ORIGINAL PAPER
Polish Journal of Microbiology
2025, Vol. 74, No 2, 206-217
https://doi.org/10.33073/pjm-2025-017

Roles of Gut Microbiota and Associated Metabolites in *Clostridioides difficile* Infection

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Submitted 31 December 2024, accepted 25 April 2025, published online 16 June 2025

Abstract

Clostridioides difficile infection (CDI), is the most common healthcare problem primarily involving the colon of individuals who's gut microbiota has been disrupted. Proteobacteria (officially updated and recognized as Pseudomonadota), a minor gut-associated microbial community within a healthy host, could serve as a metric for CDI. However, the alterations of specific members of Proteobacteria in the context of CDI are not thoroughly understood. Based on the summary data of microbiome from 7,738 participants in the Dutch cohort, linkage disequilibrium score regression (LDSC) was used to explore the causal effect of 207 gut microbiome on CDI. Secondly, we performed a Mendelian randomization analysis to investigate the causal relationship between 31 microbiota taxa affiliated with Proteobacteria and CDI. Finally, three significant taxa ($p < 0.05$, OR > 1) were utilized to conduct the mediation analysis of 1,400 metabolites based on a two-step Mendelian randomization study (two-step MR). The inverse-variance weighted method was conducted as a primary analysis to estimate the causal effect, and the robustness of the results was tested via sensitivity analysis using multiple methods. Bivariate LDSC analysis identified a strong correlation between four populations affiliated with Proteobacteria (*Pasteurellaceae*, *Haemophilus*, Pasteurellales and *Haemophilus parainfluenzae)* and CDI. In two-step MR, Burkholderiales order exerted detrimental effects on CDI by decreasing the levels of 3-hydroxylaurate (OR 0.896; 95%CI, 0.803-0.998; $p = 0.047$), indicating that metabolite did act as mediator between gut microbiota and CDI. We conducted a study to assess the relations between genetically predicted gut microbiota and metabolite levels with CDI. These results highlight the potential of targeting Burkholderiales and 3-hydroxylaurate as a new antimicrobial strategy against CDI.

Keywords: *Clostridioides difficile* infection, Proteobacteria, metabolites, Mendelian randomization, linkage disequilibrium score regression

Introduction

Clostridioides difficile is a ubiquitous anaerobic Gram-positive spore-forming and toxin-producing bacterium. *C. difficile* infection (CDI), is the most common healthcare problem in the developed world (Guh et al. 2020; Khanna 2021). It is a disease primarily involving the colon of individuals whose gut microbiome has been disrupted and propagated by risk exposure factors. Alterations in the structure and function of the gut microbiome in the context of CDI create a favorable metabolic microenvironment for the life cycle of *C. difficile*, promoting the germination, expansion, and virulence of this important pathogen. The available approved antibiotic-based and microbiome-based medications for CDI currently have several drawbacks (McDonald et al. 2018). Antibiotics, such as vancomycin or fidaxomicin, may lead to collateral damage to the gut microbiome and fail to improve outcomes (Sehgal and Khanna 2021). Fecal microbiota transplantation, which addresses the pathophysiology of CDI, remains available in limited clinical settings as part of unclear exact mechanisms (Bednárik et al. 2025). Therefore, novel microbiome restoration therapies remain vital to curb the unmet CDI management needs.

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Changes in the gut-associated microbial community composition are associated with CDI, but the mechanisms underlying this imbalance are not thoroughly understood. The main taxa within the balanced gut microbial community of human hosts are the classes Bacteroidetes (officially updated and recognized as Bacteroidia) and Clostridia (phylum Bacillota) (Tap et al. 2009). An increased abundance of Proteobacteria (officially updated and recognized as Pseudomonadota) has been proposed to characterize gut microbial imbalance (Shin et al. 2015). Proteobacteria are a minor gut-associated microbial community within a healthy host. However, a bloom of Proteobacteria is observed in patients with inflammatory bowel disease (Morgan et al. 2012), colorectal cancer (Wang et al. 2012) or necrotizing enterocolitis (Normann et al. 2013). Previous studies have also shown that reduced abundance of Bacteroidetes and Firmicutes (officially updated and recognized as Bacillota) as well as expansion of Proteobacteria were exhibited in the gut microbiota of CDI patients (Reeves et al. 2011; Mooyottu et al. 2017). A systematic search of fecal microbiota transplantation (FMT) found an increased abundance in Bacteroidetes to the detriment of Proteobacteria after fecal microbiota transplantation for CDI treatment (van Nood et al. 2013). This indicates that Proteobacteria could also serve as a metric for CDI, offering insights into an individual's susceptibility to CDI. However, the alterations of specific members of Proteobacteria in the context of CDI are not entirely understood.

Gut microbiota can participate in critical metabolic processes of the host and shape the metabolic environment (van Prehn et al. 2021). The dominance of Bacteroidetes and Firmicutes in the human intestine ensures the production of metabolites that maintain gut homeostasis. Bacteroidetes can break down glycans and non-digestible carbohydrates for sugar harvest. Firmicutes can ferment complex carbohydrates and amino acids into short-chain fatty acids (SCFAs) (Hou et al. 2022; Gurung et al. 2024). Risk factors, such as antibiotic usage, alter gut microbiota's structure, causing changes in amino acids, fatty acids, and bile acids and increasing susceptibility to CDI (Vliex et al. 2024). Therefore, in addition to the specific gut microbiome, metabolites could serve as the hallmark of gut microbiota dysbiosis in CDI.

Nevertheless, the results of previous studies are not always consistent. For example, in a fecal metabolome study from the 186-person cohort, 4-MPA (4-methylpentanoic acid) was identified to be elevated in patients with CDI, which was consistent with its production by *C. difficile* from leucine during Stickland metabolism. Noncanonical, unsaturated bile acids were also depleted in patients with CDI (Robinson et al. 2019). Another cohort study demonstrated that levels of primary bile acids, some amino acids, and fatty acids metabolites increased in feces from 30 cases of CDI compared with 25 non-CDI patients (Gu 2016). Diverse general population attributes and different analytical approaches across studies may explain these inconsistencies. Conducting studies that are less susceptible to selection bias, reverse causation, and confounding effect are critical.

We speculated that a specific metabolite might mediate the effect of Proteobacteria on CDI. Large-sample genome-wide association studies (GWAS) have identified hundreds of human single nucleotide polymorphisms (SNPs) associated with gut microbiota, facilitating the exploration of causal associations between Proteobacteria and CDI using Mendelian randomization (MR). In MR analysis, the alleles are randomly transferred from parents to offspring when the gamete is formed (Burgess et al. 2015).

Thus, in order to better characterize specific members of Proteobacteria and associated metabolite markers related to CDI susceptibility, we applied genetic instruments to assess the relations between genetically predicted gut microbiota and metabolite levels with CDI. Specifically, we applied bivariate linkage disequilibrium score regression analysis (LDSC) and MR analysis leveraging data from different populations of two ethnicities. Two-step MR extends this approach to ease mediation analysis within an MR framework. The present study aimed to investigate the detailed microbial signature of the CDI environment, especially Proteobacteria and the associated metabolic microenvironment, which will highlight therapeutic strategies targeting microbes or molecules that disrupt or enforce metabolic networks associated with CDI.

Experimental

Materials and Methods

Study design. Firstly, LDSC was used to explore the causal effect of 207 human gut microbial taxa on *C. difficile*. Secondly, we performed MR analysis from 31 microbiota taxa affiliated with Proteobacteria (7 Deltaproteobacteria class, 10 Gammaproteobacteria class, and 14 Betaproteobacteria class) to *C. difficile*. Finally, three significant taxa ($p < 0.05$, OR > 1) were utilized

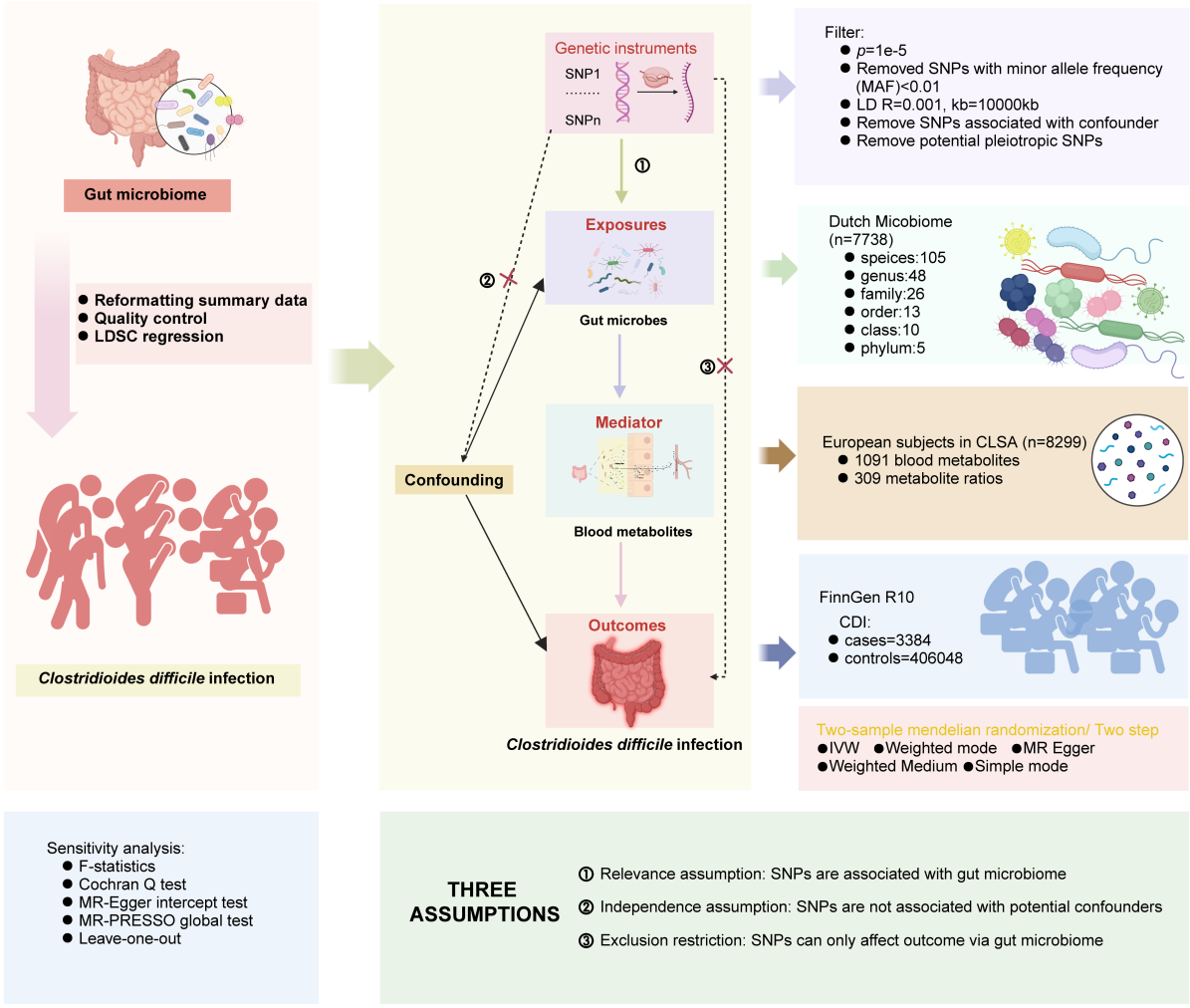

Fig. 1. Assumptions and design of the bidirectional mediation Mendelian randomization (MR) analysis.
Firstly, a two-sample bidirectional MR was performed to investigate the causal relationships between gut microbiota (exposures) and *Clostridioides difficile* infection (outcomes). Secondly, 1,400 blood metabolites (mediator) were selected for subsequent mediation analysis. Finally, a two-step MR analysis was conducted to detect potential mediating metabolites (Step 1, the effect of gut microbiota on metabolites; Step 2, the effect of metabolites on CDI). LDSC – linkage disequilibrium score regression; CLSA – Canadian Longitudinal Study on Aging; IVW – inverse variance weighted; CDI – C. difficile infection

to conduct the mediation analysis of 1,400 metabolites based on a two-step MR study. OR > 1 refers to the odds ratio (OR) of (CDI) occurrence associated with a per-unit increase in the abundance of specific gut microbial taxa, which indicates that a higher abundance of the taxon is correlated with an elevated risk of CDI. The diagram of the study design, the causal interpretation of Mendelian randomization, and three necessary assumptions were illustrated in Fig. 1. The instrumental variables (IVs) must be strongly associated with the exposure factor. Then, the IVs should not be associated with any confounders of the exposure-outcome association. The IVs can only influence the outcome variable through the exposure factor (Lawlor et al. 2008; Davies et al. 2018). Our study is reported following the Strengthening the Reporting of Observational Studies in Epidemiology Using Mendelian randomization guidelines (STROBE-MR) (Skrivankova et al. 2021) (Table SI).

Data sources. The summary data of microbiome was sourced from the study by Lopera-Maya et al. (2022), reporting 207 taxa and 205 pathways involving 7,738 participants in the Netherlands cohort, spanning across five phyla, ten classes, 13 orders, 26 families, 48 genera, and 105 species. Circulating plasma metabolites originated in the study by Chen et al. (2023), analyzing 8,299 unrelated European subjects in the platform of Canadian Longitudinal Study on Aging (CLSA) (Raina et al. 2019; Chen et al. 2023). Summary statistics were deposited in the GWAS Catalog (https://www.ebi.ac.uk/gwas) (Cerezo et al. 2025). In the genome-wide association analysis, accession num-

bers for European GWASs: GCST90199621-90201020, which included 1,091 blood metabolites (850 known substances and 241 unknown entities) and 309 metabolite ratios. The dataset for CDI was obtained from the FinnGen R10 (https://storage.googleapis.com/finngen-public-data-r10/summary_stats/finngen_R10_C_DIFFICILE_ENTEROCOLITIS.gz), which included 3,384 CDI patients and 406,048 controls. Detailed information on data sources was provided in Fig. 1 and Table I.

Selection of genetic instrumental variables. In order to ensure the accuracy of results on the causal link between gut microbiome and CDI, the following quality control steps were used to select the superior instrumental variables (Xiang et al. 2021). Instrumental variables (IVs) associated with microbiota traits were identified using a genome-wide significance threshold of $p < 1 \times 10^{-5}$ across six taxonomic levels: phylum, class, order, family, genus, and species. To ensure robust and unbiased results, several filtering criteria were applied. SNPs located on chromosome 23 and those with multiple alleles (> 2) were excluded to prevent confounding effects. Additionally, SNPs with a minor allele frequency (MAF) < 0.01 were removed. To account for linkage disequilibrium (LD), we excluded SNPs with $r^2 < 0.001$ and a genomic distance > 10,000 kb (Sudmant et al. 2015; Myers et al. 2020). Furthermore, to minimize horizontal pleiotropy, SNPs significantly associated ($p < 5 \times 10^{-8}$) with confounders or outcome traits were excluded, though no such SNPs were detected in our analysis. Lastly, to mitigate weak instrument bias, we calculated the *F*-statistic for each exposure and retained only SNPs with an *F*-statistic > 10 (Ning et al. 2022). These stringent selection criteria ensured that the chosen IVs were robust and suitable for Mendelian randomization analysis.

Table I
Detailed information of studies and datasets used for analysis.

Data source	Phenotype	Sample size	Cases	Population
Dutch Microbiome	Gut microbial	7,738	/	Netherlands
European subjects in CLSA	Metabolites	8,299	/	European
FinnGen R10	CDI	409,432	3,384	European

CLSA – Canadian Longitudinal Study on Aging; CDI – C. difficile infection

Genetic analysis to elucidate causality by linkage disequilibrium score regression. In order to show the genetic correlation between gut microbiota and CDI, we performed bivariate LDSC using summary statistics. The genetic correlation between two traits was estimated by regressing the LD score of each SNP against the effect size of the two traits (Bulik-Sullivan et al. 2015b). This method could generate a score reflecting whether the test statistic of a biologically relevant variant correlates with nearby variants in high linkage disequilibrium without sample overlap bias (Bulik-Sullivan et al. 2015a; Wielscher et al. 2021).

Genetic analysis to elucidate causality by bidirectional Mendelian randomization. Based on LDSC analysis, Proteobacteria were identified as related to CDI. In order to further explore a causal relationship, we conducted a bidirectional MR analysis to explore the causal relationship between the Proteobacteria and CDI, including 31 taxons affiliated with Proteobacteria (7 Deltaproteobacteria class, 10 Gammaproteobacteria class, 14 Betaproteobacteria class). The inverse variance weighted (IVW) method is considered as the most accurate and powerful method for estimating causal effects compared to other methods when the number of SNPs is ≥ 2 (Burgess et al. 2013; Bowden et al. 2016; Choi et al. 2019). We obtained an overall estimate of the impact of the microbiome on the risk of CDI through the IVW method. If only one SNP was available, the Wald ratio method was selected. Additionally, weighted median, MR Egger, weighted mode, simple mode methods were complemented, which were also reported in beta (β) value with standard error for the continuous outcome and odds ratio (OR) with a 95% confidence interval (CI); $p < 0.05$ was considered significant. The weighted median method can provide consistent estimates of the causal effects though the weight of invalid IVs reaches 50% (or < 50%) (Bowden et al. 2016). Although others do not meet the requirements for causal inference using MR analysis, weighted mode is still available when most SNPs with similar

individual causal effect estimates are valid instruments (Ooi et al. 2019; Lopera-Maya et al. 2022).

Mediation analysis link "gut microbiota-blood metabolites-CDI". Summary statistics of blood metabolites obtained from 8,299 individuals of European ancestry covering 1,091 metabolites and 309 metabolite ratios were utilized. To identify potential novel metabolites as mediators between gut microbiome and CDI, we performed a two-step MR to decompose the direct and indirect effects of the gut microbiome and blood metabolites on CDI. The two-step MR assumes no interaction between exposure and mediator (Wang et al. 2023). Two estimates were calculated: the causal effect of the gut microbiota on the blood metabolites and the causal effect of the blood metabolites on CDI.

Sensitivity analysis. We assessed horizontal pleiotropy using the MR-Egger intercept and MR-PRESSO global tests (Bowden et al. 2015; Verbanck et al. 2018). The MR-PRESSO test helped to identify and exclude SNPs that might introduce bias. While the deviation of MR-Egger intercept from the origin suggested no evidence of horizontal pleiotropy among the selected IVs if p-value ≥ 0.05 (Li et al. 2023). We also assessed heterogeneity using Cochran's Q test**,** with a p-value ≥ 0.05 indicating the absence of heterogeneity (Bowden et al. 2019). Besides, leave-one-out analysis was used to evaluate whether the significant results were driven by a single SNP (Xu et al. 2022). All MR analyses were conducted in R (version 4.3.2) (R Core Team 2023), using "LdlinkR packages" (Myers et al. 2020), "ggplot2" (Wickham 2016), "TwoSampleMR" (Hemani et al. 2018), "tidyverse" (Wickham et al. 2019), and "MR-PRESSO packages" (Verbanck et al. 2018).

Ethics approval and consent to participants. Our analysis used publicly available GWAS summary data. Ethical approval was not required. All participants have duly provided their consent forms.

Results

Genetic instrumental variables without bias were selected. Following the criteria for IVs selection, we selected several SNPs used as IVs ranging from 3 to 14 (median, 7) for Proteobacteria, which included 31 taxons belonging to it from a pool of 7,738 Dutch participants (Table SII). We extracted these SNPs' effect allele, other allele, beta, SE, and p-value for MR analysis. Importantly, all IVs exhibited F-statistics greater than 10, indicating the absence of weak IVs in this study.

Linkage disequilibrium score regression analysis confirmed the genetic correlation between Proteobacteria and CDI. Bivariate LDSC analysis was performed to evaluate the genetic correlation between 207 species-level gut microbiota and CDI. Owing to limitations such as low heritability and sample size, some species cannot be used for the above analysis (Xu et al. 2022). Finally, we researched the estimations of genetic correlation between 113 species and CDI. Bivariate LDSC analysis identified a strong correlation between four taxons affiliated with Proteobacteria (*Pasteurellaceae*, *Haemophilus*, Pasteurellales and *Haemophilus_parainfluenzae),* and CDI, *Veillonellaceae*, affiliated with Firmicutes and CDI in Fig. 2 and Table SIII.

MR analysis identified a potential causal relationship between specific taxa affiliated with Proteobacteria and CDI. In MR analysis, we evaluated the relationships between 31 microbiomes affiliated with Proteobacteria and CDI based on the IVW method (Fig. 3A). In order to explore the risk of Proteobacteria to CDI, we studied the microbiome with OR > 1. Significant taxa were *Sutterellaceae* (OR 1.284; 95%CI, 1.041-1.583; p = 0.020), Betaproteobacteria (OR 1.355; 95%CI, 1.137-1.614; p = 0.001) and Burkholderiales (OR 1.247; 95%CI, 1.052-1.478; p = 0.011) (Fig. 3B). They were all positively associated with CDI. In addition, only one taxon affiliated with Proteobacteria was negatively correlated with CDI, *Parasutterella excrementihominis* was demonstrated to exert a potent protective effect on CDI (OR 0.861; 95%CI, 0.744-0.995; p = 0.043). We did not detect significant horizontal pleiotropy and heterogeneity in the MR-Egger and Cochrane's Q tests. The p-values of the MR-Egger intercepts were between 0.09 and 0.569 (Table SIV). The Q-statistics of the IVW test and MR-Egger indicated no notable heterogeneity (p-values between 0.445 and 0.896) (Table SV). The "leave-one-out" analysis results underscored the robustness of the association between gut microbiome and the risk of CDI, which did not reveal any interference with the results attributable to a single SNP (Fig. S1–S3). The scatter plots illustrated the microbiome's overall effect on CDI (Fig. S4–S6). Besides, the forest plots indicated the causal associations between the intestinal microbiome and CDI (Fig. S7–S9).

Mediation analysis elucidated that Burkholderiales order affiliated with Proteobacteria exerted detrimental effects on CDI by decreasing blood metabolite 3-hydroxylaurate. In this two-step MR analysis,

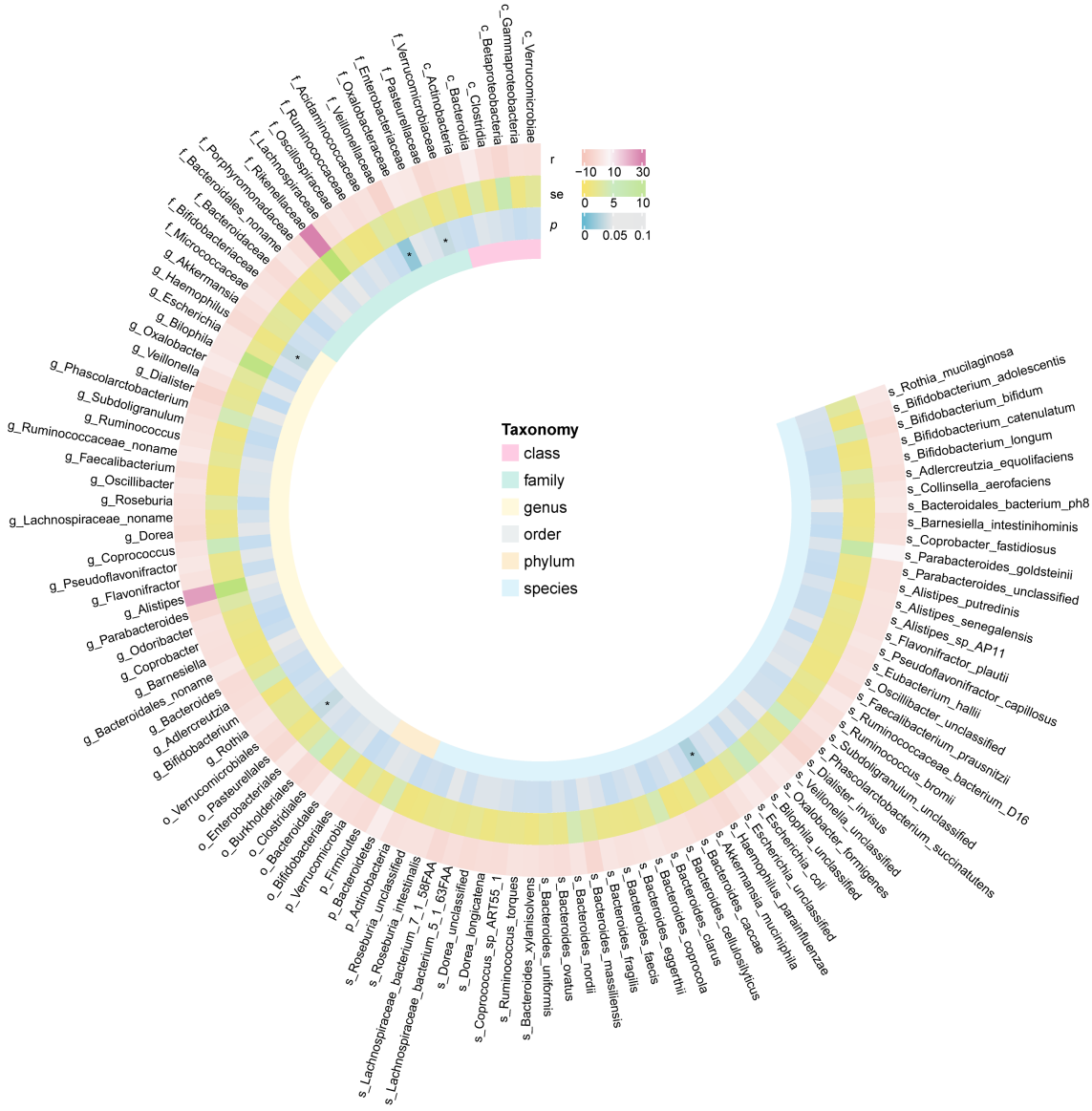

Fig. 2. Circular heatmap of suggestive genetic correlation between gut microbes and *Clostridioides difficile* infection.

blood metabolites played a mediating role from gut microbiota to CDI. Firstly, we mediated three significant populations (*Sutterellaceae*, Betaproteobacteria, and Burkholderiales) with 1,400 metabolites from the cohort of 8,299 individuals of European ancestry from the Canadian Longitudinal Study on Aging via two-step MR. Then, we conducted a causal analysis of significant metabolites with CDI. Among the three taxa causally associated with CDI, Burkholderiales order was significantly associated with metabolites based on the IVW method (Table II). It exerted detrimental effects on CDI by decreasing the levels of 3-hydroxylaurate (OR 0.896; 95%CI, 0.803-0.998; $p = 0.047$) (Table SVI–SVII), indicating that the metabolite acted as a mediator between gut microbiota and CDI.

Discussion

In summary, our findings suggested that Proteobacteria was genetically correlated with CDI by bivariate LDSC analysis. MR analysis indicated a suggestive genetic correlation between *Sutterellaceae*, Betaproteobacteria, Burkholderiales (affiliated with Proteobacteria), and CDI. Regarding a potential mechanism of metabolites, we uncovered 1,400 blood metabolites associated with the three gut microbiome taxa and CDI using two-step MR as mediation analysis. It is suggested that Burkholderiales order exerted its detrimental effects on CDI by decreasing 3-hydroxylaurate, which may provide references for the development of future interventions and potential therapeutic targets.

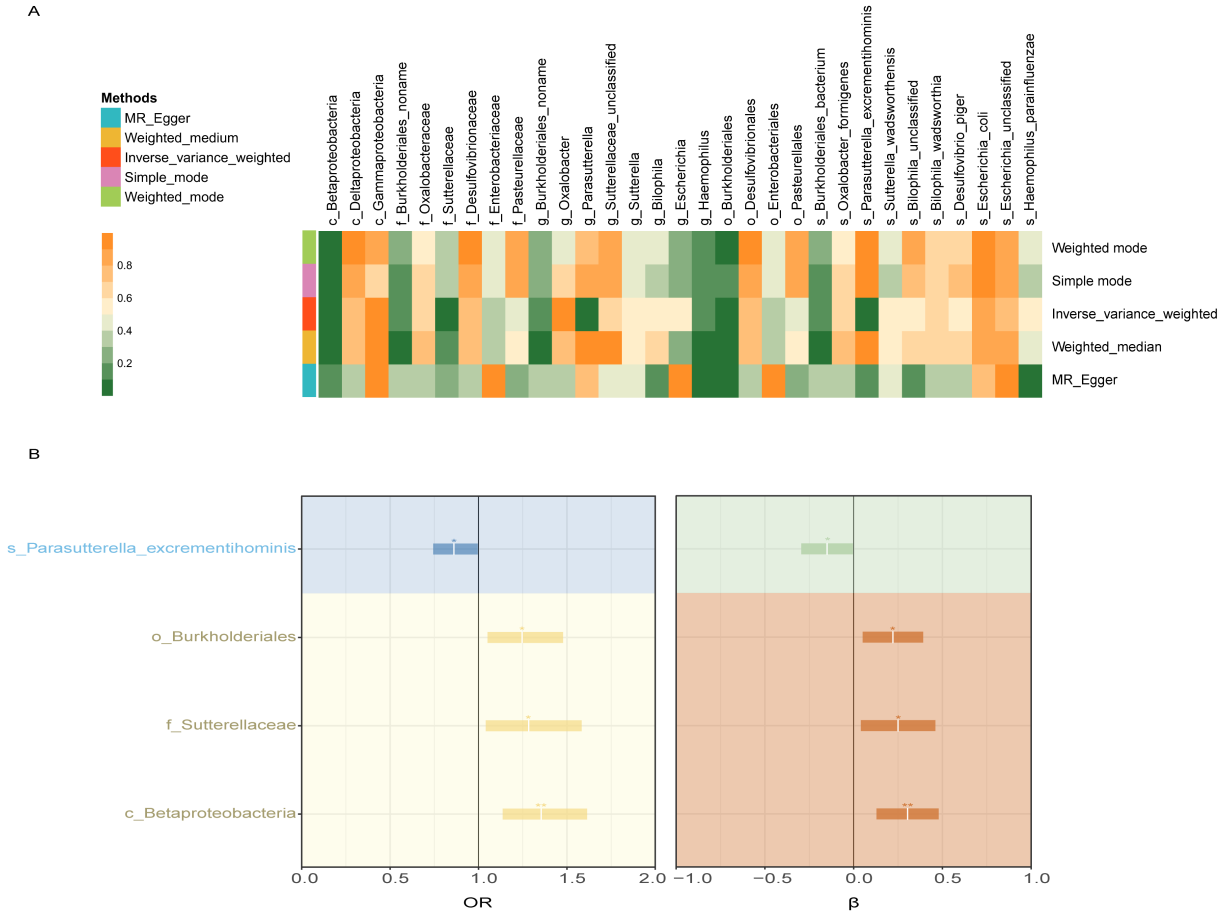

Fig. 3. Suggestive causal effects of Proteobacteria on *Clostridioides difficile* infection (CDI).
A) MR results of casual association between gut microbes belonging to Proteobacteria and CDI;
B) Significant casual estimates from genetically predicted Proteobacteria to CDI. MR – Mendelian randomization; OR – odds ratio

Previous studies have explored the association between increased Proteobacteria and CDI. A significant increase of Proteobacteria in 11 CDI patients compared with eight healthy donors enrolled in IHU-Méditerranée Infection, Marseille, France, was shown in a metagenomic analysis of gut microbiota (Amrane et al. 2019). Moreover, after fecal microbiota transplantation for recurrent CDI treatment, patients (16 patients in the infusion group) from the Academic Medical Center in Amsterdam, the Netherlands, showed an overall decrease of Proteobacteria species (Ng et al. 2020). However, these studies were performed in limited cases and in different populations. Therefore, we conducted a bivariate LDSC analysis to detect the causal relationship between gut microbiome and CDI based on 7,738 participants in the Netherlands cohort and 3,384 CDI patients. We utilized single nucleotide polymorphisms (SNPs) from GWAS summary statistics, incorporating 7,738 microbiome samples from the Netherlands and 409,432 European participants with (CDI), ensuring robust statistical power. Linkage disequilibrium score regression (LDSC) was employed to estimate genetic heritability and correlations by leveraging LD patterns from GWAS data (Bulik-Sullivan et al. 2015). First, univariate LDSC was used to assess the heritability of microbial taxa based on human ge-

Table II
Metabolites as intermediates in causal effects of gut microbiota on *Clostridioides difficile* infection (CDI).

Exposure	$\beta_{e\text{-}i}$	$OR_{e\text{-}i}$	$P_{e\text{-}i}$	Intermediate	$\beta_{i\text{-}o}$	$OR_{i\text{-}o}$	$P_{i\text{-}o}$	Outcome	$\beta_{e\text{-}o}$	$OR_{e\text{-}o}$
o_Burkholderiales	−0.110	0.896	0.047	3-hydroxylaurate	−0.616	0.540	0.010	CDI	0.220	1.247

netic variation. Subsequently, bivariate LDSC quantified the genetic correlation between microbial taxa and CDI, accounting for population stratification and confounding factors that could bias GWAS estimates. This approach ensures that our findings reflect host genetic influences on microbiome composition and disease risk rather than bacterial genomic variation (Bulik-Sullivan et al. 2015). *Pasteurellaceae* has been reported to be positively correlated with new onset, treatment-naive Crohn's disease (Clemente et al. 2018). Patients with ulcerative colitis and CDI contained a higher relative abundance of the genus *Haemophilus* than patients with ulcerative colitis only. Besides, *Veillonellaceae*, affiliated with Firmicutes, was also correlated with CDI, which was also positively correlated with new onset, treatment-naive Crohn's disease (Clemente et al. 2018). The important role of Proteobacteria in CDI is associated with the disruption of the gut microbiome and proinflammation of the intestine. The deficiency in specific IgA targeting Proteobacteria is correlated with the persistence of Proteobacteria in the inflamed gut (Mirpuri et al. 2014). However, more evidence is needed to illustrate the potential mechanisms involved.

Proteobacteria is one of the most extensively studied bacterial phyla in various body sites, including the human gut and stool. Understandably, we did not identify the common species of Proteobacteria from bivariate LDSC analysis and MR analysis. MR analysis showed that intestinal taxa *Sutterellaceae*, Betaproteobacteria and Burkholderiales correlated positively with the incidence of CDI. In a murine model, antibiotic amoxicillin-associated enterotypes exhibited severe inflammation characterized by abundant intestinal opportunistic pathogens, including *Sutterellaceae* (Zhao et al. 2023). Antibiotics usage is one of the most important risk factors for the occurrence of CDI. Thus, it is rational that *Sutterellaceae* plays a critical detrimental role in the development of CDI. Although an elevated abundance of *Sutterellaceae* was observed in a study of FMT performed in 17 CDI patients, the discrepancy may be caused by limited cases (Konturek et al. 2016). Since the late 1970s, the order Burkholderiales has become prevalent in clinical settings (Hobson et al. 1995; Voronina et al. 2015). It is reported that Burkholderiales affiliated with Betaproteobacteria were highly prevalent in inflammatory bowel disease mucosa (Rudi et al. 2012). The human lysozyme milk consumption before and during enterotoxigenic *Escherichia coli* infection in young pigs presented decreased abundance of Burkholderiales, which was accompanied by alleviated severity of diarrhea and mitigated inflammation of intestinal mucosa (Garas et al. 2017). Although, the studies of direct correlation between Burkholderiales and CDI remains limited, we speculated that Burkholderiales affiliated with Betaproteobacteria play an important role in the diarrhea and inflammation of CDI. In addition, *P. excrementihominis* affiliated with Proteobacteria was negatively correlated with CDI, which exerts potential protective effect. It is demonstrated that *P. excrementihominis* colonizing the mice intestine up-regulated the levels of secondary bile acids, ursodeoxycholic acid (UDCA) in peripheral blood (Zhou et al. 2023). Besides, UDCA was known to be able to arrest various aspects of *C. difficile* life cycles *in vitro* and attenuate CDI related symptoms in the early stage of disease *in vivo* (Thanissery et al. 2017; Winston et al. 2020). The previous studies may explain the potent protective effect of *P. excrementihominis* on CDI.

The mediation analysis indicated that Burkholderiales exerted detrimental effects on CDI through 3-hydroxylaurate. As a predominant component in the fatty acid analysis, 3-hydroxylaurate was reported to be acylated with *Leptospira interrogans* lipid A. Then the complex could stimulate the production of tumor necrosis factor of mouse RAW264.7 cells (Que-Gewirth et al. 2004). The direct correlation among 3-hydroxylaurat, Burkholderiales and CDI has not been illustrated. Although two-step MR mimics the randomization process used in randomized controlled trials, the association should be confirmed in larger datasets from other genetic backgrounds. Future research in multi-omics investigations is also required to elucidate the underlying mechanism.

However, our findings have some limitations. First, genetic instrumental variables were selected reaching the threshold of $p < 1 \times 10^{-5}$ in order to obtain more comprehensive results, which did not meet the traditional significance standard ($p < 5 \times 10^{-8}$) (Cui et al. 2023). In addition, the majority of people studied were of European ancestry, which limited the generalizability of the research findings to other ethnic groups. Moreover, further subgroup analysis was impossible due to the lack of demographic data such as age, gender, and so on. Hierarchical independence control and statistical correction strategies could be applied to address taxonomic dependencies. Specifically, the integration of nonindependence in cross-species comparative analysis, such as analysis of phylogenetically independent contrasts (Felsenstein 1985), generalized least squares (Pagel 1999), phylogenetic autoregression (Gittleman and Mark 1990) and phylogenetic mixed

models (Housworth et al. 2004), may help ensure hierarchical independence in Mendelian randomization analysis. We plan to incorporate these approaches in future analyses to enhance the robustness of our findings. Since Mendelian randomization is a hypothesis-driven approach (Hu et al. 2024), our results require further validation through mechanistic experiments and clinical studies to establish biological relevance.

Conclusions

In summary, we investigated the genetic causal effects of gut microbiota, metabolites, and CDI. Our findings revealed some significant new causal associations, including the negative effects of Burkholderiales on CDI through 3-hydroxylaurate. It may help us better understand the causal effects and identify potential therapeutic targets.

Abbreviations

CDI – *Clostridioides difficile* infection
FMT – Fecal microbiota transplantation
SCFA – Short chain fatty acids
GWAS – Genome-wide association studies
SNP – Single nucleotide polymorphisms
MR – Mendelian randomization
LDSC – Linkage disequilibrium score regression
IVs – Instrumental variables
CLSA – Canadian Longitudinal Study on Aging
LD – Linkage disequilibrium
IVW – Inverse variance weighted
OR – Odds ratio
CI – Confidence interval
UDCA – Ursodeoxycholic acid

Availability of data and material

The data of gut microbiota are openly available from Lopera-Maya et al. (2022). The data of CDI are openly available in FinnGen R10 (https://www.finngen.fi/en/access_results). The GWAS data for the 1,400 metabolites were obtained from Chen et al. (2023).

Acknowledgements

We thank all the GWAS for making the summary data publicly available, and we are grateful for all the investigators and participants who contributed to those studies. We appreciate the BioRender (https://www.biorender.com) for the help of the figures production during the preparation of this manuscript (Agreement number: NG27ZTULU5).

Author contributions

Study conception: L.M. and J.S., study design: L.M., J.S., Y.G. and J.M., data analysis: Y.G., J.M., K.W., K.M. and W.Z., manuscript drafting: Y.G. and J.M. All of the coauthors have approved the submitted final version and agreed to the publication.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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