minutes per week[d] | 1422 (3762) | 1949 (3336) | 0.341 |
| Body Mass Index (kg/m$^2$) | 31 (11) | 23 (5) | < 0.001 |
| Waist circumference average (cm) | 102 (22) | 79 (13) | < 0.001 |
| Average systolic BP (mmHg) | 122 (16) | 115 (13) | < 0.001 |
| Average diastolic BP (mmHg) | 74 (12) | 69 (10) | < 0.001 |
| MRI liver fat average (%) | 7.0 (8.15) | 1.3 (0.8) | < 0.001 |
| Steatotic Liver Disease | 100 | | |
| Mild–moderate SLD (VLFF 3.56–13.4) | 76 (76%) | | |
| Severe SLD (VLFF > 13.4) | 24 (24%) | | |
| Glucose (mg/dL)[a] | 90 (9) | 84.6 (9) | < 0.001 |
| Insulin (mU/L)[e] | 10 (6) | 5 (2) | < 0.001 |
| HOMA-IR[a] | 2.3 (1.6) | 1 (0.7) | < 0.001 |
| Cholesterol (mmol/L)[f] | 5.1 (1.4) | 4.7 (1.1) | 0.004 |
| Triglycerides (mmol/L)[f] | 1.3 (0.8) | 0.8 (0.5) | < 0.001 |
| Low Density Lipoprotein cholesterol (mmol/L)[f] | 3.2 (1.1) | 2.7 (1) | < 0.001 |
| High density lipoprotein cholesterol (mmol/L)[f] | 1.2 (0.2) | 1.5 (0.4) | < 0.001 |
| High sensitivity C-reactive protein (mg/L)[e] | 2.5 (5.8) | 0.7 (1.4) | < 0.001 |
| ALT (U/L)[f] | 45 (31) | 23 (14) | < 0.001 |
| AST (U/L)[g] | 30 (14) | 26 (8) | < 0.001 |
| PPI use[e] | 1 (1%) | 4 (0.8%) | 0.858 |
| Antibiotic use[g] | 14 (14%) | 60 (12.3%) | 0.656 |

*Note:* Data are presented as median value (interquartile range [IQR]), or as number (percentage). $p$ value assessed using Mann-Whitney or Chi-squared tests.
Abbreviations: BP, blood pressure; HOMA-IR, homeostasis model of insulin resistance (Fasting glucose (mmol/L) × fasting insulin (mU/L)/22.5) and proton pump inhibitor (PPI); IPAQ, International Physical Activity Questionnaires, Total metabolic equivalent task minutes per week; SLD, steatotic liver disease.
[a]Data for $n = 580$ participants.
[b]Data for $n = 542$ participants.
[c]Data for $n = 586$ participants.
[d]Data for $n = 584$ participants.
[e]Data for $n = 582$ participants.
[f]Data for $n = 581$ participants.
[g]Data for $n = 579$ participants.

(NMDS) plots using PRIMER. Bacterial relative and absolute abundances were compared using Linear models for differential abundance (LinDA), adjusting for sex, BMI and alcohol intake and applying a False-Discovery Rate (FDR) threshold $<0.10$ [27]. For all other statistical tests, a $p$ value $<0.05$ was considered statistically significant.

## 3 | Results

### 3.1 | Participant Characteristics

Nine hundred and sixty-nine participants of the Raine Study Gen 2 underwent an MRI liver at the age of 27 years (Table S1). Of these, 588 were eligible for inclusion based on the availability of clinical, health and lifestyle data and faecal samples (Table 1; Figure 1). Median participant BMI was 24 kg/m$^2$ [range 25–50, IQR 6] and 305 of the 588 participants (51.9%) were female.

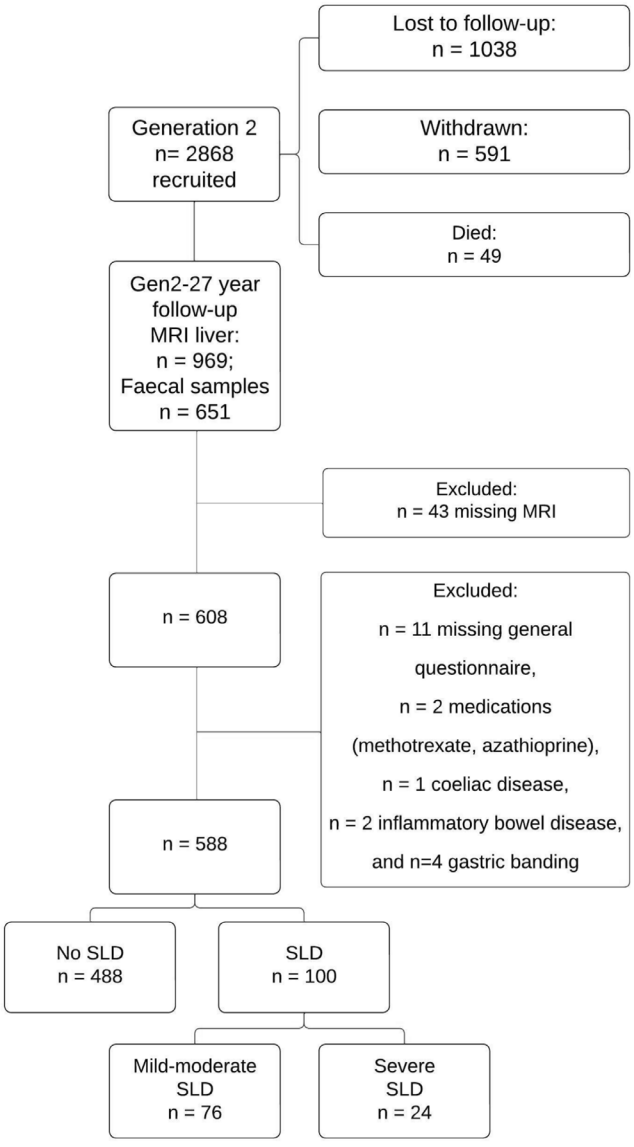


**FIGURE 1** | The Raine Study Generation 2 (Gen2) participant flow diagram including the Gen2-27 year follow-up.

One hundred participants (17%) had SLD, of whom 76 (12.9%) were classified as having mild–moderate SLD and 24 (4.1%) were classified as having severe SLD. Participants with SLD displayed significantly greater adiposity, metabolic dysfunction (higher blood pressure, insulin resistance and lipid levels) and liver enzyme levels. The proportion of female participants was significantly lower in the SLD group compared to those without SLD (39% vs. 54.5%, $p=0.005$). Alcohol consumption was not significantly different between those with or without SLD. Whilst alcohol consumption was lower in those with severe SLD compared to those with no, or mild–moderate, SLD, this association was not statistically significant ($p=0.100$). Due to the low number of participants with MASLD and increased alcohol intake (met-ALD) and alcohol-associated liver disease (ALD) (Table S2), statistical power was lacking to perform a sub-analysis comparing microbiome characteristics between MASLD, met-ALD and ALD.

### 3.2 | Gut Microbiota Composition in Relation to SLD Status

Consistent with other community-based adult cohort studies, commensal obligate anaerobic bacteria dominated the core stool microbiota (taxa being present in at least 80% of participants and with a minimum relative abundance of at least 0.01), including common fermentative genera, such as *Bacteroides*, *Blautia*, *Faecalibacterium* and *Anaerostipes* (Table S3; Figure S1). Compared to no SLD, those with SLD exhibited lower alpha diversity (Pielou's evenness and Shannon diversity) ($p<0.05$) (Figure 2A–C). Faecal microbiota composition differed significantly between those with and without SLD ($p=0.0001$). This relationship remained significant after adjusting for BMI, sex and alcohol intake ($p=0.001$). There was no significant difference in microbiota dispersion between those with and without SLD ($p$[perm] $=0.851$).

Whilst a sex effect was identified in relation to microbiota dispersion ($p=0.0011$), no significant difference in microbiota composition was identified between those with and without SLD when stratified by sex [$p$[perm] 0.171]. Significantly lower Pielou's evenness was noted in females with SLD compared to females without SLD (median 0.912 versus 0.920, respectively, $p=0.0414$). However, no other sex effects were noted in alpha diversity metrics between those with and without SLD (Figure S2).

Nineteen bacterial taxa were identified that displayed significant differences in relative abundance between SLD and no SLD following FDR correction (FDR corrected $<0.1$) (Table 2; Figure 2,3). Of these, *Lachnoclostridium* and *Prevotella* displayed higher relative abundance in those with SLD compared to those without (FDR corrected $p<0.1$). The other 17 taxa, which included *Faecalibacterium*, Bacilli genus RF39, *Lachnospiraceae* FCS020 group and *Eubacterium siraeum* group, all displayed lower relative abundance in those with SLD compared to those without (FDR corrected $<0.1$) (Table 2). Intergroup differences in taxon prevalence were not significant following adjustment for BMI, sex and alcohol intake. Total bacterial abundance did not differ between those with and without SLD (median $1.3\times10^8$ copies/mL and $1.3\times10^8$ copies/mL; $p=0.939$).

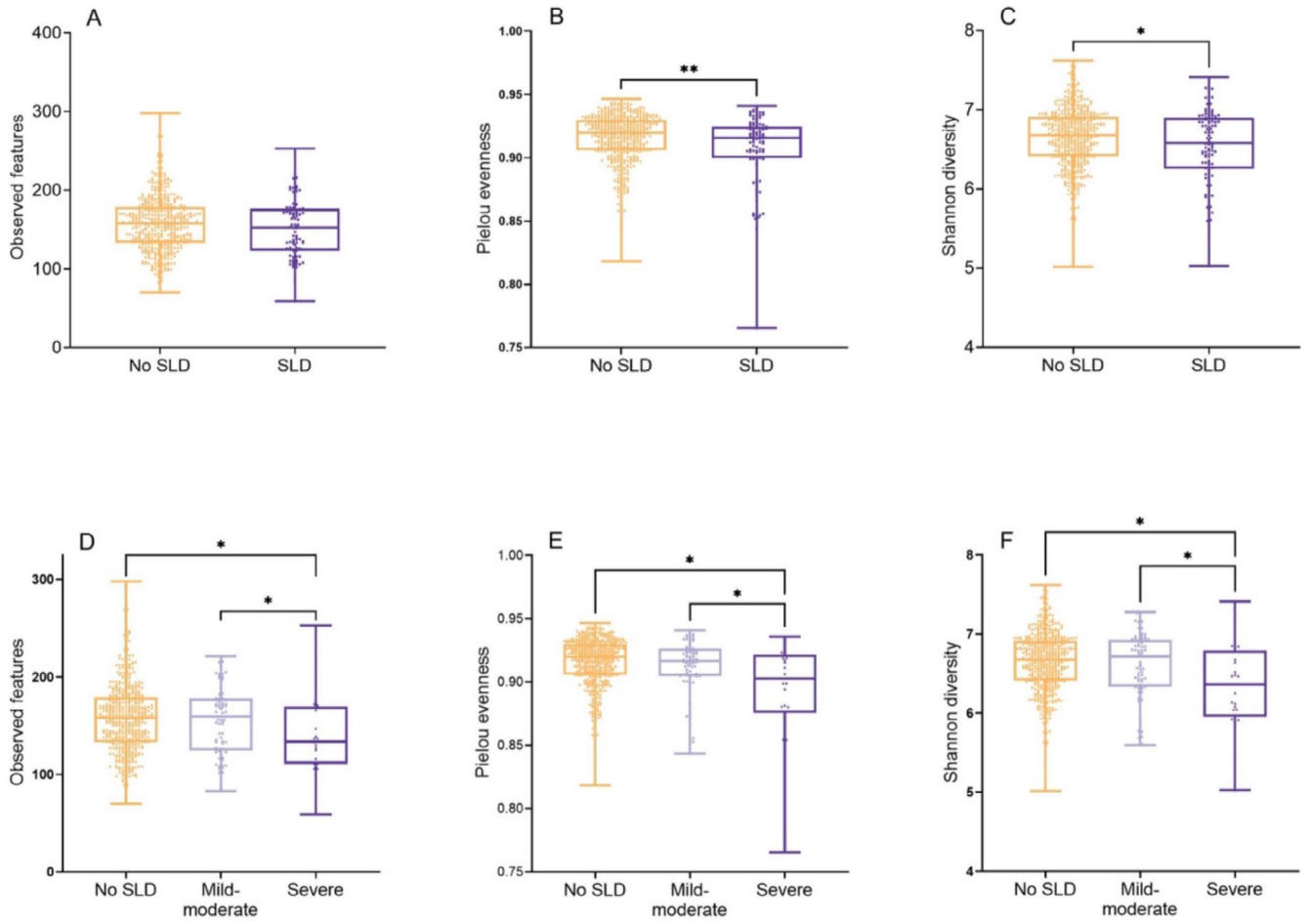


**FIGURE 2** | Alpha diversity metrics between those with and without steatotic liver disease (SLD) (A–C) and between SLD severity groups (those without SLD, mild–moderate and severe SLD) (D–F). *$p \leq 0.05$; **$p \leq 0.01$.

### 3.3 | Gut Microbiota Composition in Relation to SLD Severity

Microbiota characteristics differed significantly between participants with mild–moderate and severe SLD (Figure 2D–F). Severe SLD was associated with significantly lower observed taxa, Pielou's evenness and Shannon diversity scores ($p < 0.05$). Beta-diversity analysis also showed differences in overall faecal microbiota composition between those with no SLD and those with mild–moderate SLD ($p = 0.007$) and severe SLD ($p = 0.0002$) (Figure 4A). These differences remained significant following adjustment for BMI, sex and alcohol intake (no SLD versus mild–moderate: $p = 0.004$; no SLD versus severe SLD: $p = 0.001$). There was no significant difference in microbiota dispersion between SLD groups.

In keeping with our initial microbiota composition analysis by SLD status, no significant interaction existed between SLD severity groups and sex [$p$[perm] 0.225] (Figure 4B). However, Pielou's evenness and Shannon diversity was lower in females with severe SLD compared to females without SLD (median 0.881 and 6.71 versus 0.920 and 6.05, respectively, $p = 0.0013$ and $p = 0.0024$) and mild–moderate SLD versus severe SLD (median 0.917 and 6.73 versus 0.881 and 6.05, respectively, $p = 0.0413$ and 0.0200). No other sex effects were noted in alpha diversity metrics between SLD severity groups.

Twenty bacterial genera were identified that displayed significant differences in relative abundance between the no SLD and severe SLD groups (FDR corrected $< 0.1$) (Table 3; Figure S3). Compared to the no SLD group, the severe SLD group exhibited a lower relative abundance of bacterial taxa implicated in SCFA production, including *Christensenellaceae* R7 group, *Lachnospiraceae* ND3007 group, *Lachnospiraceae* NK4A136 group, *Eubacterium xylanophilum* group, *Eubacterium eligens* group, *Anaerobutyricum hallii* group, *Alistipes*, *Coprococcus and Clostridia vadin* BB60 group (FDR corrected $< 0.1$) (Figure 3B). Other bacterial taxa, including Family XIII UCG-001, *Oscillospiraceae* UCG-002 and *Oscillospiraceae* UCG-005, were also significantly less abundant in severe SLD compared to the no SLD group. In contrast, members of the severe SLD group displayed increased levels of *Lachnoclostridium* and *Ruminococcus gnavus* group (FDR corrected $< 0.1$) (Figure 3B). Median relative abundance of *Clostridia vadin* BB60 group was also noted to be significantly different between those with no SLD and mild–moderate SLD (FDR 0.0845; $p < 0.001$).

Between-group differences in *Faecalibacterium*, *Coprococcus* and *Oscillospiraceae* UCG-002 remained significant following adjustment for BMI, sex and alcohol intake (FDR corrected $< 0.1$) (Figure 3B). Significant differences in median relative abundance of *Odoribacter* between those without SLD and severe SLD were evident following adjustment (FDR corrected

**TABLE 2** | Median relative abundance of genera between those with and without steatotic liver disease.

| Bacterial taxon | No SLD $N=488$ | SLD $N=100$ | FDR; $p$ |
|---|---|---|---|
| *Lachnoclostridium* | 0.008 (0.004–0.013) | 0.013 (0.006–0.021) | 0.00485; <0.0001 |
| *Clostridia vadin* BB60 group | 0.000 (0.000–0.002) | 0.000 (0.000–0.001) | 0.00364; <0.0001 |
| *Alistipes* | 0.025 (0.016–0.034) | 0.019 (0.013–0.026) | 0.00429; <0.0001 |
| *Christensenellaceae* R7 group | 0.010 (0.002–0.030) | 0.004 (0.000–0.015) | 0.00485; <0.0001 |
| *Eubacterium xylanophilum* group | 0.001 (0.000–0.005) | 0.000 (0.000–0.002) | 0.0272; 0.000905 |
| *Faecalibacterium* | 0.056 (0.037–0.073) | 0.047 (0.029–0.065) | 0.0167; 0.000487 |
| *Oscillospiraceae* UCG 005 | 0.004 (0.000–0.012) | 0.002 (0.000–0.008) | 0.0288; 0.00108 |
| Bacilli genus RF39 | 0.000 (0.000–0.003) | 0.000 (0.000–0.000) | 0.0601; 0.00275 |
| *Lachnospiraceae* FCS020 group | 0.001 (0.000–0.002) | 0.000 (0.000–0.002) | 0.0717; 0.00448 |
| *Coprococcus* | 0.020 (0.013–0.030) | 0.016 (0.010–0.026) | 0.00569; 0.000119 |
| *Oscillospiraceae* UCG 002 | 0.014 (0.006–0.025) | 0.011 (0.001–0.019) | 0.0480; 0.00200 |
| *Eubacterium siraeum* group | 0.001 (0.000–0.007) | 0.000 (0.000–0.002) | 0.0643; 0.00375 |
| *Clostridia* UCG 014 | 0.00393 (0.000–0.0173) | 0.000880 (0.000–0.00889) | 0.0943; 0.00728 |
| *Lachnospiraceae* NK4A136 group | 0.00903 (0.00340–0.0175) | 0.00716 (0–0.0140) | 0.0130; 0.000326 |
| *Megasphaera* | 0.000 (0.000–0.000) | 0.000 (0.000–0.000) | 0.0943; 0.00686 |
| *Prevotella* | 0.000 (0.000–0.0156) | 0.000528 (0.000–0.0884) | 0.0643; 0.00354 |
| *Barnesiellaceae*; *genus uncultured* | 0.000 (0.000–0.000587) | 0.000 (0.000–0.000) | 0.0643; 0.00341 |
| *Marvinbryantia* | 0.000 (0.000–0.00106) | 0.000 (0.000–0.000) | 0.0943; 0.00660 |
| *Succinatimonas* | 0.000 (0.000–0.000) | 0.000 (0.000–0.000) | 0.0943; 0.00747 |

*Note:* Data are presented as median value (25th percentile [P25] – 75th percentile [P75]). $p$ value assessed using False-Discovery Rate [FDR] corrected <0.1.
Abbreviation: SLD, steatotic liver disease.

<0.1). Whilst total bacterial abundance did not differ between those with severe SLD and those without SLD, estimates of the absolute abundance of *Faecalibacterium*, *Coprococcus*, *Oscillospiraceae* UCG-002 and *Odoribacter* remained significantly lower in those with severe SLD following adjustment for BMI, sex and alcohol intake (Table S4).

## 4 | Discussion

The need for effective strategies to predict and prevent SLD onset and progression is urgent. Despite pre-clinical studies providing strong evidence of causal links between changes in intestinal microbiology and SLD pathogenesis [28, 29], identification of

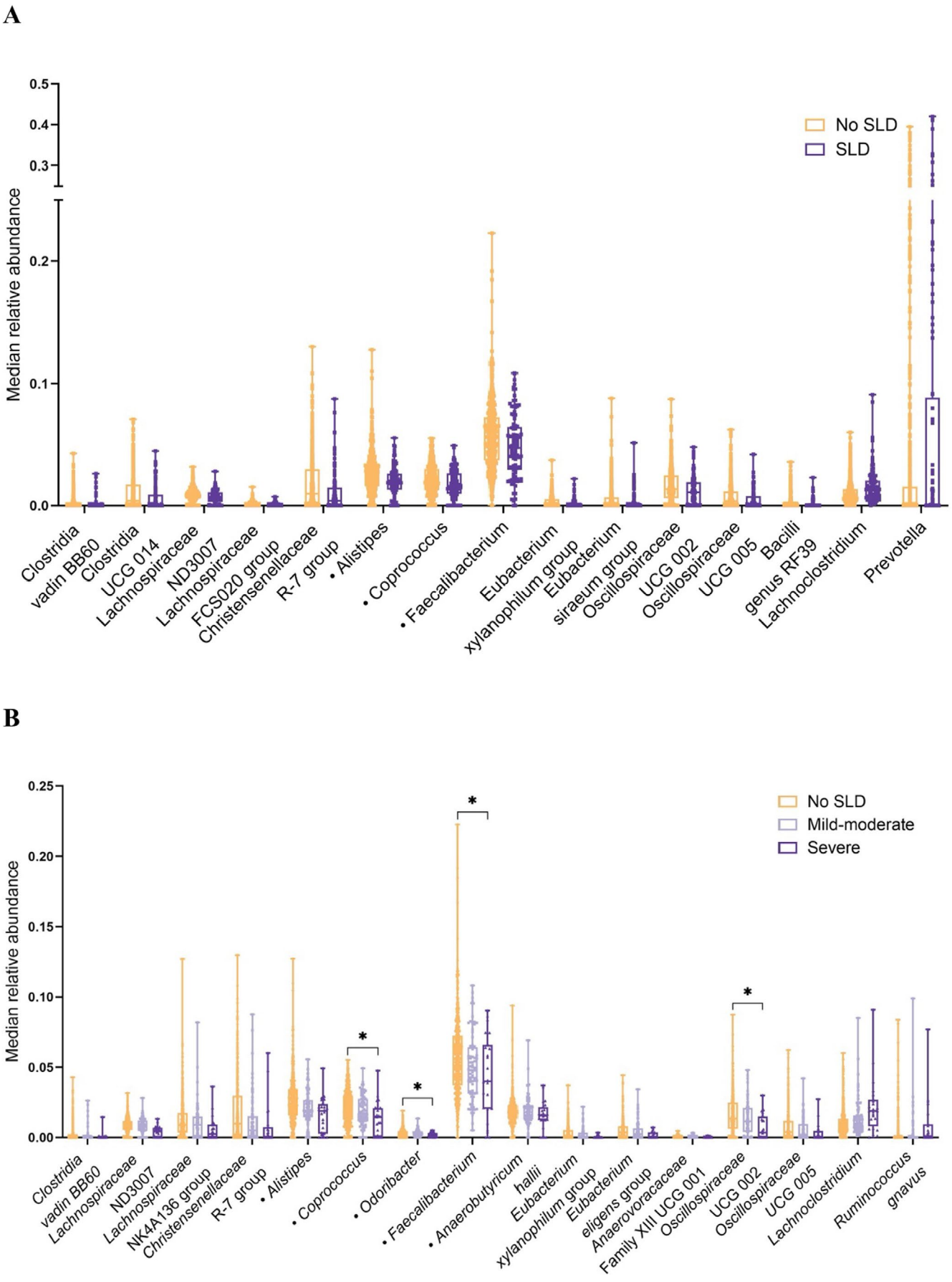


**FIGURE 3** | Legend on next page.

**FIGURE 3** | (A) Median relative abundance of genera in those with and without steatotic liver disease (SLD) (unadjusted model; no significant difference was noted following adjustment for body mass index (BMI), sex and alcohol intake) (FDR corrected <0.1). • Butyrate producers. (B) Median relative abundance of genera between SLD groups (no SLD, mild to moderate SLD and severe SLD) (unadjusted model; FDR corrected <0.1). Significant differences noted between those without SLD and those with severe SLD following adjustment for sex, BMI and alcohol intake as indicated by * (FDR corrected <0.1). • Butyrate producers. *Megasphaera*, *Barnesiellaceae*; genus uncultured, *Marvinbryantia*, *Succinatimonas*, *Rhodospirillale*; genus uncultured, *Fusobacterium* and *Veillonellacea*, genus uncultured not included in the figures.

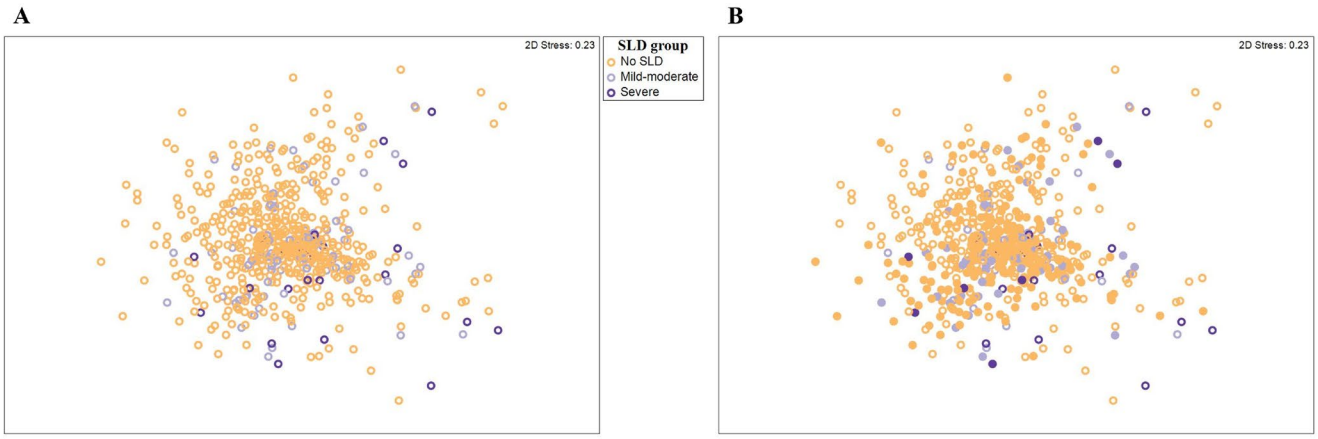


**FIGURE 4** | (A) Non-metric Multi-Dimensional Scaling (NMDS) plots of microbiota composition by steatotic liver disease (SLD) groups. (B) NMDS plots of microbiota composition by SLD group and sex, filled circles, males.

analogous relationships in human cohorts has been hampered by small cohort sizes and a lack of control for potentially confounding factors. To address this, we utilised a large, representative and well-characterised cohort of younger adults, which included participants with hepatic steatosis, as assessed using an accurate and validated MRI methodology. In so doing, we report SLD in younger adults to be associated with significant reductions in intestinal microbial diversity compared to those without SLD, including a depletion of specific commensal taxa that is consistent with other conditions involving chronic inflammation, including obesity, cardiovascular disease, colorectal cancer and inflammatory bowel disease [30–33]. We identify a lower abundance of predominantly butyrogenic bacteria, *Faecalibacterium*, *Coprococcus* and *Odoribacter* in those with severe SLD following adjustment (FDR corrected $p < 0.1$). This novel finding points to a potential role of butyrate in SLD development and progression, representing an important extension of current understanding.

The observed association between SLD and reduced lower microbial diversity is consistent with a recent systematic review and meta-analysis [11]. In particular, the depletion of obligate anaerobic bacteria, such as *Coprococcus*, *Odoribacter*, *Faecalibacterium*, *Alistipes*, *Anaerobutyricum hallii* group, *Christensenellaceae* R7 group and family *Lachnospiraceae* has been noted in SLD [34], chronic liver disease [35], metabolic conditions such as obesity [30] and cardiovascular disease [31], as well as inflammatory bowel disease [33] and colorectal cancer [32]. A consistent feature of these conditions is a chronic systemic inflammatory state.

In metabolic conditions, chronic inflammation is thought to lead to cytokine hypersecretion and insulin resistance with the latter further augmenting inflammation by promoting proinflammatory cytokines [36]. Many of the obligate anaerobic bacteria that we observed to be less prevalent in those with steatosis contribute to SCFA biosynthesis, including butyrogenic genera such as *Coprococcus*, *Odoribacter and Faecalibacterium* [37]. The capacity of SCFAs to downregulate systemic inflammation suggest that depletion of SCFA-producing gut bacteria in steatosis could contribute to SLD development [38]. Butyrate biosynthesis by gut bacteria also acts to reduce intestinal permeability [39] by promoting tight junction assembly through AMP-activated protein kinase (AMPK) [40]. A reduction in the relative abundance of these taxa could therefore contribute to increased translocation of pathogenic bacteria and by-products to the liver through the portal circulation.

The lower relative abundance of *Christensenellaceae* R7 group (family *Christensenellaceae*, phylum Bacillota [Firmicutes]) that we observed in severe SLD aligns with the reported contribution of the *Christensenellaceae* family to human health [41]. *Christensenellaceae* abundance has been shown to be inversely associated with BMI and differences have been observed in other disease states, such as obesity and inflammatory bowel disease [42]. Within our study, participants with SLD exhibited a lower relative abundance of *Christensenellaceae* R7 group prior to adjustment and displayed significantly higher levels of adiposity and metabolic dysfunction (higher blood pressure, insulin resistance and lipid levels).

Following initial analysis based on the presence of SLD, we investigated the relationship between microbiota characteristics and SLD severity. Prior to adjustment for sex, BMI and alcohol intake, participants in the severe SLD group displayed significantly increased levels of *Lachnoclostridium* and *Ruminococcus*

**TABLE 3** | Relative abundance of genera between steatotic liver disease groups with significant differences noted between the no steatotic liver disease and severe steatotic liver disease groups but not between the no steatotic liver disease and mild–moderate steatotic liver disease groups.

| Bacterial taxon | No SLD Median (P25–P75) | Mild–moderate Median (P25–P75) | Severe SLD Median (P25–P75) | No SLD versus Severe SLD FDR; *p* |
|---|---|---|---|---|
| *Alistipes* | 0.025 (0.016–0.034) | 0.019 (0.0.014–0.027) | 0.019 (0.003–0.024) | 0.000408; 0.0000510 |
| *Coprococcus* • | 0.020 (0.013–0.030) | 0.017 (0.0117–0.0276) | 0.015 (0.00038–0.0210) | 0.0000623; <0.0001 |
| *Lachnoclostridium* | 0.008 (0.004–0.013) | 0.010 (0.006–0.016) | 0.019 (0.008–0.027) | 0.0944; 0.00748 |
| *Odoribacter* • | 0.003 (0.001–0.005) | 0.003 (0.001–0.004) | 0.001 (0.001–0.003) | 0.151; 0.0151 |
| *Faecalibacterium* • | 0.0557 (0.037–0.0725) | 0.0479 (0.0312–0.0643) | 0.0401 (0.0204–0.0659) | 0.000169; <0.0001 |
| *Lachnospiraceae*, ND3007 group | 0.009 (0.005–0.012) | 0.008 (0.005–0.012) | 0.004 (0.000–0.007) | 0.00771; 0.000225 |
| *Lachnospiraceae*, NK4A136 group | 0.009 (0.003–0.018) | 0.009 (0.001–0.015) | 0.003 (0.000–0.009) | 0.00266; <0.0001 |
| *Oscillospiraceae*, UCG-002 • | 0.014 (0.006–0.025) | 0.012 (0.004–0.021) | 0.003 (0.000–0.015) | 0.00266; <0.0001 |
| *Christensenellaceae*, R-7 group | 0.010 (0.002–0.0298) | 0.005 (0.001–0.015) | 0.000 (0.000–0.007) | 0.000448; <0.0001 |
| *Oscillospiraceae*, UCG-005 | 0.004 (0.000–0.012) | 0.002 (0.000–0.010) | 0.000 (0.000–0.005) | 0.00926; 0.000309 |
| *Eubacterium eligens* group | 0.004 (0.000–0.008) | 0.002 (0.000–0.007) | 0.000 (0.000–0.004) | 0.0366; 0.00244 |
| *Clostridia vadin*, BB60 group | 0.000 (0.000–0.002) | 0.000 (0.000–0.001) | 0.000 (0.000–0.001) | 0.0980; 0.00817 |
| Anaerovoracaceae Family XIII UCG-001 | 0.000 (0.000–0.001) | 0.000 (0.000–0.001) | 0.000 (0.000–0.000) | 0.0249; 0.00121 |
| *Eubacterium xylanophilum* group | 0.001 (0.000–0.005) | 0.000 (0.000–0.003) | 0.000 (0.000–0.001) | 0.0309; 0.00175 |
| *Rhodospirillales*, uncultured | 0.000 (0.000–0.009) | 0.000 (0.000–0.007) | 0.000 (0.000–0.000) | 0.0249; 0.00114 |
| *Ruminococcus gnavus* group | 0.000 (0.000–0.000) | 0.000 (0.000–0.001) | 0.001 (0.000–0.009) | 0.0249; 0.00124 |
| *Megasphaera* | 0.000 (0.000–0.000) | 0.000 (0.000–0.000) | 0.000 (0.000–0.000) | 0.0363; 0.00227 |
| *Fusobacterium* | 0.000 (0.000–0.000) | 0.000 (0.000–0.000) | 0.000 (0.000–0.000) | 0.0516; 0.00365 |
| *Anaerobutyricum hallii* | 0.0182 (0.0133–0.0229) | 0.0179 (0.0128–0.0231) | 0.0158 (0.0123–0.0214) | 0.0944; 0.00735 |
| *Veillonellacea*, uncultured | 0.000 (0.000–0.000) | 0.000 (0.000–0.000) | 0.000 (0.000–0.000) | 0.0124; 0.000464 |

*Note:* Data are presented as median value (25th percentile [P25] – 75th percentile [P75]). *p* value corrected using False-Discovery Rate [FDR] <0.1. Significant differences noted in relative abundance and absolute abundance between those without steatotic liver disease (SLD) and those with severe SLD following adjustment for sex, body mass index and alcohol intake as indicated by •.

*gnavus* group (FDR corrected < 0.1), suggesting that these factors potentially mediate the association with disease. Notably, associations with cardiovascular disease have been reported previously for both *Lachnoclostridium* and *R. gnavus* group [43, 44]. Whilst not overtly pathogenic, higher levels of *R. gnavus* have been reported in those with steatotic liver disease in older populations [12], as well as in the contexts of chronic cardiac failure [45], inflammatory bowel disease [46] and spondyloarthritis [47]. The overrepresentation of *R. gnavus* in inflammatory conditions appears to relate to its production of inflammatory glucorhamnan polysaccharide, which is thought to induce dendritic cells to secrete inflammatory cytokine Tumour Necrosis Factor alpha [48]. *R. gnavus* may also contribute to a reduction in the integrity of the gastrointestinal tract barrier through its utilisation of mucin as a carbon source, thereby promoting the translocation of bacteria and harmful microbial metabolites to the liver [49, 50].

Our study had limitations that should be considered. Whilst we assessed relationships between faecal microbiota composition and markers of SLD, we were not able to investigate potential mediators of these relationships directly. The cross-sectional design of our study also limited our ability to derive evidence of causality in relation to observed associations. Markers of liver fibrosis at the Gen2-27 year follow-up were not available and so we cannot comment on whether participants had advanced liver disease, although this is unlikely in this age group. As mentioned in the results, statistical power was lacking to perform a sub-analysis comparing microbiome characteristics between SLD subtypes (MASLD, met-ALD and ALD), which should be considered in future cohort follow-up analyses. Finally, LinDA, the regression method used, accepts a log linear regression model on absolute abundance. As a result, our approach may underestimate the extent of interactions between microbiota and host [27].

By conducting the first population-based microbiome study in younger adults with SLD, we provide important insight into the changes that occur to the gut microbiome prior to the onset of advanced disease. Overall, we show SLD in younger adults to be associated with reduced intestinal microbial diversity, including the depletion of specific bacteria that is consistent with other chronic inflammatory states. The potential overlap in microbiome features between SLD and other chronic inflammatory conditions requires ongoing investigation to determine causality and the potential for microbiome-targeted interventions. However, the significant reduction in butyrogenic bacterial taxa in SLD highlights the need for further exploration of therapeutics that target butyrate production as potential disease modifiers.

### Author Contributions

Study design, concept, research process, statistical analysis, first draft and editing of the manuscript: Y.T. Study design, concept and editing of the manuscript: A.J.W., K.R.M., L.A.A. and G.B.R. Study design, statistical analysis and editing of the manuscript: J.M.C. Research process: A.R., Z.W., L.C.-B. and B.A. Statistical analysis and editing the manuscript: E.V., S.M. and S.L.T. Statistical analysis: R.W. Concept and editing the manuscript: K.L.I. Editing the manuscript: L.J.B., T.A.M., O.T.A. and J.K.O.

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### Ethics Statement

The Raine Study was conducted in accordance with the Declaration of Helsinki and was approved by the University of Western Australia Human Research Ethics committee (reference number: 2022/ET000237).

### Consent

All participants provided written, informed consent.

### Conflicts of Interest

The authors declare no conflicts of interest.

### Data Availability Statement

All sequencing data has been submitted to the publicly accessible NCBI SRA database (Bioproject ID PRJNA1182921). Clinical data can be requested from the Raine Study Investigator Team (https://rainestudy.org.au/home-beta-2/information-for-researchers); (rainestudy@uwa.edu.au).

### Permission to Reproduce Material From Other Sources

No data from other sources has been used in this manuscript.

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**Supporting Information**

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