Gut microbial metabolism is linked to variations in circulating non-high density lipoprotein cholesterol

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Summary

Background Non-high-density lipoprotein cholesterol (non-HDL-c) was a strong risk factor for incident cardiovascular diseases and proved to be a better target of lipid-lowering therapies. Recently, gut microbiota has been implicated in the regulation of host metabolism. However, its causal role in the variation of non-HDL-c remains unclear.

Methods Microbial species and metabolic capacities were assessed with fecal metagenomics, and their associations with non-HDL-c were evaluated by Spearman correlation, followed by LASSO and linear regression adjusted for established cardiovascular risk factors. Moreover, integrative analysis with plasma metabolomics were performed to determine the key molecules linking microbial metabolism and variation of non-HDL-c. Furthermore, bi-directional mendelian randomization analysis was performed to determine the potential causal associations of selected species and metabolites with non-HDL-c.

Findings Decreased *Eubacterium rectale* but increased *Clostridium* sp *CAG_299* were causally linked to a higher level of non-HDL-c. A total of 16 microbial capacities were found to be independently associated with non-HDL-c after correcting for age, sex, demographics, lifestyles and comorbidities, with the strongest association observed for tricarboxylic acid (TCA) cycle. Furthermore, decreased 3-indolepropionic acid and N-methyltryptamine, resulting from suppressed capacities for microbial reductive TCA cycle, functioned as major microbial effectors to the elevation of circulating non-HDL-c.

Interpretation Overall, our findings provided insight into the causal effects of gut microbes on non-HDL-c and uncovered a novel link between non-HDL-c and microbial metabolism, highlighting the possibility of regulating non-HDL-c by microbiota-modifying interventions.

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Keywords: Gut microbiota; Non-high-density lipoprotein cholesterol; Mendelian randomization; 3-Indolepropionic acid; N-methyltryptamine

Introduction

Accumulating evidence implicates that non-high-density lipoprotein cholesterol (non-HDL-c) functions as an important modifiable risk factor associated with atherosclerotic cardiovascular diseases (ASCVDs).[1] Despite an optimal control of low-density lipoprotein cholesterol (LDL-c), subjects with discordantly high non-HDL-c are still at a higher risk for adverse cardiovascular events.[2] Compared to LDL-c, targeting non-HDL-c takes into account more residual risks for ASCVDs, especially several proatherogenic lipoproteins containing apolipoprotein B (apoB).[3] Thus, identifying novel therapeutic approaches for deceasing non-HDL-c has the potential to ameliorate the global burden of ASCVDs.

Gut microbiota has been reported to be a central regulator in host metabolism and immune homeostasis.[4] Growing evidence from both human studies and animal models suggested a pathophysiological role of gut microbiota in lipid metabolism through several

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Research in context

Evidence before this study

Non-high-density lipoprotein cholesterol (non-HDL-c) is a major residual risk for atherosclerotic cardiovascular diseases. So far, however, no effective therapeutic strategies exist. Gut microbiota is a central regulator of host metabolism. Despite several small-scale human studies and murine models implicating a critical role of gut microbiota in lipid metabolism, currently whether and how microbial metabolism are causally involved in the regulation of non-HDL-c remain largely unknown.

Added value of this study

Utilizing a deep characterization of gut microbiota by shotgun metagenomics, metabolomics and host genotyping in 1361 community residents free of cardiovascular disease and lipid-lowering therapies, we demonstrate a causal role of *Eubacterium rectale* and *Clostridium* sp *CAG_299* in the variation of circulating non-HDL-c. Moreover, through the integration of multi-omics and Mendelian Randomization analysis, depletion of 3-indolepropionic acid and N-methyltryptamine are proved to be the key effectors linking gut microbiota and elevated non-HDL-c.

Implications of all the available evidence

These findings highlight the potential of targeting gut microbiota to control non-HDL-c, and demonstrate that 3-indolepropionic acid and N-methyltryptamine may serve as postbiotics for the management of atherogenic lipids.

mechanisms, including increased gut permeability and endotoxemia, changes in production of short-chain fatty acids (SCFAs) and choline, and perturbation of bile acid (BA) metabolism.[5] Compared to conventionally raised mice, germ free mice demonstrated an enhanced clearance of triglycerides (TG) from the circulation,[6] whereas, circulating TG and cholesterols were increased in mice receiving fecal microbial transplantation from obese donors.[7] Beneficial effects of probiotics supplementation, such as *Bifidobacterium* spp. and *Lactobacillus*, on lipid profiles provided further evidence for a role of gut microbiota in the regulation of lipid homeostasis.[8,9] Compositional changes of gut microbiota have also been observed in humans. Reduced microbial gene richness was found to be associated with increased overall adiposity and dyslipidaemia,[10] whereas, an energy-restricted diet intervention was reported to decrease serum lipids through augmenting microbial gene counts.[11] Additionally, a cohort study from the Netherlands suggested that up to 6% of the variance in serum TG and 4% in HDL-c could be attributed to gut microbiota.[12] However, most of the studies used 16S rRNA gene sequencing, which was limited by a lack of taxonomic and functional resolution. Moreover, despite recent progress in establishing the associations between microbial species and lipid metabolism, whether and how microbial metabolism are causally involved in the variation of non-HDL-c remains unclear.

To address the questions above, we first performed metagenomic and Mendelian randomization (MR) analysis in a large cohort of 1361 community residents, and found that *Eubacterium rectale* (*E. rectale*) and *Clostridium* sp *CAG_299* were causally involved in non-HDL-c variation. Subsequently, integrative analysis with both metabolomics profiling and targeted analysis was employed to determine the key molecular transducers linking selected microbial species to the variation of circulating non-HDL-c.

Methods

Study participants

This study was approved by the Ethics Committee of School of Public Health, Sun Yat-Sen University (L2017-001), and was in accordance with the principles of the Declaration of Helsinki. Written informed consents were obtained from each individual. Local residents, who have lived in Guangdong Province, China for over 5 years, were invited for health screening through flyers and advertisement at the Community Healthcare Center of Chashan Town (Dongguan City, Guangdong, China). Fecal metagenomics and plasma metabolomics data were available from 1415 participants aged between 35 and 74 years. Detailed inclusion and exclusion criteria for this study were as follows:

Inclusion criteria

(i) No severe disability, and absence of any malignant tumors, thyroid disorder, biliary acute or chronic viral hepatitis, cirrhosis, chronic renal insufficiency, acute or chronic inflammatory disease; (ii) not pregnant and (iii) able to understand the nature and possible consequence of the study.

Exclusion criteria

(i) Ongoing treatment for hyperlipidemia (n = 43); (ii) use of antibiotic drugs within 3 months at sample collection (n = 7); and (iii) gastrectomy, infectious disease or known history of coronary heart disease (n = 4). Finally, a total of 1361 participants were included in current analysis (Fig. 1). More details about metadata collection were provided in Supplementary Materials.

Determination of lipid profiles

Blood samples after an overnight fast for 10–12 h were collected and analyzed immediately at local laboratory. Measurements of total cholesterol (TC), TG, HDL-c and LDL-c were assessed by enzymatic methods on a Microplate Reader (Mindray BS800M; Mindray,

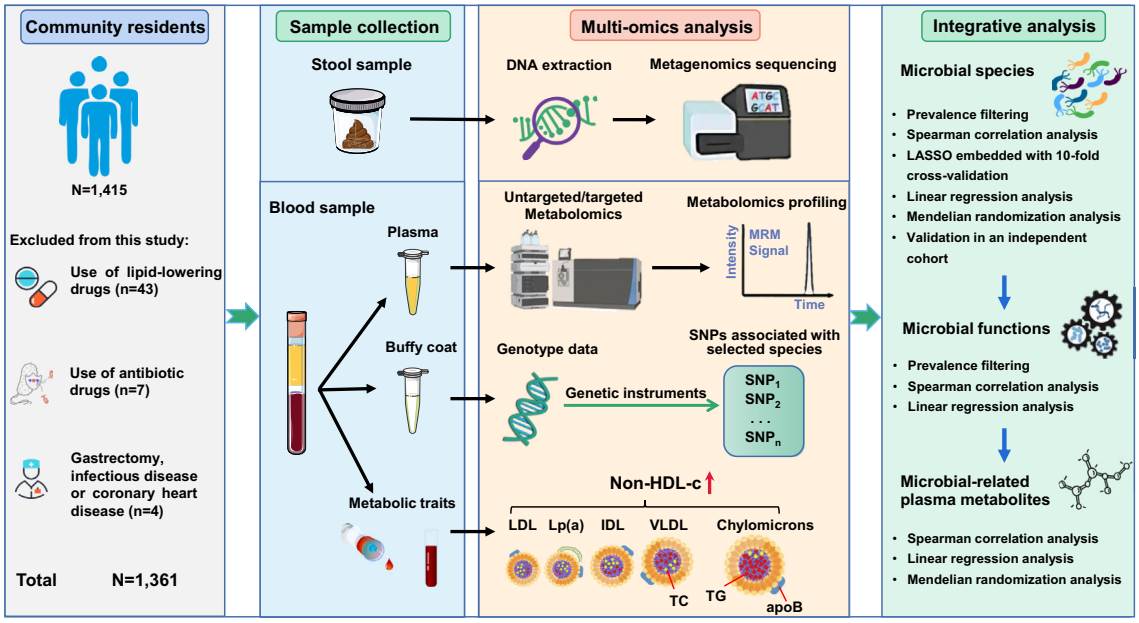

***Fig. 1:* Flowchart of the study.** 1361 individuals free of lipid-lowering therapies and antibiotics use prior to sample collection were included and subjected to integrative analysis of fecal metagenomics sequencing, genotyping, and plasma metabolomics. apoB = apolipoprotein B; Lp(a) = lipoprotein(a); IDL = intermediate-density lipoprotein; LDL = low-density lipoprotein; VLDL = very low-density lipoprotein.

Shenzhen, China). Non-HDL-c was calculated by subtracting HDL-c from TC as previously described.[3] Given that no threshold or reference of non-HDL-c had been established for Asian populations, subjects were divided into two groups by the median level of non-HDL-c in this study (low non-HDL-c group defined as <3.88 mmol/L, and high non-HDL-c group defined as ≥3.88 mmol/L).

Host genotyping

Host DNA was extracted from buffy coat using TIANamp Blood DNA Kit fromTIANGEN Co., Ltd. (Beijing, China) according to the manufacturer's instruction. The dosage of DNA used for subsequent library preparation was more than 1 μg and their concentration was controlled at no less than 80 ng/μL. Genotyping was performed with Infinium Chinese Genotyping Array-24 v1.0 BeadChip at Illumina platform by WeGene Co., Ltd. (Beijing, China). Quality control was performed with PLINK (v.1.9), and we excluded single-nucleotide polymorphisms (SNPs) with (1) Minor Allele Frequency <5%; (2) Hardy–Weinberg equilibrium violation $P < 0.00001$, and (3) genotype calling rate <5%.[13] Furthermore, we calculated linkage disequilibrium (LD) between each pair of SNPs at a window of 50 SNPs, and removed one of a pair of SNPs if LDs was higher than 0.5. Finally, a total of 3,855,574 and 1,972,724 SNPs were retained in the current genome-wide association study (GWAS) with bacterial species and metabolites, respectively among 1361 participants. The threshold of $P < 1 \times 10^{-5}$ were set for identifying SNPs associated with microbiota or metabolites to maximize the amount of genetic variance explained by selected SNPs, as previously described.[13]

Fecal DNA extraction and sequencing

Participants received MGIEasy stool collection kit containing a room temperature stabilizing reagent and detailed instructions at the community center during study visit. Stool samples were collected on the same day of blood drawing and stored at –20 °C at the community center for a maximum of one day before transportation to the central freezer at –80 °C until analysis. Stool DNA was extracted from frozen stools using MagMAXTM Microbiome Ultra Nucleic Acid Isolation kit (Thermo Fisher Scientific, MA, USA). All samples were sequenced on the Illumina NovaSeq 6000 paltform (Illunima, San Diego, California, USA; Paired-end; insert size, 350 bp; read length, 150 bp) by Novogene Co., Ltd. (Beijing, China). More details about quality control and data analysis for metagenomics were provided in Supplementary Materials.

Metabolomics profiling and targeted quantification of key metabolites

After at least 10 h of fasting, blood samples were collected and stored at –80 °C for further analysis. An integrated method for large-scale detection, identification, and quantification of widely targeted metabolites, was performed as previously described.[14] Furthermore,

a targeted analysis of 3-indolepropionic acid, orotic acid and N-methyltryptamine was carried out by ultra-performance liquid chromatography-tandem mass spectrometry. More details concerning metabolomics profiling and data analysis, as well as targeted metabolomics analysis were provided in Supplementary Materials.

Statistical analysis

General characteristics

All statistical analyses were performed using R software version 4.0.3 (R Foundation for Statistical Computing, Vienna, Austria). Continuous parameters were examined for normality using the Shapiro–Wilk test. Since all continuous variables were non-normally distributed, they were presented as median with interquartile range (IQRs), and analyzed with Wilcoxon rank-sum test. Categorical variables were presented as counts and percentages (%) and analyzed with χ^2 test or Fisher exact test, as appropriate. For variables with missing values, multiple imputation was performed with "mice" package before subjecting to further analysis. Spearman rank correlation coefficient was calculated using cor.test. Benjamini-Hochberg's false-discovery rate-corrected *P*-value (P_{fdr}) was applied for all multiple comparisons.

Metagenomics analysis

After filtering out low-prevalence microbiome features (defined as a detection rate less than 5% for species, and 20% for metabolic functions in all subjects), Spearman correlation analysis was firstly applied to identify species and metabolic functions significantly associated with non-HDL-c ($P_{fdr} < 0.05$). Subsequently, a feature selection strategy based on repeated least absolute shrinkage and selection operator (LASSO) with 10-fold cross-validation was implemented to screen for key species associated with non-HDL-c using the R package 'glmnet' (v.4.1-3).[15] Furthermore, general linear regression was employed to assess the independent associations of core species with the variation in circulating non-HDL-c after adjustment for (i) model 1: age and sex; (ii) model 2: model 1 plus smoking, drinking, diet diversity and physical activity; (iii) model 3: model 2 plus overweight, diabetes and hypertension ($P_{fdr} < 0.1$). Due to a lack of information on physical activity, the independent associations between selected species and non-HDL-c in the independent validation cohort (Xu et al.[16]) were determined by general linear regression adjusted for age, sex, overweight, smoking, drinking and diet diversity.

Mendelian randomization analysis

To interrogate the causal effects of selected microbial species and microbial metabolites on the variation of non-HDL-c, bi-directional MR analysis was performed based on R package "Mendelian Randomization". SNPs with F statistic ($beta^2/SE^2$) >10 were considered as strong genetic IVs, and used for subsequent MR analysis. MR estimates were calculated using inverse-variance-weighted (IVW) methods. In addition, we report MR estimates using weighted median, simple median and the MR-Egger regression methods. To ensure the validity of the results, several sensitivity analyses were performed: (1) check out the horizontal pleiotropy (MR-Egger intercept $P > 0.05$); (2) check out the heterogeneity (Cochran's Q-test $P > 0.05$).[17] In addition, to identify potential heterogeneous SNPs, the "leave-one-out" analysis was performed by omitting each instrumental SNP in turn. Furthermore, we performed reverse MR analysis on selected species and metabolites by the same analysis procedure to ensure its causality.

Metabolomics analysis

As the metabolomics data were extremely skewed, metabolite LC-MS peak areas were log transformed and scaled to a mean of zero and SD of one before analysis.[18] First, Spearman correlation analysis was applied to identify metabolites significantly correlated with non-HDL-c ($P_{fdr} < 0.05$). Second, general linear regression adjusted for indicated covariates, including age, sex, smoking, drinking, diet diversity, physical activity, overweight, diabetes and hypertension, was employed to screen for metabolites significantly associated with at least one of two species causally linked to the variation of non-HDL-c ($P_{fdr} < 0.05$). Moreover, the associations of 3-indolepropionic acid, orotic acid and N-methyltryptamine determined by targeted metabolomics analysis and non-HDL-c were determined by linear regression analysis.

Identification of microbial enzymes involved in the production of selected metabolites

To delineate how *Clostridium* sp *CAG_299* and *E. rectale* influence the level of 3-indolepropionic acid, orotic acid and N-methyltryptamine, a BLASTP search was performed with the protein sequence of enzymes known to be involved in tricarboxylic acid (TCA) cycles, and its closely related pathways, as the query against all open reading frames of *Clostridium* sp *CAG_299* and *E. rectale*.

Role of funders

The funder of this study had no role in study design, data collection, data analysis, data interpretation or the writing of the report.

Results

Microbial species contribute to the variation in non-HDL-c

A total of 1361 participants with no history of cardiovascular disease, any lipid-lowering or antibiotics treatment were analyzed. The median age was 50

(IQR: 44–56) years-old, and 54.7% of them were female. Overall, men had a higher level of non-HDL-c than women (Supplementary Fig. S1). According to the median level of non-HDL-c in this cohort, individuals with high non-HDL-c were older, more likely to be men and had worse lipid profiles, in addition to greater proportions of metabolic disorders (Table 1).

We then analyzed gut microbial profiles using shotgun metagenome sequencing in fecal samples. A total of 23 species were found to be significantly associated with non-HDL-c ($P_{fdr} < 0.05$, Fig. 2a). To further identify key species closely related to circulating non-HDL-c, LASSO embedded with 10-fold cross-validation was employed, and a panel of 8 species, mainly from *Firmicutes* and *Bacteroidetes* phylum, were identified (Fig. 2b and c). After correcting for potential covariates which may affect gut microbiota, including age, sex, smoking, drinking, diet diversity, physical activity, overweight, diabetes and hypertension, 5 species out of this selected panel remained significantly associated with non-HDL-c (Fig. 2d, all $P_{fdr} < 0.1$). Among them, several SCFAs-producing bacteria,[19] such as *Prevotella disiens* (*P. disiens*), *E. rectale*, and *Faecalibacterium prausnitzii* (*F. prausnitzii*), accounted for the largest contribution to the variance of non-HDL-c, followed by *Parabacteroides goldsteinii* (*P. goldsteinii*) and *Clostridium* sp *CAG_299* (Fig. 2d). Compared to individuals with elevated non-HDL-c, *F. prausnitzii* was substantially higher, while *Clostridium* sp *CAG_299*, a genus previously reported to be enriched in patients with coronary atherosclerosis,[20] was remarkably lower in subjects with low non-HDL-c (Fig. 2e). Consistently, *F. prausnitzii*, *E. rectale* and *P. disiens* demonstrated an inverse correlation with non-HDL-c and other established risk factors for ASCVDs,[21] such as atherogenic lipids, increased homeostatic model assessment for insulin resistance (HOMA-IR) and waist circumference (Fig. 2a). On the contrary, *Clostridium* sp *CAG_299* and *P. goldsteinii*

Characteristics	Non-HDL-c			
	Overall	Low (<3.88 mmol/L)	High (≥3.88 mmol/L)	*P* value
N, (n%)	1361	680 (50.0)	681 (50.0)	
Demographics				
Age, yrs	50.0 (44.0–56.0)	48.0 (43.0–55.0)	52.0 (46.0–57.0)	<0.001
Male, n (%)	615 (45.3)	281 (41.0)	334 (49.0)	0.004
Laboratory measures				
SBP, mm Hg	124.0 (114.0–135.0)	121.0 (111.0–133.0)	126.0 (117.0–139.0)	<0.001
DBP, mm Hg	80.5 (74.0–87.8)	79.0 (73.0–87.0)	82.0 (76.0–89.0)	<0.001
Waist circumference, cm	83.5 (76.5–91.0)	81.0 (74.0–89.0)	86.0 (80.0–92.0)	<0.001
Triglyceride, mmol/L	1.16 (0.83–1.67)	0.94 (0.71–1.37)	1.40 (1.02–1.95)	<0.001
TC, mmol/L	5.24 (4.68–5.91)	4.67 (4.31–5.05)	5.90 (5.45–6.49)	<0.001
HDL-c, mmol/L	1.37 (1.16–1.62)	1.43 (1.23–1.70)	1.32 (1.11–1.55)	<0.001
LDL-c, mmol/L	3.15 (2.67–3.67)	2.70 (2.34–2.94)	3.65 (3.37–4.08)	<0.001
Non-HDL-c, mmol/L	3.88 (3.27–4.52)	3.27 (2.85–3.59)	4.51 (4.15–5.04)	<0.001
Glucose, mmol/L	4.70 (4.28–5.19)	4.61 (4.23–5.06)	4.79 (4.34–5.30)	<0.001
HOMA-IR	1.80 (1.16–2.94)	1.58 (0.99–2.51)	2.14 (1.35–3.43)	<0.001
Comorbidities				
Overweight, n (%)	657 (48.3)	272 (40.0)	385 (57.0)	<0.001
Diabetes, n (%)	82 (6.1)	27 (4.0)	55 (8.1)	0.001
Hypertension, n (%)	456 (33.5)	197 (29.0)	259 (38.0)	<0.001
Lifestyles				
Diet diversity	5 (5–6)	5 (5–6)	5 (5–6)	0.761
MET, min/w	1764.0 (693.0–3612.0)	1533.0 (693.0–3892.0)	1893.0 (840.0–3465.0)	0.300
Drinking, n (%)	161 (11.8)	78 (11.0)	83 (12.0)	0.700
Cigarettes				0.110
Never, n (%)	1092 (80.2)	559 (82.0)	533 (78.0)	
Quit smoking, n (%)	89 (6.5)	44 (6.5)	45 (6.6)	
Current smoking, n (%)	180 (13.3)	77 (11.0)	103 (15.0)	

Data were expressed as median (interquartile range) or n (%), and *P* Value were determined by Wilcoxon rank-sum test, χ^2 test or Fisher exact test, as appropriate. Number of missing variables: Waist circumference (n = 4), Diet diversity (n = 11), MET (n = 18). SBP = systolic blood pressure; DBP = diastolic blood pressure; TC = total cholesterol; HDL-c = high-density lipoprotein cholesterol; LDL-c = low-density lipoprotein cholesterol; Non-HDL-c = non-high-density lipoprotein cholesterol; HOMA-IR = homeostatic model assessment of insulin resistance; MET = metabolic equivalent.

Table 1: **Baseline characteristics of study participants.**

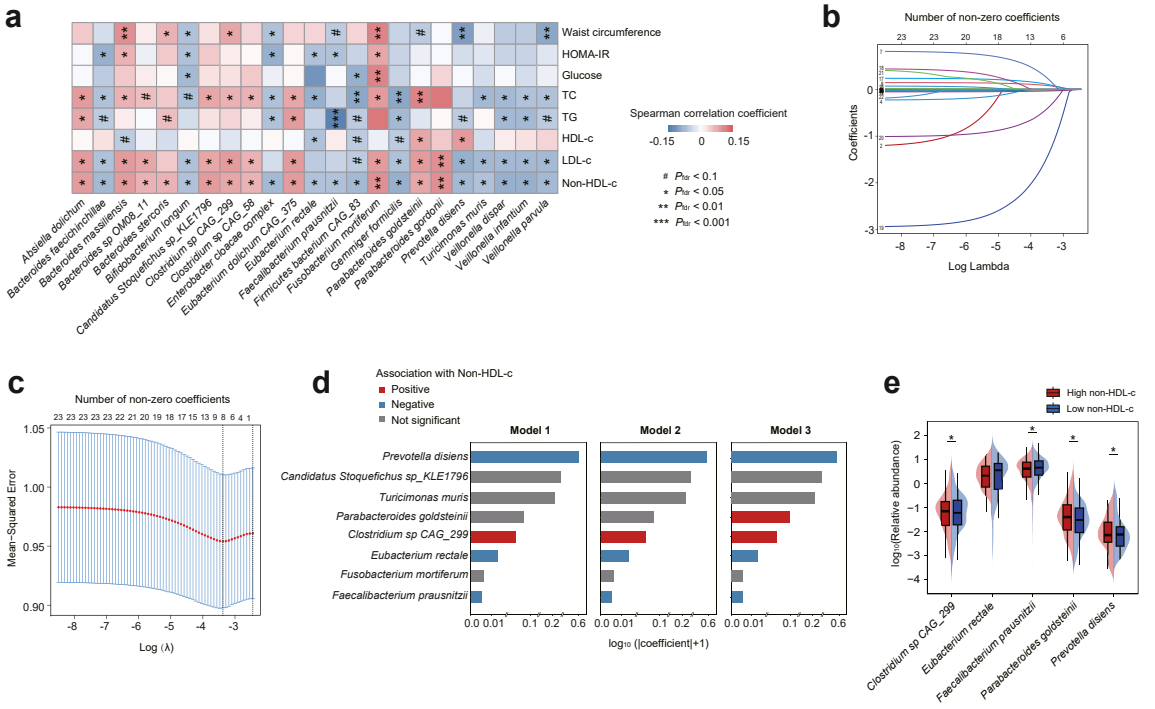

***Fig. 2:* Microbial species contributed to non-HDL-c variation.** (a) Heatmap of the Spearman's correlation coefficients between 23 species and clinical indices (n = 1361). $^{\#}P_{fdr} < 0.1$, $^{*}P_{fdr} < 0.05$, $^{**}P_{fdr} < 0.01$, and $^{***}P_{fdr} < 0.001$. (b) Tuning parameter selection via 10-fold cross-validation with minimum criteria in the LASSO model. (c) LASSO coefficient profiles of 23 candidate key species. (d) The independent association of 8 species selected by LASSSO with non-HDL-c adjusted for (i) Model 1: age and sex; (ii) Model 2: Model 1 plus lifestyles including smoking, drinking, diet diversity and physical activity; (iii) Model 3: Model 2 plus overweight, diabetes, and hypertension. Red, blue and gray indicate positive, negative and insignificant correlations with non-HDL-c, respectively (n = 1361). (e) Distribution of 5 species independently associated with non-HDL-c in subjects with lower (n = 680) or higher (n = 681) non-HDL-c. Red and blue indicate two groups above and below the median level of non-HDL-c, respectively. $^{*}P_{fdr} < 0.05$ by Wilcoxon rank-sum test.

exhibited positive associations not only with non-HDL-c but also with LDL-c and TC (Fig. 2a). Taken together, these findings indicated that microbial species were closely related to circulating non-HDL-c.

E. rectale and *Clostridium* sp *CAG_299* are causally linked to circulating non-HDL-c

Furthermore, bi-directional MR analysis was employed to explore the causal associations between selected species and the variation of non-HDL-c. Of the 5 species independently associated with non-HDL-c, only *Clostridium* sp *CAG_299* and *E. rectale* were found to be associated with non-HDL-c in three MR methods, including IVW, simple median and weighted median (Fig. 3a and b). IVW estimate suggested that *Clostridium* sp *CAG_299* enhanced (OR_{IVW} = 1.238, 95% CI = 1.016–1.509, $P_{fdr} < 0.1$), while *E. rectale* suppressed the level of circulating non-HDL-c (OR_{IVW} = 0.924, 95% CI = 0.873–0.979, $P_{fdr} < 0.1$, Supplementary Fig. S2). On the contrary, IVW estimate did not support any causal associations of *F. prausnitzii*, *P. disiens* and *P. goldsteinii* with non-HDL-c (Fig. 3c–e). Importantly, for the two causal associations observed, the *F*-statistics of the IVs were all greater than 10, eliminating the bias of weak IVs, and the results of Cochran's IVW *Q* test showed no significant heterogeneity of these IVs. Additionally, according to the results of the MR-Egger regression intercept analysis, no significant directional horizontal pleiotropy was found for these two causal relationships (Supplementary Fig. S2). Moreover, leave-one-out analysis further suggested that the causal links between *Clostridium* sp *CAG_299*, *E. rectale* and non-HDL-c were not driven by any single SNP (Supplementary Fig. S3). Furthermore, reverse MR analysis demonstrated that there were no significant causal effects of non-HDL-c on all the 5 species in any MR method (Fig. 3f–j), suggesting that *Clostridium* sp *CAG_299* and *E. rectale* were likely to be causally involved in the variation of circulating non-HDL-c. Of Note, despite a younger age and better metabolic status in terms of lower blood pressure and more favorable lipid profiles (Supplementary Table S1), the negative associations of *Clostridium* sp *CAG_299* and *E. rectale* with non-HDL-c remained significant after adjustment for age, sex, overweight,

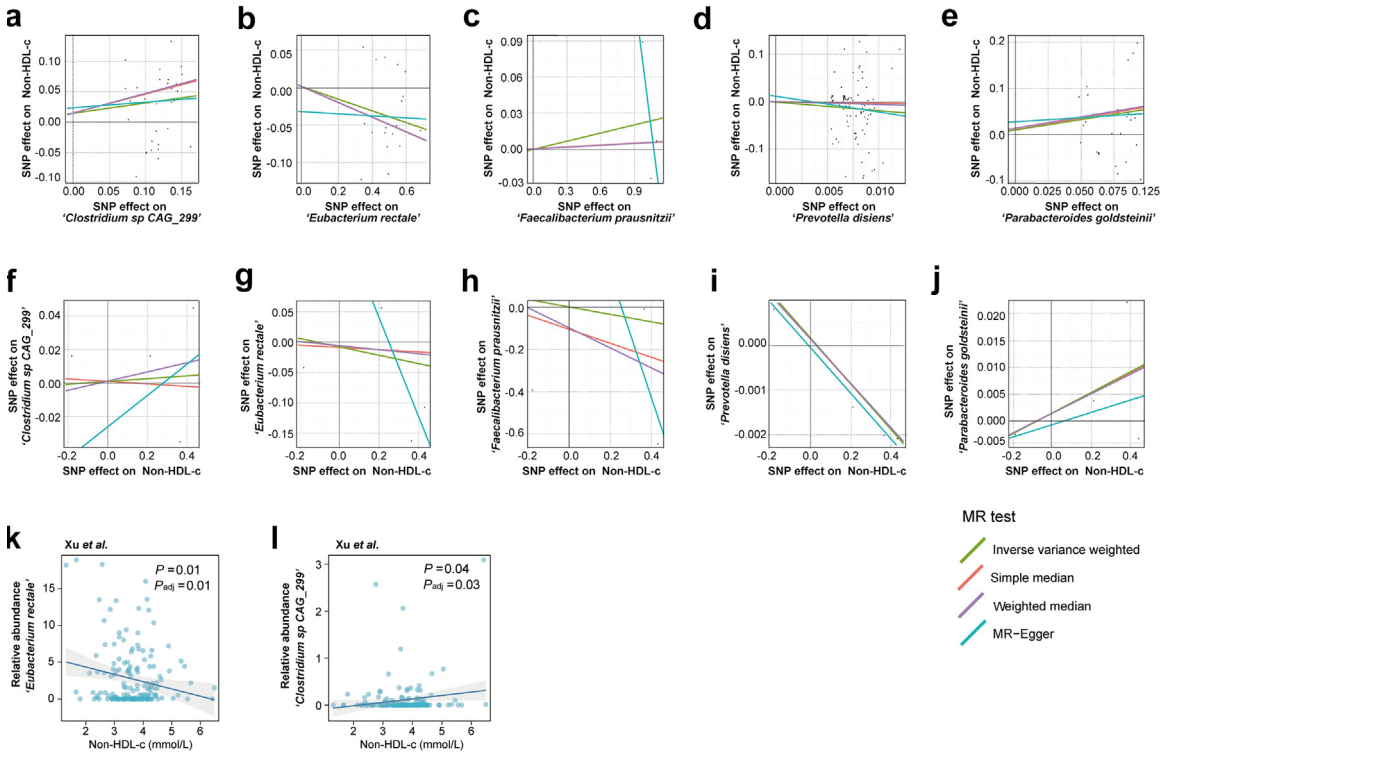

Fig. 3: **Causal links between selected species and non-HDL-c.** (a–e) Scatterplot of associations between genetic variants and (a) *Clostridium sp CAG_299* (n = 1361), (b) *Eubacterium rectale* (n = 1361), (c) *Faecalibacterium prausnitzii* (n = 1361), (d) *Prevotella disiens* (n = 1361), and (e) *Parabacteroides goldsteinii* (n = 1361) versus between genetic variants and non-HDL-c. (f–j) Scatterplot of associations between genetic variants and non-HDL-c versus between genetic variants and (f) *Clostridium sp CAG_299* (n = 1361), (g) *Eubacterium rectale* (n = 1361), (h) *Faecalibacterium prausnitzii* (n = 1361), (i) *Prevotella disiens* (n = 1361), and (j) *Parabacteroides goldsteinii* (n = 1361). The slope of each line corresponded to the estimated MR effect. Associations between the abundance of (k) *Eubacterium rectale*, or (l) *Clostridium sp CAG_299* and circulating non-HDL-c in an independent validation cohort.

smoking and drinking in an independent validation cohort (Fig. 3k and l).[16] Taken together, the above findings demonstrated that *Clostridium* sp *CAG_299* and *E. rectale* may function as major regulators in non-HDL-c.

Alterations of metabolic capacities of gut microbiota account for the variation in non-HDL-c

To further explore how gut microbiota modulates host metabolism and contributes to the variation of non-HDL-c, we then annotated microbial genes to MetaCyc database. After quality control, a total of 26 metabolic capacities were found to be significantly correlated with non-HDL-c (Supplementary Table S2 and Supplementary Fig. S4, all $P_{fdr} < 0.05$). After correcting for demographics, lifestyles, and metabolic comorbidities, 16 metabolic pathways, spanning TCA cycle, sugar acid degradation and vitamin biosynthesis, remained significantly associated with non-HDL-c (Fig. 4a, all $P_{fdr} < 0.1$). Among them, TCA cycles (PWY-5392, P23-PWY) and adenosylcobalamin biosynthesis from cobyrinate a,c-diamide I (PWY-5509), a key step in the biosynthesis of vitamin B_{12}, showed the strongest association with circulating non-HDL-c. When subjects were categorized into binary groups according to the median of non-HDL-c in this cohort, D-fructuronate degradation (PWY-7242) and superpathway of hexuronide and hexuronate degradation (GALACT-GLUCUROCAT-PWY), belonging to sugar acid degradation were markedly suppressed in individuals with high non-HDL-c (Fig. 4b). Interestingly, in line with previous report that the end-product of sugar acid degradation would eventually be transported into mitochondria as the primary fuel for TCA cycle carbon flux,[22] TCA cycle-related pathways (P23-PWY, PWY-5392) exhibited a negative correlation with non-HDL-c. Moreover, consistent with the beneficial effects of TCA cycle metabolism on stimulating insulin secretion,[23] microbial reductive TCA cycles also displayed a negative association with adverse lipid profiles and HOMA-IR (Fig. 4c). Additionally, given the fact that D-galactose, a nerve poison,[24] has been implicated in the activation of oxidative stress and

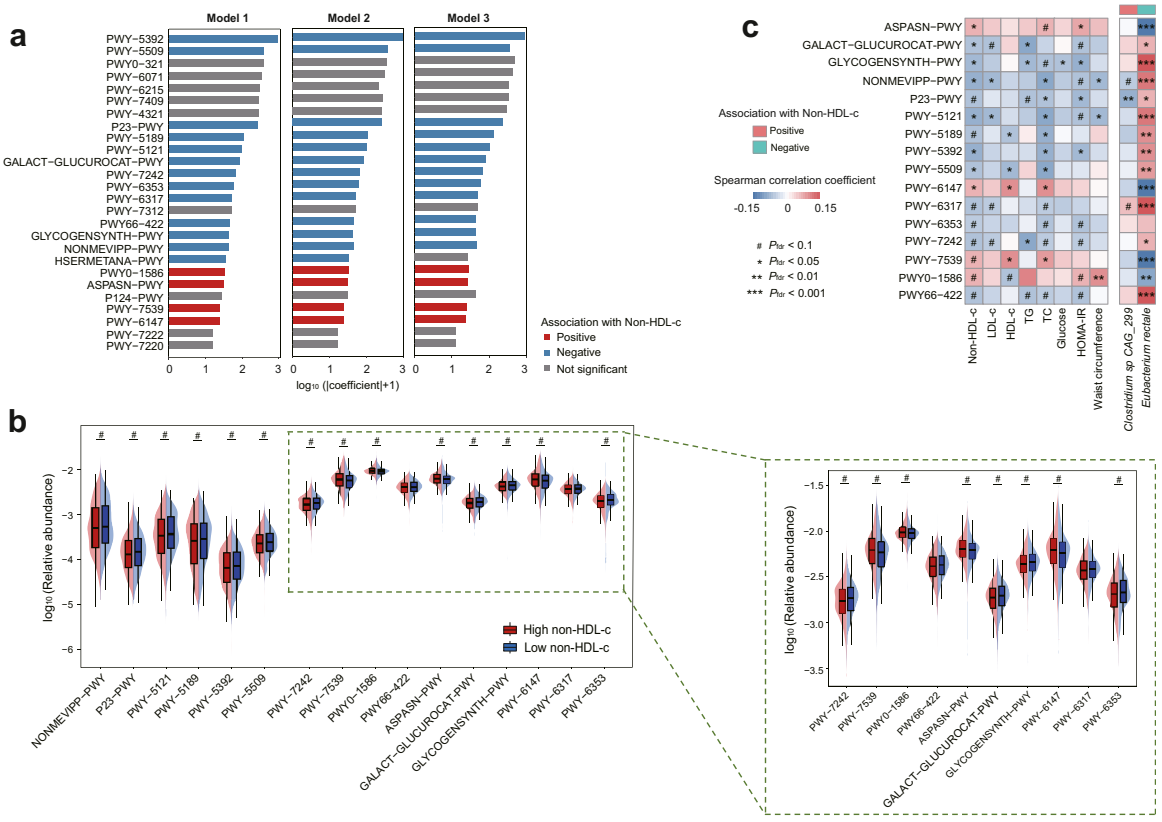

***Fig. 4:* Microbial functions are closely associated with non-HDL-c variation.** (a) The independent associations of 26 microbial pathways with non-HDL-c adjusted for (i) Model 1: age and sex; (ii) Model 2: Model 1 plus lifestyles including smoking, drinking, diet diversity and physical activity; (iii) Model 3: Model 2 plus overweight, diabetes and hypertension (n = 1361). Red, blue and gray indicate positive, negative and insignificant correlations with non-HDL-c, respectively. (b) Distribution of 16 pathways in subjects with lower (n = 680) or higher (n = 681) levels of non-HDL-c. Red and blue indicate two groups above and below the median level of non-HDL-c, respectively. $^{\#}P_{fdr} < 0.1$, and $^{*}P_{fdr} < 0.05$ by Wilcoxon rank-sum test. (c) Heatmap of the Spearman's correlation coefficients among selected microbial pathways, clinical indices (*left*) as well as *Clostridium sp CAG_299* and *Eubacterium rectale* (*right*) (n = 1361). $^{\#}P_{fdr} < 0.1$, $^{*}P_{fdr} < 0.05$, $^{**}P_{fdr} < 0.01$, and $^{***}P_{fdr} < 0.001$. Pink and green indicate positive and negative correlations with non-HDL-c, respectively.

metabolic inflammation,[25] it was not surprising to find that metabolic capacities involved in D-galactose degradation (PWY-6317, PWY66-422) were strongly associated with lower levels of non-HDL-c, and displayed an inverse correlation with TC and insulin resistance in our results (Fig. 4c). Collectively, the above findings implied that differential capacities for microbial metabolism are closely related to the alteration of non-HDL-c.

Microbiota-related metabolites are closely associated with circulating non-HDL-c

As metabolites always function as effectors for gut microbiota to act on target organs,[26] an integrative analysis of metagenomics and untargeted metabolomics were further employed to interrogate how microbial metabolites modulate non-HDL-c. A total of 210 metabolites were found to be significantly correlated with non-HDL-c (Supplementary Table S3, all $P_{fdr} < 0.05$), among which, only 8 molecules were found to be significantly associated with at least one of the two species causally related to non-HDL-c, after adjustment for age, sex, lifestyles and metabolic comorbidities ($P_{fdr} < 0.05$). Of these 8 potential microbial effectors, L-cystine, phenylsulfate, and hyodeoxycholic acid were upregulated while the other 5 microbiota-related metabolites were downregulated in participants with high non-HDL-c (Fig. 5a). In line with the antioxidant,[27] anti-inflammatory[28] and hypoglycemic[29] effect previously reported, cinnamoylglycine was found to be remarkably lower in individuals with elevated non-HDL-c (Fig. 5a), and was negatively associated with atherogenic lipids, HOMA-IR and *Clostridium* sp *CAG_299* (Fig. 5b). On the contrary, phenylsulfate, a uremic toxin,[30] which was capable of inducing reactive oxygen species production, rendering cells vulnerable to oxidative stress,[31] demonstrated a positive correlation with non-HDL-c, TC and *Clostridium* sp *CAG_299* (Fig. 5b). Of note, such an observation was further supported by a positive

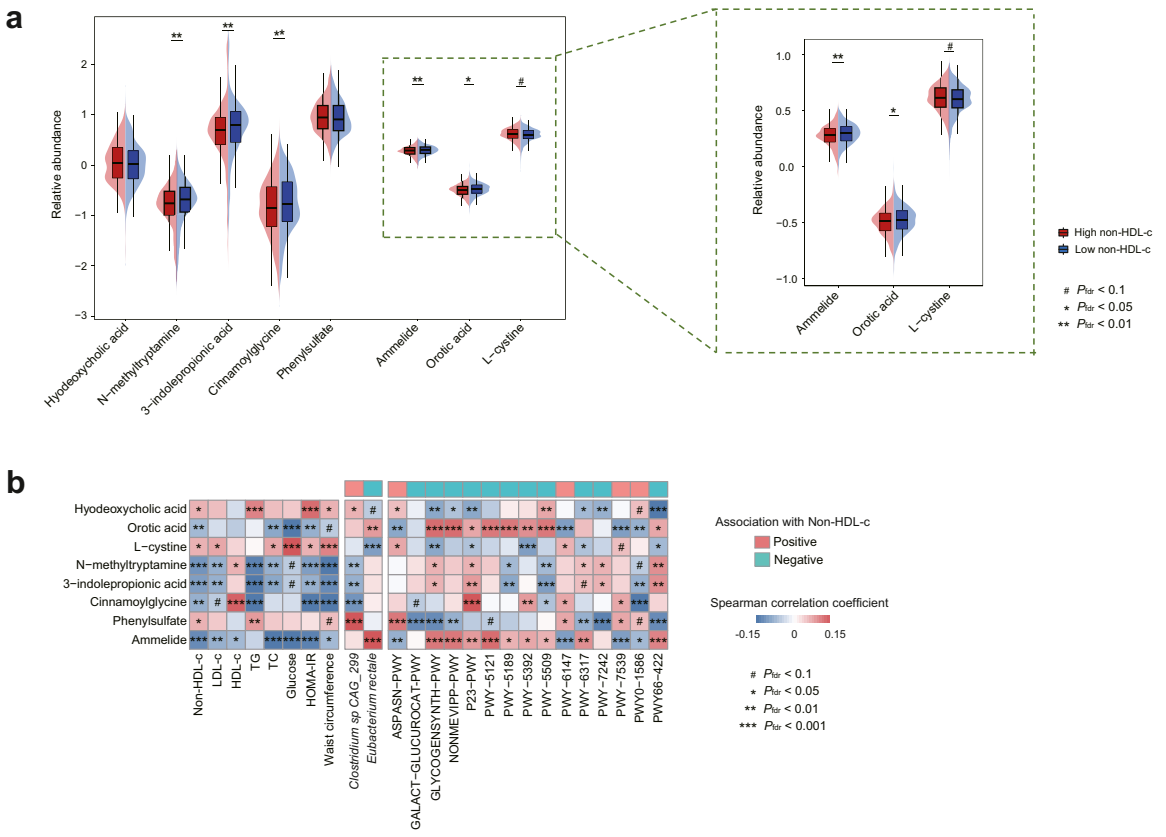

Fig. 5: **Correlation between non-HDL-c-related microbial features and plasma metabolites.** (a) Distribution of 8 metabolites highly associated with non-HDL-c and at least one of the two species causally associated with non-HDL-c in subjects with lower (n = 680) or higher (n = 681) levels of non-HDL-c. Red and blue indicate two groups above and below the median level of non-HDL-c, respectively. $^{\#}P_{fdr} < 0.1$, $^{*}P_{fdr} < 0.05$, $^{**}P_{fdr} < 0.01$, and $^{***}P_{fdr} < 0.001$ by Wilcoxon rank-sum test. (b) Heatmap of the spearman's correlation coefficients among selected plasma metabolites and clinical indices (*left*), two selected species (*middle*), and microbial capacities (*right*) (n = 1361). $^{\#}P_{fdr} < 0.1$, $^{*}P_{fdr} < 0.05$, $^{**}P_{fdr} < 0.01$, and $^{***}P_{fdr} < 0.001$. Pink and green indicate positive and negative correlations with non-HDL-c, respectively.

association between higher levels of phenylsulfate and exacerbation of diabetes.[32] Additionally, consistent with the association between L-cystine and enhanced risk of mortality in patients with coronary artery disease,[33] L-cystine was notably higher in subjects with elevated non-HDL-c and demonstrated a positive association with LDL-c, TC, fasting glucose, waist circumference and decreased *E. rectale* (Fig. 5a and b). Collectively, these findings further supported the biological relevance of microbial metabolites in the variation of non-HDL-c.

3-Indolepropionic acid and N-methyltryptamine act as the key microbial effectors to non-HDL-c variation

Intriguingly, *glutamate synthase*, a key enzyme involved in L-glutamate biosynthesis, can be annotated in the genome of *Clostridium* sp *CAG_299* through protein sequence BLAST (Fig. 6a). As an important substrate for the production of glutamate, activation of L-glutamate biosynthesis due to high abundance of *Clostridium* sp *CAG_299* in subjects with high non-HDL-c consumed a large amount of 2-oxoglutarate. Notably, also functioning as a key intermediate metabolite in the TCA cycle (P23-PWY), reduced 2-oxoglutarate subsequently hampered the TCA cycle and led to a decreased abundance of the intermediate metabolites in this pathway, such as oxaloacetate. On the one hand, reduced level of 2-oxoglutarate inhibited L-tryptophan degradation XIII pathway, and finally led to a lower biosynthesis of 3-indolepropionic acid (Fig. 6b). On the other hand, decreased abundance of oxaloacetate suppressed the biosynthesis of L-aspartate, which in turn led to an attenuated metabolic cascade including L-asparagine biosynthesis, glycine production, and L-serine biosynthesis, Glycine, serine and threonine metabolism, and tryptophan metabolism, and finally resulted in a reduced production of N-methyltryptamine (Fig. 6c). In line with the functional inference, most of the intermediate metabolites along these pathways demonstrated a lower abundance in subjects with elevated non-HDL-c

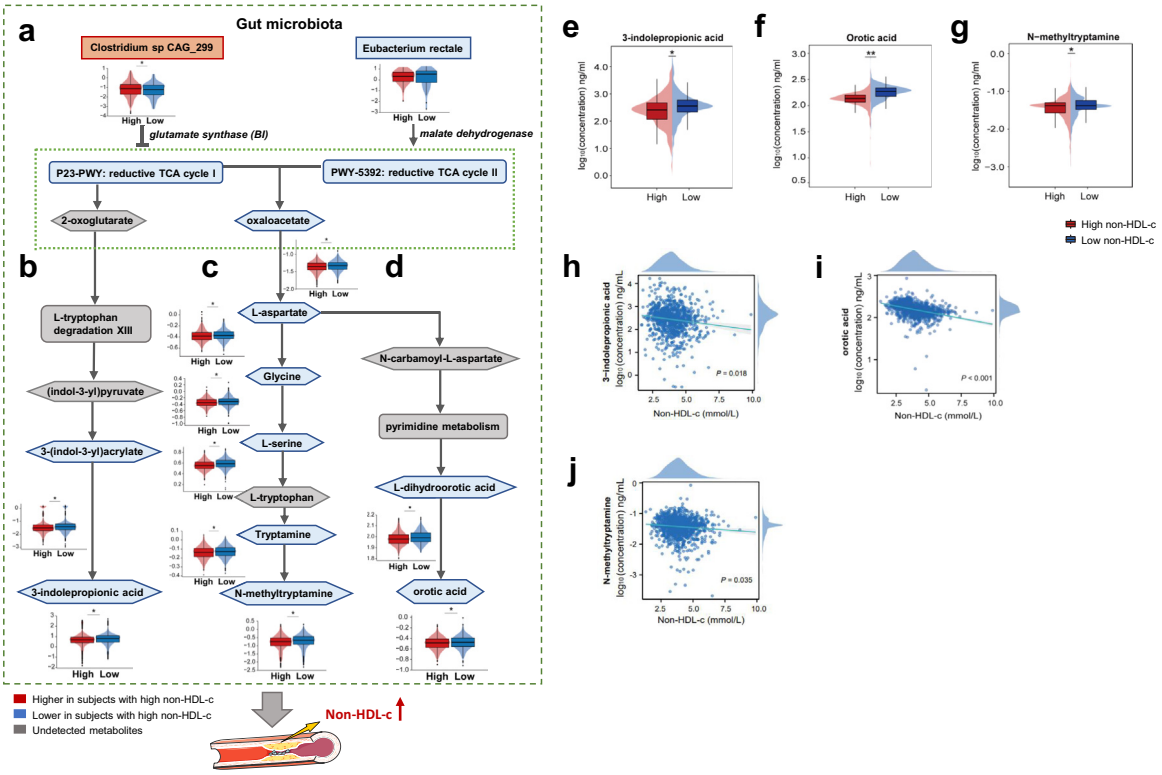

***Fig. 6:* Microbial metabolites serve as effectors to non-HDL-c variance.** (a–d) Illustration of how (a) high *Clostridium sp CAG_299* but low *Eubacterium rectale*, as well as suppressed reductive tricarboxylic acid (TCA) cycle (n = 1361), and subsequent decreased (b) 3-indolepropionic acid (n = 1361), (c) N-methyltryptamine (n = 1361) and (d) orotic acid (n = 1361) production, contribute to the elevation of non-HDL-c in the circulation. Grey, red and blue indicated undetected, increased and decreased species/functions/metabolites in individuals with high non-HDL-c. (e–g) Distribution of (e) 3-indolepropionic acid, (f) orotic acid and (g) N-methyltryptamine in subjects with lower (n = 680) or higher (n = 681) levels of non-HDL-c by targeted metabolomics analysis. Red and blue indicated above or below the median level of non-HDL-c, respectively. $^{*}P_{fdr} < 0.05$ and $^{**}P_{fdr} < 0.01$ by Wilcoxon rank-sum test. (h–j) Scatterplot of associations of (h) 3-indolepropionic acid (n = 1361), (i) orotic acid (n = 1361), and (j) N-methyltryptamine (n = 1361) with circulating non-HDL-c by targeted metabolomics.

(Fig. 6a and b). Additionally, both N-methyltryptamine and 3-indolepropionic acid demonstrated an inverse correlation with *Clostridium* sp *CAG_299* and multiple ASCVD risk factors, such as proatherogenic lipids and insulin resistance (Fig. 5b).

In a similar fashion, *malate dehydrogenase*, a key enzyme involved in TCA cycle (PWY-5392) can be annotated in the genome of *E. rectale* (Fig. 6a). Decreased abundance of *E. rectale* in subjects with high non-HDL-c led to a lower expression of *malate dehydrogenase*, which subsequently suppressed TCA cycle and resulted in a reduced production of oxaloacetate. As an intermediate metabolite in the TCA cycle, decreased oxaloacetate restrained L-aspartate biosynthesis and led to a lower production of the downstream metabolites including N-carbamoyl-L-aspartate and L-dihydroorotic acid, which in turn hampered pyrimidine metabolism and resulted in a lower production of orotic acid (Fig. 6d). Consistently, orotic acid was found to be inversely associated with cardiometabolic risk factors, including TC, fasting glucose and HOMA-IR, while positively associated with *E. rectale* (Fig. 5b).

Furthermore, targeted metabolomics analysis showed that the median levels of 3-indolepropionic acid, orotic acid and N-methyltryptamine were 517.88 ng/mL, 195.61 ng/mL and 0.05 ng/mL, respectively, in individuals with low levels of non-HDL-c, which were considerably higher than the level observed in subjects with high non-HDL-c (Fig. 6e–g). Additionally, all the three microbial metabolites demonstrated a significant negative correlation with non-HDL-c in the circulation (Fig. 6h–j, all $P < 0.05$), further suggesting that orotic acid, 3-indolepropionic acid and N-methyltryptamine might be key players in the regulation of non-HDL-c. To further determine the causal associations of these three metabolites and the variation of non-HDL-c, bi-directional MR analysis was applied. Though all these 3 metabolites were all found to be associated with non-HDL-c in at least two MR methods, including IVW, simple median and weighted median (Fig. 7a–c, and Supplementary Fig. S5), reverse MR analysis also supported a significant causal effect of non-HDL-c on orotic acid (Fig. 7d–f), suggesting that only 3-indolepropionic

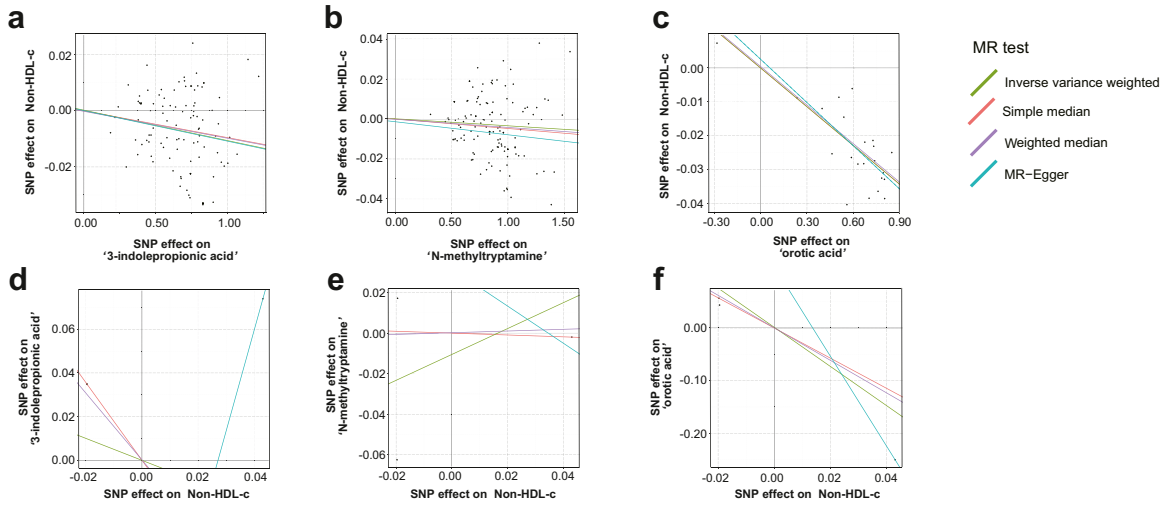

***Fig. 7:* Causal links between selected metabolites and non-HDL-c.** (a–c) Scatterplot of associations between genetic variants and (a) 3-indolepropionic acid (n = 1361), (b) N-methyltryptamine (n = 1361), and (c) orotic acid (n = 1361) versus between genetic variants and non-HDL-c. (d–f) Scatterplot of associations between genetic variants and non-HDL-c versus between genetic variants and (d) 3-indolepropionic acid (n = 1361), (e) N-methyltryptamine (n = 1361), and (f) orotic acid (n = 1361). The slope of each line corresponded to the estimated MR effect.

acid and N-methyltryptamine were causally linked to reduced non-HDL-c. Importantly, for the two causal associations observed, the *F*-statistics of the IVs were all greater than 10, eliminating the bias of weak IVs, and the results of Cochran's IVW *Q* test showed no significant heterogeneity of these IVs. Additionally, according to the results of the MR-Egger regression intercept analysis, no significant directional horizontal pleiotropy was found for these two causal relationships (Supplementary Fig. S5). Moreover, leave-one-out analysis further suggested that the causal links between 3-indolepropionic acid, N-methyltryptamine and non-HDL-c were not driven by any single SNP (Supplementary Fig. S6). Collectively, the above findings demonstrated that decreased 3-indolepropionic acid and N-methyltryptamine served as key microbial effectors of *E. rectale* and *Clostridium* sp *CAG_299* to elevated non-HDL-c.

Discussion

Despite several small-scale human studies and murine models implicating a critical role of gut microbiota in lipid metabolism,[5,34] evidence concerning the effect of microbial metabolism in non-HDL-c variation remains limited. Here, utilizing a deep characterization of gut microbiota by shotgun metagenomics, metabolomics and host genotyping in 1361 community-dwelling participants free of lipid-lowering therapies, we demonstrated a causal role of *E. rectale* and *Clostridium* sp *CAG_299* in non-HDL-c variation and uncovered a strong correlation between microbial reductive TCA cycle and circulating non-HDL-c. Moreover, through the integration of multi-omics and MR analysis, we further identified decreased production of 3-indolepropionic acid and N-methyltryptamine as key molecular transducers to the elevation of circulating non-HDL-c.

Emerging evidence implies that gut microbiota may affect various physiological processes in the host, including lipid metabolism.[35] Similar to our observation, a recent study in 2309 individuals from Europe identified a close association of 32 microbial families and genera with various lipoprotein particles, including very-low-density and high-density subfractions.[36] Moreover, a strong association between family *Clostridiaceae/Lachnospiracease* and serum lipids was also found in a study conducted in northern Netherlands.[12] However, these studies were primarily performed in Caucasians and didn't take into account the confounding effect of comorbidities.[12,36] Due to a substantial influence of genetic backgrounds, diet, lifestyles, and geographic differences on gut microbiota, core microbial features and their associations with host metabolism were quite different across ethnics.[37] In this regard, we were the first to delineate the microbial metabolism and non-HDL-c in Chinese, with a relatively large and medication-naïve cohort. Moreover, targeting of 16S variable regions with short-read sequencing platforms is low in taxonomic resolution.[38] In this connection, with the advantage of metagenomics, our findings from large-scale cohort lend further support to the observation that perturbations in gut microbiota contribute to the variation in non-HDL-c, and proved that decrease in SCFAs-producers and increase of *Clostridium* sp *CAG_299* and *P. goldsteinii* remained independently associated with the variation in non-HDL-c after

extensive adjustment for potential confounders, including demographics, lifestyles and metabolic comorbidities. Moreover, taking advantage of the causal inference potential of MR analysis, we expanded existing evidence by proving that *E. rectale* and *Clostridium* sp *CAG_299* were causally associated with non-HDL-c in the circulation. As a predominant atherogenic agent, our findings demonstrated that interventions targeting gut microbiota to reduce non-HDL-c might provide additional benefits for ASCVDs control.

Given the ability of bile salt hydrolase (BSH) to hydrolyze conjugated bile salts into deconjugated BAs, which contributes to the maintenance of cholesterol balance, the genera *Eubacterium*, a major reservoir of BSHs,[39] was found to play a critical role in lipid absorption via catalyzing BSH activity. In a similar fashion, a butyrate-mediated inhibitory effect on the progression of atherosclerosis provided by *E. rectale* had also been found in murine models.[40] Therefore, it was not surprising to find that *E. rectale* was causal in decreasing non-HDL-c in our study. On the contrary, *Clostridium* sp *CAG_299* was found to be causal in promoting non-HDL-c levels and was positively associated with LDL-c, TC and waist circumference. Such an observation was supported by previous findings that *Clostridium OTUs* were more abundant upon exposure to high-cholesterol diet.[5] Intriguingly, the genus *Clostridium* had been reported to disrupt the balance of BAs metabolism, which in turn led to an increase in circulating cholesterol.[41] In addition, consistent with the positive correlation between *F. prausnitzii*, *E. rectale*, *Prevotella* gena and increasing adherence to Mediterranean diet,[19] *F. prausnitzii* and *P. disiens* were also found to be negatively associated with non-HDL-c in our study. Similar to the observation that *F. prausnitzii*, a well-known butyrate producer, could reduce hepatic fat accumulation[42] and exert anti-inflammatory effects,[43] it was found to be significantly lower in subjects with elevated non-HDL-c and negatively correlated with TG and insulin resistance. Additionally, as a propionate-producer, the genera *Prevotella* was reported to be enriched in response to high-fiber diets and promote the differentiation of anti-inflammatory Treg/Tr1 cells in the gut.[44] Consistent with previous reports that propionate was able to decrease intestinal cholesterol absorbance via an immunomodulatory pathway,[45] *P. disiens* exhibited an inverse correlation with atherogenic lipids, but a positive association with HDL-c in our cohort. Thus, the above findings further support the notion that gut microbiota might be a promising therapeutic target for reducing non-HDL-c.

Moreover, different from previous large-scale studies using 16S rRNA sequencing, which lacks functional resolution,[46] with the advantage of metagenomics technique, we found that capacities for microbial reductive TCA cycle were substantially suppressed in subjects with elevated non-HDL-c. More importantly, integrative analysis of metagenomics and metabolomics demonstrated that consistent with a reduced capacity for TCA cycle and a decrease of TCA cycle intermediates, 3-indolepropionic acid, whose levels were completely depended on microbial catabolism of tryptophan,[47] was found to be remarkably lower in individuals with high non-HDL-c. Notably, 3-indolepropionic acid had been reported to have an anti-atherogenic effect. Mechanistically, elevated 3-indolepropionic acid alleviated atherosclerotic plaque development through enhancing macrophage reverse cholesterol transport in miR-142-5p/ABCA1 dependent signalling.[48] In line with previous reports that 3-indolepropionic acid inhibited lipid accumulation[5] and was strongly associated with lower risk of type 2 diabetes,[49,50] we also found a negative correlation between 3-indolepropionic acid and several risk factors for cardiometabolic diseases, such as adverse lipid profiles and insulin resistance, as well as *Clostridium* sp *CAG_299* in our cohort. Likewise, the suppressed capacity for TCA cycle also led to an inhibition of oxaloacetate and L-aspartate biosynthesis, which in turn resulted in a dampened generation of L-dihydroorotic acid and reduced level of orotic acid in individuals with high non-HDL-c. Moreover, as a key intermediate metabolite in the biosynthetic pathway of pyrimidines, which had a beneficial effect on post-ischemic myocardial function,[51] it was not surprising to find a strong correlation between orotic acid and lower levels of TC and insulin resistance, as well as elevated SCFA-producers in our study. Such an observation was further supported by animal studies that dietary supplementation with orotic acid lowered the levels of pro-atherogenic lipids and maintained lipid homeostasis in cardiomyocytes, via the activation of peroxisome proliferator-activated receptor alpha.[52] In addition, decreased biosynthesis of oxaloacetate and L-aspartate inhibited the generation of glycine and L-serine, which eventually led to a suppression of tryptamine biosynthesis and a lower level of N-methyltryptamine in participants with high non-HDL-c. Notably, as a precursor of N-methyltryptamine, tryptamine contributed to metabolic health through attenuating pro-inflammatory cytokine responses in an aryl hydrocarbon receptor-dependent manner.[47] Consistently, a close correlation between N-methyltryptamine and lower levels of adverse lipids, high level of HDL-c, and a depletion of *Clostridium* sp *CAG_299* was found in our cohort. However, according to results of bi-directional MR analysis, only 3-indolepropionic acid and N-methyltryptamine were likely to be causally involved in the variation of circulating non-HDL-c. Though the molecular mechanisms underlying the beneficial effects of these metabolites on non-HDL-c remain unclear and warrants further investigation in animal models, our integrative analysis suggested that 3-indolepropionic acid and N-methyltryptamine may function as potential postbiotics for modulating non-HDL-c.

Limitations of this study

Though our findings remained robust after adjustment for a range of potential confounders, this study is not exempt from limitations. First, this is a single-center study that only included Chinese participants, which might limit the generalizability of our findings to other ethnicities. Second, though causal effects of gut microbiota and metabolites on non-HDL-c had been proved by MR analysis, more validations in preclinical models and prospective cohorts are needed before moving from potential to action.

Conclusions

In summary, our study uncovers a causal effect of gut microbiota on non-HDL-c and identify 3-indolepropionic acid and N-methyltryptamine as key effectors of gut microbiota to the variation of non-HDL-c. These findings highlight the potential of targeting gut microbiota to control non-HDL-c and improve the efficacy of ASCVDs prevention.

Contributors

Yan Liu and Min Xia conceived and designed this study. Bingqi Ye and Ludi Liu established the platform for omics data analysis. Shiyi Zhou carried out the analysis. Jingmeng Ju and Jialu Yang helped with recruitment of study participants and sample collection. Shanshan Zhu, Yingxi Xu, Yi You and Wenkang Li helped with the processing of biological samples. Shiyi Zhou, Yan Liu and Min Xia interpreted the results. Shiyi Zhou, Yan Liu and Min Xia drafted and revised the manuscript for intellectual content. Yan Liu and Min Xia obtained fundings and supervised this study. All authors made substantial contributions to the intellectual content of the paper and approved the final version of the manuscript.

Data sharing statement

Metagenomic sequencing data for all samples were deposited at NCBI Sequencing Read Archive (SRA Accession: PRJNA855026 and PRJNA851599). The analysis pipelines and codes used in this study have been made publicly available at https://github.com/Xia-Liu-Lab/Non-HDL-c-Project. Other data that support the findings of this study were available from the corresponding authors upon reasonable request.

Declaration of interests

The authors have no conflicts of interest to report.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2024.105150.

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