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Interaction between the gut flora and immunity in intestinal diseases

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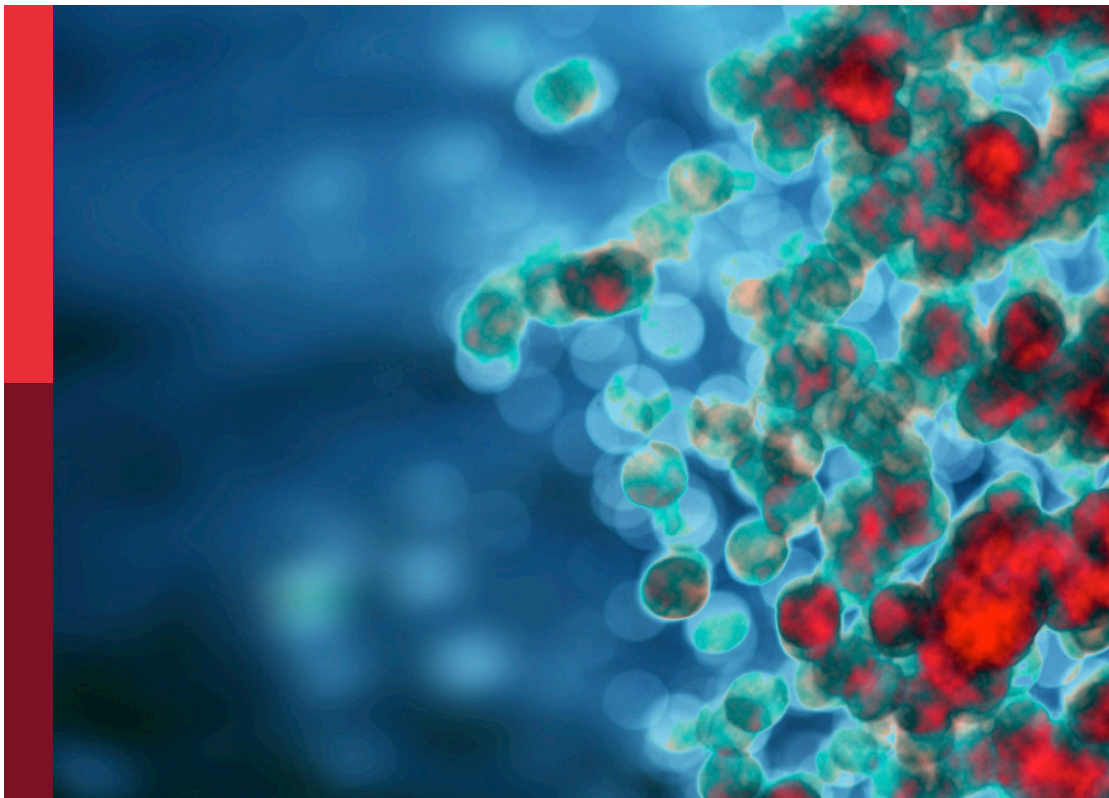

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Interaction between the gut flora and immunity in intestinal diseases

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Editorial: Interaction between the gut flora and immunity in intestinal diseases

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Editorial on the Research Topic
Interaction between the gut flora and immunity in intestinal diseases

It is widely recognized that the gut microbiota influences the host's health, especially concerning immune homeostasis. The microbiome plays critical roles in the training and development of major components of innate and adaptive immune systems, with immunity regulating the equilibrium of the host-microbe relationship. Comprehending the symbiotic relationship between the gut microbiota and our immune system is crucial for both the discipline of immunology and for gaining insights into the pathogenesis of intestinal diseases. Gut microbiota disruptions are associated with a range of intestinal diseases, including immunological diseases inflammatory bowel disease (IBD) consists of two major subtypes, Crohn's disease (CD) and ulcerative colitis (UC) (1); irritable bowel syndrome (IBS)and colorectal cancer (CRC) (2, 3).

Recently, researchers have explored microbiome-immune interactions in the development and advancement of intestinal diseases, including disease-specific bacterial lineage strains and the core microbiota in the onset and progression of intestinal diseases (1, 4, 5). The evaluation of clinical models and microbiome-immune biomarkers is predictive of the prognosis of intestinal disorders. An integrated analysis of the microbiome and metabolome uncovers distinct profiles associated with intestinal diseases (6). Metabolic characteristics are considered to act as mediators originating from the gut microbiota and are crucial in influencing the differentiation of immune cells. This is due to the capacity of bacteria to generate distinct molecules that are not produced by humans, with numerous immune cells in the intestinal tract expressing receptors for these molecules (7). The interaction between the microbiome and the immune system presents a crucial and challenging area of study, particularly in understanding the causal relationship between gut flora and the immune system in intestinal diseases. Mendelian Randomization offers a viable approach to investigating the causal relationships between microbial factors and intestinal disease (8). The management of intestinal disorders through microbiota approaches and immunotherapy strategies

encompasses various interventions such as dietary modification, probiotics, synbiotics, fecal microbiota transplantation, antibiotics, prebiotics, postbiotics, modified phage therapy, and genetically engineered bacteria. Correlation studies suggest that identifying intestinal microecological regulators in clinical practice is increasingly feasible (9).

In this context, the Research Topic has proven to be relevant and has attracted considerable interest. Specifically, our research focuses on the causal link between the microbiome and immunity. Our aim is to identify the key microbiota that play a role in the development and advancement of intestinal diseases, elucidate the molecular mechanisms underlying interactions between the host immune system and the microbiome, and propose innovative pharmacological interventions targeted at intestinal diseases. The articles included in this Research Topic present original research that aligns with these objectives.

Mendelian randomization is a useful tool for exploring the causal relationships between gut microbiota and various diseases. Zhang et al. reveal that certain gut microbiota, such as phylum *Lentisphaerae*, class *Lentisphaeria*, and order *Victivallales* are associated with a lower risk of sepsis, while other microbiota, including phylum *Tenericutes* and class *Mollicutes*, are related to an increased risk of sepsis. Additionally, C-reactive protein (CRP) has been confirmed as a potential intermediate factor for the influence of the gut microbiome on sepsis. Moreover, Gao et al. identified 21 bacterial features that have a causal relationship with sepsis and its related outcomes, such as sepsis requiring intensive care and 28-day mortality. The findings of these studies contribute to the development of microbiome-based therapeutic strategies for sepsis, aimed at reducing the incidence and mortality rates of the condition.

Gut leakage and bacterial translocation are closely associated with sepsis, as well as with chronic complex disorders. Martin et al. investigate the relationship between gut barrier function and inflammation in fibromyalgia (FM) and myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) by analyzing circulating biomarkers and self-reported symptoms. FM and ME/CFS patients had significantly higher levels of biomarkers associated with increased gut permeability (anti-beta-lactoglobulin antibodies, ZO-1) and bacterial translocation (LPS, sCD14) compared to healthy controls. Zhao et al. identify 14 specific bacterial genera associated with various peptic ulcer diseases (PUDs) types. Certain bacteria, such as *Eubacterium hallii* and *Flavonifractor*, are causally linked to esophageal ulcers, while others like *Lachnospiraceae UCG004* are associated with gastric ulcers, providing insights into the role of gut flora in PUD development. In another common gastrointestinal disorder, Wang et al. identified genetic associations between variations in gut microbiota abundance and the risk of gastroesophageal reflux disease (GERD). Specifically, the *Clostridiales Vadin BB60 group*, the genus *Lachnospiraceae UCG004*, the *Methanobrevibacterium*, and the phylum *Actinobacteria* were found to potentially have a protective effect against GERD, while the class *Mollicutes*, the genus *Anaerostipes*, and the phylum *Tenericutes* may increase the risk of GERD. Additionally, GERD was found to cause the dysregulation of 13 different gut microbiota taxa. There has been a steady growth in the number of publications in the field of gut microbiota and IBD, with a particularly significant increase in recent years, Zhang et al. conduct a bibliometric analysis of the literature in the field of gut microbiota and IBD over the past two decades. Analyzing 10,479 relevant documents from the Scopus database reveals the importance of gut microbiota in IBD research and highlights the research hotspots and frontiers in this field.

The interaction between the gut microbiota and the immune system also plays a significant role in the occurrence and development of vascular diseases. Jiang et al. demonstrate that the presence of specific gut bacteria in the host is causally associated with atherosclerosis. Different types of atherosclerosis (cerebral, coronary, and peripheral) are linked to specific gut microbiota. For example, *Ruminiclostridium* was found to have a protective effect on cerebral atherosclerosis, while *Rikenellaceae*, *Streptococcaceae*, *Paraprevotella*, and *Streptococcus* were associated with increased risk. Chen et al. investigate the causal relationship between gut microbiota and granulomatosis with polyangiitis (GPA). The study identified that one phylum, one family, and nine genera of microbiota were significantly associated with GPA, and it established that various immune cell characteristics mediated the impact of gut microbiota on GPA. For example, the family *Defluviitaleaceae* and the genus *Defluviitaleaceae UCG011* affected GPA by influencing the expression of CD11c in granulocytes.

Recently, the immunomodulatory mechanisms of some probiotics or bacterial metabolic synthesis have also attracted widespread attention. Lee et al. investigate the immunomodulatory effects of paraprobiotics derived from heat-killed *Bacillus velezensis* GV1. The study demonstrates that GV1 effectively enhances immune responses *in vitro* and *in vivo*, particularly in immunosuppressed mice treated with cyclophosphamide. Gubernatorova et al. explore the complex and controversial role of *Akkermansia muciniphila*, a mucin-degrading bacterium, in colorectal cancer. Experimental variations, such as antibiotic use, the form, or the dosage, significantly impact outcomes. The key seems to be moderation, as lower doses of A. muciniphila or its derivatives, administered without disrupting the gut microbiota, may protect against colorectal cancer. Sun et al. highlight the multifaceted role of bacterial extracellular vesicles (BEVs) in gut health and disease. BEVs, which are released by both gram-negative and gram-positive bacteria, have been traditionally viewed as waste products. However, emerging research demonstrates their significant impact on various aspects of gut homeostasis and pathogenesis.

In summary, existing evidence suggests a significant bidirectional relationship between perturbations in the microbiome and dysregulation of the immune system. The intricate communication between gut microbiota and host immunity has not been fully elucidated in the contexts of maintaining homeostasis and the progression of diseases. Therefore, comprehensive mechanistic investigations are warranted to delve deeper into the impact of microbial

manipulation on host immunity and the immune response to dysbiosis of the microbiome in intestinal disorders.

Author contributions

YL: Writing – original draft, Writing – review & editing. ST: Writing – review & editing. LG: Writing – review & editing. DS: Writing – review & editing, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Gut microbiota and sepsis: bidirectional Mendelian study and mediation analysis

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Background: There is a growing body of evidence that suggests a connection between the composition of gut microbiota and sepsis. However, more research is needed to better understand the causal relationship between the two. To gain a deeper insight into the association between gut microbiota, C-reactive protein (CRP), and sepsis, we conducted several Mendelian randomization (MR) analyses.

Methods: In this study, publicly available genome-wide association study (GWAS) summary statistics were examined to determine the correlation between gut microbiota and sepsis, including various sepsis subgroups (such as under 75, 28-day death, Critical Care Units (ICU), 28-day death in ICU). Initially, two-sample and reverse Mendelian randomization (MR) analyses were conducted to identify causality between gut microbiota and sepsis. Subsequently, multivariable and two-step MR analyses revealed that the relationship between microbiota and sepsis was mediated by CRP. The robustness of the findings was confirmed through several sensitivity analyses.

Findings: In our study, we revealed positive correlations between 24 taxa and different sepsis outcomes, while 30 taxa demonstrated negative correlations with sepsis outcomes. Following the correction for multiple testing, we found that the Phylum Lentisphaerae (OR: 0.932, p = 2.64E-03), class Lentisphaeria, and order Victivallales (OR: 0.927, p = 1.42E-03) displayed a negative relationship with sepsis risk. In contrast, Phylum Tenericutes and class Mollicutes (OR: 1.274, p = 2.89E-03) were positively related to sepsis risk and death within 28 days. It is notable that Phylum Tenericutes and class Mollicutes (OR: 1.108, p = 1.72E-03) also indicated a positive relationship with sepsis risk in individuals under 75. From our analysis, it was shown that C-reactive protein (CRP) mediated 32.16% of the causal pathway from Phylum Tenericutes and class Mollicutes to sepsis for individuals under 75. Additionally, CRP was found to mediate 31.53% of the effect of the genus Gordonibacter on sepsis. Despite these findings, our reverse analysis did not indicate any influence of sepsis on the gut microbiota and CRP levels.

Conclusion: The study showcased the connection between gut microbiota, CRP, and sepsis, which sheds new light on the potential role of CRP as a mediator in facilitating the impact of gut microbiota on sepsis.

KEYWORDS

C-reactive protein, gut microbiota, mediator, Mendelian randomization, sepsis

1 Background

Sepsis is a complex syndrome characterized by an unbalanced immune response to various infections (1), which can lead to malfunctioning of multiple organ systems such as the cardiopulmonary, renal, and digestive systems (2). According to epidemiological studies, sepsis rates of prevalence and mortality range from 25% to 30% in hospitals (3). Despite our growing understanding of the biological mechanisms behind sepsis, current treatments have proven ineffective in correcting the dysregulated immunity in patients (4), making it essential to develop targeted prevention and treatment strategies.

The gut microbiome has been found to contribute to the severity of sepsis and prognosis of treatment (5). Preclinical studies have shown that gut microbiota plays a pivotal role in the immune response to systemic inflammation and that disruption of this symbiosis increases susceptibility to sepsis (6). Additionally, the use of omic technologies to analyze the gut microbiota has confirmed the alteration of composition related to septic dysfunction across organs (7).

Although probiotic supplementation has reported some positive effects (8–10), their efficacy and safety remain a subject of controversy (11, 12). Therefore, more research is necessary to identify the specificity and safety of probiotic supplements.

In addition to being a biomarker of acute-phase inflammation, CRP has a role in defending against infections as it can bind to cells and some bacteria, triggering the complement system and helping to remove dead cells (13, 14). However, prospective studies have also revealed that elevated CRP levels correlate with a higher risk of infections in adults (15).

Mendelian randomization (MR) involves using genetic variants to construct instrumental variables of exposure and estimate the causal association between exposure and outcome (16). As the random distribution of alleles is not affected by common confounding factors, a causal relationship is generally considered to be reliable (17). However, in previous studies, we were unable to find any MR studies examining the relationship between gut microbiota, sepsis, and their association with CRP. Therefore, we conducted multiple MR analyses based on genome-wide association study (GWAS) summary statistics to evaluate the causal association among gut microbiota, CRP, and sepsis.

2 Method

2.1 Study design

In this study, we conducted a two-sample and bidirectional Mendelian randomization (MR) to examine the causal relationship between gut microbiota and sepsis. We then used a two-step and multivariable MR approach to identify the mediation effect of CRP on the relationship between gut microbiota and sepsis. A summary of the study design is illustrated in Figure 1. Study used publicly available summary statistics for gut microbiota, C-reactive protein (CRP), and sepsis from previously published studies or consortiums. All of these studies were approved by their respective institutional review boards (IRBs), and hence, there was no need to re-apply for approval by the IRB.

2.2 Data sources

The gut microbiota data used in this study were sourced from the MiBioGen consortium (18). This consortium has curated and analyzed genome-wide genotypes and 16S fecal microbiome data from 18,340 individuals across 24 cohorts, which includes 14,306 European individuals from 18 cohorts. The consortium performed adjustments for age, sex, genetic principal components, technical covariates such as stool DNA isolation methods, 16S domain to reduce heterogeneity among the cohorts. However, the study did not account for other potential confounders like diet, medication use, and lifestyle factors, as this information was not consistently available for all cohorts (Supplementary Table 1).

C-reactive protein was derived from 1,000 individuals in the population-based KORA (Cooperative Health Research in the Region of Augsburg) study (19). The study used a highly multiplexed, aptamer-based, affinity proteomics platform (SOMAscan) to quantify levels of 1,124 proteins in blood plasma samples.

The sepsis data and sepsis subgroups (under 75, 28-day death, Critical Care Units (ICU), 28-day death in ICU) was collected from the IEU Open GWAS with summary-level data obtained from the UK Biobank which included 11643,11568,1896,1380, and 347 sepsis cases and 474841,451301,484588, 429985, 431018 controls respectively. The study use Regenie v2.2.4 to analyze GWAS data, and adjusted for age, sex, chip, and the first 10 Principal Component Analysis (https://gwas.mrcieu.ac.uk/datasets/ieu-b-4980/).

2.3 SNP selection

We utilized MR analysis to investigate potential causal relationships between gut microbiota and sepsis, using genetic variants as instrumental variables (IVs). The validity of an MR analysis is contingent upon three key assumptions: (1) IVs are not associated with any confounding variables; (2) IVs are strongly associated with the exposure; and (3) IVs influence the outcome solely through the exposure (20).

Initially, we selected single nucleotide polymorphisms (SNPs) from the genome-wide association study (GWAS) summary data for exposures that exhibited a genome-wide significant association ($p < 5\times10^{-8}$) with the traits as IVs. In instances where the number of IVs was limited, we relaxed the significance threshold to 5×10^{-5} to prevent inaccurate results due to insufficient SNPs. The selection of other SNPs followed the same threshold. Subsequently, we employed linkage disequilibrium clumping to exclude certain undesirable SNPs ($r^2 < 0.01$, window size > 10,000 kb) (21). Finally, we harmonized the exposure and outcome datasets and eliminated palindromic SNPs with allele frequencies close to 0.5. All the selected SNPS are placed in the Supplementary Table 2.

We ensured the strength of genetic instruments for exposures by calculating the F statistic using the formula:$F = (n - k - 1)/k\times(R^2/$

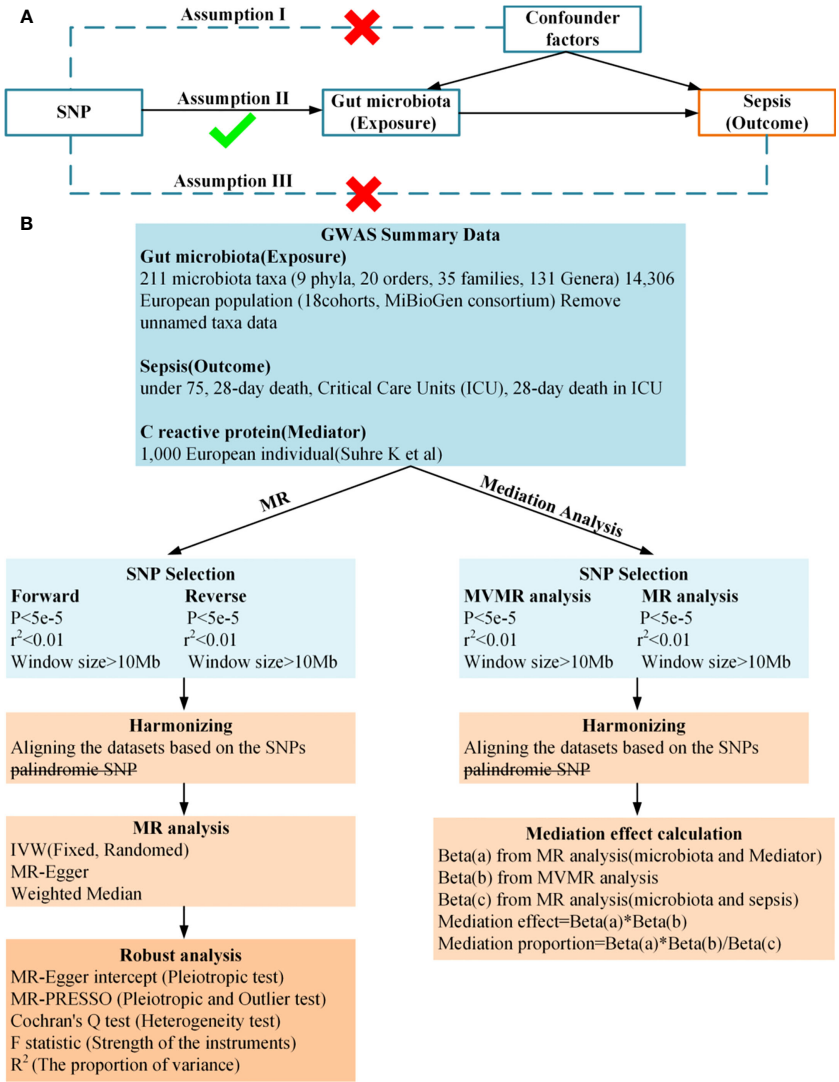

FIGURE 1
(A), Principles of Mendelian Randomization: I) Independence: The genetic variants utilized in the analysis are not associated with any confounders that could potentially influence the relationship between the exposure and the outcome. II) Relevance: The genetic variants selected as instrumental variables have a strong association with the exposure. III) Exclusion Restriction: The genetic variants influence the outcome solely through their effect on the exposure, and not through any alternative pathways; **(B)**, Flowchart of Bidirectional Two-Sample Mendelian Randomization and mediation Analysis.

$1-R^2$) (22), where R^2 represents the cumulative explained variance in the selected SNPs, N is the sample size, and k is the number of SNPs in the analysis. An F statistic greater than 10 indicates sufficient strength to avoid the issue of weak instrument bias in the two-sample model (23).

2.4 Statistical analysis

We conducted bidirectional two-sample MR analyses to assess the relationship between gut microbiota and sepsis. Our primary analysis employed an inverse variance-weighted (IVW) meta-analysis approach, which is a robust method for MR analysis (17). We also performed secondary analyses using the weighted median (24), and MR-Egger regression approaches. We evaluated the potential impact of directional pleiotropy by testing the intercept value of the MR-Egger regression (25). We used Cochran's Q test to assess heterogeneity (26). In cases of heterogeneity, we opted for a random-effects IVW for the primary analysis. At each feature level (phylum=9, class=15, order=19, family=30, and genus=117), according to previous reports (27), we used a multiple-testing significance threshold of $p < 0.05/n$ (where n represents the effective number of independent bacterial taxa at the corresponding taxonomic level).

In mediation terms, the total effect of an exposure on the outcome is estimated by univariable MR. Multivariable MR (MVMR) and two-step MR is used to decompose direct and indirect effects. The first step is to evaluate the effect of exposure on the mediator with univariable MR. The second step estimating the effect of the mediator on each outcome was carried out with

MVMR. For this second step, MVMR has not been used in previous literature (28, 29), and a univariable MR has been proposed for calculating the mediator's effect. However, in the case of MVMR, the mechanism of the mediator's effect on the outcome can be ensured to be independent of the effect of the exposure (30). Furthermore, it exerts a direct effect on exposure. The indirect effect is estimated by multiplying the two-step (MR) estimates. Stepwise regression was used to select exposures and mediators with true effects (31).

3 Result

3.1 Two-Sample and bidirectional MR analysis of gut microbiota and sepsis, sepsis subgroups risk

Four MR approaches were utilized to investigate the association between gut microbiota and sepsis (Figure 2 and Supplementary Table 3). Positive associations were observed for the genera Actinomyces, Enterorhabdus, Gordonibacter, and Ruminococcaceae UCG014, and the families Coriobacteriaceae and Prevotellaceae, with various outcomes. For example, the genus Actinomyces was associated with an increased likelihood of critical care units (OR = 1.21, p = 2.58E-02) and 28-day death in critical care units (OR = 1.46, p = 2.58E-02). The genus Fusicatenibacter demonstrated a strong positive association with 28-day death in critical care units(OR = 1.49, p = 3.90E-02). In contrast, several taxa showed negative associations with sepsis outcomes. For instance, the genera Anaerotruncus, Coprococcus1, Coprococcus2, Dialister, Dorea, Eubacterium ventriosum group, Eubacterium xylanophilum group, Faecalibacterium, Intestinimonas, Lachnospiraceae UCG001, Lachnospiraceae UCG004, and Peptococcus, and the family Enterobacteriaceae, all demonstrated negative associations with various outcomes.

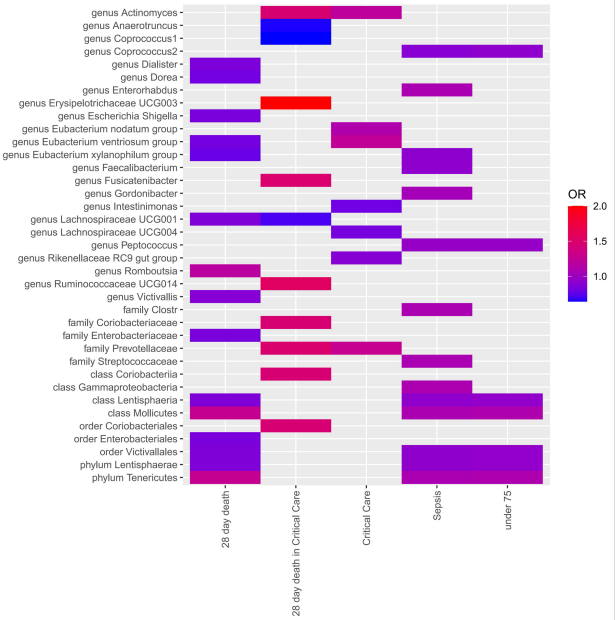

FIGURE 2
Each cell in the heatmap corresponds to a specific micrbiota taxa-sepsis pair. The color of the cell indicates the OR value associated with that pair, with a color scale used to differentiate between positive, negative, and zero beta values.

Notably, the genus Erysipelotrichaceae UCG003 displayed a particularly strong positive association with 28-day death in ICU (OR = 4.97, p = 2.43E-02), suggesting a potential role in severe sepsis outcomes. The genus Eubacterium xylanophilum group showed negative associations with both 28-day death (OR = 0.78, p = 3.26E-03) and sepsis (OR = 0.92, p = 1.68E-02), suggesting a protective role.

Multiple-testing correction was taken into account by setting significance thresholds as follows: phylum $p = 5.56\times10^{-3}$ (0.05/9), class $p = 3.13\times10^{-3}$ (0.05/16), order $p = 2.63 \times 10^{-3}$ (0.05/19), family $p = 1.67 \times 10^{-3}$ (0.05/30), genus $p = 4.27 \times 10^{-4}$ (0.05/117). As the SNPs within a class might overlap with those in a related phylum and other subcategories, the MR results would remain similar if a class was considered a subcategory of a phylum or another subcategory.

Based on the results of IVW fixed-effects analyses Table 1, phylum Lentisphaerae (OR = 0.932, 95% CI = 0.89-0.98, p = 2.64E-03), class Lentisphaeria and order Victivallales (OR = 0.927, 95% CI = 0.88-0.97, p = 1.42E-03) were negatively associated with the risk of Sepsis. Conversely, phylum Tenericutes and class Mollicutes (OR = 1.274, 95% CI = 1.09-1.49, p = 2.89E-03) were positively correlated with the risk of Sepsis (28 day death). Interestingly, Phylum Tenericutes and class Mollicutes (OR = 1.108, 95% CI = 1.04-1.18, p = 1.72E-03) were positively correlated with the risk of Sepsis (under 75 years) as well. No effect of sepsis on gut microbiota was found in the reverse analysis (Supplementary Table 4).

For additional confirmation of the robustness of the results, several sensitivity tests were conducted (Supplementary Table 4). Most of the results were consistent in sensitivity analyses, though with wider confidence intervals. In addition, all results of Cochran's Q test were above 0.05, signifying that there was no significant heterogeneity. The MR-PRESSO analysis also corroborated this, demonstrating no outlier of SNPs. Moreover, the MR-Egger intercept test and the global test p-values both revealed no statistically significant results, suggesting no presence of horizontal pleiotropy.

3.2 Gut microbiota and C-reactive protein level

Similarly, we conducted two-sample analyses to examine the relationship between gut microbiota and C-reactive protein (CRP). The IVW fixed-effects analyses Table 2 showed that family Coriobacteriaceae, order Coriobacteriales, class Coriobacteriia had a negative correlation with CRP levels (Beta = -0.502 p = 0.046), However, Phylum Tenericutes, class Mollicutes, genus Dialister, genus Gordonibacter had a positive correlation with CRP levels, and WM analysis also obtained similar causal estimates.

A series of sensitivity analyses, including WM, Cochran's Q test, MR-Egger regression, intercept test were conducted Table 2. These

TABLE 1 MR result of gut microbiota on sepsis.

Exposure	Methods	Number of SNPs	OR	95%CI	*p* val	Cochran's Q statistic (*p* val)	Egger intercept(*p* val)	F
Sepsis(Outcome)								
phylum Lentisphaerae	IVW-FE	41	0.932	0.89-0.98	**2.64E-03**	0.31	0.979	19.67
	IVW-RE		0.932	0.89-0.98	**4.11E-03**			
	MR Egger		0.93	0.78-1.11	0.429			
	WM		0.969	0.91-1.04	0.347			
class Lentisphaeria order Victivallales	IVW-FE	40	0.927	0.88-0.97	**1.42E-03**	0.663	0.982	19.56
	IVW-RE		0.927	0.88-0.97	**1.42E-03**			
	MR Egger		0.929	0.78-1.1	0.406			
	WM		0.958	0.89-1.03	0.234			
Sepsis (28 day death)(Outcome)								
phylum Tenericutes class Mollicutes	IVW-FE	46	1.274	1.09-1.49	**2.89E-03**	0.691	0.489	19.45
	IVW-RE		1.274	1.09-1.49	**2.89E-03**			
	MR Egger		1.099	0.7-1.71	0.68			
	WM		1.288	1.02-1.63	0.034			
Sepsis(under 75)(Outcome)								
phylum Tenericutes class Mollicutes	IVW-FE	46	1.108	1.04-1.18	**1.72E-03**	0.204	0.27	19.5
	IVW-RE		1.108	1.03-1.19	**3.73E-03**			
	MR Egger		1	0.83-1.21	0.999			
	WM		1.105	1-1.22	0.047			

IVW-FE, Inverse variance weighted-Fixed model; IVW-RE, Inverse variance weighted-Random model; WM, weight median; F is the value of F statistics to examine the weak instrument bias; Significant p-values were bold after multiple-testing correction [phylum $p = 5.56\times10^{-3}$ (0.05/9), class $p = 3.13\times10^{-3}$ (0.05/16), order $p = 2.63\times10^{-3}$ (0.05/19), family $p = 1.67\times10^{-3}$ (0.05/30), genus $p = 4.27\times10^{-4}$ (0.05/117)].

results were consistent in sensitivity analyses, though some with wider confidence intervals. Additionally, all *p* values from both the Cochran's Q test and the MR-Egger intercept test were greater than 0.05, indicating the absence of heterogeneity and horizontal pleiotropy. The reverse analysis did not find any effect of CRP on gut microbiota (Supplementary Table 5).

3.3 C-reactive protein level and sepsis, sepsis subgroups

Initially, we conducted two-sample MR analyses (Table 3) to examine the effect of C-reactive protein levels on sepsis and its subgroups. The table presents the results, and the IVW fixed-effects analyses showed a positive correlation between CRP levels and Sepsis and Sepsis (under 75). No effect of sepsis on CRP was found in the reverse analysis (Supplementary Table 6). Furthermore, a series of sensitivity analyses validated the robustness of the findings.

Secondly, we utilized MVMR (as shown in Table 4) to assess the independent impact of CRP on sepsis, which was independence of gut microbiota. The results indicate a significant positive association between CRP levels and a higher risk of sepsis as well as sepsis under 75 years old.

As shown in Table 5, the mediation analysis revealed that CRP plays a significant role (32.02% mediation effect) in the causal pathway from Phylum Tenericutes and class Mollicutes to sepsis (in individuals under 75 years old). And CRP mediate 31.53% effect of genus Gordonibacter on sepsis.

4 Discussion

Over the past decade, numerous studies have confirmed the diverse biological functions of gut microbes, including aiding in food digestion, hormone production, and enhancing the immune system, among others (32–34). In this study, we collected data from the largest GWAS to date on gut microbiota and sepsis, and evaluated the causal relationship between all gut microbiota taxa and sepsis. We found that 24 taxa were positively associated with various sepsis outcomes, 30 taxa were negatively associated with sepsis outcomes. In total, we identified 37 unique taxa, including 23 at the genus level, 5 at the family level, 3 at the order level, 4 at the class level, and 2 at the phylum level. After multiple-testing correction, phylum Lentisphaerae, class Lentisphaeria, and order Victivallales were still associated with a reduced risk of sepsis, while Phylum Tenericutes and class Mollicutes were linked to an

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TABLE 2 MR result of gut microbiota on CRP.

Exposure	Methods	Number of SNPs	Beta	Se	*Pval*	Cochran's Q statistic (*P*-value)	Egger intercept(*P*-value)	F
CRP(Outcome)								
family Coriobacteriaceae	IVW-FE	12	-0.502	0.252	**4.59E-02**	0.642	0.582	19.388
class Coriobacteriia	IVW-RE	12	-0.502	0.252	**4.59E-02**	0.642	0.582	19.388
order Coriobacteriales	MR Egger	12	-1.304	1.430	3.83E-01	0.642	0.582	19.388
	WM	12	-0.492	0.335	1.42E-01	0.642	0.582	19.388
class Mollicutes	IVW-FE	6	0.736	0.292	**1.17E-02**	0.878	0.760	18.688
phylum Tenericutes	IVW-RE	6	0.736	0.292	**1.17E-02**	0.878	0.760	18.688
	MR Egger	6	0.408	1.043	7.16E-01	0.878	0.760	18.688
	WM	6	0.819	0.370	**2.68E-02**	0.878	0.760	18.688
genus Dialister	IVW-FE	9	0.585	0.238	**1.41E-02**	0.267	0.463	18.297
	IVW-RE	9	0.585	0.266	**2.80E-02**	0.267	0.463	18.297
	MR Egger	9	1.523	1.238	2.58E-01	0.267	0.463	18.297
	WM	9	0.410	0.335	2.22E-01	0.267	0.463	18.297
genus Gordonibacter	IVW-FE	9	0.293	0.136	**3.10E-02**	0.927	0.822	18.667
	IVW-RE	9	0.293	0.136	**3.10E-02**	0.927	0.822	18.667
	MR Egger	9	0.162	0.574	7.86E-01	0.927	0.822	18.667
	WM	9	0.188	0.178	2.89E-01	0.927	0.822	18.667

IVW-FE, Inverse variance weighted-fixed model; IVW-RE, Inverse variance weighted-random model; WM, weight median; CRP, C reactive protein; F is the value of F statistics to examine the weak instrument bias.
Bold means that the p-value is less than 0.05.

increased risk of sepsis (particularly in individuals under 75 years old) and 28-day mortality. Notably, we did not observe any significant association between sepsis and these gut microbiota. Taken together, our findings provide valuable insights into the role of gut microbiota in sepsis treatment, including reducing the risk of sepsis, minimizing mortality, and improving sepsis prognosis.

Tenericutes and Mollicutes are primarily associated with infections in pregnant women and newborns. Several studies have shown that mycoplasma infections can cause puerperal sepsis (35), and in newborns, these infections are linked to an increased risk of bronchopulmonary dysplasia, early-onset neonatal sepsis, and meningitis (36, 37). In contrast, Lentisphaerae (phylum), Lentisphaeria (class), and Victivallales (order) are relatively under-studied bacterial groups. However, recent research suggests that these microbial communities are closely associated with immune regulation. Lentisphaerae, for instance, has been found to be more abundant in cases of inflammatory bowel diseases (38), while its abundance is reduced in patients with rosacea (39). Furthermore, in patients diagnosed with post-traumatic stress disorder, Lentisphaerae has been associated with a decrease in symptom severity scores (40). genus Gordonibacter is primarily found to be excessively increased in patients with Crohn's disease and Rheumatoid Arthritis (RA), which indicates its close relationship with immunity and inflammation (41). This also indirectly confirms its association with the increase in CRP.

Previous MR analyses have suggested that gut microbiota and their metabolites can impact Systemic Lupus Erythematosus, inflammatory bowel diseases, and blood metabolites (42–44). These findings emphasize the significance of these bacterial groups in regulating inflammation in the human body. Their presence and abundance in various disease conditions imply a potential role in modulating immune responses and contributing to the development or resolution of inflammation-related disorders.

Recently, a study employed regression analysis to investigate the potential impact of the interaction between gut microbiota and CRP using individual level genotype data from UK Biobank (45). Nonetheless, due to the insufficient research on the relationship between gut microbiota and serum inflammation, we examined the effect of CRP, an inflammation protein linked to a higher risk of infections in adults (15), in the association between gut microbiota and sepsis. Our findings indicate that Phylum Tenericutes and class Mollicutes are strongly associated with increasing levels of C-reactive protein. Previous studies have shown elevated levels of CRP in patients with mycoplasma infection. Taken together with our results, this implies that CRP might not only work as a biomarker for mycoplasma infection but also play a role in

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TABLE 3 MR result of CRP on sepsis.

Exposure	Methods	Number of SNPs	OR	95%CI	*P*val	Cochran's Q statistic (*P*-value)	Egger intercept (*P*-value)	F
Sepsis(Outcome)								
CRP	IVW-FE	21	1.046	1.01-1.08	**0.006**	0.608	0.497	29.45
	IVW-RE	21	1.046	1.01-1.08	**0.006**			
	MR Egger	21	1.077	0.99-1.18	0.114			
	WM	21	1.036	0.99-1.09	0.135			
Sepsis (28 day death)(Outcome)								
CRP	IVW-FE	21	1.042	0.96-1.13	0.305	0.448	0.108	29.45
	IVW-RE	21	1.042	0.96-1.13	0.307			
	MR Egger	21	1.238	1-1.53	0.067			
	WM	21	1.073	0.96-1.2	0.215			
Sepsis (28 day death in Critical Care Units)(Outcome)								
CRP	IVW-FE	21	1.0823696	0.9	1.302	0.414	0.027	29.45
	IVW-RE	21	1.0823696	0.9	1.307			
	MR Egger	21	1.9199409	1.2	3.176			
	WM	21	1.137025	0.9	1.485			
Sepsis (under 75)(Outcome)								
CRP	IVW-FE	21	1.046	1.01-1.08	**0.005**	0.729	0.597	29.45
	IVW-RE	21	1.046	1.01-1.08	**0.005**			
	MR Egger	21	1.069	0.98-1.17	0.142			
	WM	21	1.054	1.01-1.1	**0.02**			

IVW-FE, Inverse variance weighted-fixed model; IVW-RE, Inverse variance weighted-random model; WM, weight median; CRP, C reactive protein; F is the value of F statistics to examine the weak instrument bias.
Bold means that the p-value is less than 0.05.

mediating the pathogenic mechanisms of mycoplasma. These results establish the role of certain gut microbiota in systemic inflammation and immune response.

Current research on the effects of serum substances on sepsis has primarily focused on lipid and iron metabolism (46, 47). Several cross-sectional studies have demonstrated that elevated CRP levels are linked to increased morbidity and mortality in sepsis (48–50). In our examination of the relationship between CRP and sepsis, we found that CRP is associated with a higher incidence of sepsis and sepsis-related deaths among those under 75 years of age. Reverse analysis revealed no effect on CRP. Meanwhile, mediation analysis found that CRP mediates 32% of the effects of Phylum Tenericutes and class Mollicutes on sepsis (under 75 years). Based we used multivariate MR, the effect of CRP on sepsis were independent of the effect of the exposure (17). Our Mendelian randomization study on the relationship between our microbiota and the risk of

TABLE 4 MVMR result of gut microbiota and CRP on sepsis.

Exposure	Number of SNPs	OR	95%CI	*pval*
Sepsis (under 75)(Outcome)				
phylum Tenericutes/ class Mollicutes	23	1.1	0.93-1.3	0.26
CRP	23	1.05	1.01-1.08	**0.011**
Sepsis(Outcome)				
genus Gordonibacter	25	0.98	0.9-1.07	0.692
CRP	25	1.05	1.01-1.08	**0.011**

MVMR, Multivariable Mendelian randomization; IVW-FE, Inverse Variance Weighted-Fixed model; IVW-RE, Inverse Variance Weighted-Random model; CRP, C reactive protein; WM, Weight Median; Significant P-values were bold.

TABLE 5 Two-step Mendelian randomization.

Exposure	Mediation	Total effect (Beta)	A (Beta)	B (Beta)	Indirect effect (Beta)	Mediation effect/ Total effect
Sepsis (under 75)(Outcome)						
phylum tenericutes/ class Mollicutes	CRP	0.102	0.736	0.044	0.033	32.02%
Sepsis(Outcome)						
genus Gordonibacter	CRP	0.045	0.293	0.045	1.32%	31.53%

A, the effect of Exposure on Mediation; B, the effect of Mediation on Outcome is independent of the effect of the exposure.

developing and dying from sepsis will help us understand how changes in the gut microbiome lead to immune dysregulation in sepsis, which in turn can aid in improving sepsis management.

Firstly, our study used multiple sensitive analysis, thereby bolstering the reliability of our findings. The consistency between the most of the WM and MR-Egger methods with those from the IVW method attests to the robustness of our results. Despite the presence of wide confidence intervals in some results, the overarching pattern of associations remained consistent. Secondly, we implemented the MR-PRESSO technique to identify and exclude potential outliers that could introduce bias into our findings, enhancing the reliability of our results. Thirdly, our study was instrumental in spotlighting certain genera that showed a more significant association with sepsis compared to other microbial classes. Even though these associations didn't retain their statistical significance after multiple testing adjustment, they still constitute crucial preliminary observations and may be indicative of underlying biological phenomena. Fourthly, through the use of PhenomeScan, we found that no SNPs from the microbiota, CRP and sepsis were associated with infections, malignant diseases, or antibiotic use. This suggests that the observed links among the microbiota, CRP, and sepsis were unlikely to be confounded by the genetic predispositions that are typically represented by SNPs. Lastly, given that both the exposure and outcome populations were of European descent, the potential for bias resulting from population stratification was minimized.

However, there are several limitations to our study. Firstly, a limited amount of non-European population data on gut microbiota was obtained, which may have biased our findings. Secondly, we were unable to discern any non-linear correlations among mcirbiota, CRP and sepsis, such as U-shaped, J shaped patterns. Thirdly, the number of loci related to CRP is relatively small compared to those associated with sepsis and gut microbiota. Fourthly, our Mendelian randomization study was unable to access individual-level data, which posed a constraint on the depth of our analysis. For instance, we were unable to perform a hierarchical analysis, specifically in the case of sepsis. Ideally, we would have liked to divide the sepsis data into two groups according to the Sepsis-2 and Sepsis-3 guidelines, which could provide insights into the differences between these two classifications. However, due to the unavailability of the required individual-level data, we were unable to conduct such an analysis.

5 Conclusion

In conclusion, our bi-directional Mendelian randomization analysis has clearly indicated a causal relationship between the 37 unique gut microbiota taxa and increased risk of sepsis, whereas the reverse causality hypothesis did not hold. Importantly, our findings suggest that C-reactive protein (CRP) acts as a mediator of the impact of the gut microbiota on sepsis. For a more nuanced understanding of the observed association between the gut microbiota and sepsis, future research should focus on potential mechanistic pathways, while also attempting to adjust for potential confounders such as diet, lifestyle, and medication, provided these data are available. Furthermore, an analysis of sepsis as a heterogeneous condition, acknowledging its multi-stages and variations as defined by the sepsis-3 criteria, would be beneficial, throught acquire individual-level data in future. Our work constitutes a significant stride in deciphering the relationship between gut microbiota and sepsis, however, more experimental and clinical studies are warranted to verify and extend our findings. It is our hope that our study acts as a catalyst for further exploration in this field, and thereby contribute to the ceaseless enhancement of patient care in intensive care units.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

ZZ, LC, and DN designed the study and collected and collated the data. DN analyzed the data, LC and ZZ drafted the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1234924/full#supplementary-material

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Increased gut permeability and bacterial translocation are associated with fibromyalgia and myalgic encephalomyelitis/chronic fatigue syndrome: implications for disease-related biomarker discovery

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Background: There is growing evidence of the significance of gastrointestinal complaints in the impairment of the intestinal mucosal barrier function and inflammation in fibromyalgia (FM) and in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS). However, data on intestinal permeability and gut barrier dysfunction in FM and ME/CFS are still limited with conflicting results. This study aimed to assess circulating biomarkers potentially related to intestinal barrier dysfunction and bacterial translocation and their association with self-reported symptoms in these conditions.

Methods: A pilot multicenter, cross-sectional cohort study with consecutive enrolment of 22 patients with FM, 30 with ME/CFS and 26 matched healthy controls. Plasma levels of anti-beta-lactoglobulin antibodies (IgG anti-β-LGB), zonulin-1 (ZO-1), lipopolysaccharides (LPS), soluble CD14 (sCD14) and interleukin-1-beta (IL-1β) were assayed using ELISA. Demographic and clinical characteristics of the participants were recorded using validated self-reported outcome measures. The diagnostic accuracy of each biomarker was assessed using the receiver operating characteristic (ROC) curve analysis.

Results: FM patients had significantly higher levels of anti-β-LGB, ZO-1, LPS, and sCD14 than healthy controls (all $P < 0.0001$). In ME/CFS patients, levels of anti-β-LGB, ZO-1, LPS, and sCD14 were significantly higher than controls, but lower than in FM (all $P < 0.01$), while there was no significant difference in IL-1β level. In the FM and ME/CFS cohorts, both anti-β-LGB and ZO-1 correlated significantly with LPS and sCD14 ($P < 0.001$ for both). In the FM group, both anti-β-LGB and ZO-1 were correlated significantly with physical and mental health components on the SF-36 scale ($P < 0.05$); whereas IL-1β negatively correlated with the COMPASS-31 score ($P < 0.05$). In the ME/CFS cohort, ZO-1 was positively correlated with the COMPASS-31 score ($P < 0.05$). The ROC curve analysis indicated a strong ability of anti-β-LGB, ZO-1, LPS and sCD14 to predictively distinguish between FM and ME/CFS from healthy controls ($P < 0.0001$).

Conclusion: Biomarkers of intestinal barrier function and inflammation were associated with autonomic dysfunction assessed by COMPASS-31 scores in FM and ME/CFS respectively. Anti-β-LGB antibodies, ZO-1, LPS, and sCD14 may be putative predictors of intestinal barrier dysfunction in these cohorts. Further studies are needed to assess whether these findings are causal and can therefore be applied in clinical practice.

KEYWORDS

anti-beta-lactoglobulin, chronic fatigue syndrome, fibromyalgia, intestinal permeability, myalgic encephalomyelitis, zonulin

Introduction

Fibromyalgia (FM) and myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) constitute a major public health issue worldwide, imposing a serious burden on patients, caregivers, and society, and exerting a substantial economic impact (1–4). Both are complex disabling multisystem disorders without an established aetiology characterized by a wide range of overlapping symptoms such as widespread pain, post-exertional fatigue, cognitive dysfunction, dysautonomia, and gastrointestinal complaints (5, 6). No simple diagnostic tests are available, nor any curative treatment (7, 8).

While FM and ME/CFS share common symptoms and biological abnormalities of unknown cause, a growing body of evidence suggests the existence of multiple pathophysiology mechanisms underlying the association between impaired gut barrier function and local and systemic inflammation in these conditions. Assessing gut barrier function in these conditions is challenging and has been a matter of debate for many years (9–11). Recently, it was proposed that increased intestinal permeability and gut dysbiosis allows the entry of bacterial endotoxins (reflected by high levels of specific anti-LPS antibodies) into the bloodstream and may trigger systemic inflammation and sustained immune hyperactivation (in the form of imbalances of inflammatory factors such as IL-1β, IL-6, TNF-α, IFN-γ, IL-10, IL-13, IL-16, IL-17A and C-reactive protein), contributing to the development and perpetuation of chronic widespread pain and post-exertional malaise in these illnesses (12–14).

Previous studies conducted in FM and ME/CFS have speculated about a possible association between intestinal function biomarkers (zonulin, LPS and sCD14) and compromised intestinal barrier integrity; however, more research is needed to understand the exact role and the connections between them (10, 15). Consequently, the growing evidence of a potential role of gut-brain axis in triggering neuroinflammation in FM and ME/CFS has identified several intestinal barrier function biomarkers which may contribute to the onset and illness severity. For instance, these studies have linked significant high levels of zonulin, LPS and its receptor sCD14 with increased intestinal permeability and microbial translocation in FM and ME/CFS (10, 15–22). Although the ability of these biomarkers to suggest the presence of compromised intestinal barrier in these conditions is well established, their use in clinical practice and research remains limited.

Although the potential involvement of an antibody-mediated autoimmune signature and illness severity has been proposed in the pathophysiology of these conditions (23), to date the circulating anti-beta-lactoglobulin antibody profile (anti-β-LGB) in FM and ME/CFS has not explored. Increased antibody production against LGB, a major allergen in whey (cow's milk protein) has been observed in individuals with allergy and/or food intolerances and gastrointestinal complaints, which have also been reported in FM and ME/CFS (24, 25).

In addition, a growing number of studies linked the increased immune activation with the alteration of the gut microbiome composition in FM and ME/CFS (15, 23, 26–29), thereby suggesting

that perturbed microbiome homeostasis may induce an imbalance in tolerance induction. An intestinal barrier dysfunction characterized by increased gut permeability and microbial translocation may lead to irritable bowel syndrome (IBS), which could impair tolerance the development of tolerance and instead contribute to exacerbation of symptoms in these conditions (11, 30, 31). Furthermore, the damaged intestinal barrier-induced immunity may contribute to neuroimmune dysfunction followed by the gradual activation of innate responses in the brain via the vagus nerve (neuroglial activation) and a reduction of energy-consuming activities in FM and ME/CFS (8, 32, 33).

Further investigations on the gut microbial composition and metabolomics profiling have shown noticeable reductions in the relative microbial abundance/diversity and the production of microbiota-derived metabolites in ME/CFS (i.e., probiotic *Bifidobacterium* species and butyrate-producing *Faecalibacterium*), possibly leading to the perturbed gut barrier function and increased bacteria translocation implicated in low-grade systemic inflammation (10, 14, 27, 34, 35). Similar alterations in gut microbiota composition have been reported in FM patients, who presented significant reductions in the relative abundance of certain short-chain fatty acid-producing bacteria, while higher relative abundance was reported for other organic acids (36–39).

Therefore, this study aimed (1) to explore whether patients with FM and ME/CFS have altered intestinal barrier function and inflammation by measuring circulating gut biomarkers and (2) to examine the relationships among these intestinal function biomarkers and self-reported outcome measures provided by the study participants.

Methods

Study design and participants

A proof-of-concept multicenter, cross-sectional, prospective case-control cohort study was conducted in 22 FM patients who met the 2010 ACR diagnostic criteria for FM (40) and 30 ME/CFS who fulfilled the 2011 ICC definition for ME/CFS (41). Subjects were recruited by clinicians from two outpatient referral centres (Hospital VIAMED Santa Angela de la Cruz, Seville, Spain and Vall d'Hebron Hospital, Barcelona, Spain) from February 2018 to March 2019. The sample comprised 26 age- and sex-matched sedentary healthy volunteers, who were recruited from each local community by word of mouth. Ten FM patients, 12 with ME/CFS and 13 sedentary healthy volunteers were recruited from the hospital in Sevilla and the rest from the local hospital in Barcelona so as to avoid sampling bias in the selection of the target population. The participants were Caucasian, from the same geographical area, and had a sedentary lifestyle with a similar Mediterranean dietary pattern at the time of inclusion.

The exclusion criteria for participation in the study included current or past diagnosis of autoimmune conditions (such as coeliac disease), food allergies and/or intolerances, haematological conditions, cardiovascular diseases, metabolic and endocrine disturbances (thyroid-related conditions), infectious diseases, neuropsychiatric disorders (psychosis/major depression). Individuals were also excluded if they had morbid obesity, were smokers, were pregnant and/or breast-feeding, or had history of substance misuse or any underlying symptoms that might influence the clinicians' ability to distinguish between FM and ME/CFS diagnoses.

All study participants were informed of the research procedures and signed a written informed consent form prior to enrolment, in accordance with the 2013 Declaration of Helsinki. This study was approved by the local Research Ethics Committees (reference number: GutME-0634; on January 17, 2018). After providing consent, all participants underwent a clinical examination. Finally, data were analysed in an irreversibly anonymized fashion. A detailed summary of the participants' demographic and clinical characteristics is shown in Table 1.

Data collection and clinical outcomes measures

Participants were asked to complete validated self-reported questionnaires under the supervision of two trained investigators, who ensured compliance. These questionnaires included the Fatigue Impact Scale (FIS-40), the Composite Autonomic Symptom Score (COMPASS-31), the Pittsburgh Sleep Quality Index (PSQI), and the Short Form 36-item (SF-36) health survey, which were used to compile data on participants' demographic characteristics and current health status.

Fatigue impact scale

Fatigue was assessed by the Fatigue Impact Scale (FIS-40), a self-administered 40-item questionnaire which includes three subscales (scored from 0 to 4) reflecting the perceived feeling of fatigue in physical (10 items), cognitive (10 items), and psychosocial functions (20 items). The sum of the three scales yields a global score ranging from 0 to 160. Higher scores indicate more functional limitations resulting from fatigue; scores above 120 points are taken to indicate severe fatigue, while scores of 120 points or less are taken to reflect mild/moderate fatigue (42).

Composite autonomic symptom score

The frequency and severity of autonomic symptoms were evaluated by using the validated self-administered 31-item Composite Autonomic Symptom Score (COMPASS-31), comprising six main domains: orthostatic intolerance (4 items), vasomotor (3 items), secretomotor (4 items), gastrointestinal (12 items), bladder (3 items), and pupillomotor systems (5 items). The overall COMPASS-31 score ranges from 0 to 100, with higher scores indicating worse autonomic symptoms (43).

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TABLE 1 Demographic and clinical characteristics of the study population.

Variable	FM (n = 22)	ME/CFS (n = 30)	HC (n = 26)	*P*-value [a,b]
Age, years	57 ± 16	53 ± 10	51 ± 8	0.957/0.982
Gender, female (%)	16 (73)	24 (80)	20 (77)	0.954/0.971
BMI, kg/m^2	23 ± 3.5	24 ± 5.1	24 ± 3.7	0.875/1.00
SBP, mmHg	120 ± 11	109 ± 14	118 ± 8	0.951/0.919
DBP, mmHg	80 ± 6	75 ± 10	68 ± 6	0.754/0.874
HR, bpm	70 ± 7	78 ± 9	72 ± 11	0.925/0.832
Marital status, n (%)				
Single	6 (27)	7 (23)	6 (23)	n.s.
Separated/divorced	4 (18)	5 (17)	5 (19)	n.s.
Married	12 (55)	18 (60)	15 (58)	n.s.
Illness duration, months				
48	2	2	n/a	n.s.
72	8	3	n/a	n.s.
≥ 120	12	25	n/a	n.s.
Family members affected, n (%)				
Yes	12 (55)	9 (30)	9 (34)	n.s.
No	10 (45)	21 (70)	17 (66)	n.s.
Medication use, n (%)				
Antidepressants				
Tricyclics				
Yes	4 (14)	6 (20)	0 (0)	n.s.
No	18 (86)	24 (80)	26 (100)	n.s.
Dual				
Yes	5 (23)	14 (47)	0 (0)	n.s.
No	17 (77)	16 (53)	26 (100)	n.s.
SSRI				
Yes	2 (10)	6 (20)	0 (0)	n.s.
No	20 (90)	24 (80)	26 (100)	n.s.
Anticonvulsants				
Yes	3 (14)	21 (70)	0 (0)	n.s.
No	19 (86)	9 (30)	26 (100)	n.s.
Tramadol				
Yes	9 (41)	10 (33)	0 (0)	n.s.
No	13 (59)	20 (67)	26 (100)	n.s.
Major opioids				
Yes	2 (10)	3 (10)	0 (0)	n.s.
No	20 (90)	27 (90)	26 (100)	n.s.

(Continued)

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TABLE 1 Continued

Variable	FM (n = 22)	ME/CFS (n = 30)	HC (n = 26)	*P*-value [a,b]
Anxiolytics/sedatives				
Yes	1 (4)	6 (20)	0 (0)	n.s.
No	21 (96)	24 (80)	26 (100)	n.s.
NSAIDs				
Yes	7 (32)	14 (47)	0 (0)	n.s.
No	15 (68)	16 (53)	26 (100)	n.s.
Other analgesics				
Yes	10 (45)	19 (63)	0 (0)	n.s.
No	12 (55)	11 (37)	26 (100)	n.s.
Measures				
FIS-40				
Global score (0-160)	113 ± 19.6	140.7 ± 19.4	15.6 ± 5.4	< 0.0001
Physical	32 ± 6	36.7 ± 3.4	4.4 ± 0.3	< 0.0001
Cognitive	30 ± 5	37 ± 5.9	4.1 ± 0.7	< 0.0001
Psychosocial	51 ± 9	67 ± 12	7.1 ± 1.9	< 0.0001
COMPASS-31				
Global score (0-100)	64.5 ± 2.8	65.3 ± 1.4	6.8 ± 1.1	< 0.0001
Orthostatic intolerance	31 ± 2.8	30.6 ± 6.4	4 ± 1.1	< 0.0001
Vasomotor	2.4 ± 1.2	2.5 ± 0.4	0.0 ± 0.0	< 0.0001
Secretomotor	10.5 ± 1.7	10.7 ± 2.3	1.3 ± 0.4	< 0.0001
Gastrointestinal	12.9 ± 3.1	13.4 ± 3.8	0.7 ± 0.2	< 0.0001
Bladder	4.2 ± 3.2	4.4 ± 3.2	0.0 ± 0.0	< 0.0001
Pupillomotor	3.5 ± 0.9	3.7 ± 0.9	0.8 ± 0.2	< 0.0001
PSQI				
Global score (0-21)	5.0 ± 2.0	18.0 ± 3.1	4.0 ± 2.0	< 0.0001
Subjective sleep quality	1.0 ± 0.6	3.0 ± 0.6	1.0 ± 0.6	< 0.0001
Sleep latency	1.0 ± 0.6	3.0 ± 0.8	1.0 ± 0.8	< 0.0001
Sleep duration	0.5 ± 0.4	2.0 ± 0.4	1.0 ± 0.4	< 0.0001
Habitual sleep efficiency	0.5 ± 0.4	3.0 ± 0.3	0.0 ± 0.0	< 0.0001
Sleep disturbances	1.0 ± 0.5	2. 0 ± 0.3	1.0 ± 0.3	< 0.0001
Sleeping medication	1.0 ± 0.5	3.0 ± 0.3	0.0 ± 0.0	< 0.0001
Daytime dysfunction	0.0 ± 0.0	3.0 ± 0.5	0.0 ± 0.0	< 0.0001
SF-36				
Physical functioning	14 ± 0.8	18.3 ± 5.5	100 ± 0.0	< 0.0001
Physical role functioning	0.0 ± 0.0	0.0 ± 0.0	98 ± 7.9	< 0.0001
Bodily pain	9 ± 0.8	11 ± 9.6	100 ± 0.0	< 0.0001
General health perception	12 ± 1.9	17 ± 11.8	80 ± 15.7	< 0.0001
Vitality	10 ± 0.9	5 ± 0.9	90 ± 12.4	< 0.0001

(Continued)

TABLE 1 Continued

Variable	FM (n = 22)	ME/CFS (n = 30)	HC (n = 26)	*P*-value [a,b]
Social role functioning	14 ± 0.7	20 ± 3.9	100 ± 0.0	< 0.0001
Emotional role functioning	25 ± 3.7	26 ± 4.8	100 ± 0.0	< 0.0001
Mental health	17 ± 0.6	44 ± 12.3	92 ± 8.1	< 0.0001
PCS	24.2 ± 0.1	20.9 ± 1.0	57.0 ± 0.4	< 0.0001
MCS	7.6 ± 0.2	18.3 ± 2.5	54.3 ± 0.6	< 0.0001

Data are given as means ± standard error of the mean (SEM) for continuous variables, and as number of cases (percentages) for categorical variables, as appropriate (unless otherwise specified). P-values from Mann-Whitney U-test for continuous variables and from Fisher's exact test for categorical variables (gender, marital status, family background, medications). Bold values denote statistical significance at P < 0.05 between each cohort ([a]FM and [b]ME/CFS) with healthy controls. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; SSRI, selective serotonin reuptake inhibitors; NSAIDs, non-steroidal anti-inflammatory drugs; FIS-40, 40-item fatigue impact scale; COMPASS-31, composite autonomic symptom score; PSQI, Pittsburgh sleep quality index; SF-36, 36-item short-form health survey; PCS, physical health component summary scores; MCS, mental health component summary scores; n.s., not significant.

Pittsburgh sleep quality index

Sleep disturbances were assessed by the standardized, self-administered 19-item Pittsburgh Sleep Quality Index (PSQI) questionnaire, which comprises seven components of sleep quality assessed over a one-month interval (scored from 0 to 3): subjective sleep quality, latency, sleep duration, habitual sleep efficiency, sleep perturbations, use of sleeping medication, and daytime dysfunction. The global PSQI score can range from 0 to 21 points, with score above 5 representing poorer subjective sleep quality (44).

Short form 36-item health survey

Participants' general physical and mental health was assessed using the short form 36-item (SF-36) questionnaire, a 36-item self-report health survey conducted over a 4-week period. The SF-36 comprises eight health domains, focusing on limitations in physical activities due to health issues, limitations in social activities due to physical or/and emotional problems, limitations in everyday activities due to health and/or psychological problems, bodily pain, general mental health (including psychological distress and well-being), vitality and overall health perceptions. The eight domains were weighted and summarized in physical component summary (PCS) scores and mental component summary (MCS) scores ranging from 0 to 100. Higher scores indicate better health-related physical and mental quality of life (45).

Collection of blood samples and processing

Fasting blood samples were collected from each participant directly in K_2EDTA tubes (Vacutainer, BD Biosciences, Madrid, Spain) by venipuncture upon confirmation of the diagnosis. Plasma samples were obtained by centrifugation at 2,000 x *g* for 15 minutes at 4°C within 1 hour of the blood collection; then they were collected immediately and frozen in aliquots at −80°C until further analysis.

Quantification of intestinal barrier function biomarkers

Plasma ZO-1 (Catalog #: MBS706368, MyBioSource, San Diego, CA), LPS (Catalog #: MBS266722, MyBioSource, San Diego, CA), sCD14 (Catalog #: RK01060, Abyntek Biopharma, Vizcaya, Spain), and IL-1β (Catalog #: MBS2510385, MyBioSource, San Diego, CA) were measured using commercially available ELISA kits according to the manufacturers' instructions. Circulating human anti-β-lactoglobulin antibody levels (IgG isotypes) were assayed using a validated home-made ELISA protocol as detailed below. All the plasma samples were measured in blind duplicates for each biomarker. For all protocols, absorbance at 450 nm with 570 nm correction was measured in a microplate reader, and corrected absorbance was interpolated in each standard curve to determine the concentrations (Sigma Plot).

Detection of human anti-β-lactoglobulin antibody levels

Circulating anti-β-lactoglobulin antibody levels (IgG isotypes) were tested using a standard direct ELISA protocol (46). Briefly, microtitre 96-well polystyrene plates (Nunc, Roskilde, Denmark) were coated with 1 µg/µL β-lactoglobulin from cow's milk as target protein (Cat # L7880; Sigma Aldrich, Madrid, Spain) diluted in 50 mM Na_2CO_3/$NaHCO_3$ buffer (pH 9.6) at 4°C overnight. After blocking with 1X phosphate-buffered saline containing 0.05% Tween-20 (PBS-T) and 3% BSA for 90 minutes at room temperature, the pre-coated plates were incubated with the human plasma samples diluted in blocking buffer (dilution from 1:200 to 1:2000) for two hours at room temperature. Plates were washed out three times with PBS-T for five minutes and then each well was incubated with an HRP-conjugated mouse anti-human IgG1 Fc secondary antibody at dilution 1:5000 (Cat # A-10648; Thermo Fisher Scientific, MA, USA) for three hours at room temperature. Between incubations, plates were washed three times with PBS-T. Plates were revealed using TMB substrate (Thermo Fisher Scientific, MA, USA) for 30 minutes at room temperature in the dark and then 50 µl of stop solution (2M sulphuric acid) was

added to each well. The absorbance (O.D.) was read immediately at 450 nm with 570 nm correction using a microplate reader (Varioskan Flash, Thermo Electron Corporation, NH, USA). The results were given as antibody levels per ng/ml on a standard curve with anti-human beta-LGB chimera monoclonal antibody (clone 25I2; CABT-L2429; Creative Diagnostics, NY, USA) considering the dilution of each sample. The standard curve was prepared using serial dilutions from 8000 ng/ml to 31.25 ng/ml and included five standard concentrations within the indicated range. Based on the signal-to-noise ratio of assay, the limit of detection was 1.96 ng/ml. In each ELISA assay, endogenous negative and positive controls of human plasma samples were included. Negative controls were tested from healthy donors who had not ingested dairy products for at least one year. Positive controls were assayed from patients recently diagnosed with celiac disease, before the start of treatment, and who habitually ingested dairy products.

Statistical analysis

All the data obtained were checked for normality with the Kolmogorov-Smirnov and Shapiro-Wilk tests. Normally distributed data were presented as means ± standard error of the mean (SEM). Statistical analysis of non-parametric data in the three study groups was performed using an analysis of non-parametric data evaluated by the Kruskal-Wallis test and compared by Dunn's multiple comparison test. Scale and subscale scores were examined using the non-parametric Mann-Whitney *U* test (two-group comparisons). Fisher's exact test was used to compare the frequency of the reported categorical variables between the groups. Box plots and ROC curves were generated using GraphPad Prism software. The area under the curve (AUC) was calculated so as to compare the overall diagnostic accuracy of gut biomarkers for predicting FM and ME/CFS. Cut-off values with the highest accuracy were selected as the diagnostic cut-off points. Correlations were analysed using Spearman's test with the R package. Statistical significance was set at $P < 0.05$ (two-tailed). Only adjusted *P*-values presenting significant differences are shown. Statistical analyses were performed using R software version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism version 9.5.1 for Windows (GraphPad software, Boston, MA, USA).

Results

Baseline demographics and clinical characteristics of the study participants

Seventy-eight participants, comprising 22 FM patients, 30 ME/CFS patients and 26 healthy-matched controls were included in the study. Table 1 shows the demographics and clinical characteristics of the study population. No significant differences were observed for age, gender, BMI, hemodynamic variables, marital status, illness duration, family background, and concomitant medication between the study cohorts from each site. In this group, most patients with FM (n = 12) and ME/CFS (n = 25) had an illness duration of more than 120 months (10 years), which was self-reported as long-lasting fatigue and chronic pain (*data not shown*). More than half of the patients reported frequent medication use, including analgesics, non-steroidal anti-inflammatories, anticonvulsants, anxiolytics and antidepressants, but none of the controls were taking medication.

The clinical assessment based on self-reported outcome measures showed that patients with FM and ME/CFS had significantly higher scores on the FIS-40, COMPASS-31 and PSQI questionnaires than controls (all $P < 0.0001$), whereas healthy controls had significantly higher SF-36 scores than the patients' groups (all $P < 0.0001$).

Profile of biomarkers of intestinal barrier function and inflammation

The measurement of intestinal biomarkers proposed in this study revealed that FM patients had higher presence of increased gut permeability and microbial translocation than ME/CFS patients and matched controls. As shown in Figure 1, plasma levels of intestinal barrier function biomarkers and inflammation were as follows: IgG anti-β-LGB antibodies (A), ZO-1 (B), LPS (C), sCD14 (D), and IL-1β (E) in individuals with FM, ME/CFS, and healthy controls. FM patients had significantly higher levels of IgG anti-β-LGB antibodies, ZO-1, LPS and sCD14 than ME/CFS and controls ($P < 0.001$ for all). In ME/CFS patients, plasma levels of anti-β-LGB, ZO-1, LPS, and sCD14 were significantly higher than in controls ($P < 0.01$), but lower than in FM cases ($P < 0.001$). There were no significant differences in IL-1β levels in patients with FM and ME/CFS and healthy controls.

Correlation analysis between intestinal function biomarkers and self-reported outcome measures

Correlations between the proposed biomarkers of intestinal barrier function and inflammation and self-reported outcome measure scores in the study cohorts are displayed in Figure 2. Briefly, in the FM cohort, ZO-1 was significantly correlated with anti-β-LGB antibodies (r = 0.91; $P < 0.001$), LPS (r = 0.83; $P < 0.001$) and sCD14 (r = 0.65; $P < 0.01$), and with physical and mental health component scores on the SF-36 questionnaire (r = 0.51 and r = -0.51; both $P < 0.05$) respectively. In this cohort, anti-β-LGB was strongly correlated with ZO-1 (r = 0.91; $P < 0.001$), LPS (r = 0.91; $P < 0.001$), and sCD14 (r = 0.86; $P < 0.001$) and also with physical health component scores on the SF-36 questionnaire (r = 0.43; $P < 0.05$). In contrast, IL-1β negatively correlated with overall COMPASS-31 scores (r = -0.45; $P < 0.05$) (Figure 2A).

Analysis of the ME/CFS cohort showed significant positive correlations between ZO-1 and anti-β-LGB (r = 0.82; $P < 0.001$), LPS (r = 0.91; $P < 0.001$) and sCD14 (r = 0.61; $P < 0.001$), and COMPASS-31 scores (r = 0.45; $P < 0.05$), whereas anti-β-LGB was positively correlated with ZO-1 (r = 0.82; $P < 0.001$), LPS (r = 0.89; $P < 0.001$) and sCD14 (r = 0.65; $P < 0.001$) (Figure 2B).

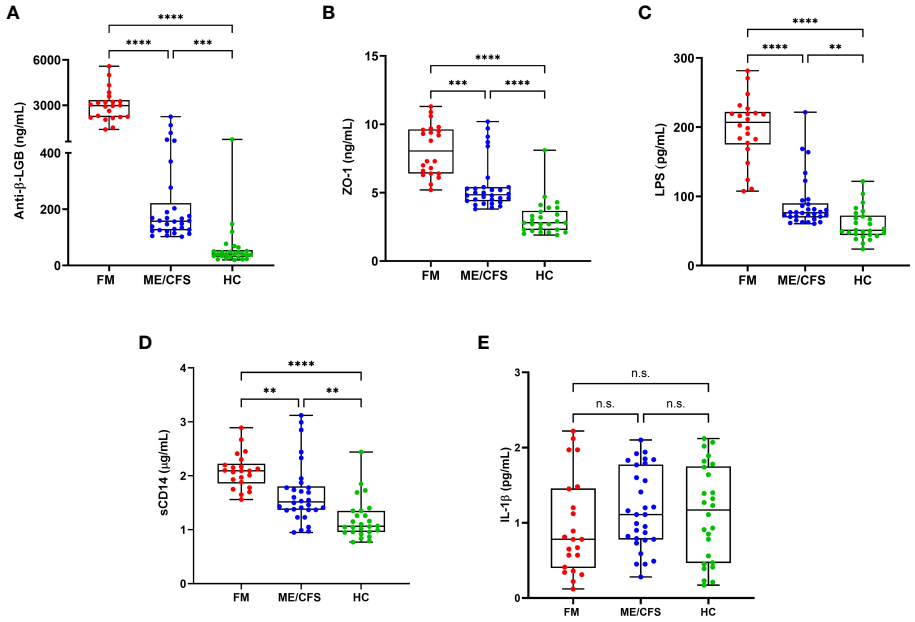

FIGURE 1
Circulating biomarkers of intestinal permeability, bacterial translocation and inflammation in the study participants. Plasma levels of anti-β-LGB **(A)**, ZO-1 **(B)**, LPS **(C)**, sCD14 **(D)**, and IL-1β **(E)** in patients with FM (n = 22), ME/CFS (n = 30) and healthy controls (n = 26). Each dot denotes a single participant. Values are shown as mean ± SEM of duplicates and are representative of two independent experiments. The box extends from the 25th to 75th percentiles, the line represents the mean, and the whiskers indicate the range of minimum and maximum values. Significance at $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ was calculated using the Kruskal-Wallis signed-rank test on normalized data. Anti-β-LGB, anti-beta-lactoglobulin antibodies; ZO-1, zonulin-1; LPS, lipopolysaccharides; sCD14, soluble CD14; IL-1β, interleukin-1 beta. n.s., not significant

In healthy controls, ZO-1 was positively and significantly correlated with sCD14 (r = 0.42; $P < 0.05$) and opposed with physical healthy component scores on the SF-36 questionnaire (r = -0.46; $P < 0.05$); while anti-β-LGB was negatively correlated with physical and mental health component scores on the SF-36 questionnaires (r = -0.46 and r = -0.48; both $P < 0.05$), respectively (Figure 2C). Multipanel scatter dot plots for statistically significant correlations between intestinal barrier function biomarkers and self-reported outcome measures in FM, ME/CFS and healthy controls are depicted (Supplementary Figs S1-S3).

ROC analysis for each intestinal barrier function biomarker in FM and ME/CFS

Analyses of the diagnostic power of each circulating gut function biomarker with regard to predictively distinguishing FM

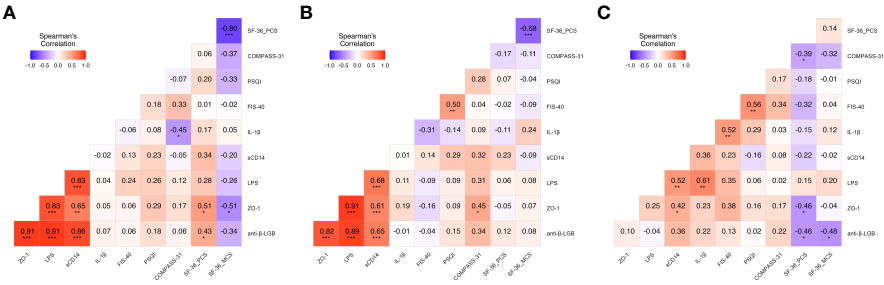

FIGURE 2
Heatmap depicting color-coded Spearman's correlation coefficients of circulating intestinal barrier function biomarkers and clinical outcome measures in the study participants. Correlation analysis of patients with FM **(A)**, ME/CFS **(B)** and healthy controls **(C)** were evaluated using Spearman's rank correlation test and FDR-adjusted $P < 0.05$. Pairwise Spearman's rank correlation coefficients (rho) are depicted for each correlation and is presented by color intensity scale (at the top left of each panel). Heat color show standardized Z-scores (adjusted rho) across biomarkers and outcome measures. The color intensity is proportional to the strength of the association (rho value) ranging from red (positive correlations) to blue (negative correlations). Statistical significance was assessed using the Kruskal-Wallis test. FDR was calculated using Benjamini-Hochberg method. Statistical significance was set at $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Anti-β-LGB, anti-beta-lactoglobulin antibodies; ZO-1, zonulin-1; LPS, lipopolysaccharides; sCD14, soluble CD14; IL-1β, interleukin-1-beta.

and ME/CFS from healthy controls are displayed in Figures 3 and 4 respectively. As shown in the ROC curve analysis for FM, compared to the reference, anti-β-LGB (AUC = 1.00; 95% CI: 1.00-1.00; $P < 0.0001$), ZO-1 (AUC = 0.980; 95% CI: 0.94-1.00; $P < 0.0001$), LPS (AUC = 0.996; 95% CI: 0.98-1.00; $P < 0.0001$), and sCD14 (AUC = 0.949; 95% CI: 0.88-1.00; $P < 0.0001$) were able to distinguish between FM patients and healthy controls as demonstrated by the AUC values using a univariate model (Figures 3A-E).

ROC curve analysis for ME/CFS showed that compared to the reference, anti-β-LGB (AUC = 0.943; 95% CI: 0.86-1.00; $P < 0.0001$), ZO-1 (AUC = 0.934; 95% CI: 0.86-1.00; $P < 0.0001$), LPS (AUC = 0.806; 95% CI: 0.68-0.93; $P < 0.0001$), and sCD14 (AUC = 0.794; 95% CI: 0.68-0.92; $P < 0.0001$) were able to distinguish between ME/CFS patients and healthy controls as demonstrated by the AUC values using a univariate model (Figures 4A-E).

Also, an ROC curve analysis to predictively distinguish patients with FM and ME/CFS is shown in Figure 5. It showed that compared to the reference, anti-beta-LGB (AUC = 0.991; 95% CI: 0.97 to 1.00; $P < 0.0001$), ZO-1 (AUC = 0.882; 95% CI: 0.78 to 0.97; $P < 0.0001$), LPS (AUC = 0.953; 95% CI: 0.89 to 1.00; $P < 0.0001$), and sCD14 (AUC = 0.800; 95% CI: 0.67 to 0.92; $P = 0.0002$) were able to distinguish between patients with FM and ME/CFS as demonstrated by the AUC values using a univariate model (Figures 5A-E).

Discussion

This is a proof-of-concept study to investigate the relationship between circulating intestinal function biomarkers and inflammation and self-reported clinical symptoms in Spanish patients with FM and ME/CFS, and also to evaluate the suitability of these gut barrier function biomarkers as potential suggestive predictors of diagnosis in these cohorts which replicate prior studies (14–16, 19, 47–51). Our findings corroborate those of previous studies (9, 12, 14, 19, 47, 48, 52) reporting the presence of significantly increased levels of suggestive biomarkers of intestinal permeability (IgG anti-β-LGB antibodies and ZO-1), and microbial translocation (LPS and sCD14) in FM and ME/CFS compared to healthy controls. Interestingly, these biomarkers were markedly higher in individuals with FM than in those with ME/CFS.

Further analysis indicated that the proposed novel intestinal permeability biomarkers (anti-β-LGB antibodies and ZO-1) significantly correlated with indices of microbial translocation (LPS and sCD14) in FM and ME/CFS. In addition, these measures were correlated with scores of self-reported outcome measures determined by COMPASS-31 and SF-36 questionnaires in FM and ME/CFS. Specifically, in FM the IL-1β levels were associated with measures of physical and mental health components on the SF-36 questionnaire; whereas the frequency and severity of autonomic symptoms evaluated by COMPASS-31 scores was positively correlated with the ZO-1 in the ME/CFS cohort. Further analysis of covariates indicated a significant correlation between age and anti-β-LGB and ZO-1, as well as LPS and sCD14 in ME/CFS patients. Finally, the ROC curve analysis of the diagnostic accuracy of the biomarkers measured demonstrated a high predictive capacity of anti-β-LGB, ZO-1, LPS and sCD14 for distinguishing FM and ME/CFS cases from healthy controls.

Recently, a growing number of studies have reported gut dysbiosis and increased intestinal permeability in ME/CFS

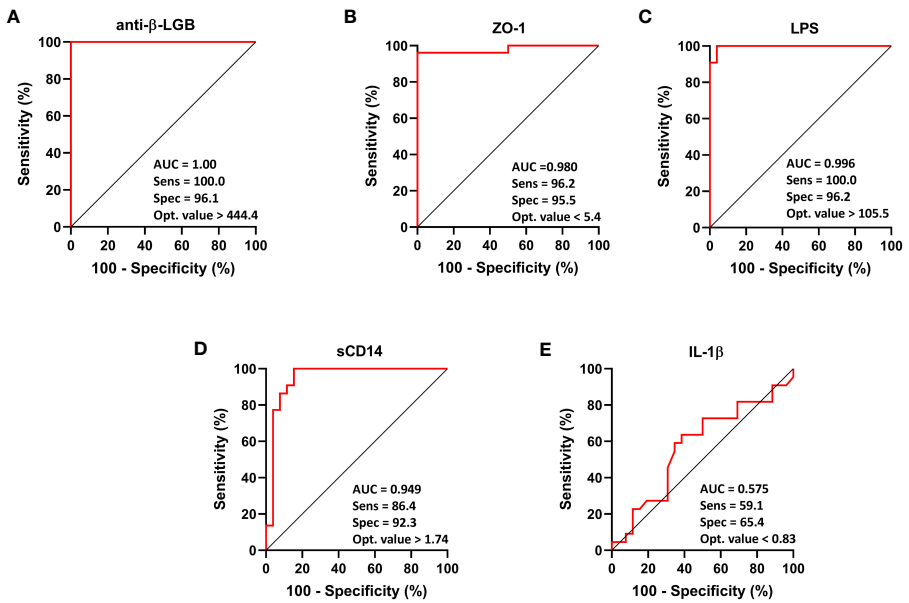

FIGURE 3
ROC curve analysis of each intestinal barrier function biomarker to discriminate FM patients from healthy controls. IgG anti-β-LGB antibodies **(A)**, ZO-1 **(B)**, LPS **(C)**, sCD14 **(D)**, and IL-1β **(E)**. ROC curves were used to explore the accuracy of each biomarker to discriminate between FM subjects and healthy controls. Cut-off values are shown for each biomarker with their respective sensitivity, specificity and optimal value. AUC values close to 1 indicate that a high true positive rate was achieved with false positive rate (ideal performance), while AUC values close to 0.5 indicate random performance. Anti-β-LGB, anti-beta-lactoglobulin antibodies; ZO-1, zonulin-1; LPS, lipopolysaccharides; sCD14, soluble CD14; IL-1β, interleukin-1-beta.

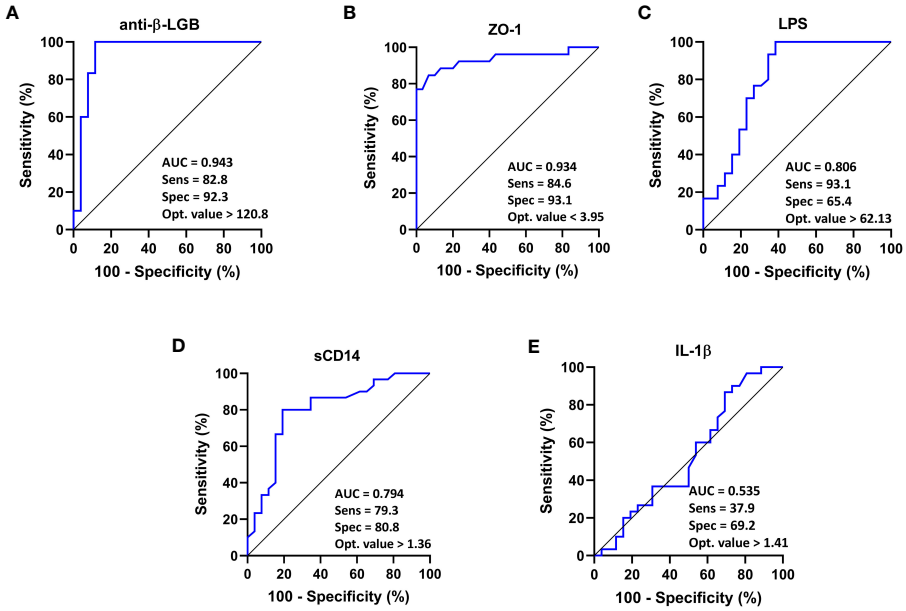

FIGURE 4
ROC curve analysis of each intestinal barrier function biomarker to discriminate ME/CFS patients from healthy controls. IgG anti-β-LGB antibodies **(A)**, ZO-1 **(B)**, LPS **(C)**, sCD14 **(D)**, and IL-1β **(E)** are displayed. ROC curves were used to analyze the accuracy of each biomarker to discriminate between ME/CFS subjects and healthy controls. Cut-off values are shown for each biomarker with their respective sensitivity, specificity and optimal value. AUC values close to 1 indicate that a high true positive rate was achieved with false positive rate (ideal performance), while AUC values close to 0.5 indicate random performance. Anti-β-LGB, anti-beta-lactoglobulin antibodies; ZO-1, zonulin-1; LPS, lipopolysaccharides; sCD14, soluble CD14; IL-1β, interleukin-1-beta.

(9,10, 53), while in FM this phenomenon is still to be confirmed. The study by Goebel et al. of FM patients with complex regional pain syndromes who reported alterations of gut barrier integrity in the form of increased gastroduodenal and small intestinal permeability found that these conditions coincided with typical IBS symptoms which were recorded in up to 18% of FM patients (16, 54). Although few overlapping mechanisms explaining the high prevalence of GI symptoms in FM patients have been proposed,

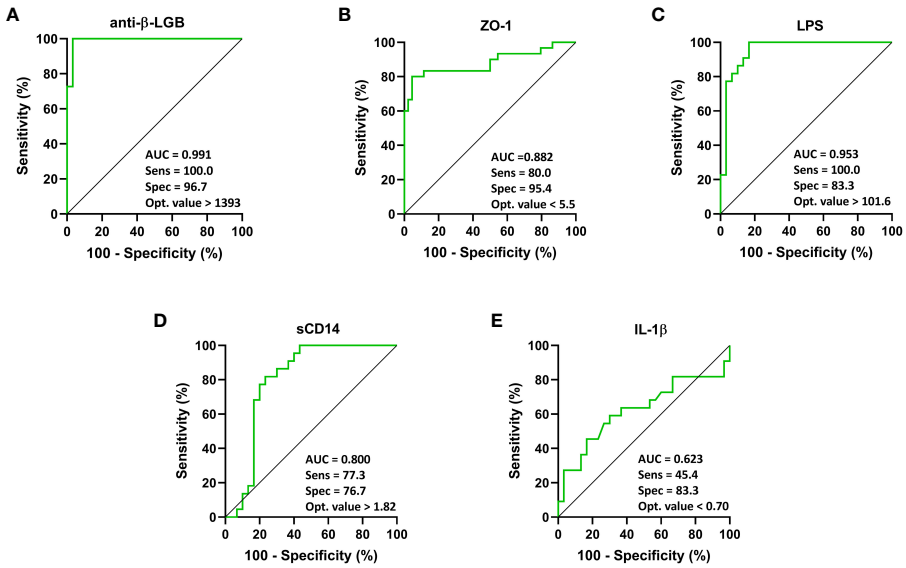

FIGURE 5
ROC curve analysis of each intestinal barrier function biomarker to discriminate individuals with FM from ME/CFS. IgG anti-β-LGB antibodies **(A)**, ZO-1 **(B)**, LPS **(C)**, sCD14 **(D)**, and IL-1β **(E)** are displayed. ROC curves were used to analyze the accuracy of each biomarker to discriminate between ME/CFS subjects and healthy controls. Cut-off values are shown for each biomarker with their respective sensitivity, specificity and optimal value. AUC values close to 1 indicate that a high true positive rate was achieved with false positive rate (ideal performance), while AUC values close to 0.5 indicate random performance. Anti-β-LGB, anti-beta-lactoglobulin antibodies; ZO-1, zonulin-1; LPS, lipopolysaccharides; sCD14, soluble CD14; IL-1β, interleukin-1-beta.

immune activation and neurotransmitter disruption have recently attracted attention (54).

Additionally, in ME/CFS it has been shown that elevated levels of bacterial wall components such as LPS, followed by an increased presence in the intestine of Gram-negative bacteria and also plasmacytoid dendritic cells as uniquely immunoreactive to antibodies against HERV proteins that damage the gut epithelial barrier and infiltrate in the bloodstream can provoke an immune response, ultimately leading to the establishment of low-grade chronic systemic inflammation (10, 55). Separately, the significant increases in Gram-positive facultative anaerobic bacteria reported in ME/CFS, including D-lactic acid-producing Enterococcus and Streptococcus spp., suggest that these bacteria are a more significant source of lactate than Gram-negative Escherichia coli. Gram-positive bacteria may thus contribute to the cognitive symptoms and also the mitochondrial dysfunction resulting from the lactic acidosis in this cohort (53).

Although intestinal damage may arise due to various pathomechanisms and may involve several factors, the evidence to date highlights its involvement in the context of FM and ME/CFS. For instance, disturbances in the gut wall may increase intestinal permeability, which can induce inflammatory changes that lead to comorbid chronic diseases. Loss of gut barrier integrity may contribute to bacterial translocation into the systemic circulation, followed by increased levels of autoantibodies IgA and IgM against LPS and more severe ME/CFS symptoms (22). In addition, changes in the gut microbiome in ME/CFS, with noticeable decreased bacterial diversity (in particular, a reduction in the relative abundance of members belonging to the Firmicutes phylum) may increase the predisposition to gut inflammation (14).

In the present study, both FM and ME/CFS patients had significantly higher levels of intestinal function biomarkers that indicate increased gut permeability and bacterial translocation than healthy controls. These observations are consistent with those of an earlier study that reported the presence of gut dysbiosis as demonstrated by increased levels of LPS, sCD14, endotoxins and lipid binding proteins (LBPs), which was positively correlated with illness severity in patients with chronic fatigue (48). A recent study, however, despite showing the presence of the antibody-induced responses to both microbial and dietary antigens along with greater epithelial cell damage and turnover rate confirmed by higher FABP-2 levels, failed to report any significant differences in LBPs or sCD14 levels due to a suppressed anti-microbial response in ME/CFS compared with controls (50). Interestingly another study, which reported increased levels of LPS and sCD14 along with some other biomarkers not included in this analysis (such as LBP, I-FABP, MCP-1 and C-reactive protein) was able to correctly discriminate between ME/CFS and controls with a cross-validation accuracy of 82.9% (14).

The findings reported here are of particular interest, because they confirm an association between the changes in indicators of increased intestinal permeability and bacterial translocation and their association with clinical outcomes measures in FM and ME/CFS. Besides, the present data add to the evidence that a gut barrier integrity injury may be involved in the pathophysiology of these illnesses, even if it is not always detectable.

To our knowledge, this is the first confirmatory study to explore the use of intestinal barrier function biomarkers related to increased gut permeability, such as anti-β-LGB antibodies in a Spanish FM and ME/CFS cohort. The main strength of the study is that data on the participants were obtained from a well-phenotyped cohort of Spanish FM and ME/CFS patients from two Spain outpatient referral centres, applying updated diagnostic case criteria and validated self-reported symptom questionnaires in these conditions. However, this study has several limitations, including its small sample size, its cross-sectional nature, and limited measures of inflammatory cytokine/chemokine and growth factor markers which are unable to establish causation between disrupted gut mucosal barrier and inflammation severity status.

In addition, self-reporting outcome measures do not use "*in vivo*" differential urinary multi-sugar excretion test for small bowel and colonic permeability assessment. It should be also noted that the bacterial DNA load assessed by culturing bacteria directly from blood and stool was not measured in these populations, and so the presence of potential infection cannot be conclusively ruled out, or its potential influence on the bacterial translocation biomarkers LPS and sCD14 (21). Finally, no information was available on other confounding factors related to lifestyle habits, previous infections, use of high-dose antibiotics, concomitant drugs, psychological stress, air pollutants, and others comorbid health conditions such as IBS, and/or anxiety/depression.

Further multisite longitudinal studies with larger numbers of participants who are representative of the general population should be conducted to confirm the observations reported here. Future studies should also expand the use of other validated inflammatory biomarkers in stool and blood in order to fully define the specific role of disturbed intestinal barrier function and inflammation in FM and ME/CFS. These studies should also include lactulose breath testing (SIBO) by collecting longitudinal faecal samples to explore gender-dependent microbiota composition in these conditions (29, 56).

In conclusion, by demonstrating the associations between the putative biomarkers of gut barrier dysfunction and bacterial translocation, and self-reported clinical outcomes assessed by the COMPASS-31 score, our findings add to the existing evidence of the potential role of increased intestinal permeability in FM and ME/CFS. Future research should aim to confirm the applicability of these findings in clinical practice by targeting gastrointestinal complaints in FM and ME/CFS and assessing the usefulness of interventions focused on the restoring gut microbiota homeostasis and enhancing intestinal barrier function. If future studies show this strategy to be valid, it may offer new therapeutic benefit and provide an opportunity to reduce gastrointestinal symptoms and restore the quality of life of these patients.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

This study was approved by the local Research Ethics Committees (reference number: GutME-0634; on January 17, 2018). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

Conception and design of the study: FM, MB-S, JA-M, OC, and JC-M. Biostatistical analysis: BL, JCD, JJ, and JC-M. Acquisition of data: FM, PZ, and JC-M. Analysis and interpretation of data: FM, BL, JCD, JJ, and JC-M. Drafting the manuscript: FM, AG-C, JJ and JC-M. Review and editing of manuscript: FM, MA, BL, JS, JJ, and JC-M. Supervision: MB-S, JJ and JA-M. Project administration: FM, PZ, and JC-M. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1253121/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Multipanel scatter dot plots depicting the statistically significant between intestinal barrier function biomarkers and self-reported outcome measures in fibromyalgia patients. Each dot corresponds to an individual. Spearman's correlation scatter plots with linear regression (black line) and the 95% confidence interval (brown band) was used to calculate the association. Square Spearman's rank correlation coefficient (rho^2) and statistically significant p-values are shown in each panel.

SUPPLEMENTARY FIGURE 2
Multipanel scatter dot plots depicting the statistically significant between intestinal barrier function biomarkers and self-reported outcome measures in ME/CFS patients. Each dot corresponds to an individual. Spearman's correlation scatter plots with linear regression (black line) and the 95% confidence interval (brown band) was used to calculate the association. Square Spearman's rank correlation coefficient (rho2) and statistically significant p-values are shown in each panel.

SUPPLEMENTARY FIGURE 3
Multipanel scatter dot plots depicting the statistically significant between intestinal barrier function biomarkers and self-reported outcome measures in healthy controls. Each dot corresponds to an individual. Spearman's correlation scatter plots with linear regression (black line) and the 95% confidence interval (brown band) was used to calculate the association. Square Spearman's rank correlation coefficient (rho2) and statistically significant p-values are shown in each panel.

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From trash to treasure: the role of bacterial extracellular vesicles in gut health and disease

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Bacterial extracellular vesicles (BEVs) have emerged as critical factors involved in gut health regulation, transcending their traditional roles as byproducts of bacterial metabolism. These vesicles function as cargo carriers and contribute to various aspects of intestinal homeostasis, including microbial balance, antimicrobial peptide secretion, physical barrier integrity, and immune system activation. Therefore, any imbalance in BEV production can cause several gut-related issues including intestinal infection, inflammatory bowel disease, metabolic dysregulation, and even cancer. BEVs derived from beneficial or commensal bacteria can act as potent immune regulators and have been implicated in maintaining gut health. They also show promise for future clinical applications in vaccine development and tumor immunotherapy. This review examines the multifaceted role of BEVs in gut health and disease, and also delves into future research directions and potential applications.

KEYWORDS

bacterial extracellular vesicles, outer membranes vesicles, gut health, intestinal barriers, immune barrier, inflammatory bowel disease, cancer, gut disease treatments

Introduction

The gut is an intricate and dynamic ecosystem that plays a pivotal role in human health and disease (1–3). Housing approximately 100 trillion organisms, the influence of the gut microbiota extends beyond simple digestion (1, 4). They can shape metabolic functions, influence epithelial barrier integrity, regulate immune responses (5–7). These microorganisms interact with host cells in numerous ways, from direct cellular adhesion or invasion to the release of cell wall components and the secretion of metabolically functional products (8–10). Emerging research recognizes that bacteria can modulate gut health via producing bacterial extracellular vesicles (BEVs) (11).

BEVs represent a class of cellular products secreted by both gram-negative and positive bacteria (12–14). These vesicles are usually 20 – 400 nm in diameter and have a bilayer lipid membrane structure with a similar composition to that of the parent membrane (15). Protected by the membrane, BEVs encapsulate various substances including virulence factors, proteins, nucleic acids, and lipids (13). The primary function of BEVs are considered as an excretion

system for the disposal of unwanted metabolites and misfolded proteins (16). Moreover, BEVs are found to function as signal and material transmission tools that mediate bacteria-bacteria and bacteria-host interactions (13, 15). BEVs can aid bacteria in nutrient acquisition, resistance to antibiotics or antimicrobial peptides (AMPs), and elimination of specific microbes (17). Meanwhile, BEVs can deliver virulence factors and toxins to host cells, thereby disrupt barrier integrity, induce inflammation, and even promote carcinogenesis (18). Nevertheless, the BEVs from certain beneficial or commensal bacteria are contribute to host health maintenance by triggering a host defence response or immune activation (11).

In this review, we consolidate the published evidence demonstrating the impact of BEVs on gut health, particularly their role in regulating the integrity and function of the intestinal barrier. We also highlight the significant roles of BEVs in various gut diseases, including infection, inflammatory bowel disease (IBD), gut-related metabolic diseases, and gastrointestinal tumors. We discuss the limitations of current research on BEVs in the gut, while concurrently exploring their potential therapeutic applications in gut disease treatment.

Biogenesis and types of BEVs in the gut

The gastrointestinal tract harbors a dynamic and symbiotic microbial ecosystem (19). These microorganisms exhibit remarkable metabolic abilities and continuously secrete BEVs into the lumen. Recent studies have reported a significant concentration of 8×10^{12} BEVs per milliliter in a solution containing 20 g of stool resuspended in 100 ml phosphate-buffered saline (20). Typically, these BEVs are classified into outer membrane vesicles (OMVs) and cytoplasmic membrane vesicles (CMVs), based on their constituent parts and unique biogenesis pathways (13).

The ability of gram-negative bacteria to secrete membrane vesicles originating from their outer membranes, termed as OMVs, was discovered over fifty years ago (14, 21). Subsequent research has revealed that gram-negative bacteria generate several types of BEVs under various conditions, including OMVs, outer inner membrane vesicles (OIMVs), and explosive outer membrane vesicles (EOMVs) (14, 22). Traditional OMVs are formed through a process known as "blebbing" (or the non-lytic route), resulting in a vesicle encapsulated in a single membrane bilayer (Figure 1A) (22). OMV generation is attributed to several mechanisms, including reduced outer membrane-peptidoglycan connection linkages, increased membrane curvature, increased periplasmic pressure, and flagellar rotation (22–24). Additionally, during genotoxic stress, gram-negative bacteria may utilize explosive cell lysis (or the lytic route) to produce OIMVs and EOMVs (13, 22). The prominent feature of these vesicles is both OIMVs and EOMVs contain many cytoplasmic components; moreover, OIMVs have two membrane bilayers, derived from the outer and inner membranes. (Figure 1A) (25).

Although enveloped in a dense peptidoglycan layer, gram-positive bacteria have evolved to generate their own types of vesicles, termed as CMVs (14). Similar to OMVs, these vesicles are

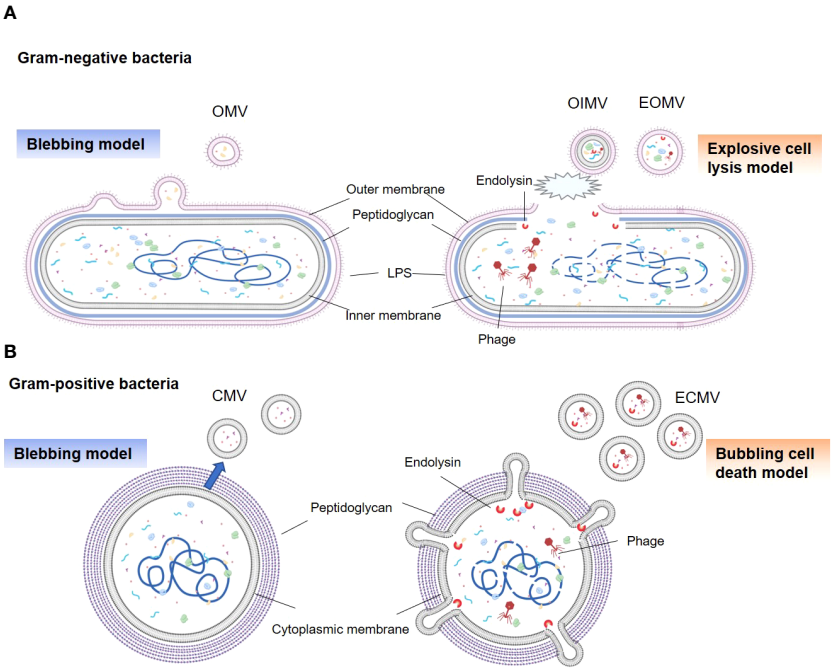

FIGURE 1
Types and generation models of BEVs. **(A)** Gram-negative bacteria can release OMVs by blebbing of the outer membrane (left panel). Vesicles produced by explosive cell lysis are named explosive outer membrane vesicles (EOMVs) and outer-inner membrane vesicles (OIMVs), which are triggered by phage-derived endolysin that degrades the peptidoglycan layer (right panel). EOMVs and OIMVs randomly contain cytoplasmic components, while OMVs don't directly package cytoplasmic components. **(B)** Gram-positive bacteria can secret cytoplasmic membrane vesicles (CMVs) (left panel). Stress induced Gram-positive bacteria lysis, named "bubbling cell death", can lead to the release of ECMV (right panel).

encased in a lipid bilayer derived from the cytoplasmic membrane of the parent bacteria and exhibit a comparable size range (Figure 1B). The precise process underlying CMVs biogenesis remains elusive; however, a series of pivotal steps have been identified (26–28). First is cytoplasmic membrane budding, prompted by the accumulation of specific phospholipids in the outer leaflet of the membrane (26). Next is the formation and release of CMVs from the plasma; this is influenced by lipoprotein content reduction, which increases membrane fluidity, and accumulation of phenol-soluble modulins, which disrupt membranes due to their surfactant-like properties and amphipathic helical structure (26, 29). The final step is the passage of CMV through the cell wall. This process is facilitated by peptidoglycan-degrading enzymes (27, 29). In addition, explosive CMVs (ECMVs) can be formed in gram-positive bacteria via "bubbling cell death", which is similar to EOMV biogenesis (13). In this process, the release of CMVs under SOS response-inducing conditions is facilitated via prophage-derived endolysins (Figure 1B) (13, 30). However, the comprehensive elucidation of CMVs biogenesis in gram-positive bacteria remains unclear.

In addition, evidence suggests that BEV generation is accurately regulated. Recent studies on *Salmonella enterica* have indicated that the production of OMVs is upregulated by its PhoPQ system when attacked by host innate immunity (31). Antibiotic-induced oxidative stress in *S. aureus* triggers CMV production via increasing permeability of the peptidoglycan layer. Genetic regulation of vesiculation has also been investigated, with disruptions in gene encoding factor σ B (sigB) in *Listeria monocytogenes* (*L. monocytogenes*) (32) or the two-component system CovRS in *Streptococcus pyogenes* (33) resulting in altered CMVs production, which indicates a regulatory role in vesicle biogenesis. Furthermore, the cargos contained in OMVs are rigorously controlled (34, 35). The lipoprotein composition between the outer membrane of *Bacteroides thetaiotaomicron* and its OMVs were found to be significantly different (36). Moreover, a study showed that the exposure of *Pseudomonas aeruginosa* (*P. aeruginosa*) to the epoxide epibromohydrin resulted in the significant upregulation of the epoxide hydrolase (Cif) and outer membrane protein OprF in its OMVs (37).

Role of BEVs in gut homeostasis

Gut homeostasis is fundamentally reliant on an intact barrier function composed of microbial, chemical, physical, and immune barriers that work together to form a defense line from the lumen to the basal layer (38–41). BEVs, which are products of gut commensal bacteria (42), serve as key messengers and regulators in this environment. They facilitate a range of interactions with the gut barrier that contribute to the maintenance of gut health (Figure 2).

Microbial barrier

The healthy gut microbiota is referred to as the microbial barrier, and comprises various species of commensal intestinal bacteria (43). These species either compete or cooperate to establish a balanced microbial community (43), which is critical for resisting the colonization, growth, and invasion of pathogenic microorganisms (44). BEVs can modulate the equilibrium of the gut microbiota in several ways (Figure 2B). First, BEVs promote the survival of their parent bacterium or other bacteria. For instance, *P. aeruginosa* OMVs carry many *Pseudomonas* quinolone signals, which can bind iron, an essential element for bacterial viability, and bring it to the outer membrane of the parent bacterium via fusion (18). Similarly, OMVs from *Akkermansia muciniphila* (*A. muciniphila*) can restore the disturbed balance of gut microbiota via selectively promoting the proliferation of beneficial bacteria through membrane fusion (45). Second, bacteria release BEVs as a defense mechanism against phage infections. For example, Manning et al. reported that the co-incubation of OMVs collected from *Escherichia coli* (*E. coli*) and T4 bacteriophages resulted in a significant reduction in the active phage number (46). Similarly, Reyes-Robles et al. found that *Vibrio cholerae* (*V. cholerae*) secreted OMVs carrying phage receptors as a defense mechanism that conferred protection against phage predation (47). Finally, BEVs function as tools to eliminate other bacteria. Li et al. reported that OMVs from 15 strains of gram-negative bacteria, including many commensal or pathogenic gut bacteria, such as *Enterobacter*, *Escherichia*, *Morganella*, *Salmonella*, and *Shigella* strains, could lyse many gram-positive and gram-negative cultures. Peptidoglycan hydrolases associated with BEVs are thought to account for bacterial lysis (48). Growing evidence has supported the antimicrobial functions of BEVs. For instance, OMVs from *P. aeruginosa* can kill competitor species such as *S. aureus* via peptidoglycan hydrolases, antimicrobial 4-hydroxy-3-methyl-2-(2-non-enyl)-quinoline, and rhamnolipid (49). OMVs from *Lysobacter* and *Myxococcus* contain a toxic mixture of bioactive compounds and lytic enzymes capable of killing the surrounding microbes (50).

Chemical barrier

The intestinal chemical barrier is composed of AMPs and other antibacterial substances, such as bile acids (41), and inhibits growth of certain bacteria and segregates intestinal bacteria from intestinal epithelial cells. Several studies have suggested that BEVs can disrupt the function of the chemical barrier (Figure 2C). Nakayama-Imaohji et al. reported that a *Bacteroides fragilis* (*B. fragilis*) strain with hypervesiculating mutants (which release more OMVs) showed higher resistance to treatment with AMPs, such as LL-37 and defensin-2 (51). Similarly, Urashima et al. found that the outer membrane protein T, which was specifically enriched in the OMVs of enterohemorrhagic *E. coli* (EHEC), broke down LL-37 and inhibited its antimicrobial activity, thereby enhancing EHEC survival and adaptation to the host gut environment (52). Moreover, the exposure of *P. aeruginosa* to lysozyme significantly enhanced OMVs release (by approximately 100-fold) (53). Analogously, *in vitro* studies have shown that *E. coli* upregulates OMVs secretion upon encountering AMPs, and the addition of *E. coli* OMVs has been demonstrated to increase bacterial survival *in vitro* when challenged with antibiotics, such as Polymyxin B and

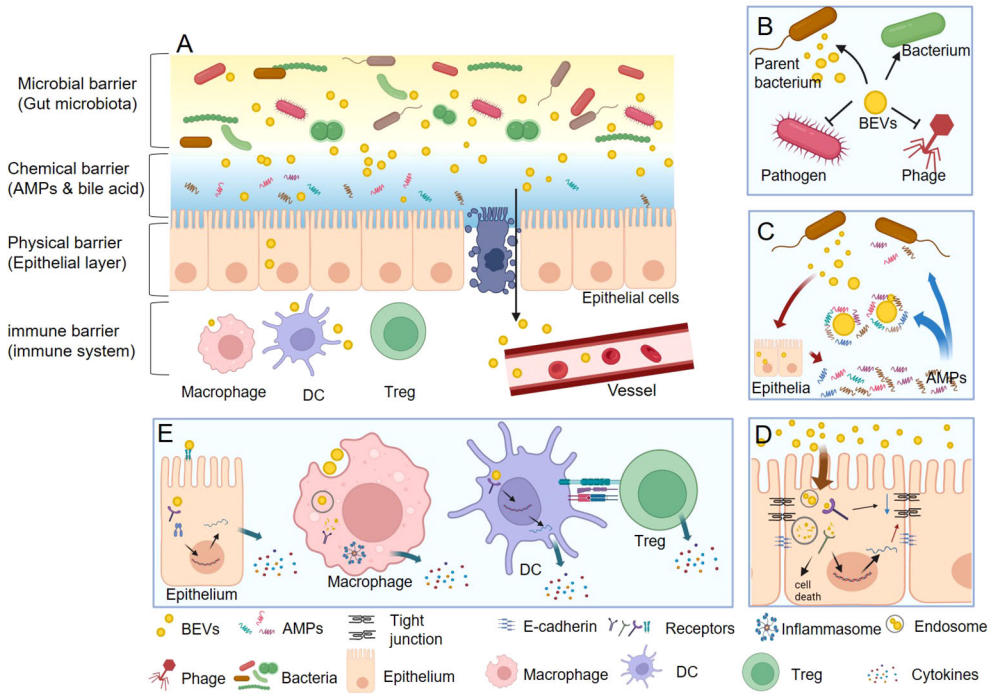

FIGURE 2
The functions of BEVs in gut homeostasis. **(A)** BEVs regulate gut health by interacting with microbial, chemical, physical, and immune barriers. **(B)** In microbial barrier, BEVs can promote the survival of their parent bacterium or other bacteria, protect against phage infection, and kill competitor species. **(C)** In chemical barrier, BEVs can neutralize the function of AMPs. Nevertheless, some BEVs also act as stimulators that induce the intestine to express more AMPs thus enhance the chemical barrier. **(D)** In physical barrier, BEVs can damage the integrality of epithelial barrier via reducing the tight junction protein and E-cadherin, or causing epithelial cell death. On the contrary, the BEVs from some beneficial bacteria could enhance the physical barrier function. **(E)** In immune barrier, BEVs could stimulate epithelial cell to secret cytokines through both cell surface receptors (such as TLR4) and inter intracellular receptors (such as NOD1). Macrophage can directly recognize and uptake BEVs and then activate inflammasome and secret cytokines. DCs can detect the polysaccharide (PSA) from OMV then result to promote the differentiation Tregs and the anti-inflammatory cytokine IL-10.

colistin (54). All in all, BEVs can digest or neutralize AMPs, potentially weakening the chemical barrier function.

Contrarily, some evidences indicated that BEVs act as stimulators, inducing the intestine to increase AMP expression, thereby enhancing the chemical barrier (Figure 2C). For instance, Kaparakis et al. discovered that OMVs from *P. aeruginosa* and *Helicobacter pylori* (*H. pylori*), which contained peptidoglycans, could induce epithelial cells to express human β-defensins (HBD), such as HBD2 and HBD3 (55). *Lactobacillus* derived CMVs have also been reported to stimulate the expression of the AMP REG3G, a c-type lectin, thus promoting the chemical barrier of the gastrointestinal tract and providing protection against pathogens (56). OMVs released from *A. muciniphila* were recently reported to stimulate goblet cells to produce mucus (45), which resisted the adhesion and stimulation of pathogenic bacteria to gut epithelial cells. These findings suggest that some BEVs can stimulate intestinal cells, leading to increased AMP and mucus production, thereby enhancing the chemical barrier.

Physical barrier

The intestinal epithelial barrier, a physical partition separating the body's internal environment from the lumen, is composed of a single layer of epithelial cells interconnected via tight junction proteins, such as occludin, claudins, and zonula occludens (39, 57). Although this physical barrier effectively limits the intrusion of most harmful substances, BEVs have been shown to internalize or permeate it (58). BEVs penetrate non-phagocytic host cells via five primary mechanisms: clathrin-mediated endocytosis, caveolin-mediated endocytosis, lipid raft-mediated endocytosis, macropinocytosis, and membrane fusion (17, 59). BEVs can concurrently utilize one or more pathways to infiltrate host cells, depending on their size and components. For example, OMVs derived from *H. pylori* were found to enter epithelial cells via four different mechanisms (55, 60, 61).

Once internalized, BEVs traverse the endolysosomal pathway and are subsequently degraded in lysosomes or autophagosomes (62); however, recent studies suggest that some BEVs can escape degradation and deliver their cargos into cells. Bielaszewska et al. demonstrated that after the OMVs of EHEC O157 were internalized in early endosomes through a process reliant on dynamin-dependent endocytosis, virulence factors, including Shiga toxin 2a (Stx2a), cytolethal distending toxin V (CdtV), and EHEC hemolysin, were separately transported from the vesicles via intracellular trafficking (63). Although the precise mechanisms of BEVs internalization and cargo transport remain unclear, BEVs are surmised act as a significant cargo delivery system to intestinal

epithelial cells, influencing the function and integrity of the physical barrier of the gut.

Evidence suggests that some gut pathogenic bacteria can damage the intestinal barrier via BEVs (Figure 2D). Upon internalization in human intestinal epithelial cells, gram-negative bacterial OMVs release lipopolysaccharides (LPS) into the cytosol (64), facilitated by sorting nexin 10 (SNX10), which activates caspase-5. This leads to Lyn phosphorylation, subsequently down-regulating E-cadherin expression and impairing the intestinal barrier (64). EHEC O157 OMVs can disrupt the barrier through two pathways: the release of hemolysin, which increases mitochondrial permeability and triggers apoptosis (65), and the discharge of CdtV-B, which causes DNA damage and induces apoptosis (66). Moreover, OMVs of the pathogen *Fusobacterium nucleatum* (*F. nucleatum*) can activate the FADD-RIPK1-cCASP-3 signaling pathway, decreasing ZO-1 protein and increasing apoptosis, thereby damaging the epithelial barrier (67). Similarly, *Campylobacter jejuni* releases OMVs containing toxins that harm cellular DNA and impair the intestinal barrier (68–70). *V. cholerae* OMVs carry active proteases that induce apoptosis or necrosis, causing epithelial barrier loss during infection (71). Finally, Enterotoxigenic *B. fragilis* releases OMVs along with *B. fragilis* toxin, which disrupt the intestinal barrier via cleaving E-cadherin and affecting the zonula adherens and tight junctions in the intestinal epithelium (72).

Despite their disruptive potential, BEVs do not always impair the intestinal epithelial barrier (Figure 2D). The probiotic *E. coli* Nissle 1917 and commensal ECOR63 enhance barrier function via increasing tight junction protein expression (73, 74). Furthermore, OMVs produced by *E. coli* C25, a commensal bacterium, trigger a moderate release of the proinflammatory interleukin 8 (IL-8) and stimulate the transcriptional upregulation of Toll-like receptors (TLRs) in intestinal epithelial cell lines, subsequently enhancing the barrier function of epithelial cells and inhibiting bacterial internalization (75). Similarly, OMVs released from *A. muciniphila* help maintain the integrity of the intestinal barrier via penetrating the intestinal epithelial cells and boosting the expression of tight junction proteins and mucus (45, 76).

Immune barrier

The gut immune barrier, primarily comprising immune cells including macrophages, dendritic cells (DCs), lymphocytes, mast cells, and natural killer cells, resides in the lamina propria or Peyer's patch, situated beneath the physical barrier (77). They can gather information from the intestinal epithelial cells which produce a range of immunoregulatory signals (78). Furthermore, they directly recognize and accept certain bacterial components that permeate this barrier (79).

BEVs can stimulate intestinal epithelial cells to secrete various cytokines and chemokines that play pivotal roles in modulating intestinal immune functions (Figure 2E). For instance, *F. nucleatum* releases OMVs that stimulate epithelial cells, thereby increasing the activation of p-ERK, p-CREB, and NF-κB signaling pathways. This activation subsequently upregulates proinflammatory cytokines, including tumor necrosis factor, keratinocyte chemoattractant, IL-6, interferon (IFN)-γ, and monocyte chemoattractant protein (MCP)-1 (55). Thapa et al. analyzed the effect of BEVs derived from 32 different gut bacteria (26 gram-negative and six gram-positive bacteria) on intestinal epithelial cells. Their findings revealed that BEVs could induce species-specific immune responses in these cells. OMVs from gram-negative bacteria were found to trigger a stronger proinflammatory response than CMVs from gram-positive bacteria. A large proportion of the BEVs induced a significant increase in CCL20, IL-8, and CXCL1 levels in epithelial cell lines. Their research also identified LPS as the dominant proinflammatory bacterial effector that activated the caspase- and RIPK2-dependent pathways (80). OMVs can stimulate immune responses via cell surface and intracellular receptors in epithelial cells. For example, EHEC O157 OMVs induce IL-8 production in human intestinal epithelial cells via stimulating TLR4 and TLR5 (cell surface receptors), thus activating the nuclear factor NF-κB (81). In addition, these OMVs can deliver peptidoglycan into the host cell cytosol, thereby inducing innate immune responses through a NOD1 (intracellular receptor)/NF-κB dependent, but TLR-independent, mechanism (55, 59).

BEVs can also directly engage with intestinal immune cells (59), particularly macrophages, which play vital roles in the immune barrier (Figure 2E). Research shows that macrophages can uptake gram-negative OMVs via clathrin-mediated endocytosis. LPS from these OMVs can escape from early endosomes into the cytosol, triggering the caspase-11-dependent release of IL-1β and cell death in a dose-dependent manner (82). Previous studies found that guanylate-binding proteins recognized LPS, bound to the OMV surface, and mediated activation of the caspase-11 non-canonical inflammasome (83). Similarly, Bitto et al. reported that OMVs from *P. aeruginosa* directly activated the inflammasome in macrophages (84), which were dependent on caspase-5, a human homolog of murine caspase-11, highlighting another pathway of activation of immune responses in mice and humans via OMVs (83). Moreover, gram-positive bacteria can also initiate an immune response in macrophages via signaling pathways that differ significantly from those used by OMVs. Wang et al. discovered that *S. aureus* released CMVs that interacted with TLR2, thereby activating the NLRP3 inflammasome via potassium efflux. This led to the recruitment of apoptosis-associated speck-like protein containing a caspase recruitment domain and caspase-1 activation, resulting in the cellular release of mature cytokines IL-1β and IL-18 and the induction of pyroptosis (85). Conversely, CMVs from *Pediococcus pentosaceus* demonstrated potent anti-inflammatory properties. These CMVs facilitate the differentiation of bone marrow precursors into myeloid-derived suppressor-like cells and promote M2 macrophage polarization *in vitro* and *in vivo* (85, 86).

DCs are phagocytes and antigen-presenting cells that regulate the activation of adaptive immune responses, particularly T-helper and regulatory T (Tregs) cells (Figure 2E) (87). Shen et al. and Chu et al. demonstrated that DCs could detect polysaccharide found in *B. fragilis* OMVs via TLR2, which then activated growth arrest and DNA damage-inducible protein (Gadd45a) signaling, resulting in increased proliferation of Tregs and secretion of the anti-

inflammatory cytokine IL-10 (88). This immune response process protected mice from severe experimental colitis (89). However, deficiencies in ATG16L1 or NOD2, two genes associated with IBD, disrupt DC-Treg cell interactions, thereby obstructing the protective function of *B. fragilis* OMVs (89). Additionally, *E. coli* OMVs induce DCs to generate T-helper cell responses in a strain-specific manner. Non-pathogenic *E. coli* strains, *E. coli* Nissle 1917 (probiotic) and ECOR63 (commensal), trigger increased secretion of Th1 polarizing cytokines (IFN-γ and IL-12) from DCs. Conversely, OMVs from ECOR12 (commensal) or ECOR53 (pathogenic) stimulate the production of higher levels of Treg-related cytokines (IL-10 and TGF-β). Despite the differences between strains, all OMVs enhance the secretion of Th17/Th22 priming cytokines (IL-6, IL-23, tumor necrosis factor-α, and IL-1β) (58).

What's more, BEVs can access Peyer's patches and then directly interact and activate the immune cells. Wang et al. found *A. muciniphila* OMVs are able to enter Peyer's patches after direct delivery into the intestinal lumen, and induce higher production of immune active DCs with CD80 expression (45). Consequently, with the help of activated DCs, the productions of $CD69^+$ B cells and IgA^+ plasma cells along with total B cells are significantly augmented, thereby increasing intestinal IgA concentration (45, 90). This process is believed to reduce the relative abundance of harmful pathogens in the gut microbiota.

In conclusion, the influence of BEVs on intestinal barrier regulation is complex, with some enhancing barrier function, and others contributing to its impairment. This highlights the intricate interplay between the gut microbiota and their multifaceted effects on human health and diseases.

BEVs and gut related diseases

Considering the significant roles of BEVs in maintaining gut homeostasis, investigation of their possible involvement in the onset and progression of gut-related diseases is appropriate. Current research indicates a key role of BEVs in various diseases related to the gut. These conditions include infections, IBD, metabolic disorders, and cancer (Table 1). BEVs, with their diverse biological functions and intricate interactions with host cells, may be pivotal in the pathogenesis of these conditions. Details of the specific roles of BEVs in each of these disease categories are discussed in the following sections.

Infections

Numerous studies have highlighted the role of gut bacteria in exploiting BEVs to infect and harm hosts. BEVs can neutralize AMP activation, potentially undermining the effectiveness of the host chemical barriers and enhancing their susceptibility to pathogenic infections (53, 54). Furthermore, BEVs can foster the formation of biofilms and complex microbial communities, which are implicated in gastrointestinal infections and other diseases (22, 104, 105). A significant proportion of biofilm matrix-associated proteins originate from BEVs such as OMVs produced by *P. aeruginosa* (91). Several intestinal pathogens employ BEVs as vehicles for delivering toxins to gut cells during infection. *V. cholerae*, a noninvasive gram-negative pathogen, causes cholera via colonizing the small intestine and releasing a potent enterotoxin called cholera toxin (CT). Chatterjee et al. discovered that *V. cholerae* OMVs carried copious amounts of CT and could be internalized by intestinal epithelial cells, subsequently increasing cyclic adenosine monophosphate levels in a ganglioside GM1 (CT receptor)-dependent manner (92). Similarly, *L. monocytogenes*, a gram-positive intracellular pathogen, utilizes CMVs to release toxins, including listeriolysin O and phosphatidylinositol-specific phospholipase C, which cause mammalian cytotoxicity (93).

However, not all BEV effects on infections are harmful. Evidence suggests that BEVs can bolster gut defense against bacterial infections. During these infections, OMVs deliver LPS to macrophages, inducing a caspase-11-mediated inflammatory reaction that aids the host in pathogen clearance (82). Patten et al. observed that pre-incubation of intestinal epithelial cells with *E. coli* C25-derived OMVs impeded the internalization of the parent bacterium. They suggested that this was due to the mild proinflammatory response induced by OMVs in epithelial cells, which enhanced their ability to combat infection (75).

Moreover, BEVs are critical mediators in immune training and fortifying antiviral defenses. Bhar et al. found that co-inoculation of BEVs with murine norovirus led to the enhanced production and release of proinflammatory cytokines in macrophages, suggesting the potential role of BEVs in promoting an antiviral response (94). Erttmann et al. reported that gut microbiota depletion lowered systemic tonic IFN-I levels and antiviral priming, rendering the mice more susceptible to systemic viral infections. They found that the gut microbiota released DNA-containing BEVs that could permeate the intestinal barrier and circulate in the blood, delivering foreign DNA to distal host cells, thereby activating the cGAS-STING-IFN-I-dependent pathway to protect against RNA viruses (95). Additionally, Frantz et al. identified a specific small RNA (sRNA), rli32, partially derived from *L. monocytogenes* CMVs, that could infiltrate mammalian cell lines and increase IFN-I expression in a RIG-I-dependent manner (96).

IBD

Numerous studies have suggested that gut barrier dysfunction can exacerbate IBD progression. Current research indicates that BEVs contribute to IBD via damaging both physical and immune barriers, particularly the epithelial and immune cells (Figure 3A). The increased proportion of gram-negative bacteria observed in patients with IBD typically release excessive OMVs laden with LPS (64). These OMVs infiltrate epithelial cells and their LPS translocate into the cytosol, instigating immune reactions, downregulating E-cadherin expression, and causing intestinal barrier dysfunction (64). Tulkens et al. clinically investigated and revealed a significant correlation between the levels of BEV-associated LPS in the plasma and impaired barrier integrity in patients with intestinal mucositis, including IBD (20, 106). As a result, bacterial

TABLE 1 The function of BEVs in gut related disease.

Disease	Beneficial or harmful	Parent bacteria	Effective component	Target barrier layer	Influence	Reference
Gut infections	Harmful	Enterohemorrhagic *E. coli*	OmpT protease	Chemical barrier	Breaking down gut AMPs	(52)
		P. aeruginosa	Phospholipid bilayer	Chemical barrier	Absorbing and neutralizing AMPs	(53)
		E. coli	Phospholipid bilayer	Chemical barrier	Absorbing and neutralizing AMPs	(54)
		P. aeruginosa	Biofilm matrix-associated proteins	Physical barrier	Helping bacteria cope with stressful host environments by facilitating biofilm formation	(91)
		V. cholerae	Cholera toxin	Physical barrier	Delivering cholera toxin to epithelial cell and up-regulating cAMP	(92)
		L. monocytogenes	LLO and PI-PLC	Physical, immune barrier	Delivering a concentrated and varied toxin cargo to host cells.	(93)
	Beneficial	Gram-negative bacteria	LPS	Immune barrier	Eliciting caspase-11-mediated inflammatory reaction and helping the host to promote pathogens clearance	(82)
		E. coli C25	Unidentified	Physical barrier	Inhibiting the internalization of the parent bacterium	(75)
		Universal gut bacteria	Unidentified	Immune barrier	Stimulating immune cell to release of pro-inflammatory cytokines and promote an antiviral response	(94)
		Universal gut bacteria	DNA	Immune barrier	Activating the cGAS-STING-IFN-I dependent pathway to protect against RNA virus	(95)
		L. monocytogenes	sRNAs rli32	Immune barrier	Increase type I IFN expression in RIG-I-dependent manner	(96)
IBD	Harmful	Gram-negative bacteria	LPS	Physical barrier	Down-regulating E-cadherin expression	(64)
		F. nucleatum	Unidentified	Physical barrier	Activating RIPK1 and RIPK3 inducing epithelial necroptosis	(67)
				Immune barrier	Activating TLR4 and promoting pro-inflammatory cytokine production and leading to increased immune cell infiltration	(67)
	Beneficial	*E. coli* Nissle 1917	Unidentified	Physical barrier	Up-regulating tight junction proteins ZO-1, ZO-2, and claudin-14	(73)
		B. fragilis	Polysaccharide	Immune barrier	Interacting with DC cells and causeing immune tolerance	(88)
		A. muciniphila	Unidentified	Microbial, physical, and immune barrier	Restoring disturbed balance of the gut microbiota, maintaining the integrity of the intestinal barrier, activating B cells and DCs	(45)
Metabolism disease	Harmful	*P. panacis*	Unidentified	–	Blocking the insulin signaling pathway	(97)
	Beneficial	*A. muciniphila*	Unidentified	Physical barrier	Ameliorateing HFD-induced intestinal barrier dysfunction	(98)
Gastrointestinal Cancer	Harmful	*E. coli* MG1655	Ile-tRF-5X	–	Promoting the expression of the MAK3K4 gene, enhancing cell proliferation	(99)
		F. nucleatum	Unidentified	Physical and immune barrier	Inducing IL-8 expression and reducing E-cadherin and cadherin-1 gene expression	(100–102)

(Continued)

TABLE 1 Continued

Disease	Beneficial or harmful	Parent bacteria	Effective component	Target barrier layer	Influence	Reference
	Beneficial	Gram-negative bacteria	Unidentified	–	OMVs specifically targeted and accumulated in tumor tissues of syngeneic mouse colonic tumor model, subsequently triggering the production of antitumor cytokines	(103)
		A. muciniphila	Unidentified	Immune barrier	Enhance PD-1–based immunotherapy of CRC in a mouse model	(45)

BEVs significantly stimulate peripheral blood mononuclear cells to secrete proinflammatory cytokines such as IL-6, IL-8, MCP-1, and macrophage inflammatory protein-1α. Specific OMVs released by gut pathogens are also associated with IBD (20). Liu et al. reported that *F. nucleatum* OMVs significantly exacerbated dextran sulfate sodium (DSS)-induced colitis symptoms in mice via activating receptor-interacting protein kinases 1 and 3 and inducing epithelial necroptosis. This process resulted in significant epithelial barrier loss and oxidative stress-related damage (67). Engevik et al. supported this result and, in addition, they found that *F. nucleatum* OMVs also activated TLR4 and downstream targets signal-regulated kinase, cAMP response element binding Protein, and NF-κB, thereby promoting proinflammatory cytokine production and leading to increased immune cell infiltration (107).

Conversely, several studies have reported the protective role of BEVs against IBD (Figure 3B). The probiotic *E. coli* Nissle 1917 enhances gut physical barrier integrity via upregulating the tight junction proteins ZO-1, ZO-2, and claudin-14, thereby attenuating DSS colitis in mice (73). The commensal bacterium *B. fragilis* secretes OMVs that interact with DCs, triggering immune tolerance and thereby protecting animals from 2,4,6-trinitrobenzenesulfonic acid solution-induced colitis and intestinal inflammation (88). *A. muciniphila* OMVs are reported to ameliorate DSS-induced colitis using several mechanisms, including restoring the disturbed balance of the gut microbiota, maintaining the integrity of the intestinal barrier, and activating B cells and DCs (45). The IBD-associated genes ATG16L1 and NOD2 are crucial for OMV-mediated activation of colitis protection. ATG16L1 T300A transgenic mice did not exhibit protection from 2,4-dinitrobenzene sulfonic acid-induced colitis. Individuals with Crohn's disease, a subtype of IBD, typically carry the ATG16L1 major risk variant T300A. This finding suggests a potential target for the early genetic diagnosis of IBD (89).

Metabolic diseases

The balance of gut microbiota significantly influences host metabolic homeostasis, and BEVs are crucial in this process. A significant increase in OMVs from *Pseudomonas panacis* was

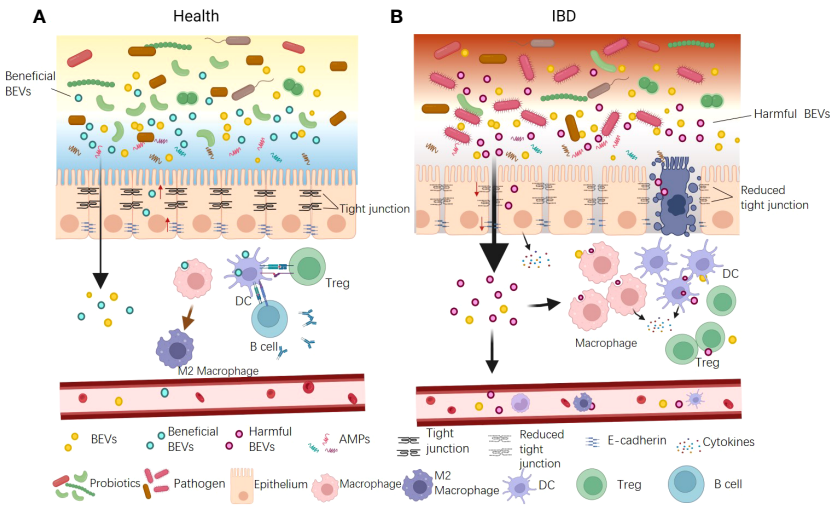

FIGURE 3
The dual functions of BEVs in IBD pathogenesis. **(A)** Some beneficial OMVs play roles in inhibiting colitis. The mechanisms include: ① enhancing gut physical barrier integrity by up-regulating tight junction proteins; ② interacting with DCs and increasing production of Tregs and the anti-inflammatory cytokine IL-10; ③ promoting anti-inflammation M2 macrophage polarization. ④ activating B cells to product mucosal immunoglobulin A. **(B)** The BEVs from pathogens can promote IBD. The mechanisms include: ① causing intestinal barrier dysfunction by down-regulating tight junction proteins and E-cadherin and inducing epithelial cell death; ② promoting pro-inflammatory cytokine production and leading to increased immune cell infiltration. In addition, BEVs could enter the blood system and stimulate peripheral blood mononuclear cells to aggravate systemic immune activation.

observed in a high-fat diet-induced type 2 diabetes mouse model. Subsequent studies confirmed that these OMVs could block the insulin signaling pathway in skeletal muscles and adipose tissues (97). Conversely, *A. muciniphila*-derived OMVs ameliorate high-fat diet-induced obesity via various mechanisms, which include improved intestinal barrier integrity, reduced inflammation, balanced energy, and improved blood parameters (98). This contrasting effect of different BEVs on metabolic homeostasis emphasizes the complex and multifaceted roles of these entities in maintaining host health. It is thought that the characteristics of the parent bacteria determine whether their BEVs are harmful or beneficial, and normal quantity of BEVs could maintain immunological activity while excess amounts would be harmful.

Gastrointestinal cancer

Numerous reports have highlighted the influence of BEVs on cancer development and metastasis in the gastrointestinal tract. OMVs from *E. coli* MG1655 have been shown to deliver a tRNA fragment termed as Ile-tRF-5X, into human colorectal carcinoma cells (HCT116). This interaction promotes the expression of mitogen-activated protein kinase 3, thereby enhancing cell proliferation (99). *F. nucleatum*, widely recognized as a pathogen that promotes colorectal cancer (CRC) development, utilizes various mechanisms for this process, including OMVs. Proteomic analysis using mass spectrometry revealed an abundance of virulence factors and biologically active proteases present or selectively enriched in these OMVs (100). The specific roles of OMVs in CRC include inducing IL-8 expression (100, 101), which fosters a pro-inflammatory microenvironment favoring tumor growth; and reducing E-cadherin and cadherin-1 gene expression to promote an epithelial-to-mesenchymal transition-like genotype in tumor cells (100, 102), which ultimately promotes the migration and invasion of cancer cells *in vivo* (108).

Conversely studies have explored the potential of BEVs as therapeutic agents for cancer treatment via immunotherapy. Kim et al. found that gram-negative bacterial OMVs specifically targeted and accumulated in tumor tissues of a syngeneic mouse colonic tumor model, subsequently triggering the production of antitumor cytokines CXCL10 and IFN-γ. This indicates that BEVs represent a promising new approach for cancer immunotherapy (103). Additionally, *A. muciniphila* OMVs have been found to enhance programmed cell death protein-1-based immunotherapy of CRC in mouse models. This suggests a potential clinical application of OMVs in improving the efficacy of immunotherapy by targeting programmed cell death protein-1 (45). These varied findings demonstrate the significant and multifaceted roles of BEVs in gastrointestinal cancer progression and potential therapeutic strategies.

Current challenges and future perspectives

Despite substantial evidence supporting the process of BEVs generation are positive controlled, the regulation of BEVs production and cargo selection remains unclear. Further research is required to elucidate these mechanisms, which will significantly facilitate basic research on BEVs functions in bacteria-bacteria and bacteria-host communication and its translational application. Moreover, the dual role of BEVs in gut health and the pathogenesis of intestinal-related diseases remains unclear. Furthermore, the precise active components of BEVs, their receptors, and the induced signaling pathways in host cells remain unidentified. The investigation of their impact on other intestinal cell types, such as intestinal stromal and neuronal cells is also required.

Considering their ability to penetrate the intestinal barrier and their correlation with various gut diseases, BEVs are potential diagnostic biomarkers for intestinal disorders (106). Their capacity to regulate host immune responses indicates their potential as vaccines against intestinal infections and inflammatory disorders. Preliminary studies have suggested that some BEVs can induce an antitumor immune response and inhibit tumor growth, suggesting their role in cancer immunotherapy (45, 103). Further studies are required to validate these findings and translate them into clinical applications.

Despite the encouraging findings on BEVs, this field of research still remains largely unexplored, and requires more comprehensive investigations a deeper understanding of BEV biogenesis, cargo selection, and their interaction mechanisms with host cells is crucial. With this knowledge, the full potential of BEVs in diagnostics, therapeutics, and vaccine development can be harnessed, thereby opening new frontiers for microbiome-related biomedical applications.

Author contributions

DS: Conceptualization, Data curation, Funding Acquisition, Writing – original draft, Writing – review & editing. PC: Data curation, Software, Visualization, Writing – original draft. YX: Data curation, Funding acquisition, Writing – review & editing. JS: Conceptualization, Data curation, Funding acquisition, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Genome-wide Mendelian randomization identifies putatively causal gut microbiota for multiple peptic ulcer diseases

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Objective: The pathogenesis of peptic ulcer diseases (PUDs) involves multiple factors, and the contribution of gut microbiota to this process remains unclear. While previous studies have associated gut microbiota with peptic ulcers, the precise nature of the relationship, whether causal or influenced by biases, requires further elucidation.

Design: The largest meta-analysis of genome-wide association studies was conducted by the MiBioGen consortium, which provided the summary statistics of gut microbiota for implementation in the Mendelian randomization (MR) analysis. Summary statistics for five types of PUDs were compiled using the FinnGen Consortium R8 release data. Various statistical techniques, including inverse variance weighting (IVW), MR-Egger, weighted median (WM), weighted mode, and simple mode, were employed to assess the causal relationships between gut microbiota and these five PUDs.

Result: In the intestinal microbiome of 119 known genera, we found a total of 14 causal associations with various locations of PUDs and reported the potential pathogenic bacteria of *Bilophila* et al. Among them, four had causal relationships with esophageal ulcer, one with gastric ulcer, three with gastroduodenal ulcer, four with duodenal ulcer, and two with gastrojejunal ulcer.

Conclusion: In this study, the pathogenic bacterial genera in the gut microbiota that promote the occurrence of PUDs were found to be causally related. There are multiple correlations between intestinal flora and PUDs, overlapping PUDs have overlapping associated genera. The variance in ulcer-related bacterial genera across different locations underscores the potential influence of anatomical locations and physiological functions.

KEYWORDS

Mendelian randomization, gut microbiota, peptic ulcer, causal relationship, genus

1 Introduction

Peptic ulcer diseases (PUDs) represent a prevalent clinical condition characterized by multifactorial etiology and extremely complex pathogenesis, primarily related to *Helicobacter pylori* infection (1). The incidence of PUDs is common among individuals between the age of 25 and 64 years and increases with age. These ulcers are predominantly located near the stomach or duodenum but can also occur in the esophagus or Meckel's diverticulum (2). In the general population, the lifetime prevalence of PUDs is estimated to range between 5% and 10%, while the annual incidence rate ranges from 0.1% to 0.3% (3). The continued prevalence of peptic ulcers within the stomach and duodenum poses a significant threat to global public health. The diagnosis and treatment of PUDs remain a major healthcare problem with a significant disease burden (2).

The human gut harbors an intricate and diverse microbial community that plays a crucial role in both health and diseases (4, 5), for instance, digestion and absorption of substances, synthesis of essential vitamins such as B and K, catabolism of compounds *in vivo*, coordination of innate and cell-mediated immune responses, and maintenance of intestinal barrier function (6). The symbiotic relationship between these microbes and the host is indispensable for maintaining overall homeostasis; disruptions in this ecological equilibrium can lead to adverse health outcomes (7). The correlation between alterations in the gut microbiome and peptic ulcers has been studied for a long time. Several studies have demonstrated the mechanism underlying *H. pylori*-induced PUDs (8). At the same time, histological techniques have been utilized to examine the microbiome and metabolome of gastric biopsy tissues, identifying a distinct correlation between gastrointestinal ulcers and gastrointestinal bacteria (6). Moreover, PUDs were significantly associated with abnormal microbiota compositions in the oropharynx, esophagus, and gastrointestinal tract (9). Therapies to protect, adapt, shape, or restore the balance of the microbiome are critical aspects of the current and prospective approaches to gastrointestinal ulcer management (10). However, the causal relationship between PUDs at different anatomical sites and the gut microbiota remains unclear and requires further elucidation.

The genome-wide association study (GWAS) has gained widespread acceptance as a pivotal approach for exploring potential genetic variants linked to diverse and complex traits and diseases (11, 12). Mendelian randomization (MR) analysis introduces an innovative paradigm to explore the potential causal association between exposure and outcome independent of confounding factors and ethical considerations. Through MR, genetic variants are leveraged as instrumental variables (IVs) for exposure, enabling the estimation of causality between the exposure and the resultant outcome (13, 14). An MR study mimics a randomized controlled trial (RCT), as genetic variations are randomly assigned during fertilization (15). Furthermore, genotype formation occurs prior to disease onset and is typically unaffected by disease progression, reducing the likelihood of confounding influences.

Here, we employed MR analysis to investigate the correlation between gut microbiota and PUDs. We further explored the potential therapeutic implications of selective support or disorder of the gut microbiota.

2 Methods

2.1 Study design

The basic logic and analysis flow of the entire procedure were briefly described in Figure 1. The causal effects of the gut microbiota on five PUDs, including esophageal ulcer (OESU), gastric ulcer (GU), gastroduodenal ulcer (GASTRODU), duodenal ulcer (DU), and gastrojejunal ulcer (GJU), were evaluated. To comprehensively investigate the role of the gut microbiota in PUDs, MR analysis was performed at the classification of genera. The population information involved in the MR was detailed in Table 1.

2.2 Data sources

A two-sample MR study was undertaken to explore the potential relationship between genus-level gut microbiota and PUDs, utilizing GWAS summary data. Studies received prior approval from their respective institutional review boards (IRBs), and informed consent was obtained from all participants and/or their legal guardians.

To obtain GWAS summary statistics for the gut microbiota, data from the MiBioGen consortium, the largest GWAS dataset published to date, were utilized (16). This dataset consisted of 18,340 individuals spanning 24 population-based cohorts of diverse ancestry, including European, Middle Eastern, East Asian, American Hispanic/Latino, and American African. Microbial composition profiling and taxonomic classification were performed using direct taxonomic binning, targeting variable regions V4, V3–V4, and V1–V2 of the 16S rRNA gene. A microbiota quantitative trait loci (mbQTL) mapping analysis was employed to identify host genetic variants associated with the abundance of bacterial taxa within the gut microbiota. The genus with the least classification in the GWAS data for gut microbiota was selected for preprocessing. Out of 131 identified genera with an average abundance surpassing 1%, 119 genera were included for analysis, while 12 genera remain uncharacterized.

For the GWAS summary statistics of the five peptic ulcer types, data were obtained from the FinnGen consortium R8 release (https://www.finngen.fi/fi). The FinnGen consortium is a large public–private partnership aiming to collect and analyze genomic and health data from 500,000 Finnish biobank participants (12). The dataset available for analysis was up to December 2022. The peptic ulcer types included were OESU (NCase = 1,840, NControl = 292,256), GU (NCase = 5,277, NControl = 292,256), GASTRODU (NCase = 8,240, NControl = 292,256), DU (NCase = 3,164, NControl = 292,256), and GJU (NCase = 288, NControl = 292,256). The classification of OESU, GU, GASTRODU, DU, and GJU adhered strictly to the guidelines outlined by the International Classification of Diseases 10th Revision (ICD-10) code.

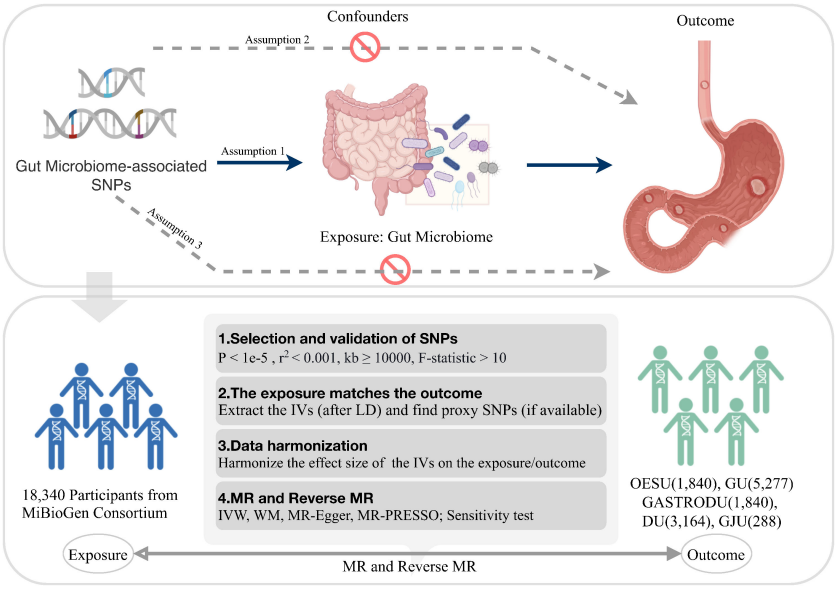

FIGURE 1
Overview of the study logic and workflow. SNP, single nucleotide polymorphism; LD, linkage disequilibrium; IVW, inverse variance weighted; WM, weighted median; MR-PRESSO, Mendelian Randomization Pleiotropy RESidual Sum and Outlier; MR, Mendelian randomization. Created with BioRender.com.

2.3 Instrumental variable selection

The process of IV selection from the GWAS summary statistics of the gut microbiome adhered to the following criteria: 1) Single-nucleotide polymorphisms (SNPs) associated with each genus at the locus-wide significance threshold (P < 1e–5) were considered as potential IVs (17); 2) Using reference panel data from the 1000 Genomes project European samples, linkage disequilibrium (LD) between SNPs was calculated. Among the SNPs with R^2 < 0.001 (clumping window size = 10,000 kb), only those with the lowest P-values were retained to minimize biased genetic variation arising from residual LD; 3) The intensity of each IV and exclusion of weak instruments were evaluated by calculating the F-statistic (F > 10); 4) In the presence of palindromic SNPs, the alleles on the forward strand were deduced by utilizing information on allele frequencies; 5) In cases where exposure-associated SNPs were absent in outcome data, suitable proxy SNPs (r^2 > 0.8) were identified and included in subsequent analyses; and 6) To address confounding, SNPs related to *H. pylori* infection, bile reflux, obesity, alcoholism, smoking, and stress factors were systematically removed during the MR analysis.

2.4 Statistical analysis

Five popular MR methods were utilized to analyze valid IVs: inverse variance-weighted (IVW) test, MR-Egger regression, weighted median, weighted mode, and simple mode (Figure 2). Among these, IVW was predominantly used due to its slightly higher power under certain conditions (18). The IVW method utilized the inverse of the outcome variance as weights for fitting, regardless of the presence of an intercept term in the regression. Complementary assessments were performed using the remaining four methods, each of which was based on different assumptions about potential pleiotropy. If the results obtained by these

TABLE 1 The population information involved in this Mendelian randomization.

Exposure/ Outcome	Ethnic origin	Sample size (case/ control)	Gender	Registry filter (ICD-10)	Public release	Data source
Gut microbiota (genus)	European, Hispanic, Middle Eastern, Asian and African	18,340	Mixed sex	—	2018	MiBioGen consortium
OESU	Europe	1,840/292,256	Mixed sex	K22.1	2022	FinnGen R8
GU	Europe	5,277/292,256	Mixed sex	K25	2022	FinnGen R8
GASTRODU	Europe	8,240/292,256	Mixed sex	K2[5-8]	2022	FinnGen R8
DU	Europe	3,164/292,256	Mixed sex	K26	2022	FinnGen R8
GJU	Europe	288/292,256	Mixed sex	K28	2022	FinnGen R8

OESU, esophageal ulcer; GU, gastric ulcer; GASTRODU, gastroduodenal ulcer; DU, duodenal ulcer; GJU, gastrojejunal ulcer; IVW, inverse variance weighted; SNP, single nucleotide polymorphism.

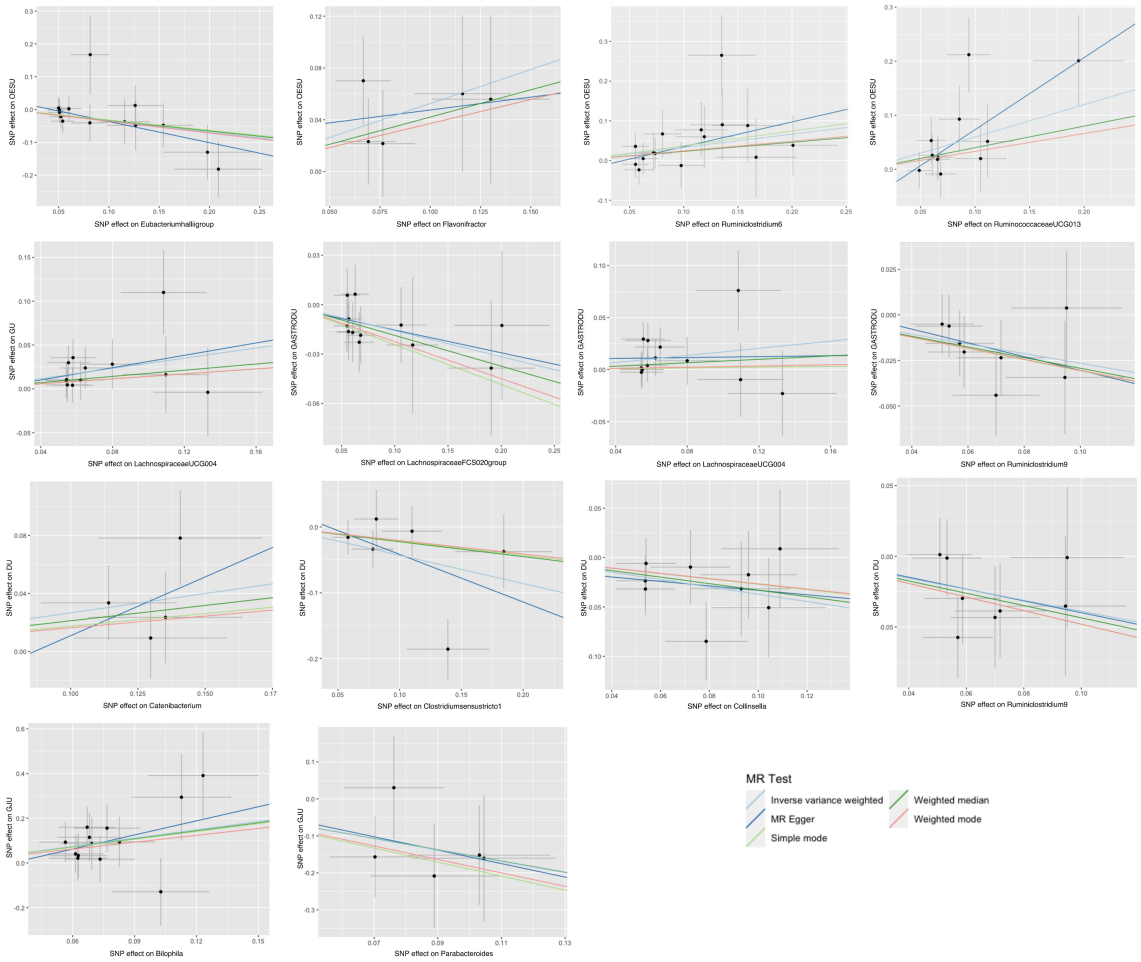

FIGURE 2
Scatter plots for the causal association between gut microbiota and peptic ulcer.

complementary methods are consistent with the IVW estimation results, the robustness of the effect estimation can be reinforced.

Multiple methods of sensitivity analyses were undertaken to ensure robustness. Initially, Cochran's Q statistics was applied to assess heterogeneity across diverse studies (19). Statistically significant Cochran's Q-test would indicate significant heterogeneity in the analytical outcomes. Secondly, MR-Pleiotropy Residual Sum and Outlier (MR-PRESSO) was used to detect instances of horizontal pleiotropy, with SNPs demonstrating horizontal pleiotropy outliers being systematically excluded to minimize pleiotropy-induced effects (20). In cases where significant horizontal pleiotropy was detected in the MR-PRESSO global test, outliers with $P < 0.05$ were removed, and the remaining SNPs were reanalyzed with the IVW analysis. Thirdly, the MR-Egger regression intercept was employed to estimate the potential pleiotropy of SNPs, with a P-value >0.05 indicating no horizontal pleiotropy (21). Fourthly, a leave-one-out analysis was performed to assess the impact of each SNP on the causal signal. Finally, funnel and forest plots were constructed to visually examine the presence of horizontal pleiotropy in the MR analysis, with $P < 0.05$ indicating potential causal associations. The statistical analyses were carried out using the R packages: two-sample MR (22) and MR-PRESSO (20).

3 Results

3.1 Genetic correlations between gut microbiota and PUDs

Initially, 1,698 SNPs were screened as possible IVs for 131 bacterial genera including 12 unidentified genera. The genetic variants were then eliminated based on specific criteria. All F-statistic exceeded 10, suggesting the absence of weak ins. Following validation through the PhenoScanner database, the remaining SNPs exhibited no discernible associations with *H. pylori* infection, bile reflux, obesity, alcoholism, smoking, and stress factors, indicating that IVs were not resolved by confounding factors. Concurrently, the elimination of palindromic SNPs was performed. GWAS data for patients with PUDs were derived from corresponding cohorts.

3.2 Bidirectional causal relationship of gut bacteria on PUD development

Our results demonstrated an association of four bacterial genera with OESU, one with GU, three with GASTRODU, four with DU, and two with GJU. Reverse MR analysis demonstrated that peptic ulcers did not change the abundance of the above bacteria. The leave-one-out sensitivity test highlighted some continuity around the midpoint. Crossing the zero line indicated that the result may be insignificant or unstable. The overall assessment indicated an absence of SNPs having a dominant impact. Furthermore, the selected SNPs exhibited no significant heterogeneity, as indicated by Cochran's Q statistics. Leave-one-out analysis did not identify a single SNP driving the association (Supplementary Figure S1). The application of MR-PRESSO yielded no outliers. The findings from the MR-Egger regression intercept analysis further corroborated the absence of significant directional horizontal pleiotropy (Table 2). The three main statistical results of the MR analysis were shown in Supplementary Table S1. Given the absence of significant statistical difference in reverse causality, relevant results were presented in Supplementary Table S2.

3.2.1 Causal relationship of gut bacteria on OESU

Notably, the two-sample MR analysis unveiled a causal linkage between *Eubacterium hallii* and OESU [IVW odds ratio (OR) = 0.71, 95% CI: 0.53–0.95, P = 0.024]. Furthermore, three bacterial features exhibited potential associations with an increased OESU risk: *Flavonifractor* (IVW OR = 1.69, 95% CI: 1.08–2.64, P = 0.020), *Ruminiclostridium 6* (IVW OR = 1.39, 95% CI: 1.03–1.88, P = 0.030), and *Ruminococcaceae UCG013* (IVW OR = 1.82, 95% CI: 1.27–2.61, P = 0.001).

3.2.2 Causal relationship of gut bacteria on GU

Our results underscored a robust causal relationship between *Lachnospiraceae UCG004* and GU (IVW OR = 1.34, 95% CI: 1.09–1.65, P = 0.006).

TABLE 2 MR estimates for the association between gut microbiota and PUDs.

Exposure	Outcome	SNP (n)	IVW		Cochran's Q (MR-Egger)		Pleiotropy_test			F-statistic (median)
			OR (95% CI)	P-value	Q	Q_pval	Egger intercept	Se	P-value	
Eubacterium hallii	OESU	14	0.71 (0.53-0.95)	0.024	5.935	0.92	0.028	0.026	0.30	21.10
Flavonifractor	OESU	5	1.69 (1.08-2.64)	0.020	1.349	0.72	0.028	0.074	0.73	21.93
Ruminiclostridium 6	OESU	15	1.39 (1.03-1.88)	0.030	8.677	0.80	-0.027	0.033	0.42	20.91
Ruminococcaceae UCG013	OESU	11	1.82 (1.27-2.61)	0.001	7.770	0.56	-0.060	0.040	0.17	21.52
Lachnospiraceae UCG004	GU	12	1.34 (1.09-1.65)	0.006	5.900	0.82	-0.004	0.028	0.89	21.26
Lachnospiraceae FCS020	GASTRODU	12	0.85 (0.73-0.99)	0.040	2.979	0.98	-0.002	0.015	0.92	21.66
Lachnospiraceae UCG004	GASTRODU	12	1.19 (1.00-1.40)	0.048	8.217	0.61	0.010	0.023	0.68	21.26
Ruminiclostridium 9	GASTRODU	8	0.77 (0.61-0.96)	0.019	2.762	0.84	0.007	0.035	0.84	21.46
Catenibacterium	DU	4	1.31 (1.05-1.63)	0.018	2.395	0.30	-0.070	0.199	0.76	21.28
Clostridium sensu stricto 1	DU	6	0.65 (0.42-1.00)	0.048	9.815	0.04	0.031	0.062	0.65	20.32
Collinsella	DU	9	0.69 (0.50-0.95)	0.024	3.549	0.83	-0.011	0.043	0.81	20.78
Ruminiclostridium 9	DU	8	0.68 (0.48-0.97)	0.031	3.555	0.74	0.002	0.055	0.97	21.46
Parabacteroides	GJU	5	0.22 (0.06-0.84)	0.027	1.590	0.66	0.025	0.374	0.95	21.55
Bilophila	GJU	13	3.45 (1.52-7.81)	0.003	7.230	0.78	-0.064	0.150	0.68	21.02

OESU, esophageal ulcer; GU, gastric ulcer; GASTRODU, gastroduodenal ulcer; DU, duodenal ulcer; GJU, gastrojejunal ulcer; IVW, inverse variance weighted; SNP, single nucleotide polymorphism.

3.2.3 Causal relationship of gut bacteria on GASTRODU

We found that *Lachnospiraceae UCG004* (IVW OR = 1.19, 95% CI: 1.00–1.40, P = 0.048) was associated with an increased GASTRODU risk. Moreover, *Lachnospiraceae FCS020* (IVW OR = 0.85, 95% CI: 0.73–0.99, P = 0.040) and *Ruminiclostridium 9* (IVW OR = 0.77, 95% CI: 0.61–0.96, P = 0.019) were associated with a lower GASTRODU risk.

3.2.4 Causal relationship of gut bacteria on DU

We found potential associations between one bacterial feature, *Catenibacterium* (IVW OR = 1.31, 95% CI: 1.05–1.63, P = 0.018), and increased DU risk. Meanwhile, three bacterial features, *Clostridium sensu stricto 1* (IVW OR = 0.65, 95% CI: 0.42–1.00, P = 0.048), *Collinsella* (IVW OR = 0.69, 95% CI: 0.50–0.95, P = 0.024), and *Ruminiclostridium 9* (IVW OR = 0.68, 95% CI: 0.48–0.97, P = 0.031), were associated with a reduced DU risk.

3.2.5 Causal relationship of gut bacteria on GJU

Furthermore, we found an association between *Parabacteroides* (IVW OR = 0.22, 95% CI: 0.06–0.84, P = 0.027) and a lower GJU risk, while *Bilophila* (IVW OR = 3.45, 95% CI: 1.52–7.81, P = 0.003) showed a correlation with an elevated risk of GJU.

4 Discussion

Prior to the recognition of *H. pylori* infection and the extensive utilization of nonsteroidal anti-inflammatory drugs (NSAIDs) during the latter time frame of the 20th century, PUDs were primarily attributed to a hypersecretory acidic environment, coupled with dietary factors or stress (23, 24). However, there is growing recognition that the etiology of PUDs extends beyond *H. pylori* infection in the stomach (6). Rather, the genesis and progression of PUDs emerge as a result of the interplay of multiple factors, encompassing the presence of different *H. pylori* virulence proteins, ensuing human immune reactions, and imbalances in the gastrointestinal microbiota (1, 25). The role of intrinsic gut bacteria in PUD development is also noteworthy. Our findings emphasized the causal involvement of specific bacterial characteristics' abundance in modulating the susceptibility to diverse peptic ulcer types. Remarkably, this study represents the first MR analysis to illuminate the multiple connections between gut microbiota and PUDs. We regarded it as a longitudinal microbiome investigation conducted before the onset of PUDs in humans. This study effectively identified robustly gene variants through the largest gut microbiome GWAS.

One pivotal role played by gut microbiota involves the synthesis of short-chain fatty acids (SCFAs), which can directly regulate host health through energy regulation, intestinal mucosal barrier, immune regulation, and induction of tumor cell differentiation and apoptosis (26, 27). Dysregulation in the equilibrium of SCFAs within the body results in a cascade of disease manifestations (28). Moreover, SCFAs can promote the expression of tight junction proteins, such as claudin, occludin, and Zonula occludens (ZOs) within the intestinal tract, decrease intestinal permeability, promote the proliferation of intestinal mucosal cells, and improve the mechanical barrier function of the intestine in animal models (29, 30). In this MR study, SCFA-producing bacteria included *E. hallii* (31), *Flavonifractor* (32), *Ruminiclostridium* (33), *Ruminococcaceae* (33), *Collinsella* (34), and *Parabacteroides* (35).

A previous study revealed reduced levels of *Collinsella* in patients with inflammatory bowel disease (IBD) and gut microbiota dysbiosis (36). This finding was consistent with our results, implicating a potentially beneficial role for *Collinsella* in gut health and highlighting its association with a reduced DU risk. Researchers have found that *Parabacteroides* could produce a molecule named rhamnose in the mouse gut to facilitate the repair and maintenance of the intestinal mucosal barrier in mice (37). This suggested that *Parabacteroides* may benefit gut health, aligning with our findings that it exhibited a negative association with GJU. *E. hallii* is a high-yielding butyrate producer in the gut, contributing significantly to the maintenance of intestinal metabolic equilibrium (38). In the context of aging populations characterized by a decrease in microbiota diversity, a reduction in the abundance of *E. hallii* has been noted, accompanied by decreased production of SCFAs and increased intestinal inflammation (39). Consistent with these studies, our results demonstrated a negative link with OESU. The presence of *Eubacterium* in the gut is primarily associated with increased dietary fiber intake. As previously reported, it may improve the intestinal mucosal barrier and metabolic diseases, making it a potential candidate strain for a new generation of probiotics (40).

Previous studies have shown an increased level of *Flavonifractor* in patients with early-onset colon cancer, while *Lachnospiraceae UCG004* was significantly increased in patients with postmenopausal osteoporosis (PMO). While the adverse effects of *Flavonifractor* remain relatively underexplored, several studies hint at its role in stabilizing gut intestinal flora and immune modulation (41). Our study uncovered a positive correlation between *Flavonifractor* and OESU, hinting at its potential as a risk factor. *Ruminiclostridium*, a common anaerobic intestinal bacterium, plays a pivotal role in polysaccharide degradation and SCFA production, thereby influencing intestinal peristalsis, intestinal health, and immune modulation (42). In addition, *Ruminiclostridium 9* inhibits the growth of other harmful bacteria, which is crucial for preserving gut microbiota equilibrium (43). Recent studies have shown that reduced *Ruminiclostridium 9* abundance is also associated with some intestinal diseases, such as IBD and obesity (10). Our MR study demonstrated it with the negative causal relationship between GASTRODU or DU, thus highlighting the potential protective role of *Ruminiclostridium 9* during the development of PUDs. Despite the positive correlation between *Ruminiclostrium 6* and OESU, we did not observe any significant negative effect in the gut. *Ruminococcaceae* and *Lachnospiraceae* are typical intestinal flora that are important in maintaining intestinal health (44). However, some studies have shown the increased abundance of *Ruminococcaceae* or *Lachnospiraceae* in metabolic disorders such as obesity and diabetes (45). Additionally, certain members of these genera have been associated with the production of inflammatory mediators, enterotoxins, and other harmful

substances related to the occurrence and development of intestinal diseases (46, 47). Our study found that *Ruminococcaceae UCG013* was positively associated with OESU, while *Lachnospiraceae UCG004* was positively correlated with GU and GASTRODU, suggesting their potential role as risk factors. Intriguingly, *Lachnospiraceae FCS020* exhibited a negative causal link with OESU.

C. sensu stricto, a beneficial intestinal bacterium, has many vital physiological and metabolic functions, such as participating in the metabolism of glucose and lactose as well as promoting the synthesis of biotin and vitamin K (48). *C. sensu stricto* can also promote the integrity of the intestinal mucosal barrier, regulate the intestinal immune response, and reduce intestinal inflammation (48). Our MR analysis corroborates its protective effect, revealing a negative correlation between *C. sensu stricto 1* and DU. Conversely, *Catenibacterium* shows a positive causal association with DU. *Catenibacterium* is a Gram-positive bacterium (49). Although many species of *Catenibacterium* are unknown, different genera play different roles in intestinal diseases. For instance, certain strains have been implicated in the occurrence and development of IBD, wherein harmful substances, such as enterotoxins, contribute to intestinal mucosal barrier disruption and aggravated inflammatory responses (50). Our findings implicate *Bilophila* as a potential risk factor for GJU, which used to be mainly associated with metabolic diseases (51). *Bilophila*'s role in the gut requires further understanding; however, studies suggest that some members of *Bilophila* may be involved in the occurrence and development of intestinal inflammation. Some of these strains can produce harmful substances, such as hydrogen sulfide, breaking the intestinal mucosal barrier and increasing inflammatory responses (52). Notably, PUDs exhibited no significant association with the aforementioned bacteria in reverse MR analysis.

The variance in ulcer-related bacterial genera across different locations underscores the potential influence of anatomical locations and physiological functions. Interestingly, ulcers with overlapping sites, such as gastroduodenal ulcer, gastric ulcer, and duodenal ulcer, exhibited similar bacterial flora and associations, confirming the reliability of the results. Importantly, these findings prompted an exploration into the pathological mechanism underlying overlapping bacterial flora in ulcer development. Furthermore, there was no evidence of reverse causality between PUDs and gut bacterial genera. Overall, gene-based analysis from 119 gut bacterial genera revealed specific genera associated with PUDs in different locations and explained the multiple correlations between them. These findings supported the influence of gut microbiota on the development of PUDs and highlighted the putative association between specific bacterial genera and site-specific PUDs. Ultimately, these findings extended valuable implications for the clinical management of patients afflicted with PUDs.

5 Article summary

This study presented a comprehensive analysis of the causal relationships between 121 known gut bacterial genera and PUDs, utilizing both forward and reverse MR analyses. This approach effectively mitigated the influence of confounding variables and causal inference's challenge of reverse causation. Notably, the genetic variants associated with gut microbiota were derived from the most extensive GWAS meta-analysis, enhancing robust IVs for the MR analysis. Multiple statistical methods were used to test the sensitivity, pleiotropy, and heterogeneity of this study.

However, despite our efforts to minimize confounding influences, the complete elimination of horizontal pleiotropy remains a challenge, largely attributed to our limited understanding of the disease. As knowledge and awareness evolve over time, perceptions about confounding factors may change. In the future, extending MR investigations on the causal relationship between gut microbiota and peptic ulcers in diverse European and non-European populations would enhance the generalization of our findings. Additionally, while this article only explores the problem from the perspective of genetics, higher-level RCTs are necessary to validate the causal relationship while also into the intricate mechanisms underlying specific bacterial contributions.

In summation, through a systematic investigation, this study constituted a pioneering MR analysis focused on gut microbiota and PUDs. Our findings shed light on the multiple correlations between gut microbiota genera and five PUD types. Moreover, it has established definitive links between 14 specific bacterial genera and their corresponding ulcer manifestations, the pathogenic intestinal bacteria deserved more attention. These findings hold significant implications for understanding the role of gut flora in PUDs, offering valuable insights for the formulation of preventive strategies in patients with this condition. Novel treatment avenues targeting specific intestinal bacterial genera may represent new treatment options for PUDs.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: MiBioGen Consortium, https://mibiogen.gcc.rug.nl/ and FinnGen Consortium R8, https://r8.finngen.fi/.

Ethics statement

All studies were previously approved by respective Institutional Review Boards (IRBs). No new IRB approval was required. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

JZ: Conceptualization, Data curation, Formal Analysis, Methodology, Software, Validation, Writing – original draft,

Writing – review & editing. YH: Data curation, Software, Writing – original draft, Writing – review & editing. YiZ: Conceptualization, Writing – original draft, Writing – review & editing. TX: Conceptualization, Data curation, Software, Writing – original draft. XF: Data curation, Formal Analysis, Software, Writing – original draft. ZY: Conceptualization, Investigation, Supervision, Visualization, Writing – original draft. YaZ: Funding acquisition, Investigation, Resources, Software, Supervision, Writing – review & editing. WG: Funding acquisition, Resources, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1260780/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Sensitivity test performed by leave-one-out analysis.

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Emerging insights into inflammatory bowel disease from the intestinal microbiota perspective: a bibliometric analysis

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Background: Inflammatory bowel disease (IBD) has caused severe health concerns worldwide. Studies on gut microbiota have provided new targets for preventing and treating IBD. Therefore, it is essential to have a comprehensive understanding of the current status and evolution of gut microbiota and IBD studies.

Methods: A bibliometric analysis was performed on documents during 2003-2022 retrieved from the Scopus database, including bibliographical profiles, citation patterns, and collaboration details. Software programs of VOSviewer, CiteSpace, and the Bibliometrix R package visually displayed the mass data presented in the scientific landscapes and networks.

Results: 10479 publications were retrieved, showing a steadily growing tendency in interest. Xavier Ramnik J. group led the total number of publications (73 papers) and 19787 citations, whose productive work aroused widespread concern. Among the 1977 academic journals, the most prolific ones were *Inflammatory Bowel Diseases*, *Frontiers in Immunology*, and *Nutrients*. Research outputs from the United States (US, 9196 publications), China (5587), and Italy (2305) were highly ranked.

Conclusion: Our bibliometric study revealed that the role of gut microbiota has become a hot topic of IBD research worldwide. These findings are expected to improve understanding of research characteristics and to guide future directions in this field.

KEYWORDS

intestinal microbiota, inflammatory bowel disease (IBD), bibliometric analysis, citation, research trends

Introduction

Inflammatory bowel disease (IBD) comprises a heterogeneous group of inflammatory disorders that are immune-mediated and primarily affect the gastrointestinal tract, with Crohn's disease (CD) and ulcerative colitis (UC) being the two main subtypes (1, 2). IBD significantly impacts daily life and is a significant risk factor for the development of gastrointestinal cancers (3). The rapid evolution of social norms, lifestyles, diets, and the environment resulting from contemporary human behavior may instigate or contribute to the escalating prevalence of IBD, rendering it an emerging global concern (4). Increasing evidence suggests that the gut microbiome plays a crucial role in the development of IBD (5). IBD is associated with alterations in the gut microbiome, characterized by a consistent reduction in bacterial diversity (6). Meanwhile, fecal microbiota transplantation has been shown to restore intestinal microecological balance and treat IBD effectively (7).

Microbiota in the human digestive tract make up a complex ecological system. To date, over 3000 species have been detected in human feces; only 30% of this bacterial population is the typical core microflora shared between different individuals (8, 9). Investigations have indicated that gut microbiota is crucial in the maintenance of intestinal physiological function (10). The dynamic composition of the microbiota is influenced and regulated by a combination of endogenous and exogenous factors (11). Diet, hormones, medication, and health conditions of the host may affect the numbers and diversity of microflora in the gastrointestinal tract (10, 11). Dramatic perturbations like these may result in dysbiosis characterized by an altered composition and reduced stability (12). Moreover, microbiota dysbiosis could induce various human diseases like IBD in the pathological processes (13, 14).

Bibliometric analysis is an approach to evaluate the trends and characteristics of published literature in a particular domain over time. It provides an easy and direct way for scientists and researchers to access the field's developing trends and research interests. The academic influence of leading publications and literature distributions from different origins is clearly present (15, 16). The conventional classification and summarization of literature heavily rely on the subjective judgment of authors, making it challenging to analyze a large volume of literature comprehensively and accurately. To address this issue, scientific cartography based on bibliometric quantitative analysis can be employed to examine the structure and development of research fields. This method facilitates the summarization and analysis of applied literature while uncovering key application areas and enables topic clustering using CiteSpace or VOSviewer software (17). Currently, bibliometric analysis has garnered increasing attention due to its distinctive advantages that enable investigators to delve into specific fields of study through the visualized analysis of citations, co-citations, geographic distribution, and term frequency, yielding highly valuable insights (18).

In this study, on the hotspot of gut microbiota and IBD, we conducted a bibliometric analysis of publications in the Scopus database during the past two decades to capture its research state and trends. Using software programs VOSviewer, CiteSpace, and Bibliometrix, we mapped the literature landscape and distribution layouts of active authors, journals, institutions, and countries. We also visualized the patterns of cooperation and citation. This study presents an overview and summary of the evolution of gut microbiota and IBD studies, and analyzes the current research state and future trends, aiming to assist researchers and policymakers gain a comprehensive understanding of the study on this topic and better grasp future directions.

Methods

Data source

A bibliometric search of research output on the gut microbiome and IBD, published from 2003 to 2022, was performed on July 10, 2023, using the Scopus database. Scopus by Elsevier is known to be the most comprehensive data source for detailed bibliometric evaluation from a quantitative and qualitative point of view (19–21). With the comprehensive coverage of scientific journals and the powerful performance of analytical tools, Scopus was selected as the literature source to retrieve abstracts, citations, and other bibliometric data at the initial stage.

Search strategy

Aiming to ensure reliable and accurate records, our primary keywords used in the literature search focused on gut microbiota and inflammation, along with the relevant synonyms based on Medical Subject Headings (MeSH) in MEDLINE (22). The terms "gastrointestinal microbiomes," "gut microflora," "gut microbiota," "gastrointestinal flora," "gut flora," "gastrointestinal microbiota," "gut microbiome," "gastrointestinal microflora," "intestinal microbiome," "intestinal microbiota," "intestinal microflora," "intestinal flora" and "enteric bacteria" were used as the keywords of gut microbiota; the primary keywords of IBD were "inflammatory bowel disease" and "IBD" Meanwhile also includes "ulcer colitis", "UC", "Crohn disease" and "CD". The two sets of keywords with the AND logic were searched in the field of "Article title/Abstract/Keywords." The search was conducted in Scopus using the following terms: (TITLE-ABS-KEY (gastrointestinal AND microbiomes) OR TITLE-ABS-KEY (gut AND microflora) OR TITLE-ABS-KEY (gut AND microbiota) OR TITLE-ABS-KEY (gastrointestinal AND flora) OR TITLE-ABS-KEY (gut AND flora) OR TITLE-ABS-KEY (gastrointestinal AND microbiota) OR TITLE-ABS-KEY (gut AND microbiome) OR TITLE-ABS-KEY (gastrointestinal AND microflora) OR TITLE-ABS-KEY (intestinal AND microbiome) OR TITLE-ABS-KEY (intestinal AND microbiota) OR TITLE-ABS-KEY (intestinal AND microflora) OR TITLE-ABS-KEY (intestinal AND flora) OR TITLE-ABS-KEY (enteric AND bacteria) AND PUBYEAR > 2002 AND PUBYEAR < 2023) AND (TITLE-ABS-KEY (inflammatory bowel disease) OR TITLE-ABS-KEY (ulcer colitis) OR TITLE-ABS-KEY (crohn disease) OR TITLE-ABS-KEY (IBD) OR TITLE-ABS-KEY (UC) OR TITLE-ABS-KEY (CD) AND PUBYEAR > 2002 AND PUBYEAR < 2023).

Data analysis

The search outcomes from the Scopus database were exported into CSV format for further analysis, including bibliographical profiles, citation patterns, collaboration details, and other retrieved publications. We established the inclusion criteria as follows: 1. The literature pertains to topics of IBD and inflammation; 2. Articles published within the past two decades (2003-2022). Exclusions encompassed: 1. Incomplete or duplicated literature; 2. Non-academic documents such as conference proceedings, calls for papers, news reports, patent achievements, and newspaper abstracts.

Microsoft Excel and GraphPad Prism (Version 9.5.0, San Diego, CA, US) were applied to conduct statistical procedures, generating frequency distribution, sum, and average data. Further investigations were performed to determine the top, most prolific authors, journals, countries, institutions, and the most cited papers according to the standard competition ranking (SCR, also known as the 1-2-2-4 rule). We calculated the H-index to assess the number and level of academic output of researchers. In addition, we also calculate the G index and M-index as a supplement to the H index. The calculation method of G-index is as follows: the papers are sorted in descending order according to the number of citations, and the number of citations is superimposed according to the serial number. When the cumulative number of citations is equal to the square of the serial number, the serial number value is the G-index (23). The M-index is derived from the H-index of academic tenure, calculated by dividing the H-index by the number of years since an author's initial publication (24).

Visualization analysis was applied for presenting a mass of data to display scientific landscapes and networks using software programs of VOSviewer (v.1.6.17) (25), CiteSpace (v.5.8.R2) (26), and the R package of Bibliometrix (27). VOSviewer conducts a visual analysis of country, institutional, author, and collaboration distribution, as well as keyword collaboration networks. Clustering is automatically completed using the similarity matrix and mapping techniques of VOSviewer, with corresponding labels added by the authors based on content. CiteSpace was utilized to analyze the distribution and collaboration among countries, institutions, keyword timelines, and reference data. Additionally, we employ R studio Desktop Software (v.2023.6.1.0) linked to the R Software (v.4.3.1) and converted into an R data frame. The Bibliometrix R package, which provides a web interface, was used for statistical analysis of the number of publications, references, and other data and visual analysis of national distribution and cooperation (27). The flow diagram for the searching and sorting process of related articles is shown in Figure 1. All raw data utilized in this study were sourced from publicly available databases, thus exempting the need for ethical review.

Results

Overview and trends in research literature production

The 20-year period 2003-2022 saw the publication of 10479 articles in gut microbiota and IBD research. Global trends in the number of annual and accumulated publications related to this topic are shown in Figure 2A. During the first four years, 2003-2006, the number of annual articles ranged from117 to 156 and was relatively stable, implying that this crossover domain was not so attractive to scientists at that moment. Moreover, within a span of only two years (2007-2008), two important programs of the Human Microbiome Project (HMP) were launched by the United States National Institutes of Health (NIH) in 2007 (28), and Metagenomics of the Human Intestinal Tract (MetaHIT) by European Union in 2008 (29), and the number of annual articles increased to 209. Since then, the number has continued to rise, reaching 1813 publications in 2022 (or 906.5% of the articles in 2008). The active interest and intensive efforts from worldwide research communities in the past ten years have led to the enormous growth of this field, which is supported by a significant increase in the number of related publications. The number of articles published on this topic in the recent decade is more than 5.93 times in the first ten years since 2003. Furthermore, it is noted that the percentage of annual intestinal microbiota-related publications in the domain of inflammation research was increasing gradually, starting from 2009, when an increase from 0.9%-0.11% was observed (Figure 2B). Therefore, the combination of gut microbiota and inflammation as an immediate area of the research focus has attracted significant attention from scientists worldwide.

Among the retrieved documents, the research articles (6544, 52.36%) and reviews (4629, 37.04%) made up the majority, while the others (10.60%) were conference papers, book chapters, short surveys, notes, and editorials, etc. (Figure 3A). Journals were the primary source of documents, accounting for 95.40% of all the publications (Figure 3B). In terms of subject distribution (Figure 3C), 8334 documents (66.69%) were related to Medicine, 2936 (23,49%) to Biochemistry, Genetics, and Molecular Biology, 2956 (23.65%) to Immunology and Microbiology, and 1204 (9.63%) to Agricultural and Biological Sciences. Of the 26 languages published, English was predominant (11857, 94.88%), followed by Chinese (236, 1.89%). Other languages, like German, Russian, French, etc., only covered less than 1% of publications (Figure 3D).

Due to the source type and language heterogeneity among the retrieved documents, we set the inclusion criteria to limit publications to only research articles written in English to perform further analyses. Thus, papers of document types besides research articles and papers written in other languages were excluded (Figure 1). As a result, a total of 10479 English papers were included for the following analyses.

Analysis of the most cited articles

Although many factors may influence the citation impact of publications, it is widely regarded as a vital evaluation index for scientific documents. Table 1 presents the 20 most commonly cited papers between 2003 and 2022 (30–49). "Metabolic endotoxemia initiates obesity and insulin resistance," published in *Nature* by *David L.A. et al.*, was the most frequently cited article (5975 times) (30). In addition, the top journals were represented among the most

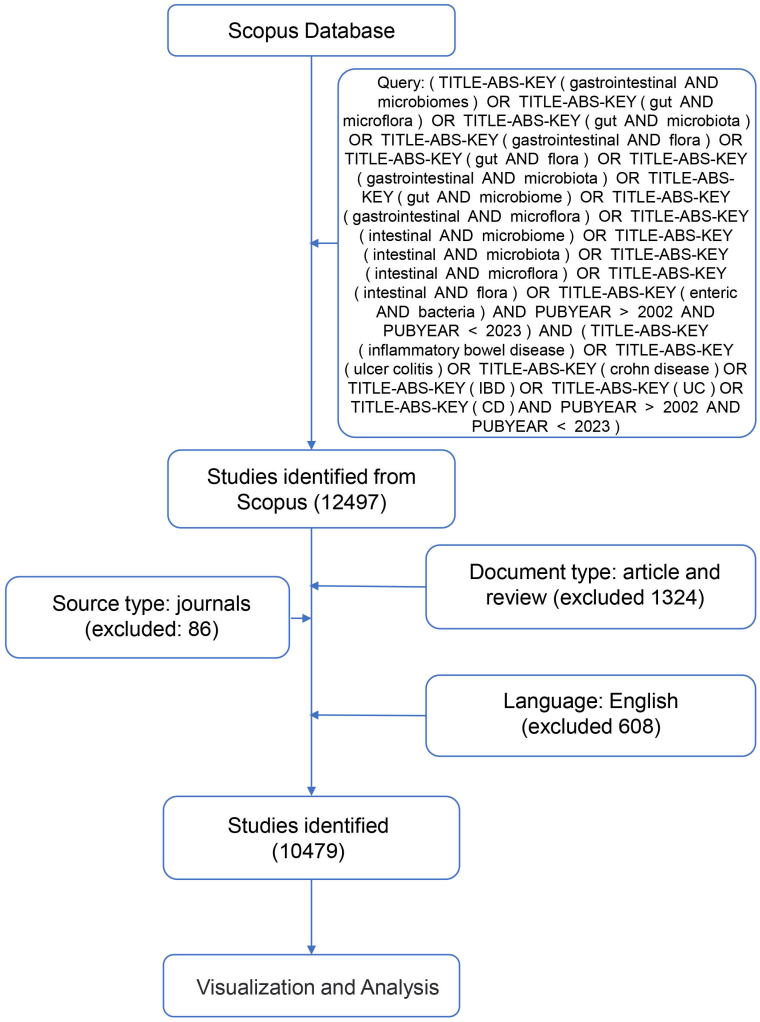

FIGURE 1
Flow diagram of the literature selection process in this study.

cited articles in this field. Of the 20 most cited papers, four were published in *Nature*, and three were published in *Proceedings of the National Academy of Sciences of the United States of America* (*PNAS*), two from *NEJM* and two from *Cell*, respectively.

Contribution of author performance

A total of 36335 different authors contributed to the 10479 papers included in this study. Further analysis revealed that the 722 most prolific authors who published ten articles or more accounted for 2.0% of all contributors. We created a historical map of the related publications and authors in the format of a bubble chart. This chart demonstrated the 20 most productive authors by year, as shown in Figure 4. Since 2003, Sartor R. Balfour, Colombel Jean Frederick, and Shanahan Ferguson have been pioneers in exploring the field of gut microbiota and inflammation, but at that time there were relatively few related papers on this topic. After a 5-year significant increase from 2007 to 2012, this emerging field witnessed explosive publication growth from 2013. More specifically, Xavier Ramnik J. group led in the total number of publications (73 papers), followed by Sokol Harry (67), Sartor R. Balfour (50), Colombel Jean-Frederic (49), and Huttenhower Curtis (42).

The citation of the 234 most prolific authors with at least twenty publications was quantified and subjected to analysis, while the top 10 authors with the most published papers and the top 10 most cited authors on gut microbiota and IBD from 2003 to 2022 were presented in Table 2. Xavier, Ramnik J. led in the first place (19787 cited), followed by Sokol, Harry (13933) and Huttenhower, Curtis (11575). In addition, Xavier, Ramnik J. had the most citations (19787) among the top 20 most cited authors, followed by Sokol, Harry (13933) and Knight, Rob (12811). The 234 most prolific authors were also integrated into collaborative networks, as shown in Figure S1. The link thickness between any two authors indicates the extent of co-authorships (collaboration). The clusters in Figure S1 revealed a strong correlation between the number of publications produced by an author and co-authorship. In other words, the more muscular the total link strength of scientific collaboration, the more authored publications.

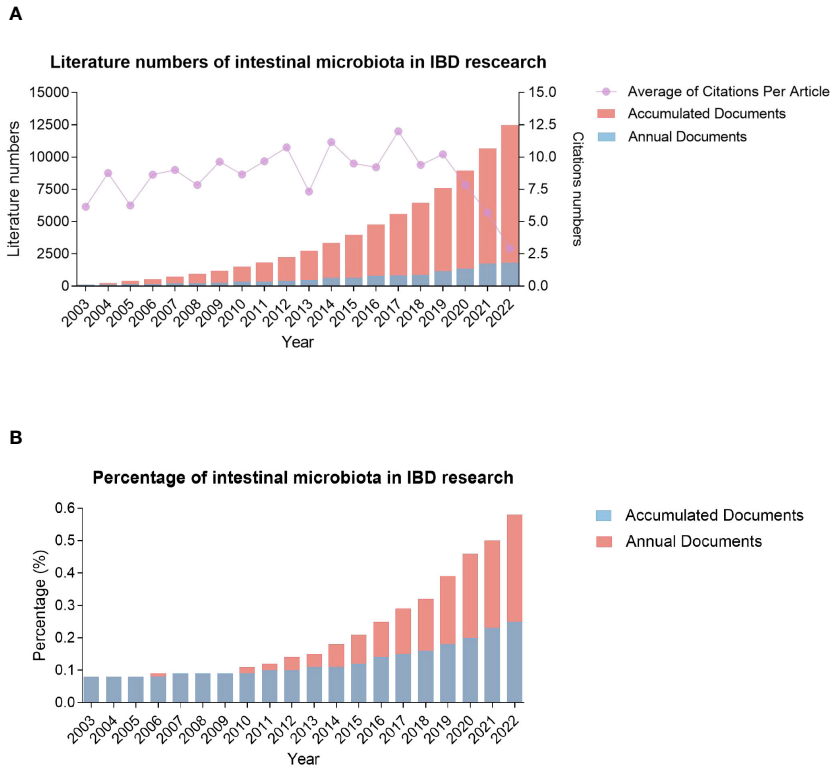

FIGURE 2
Global trends in the number of published articles related to gut microbiota and IBD over the past two decades from 2003 through 2022. **(A)** annual and accumulated publications of intestinal microbiota and IBD; **(B)** the percentage of intestinal microbiota-related publications in the IBD research.

Contribution of journal production

The retrieved articles were published in 1977 different academic journals. Table 3 presents the top 20 active journals publishing articles on intestinal microflora and IBD, which produced 3269 articles (31.1%). Among them, *Inflammatory Bowel Diseases* took the leading position with 324 papers, followed by *Frontiers in Immunology* (301) and *Nutrients* (259). These three journals issued 8.5% of the total publications. According to IF, *Nature Reviews Gastroenterology and Hepatology* held the top position by an overwhelming high value of 65.1, owing to its excellent specialization in this field. In addition, Bradford's law of scattering was applied here to reveal the distribution of the scientific literature in the research on gut microbiota and inflammation. Bradford zones acted as concentric zones of publication productivity with decreasing correlation, while each zone involved a similar number of articles. As shown in Table 4 and Figure S4, a total of 1977 journals were distributed in 3 Bradford's zones in the field of gut microbiota and IBD. The average number of articles in each zone was 3493.

The number of citations also indicates the power and authority of journals in the field. Citation analysis of 380 journals with minimum productivity of 5 publications was presented in Figure 5A. Articles on gut microbiota and IBD published in *Gut* received the highest number of citations (28430), while those published in *Nature* (25407) and *Inflammatory bowel disease* (23871) ranked second and third, respectively. The dot size is proportionate to the number of citations, with yellow indicating higher average citations and blue indicating lower average citations (Figure 5A).

Analyzing the top 20 most cited journals by year provided further insight into the level of journals directed to topic interest in Figure 5B. It was apparent that the journal citations have increased enormously, based on the great concentration in this area from 2008, which was consistent with patterns shown in Figure 1. Among the top 20 most cited journals, the quantity of publications regarding intestinal microflora and IBD has exhibited a consistent upward trend from 2003 to 2022. *Frontiers in Immunology*, *Nutrients*, and *Frontiers in Microbiology* experienced the most significant surge, demonstrating the most published research topics in intestinal microflora and IBD between 2003-2022.

Global contribution and leading countries/regions

The geographical distribution of research productivity from 217 countries/regions on six continents was presented in Figure 6. In the

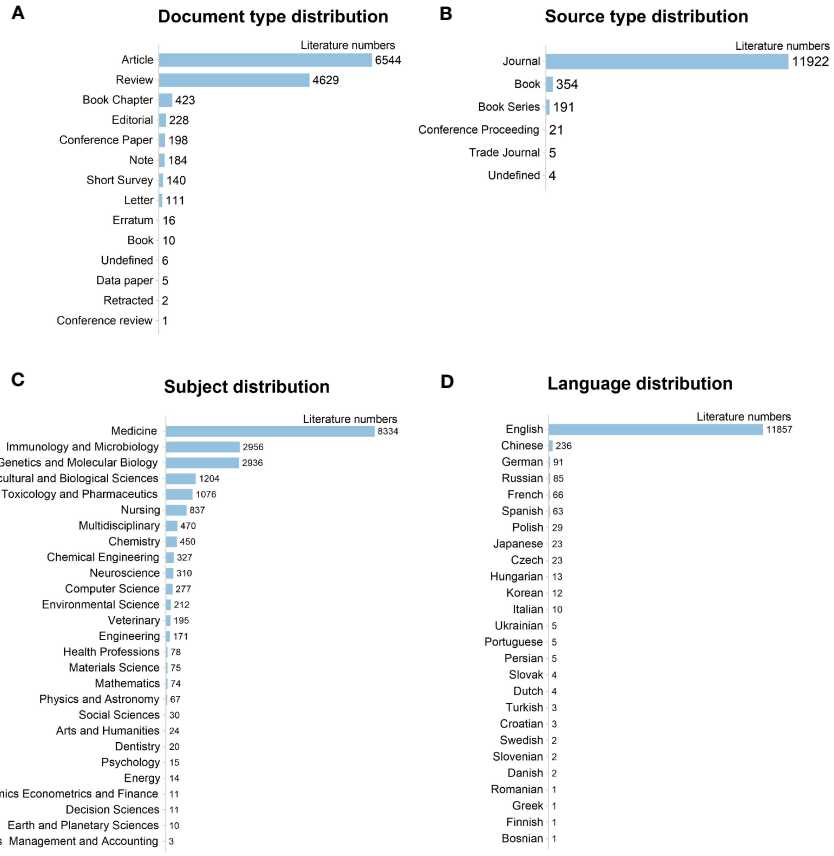

FIGURE 3
General information of retrieved 10479 publications on gut microbiota and IBD. **(A)** Document type distribution; **(B)** Source type distribution; **(C)** Subject distribution; **(D)** Language distribution.

map, the darker blue color represented countries/regions with the higher productivity of gut microflora and IBD articles. The intensity of the color is directly proportional to the quantity of publications. Among the most productive countries, the United States (US) contributed most to the research productivity (9196 publications), followed by China (5587), Italy (2305), the United Kingdom (1806), and France (1740). International collaboration of active countries/regions was also assessed and presented in a network visualization map (Figures 6, S2). The thickness of the link between any two countries/regions indicated the strength of collaboration, while the density of the threads assigned for that country/region indicated the extent of international collaboration. The network visualization map shows that the most vigorous collaboration was between the US and China. With the densest line, the US implied the greatest extent of international collaboration with 80 countries/regions due to the wide range of its publications. From the cluster analysis in the map, countries/regions such as the US and Canada were observed in a close cluster, while Germany, Sweden, and Denmark were found in another close cluster. The ranking of production and national collaborations between the corresponding author's countries are shown in Table S1. Citations analysis for countries in Table S2 showed that the US had been the most highly cited globally.

Contribution of academic institutions

Table 5 lists the top 20 prolific institutions publishing papers on gut flora and IBD. Harvard Medical School ranked first in productivity with 336 scientific publications, followed by the University of California (274) and University College Cork (208). The 20 most active institutions are primarily in North America, with 10 in the US and 4 in Canada. The other six institutions are widely distributed in Europe (Ireland, Belgium, Denmark), Oceania (Australia), and Asia (China). The analysis of 277 organizations with high citations of more than 1000 times is shown in Figure S3. The Division of Biology, California Institute of Technology, Pasadena, CA, United States received the highest citation number (7833 citations), while the FAS Center for Systems Biology, Harvard University, Cambridge, MA, in the United States (6599) and Laboratory of Microbiology, Wageningen University Wageningen

TABLE 1 Top 20 most cited articles on gut microbiota and IBD from 2003 to 2022.

SCR	Authors	Title	Year	Journals	Citations
1st	David et al.	Diet rapidly and reproducibly alters the human gut microbiome	2014	*Nature*	5975
2nd	Kaper et al.	Pathogenic Escherichia coli	2004	*Nat Rev Microbiol*	3500
3rd	Round et al.	The gut microbiota shapes intestinal immune responses during health and disease	2009	*Nat Rev Immunol*	3402
4th	Frank et al.	Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases	2007	*PNAS*	3341
5th	Lozupone et al.	Diversity, stability and resilience of the human gut microbiota	2012	*Nature*	3247
6th	Sokol et al.	Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients	2008	*PNAS*	3034
7th	Guarner et al.	Gut flora in health and disease	2003	*Lancet*	2528
8th	Clemente et al.	The impact of the gut microbiota on human health: An integrative view	2012	*Cell*	2405
9th	Cho et al.	The human microbiome: At the interface of health and disease	2012	*Nat Rev Genet*	2185
10th	Abraham et al.	Inflammatory bowel disease	2009	*NEJM*	2184
11st	Gevers et al.	The treatment-naive microbiome in new-onset Crohn's disease	2014	*Cell Host and Microbe*	2025
12nd	O'Hara et al.	The gut flora as a forgotten organ	2006	*EMBO Reports*	1948
13rd	Lynch et al.	The human intestinal microbiome in health and disease	2016	*NEJM*	1861
14th	Morgan et al.	Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment	2012	*Genome biology*	1828
15th	Khor et al.	Genetics and pathogenesis of inflammatory bowel disease	2011	*Nature*	1765
16th	Mazmanian et al.	A microbial symbiosis factor prevents intestinal inflammatory disease	2008	*Nature*	1746
17th	Manichanh et al.	Reduced diversity of fecal microbiota in Crohn's disease revealed by a metagenomic approach	2006	*Gut*	1683
18th	Round et al.	Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota	2010	*PNAS*	1617
19th	Roberfroid et al.	Prebiotic effects: Metabolic and health benefits	2010	*British Journal of Nutrition*	1510
20th	Elinav et al.	NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis	2011	*Cell*	1493

SCR, standard competition ranking; PNAS, Proceedings of the National Academy of Sciences of the United States of America; NEJM, New England Journal of Medicine; Nat Rev Microbiol, Nature Reviews Microbiology; Nat Rev Immunol, Nature Reviews Immunology; Nat Rev Genet, Nature Reviews Genetics; IF, impact factor.
*Data extracted from Journal Citation Reports, Thomson Reuters, 2022.

in Netherlands (6272) were in the second and third place, respectively.

Analysis of research interests in terms of frequency

This thematic analysis was performed on the terms that appeared in the information sources of retrieved publications from 2003-2022. The terms were mainly from the title, abstract, and keyword fields of academic literature, representing the authors' main concepts and research interests for communication. A density visualization map is used to display which terms occur more often and how the terms interconnect (Figure 7). The larger the character fonts, the more frequently the terms are applied. A total of 525 terms that occurred more than 100 times were presented and divided into three groups with their interconnections. As a result, terms such as inflammatory bowel disease(IBD) were by far the most prevalent (8168 times), followed by patient (3202), gut microbiota (2651), microbiota (2346), and inflammation (2210).

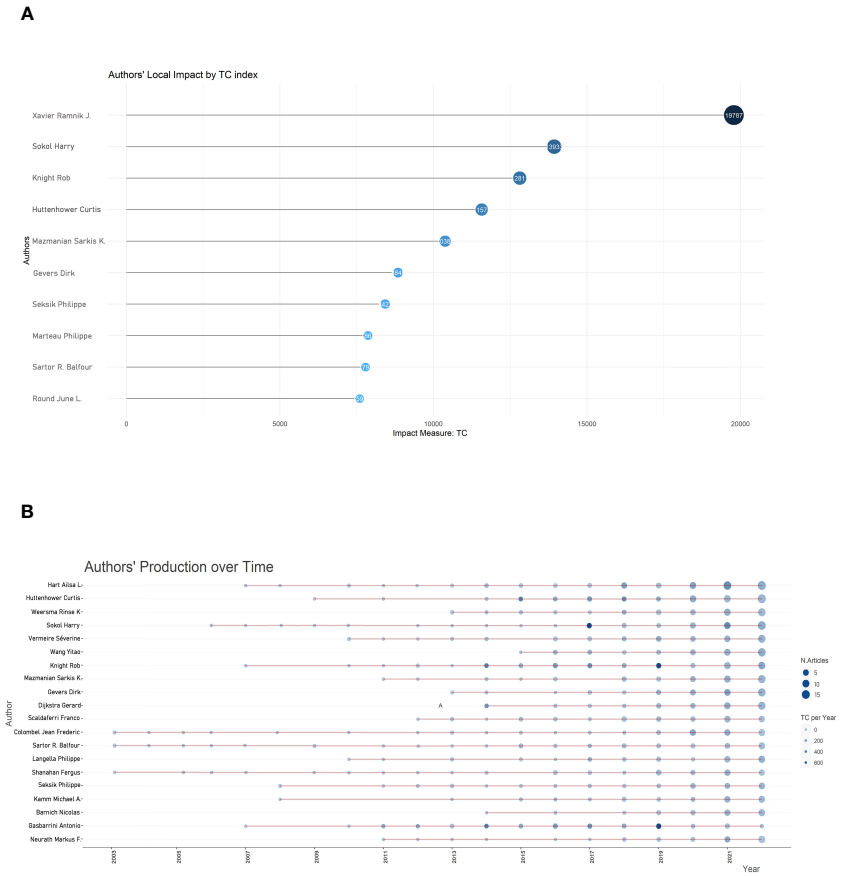

FIGURE 4
Bubble diagram depicting the influence of authors. **(A)** Top 10 authors with the highest citations; **(B)** Annual publication count and citation frequency. Larger shapes display more publications, and darker blue displays more citations.

TABLE 2 Top 10 prolific authors and top 10 most cited authors on gut microbiota and IBD from 2003 to 2022.

Rank	Author	H-index	G-index	M-index	TC	NP	PY start
1st	Xavier Ramnik J.	48	73	2.824	19787	73	2007
2nd	Sokol Harry	39	67	2.167	13933	67	2006
3rd	Sartor R. Balfour	33	50	1.571	7791	50	2003
4th	Colombel Jean Frederic	33	49	1.571	6200	49	2003
5th	Shanahan Fergus	32	43	1.524	7425	43	2003
6th	Huttenhower Curtis	35	42	2.692	11575	42	2011
7th	Vermeire Séverine	26	38	1.368	5395	38	2005
8th	Seksik Philippe	25	37	1.25	8427	37	2004
9th	Kamm Michael A.	28	37	1.333	5703	37	2003
10th	Neurath Markus F.	25	37	1.19	4972	37	2003
1st	Xavier Ramnik J.	48	73	2.824	19787	73	2007
2nd	Sokol Harry	39	67	2.167	13933	67	2006
3rd	Knight Rob	24	33	2	12811	33	2012
4th	Huttenhower Curtis	35	42	2.692	11575	42	2011

(Continued)

TABLE 2 Continued

Rank	Author	H-index	G-index	M-index	TC	NP	PY start
5th	Mazmanian Sarkis K.	12	13	0.706	10381	13	2007
6th	Gevers Dirk	19	20	1.462	8848	20	2011
7th	Seksik Philippe	25	37	1.25	8427	37	2004
8th	Marteau Philippe	19	22	0.95	7863	22	2004
9th	Sartor R. Balfour	33	50	1.571	7791	50	2003
10th	Round June L.	10	12	0.625	7599	12	2008

TC, total cited; NP, number of publications; PY start, publication year start.
*Data extracted from Journal Citation Reports, Thomson Reuters, 2022.

Discussion

General research directions

IBD significantly impacts daily life and poses a significant risk for the development of gastrointestinal malignancies (3). Due to its clinical refractory nature, treating IBD has become a prominent topic in healthcare. However, the in-depth pathogenesis that accounts for IBD has been largely debated. The role of commensal microbiota in the onset and development of IBD has attracted increasing attention (6). Aberrant microbiota community structure and dysbiosis of the host's microbiota may affect the gut's immunological function and

TABLE 3 Top 20 prolific journals in publishing papers on intestinal microflora and IBD.

SCR	Journals	Documents	% N=10497	IF 2022
1st	*Inflammatory Bowel Diseases*	324	3.1%	4.9
2nd	*Frontiers in Immunology*	301	2.9%	7.3
3rd	*Nutrients*	259	2.5%	5.9
4th	*World Journal of Gastroenterology*	220	2.1%	4.3
5th	*Gut Microbes*	196	1.9%	12.2
6th	*Frontiers in Microbiology*	193	1.8%	5.2
7th	*Plos One*	190	1.8%	3.7
8th	*International Journal of Molecular Sciences*	179	1.7%	5.6
9th	*Gastroenterology*	139	1.3%	29.4
10th	*Gut*	128	1.2%	24.5
11th	*Journal of Crohn'S and Colitis*	100	1.0%	8.0
11th	*Scientific Reports*	100	1.0%	4.6
13rd	*Current Opinion in Gastroenterology*	98	0.9%	2.5
14th	*Frontiers in Cellular and Infection Microbiology*	92	0.9%	5.7
15th	*Mucosal Immunology*	85	0.8%	8.0
16th	*Digestive Diseases and Sciences*	77	0.7%	3.1
17th	*Food and Function*	76	0.7%	6.1
18th	*Frontiers in Pharmacology*	74	0.7%	5.6
19th	*Nature Reviews Gastroenterology and Hepatology*	73	0.7%	65.1
20th	*Cells*	67	0.6%	6.0

TABLE 4 Distribution of the journals in Bradford's zones.

Bradford's Zones	Number of Journals	% Journals	Number of articles	Bradford's multiplier
1	29	1.4	3462	
2	199	10.1	3492	1.80
3	1749	88.5	3525	1.68
Total number of journals = 1977 Average number of articles in each zone = 3493				

immune homeostasis (50). As metabolites of gut microbiota, short-chain fatty acid (SCFA) and bile acid have been shown to work on immune homeostasis through cell signaling receptors or epigenetic regulations (50, 51). The dysfunction of the intestinal barrier leading to pathological bacterial translocation also contributes to IBD (1).

The perspective of intestinal microbiota may shed new light on the investigation of IBD. Lifestyle and environmental factors could modulate the gut microbiota composition and bacterial diversity (52). Unhealthy dietary patterns result in microbiota dysbiosis, which becomes the facilitator of IBD (4). Gut microbiota-related approaches may have the potential to ameliorate IBD (7). Fecal microbiota transplantation has been shown to have beneficial effects, including alleviating colonic inflammation and promoting the restoration of intestinal homeostasis through multiple immune-mediated pathways (53). However, more in-depth investigations are needed to reach a thorough understanding of the interplay between intestinal microbiota and IBD.

Bibliometric analysis is a quantitative study of bibliographic information, including authors, institutions, publication types, source countries, funding and citation information, etc. (54). This approach can assess the academic performance of journals, authors, or countries and provide a comprehensive overview of a particular research domain. This study aimed to present a complete picture of intestinal microbiota and IBD research during the past two decades. The significance of gut microbiota in IBD-related research was investigated using a bibliometric method for the first time, and the scientific production and global trends of this field were also evaluated.

As revealed in the results, since 2003, Sartor R. Balfour, Colombel Jean Frederick, and Shanahan Ferguson have pioneered the field of gut microbiota and inflammation study, which has demonstrated sustained and exponential growth over the ensuing two decades. Especially in the last few years, the annual number of documents has been soaring since 2007, which coincides with the launch of the HMP and MetaHIT projects (55, 56). In recent years, gut microbiota research has been drawing increased attention to provide a new perspective on many complicated issues, it also accelerates the understanding of the origin and mechanism of IBD. The steady growth in the percentage of intestinal microbiota-related publications on IBD research indicates a promising future in this domain.

Hotspots and Frontiers

Through the utilization of keyword cluster analysis and timeline view, the current research hotspots pertaining to IBD and intestinal flora can be primarily categorized into two aspects: the microscopic mechanisms underlying IBD and its clinical treatment, with a particular emphasis on exploring the inflammatory pathways mediated by intestinal flora. The intestinal barrier and mucosal

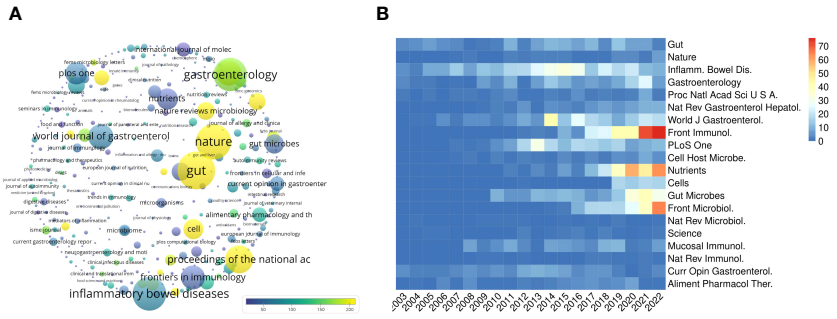

FIGURE 5
Citation analysis of journals. **(A)** Density map of 380 journals with a minimum productivity of 5 publications in this field. Journals with a higher number of citations have a yellower spot. **(B)** Heatmap illustrated the publication volume distributions per year of the top 20 most cited journals. The intensity of the blue hue is inversely proportional to the quantity of published papers, while the intensity of the red hue is directly proportional to the quantity of published papers.

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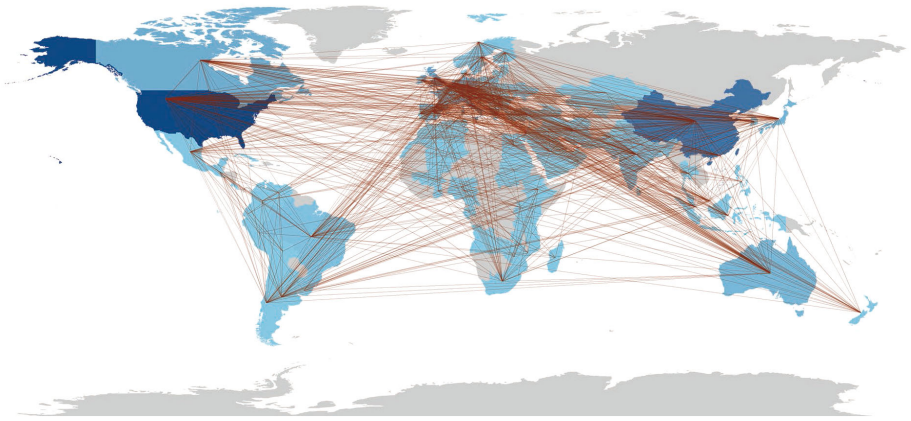

FIGURE 6
Trends in the publications on intestinal microbiota in IBD research involved 217 countries/regions over six continents. The interconnection among nations signifies collaborative efforts.

immunity play crucial roles in connecting gut microbiota with IBD. The TNF-α, TLR4, and other signaling pathways, as well as the metabolic mechanisms of intestinal flora such as SCFA, have garnered increasing attention in recent years. In studies of gut microbes associated with IBD, proteobacteria, and parabacteroides were the most heavily investigated in the past two decades, which have been reported in 287 and 154 articles, respectively, revealing the research hotspots in this field. Human-centered clinical study is experiencing steady growth and is poised to become a prominent topic in this field.

TABLE 5 Top 20 prolific institutions in publishing papers on gut flora and IBD.

SCR	Institution	Documents	Country	% N=10497
1st	Harvard Medical School	336	USA	3.2
2nd	University of California	274	USA	2.6
3rd	University College Cork	208	Ireland	2.0
4th	University of Alberta	168	Canada	1.6
5th	University of Toronto	163	Canada	1.5
6th	University of Calgary	162	Canada	1.5
7th	Baylor College of Medicine	139	USA	1.3
8th	University of North Carolina at Chapel Hill	138	USA	1.3
9th	Icahn School of Medicine at Mount Sinai	134	USA	1.3
10th	Mcmaster University	133	Canada	1.3
10st	University of Michigan	133	USA	1.3
12st	University of Pennsylvania	124	USA	1.2
13rd	Cornell University	121	USA	1.2
14th	University of Chicago	114	USA	1.1
15th	University of California San Diego	111	USA	1.1
15th	University of Copenhagen	111	Danmark	1.1
17th	Ghent University	109	Belgium	1.0
17th	Jiangnan University	109	China	1.0
19th	Zhejiang University	105	China	1.0
20th	University of New South Wales	103	Australia	1.0

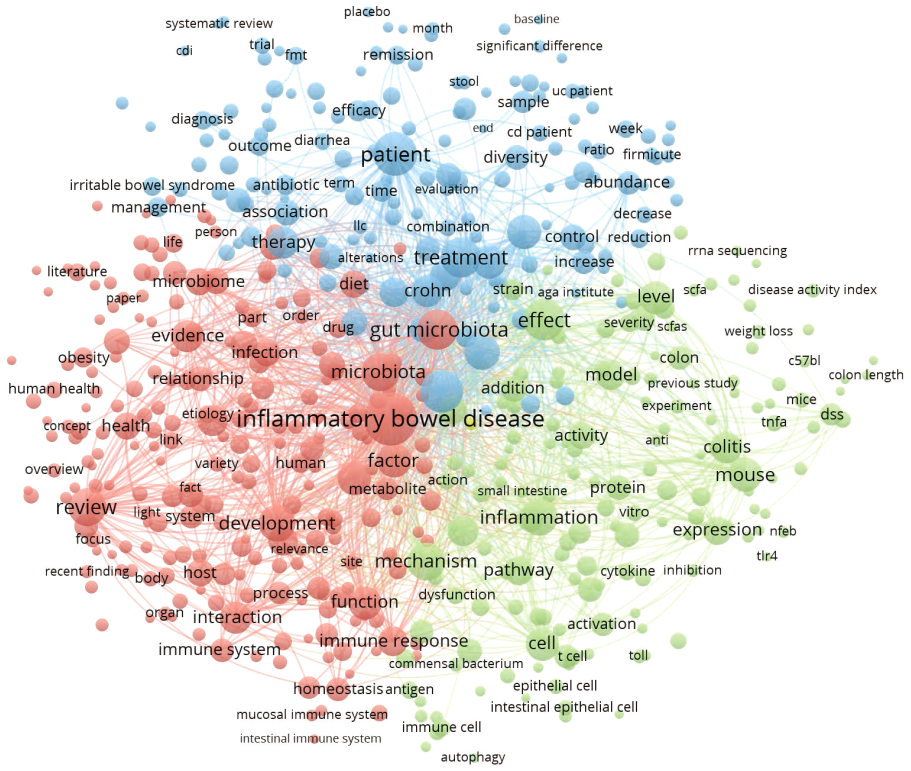

FIGURE 7
Density map of most frequently encountered terms extracted from the titles and abstracts of retrieved publications. A number of 525 terms met the threshold with a minimum number of occurrences of 100. The larger circle size or font size indicates higher occurrence.

Existing limitations

This study solely focused on publications within the Scopus database and did not encompass other databases, such as PubMed and Web of Science, which may yield marginally distinct outcomes. Despite being the largest peer-reviewed abstracts and citation database globally, it is plausible that several papers on this topic may have been published in journals not incorporated in Scopus. The other limitation of our study, which is inherent to any bibliometric approach, is that we did not examine individual article records beyond the random sample used for verifying index accuracy. Instead, we relied on MEDLINE indexes for classification purposes. Nevertheless, the manually verified 5% sample had a high level of accuracy; thus, we can confidently assert that the results obtained through bibliometric analysis are valid.

Conclusions

This extensive bibliometric study provides researchers with a global overview of academic trends, geographical distribution, and collaboration patterns in the field of gut microbiota and IBD research over the past 20 years. The comprehensive analysis and structured data presented in this study benefit scientists in screening academic interests and informing policymakers in developing policies related to this topic. Furthermore, the results reveal the significant role of gut microbiota in IBD and lay the groundwork for further explorations in the future.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

Author contributions

AZ: Writing – original draft, Writing – review & editing. FW: Writing – review & editing. DL: Writing – review & editing. C-ZW: Writing – review & editing. HY: Writing – original draft, Writing – review & editing. J-YW: Writing – review & editing, Writing – original draft. C-SY: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1264705/full#supplementary-material

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Gut microbiota and immune mediation: a Mendelian randomization study on granulomatosis with polyangiitis

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Background: The gut microbiota plays a pivotal role in influencing various health outcomes, including immune-mediated conditions. Granulomatosis with Polyangiitis (GPA) is one such condition, and its potential associations with gut microbiota remain underexplored.

Method: Using a two-sample Mendelian randomization approach, we investigated the causal links between gut microbiota and GPA. We sourced our data from multiple cohorts and consortiums, including the MiBioGen consortium. Our study design incorporated both direct associations and mediation effects of immune traits on the relationship between gut microbiota and GPA.

Results: Our analysis revealed significant associations between 1 phylum, 1 family 9 genus microbiota taxa and GPA. Furthermore, we identified several immune cell traits that mediated the effects of gut microbiota on GPA. For instance, the family Defluviitaleaceae and genus Defluviitaleaceae UCG011 influenced GPA through CD11c in granulocytes. The mediation effect proportions further elucidated the complex dynamics between gut microbiota exposures, immune markers, and their combined influence on GPA.

Conclusion: Our findings underscore the intricate relationship between gut microbiota, immune markers, and GPA. The identified associations and mediation effects provide valuable insights into the potential therapeutic avenues targeting gut microbiota to manage GPA.

KEYWORDS

granulomatosis with polyangiitis, gut microbiota, immune cell, mediation analysis, mendelian randomization

Background

Granulomatosis with Polyangiitis (GPA), previously known as Wegener's granulomatosis, is a rare form of vasculitis primarily impacting the respiratory tract and kidneys (1). Left untreated, GPA can lead to organ damage and can be life-threatening (2). The standard therapeutic approach involves immunosuppressive agents, mainly corticosteroids coupled with drugs like cyclophosphamide or rituximab (3). These therapy will bring side effect when have a long term, some report show more than 40% morbidity of side effect (1). However, the recurrence rate remains a challenge, with many patients experiencing disease flare-ups after achieving remission (4).

The genesis of GPA, although not fully elucidated, is believed to be a combination of genetic and environmental triggers (5). Recent genomic studies have identified several susceptibility loci associated with GPA, highlighting inherited factors in its onset (6). Parallel to the increasing understanding of genetic underpinnings of autoimmune disorders, there has been a burgeoning interest in the gut microbiota's role in modulating immune responses (7). The human gut is home to trillions of microbes that play a pivotal role in maintaining gut homeostasis, influencing metabolic processes, and modulating the immune system. Emerging evidence suggests that gut microbial dysbiosis can lead to aberrant immune responses, thereby suggesting its potential role in GPA pathogenesis.

Mendelian randomization (MR) analysis offers a unique approach to discerning causal relationships in observational data by leveraging genetic variants as instrumental variables (8). This method is particularly powerful in delineating the role of gut microbiota in disease pathogenesis, as it can minimize confounding and reverse causation, inherent limitations in conventional observational studies. Moreover, the immune system, with its myriad of cell types and signaling pathways, plays a central role in the pathogenesis of GPA. By analyzing a comprehensive set of 731 immune cell traits, there's an opportunity to pinpoint specific immune pathways or cells that act as mediators between gut microbial composition and the onset or progression of GPA.

In conclusion, this study aims to bridge the knowledge gap between gut microbiota, immune modulation, and the pathogenesis of GPA. By leveraging advanced genetic techniques and a comprehensive analysis of immune cell traits, the research seeks to shed light on potential therapeutic targets and provide a deeper understanding of GPA's intricate etiology, which will be useful in prevention, morbidity, recur and low side effect by intervening gut microbiota taxa and immune cell.

Method

Study design

In our study, we employed a two-sample Mendelian randomization approach (9) to investigate the possible causal links between gut microbiota and Granulomatosis with Polyangiitis (GPA). To deepen our understanding of the mediation by immune traits, we adopted a two-step (network) MR strategy (10). The study's design and progression are illustrated in Figure 1.

Data sources

Our research utilized data from multiple cohorts and consortiums to investigate the links between gut microbiota and GPA. The pivotal gut microbiota data for our investigation was sourced from the MiBioGen consortium (11). The MiBioGen consortium serves as a vast database, diligently compiling and analyzing genome-wide genotypes alongside 16S fecal microbiome data. This rich dataset includes 18,340 participants from 24 unique cohorts. Impressively, a major chunk of this data, specifically 14,306 participants, comes from 18 European-descent cohorts. The consortium has made thorough adjustments for variables such as sex, age, and genetic principal components (PCs). Additionally, incorporated alpha diversity indices and technical covariates, including DNA isolation methods and genotyping platforms. Quality control measures, such as minor allele frequency (MAF) and the removal of outliers, were also implemented. It's worth noting, however, that while diet, medication (including PPIs and antibiotics), and lifestyle are acknowledged influencers of the microbiome, they were not incorporated into our analysis. More detail information provided in Supplementary Table 1.

For GPA, data was extracted from the FinnGen R9 GWAS, comprising 413 cases and 365,533 controls, also used sex, age, genotyping batch and ten PCs as covariates to adjust (12). To further understand the genetic intricacies of immune functions, we integrated a dataset from Orrù V et al. This dataset offers insights into 731 immune cell traits, derived from an analysis of over 3,000 participants (13). To maintain uniformity, all study participants are of European descent, with comprehensive details provided in Supplementary Table 1.

SNP selection

The validity of an MR analysis hinges on three core premises (Figure 2): a) Instrumental variables (IVs) should be free from confounding; b) There should be a strong link between IVs and the exposure; c) IVs should influence the outcome exclusively through the exposure. Our initial step was to pick single nucleotide polymorphisms (SNPs) from the GWAS summary data related to exposures. Only those exposures that had a genome-wide significant association ($p < 5 \times 10^{-8}$) with the traits were chosen as IVs. Given the limited number of IVs, we relaxed the significance level to 5×10^{-5} to avoid potential errors from a limited SNP pool. For the mediation analysis, we adjusted the significance levels based on the count of selected SNPs being more than 20. We then employed linkage disequilibrium clumping to exclude specific SNPs that weren't desirable ($r^2 > 0.01$, window size < 10,000 kb) (14). Subsequently, we synchronized the datasets for exposure and outcome, and eliminated palindromic SNPs with allele frequencies close to 0.5. The chosen SNPs are elaborated in Supplementary Table 2.

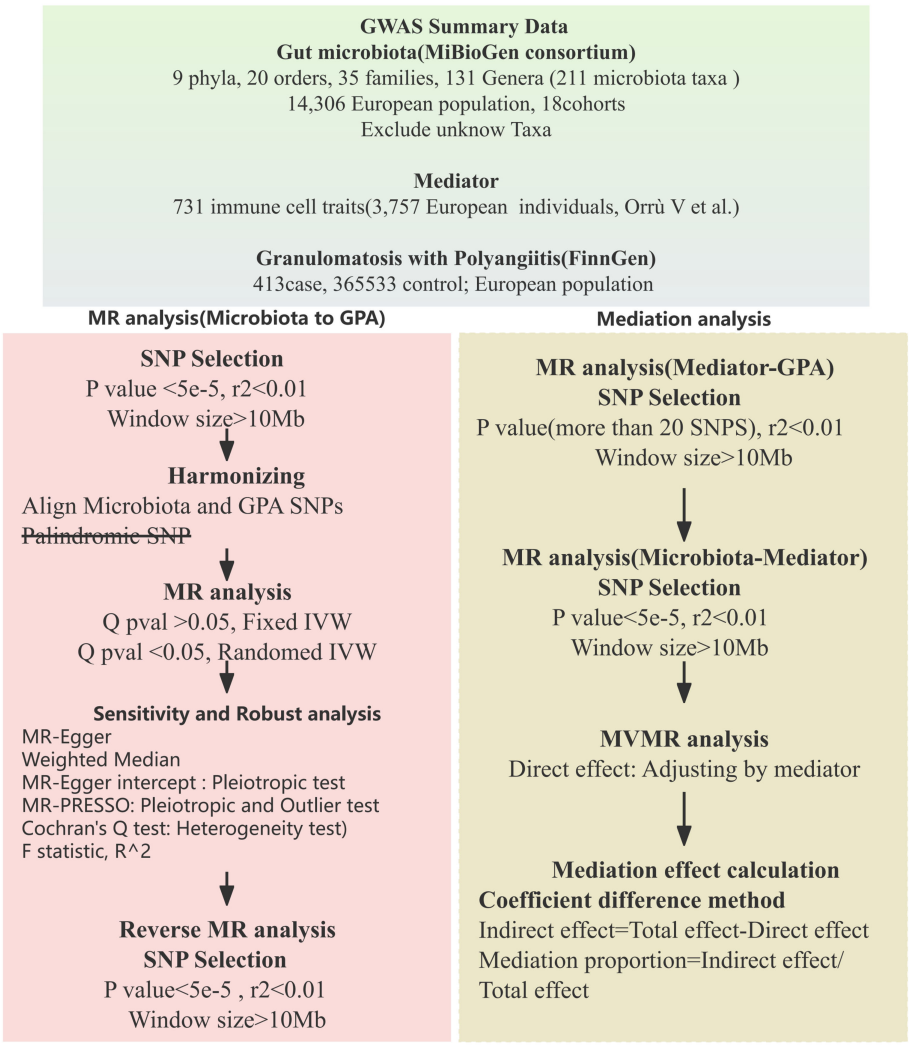

FIGURE 1
Mendelian randomization analysis flow chart.

To ensure the reliability of the genetic tools for exposures, we determined the F statistic using the given formula: $F = R^2 \times [(N - 1 - k)/k] \times (1 - R^2)$. Here, R^2 denotes the total variance explained by the chosen SNPs, N represents the sample size, and k stands for the number of SNPs considered. An F statistic above 10 suggests adequate strength, mitigating concerns of weak instrument bias in the two-sample approach (15).

Statistical analysis strategy

We conducted a bidirectional two-sample MR analysis to assess the connection between gut microbiota and GPA. Our main analysis employed the inverse variance-weighted (IVW) meta-analysis method, a well-established technique for MR studies (16). According to taxonomic classification levels, we use Bonferroni

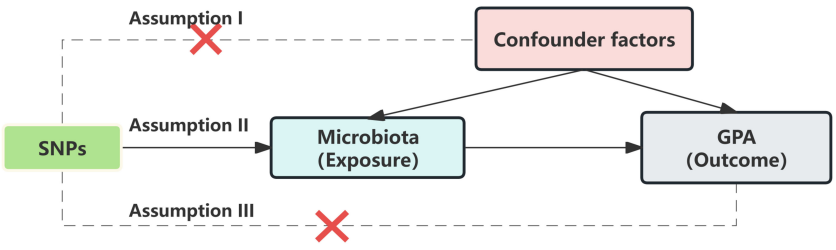

FIGURE 2
Mendelian randomization assumption.

correction respectively. To enhance the reliability of our findings, we also performed additional analyses using the weighted median (17) and MR-Egger regression methods (18). We evaluated the potential influence of directional pleiotropy by examining the intercept value in the MR-Egger regression (19). The MR PRESSO was utilized to detect pleiotropy and outliers. We gauged heterogeneity using Cochran's Q test (20). When faced with heterogeneity, we chose a random-effects IVW for our primary analysis. All statistical analyses were conducted using the R software, version 4.3.1. For our Mendelian randomization approach, we utilized the "TwoSampleMR" package available in R. This package facilitated the harmonization of our datasets and the execution of various MR methods, ensuring robust and consistent results. For generating visual representations of our findings, we employed Python-based plotting libraries.

Result

Two sample Mendelian randomization analysis between microbiota and GPA

Utilizing Mendelian randomization, we delved into the associations between specific gut microbiota taxa and Granulomatosis with Polyangiitis (GPA). As Figure 3 show Phylum Firmicutes emerged with a positive association to GPA (OR = 1.68, 95% CI: 1.19-2.37, p-value = 0.003). Genus Ruminococcus torques illustrated a protective effect (OR = 0.6, 95% CI: 0.41-0.88, p-value = 0.010). Genus Desulfovibrio and Genus Lactococcus were linked positively to GPA, presenting ORs of 1.44 and 1.31, respectively. Conversely, Genus Eubacterium oxidoreducens, Family Defluviitaleaceae, and Genus Defluviitaleaceae UCG011 showcased protective roles, with respective ORs. Other significant taxa such as Ruminococcaceae UCG004, Ruminiclostridium5, Prevotella9, and Phascolarctobacterium further elucidated the intricate relationship between the gut microbiota and GPA.

After Bonferroni correction, Phylum Firmicutes(p-value=0.029) still be significant. The variability in GPA explained by these taxa, represented by R^2 values, spanned from 4.56% to 8.82% (Supplementary Table 3). Furthermore, the robustness of our instruments was evident from the F-statistics, which consistently hovered between 18.95 and 19.98. Critically, our results demonstrated an absence of heterogeneity and pleiotropy. Sensitivity tests, such as MR Egger and Weighted Median (WM), most of them supported the primary outcomes, showcasing consistent directions. MR PRESSO analysis show no outlier and pleiotropy (Supplementary Table 3).

In our assessment through Phenoscanner, none of the included SNPs demonstrated a significant association with infections, autoimmune conditions, or antibiotic use. In reverse MR analysis, There no significant result can be found (Supplementary Table 4).

Mediator screening

In our study aimed at identifying potential mediators, we initially selected 731 immune cell traits to investigate their effects

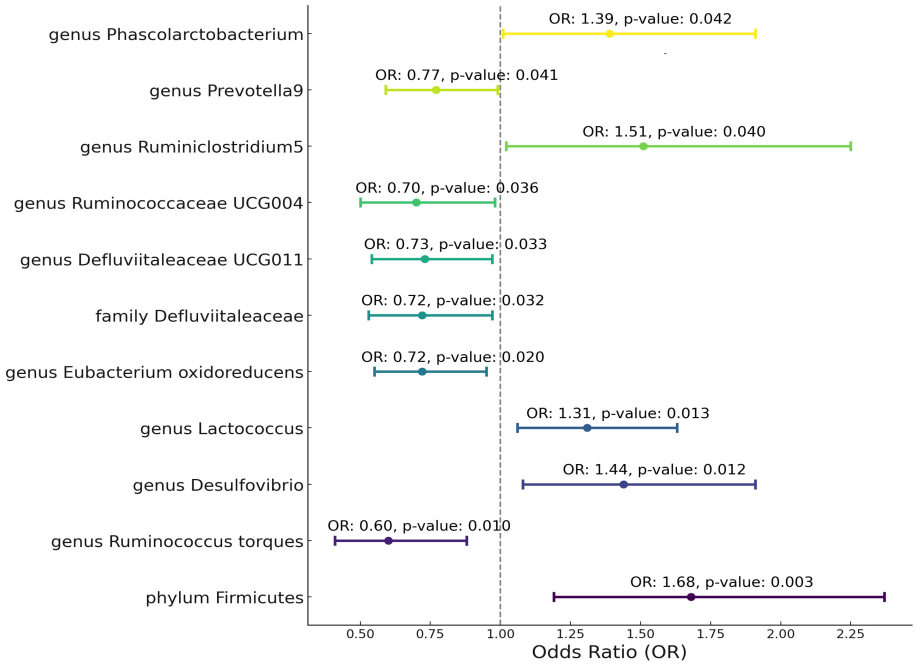

FIGURE 3
Mendelian randomization analysis between Microbiota and Granulomatosis with Polyangiitis. This plot visualizes the association between microbial exposures and Granulomatosis with Polyangiitis (GPA). Each point denotes the Odds Ratio (OR) for the exposure. Horizontal lines represent the 95% confidence intervals. The vertical dashed line at OR=1 serves as a reference for no effect. Annotations provide the OR value and p-value for statistical significance.

on GPA. In our analysis examining the association between these immune cell traits and GPA, we found several significant relationships (Figure 4). The percentage of naive-mature B cells in lymphocytes was associated with a decreased risk (OR = 0.92, p = 0.0441). Similarly, the absolute count of CD11c+ HLA DR++ monocytes, CD33- HLA DR- cells, and central memory CD4- CD8- T cells were linked with odds ratios of 1.07 (p = 0.0242), 1.09 (p = 0.0108), and 0.85 (p = 0.0094) respectively. Notably, the presence of HLA DR on CD14+ monocytes exhibited a more than twofold increased risk (OR = 2.43, p = 0.0010). Other significant associations included exposures such as CD25 on naive-mature B cells (OR = 1.25, p = 0.0396) and CD11c on granulocytes (OR = 0.72, p = 0.0017), among others. These findings highlight the intricate relationship between specific cellular markers and GPA, providing a foundation for further mediation analyses. In pleiotropy analysis (Supplementary Table 5), we find two result show the significant pleiotropy, including HLA DR on CD14+ CD16- monocyte and HLA DR on CD14+ monocyte. So, we use MR-Egger result as primary result. In heterogeneity analysis, there are a result show the heterogeneity in HLA DR on CD33+ HLA DR+ CD14-, we used random IVW as primary analysis.

Following our examination of the influence of immune cell traits on GPA, we further explored the potential mediation effects of gut microbiota exposures on these significant mediators (Figure 5). Our analysis yielded several noteworthy findings. The family Defluviitaleaceae and genus Defluviitaleaceae UCG011 were both observed to influence GPA through their impact on CD11c in granulocytes, with effect sizes of 0.13 (p = 0.0256 and p = 0.0289, respectively). Genus Desulfovibrio showcased a notable mediation effect on GPA via three different mediators: CD33- HLA DR- Absolute Count (β = 0.43, p = 0.0492), HLA DR on CD14+ CD16- monocyte (β = 0.11, p = 0.0358), and HLA DR on CD14+ monocyte (β = 0.11, p = 0.0402). Genus Eubacterium oxidoreducens demonstrated a negative mediation effect through HLA DR on both CD14+ CD16- monocyte and CD14+ monocyte, with effect sizes of -0.11 (p = 0.0264 and p = 0.0297, respectively). Several other genera, including Lactococcus, Phascolarctobacterium, Ruminiclostridium5, Ruminococcaceae UCG004, and Ruminococcus torques, also displayed varying mediation effects through a range of immune cell traits. These results underline the complex interplay between gut microbiota exposures and specific immune markers in influencing GPA, offering a deeper understanding of the pathways involved. There no significant heterogeneity were shown in analysis (Supplementary Table 6). The MR-Egger show significance while there were significant pleiotropy in three result including genus Desulfovibriogenus, Phascolarctobacterium, Ruminiclostridium5.

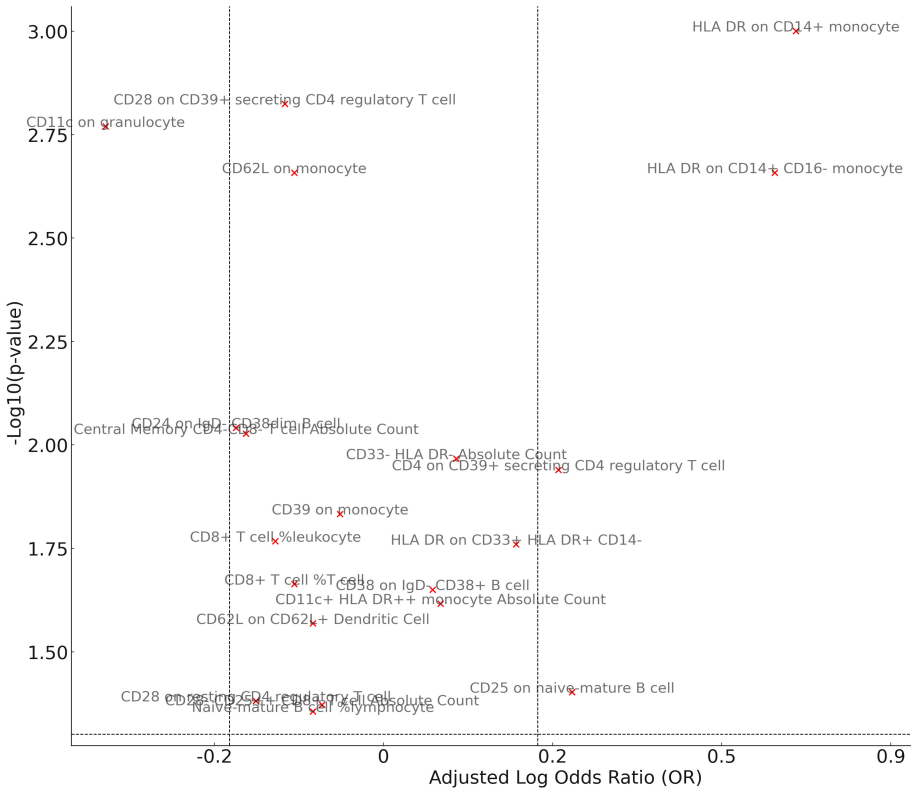

FIGURE 4
Mendelian randomization analysis between Mediator and Granulomatosis with Polyangiitis. The volcano plot visually illustrates the associations between cellular exposures and GPA. The x-axis represents the adjusted Log OR, indicating the direction and strength of the association, while the y-axis showcases the -Log10(p-value) for significance levels. Exposures are color-coded, with red dots signifying significant associations (p-value < 0.05) and grey dots denoting non-significant relationships. The plot also includes reference lines that mark OR thresholds of 1.2/0.83 and a p-value of 0.05.

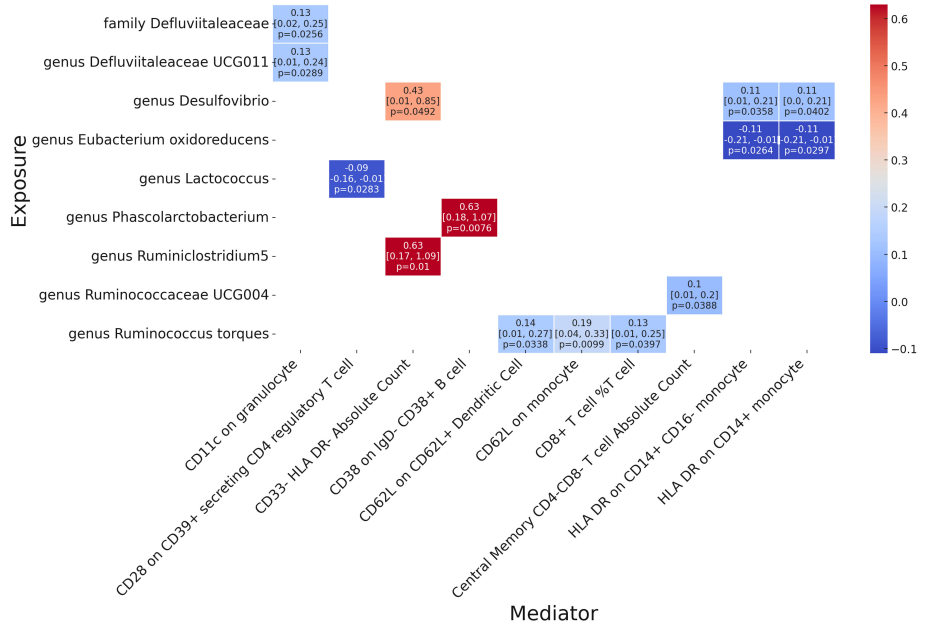

FIGURE 5
Mendelian randomization analysis between Microbiota and Mediator.

Multivariable MR and mediation analysis

After pinpointing significant mediators influencing GPA and the subsequent effects of exposure on mediation, we quantified the mediation effect proportions. This entailed calculating the indirect effect, derived from the total effect minus the direct effect, with the direct effect assessed based on the immediate influence of the gut microbiota, adjusting for the mediator in the Multivariable Mendelian Randomization (MVMR) analysis (Supplementary Table 7). Specifically, as the Figure 6 show the family Defluviitaleaceae and the genus Defluviitaleaceae UCG011 mediated their effects on GPA through CD11c on granulocytes with proportions of 14.45% and 30.83%, respectively. The genus Desulfovibrio exhibited mediation effects via CD33- HLA DR- Absolute Count (2.01%), HLA DR on CD14+ CD16- monocyte (4.76%), and HLA DR on CD14+ monocyte (35.10%). Eubacterium oxidoreducens channeled its effects through HLA DR on CD14+ CD16- monocytes (17.63%) and HLA DR on CD14+ monocytes (40.10%). Other notable mediations include Lactococcus via CD28 on CD39+ secreting CD4 regulatory T cell (13.76%), Phascolarctobacterium through CD38 on IgD- CD38+ B cell (21.60%), Ruminiclostridium5 via CD33- HLA DR- Absolute Count (15.92%), Ruminococcaceae UCG004 through Central Memory CD4-CD8- T cell Absolute Count (6.14%), and Ruminococcus torques via CD62L on monocyte (11.94%). These proportions underscore the intricate dynamics between specific gut microbiota exposures, their mediators, and their cumulative impact on GPA.

Discussion

The intricate relationship between the gut microbiota and immune-mediated diseases has been a topic of burgeoning interest in recent years. The microbiome and immune system share a complex relationship, influencing health and disease. Disruptions in this balance can lead to immune disorders (21, 22). Our study, which delved into the associations between specific gut microbiota taxa and GPA using Mendelian randomization, has provided compelling insights into this complex interplay. The phylum Firmicutes, genus Desulfovibrio, genus Lactococcus, genus Ruminiclostridium5, and genus Phascolarctobacterium have been found to be positively associated with GPA. This suggests that an increased abundance of these taxa might be linked to a higher risk of developing GPA. The genus Ruminococcus torques, genus Eubacterium oxidoreducens, family Defluviitaleaceae, genus Defluviitaleaceae UCG011, genus Ruminococcaceae UCG004, and genus Prevotella9 show a negative association with GPA. This indicates that these taxa might have a protective effect against the disease.

Notably, an enrichment of potential pathobionts (Enterobacteriacee and Streptococcaceae) was found in Eosinophilic Granulomatosis with Polyangiitis (23)., particularly in patients with active disease, while lower levels were found in patients on immunosuppression, compared with non-immunosuppressed ones. Significantly lower amounts of hexanoic acid were found in patients, compared to controls. The analysis of the immune response in the gut mucosa revealed a high frequency

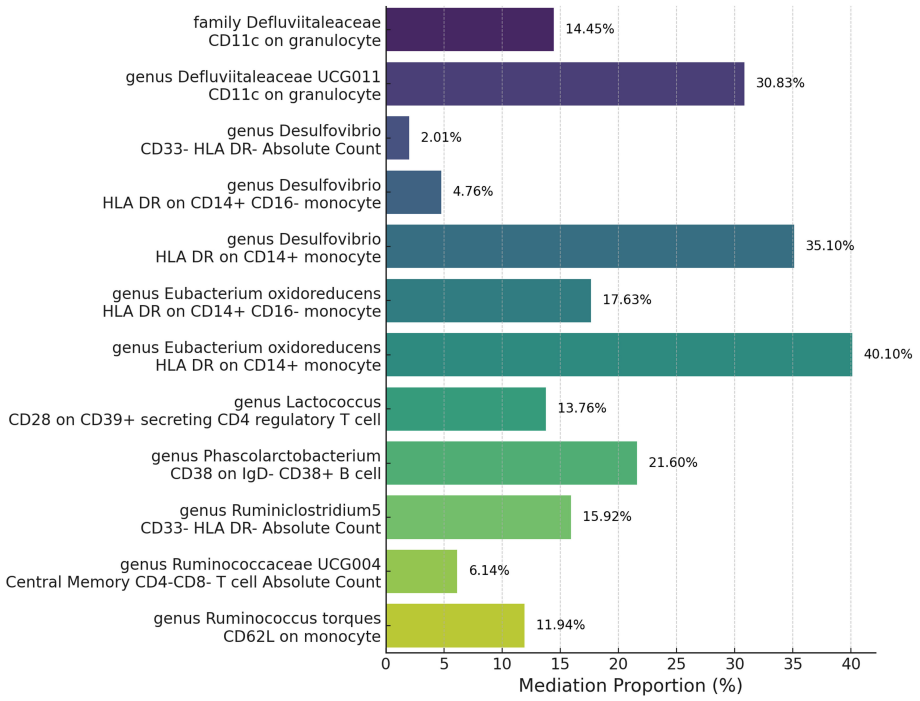

FIGURE 6
Mediation analysis of immune cell trait between Microbiota and Granulomatosis with Polyangiitis.

of IFN-γ/IL-17-producing T lymphocytes, and a positive correlation between EGPA disease activity and intestinal T-cell levels. Metagenomic sequencing demonstrated that this dysbiosis in active GPA patients is manifested by increased abundance of S. aureus and a depletion of S. epidermidis, further demonstrating the antagonist relationships between these species (24). SEED functional protein subsystem analysis identified an association between the unique bacterial nasal microbiota clusters seen mainly in GPA patients and an elevated abundance of genes associated with chorismate synthesis and vitamin B12 pathways. The richness and diversity of gut microbiota were reduced in AAV patients with kidney injury, and the alteration of gut microbiota might be related with the severity of kidney injury of AAV patients (25). All of these result show the significance between gut microbiota and immune, inflammation disease.

Gut microbiota can affect disease through immune cell or immune target. Several studies indicate that the gut microbiota can influence antitumor immunity and the effectiveness of cancer immunotherapies, particularly immune checkpoint inhibitors (26). Also. the gut microbiota affects tumor immunity by interacting with various immune cells. In the context of COVID-19, research has found a correlation between gut microbiota composition and disease severity (27), with harmful microbes linked to severe outcomes and beneficial ones to milder responses. The gut microbiota also plays a role in brain immunity, potentially influencing neurodegenerative diseases like Alzheimer's (28). Imbalances in the gut can exacerbate Alzheimer's symptoms due to impacts on intestinal and blood-brain barriers. External factors like diet and age can amplify these effects. Modifying the gut microbiota through dietary changes, probiotics, or fecal transplants may provide therapeutic avenues for Alzheimer's. A research observing significant shifts in both gut microbiota and immune cell populations (29). Analysis revealed consistent associations between specific gut bacteria and immune cell dynamics in cancer patients. The findings emphasize the considerable influence of the gut microbiota on systemic immune cell behavior, highlighting a quantifiable link between the two with potential therapeutic implications These relationship not only appear in innate immune (30), but also adapted immune (31).

In current study, the activation of immune cell, especially in granulocytes, cause the inflammation factor release, which cause GPA (32). Our mediation analysis find some immune cell trait can participate the effect of microbiota on GPA. As our result show that both the family Defluviitaleaceae and its genus Defluviitaleaceae UCG011 show mediation through CD11c on granulocytes. CD11c is an integrin commonly expressed on dendritic cells and is involved in various immune responses (33). The substantial mediation proportion, especially for the genus Defluviitaleaceae UCG011, suggests that this integrin might play a significant role in how these microbiota taxa influence GPA. HLA-DR is a major histocompatibility complex class II cell surface receptor, and its expression on monocytes indicates an activated state. The genus Desulfovibrio and Eubacterium oxidoreducens both show mediation through HLA-DR on different monocyte subsets (34). This suggests that these microbes might influence GPA by modulating monocyte activation and subsequent immune responses. The genus Lactococcus shows mediation through CD28 on a specific subset of regulatory T cells. CD28 is crucial for T cell activation, and its role in regulatory T cells suggests a potential modulation of immune tolerance (35). This could imply

that Lactococcus might influence GPA by affecting T cell-mediated immune regulation. The genus Phascolarctobacterium mediates its effect through CD38 on a specific B cell subset. CD38 is a multifunctional enzyme involved in calcium signaling and can influence B cell activation and differentiation (36). This suggests a potential role of B cell-mediated immunity in the relationship between this microbe and GPA. The genus Ruminococcaceae UCG004 shows mediation through central memory T cells, which play a crucial role in long-term immune protection. This could indicate that this microbe might influence GPA by modulating adaptive immune responses. The genus Ruminococcus torques mediates its effect through CD62L on monocytes. CD62L is involved in cell trafficking, and its expression on monocytes can influence their migration to inflammation sites (37).

Gut microbiota have several way to affect the immune system, include metabolites, Microbial components like LPS, produce immune mediator directly, influence the intestinal barrier et al (38). The gut microbiota synthesizes a vast array of metabolites, including but not limited to short-chain fatty acids (SCFAs) pivotal for immune modulation; tryptophan derivatives like indole, which interact with aryl hydrocarbon receptors; secondary bile acids influencing lipid metabolism; polyamines with anti-inflammatory properties; vitamins vital for immune function; immune-stimulating molecules like lipopolysaccharide (LPS); gases such as hydrogen sulfide (H_2S) that serve as signaling molecules; and neuroactive compounds that bridge gut-brain communication (39). In Our study, Desulfovibrio mainly utilize the dissimilatory sulfate reduction pathway for energy conversion by using hydrogen or organic compounds to reduce sulfate or oxidized sulfur compounds resulting in the production of H_2S (40, 41). H2S exhibits both pro-inflammatory and anti-inflammatory effects, depending on its concentration, cellular context, At low to moderate concentrations, H2S can possess anti-inflammatory properties (42). Conversely, in certain conditions, high concentrations of H_2S can promote inflammation. As our result of Desulfovibrio increase the monocyte, H_2S can active the monocyte and induces the synthesis of proinflammatory cytokines (43). Lactococcus primarily ferments sugars to produce lactic acid (44). Study find that regulator T cell can take up lactic acid, which will active the treg cell and increase the anti-inflammation effect of Treg through enhance PD-1 expression (45). Members of Ruminococcaceae are known for fermenting dietary fibers and producing SCFAs, primarily butyrate. Butyrate is an essential energy source for colonocytes (colon cells) and possesses anti-inflammatory properties (46). These SCFAs can increase proportion of double-negative T cells (CD4−CD8−, DNTs) (47), which can as regulatory T cells that are able to prevent immune related diseases (48). A surprisingly small number of organisms, dominated by Eubacterium appear to be responsible for the major fraction of butyrate production (49). Butyrate has been shown to possess anti-inflammatory properties, it can regulate human monocyte, decrease the IL-12 and up-regulation of IL-10 production (50). Also it can inhibits functional differentiation of human monocyte (51). Together, these metabolites illustrate the profound and multifaceted influence of microbial metabolism on host immunity and overall health.

Our research stands out due to its comprehensive methodology, integrating multiple rigorous analyses to delve into the associations between gut microbiota and GPA. The consistency of our findings across various methods, including the weighted median, MR-Egger, and the primary IVW, lends robustness to our conclusions. The application of the MR-PRESSO strategy further bolsters the credibility of our results by detecting and rectifying potential outliers, ensuring a reduced bias. Also, none of the included SNPs show a significant association with infections, autoimmune conditions, or antibiotic use, all of which can potentially impact GPA. A hallmark of our study is the detailed exploration of specific gut microbiota genera and their associations with GPA. While certain associations lost their statistical significance post adjustments for multiple testing, our inclination leans towards identifying more potential associations, even at the risk of some false positives. They provide intriguing insights into potential biological interactions. The uniformity in our study samples, predominantly of European descent, ensures a minimized bias due to population variations.

However, our study is not without its limitations. The primary constraint is the heavy reliance on European population data, which might introduce certain biases and restrict the broader applicability of our findings to other ethnic groups. Additionally, the lack of individual-level data curtailed our exploration into more intricate relationships, potentially overlooking non-linear associations between the gut microbiota, immune cell traits, and GPA. As a result, specific association patterns, such as U-shaped or J-shaped relationships, might have been overlooked.

In conclusion, our study underscores the pivotal role of gut microbiota in modulating immune responses and their potential implications in GPA. The identified associations and mediation effects pave the way for future research, emphasizing the importance of the gut-immune axis in health and disease. Potential therapeutic interventions targeting the gut microbiota could be explored as novel strategies for managing GPA and other related conditions.

Future research

The recent findings highlighting the mediation effects of immune cell traits between gut microbiota and GPA open a plethora of avenues for future research. A deeper mechanistic exploration into these mediators, such as the role of CD11c on granulocytes in relation to the family Defluviitaleaceae, could refine therapeutic strategies. Longitudinal studies would offer insights into the evolving interplay between gut microbiota, immune cell traits, and GPA progression, distinguishing causative from correlative associations. Validating these associations through functional assays in animal models and exploring dietary or probiotic interventions could pave the way for novel therapeutic approaches. Additionally, broadening the scope to other microbial taxa, considering environmental and genetic interactions, and

leveraging advanced sequencing techniques could provide a holistic understanding of GPA pathogenesis. Stratifying GPA patients based on clinical parameters and conducting global studies would further ascertain the universality of these findings. In essence, the intricate relationships unveiled between specific gut microbiota exposures, their mediators, and GPA underscore the need for comprehensive research to harness these insights for clinical advancements.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author. Gut microbiota is accessed through this link: https://gwas.mrcieu.ac.uk/datasets/ (Accsession number: ebi-a-GCST90016908 to ebi-a-GCST90017118). Immune cell trait canbe found in https://gwas.mrcieu.ac.uk/datasets/ (ebi-a-GCST90001391 to ebi-a-GCST90002121). Granulomatosis with Polyangiitis obtained from FinnGen consortium: https://storage.googleapis.com/finngen-public-data-r9/summary_stats/finngen_R9_M13_WEGENER.gz.

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

YC: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft. ST: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1296016/full#supplementary-material

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Akkermansia muciniphila - friend or foe in colorectal cancer?

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Akkermansia muciniphila is a gram-negative anaerobic bacterium, which represents a part of the commensal human microbiota. Decline in the abundance of *A. muciniphila* among other microbial species in the gut correlates with severe systemic diseases such as diabetes, obesity, intestinal inflammation and colorectal cancer. Due to its mucin-reducing and immunomodulatory properties, the use of probiotics containing *Akkermansia* sp. appears as a promising approach to the treatment of metabolic and inflammatory diseases. In particular, a number of studies have focused on the role of *A. muciniphila* in colorectal cancer. Of note, the results of these studies in mice are contradictory: some reported a protective role of *A. muciniphila* in colorectal cancer, while others demonstrated that administration of *A. muciniphila* could aggravate the course of the disease resulting in increased tumor burden. More recent studies suggested the immunomodulatory effect of certain unique surface antigens of *A. muciniphila* on the intestinal immune system. In this Perspective, we attempt to explain how *A. muciniphila* contributes to protection against colorectal cancer in some models, while being pathogenic in others. We argue that differences in the experimental protocols of administration of *A. muciniphila*, as well as viability of bacteria, may significantly affect the results. In addition, we hypothesize that antigens presented by pasteurized bacteria or live *A. muciniphila* may exert distinct effects on the barrier functions of the gut. Finally, *A. muciniphila* may reduce the mucin barrier and exerts combined effects with other bacterial species in either promoting or inhibiting cancer development.

KEYWORDS

intestinal inflammation, colorectal cancer, mucin-reducing bacteria, Akkermanisa municiphila, probiotic

Introduction

Gut microbiota plays an important role in maintaining intestinal homeostasis. Among a huge variety of gut colonizing bacteria, *Akkermansia muciniphila* (*A. muciniphila)* deserves special attention. *A. muciniphila* is a non-motile gram-negative mucin-degrading bacterium of the phylum *Verrucomicrobiota* first isolated from human faeces by Derrien et al. (1, 2). A strict anaerobe, *A. muciniphila* adapted to living in human intestine by producing mucin-degrading enzymes (α- and β-D-galactosidase, α-L fucosidase and other) to utilize mucins as a source of nitrogen and carbon (3, 4). In mucin-depleted culturing conditions *A. muciniphila* is capable of switching to glucose-driven glycolysis (5), thus utilizing the excess of glucose. Also, it was demonstrated that *A. muciniphila* utilizes circulating host lactate and urea (6), reshaping host systemic metabolism.

A. muciniphila is localized mostly in the colon mucus layer of healthy individuals with relative abundance of 3% (7). Akkermansia-like sequences were found in other anatomical regions of the human digestive tract and even in breast milk (7). *A. muciniphila* was reported as a part of commensal microbiota in other animal species, including mice (8–10), making mice a convenient animal model to study the *in vivo* functions of this microorganism (11, 12).

Over the past ten years, numerous studies addressed the role of *A. muciniphila* in health and disease. Reduced amounts of *A. muciniphila* were reported in obesity and type 2 diabetes (13) and are associated with Western-type diet. The same correlation was shown for a high-fat (14, 15) and high-sucrose diet in mice (16). It was also established that the decline in *A. muciniphila* abundance correlated with the development of intestinal inflammation, colorectal cancer, and even with cognitive disorders such as depression and anxiety (17). Thus, a promising therapeutic potential of *A. muciniphila* as a probiotic or postbiotic and gut microbiota modulator is widely recognized (18–20). However, several studies suggest that *A. muciniphila* over representation may correlate with negative prognosis of anti-cancer therapy (21). Animal studies aimed to elucidate the specific molecular mechanisms of *A. muciniphila* effects in colorectal cancer remain contradictory. In this regard, we hypothesized that the introduction of high doses of *Akkermansia* can lead to disruption of homeostasis and increased tumor growth, while moderate and gentle introduction of bacteria has a protective effect. In present article we are attempting to explain how the differences in experimental settings may affect the results in these earlier reported studies.

Regulatory of effects of *A. muciniphila* on gut homeostasis

Microbiota interacts with the immune system either directly by activating the immune cells or via production of immunomodulatory metabolites and other molecules. Recent studies suggested that *A. muciniphila* acquired mechanisms to control host metabolism in the gut and, therefore, may contribute to healthy niche maintenance. For example, protein P9 secreted by *A. muciniphila* was reported to directly promote the production of GLP-1 by the human primary intestinal epithelial cells, stimulating insulin production and fat browning (22). The most abundant outer membrane pili protein of *A. muciniphila*, Amuc_1100, was shown to provide beneficial effects in HFD-mice (23). As TLR2 activator, Amuc_1100 demonstrated effects on immune cells (24–26). Another study reported that Amuc_1100 synthesis was increased in mucin-depleted conditions (5), while Khan et al. found that increased sugar consumption in mice may lead to overgrowth of *A. muciniphila* within 1 week and its mucin-degrading activity may result in thinning of the mucus layer (27).

Bae et al. identified a lipid from *A. muciniphila's* cell membrane, diacyl phosphatidylethanolamine with two branched chains (a15:0-i15:0 PE), that can contribute to immunomodulatory activity of bacteria in TLR2-TLR1 dependent manner. Interestingly, in high doses it triggers the release of TNF and IL-6 but not IL-10 or IL-12p70 by mouse BMDCs, while in low doses it resets activation thresholds and responses for immune signaling, so that weak activating signals are ignored and strong signals are moderated, contributing to the regulation of immune response (28).

A newly described outer membrane protein, Amuc_2172, was implicated in activation of immune cells via promotion of HSP70 production in cellular microenvironments (29). Bacterial control of the host immune system may indirectly affect barrier integrity, as well as suppression of autoimmunity against symbionts. It was shown that *A. muciniphila* secretes tripeptide RKH (Arg-Lys-His), which binds to TLR4 block signal transduction, rescuing mice form lethality in a model of CLP-induced sepsis (30). RKH production represents direct immune-suppressing activity of *A. muciniphila*. Since inflammation plays a significant role in cancer development, a proper use of the evolutionarily selected functions of *A. muciniphila* in its interaction with the host may represent novel therapeutic strategies to control inflammation and tumorigenesis.

A. muciniphila in gut inflammation control

Both colorectal cancer and inflammation are influenced by many factors, such as heredity, habits and nutrition, but in recent years much attention was paid to the relationship between the microbiota and the host immune system. The development of inflammatory bowel disease correlates with an increase in opportunistic microorganisms and a decrease in beneficial *Bifidobacteria* and *Lactobacilli* (31, 32). Colonization by *A. muciniphila* is thought to occur early in life during the induction of RORγt^{+}Foxp3^{+} Tregs to ensure intestinal homeostasis (33). At the same time, a decrease in the abundance *of A. muciniphila* is characteristic for inflammatory bowel diseases (34–36), as well as for dysbiosis associated with cancer (25).

TABLE 1 Effects of *A. muciniphila* in mouse models of acute and chronic intestinal inflammation and gastrointestinal cancer.

Effects of *A. muciniphila* in mouse models of acute and chronic intestinal inflammation						
#	Bacteria introduction protocol	Form of bacteria or antigen	Dose of bacteria	Effect	Colitis induction	Reference
1	Oral administration daily for 5 days during colitis induction	**Viable** *A. muciniphila* or outer membrane vesicles from *A. muciniphila*	10^8 CFU in 100 mcl per mouse or 20 mcg *A. muciniphila* OMVs in 100 mcl per mouse	Reduced colonic inflammation with **increased production of mucus**	7 days of 5% DSS	(37)
2	Oral administration daily for 14 days before colitis induction and after **antibiotic treatment**	**Viable** *A. muciniphila*	10^8 CFU in 100 mcl per mouse	Alleviated colitis severity and depression-like symptoms with more **intensive mucus production and *Muc2* expression**	7 days of 2,5% DSS after psychological stress (restraining)	(38)
3	Oral administration daily 7 days before colitis induction and during colitis induction	**Viable** *A. muciniphila*	3×10^9 CFU in 200 mcl per mouse	Ameliorated disease severity with enhanced barrier function and **alleviated colitis-induced dysbiosis**	7 days of 2% DSS	(39)
4	Oral administration daily for 7 days after **antibiotic treatment** and before colitis induction	**Viable** *A. muciniphila*	10^9 CFU in 300 mcl per mouse	Ameliorated disease severity and body weight loss with inhibited expression of inflammatory cytokines and **higher NRLP3 activation**	8 days of 3% DSS	(40)
5	Oral administration daily during **chronic colitis induction**	**Viable** *A. muciniphila* ATCC BAA-835 strain and isolated 139 substrain	2×10^8 CFU in 200 mcl per mouse	Improved clinical parameters including spleen weight, colon inflammation index, and colon histological score with decreased expression of inflammatory cytokines and fecal lipocalin-2. ATCC BAA-835 strain was more powerful in amelioration of inflammation than murine substrain 139	Three cycles of 3 days of 3% DSS	(41)
6	Oral administration daily 14 days before the colitis induction till sacrifice	**Pasteurized** *A. muciniphila* or **recombinant surface protein Amuc_1100**	1.5×10^8 CFU in 100 mcl per mouse Or 3 mcg of protein in 100 mcl per mouse	Reduced colonic inflammation with **decreased** proportion of **CTLs** in colon	8 days of 2% DSS	(25)
7	Oral administration daily 21 days before colitis induction and during colitis induction	**Recombinant protein Amuc_2109** from *A. muciniphila*	100 mcg/kg per mouse	Ameliorated disease severity and body weight loss with **inhibited expression of inflammatory cytokines and NRLP3 activation**	7 days of 2% DSS	(42)
8	Oral administration daily for 14 days after **antibiotics treatment**	**Viable** *A. muciniphila*	10^9 CFU per mouse	**Increased the levels of M1-like monocytes** ($CD45^+Ly6C^+MHCII^+$) in colon, blood, and bone marrow	7 days of 3% DSS after antibiotics	(43)

(Continued)

TABLE 1 Continued

Effects of *A. muciniphila* in mouse models of acute and chronic intestinal inflammation						
#	Bacteria introduction protocol	Form of bacteria or antigen	Dose of bacteria	Effect	Colitis induction	Reference
9	Oral administration daily after colitis induction till sacrifice	**Viable** *A. muciniphila* or **secreting extracellular vesicles** from *A. muciniphila*	5×10^8 CFU in 1 ml per mouse or 100 mg in 1 ml per mouse of secreting extracellular vesicles	More severe body weight loss with *A. muciniphila* introduction and attenuated weight loss with secreting extracellular vesicles introduction	10 days of 3% DSS	(29)
10	Oral administration daily during colitis induction till sacrifice	**Viable** *A. muciniphila* or **extracellular vesicles** from *A. muciniphila*	5×10^8 CFU or 100 mg of extracellular vesicles per mouse	More severe body weight loss with *A. muciniphila* introduction and attenuated weight loss with extracellular vesicles introduction	5 days of 2% DSS	(44)
Differential effects of *A. muciniphila* in mouse models of gastrointestinal cancer						
#	Model of cancer	Bacteria introduction protocol	Form of bacteria or antigen	Dose of bacteria	Effect	Reference
1	AOM/DSS-induced colitis-associated colorectal cancer	Oral administration at the 0, 3, 5, and 7 days of experiment before cancer induction	**Viable** *A. muciniphila*	**High dose** (10^9 CFU) in 100 mcl per mouse	Increased number of colon tumors, more colon damage, **increased expression of inflammation markers, decreased mucus production**	(45)
2	AOM/DSS colitis-associated colorectal cancer	Oral administration every day after **antibiotic treatment** from 3 days before the DSS treatment to sacrifice but skipped the DSS treatment period	**Viable** *A. muciniphila*	**High dose** (3×10^9 CFU) in 200 mcl per mouse	Increased number of colon tumors, impaired gut barrier function, **increased expression of inflammation markers, decreased mucus production**	(46)
3	Spontaneous tumorigenesis in $Apc^{15lox/+}$ mice	Oral administration three times started at 4 weeks of age after 1 week of **antibiotic treatment** till sacrifice	**Viable** *A. muciniphila*	**High dose** (10^9 CFU) in 100 mcl per mouse	Increased number of tumors, but **more intensive mucus production**	(47)
4	AOM/DSS colitis-associated colorectal cancer	Oral administration daily 14 days before the cancer induction till sacrifice	**Pasteurized** *A. muciniphila* or **recombinant surface protein Amuc_1100**	**Low dose** (1.5×10^8 CFU) in 100 mcl per mouse or 3 mcg of protein in 100 mcl per mouse	Decreased number of colon tumors with **expanded CTLs** in the colon and MLN	(25)
5	AOM/DSS colitis-associated colorectal cancer	Oral administration daily after cancer induction till sacrifice	**Secreting extracellular vesicles** from *A. muciniphila*	100 mg in 1 ml per mouse	Decreased number of colon tumors with **increased CTLs activity**	(29)
6	Spontaneous tumorigenesis in $Apc^{Min/+}$ mice	Intraperitoneal injection twice a week for 14 weeks	**Recombinant surface protein Amuc_2172**	150 mcg/kg per mouse	Decreased number of tumors with **increased CTLs**	(29)

(Continued)

TABLE 1 Continued

Differential effects of *A. muciniphila* in mouse models of gastrointestinal cancer						
#	Model of cancer	Bacteria introduction protocol	Form of bacteria or antigen	Dose of bacteria	Effect	Reference
7	Spontaneous tumorigenesis in $Apc^{Min/+}$ mice with two cycles of 10-day 1% DSS	Oral administration every two days for three months after **antibiotic treatment** starting from 6-8 weeks of age till sacrifice	**Viable** *A. muciniphila*	**High dose** (10^9 CFU) in 300 mcl per mouse	Suppressed colonic tumorigenesis, decreased systemic inflammation through facilitated **enrichment of M1-like macrophages** in an NLRP3-dependent, TLR2-dependent manner	(43)
8	Subcutaneous injection of CT26 cells in BALB/c mice	Oral administration started when the tumor reaches size 100 mm^3 performed every day until the end of the experiment along with intraperitoneal injection of anti–PD-1	**Viable** *A. muciniphila* or **outer membrane vesicles** from *A. muciniphila*	**Low dose** (10^8 CFU) in 100 mcl per mouse or 20 mcg *A. muciniphila* OMVs in 100 mcl per mouse	Decreased tumor size with **enhanced aPD-1 therapy efficacy**	(37)
9	Subcutaneous injection of HCT116 or CT26 cells in BALB/c nude mice	Subcutaneous injection of 3×10^6 HCT116 or CT26 cells mixed with *A. muciniphila* (MOI = 10:1)	**Viable** *A. muciniphila*	**Low dose** (3×10^7 CFU) per mouse	Suppressed growth of implanted HCT116 or CT26 tumors	(43)
10	Subcutaneous injection of CT26 cells in BALB/c mice	Intratumor injection twice a week after cancer induction till sacrifice	**Recombinant surface protein Amuc_2172**	150 mcg/kg per mouse	Inhibited allografted tumors growth by promoting **CTLs**	(29)
11	Subcutaneous injection of CT-26 cells with FOLFOX (oxaliplatin, fluorouracil and calcium folinate) treatment	Oral administration started when the tumor reaches size 100 mm^3 and performed every other day after **antibiotic treatment** until sacrifice	**Viable** *A. muciniphila*	**Low dose** (10^8 CFU) per mouse	**Enhanced anti-cancer effect of FOLFOX**, presumably due to *A. muciniphila* **effect on gut metabolomics**	(48)

Studies of *A. muciniphila* in mouse models of intestinal inflammation suggested a protective role of this bacterium or its derivatives (Table 1). For example, the administration of viable *A. muciniphila* in both low (10^8 CFU) and high (3×10^9 CFU) doses reduced the severity of colitis, increased mucus production (37, 38, 40), reduced the intensity of inflammation (40, 41), and also compensated for dysbiosis associated with inflammation (39). Furthermore, administration of pasteurized bacterium, which exemplifies the concept of "postbiotic" or a preparation of inanimate microorganisms and their components that confers a health benefit on the host (49), as well as recombinant Amuc_1100 or Amuc_2109, also reduced cytotoxic cell accumulation in the intestine and NRLR3 activation, suggesting strong antigenic properties of this bacterium (25, 42). Other data, on the contrary, indicate that the introduction of live bacteria aggravates the symptoms of colitis, however, the same studies showed the protective role of extracellular vesicles of *A. muciniphila* (29, 44). Since inflammation is the key factor in the development of colorectal cancer, and *A. muciniphila* has been shown to be an effective anti-inflammatory agent, the bacterium is considered a promising probiotic that can reduce the development of cancer.

A. muciniphila in mouse models of colorectal cancer

Studies on the role of *A. muciniphila* in mouse models of gut cancer provided contradictory results (Table 1). Several reports indicated that administration of *A. muciniphila* may aggravate the development of intestinal cancer. For example, Wang F. *et al.* found that administration of *A. muciniphila* prior to induction of colorectal cancer increased the number of intestinal tumors in the AOM/DSS model in correlation with a decrease in mucus production (45). In a similar study by Wang K. et al, oral gavage with *A. muciniphila* after a course of antibiotics led to increased tumor formation with a decrease in mucin expression (46). Finally, in a model of spontaneous tumor formation in $Apc^{15lox/+}$ mice, oral gavage with *A. muciniphila* after a course of antibiotics also increased the number of tumors, but, in contrast to the data of Wang F. and Wang K., it increased mucus production (47).

At the same time, other numerous studies confirm the protective effect of the enrichment with these bacteria on colorectal cancer. In particular, oral gavage with pasteurized *A. muciniphila*, surface antigen Amuc_1100 (25) or *A. muciniphila*

secretory extracellular vesicles (29) protected mice in the AOM/DSS model by increasing cytotoxic lymphocyte activity. An increase in the activity of cytotoxic lymphocytes was also shown for another *A. muciniphila* protein - Amuc_2172 in $Apc^{Min/+}$ mice (29). Interestingly, in the $Apc^{Min/+}$ model therapeutic administration of live *A. muciniphila* following antibiotics in the context of DSS-induced inflammation also reduced tumor burden, apparently due to a TLR2-mediated, NRLP3-dependent increase in the activity of antitumor M1 macrophages (43). The protective role of *A. muciniphila* was also shown in a number of studies using a transplantable tumor model. Therapeutic administration of bacteria or bacterial secretory vesicles *per os* reduced the growth of grafted CT26 and also enhanced the effect of anti-PD-1 therapy (37), while intratumoral administration of *A. muciniphila* (43) or Amuc_2172 (29) reduced the growth of allografts due to the activation of $CD8^+IFNy^+$ cytotoxic cells. Finally, it was established that administration of *A. muciniphila per os* enhanced the effect of the antitumor drug FOLFOX (oxaliplatin, fluorouracil and calcium folinate), and, conversely, the use of FOLFOX led to a significant increase in the *A. muciniphila* abundance in the gut (48).

Taken together, there is a major controversy over the effects of *A. muciniphila* on the development of intestinal cancer.

The molecular form of *A. muciniphila* shapes the outcome

One of the factors potentially explaining the different effects of *A. muciniphila* in colorectal cancer may be related to different protocols of bacterial administration - from live or pasteurized *A. muciniphila* to recombinant peptides and extracellular vesicles derived from these bacteria. Thus, in all studies in which *A. muciniphila* aggravated tumor growth, live bacteria was used at high concentration of 10^9 CFU (45–47), and in some studies, this bacteria was introduced following a course of antibiotics (46, 47). It was shown that the high dose of *A. muciniphila* after a course of antibiotics in colorectal cancer model dramatically changed the composition of the microbiota. There was no expansion of *A. muciniphila* itself, but rather an increase in the opportunistic bacteria, including *Clostridia*. This resulted in aggravation of dysbiosis and disturbance in the metabolic profile as indicated by a decrease of bile acids and short-chain fatty acids (46). Presumably, a high dose of *A. muciniphila*, especially after the depletion of gut microbiota with antibiotics, can be interpreted by the immune system as an infection and leads to an increased inflammation due to disrupted microbiota composition and increased opportunistic pathogens in the gut. Moreover, it was shown that in antibiotic treated mice, some phylogroups of *A. muciniphila* may outcompete others, affecting the outcome of the *A. muciniphila* colonization. Distinct phylogroup-specific phenotypes of the *A. muciniphila* modulate oxygen tolerance, iron and sulfur metabolism, and bacterial aggregation differently, therefore, the genetic variations of *A. muciniphila's* strains may influence the effect of bacterial colonization after antibiotic treatment (50). Recently, it was suggested that *A. muciniphila* phylogroups, which bear mutations in *mul* gene-cluster, lack immunomodulatory effects, but are able to colonize gut in germ-free conditions (4). It can be proposed that under antibiotic treatment, "weak" variants of the microorganism can take root and mask the immunomodulatory effects (4). Assumption about the detrimental effect of the microbiota composition disruption after antibiotics is further supported by the observed decrease in mucin expression (45, 46) upon administration of *A. muciniphila* following antibiotics. The thinning of the mucin layer allows other microorganisms to penetrate the tissue more actively and aggravate cancer-promoting intestinal inflammation.

A. muciniphila has a direct effect on mucus production in the intestine. In this context, it appears important to establish whether different forms of bacteria - viable bacteria or pasteurized bacteria, providing distinct sets of antigens, affect the production of major mucins in the intestine. It turned out that both forms of *A. muciniphila* differently increased the expression of mucins in the gut (Figure 1). For example, in the colon, only pasteurized bacteria caused a significant upregulation in the expression of *Muc1* and *Muc4*, while administration of viable *A. muciniphila* alone increased the expression of *Muc2*. In the small intestine, viable, but not pasteurized, bacteria caused a slight increase in *Muc1* expression, and only pasteurized *A. muciniphila* affected the expression of *Muc2*. The expression level of *Muc3* was not affected by either form of the bacterium. Thus, the thickness of the mucus layer following *A. muciniphila* administration was significantly influenced by the form in which bacteria were administered, as well as by the tissue specificity. In the context of colorectal cancer, the thickness of the mucus layer in the large intestine is important, and the significant upregulation of mucin expression observed with the introduction of viable and pasteurized *A. muciniphila* may provide protection to tissues from microbiota invasion and inflammation (52).

In most studies administration of *A. muciniphila* protected mice from colorectal cancer. Interestingly, these studies employed experimental protocols with lower dose of *A. muciniphila* (10^7-10^8 CFU) (37, 43, 45, 48) and without the course of antibiotics. Some studies utilized recombinant bacterial proteins (25, 29) or secreted extracellular vesicles from *A. muciniphila* (29, 37) while Wang L. et al. used pasteurized bacterium (25).Only one study, which used a high dose of *A. muciniphila* after antibiotics, reported a protective effect of the bacterium (43). Thus, we propose that administration of the lower dose of *A. muciniphila* either in viable or pasteurized forms, as well as bacterial proteins or peptides, while maintaining the native composition of gut microbiota, has a clear protective effect on intestinal cancer, regardless of the carcinogenesis model.

Moderation of *A. muciniphila* is the key to inflammation control

Although the mechanisms by which *A. muciniphila* controls intestinal inflammation and colorectal cancer are not fully understood, much is known about the immunomodulatory effects

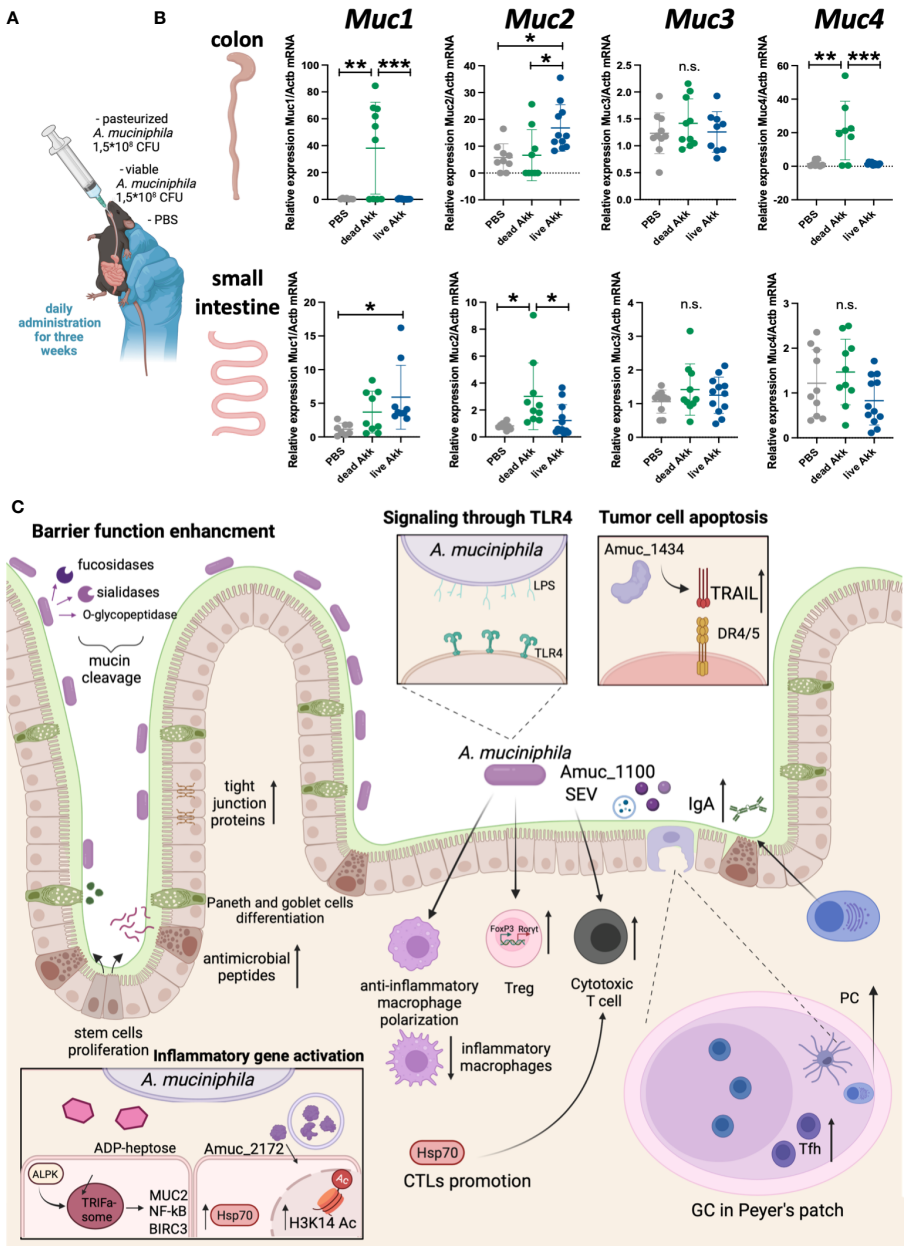

FIGURE 1
Live and pasteurized *A. muciniphila* differentially upregulate mucins expression in the gut. C57Bl/6 mice were housed in SPF conditions at the Animal Facility of the Center for Precision Editing and Genetic Technologies for Biomedicine, EIMB RAS (under the contract #075-15-2019-1660 from the Ministry of Science and Higher Education of the Russian Federation). At the age of 5-6 weeks animals of both sexes were randomly distributed between the groups and used in the experiments described below. All manipulations with animals were carried out in accordance with the protocol approved by the Bioethics Committee of the EIMB RAS (Protocol No. 3 from 27/10/22). *A. muciniphila* was grown anaerobically in the medium supplemented with porcine mucin (Sigma) and hemin (Sigma). The bacterial solution was collected at the concentration 7-8x10^7 CFU/mL, aliquoted by 1 mL and frozen at -80°C. **(A)** Scheme of experiment. To analyze the effect of bacteria inoculation on the gene expression at steady state C57Bl/6 WT mice were randomized into three groups of 7-9 individuals and then subjected to daily *per os* administration with PBS, 1.5x10^8 CFU of pasteurized (70°C, 30 min) *A. muciniphila* or 1.5x10^8 CFU of live *A. muciniphila* during 3 weeks. Fresh frozen in liquid nitrogen small intestine and colon were mechanically homogenized and lysed in ExtractRNA reagent (Evrogen, Russia). RNA was isolated by guanidinium thiocyanate-phenol-chloroform method following the manufacturer's protocol. RNA was reverse-transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo, USA) followed by quantitative real-time PCR. qPCRmix-HS SYBR+LowROX (5X) (Evrogen, Russia). Gene expression analysis was performed using Quant Studio 6 (Applied Biosystems. USA) and the following primer set: *Actb* (F: GCGCTCTTTCAGCCTTCTTT; R: TGGCATAGAGGTCCTTGCG), *Muc1* (F: TCGTCTATTTCCTTGCCCTG; R: ATTACCTGCCGAAACCTCCT), *Muc2* (F: CCCAGAAGGGACTGTGTATG; R: TTGTGTTCGCTCTTGGTCAG), *Muc3* (F: TGGTCAACTGCGAGAATGGA; R: TACGCTCTCCACCAGTTCCT), *Muc4* (F: GTCTCCCATCACGGTTCAGT; R: TGTCATTCCACACTCCCAGA). Reactions were run using the following program on the Applied Biosystems 7500: 95°C for 10 min, 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. **(B)** Relative expression level of *Muc1*, *Muc2*, *Muc3* and *Muc4* in colon and small intestine was normalized using *Actb* and calculated as 2^{-ddCt} fold change in experimental to control group (51). Each point in a diagram represents a single mouse; mean ± SD. *P < 0,05; **P < 0,01; ***P < 0,001; ns - not significant. One-way ANOVA test was used. **(C)** *A. muciniphila* in the gut inflammation and homeostasis.

of the bacterium (Figure 1C). *A. muciniphila* is known for its mucin-reducing activity, which determines its effect on the structural components of the intestine - epithelial cells, as well as Paneth cells and goblet cells. This bacterium can enhance intestinal barrier function: *A. muciniphila* increases the expression of tight junction proteins in response to disruption of epithelial integrity *in vivo* (23, 39) and *in vitro* (24, 53). In addition, *A. muciniphila* increases the proliferation of intestinal stem cells, as well as the differentiation of Paneth and goblet cells (54) with increased antimicrobial peptides (54) and mucus production (55). In addition to accelerating the renewal of the mucus in the intestine, *A. muciniphila* activates the differentiation of Tregs in the large intestine (56) and mesenteric lymph nodes (41). Not unexpectedly, induction of protective RORγt$^+$ Tregs by *A. muciniphila* is dependent on TLR4 (33, 57). *A. muciniphila*, its secretory vesicles and antigens activate a cytotoxic response in the intestine (25, 29), and, at the same time, suppress the proliferation of inflammatory macrophages (25), activate the polarization of anti-inflammatory macrophages (58). It was shown that the Amuc_1434 protein can modulate the death of tumor cells through activation of tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL) (59). *A. muciniphila* upregulates genes involved in the maintanance of intestinal barrier function via ADP-heptose-dependent activation of the ALPK1/TIFA pathway (60). Finally, *A. muciniphila* may regulate IgA production by plasma cells by affecting the number of Tfh in Peyer's patches (61), and thus influencing the microbiota composition.

Dysbiosis is a hallmark of inflammation and intestinal cancers. *A. muciniphila* is an important component of the normal microbiota, and changes in its abundance affect the course of the disease. *A. muciniphila* is capable of inducing both proinflammatory and anti-inflammatory mechanisms. Studies on the role of *A. muciniphila* in intestinal inflammation show its protective properties in barrier restoration and control of inflammation, while data obtained in colorectal cancer models remain contradictory. Some studies indicate a decrease in tumor burden, while others report an increase in tumor growth when the bacterium is introduced. We attempted to directly compare different experimental protocols using *A. muciniphila* in various models of intestinal cancer and concluded that the introduction of large amounts of *A. muciniphila*, especially after a course of antibiotics, provokes dysbiosis, disrupts the intestinal barrier functions (62), and aggravates the inflammation that provokes cancer (46). At the same time, lower doses of the bacterium or its derivatives without prior depletion of the microbiota have a positive effect on the course of the disease. This assumption is supported by the clinical study on the correlation between the presence of *A. muciniphila* and the effectiveness of checkpoint therapy. The results of this study demonstrated that moderate, but not high *A. muciniphila* load in the stool correlated with a good prognosis (21). Thus, delicate modulation of the microbiota by *A. muciniphila* may become a promising strategy for adjunctive therapy of inflammatory bowel diseases and colorectal cancer.

Data availability statement

The original contributions presented in the study are included in the article/supplementary files. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by the Bioethics Committee of EIMB RAS Protocol No. 3 from 21.09.2023. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

EG: Conceptualization, Data curation, Investigation, Visualization, Writing – original draft, Funding acquisition, Project administration. EG: Investigation, Writing – original draft, Methodology. MB: Resources, Writing – review & editing. OP: Resources, Validation, Writing – review & editing. AS: Methodology, Writing – original draft. AY: Methodology, Writing – original draft. EB: Conceptualization, Writing – review & editing. SN: Conceptualization, Funding acquisition, Resources, Supervision, Validation, Writing – review & editing. AK: Conceptualization, Resources, Supervision, Validation, Writing – review & editing. MD: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Two-sample Mendelian randomization to study the causal association between gut microbiota and atherosclerosis

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Background: According to some recent observational studies, the gut microbiota influences atherosclerosis via the gut microbiota-artery axis. However, the causal role of the gut microbiota in atherosclerosis remains unclear. Therefore, we used a Mendelian randomization (MR) strategy to try to dissect this causative link.

Methods: The biggest known genome-wide association study (GWAS) (n = 13,266) from the MiBioGen collaboration was used to provide summary data on the gut microbiota for a two-sample MR research. Data on atherosclerosis were obtained from publicly available GWAS data from the FinnGen consortium, including cerebral atherosclerosis (104 cases and 218,688 controls), coronary atherosclerosis (23,363 cases and 187,840 controls), and peripheral atherosclerosis (6631 cases and 162,201 controls). The causal link between gut microbiota and atherosclerosis was investigated using inverse variance weighting, MR-Egger, weighted median, weighted mode, and simple mode approaches, among which inverse variance weighting was the main research method. Cochran's Q statistic was used to quantify the heterogeneity of instrumental variables (IVs), and the MR Egger intercept test was used to assess the pleiotropy of IVs.

Results: Inverse-variance-weighted (IVW) estimation showed that *genus Ruminiclostridium 9* had a protective influence on cerebral atherosclerosis (OR = 0.10, 95% CI: 0.01–0.67, P = 0.018), while *family Rikenellaceae* (OR = 5.39, 95% CI: 1.50–19.37, P = 0.010), *family Streptococcaceae* (OR = 6.87, 95% CI: 1.60–29.49, P = 0.010), *genus Paraprevotella* (OR = 2.88, 95% CI: 1.18–7.05, P = 0.021), and *genus Streptococcus* (OR = 5.26, 95% CI: 1.28–21.61, P = 0.021) had pathogenic effects on cerebral atherosclerosis. For *family Acidaminococcaceae* (OR = 0.87, 95% CI: 0.76–0.99, P = 0.039), the *genus Desulfovibrio* (OR = 0.89,

95% CI: 0.80–1.00, *P* = 0.048), the *genus RuminococcaceaeUCG010* (OR = 0.80, 95% CI: 0.69–0.94, *P* = 0.006), and the *Firmicutes phyla* (OR = 0.87, 95% CI: 0.77–0.98, *P* = 0.023) were protective against coronary atherosclerosis. However, the *genus Catenibacterium* (OR = 1.12, 95% CI: 1.00–1.24, *P* = 0.049) had a pathogenic effect on coronary atherosclerosis. Finally, *class Actinobacteria* (OR = 0.83, 95% CI: 0.69–0.99, *P* = 0.036), *family Acidaminococcaceae* (OR = 0.76, 95% CI: 0.61–0.94, *P* = 0.013), *genus Coprococcus2* (OR = 0.76, 95% CI: 0.60–0.96, *P* = 0.022), and *genus RuminococcaceaeUCG010* (OR = 0.65, 95% CI: 0.46–0.92, *P* = 0.013), these four microbiota have a protective effect on peripheral atherosclerosis. However, for the *genus Lachnoclostridium* (OR = 1.25, 95% CI: 1.01–1.56, *P* = 0.040) and the *genus LachnospiraceaeUCG001* (OR = 1.22, 95% CI: 1.04–1.42, *P* = 0.016), there is a pathogenic role for peripheral atherosclerosis. No heterogeneity was found for instrumental variables, and no considerable horizontal pleiotropy was observed.

Conclusion: We discovered that the presence of probiotics and pathogens in the host is causally associated with atherosclerosis, and atherosclerosis at different sites is causally linked to specific gut microbiota. The specific gut microbiota associated with atherosclerosis identified by Mendelian randomization studies provides precise clinical targets for the treatment of atherosclerosis. In the future, we can further examine the gut microbiota's therapeutic potential for atherosclerosis if we have a better grasp of the causal relationship between it and atherosclerosis.

KEYWORDS

cerebral atherosclerosis, coronary atherosclerosis, gut microbiota, Mendelian randomization, peripheral atherosclerosis

1 Introduction

Gut microbiota are microorganisms that colonize the host gut and may affect host physiology in various ways. Increasing evidence suggests that dysregulation of the gut microbiota is associated with the pathogenesis of various cardiovascular diseases (CVD), such as atherosclerosis, heart failure, atrial fibrillation, hypertension, obesity, and dyslipidemia (1). Atherosclerosis (AS), which is characterized by lipid accumulation and immune-inflammatory changes in arterial vessels, is a major contributor to CVD and may eventually result in its clinical complications, including cerebrovascular accident, myocardial infarction, and peripheral artery embolism (2). Since the development of AS is regulated by the gut microbiota (GM) and its metabolites, scholars regard this regulation mode as the GM arterial regulation axis (2). Gut microbiota plays a role in atherosclerosis mainly in the following three ways (3): First, the infection of the gut microbiota may lead to a harmful immune inflammatory response, thereby aggravating the formation of plaque or triggering plaque rupture. Secondly, the regulation of lipid metabolism by the gut microbiota affects the progression of atherosclerotic plaques. Finally, specific components of diet and gut microbiota metabolism can have multiple effects on atherosclerosis; for example, dietary fiber is beneficial for AS, whereas trimethylamine-N-oxide (TMAO), a metabolite of gut microbiota, is thought to be detrimental.

In addition, specific commensal bacteria in the host can be protective against AS. However, pathogens or opportunistic pathogens can promote atherosclerosis. Both types regulate host metabolism and inflammatory responses directly or indirectly via their metabolites (4). For example, earlier research has established that *Akkermansia muciniphila* and *Lactobacillus* may be next-generation probiotics or live biotherapeutic products that can reduce the risk of AS (2). Treatment with Akkermansia muciniphila reduces macrophage infiltration, chemokines, and pro-inflammatory cytokines and protects the integrity of the intestinal barrier, thereby alleviating AS lesions (5). In addition, several studies have shown that alterations in the gut microbial composition in obese patients are associated with the progression of AS (6, 7), the most obvious changes were the decrease in the proportion of *Bacteroidetes phylum* and the increase in the proportion of *Firmicutes phyla*. In addition, pathogen and opportunistic pathogens including *Actinomycetes, Porphyromonas gingivalis, aggregating bacilli, Streptococcus hemolytic, Streptococcus pneumoniae, Staphylococcus aureus, Streptococcus viridans*, etc.,

which promote the transport of intestinal bacteria by destroying the integrity of the intestinal barrier and promoting the formation of atherosclerotic plaques, are considered to promote AS (8, 9).

In addition to the pathogenic role of their pathogens, gut microbes can also affect the process of atherosclerosis through their metabolites. The well-known metabolites are TMAO (10), secondary bile acids (11), short-chain fatty acids (12), and lipopolysaccharide (13) are also involved in the process of atherosclerosis. For instance, Synphytes, Clostridium, Desalinobacter, Desulfurvibrio, and members of Fusobacteriaceae have been linked to the development of AS by significantly positive correlations with TMAO (14).

Despite the rise in research linking GM and AS, it's crucial to remember that a correlation does not imply a cause-and-effect relationship. Due to possible biases including confounding and reverse causality, as well as the fact that the majority of previous research were case-control studies, it is uncertain whether these correlations are causal. Additionally, in observational research, confounding variables such as dietary patterns, age, environment, and lifestyle are easily able to influence the relationship between gut microbiota and AS (15).

Mendelian randomization (MR), a trustworthy technique for examining causal relationships, employs genetic variations as instrumental variables (IVs) to ascertain if exposure and outcome are causally related (16). Given that genotypes are randomly assigned from parents to children, common confounding variables have little impact on the relationship between genetic variation and outcome, and the causal chain is reliable (17). However, no research has utilized MR analysis to identify potential causal relationships between the gut microbiota and the risk of atherosclerosis. As a result, MR analysis was employed in this study to completely examine the potential that the gut microbiota and AS are causally related and to uncover certain pathogenic or therapeutic bacterial communities.

2 Methods

2.1 Design of the study

Throughout the study, we adhered to the principles outlined in the STROBE-MR Statement for reporting observational studies in Epidemiology (18).

Data from prior research' published genome-wide association studies (GWAS) were used in this MR analysis. The authors of the GWAS database obtained the relevant ethics and institutional review board authorizations and participant consents to permit their studies. Therefore, our MR analysis from published and anonymized data did not need further ethical approval. In this study, a GWAS summary dataset was used to evaluate the causal relationship between gut microbiota and AS, and a heterogeneity test and sensitivity analysis were carried out to ensure the reliability of the results.

An MR study needs to satisfy three core hypotheses: the correlation hypothesis, the independence hypothesis, and the exclusivity hypothesis, namely: 1. Exposure factors and instrumental variables (IVs) must be closely connected; 2. IVs cannot be correlated with any confounding variables related to the expose-outcome relationship; 3. IVs can only impact outcome variables through exposure factors (Figure 1).

2.2 GWAS summary data sources

The GWAS project opened by the IEU in 2021, which provides the largest published GWAS summary statistics on atherosclerosis, was selected for this study. GWAS data for AS were obtained from publicly available GWAS data from the FinnGen consortium, including cerebral atherosclerosis (104 cases and 218,688 controls),

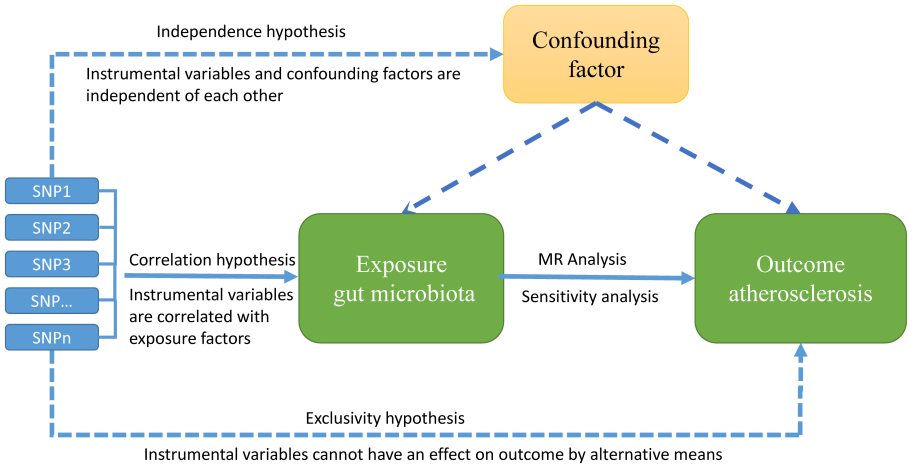

FIGURE 1
Overview of MR hypotheses, design, and procedures. There are three key hypotheses for MR study. hypotheses 1: Instrumental variables (IVs) must be strongly correlated with exposure factors; hypotheses 2: the used IVs should not be associated with any potential confounder; hypotheses 3: the IVs should influence the outcome risk merely through the exposures, not via any alternative pathway.

coronary atherosclerosis (23,363 cases and 187,840 controls), and peripheral atherosclerosis (6631 cases and 162,201 controls).

The GWAS summary microbiota statistics were mainly obtained from MiBioGen Consortium (www.mibiogen.org), 18,340 participants from 24 cohorts were included, 211 taxonomic units were recorded (35 families, 29 orders, 16 phyla, 131 genera) and 122, 110 associated SNPs (19–21), the detailed data sources showed in Table 1.

2.3 Selection and verification of IVs

First, to satisfy the first MR hypothesis that single-nucleotide polymorphisms (SNPs) need to be tightly connected to gut microbiota, SNPs that were highly related to gut microbiota were chosen at the genome-wide level (linkage disequilibrium [LD], $r^2 < 0.001$, genome-wide significance threshold $< 1\times10^{-5}$, genetic distance = 10,000 kb) (20). Second, to ensure that the second MR hypothesis, that genetic variation is not associated with potential confounding factors, we examined the phenoscanner database (22) to determine that the included SNPs were not associated with known confounding factors, such as smoking status, blood pressure, sex, family history of hypertension, dyslipidemia, diabetes, and body mass index (BMI). A heterogeneity test was used to eliminate significantly heterogeneous SNPs, and SNPs substantially linked with gut microbiota were discovered as IVs.

Palindromic SNPS may also contribute to bias in the estimate of causation (21), because the alleles of the two palindromic SNPS are not independent and may violate the MR Hypothesis. We removed palindromic SNPS from instrumental variables to ensure the validity of the results and to increase confidence in causal inference.

The F statistic is calculated to evaluate whether the selected IVs are weak. F > 10 indicates that there are no weak IVs to further verify the relevance hypothesis. The computation algorithm is $F = \beta^2_{exposure}/SE^2_{exposure}$, it is estimated according to beta and standard error. The strength of the connection between the IVs and the exposure phenotype was assessed using the F statistic; SNPs with an F-statistic < 10 should be disregarded (23). The traits of the genetic IVs for gut microbiota are listed below (Supplementary Table 1).

2.4 MR analysis

To better assess the full causal connection between gut microbiota and AS, a two-sample MR analysis was performed using IVW as the main analysis method, four more complimentary analytic techniques (MR Egger, simple mode, weighted median, and weighted mode) were also employed. In addition, a threshold of $P < 0.05$ was used to represent a significant causal relationship between gut microbiota and AS.

2.5 Pleiotropy test and heterogeneity test

First of all, the MR-PRESSO method (24) was used to detect outliers in this study. If there were outliers, they would be removed and re-analyzed. The "leave one out" sensitivity analysis (25) was carried out by removing individual SNPs at a time to assess whether the IVs drove the association between exposure and outcome. Secondly, to make it clear whether the MR analysis has horizontal polymorphism, the MR-Egger intercept item (26) is also detected in this study. If the intercepted item in the MR-Egger intercept analysis has obvious statistical significance, it indicates that the study has obvious horizontal polymorphism. Finally, this study also uses Cochran's Q measurement to test heterogeneity, it may demonstrate heterogeneity brought on by pleiotropy and other uncertain factors. IVW and MR-Egger in Cochran's Q (27) statistics have been widely used to check heterogeneity. The results of the test for pleiotropy and heterogeneity are shown in the supplementary table. $P > 0.05$ indicated no significant pleiotropy or heterogeneity.

3 Results

3.1 Selection of IVs

After a series of quality controls for cerebral atherosclerosis, we extracted 65 independent SNPs ($P < 1.0\times10^{-5}$, $r^2<0.001$) associated with five bacterial genera as IVs. For coronary atherosclerosis, we extracted 41 independent SNPS associated with 5 bacterial genera as IVs, and for peripheral atherosclerosis, we extracted 62 independent SNPs associated with 6 bacterial genera as IVs; all IVs had F statistics greater than 10, indicating that the selected SNPs all had sufficiently strong IVs effects without weak IVs bias. The results of the IVs association between AS and gut microbiota were detailed in Supplementary Tables 1, 5, 9.

MR_egger and IVW in Cochran's Q test both showed no significant heterogeneity in the genetic IVs associated with cerebral atherosclerosis, coronary atherosclerosis, and peripheral atherosclerosis (Supplementary Tables 3, 7, 11). In addition, the MR-egger intercept test showed that there was no significant

TABLE 1 Characteristics of GWAS data for gut microbiota traits and Atherosclerosis.

Trait	Sample size	Consortium	Link	Year
Gut microbiota	18,340	MiBioGen	https://mibiogen.gcc.rug.nl/	2021
Coronary atherosclerosis	211,203	FinnGen	https://gwas.mrcieu.ac.uk/datasets/finn-b-I9_CORATHER/	2021
Cerebral atherosclerosis	218792	FinnGen	https://gwas.mrcieu.ac.uk/datasets/finn-b-I9_CEREBATHER/	2021
Peripheral atherosclerosis	168832	FinnGen	https://gwas.mrcieu.ac.uk/datasets/finn-b-DM_PERIPHATHERO/	2021

GWAS, genome-wide association studies.

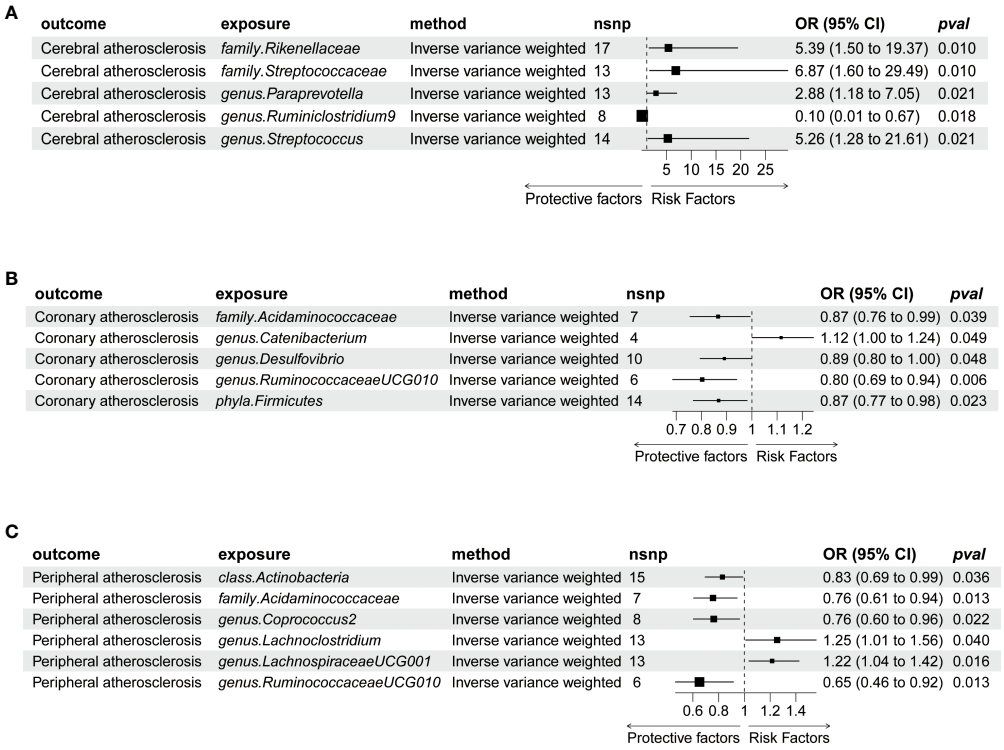

FIGURE 2
Forrest plot for summary causal effects of gut microbiota on atherosclerosis risk based on MR methods (inverse variance weighted IVW); **(A)** Represents the causal effect of gut microbiota on cerebral atherosclerosis. **(B)** Represents the causal effect of gut microbiota on coronary atherosclerosis. **(C)** Represents the causal effect of gut microbiota on peripheral atherosclerosis. MR, Mendelian randomization; nSNP, number of single-nucleotide polymorphism; OR, odds ratio.

pleiotropy of the genetic IVs related to cerebral atherosclerosis, coronary atherosclerosis, and peripheral atherosclerosis ($P > 0.05$). The results were detailed in Supplementary Tables 4, 8, 12. Therefore, the genetic IVs of all selected gut microbiota should be considered valid IVs in this MR analysis.

3.2 MR analysis

IVW estimation showed that *genus Ruminiclostridium 9* had a protective effect on cerebral atherosclerosis (OR = 0.10, 95% CI: 0.01–0.67, $P = 0.018$), while *family Rikenellaceae* (OR = 5.39, 95% CI: 1.50–19.37, $P = 0.010$), *family Streptococcaceae* (OR = 6.87, 95% CI: 1.60–29.49, $P = 0.010$), *genus Paraprevotella* (OR = 2.88, 95% CI: 1.18–7.05, $P = 0.021$), and *genus Streptococcus* (OR = 5.26, 95% CI: 1.28–21.61, $P = 0.021$) were pathogen and opportunistic pathogens to cerebral atherosclerosis (Figures 2A, 3; Supplementary Table 2). As a causal inference for coronary atherosclerosis, we found *family Acidaminococcaceae* (OR = 0.87, 95% CI: (0.76–0.99, $P = 0.039$), *genus Desulfovibrio* (OR = 0.89, 95% CI: 0.80–1.00, $P = 0.048$), *genus RuminococcaceaeUCG010* (OR = 0.80, 95% CI: 0.69–0.94, $P = 0.006$), and *Firmicutes phyla* (OR = 0.87, 95% CI: 0.77–0.98, $P = 0.023$) were protective against coronary atherosclerosis. However, the *genus Catenibacterium* (OR = 1.12, 95% CI: 1.0–1.24, $P = 0.049$) had a pathogenic and opportunistic pathogenic effect on coronary atherosclerosis (Figures 2B, 4, Supplementary Table 6). Finally, for the causal inference of peripheral atherosclerosis, we found that *class. Actinobacteria* (OR = 0.83, 95% CI: 0.69–0.99, $P = 0.036$), *family Acidaminococcaceae* (OR = 0.76, 95% CI: 0.61–0.94, $P = 0.013$), *genus Coprococcus 2* (OR = 0.76, 95% CI: 0.60–0.96, $P = 0.022$), *genus Ruminococcaceae UCG010* (OR = 0.65, 95% CI: 0.46–0.92, $P = 0.013$) for peripheral artery atherosclerosis has a protective effect. However, the *genus Lachnoclostridium* (OR = 1.25, 95% CI: 1.01–1.56, $P = 0.040$) and the *genus LachnospiraceaeUCG001* (OR = 1.22, 95% CI: 1.04–1.42, $P = 0.016$) had a pathogenic and opportunistic pathogenic effect on peripheral atherosclerosis (Figures 2C, 5, Supplementary Table 10).

3.3 No significant bias in the effect of a single SNP in gut microbiota on AS

"MR Leave-one-out" sensitivity analyses showed that the remaining SNPs after the removal of specific SNPs did not change the causal inference results (Figures 6–8), showing that no specific IVs were responsible for any of the found causal connections. Together, these results suggest that there is no significant bias in the effect of individual gut microbiota SNPs on atherosclerosis. In addition, we showed the causal effect of single SNPs by drawing forest plots, and the results showed that the effect of single SNPs was consistent with the results of the combined effect of IVW (Supplementary Figures 1–3).

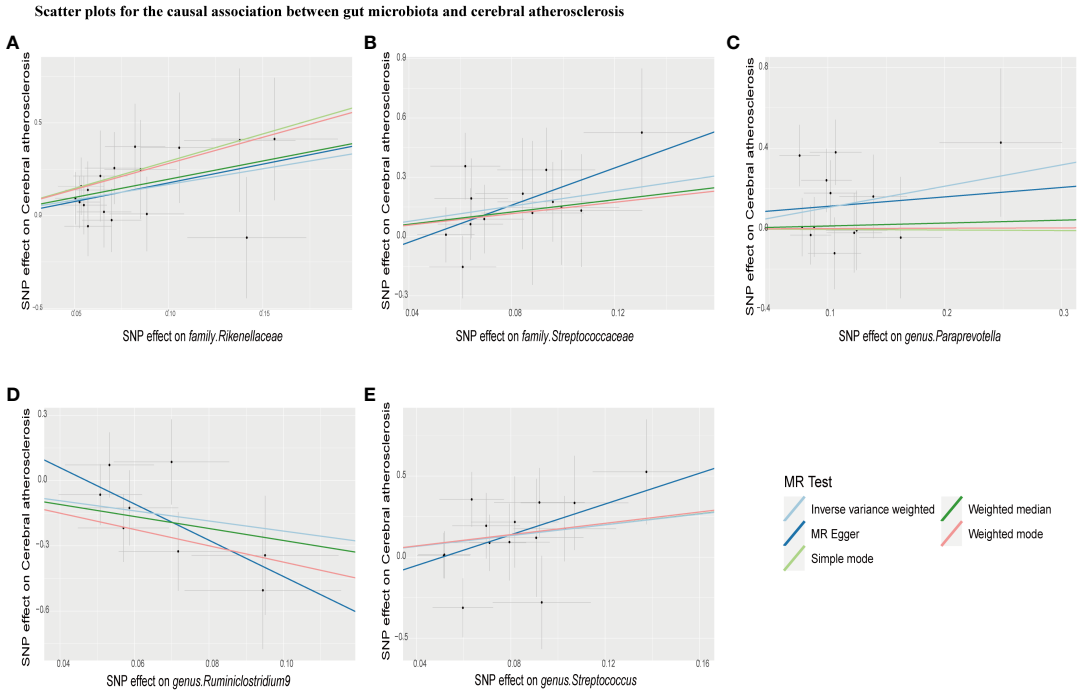

FIGURE 3
Scatter plots for causal effects of gut microbiota on cerebral atherosclerosis risk using five MR methods. **(A–E)** Represents the causal effects of family.Rikenellaceae, family.Streptococcaceae, genus.Paraprevotella, genus.Ruminiclostridium9, and genus. Streptococcus on cerebral atherosclerosis, respectively.

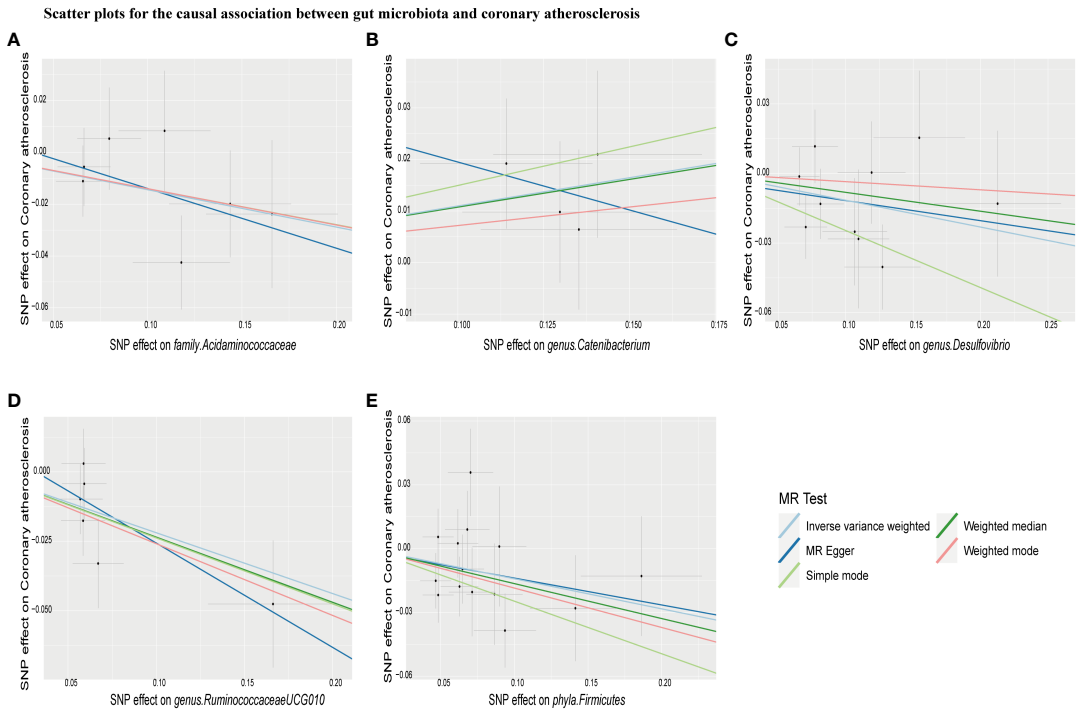

FIGURE 4
Scatter plots for causal effects of gut microbiota on coronary atherosclerosis risk using five MR methods. **(A–E)** Represents the causal effects of family.Acidaminococcaceae, genus.Catenibacterium, genus.Desulfovibrio, genus.RuminococcaceaeUCG010, and phyla.Firmicutes on coronary atherosclerosis, respectively.

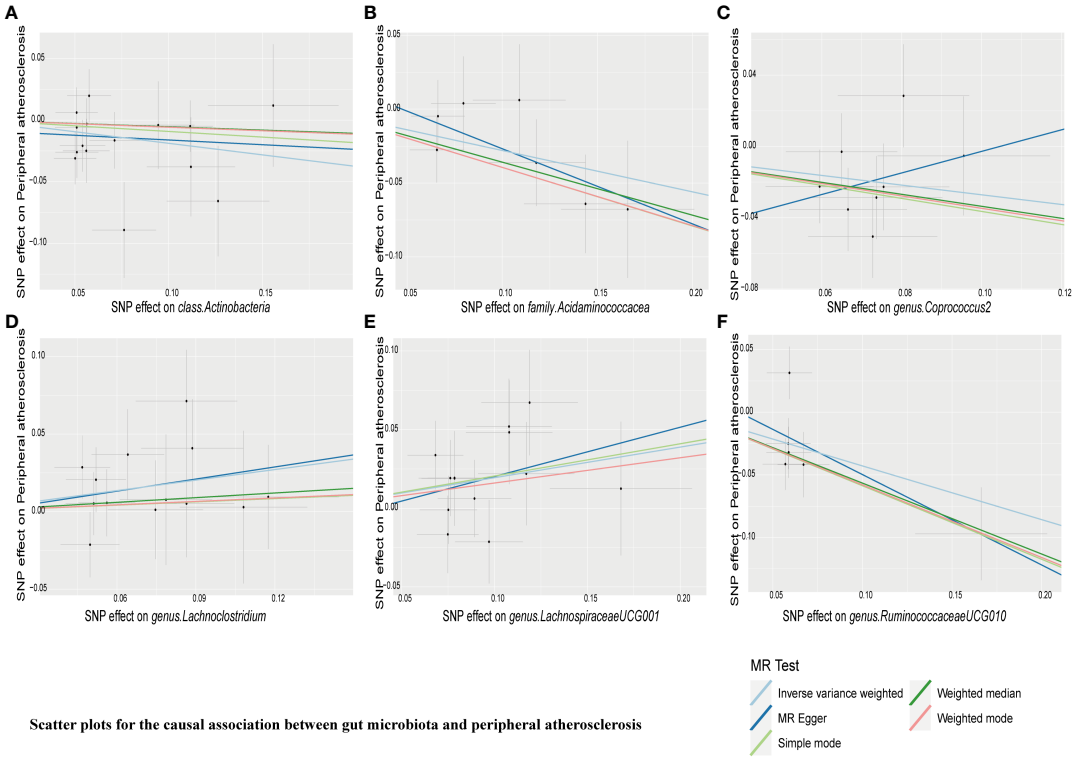

FIGURE 5
Scatter plots for causal effects of gut microbiota on peripheral atherosclerosis risk using five MR methods. **(A–F)** Represents the causal effects of class.Actinobacteria, family.Acidaminococcacea, genus.Coprococcus2, genus.Lachnoclostridium, genus.LachnospiraceaeUCG001, and genus.RuminococcaceaeUCG010 on peripheral atherosclerosis, respectively.

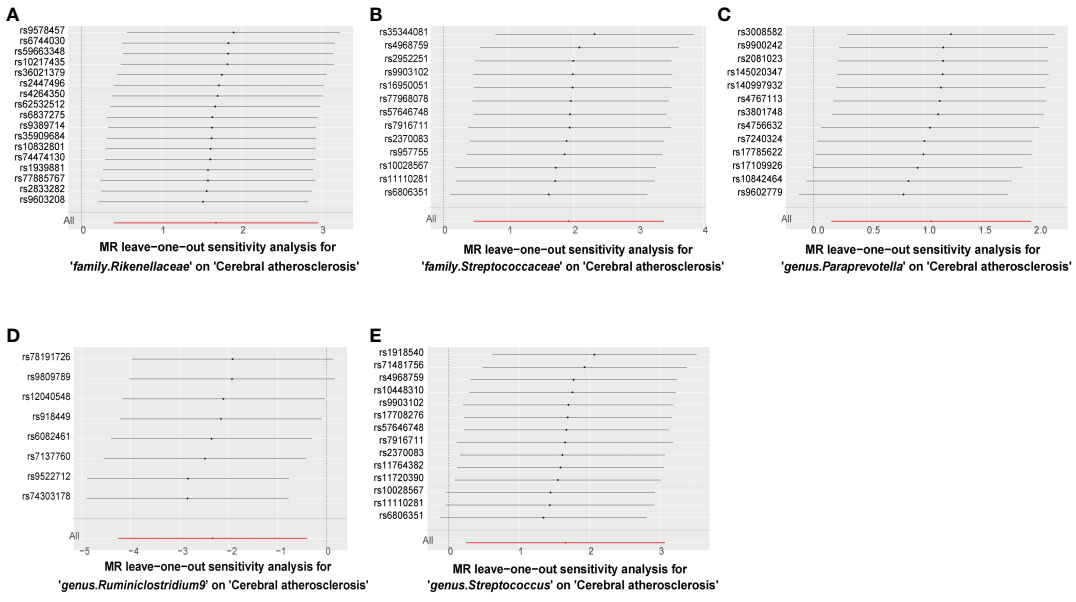

FIGURE 6
Plots for "leave-one-out" analysis for causal effect of gut microbiota on cerebral atherosclerosis risk. **(A–E)** Represents the MR leave–one–out sensitivity analysis for family.Rikenellaceae, family.Streptococcaceae, genus.Paraprevotella, genus.Ruminiclostridium9, and genus.Streptococcus on cerebral atherosclerosis, respectively.

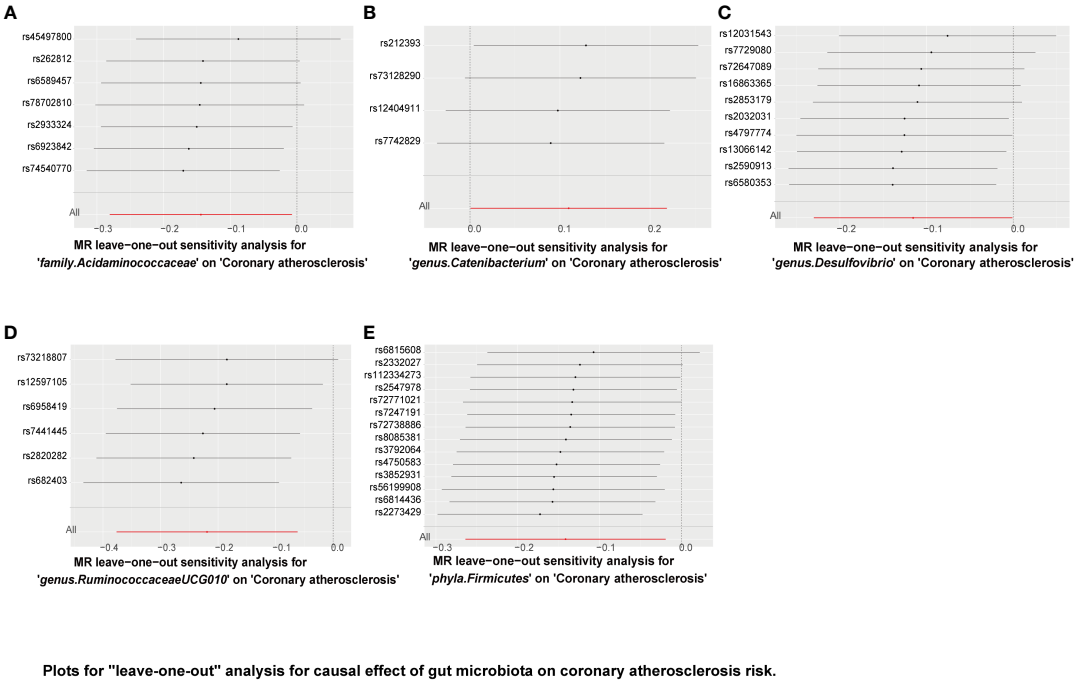

FIGURE 7
Plots for "leave-one-out" analysis for causal effect of gut microbiota on coronary atherosclerosis risk. **(A–E)** Represents the MR leave–one–out sensitivity analysis for family.Acidaminococcaceae, genus.Catenibacterium, genus.Desulfovibrio, genus.RuminococcaceaeUCG010, and phyla.Firmicutes on coronary atherosclerosis, respectively.

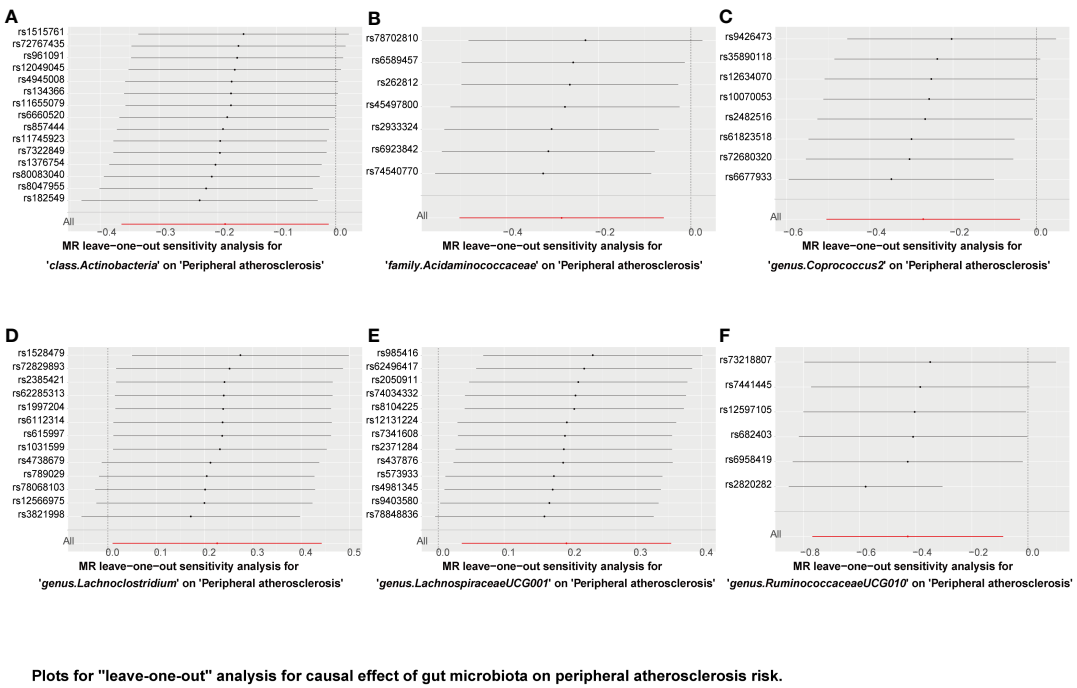

FIGURE 8
Plots for "leave-one-out" analysis for causal effect of gut microbiota on peripheral atherosclerosis risk. **(A–F)** Represents the MR leave–one–out sensitivity analysis for class.Actinobacteria, family.Acidaminococcacea, genus.Coprococcus2, genus.Lachnoclostridium, genus.LachnospiraceaeUCG001, and genus.RuminococcaceaeUCG010 on peripheral atherosclerosis, respectively.

4 Discussion

In this work, we performed two-sample MR analyses to determine the causative connection between gut microbiota and AS using summary statistics on gut microbiota from the biggest GWAS meta-analysis completed by the MiBioGen consortium and summary statistics on AS released by the FinnGen consortium. This study provides guidance for future research based on gut microbiota in the treatment of AS. As we all know, resident microbial communities in the gut are key "metabolic filters" in the diet, as these species can convert common nutrients into metabolites, and specific microbiota-associated metabolites, such as TMAO, short-chain fatty acids (SCFAs), and secondary bile acids, have been shown to influence CVD progression (28–32).

We found that specific gut microbiota may be causally linked to AS at different sites. For example, the *genus Ruminiclostridium 9* is negatively associated with the risk of cerebral atherosclerosis and has a protective effect. However, the *family Rikenellaceae*, the *family Streptococcaceae*, the *genus Paraprevotella*, and the *genus Streptococcus* are significantly linked to the risk of cerebral atherosclerosis. Therefore, These four intestinal microbes may contribute to the pathogenesis of cerebral atherosclerosis. in the family. *Acidaminococcaceae* and the *genus Ruminococcaceae (UCG010)* both have protective effects on coronary atherosclerosis and peripheral atherosclerosis. The *genus Desulfovibrio* and the *Firmicutes phyla* were specifically negatively associated with coronary atherosclerosis risk and had a protective effect, while the *genus Catenibacterium* was positively associated with coronary atherosclerosis risk and had an atherogenic effect. *class Actinobacteria* and *genus Coprococcus 2* are specifically negatively correlated with the risk of peripheral atherosclerosis and have a protective effect. *Genus Lachnoclostridium* and *genus Lachnospiraceae UCG001* specificity increased the risk of peripheral artery atherosclerosis. Our research will contribute to the theoretical foundation for AS precision therapy in the future. As atherosclerosis in different sites is associated with specific microbiota, we hypothesize that this may be because specific metabolites of the microbiota are related to the microenvironment of different arterial locations.

In addition, we also found that *Acidaminococcaceae* and *Ruminococcaceae UCG010* have the same protective effect on coronary atherosclerosis and peripheral atherosclerosis, and *Ruminiclostridium 9* has the same protective effect on cerebral atherosclerosis. Oxana M. Drapkina (33) evaluated the impact of fecal microbiota on atherosclerotic cardiovascular disease (ASCVD) and heart failure with reduced ejection fraction (HFrEF) by using bacterial culture, 16S next-generation sequencing (NGS) of the 16S rRNA gene (V3-V4), and quantitative polymerase chain reaction (qPCR). They found that *acidaminococcaceae* were significantly lower in the ASCVD and HFrEF groups, and *acidaminococcaceae* were negatively associated with ASCVD. while *Streptococcaceae* were significantly increased in ASCVD and HFrEF groups. In our study, we found that *acidaminococcaceae*, one of the commensal bacteria with an atherogenic effect, was also negatively associated with cerebral atherosclerosis and peripheral atherosclerosis. Kesavalu L (34) found that *Streptococcus mutans* infection accelerated plaque growth, macrophage invasion, and TLR4 expression after angioplasty, and *Streptococcus mutans* may also be associated with atherosclerotic plaque growth in noninjured arteries. Koren et al (35) identified Veloxella and *Streptococcus* in AS plaque samples, and several bacterial types in the gut are common in atherosclerotic plaques and correlated with cholesterol levels. Another metagenomic association study (36) showed that the abundance of *Streptococcus* in patients with atherosclerotic cardiovascular disease was higher than that in the healthy control group. Therefore, *Streptococcaceae* is considered a pathogenic bacterium and can increase the risk of atherosclerosis, which is consistent with our findings.

In addition, according to this study, the abundance of *Ruminiclostridium* in the heart failure with preserved ejection fraction (HFpEF) group was lower than that in the control group, Qiuxia Liu (37) also found that the relative abundance of *Ruminococcaceae* was positively correlated with the level of HDL through 16S ribosomal DNA sequencing. Therefore, *Ruminococcaceae* can inhibit atherosclerosis, consistent with our findings. *Ruminiclostridium 9* can alleviate the formation of cerebral atherosclerosis, and *Ruminococcaceae UCG010* can inhibit the formation of coronary and peripheral atherosclerosis. Hannelore Daniel's study (38) found that a high-fat diet caused shifts in the diversity of dominant gut bacteria and altered the proportion of *Ruminococcaceae* (decrease) and *Rikenellaceae* (increase). Our results suggest that *Rikenellaceae* can increase the risk of cerebral atherosclerosis, while *Ruminococcaceae* are negatively correlated with coronary atherosclerosis and peripheral atherosclerosis. A recent study included in the TwinsUK cohort showed that (39) *Ruminococcaceae* was negatively correlated with pulse wave velocity (PWV), which represents arterial stiffness. *Ruminococcaceae* is a bacterium that can produce butyrate, and the increase in its abundance can reduce the release of inflammatory factors and alleviate endothelial dysfunction, thus delaying the development of atherosclerosis. Our research results also support the idea that *Ruminococcaceae* belongs to the probiotic family. Additional randomized controlled studies, nevertheless, are necessary to verify these results.

Omry Koren (35) found that the atherosclerotic plaques contained significantly fewer *Firmicutes phyla* and suggested a negative correlation with the risk of atherosclerosis; low intestinal levels were associated with greater risk, whereas normal or elevated levels were protective. Some studies have shown that butyrate is usually produced by *Firmicutes phyla* (40). If the *Firmicutes phyla* population is reduced, the concentration of butyrate in the intestine will decrease, leading to reduced mucin synthesis, and a lack of sufficient mucin on the intestinal membrane will lead to increased intestinal permeability (41), which induces a chronic inflammatory state, leading to a higher intestinal inflammatory state. These included increased concentrations of IL-1 and IL-4. IL-1 is a proinflammatory cytokine (42), which is associated with atherogenesis, plaque instability, plaque rupture, and thrombosis, and increases cardiovascular risk. Therefore, *Firmicutes phyla* belong to commensal bacteria and can inhibit arterial atherosclerosis, and our results also support the role of *Firmicutes phyla* in inhibiting atherosclerosis.

Akihiro Nakajima (43) found *paraprevotella* had a positive correlation with fibrinogen in plaque and a negative correlation

with high-density lipoprotein cholesterol; *paraprevotella* were also associated with greater plaque volume. Our study also found that *paraprevotella* could promote the formation of cerebral atherosclerosis, which is consistent with our study results.

The research work of Yuan-Yuan Cai (44) provided a comprehensive metagenomic analysis of bacteria producing TMA (the precursor of TMAO) in the human gut and reported the *genus Lachnoclostridium* producing TMA for the first time. The abundance of this genus was higher in patients with atherosclerosis compared to healthy controls. They found *in vitro* that *Lachnoclostridium* can produce TMA when incubated with choline. *In vivo* studies further demonstrated that *Lachnoclostridium* could promote TMAO levels in the serum of ApoE$^{-/-}$ mice, significantly elevate aortic plaque, and accelerate plaque formation *in vivo*. Therefore, targeting *Lachnoclostridium* may serve as a potential therapeutic target for the treatment of atherosclerosis. Our findings are consistent with those of the present study, suggesting that *Lachnoclostridium* promotes atherogenesis.

In addition, we also found some new probiotics whose effects on AS have not been reported before; for example, *Coprococcus 2* and *Actinobacteria* have protective effects on AS, and their specific protective mechanisms still need to be further explored. They can be used as a new therapeutic target for anti-atherosclerosis. Of note, *Desulfovibrio* suggested a negative association with coronary atherosclerosis in our study; However, in addition, we also found some new probiotics whose effects on AS have not been reported before; for example, *Coprococcus 2* and *Actinobacteria* have protective effects on AS, and their specific protective mechanisms still need to be further explored. They can be used as a new therapeutic target for anti-atherosclerosis. Of note, *Desulfovibrio* suggested a negative association with coronary atherosclerosis in our study; however, Kun Zhang's results (45) demonstrate that D. desulfuricans can enhance the development of AS by increasing intestinal permeability and host inflammatory response, which is inconsistent with the results of our study, probably because we specifically targeted coronary atherosclerosis, while Kun Zhang's research focused on aortic atherosclerosis, Different arterial sites have different microenvironments. There are also differences in the mechanisms of gut microbiota in AS. Therefore, more in-depth research mechanisms need to be further explored.

As far as we know, bacteria are a major component of the gut microbiome, but viruses, fungi, and archaea are also present, they live symbiotic in our gut. Although intestinal flora plays an important role in atherosclerosis, enteroviruses, fungi and their metabolites are also involved in the development of atherosclerosis (46, 47). First of all, the gut microbiota of adults is mainly composed of five phyla: *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Cerrucomicrobia* (48), and changes in the components of these flora can cause ecological imbalance of intestinal flora. Several studies have confirmed the presence of bacterial DNA in atherosclerotic plaques, thereby affecting plaque stability, which may contribute to the development of cardiovascular disease (49). The main pathogenic mechanism may be the impairment of intestinal barrier function due to the imbalance of the flora (50), which leads to the change of intestinal permeability, and the absorption of metabolites of the flora and endotoxins into the blood circulation in the body. These metabolites, including trimethylamine N-oxide (TMAO), bile acids, lipopolysaccharides, and short-chain fatty acids, all have an impact on the occurrence and development of atherosclerosis (51). These changes in intestinal flora and metabolites can not only cause coronary atherosclerosis, but even cause cerebrovascular diseases through gut-brain axis, inflammatory response, etc. (52), and even rupture of cerebral aneurysms in severe cases. Research has been reported that the *genus Campylobacter* and *Campylobacter ureolyticus* may be associated with the rupture of cerebral aneurysms, the gut microbiome profile of patients with stable unruptured intracranial aneurysms and ruptured aneurysms were significantly different (53). Secondly, the imbalance of intestinal fungi can also cause metabolic disorders. Some studies reported that compared with healthy lean individuals, the fecal fungi in obese participants showed more obvious diversity, and the intestinal fungal composition changed significantly. In addition, other studies have found that the abundance of *Thermoascus* and *species Malassezia restricta* in the patients with coronary atherosclerosis was significantly lower than in healthy individuals, and the decrease of *M.restricta* might have a close association with lipid metabolism disorder in atherosclerosis patients (46), there is growing evidence that antagonistic relationships between bacteria and fungi may reduce perturbations and enhance interactions in the gut, thereby establishing a balanced microbial community (54). Finally, a growing body of research also suggests that changes in enteroviruses are associated with cardiovascular disease, after an in-depth metatenomic analysis of the viriome of the participants' fecal samples, the study found that enteroviruses in patients with cardiovascular disease were significantly different from healthy controls, for example, the *Siphoviridae* was significantly enriched in the viriome of patients with cardiovascular disease. In addition, the abundance of *Enterobacteriaceae* and *streptococcus* increased in patients with cardiovascular disease (36). As a result, the abundance of these viruses and bacteria presents a consistent level, in which the presence, absence, or abundance of viruses may regulate the progression of the disease by affecting bacteria in the host. Correlation analysis showed that *enterococcus*, *streptococcus* and *ruminococcus* were widely associated with viral operational taxonomic unit in patients with cardiovascular disease. This also reflects the fact that enteroviruses affect disease by relying on gut bacteria (47). In summary, we found that there may be a complex network among gut microbes, with interactions among bacteria, viruses, and fungi that jointly affect the occurrence and development of atherosclerosis.

The study has several advantages: MR analysis was used to establish the causal link between gut microbiota and AS, removing confounding variables' involvement and lessening the effect on causal inference. Genetic variation in the gut microbiota was obtained from the largest available GWAS summary statistics, ensuring IVs strength in the MR analysis. The IVs selected in this study were all strong IVs ($F > 10$), which had high statistical power. By utilizing the MR-PRESSO and MR-Egger regression intercept term tests, horizontal pleiotropy was identified and excluded.

However, there are some limitations to this study. Because summary statistics were used in the analysis rather than raw data,

we could not perform subgroup analyses, such as the analysis of gender differences. Since the lowest taxonomic level in the exposure dataset was genus, this limitation prevented us from further exploring the causal relationship between gut microbiota and AS at the species level. More genetic variants need to be included as IVs to perform sensitivity analyses and horizontal pleiotropy tests. Thus, the SNPs used in the analysis did not meet the traditional GWAS threshold for significance ($P < 5\times10^{-8}$).

Due to confounding by ethnic stratification, data on gut microbiota were obtained from subjects of European ancestry, thus, the findings might not be entirely relevant to participants of non-European heritage. For greater generalization in the future, MR research on the causal link between gut microbiota and AS might be addressed in other populations.

5 Conclusion

In conclusion, this two-sample MR study found that some specific gut microbiotas were causally associated with the presence of AS. Further, RCT studies are needed to elucidate the protective or pathogenic mechanisms of probiotics or pathogenic bacteria in AS.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

SJ: Conceptualization, Formal Analysis, Software, Visualization, Writing – original draft. CY: Formal Analysis, Methodology, Software, Writing – review & editing. BL: Funding acquisition, Project administration, Supervision, Writing – review & editing. SH: Funding acquisition, Project administration, Supervision, Writing – review & editing. YZ: Methodology, Writing – review & editing. WY: Data curation, Methodology, Writing – review & editing. BW: Data curation, Funding acquisition, Investigation, Supervision, Writing – review & editing. DL: Conceptualization, Data curation, Investigation, Project administration, Writing – review & editing. JL: Conceptualization, Data curation, Funding acquisition, Project administration, Supervision, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1282072/full#supplementary-material

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Causal relationship between gut microbiota and risk of gastroesophageal reflux disease: a genetic correlation and bidirectional Mendelian randomization study

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Background: Numerous observational studies have identified a linkage between the gut microbiota and gastroesophageal reflux disease (GERD). However, a clear causative association between the gut microbiota and GERD has yet to be definitively ascertained, given the presence of confounding variables.

Methods: The genome-wide association study (GWAS) pertaining to the microbiome, conducted by the MiBioGen consortium and comprising 18,340 samples from 24 population-based cohorts, served as the exposure dataset. Summary-level data for GERD were obtained from a recent publicly available genome-wide association involving 78 707 GERD cases and 288 734 controls of European descent. The inverse variance-weighted (IVW) method was performed as a primary analysis, the other four methods were used as supporting analyses. Furthermore, sensitivity analyses encompassing Cochran's Q statistics, MR-Egger intercept, MR-PRESSO global test, and leave-one-out methodology were carried out to identify potential heterogeneity and horizontal pleiotropy. Ultimately, a reverse MR assessment was conducted to investigate the potential for reverse causation.

Results: The IVW method's findings suggested protective roles against GERD for the *Family Clostridiales Vadin BB60 group* (P = 0.027), *Genus Lachnospiraceae UCG004* (P = 0.026), *Genus Methanobrevibacter* (P = 0.026), and *Phylum Actinobacteria* (P = 0.019). In contrast, *Class Mollicutes* (P = 0.037), *Genus Anaerostipes* (P = 0.049), and *Phylum Tenericutes* (P = 0.024) emerged as potential GERD risk factors. In assessing reverse causation with GERD as the exposure and gut microbiota as the outcome, the findings indicate that GERD leads to dysbiosis in 13 distinct gut microbiota classes. The MR results' reliability was confirmed by thorough assessments of heterogeneity and pleiotropy.

Conclusions: For the first time, the MR analysis indicates a genetic link between gut microbiota abundance changes and GERD risk. This not only substantiates the potential of intestinal microecological therapy for GERD, but also establishes a basis for advanced research into the role of intestinal microbiota in the etiology of GERD.

KEYWORDS

causal association, gastroesophageal reflux disease, genome-wide association study, comprehensive bidirectional mendelian randomization, gut microbiota

Introduction

Gastro-esophageal reflux disease (GERD) prevalently affects both adult and pediatric cohorts (1, 2). The worldwide incidence of GERD is rising substantially (3). The predominant phenotype of this condition is non-erosive reflux disease (NERD) (4, 5). NERD is typified by the hallmark symptoms of GERD, yet devoid of esophageal erosion. GERD syndromes encompass typical reflux symptoms, characterized by heartburn and regurgitation, potentially accompanied by belching, water brash, or nausea. Additionally, manifestations may include chest pain resembling angina and extra-oesophageal symptoms like chronic cough and laryngitis (6–8). Moreover, persistent gastroesophageal reflux may result in the transformation of the distal esophagus's stratified squamous epithelium to columnar epithelium, precipitating the onset of Barrett's esophagus (BE) (9). BE, characterized by the presence of metaplastic columnar mucosa in the distal esophagus, heightens the risk of cancer. This condition is uniquely identified as the antecedent to esophageal adenocarcinoma, a malignancy whose prevalence has surged notably in the preceding decades (10–13). Hence, numerous researchers aim to devise prevention strategies for esophageal adenocarcinoma by investigating the pathogenesis of GERD and Barrett's esophagus (14, 15). The human gastrointestinal tract is host to a complex and varied microbiota, which holds a pivotal function in health and pathophysiology. This includes processes such as the digestion and assimilation of nutrients, production of vital vitamins like B and K, *in vivo* degradation of molecules, orchestration of innate and adaptive immune reactions, and preservation of the intestinal barrier's integrity (16–18).

In recent years, numerous studies have elucidated the correlation between the onset and progression of various intestinal diseases and the intestinal flora (19). Consequently, scholars have redirected their attention to the study of esophageal microbiota, aiming to elucidate the pathogenesis, early detection, and therapeutic approaches for esophageal disorders. It has been noted that the esophageal microflora composition varies markedly between GERD-affected and normal esophagus. A preliminary research conducted by Yang in 2009 identified a potential association between modifications in the distal esophageal microbiome and disorders related to reflux. Bacterial populations from 34 patients were analyzed using 16S rRNA gene sequencing following biopsies of the distal esophagus. Based on gene analysis outcomes, the authors delineated the human esophageal microbiome into two categories. Type I esophageal microbiome corresponded more closely with the normal esophagus, whereas Type II was more associated with the pathological esophagus (20). Studies indicate a heightened colonization of Gram-negative organisms, particularly Campylobacters, in the esophageal mucosa of GERD patients compared to healthy cohorts (21). Dysregulation of the mycobiota has been implicated in the onset of visceral hypersensitivity, a condition closely associated with intractable symptoms of GERD (22). These observations prompt consideration of potential dysbiosis involvement in the pathogenesis of GERD ailments. In observational research, the relationship between the gut microbiota and GERD is susceptible to confounding variables, including dietary habits, environmental factors, age, and lifestyle. These confounders complicate the process of establishing a direct causal link between gut microbiota and GERD. Utilizing the Mendelian randomization (MR) approach allows for the inference of causative associations between exposures and subsequent outcomes (23, 24). This methodology employs genes as instrumental variables (IVs), which, due to their reliance on the random assortment of genetic variation at conception, are less prone to confounding influences (25). In the present research, we executed a two-sample MR analysis to assess the putative causal relationship between the gut microbiota and GERD. Through this endeavor, we aspire to elucidate novel perspectives on the potential involvement of the gut microbiome in the pathogenesis of GERD and discern potential pathways for preventative and therapeutic strategies. To our knowledge, this is the first time that Mendelian randomization has been used to study the pathogenic impact of the gut microbiome on the pathogenesis of GERD.

Materials and methods

Study design

In our study, we performed two-sample MR analyses with gut microbiota as the exposure and GERD as the outcome. To

investigate the causal relationship between intestinal microflora and GERD, we utilized a bi-sample MR approach, drawing on data from the MiBioGen consortium (N = 18,340) and recent GWAS (78 707 GERD cases and 288 734 controls) findings. Figure 1 depicts the MR study flowchart detailing the relationship between GM taxa and GERD. For reliable results, the MR study adhered to these three assumptions (1). They are significantly associated with the exposure (2); They don't influence the confounders linking exposure and outcome; and (3) They don't impact the outcome via alternative pathways (26). The current MR study was executed and chronicled in accordance with the STROBE-MR guidelines, established to enhance the reporting caliber of observational epidemiological investigations (27–29).

Data sources

Gut microbiota and GERD data were sourced from GWAS datasets. The intestinal microbiome information came from the MiBioGen consortium's GWAS analysis, which included 18,340 individuals spanning 24 whole-genome genotype cohorts and 16S fecal microbiome data (30). We gathered summary-level data on SNP-GERD associations from the recent publication's GWAS results. This analysis encompassed 78,707 GERD cases and 288,734 controls of European ancestry (31). GERD is characterized by abnormal esophageal acid exposure leading to GERD symptoms and/or mucosal injury due to gastro-oesophageal reflux.

Selection of SNPs

We conducted quality control procedures to select appropriate instrumental variants (IVs) (32–35). SNPs associated with each microbiota unit, meeting the locus-wide significance threshold ($P< 1.0 \times 10^{-5}$), were designated as potential IVs. The linkage disequilibrium (LD) assessment among these SNPs is as follows (36–38): LD denotes the non-random co-occurrence of alleles at distinct loci. It is evaluated via two metrics, r^2 and kb. An r^2 value spans from 0 to 1, with lower values signifying a heightened level of complete linkage equilibrium between two SNPs, suggesting a stochastic arrangement of these SNPs. An appropriate LD window size and r^2 threshold are selected to guarantee independence, given the profound impact of linkage disequilibrium. SNPs were clumped for independence using the European 1000 Genomes Project reference panel with criteria $r^2 < 0.001$ and clump distance > 10,000 kb. SNPs exhibiting a Minor Allele Frequency (MAF) of 0.01 or lower were systematically excluded from the analysis. We excluded both redundant and palindromic SNPs from our analysis. To ensure a robust association between instrumental variables (IVs) and exposure measures, the F-statistic of each SNP was employed to evaluate the strength of correlation, mitigating potential biases from weak IVs. IVs were considered devoid of bias if the F-statistic exceeded 10. To minimize the likelihood of SNPs being associated with potential confounders or risk determinants (e.g., coronary heart disease, Idiopathic pulmonary fibrosis), the Phenoscanner tool was utilized to meticulously assess and exclude such correlations.

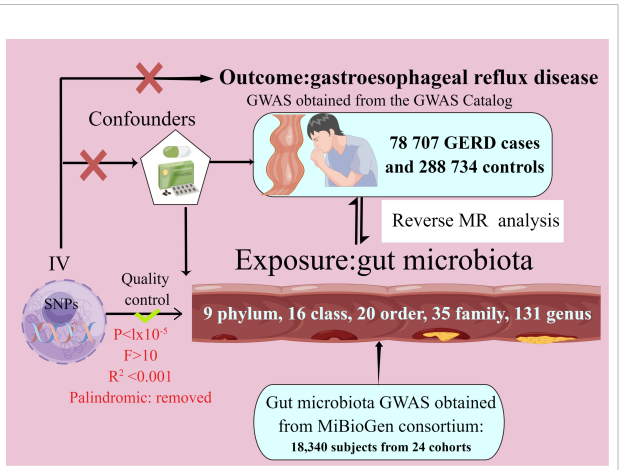

FIGURE 1
The study design of the present Mendelian randomization study of the associations of the gut microbiota and GERD risk.

MR analysis and quality assessment

We derived the primary MR estimates using the inverse-variance weighted (IVW) method. We also assessed the robustness of these IVW findings by contrasting them with results from other MR techniques, such as MR-Egger, weighted median, simple mode, and weighted mode estimation. The analyses conducted encompassed evaluations of heterogeneity, an assessment of horizontal pleiotropy, and a systematic leave-one-out examination. For the assessment of heterogeneity, the Cochrane's Q test was employed, with a *P*-value of less than 0.05 being considered indicative of significant heterogeneity. The Mendelian Randomization Pleiotropy Residual Sum and Outlier (MR-PRESSO) approach, in conjunction with the MR-Egger method, were utilized to scrutinize horizontal pleiotropy. A *P*-value of less than 0.05 was deemed indicative of the presence of horizontal pleiotropy. we performed a leave-one-out analysis to evaluate the results' sensitivity, wherein each SNP was sequentially excluded to determine if the estimates were influenced by outliers or bias. We determined the statistical power for MR analysis by utilizing the mRnd web application, accessible at https://shiny.cnsgenomics.com/mRnd/ (39). In particular, for the purpose of refining our outcomes in the context of multiple hypotheses, we employed both the Bonferroni correction method and the Hochberg's False Discovery Rate (FDR) approach. The criterion for deeming results statistically significant was established on the basis of a P-value less than 0.05, adjusted by dividing it by the effective count of unique bacterial taxa present at the respective taxonomic level, a value hereinafter referred to as 'n', An association was deemed statistically significant in instances where the p-value, after undergoing Bonferroni correction, was found to be below the threshold of 0.05. Conversely, the presence of a p-value lesser than 0.05, which nonetheless corresponded to a Bonferroni-corrected p-value exceeding 0.05, was interpreted as indicative of suggestive, rather than conclusive, evidence of an association.

Reverse MR analysis

To investigate the putative causal association between GERD and distinct bacterial genera, a reverse MR analysis was undertaken. In this context, GERD was posited as the exposure variable, while the gut microbiota composition functioned as the outcome variable. SNPs associated with GERD were utilized as instrumental variables in this analytical framework. SNPs that exhibited a statistically significant association with GERD were selected as instrumental variables, adhering to a significance threshold of $P < 5 \times 10^{-8}$.

Ethical approval

written informed consents were meticulously secured from all participating individuals. Concurrently, these investigations were granted the requisite endorsements from the pertinent ethical oversight bodies (30).

Results

In the current research, preliminary endeavors were undertaken to procure high-quality IVs through stringent quality assurance measures. Subsequently, these IVs were employed in a MR analysis to evaluate the presumptive causal association between 196 gut microbiota taxa and GERD. In each retained SNP, the F-statistic surpassed a threshold of 10, as delineated in the Supplementary Tables S1, S2. The statistical efficacy of MR analysis was greater than 70%.This indicates a robust statistical strength in the association between the IV and its respective bacterial taxa. For all MR results, we conducted comprehensive sensitivity analyses to assess both heterogeneity, as denoted by Cochran's Q statistic, and potential pleiotropic influences, as appraised via MR-Egger regression and the MR-PRESSO approach. The P-values were subjected to a more stringent Bonferroni correction, and all results were greater than 0.05.

Causal effect of gut microbiota on GERD

In the MR study on gut microbiota, employing microbiota-linked SNPs as instrumental variables, the primary IVW analysis identified seven taxa with a probable causal association to GERD onset. Through the application of the IVW analytical approach, the following associations with GERD susceptibility were discerned: The *Family Clostridiales Vadin BB60 group* (OR 0.95, 95% CI 0.91–0.99, P = 0.027), *Genus Lachnospiraceae UCG004* (OR 0.91, 95% CI 0.84–0.99, P = 0.026), *Genus Methanobrevibacter* (OR 0.95, 95% CI 0.91–0.99, P = 0.026), and *Phylum Actinobacteria* (OR 0.93, 95% CI 0.88–0.99, P = 0.019) manifested an inverse correlation with GERD vulnerability. In contrast, the *Class Mollicutes* (OR=1.09, 95% CI:1.01–1.19, P=0.037); *Genus Anaerostipes* (OR=1.09, 95% CI:1.01–1.16, P=0.017) and *Phylum Tenericutes* (OR=1.11, 95% CI:1.01–1.22, P=0.024) demonstrated association with the risk of GERD. (Figures 2, 3) The *P*-values obtained from both the Cochran Q test and the MR-Egger intercept test surpassed the 0.05 threshold. This provides robust evidence indicating an absence of heterogeneity and pleiotropy in the research (Table 1; Supplementary Table 2, Figures 2; 4–6).

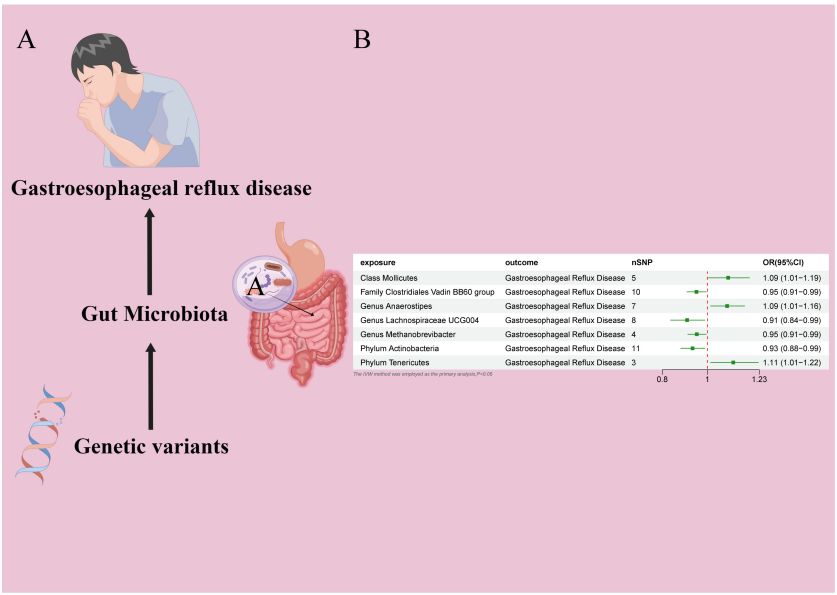

FIGURE 2
(A) Causal effect of gut microbiota with GERD Schematic representation of the MR analysis results **(B)** Forest plot of the MR analysis results.

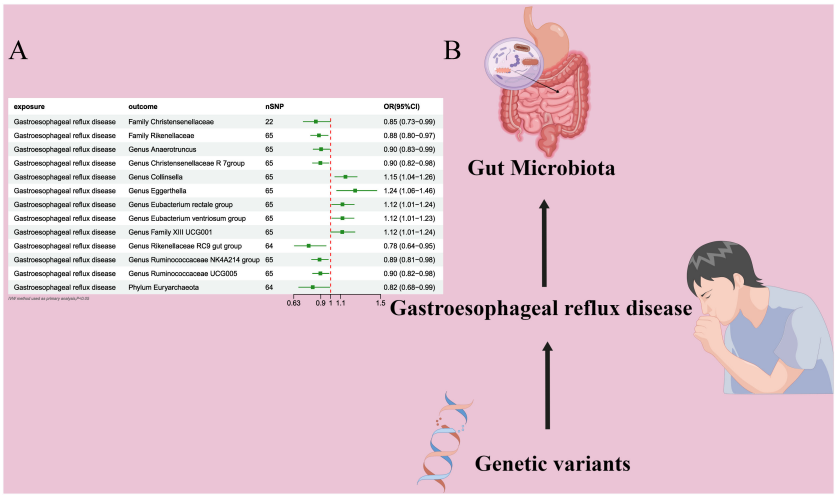

FIGURE 3
(A) Forest plot of the MR analysis results. **(B)** Forest plot of the MR analysis results Causal effect of GERD with gut microbiota Schematic representation of the Reverse MR analysis results. OR odds ratio, CI confidence interval, IVW inverse variance weighted method, Significant threshold was set at *P*-value <0.05 for the Inverse Variance Weighted method (IVW).

Causal effect of GERD on gut microbiota

In the bidirectional MR analysis, we explored the potential causal association between GERD and gut microbiota. Employing GERD as the exposure and gut microbiota as the outcome, we evaluated potential reverse causation implications. Following the MR analysis, GERD exhibited a causal influence on one Phylum, two Families, and ten Genera. Utilizing the IVW approach, several associations with the onset of GERD were identified. Specifically, a down-regulation was observed in the *Family Christensenellaceae* (OR=0.85, 95% CI:0.73–0.99, P=0.045), *Family Rikenellaceae* (OR=0.88, 95% CI:0.80–0.97, P=0.012), *Genus Anaerotruncus*

TABLE 1 Summary results of MR (Target Gut microbiome on GERD).

Taxa	Exposure	Outcome	Nsnp	Methods	Beta	SE	OR (95% CI)	*P* value	Heterogeneity		Horizontal pleiotrop	
									Cochran's Q	P value	Egger intercept P	MR-PRESSO P
Phylum	Actinobacteria	GERD	11	Inverse variance weighted	-0.068	0.029	0.93 (0.88-0.99)	0.019	6.835	0.740	0.579	0.78
Phylum	Tenericutes	GERD	3	Inverse variance weighted	0.108	0.048	1.11 (1.01-1.22)	0.024	2.468	0.291	0.364	NA
Family	Clostridiales vadin BB60 group	GERD	10	Inverse variance weighted	-0.049	0.022	0.95 (0.91-0.99)	0.027	4.406	0.882	0.490	0.85
Class	Mollicutes	GERD	5	Inverse variance weighted	0.087	0.042	1.09 (1.01-1.19)	0.037	6.032	0.196	0.745	0.27
Genus	Anaerostipes	GERD	7	Inverse variance weighted	0.083	0.035	1.09 (1.01-1.16)	0.017	5.506	0.480	0.246	0.49
Genus	Lachnospiraceae UCG004	GERD	8	Inverse variance weighted	-0.09	0.042	0.91 (0.84-0.99)	0.026	13.72	0.056	0.789	0.14
Genus	Methanobrevibacter	GERD	4	Inverse variance weighted	-0.047	0.021	0.95 (0.91-0.99)	0.026	0.333	0.953	0.931	0.95

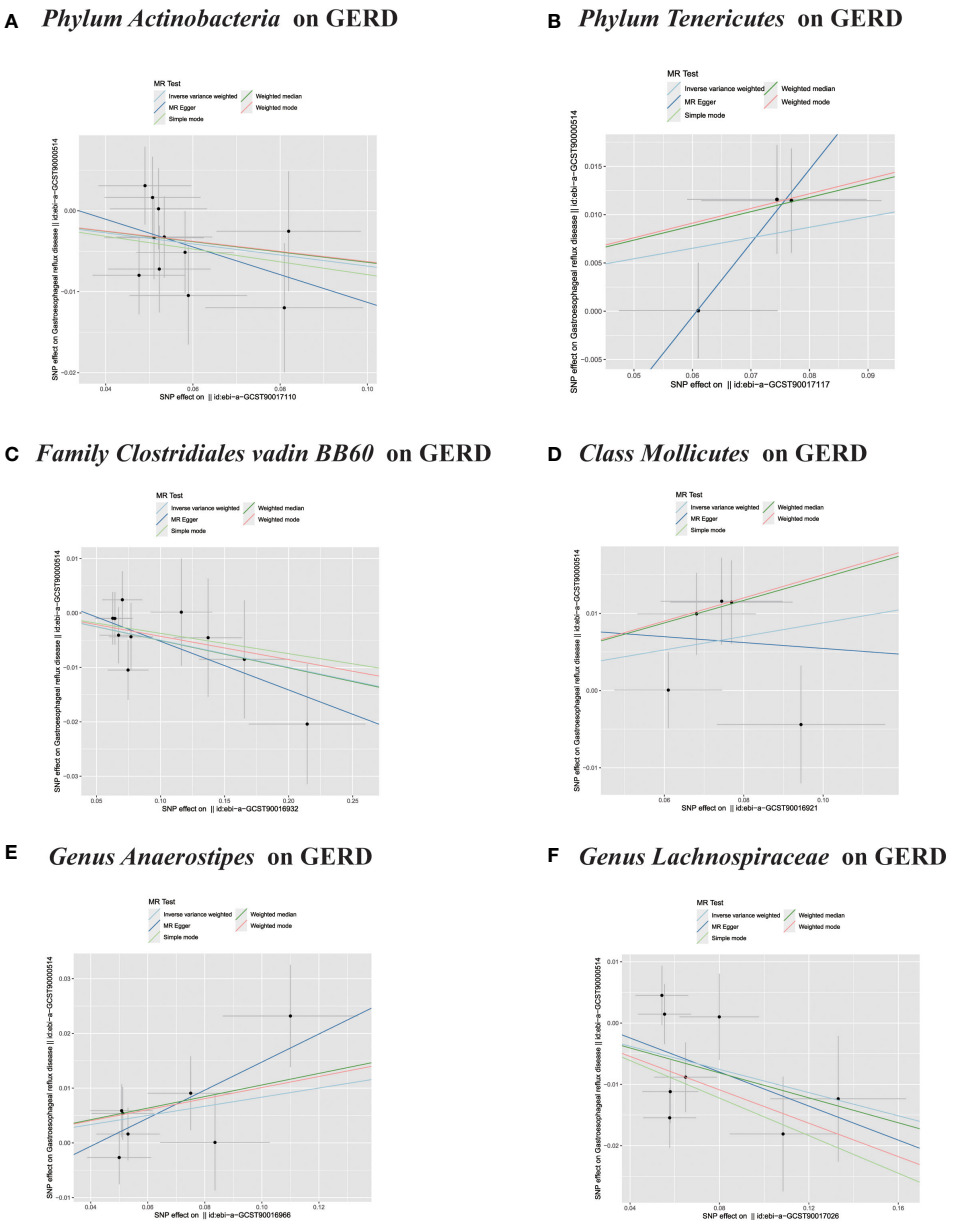

FIGURE 4
(A–F) Scatter plots of significant causality of the GM and GERD.

(OR=0.90, 95% CI:0.83–0.99, P=0.028), *Genus Christensenellaceae R 7 group*(OR=0.90, 95% CI:0.83–0.99, P=0.018), *Genus Rikenellaceae RC9 gut group* (OR=0.78, 95% CI:0.64–0.95, P=0.015), *Genus Ruminococcaceae NK4A214 group* (OR=0.89, 95% CI:0.81–0.98, P=0.013), *Genus Ruminococcaceae UCG005* (OR=0.90, 95% CI:0.82–0.98, P=0.019), and *Phylum Euryarchaeota* (OR=0.82, 95% CI:0.68–0.99, P=0.039). Conversely, an up-regulation post GERD onset was documented for *Genus Collinsella* (OR=1.15, 95% CI:1.04–1.26, P=0.005), *Genus Eggerthella* (OR=1.24, 95% CI:1.06–1.46, P=0.007), *Genus Eubacterium rectale group* (OR=1.12, 95% CI:1.01–1.24, P=0.029), *Genus Eubacterium ventriosum group* (OR=1.12, 95% CI:1.01–1.23, P=0.026), and *Genus Family XIII UCG001* (OR=1.12, 95% CI:1.01–1.24, P=0.046) (Figures 4, 5). Within the IVs, neither weak instrument bias nor significant heterogeneity metrics were identified. Further, the MR-PRESSO evaluation indicated no discernible outliers. The data's robustness was further affirmed by the leave-one-out analysis (Table 2; Figures 3, 6–10).

Discussion

To our knowledge, this is the first MR study to assess the causal relationship between the gut microbiome and susceptibility to gastroesophageal reflux disease. Using GWAS summary data, we confirmed an association between GERD and the gut microbiome.

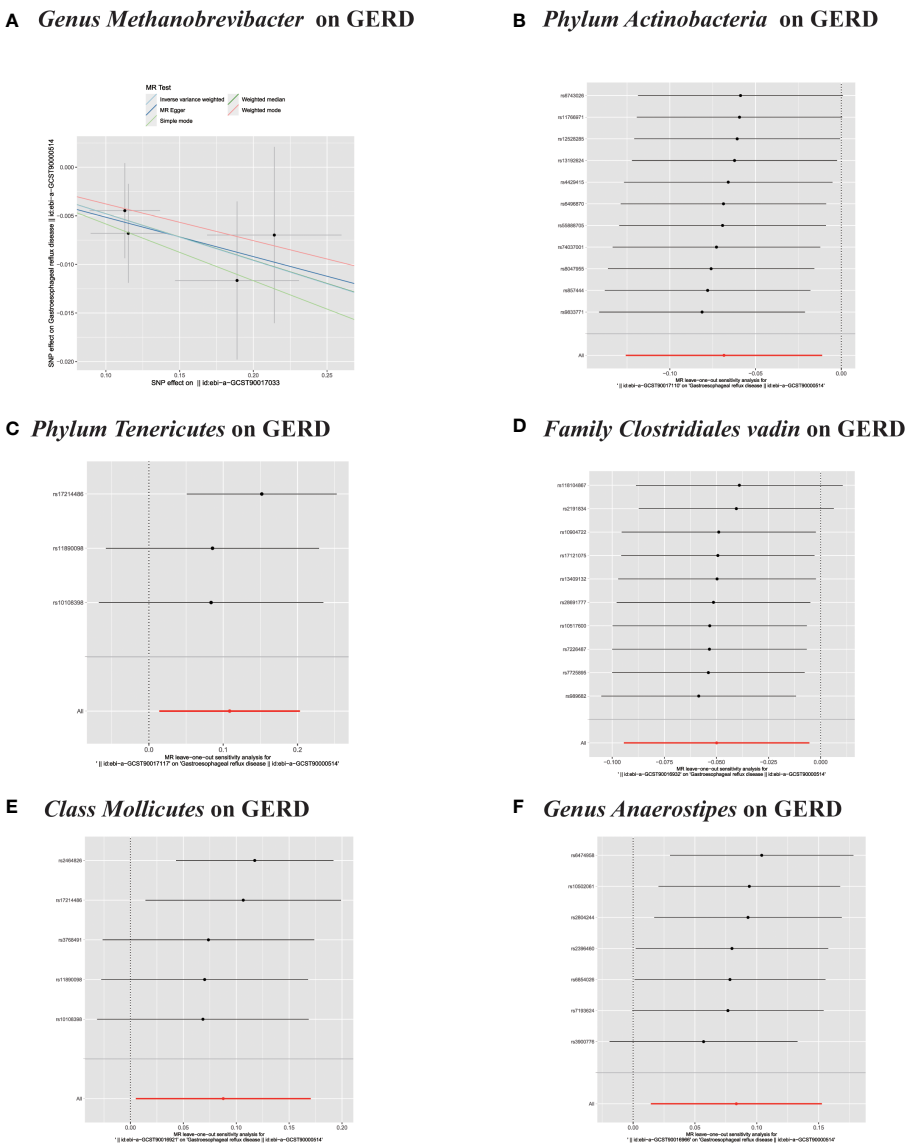

FIGURE 5
(A) Scatter plots of significant causality of the GM and GERD. **(B–F)** Leave-one-out analysis for the impact of individual SNPs on the association between GM and GERD risk.

Our research findings are consistent with extant academic literature, revealing a bidirectional relationship between GERD and the gut microbiome. We identified specific risk factors, including the *Class Mollicutes, Genus Anaerostipes and Phylum Tenericutes*. In contrast, protective factors, such as the *Family Clostridiales Vadin BB60 group,Genus Lachnospiraceae UCG004, Genus Methanobrevibacter and Phylum Actinobacteria*, were observed to be linked with GERD within the gut microbiome. The emergence of GERD manifested alterations in the gut microbiome composition. Following the MR analysis, GERD exhibited a causal influence on one Phylum, two Families, and ten Genera. Furthermore, the Phylum Actinobacteria, Family Clostridiales Vadin, and Genus Methanobrevibacter have been identified as contributors to the biosynthesis of Short-chain fatty acids (SCFAs). SCFAs emerge from the bacterial fermentation of indigestible dietary fibers within the gastrointestinal tract. The primary constituents of SCFAs are acetate, propionate, and butyrate. These acids not only serve as a principal energy source for colonocytes but also play a pivotal role in the dual-directional regulation of colonic motility, the preservation of intestinal homeostasis, and the enhancement of the integrity of the intestinal barrier (40–42). The human gastrointestinal epithelium is inhabited by a myriad of microbial entities that are instrumental in multiple physiological processes. An imbalance within this

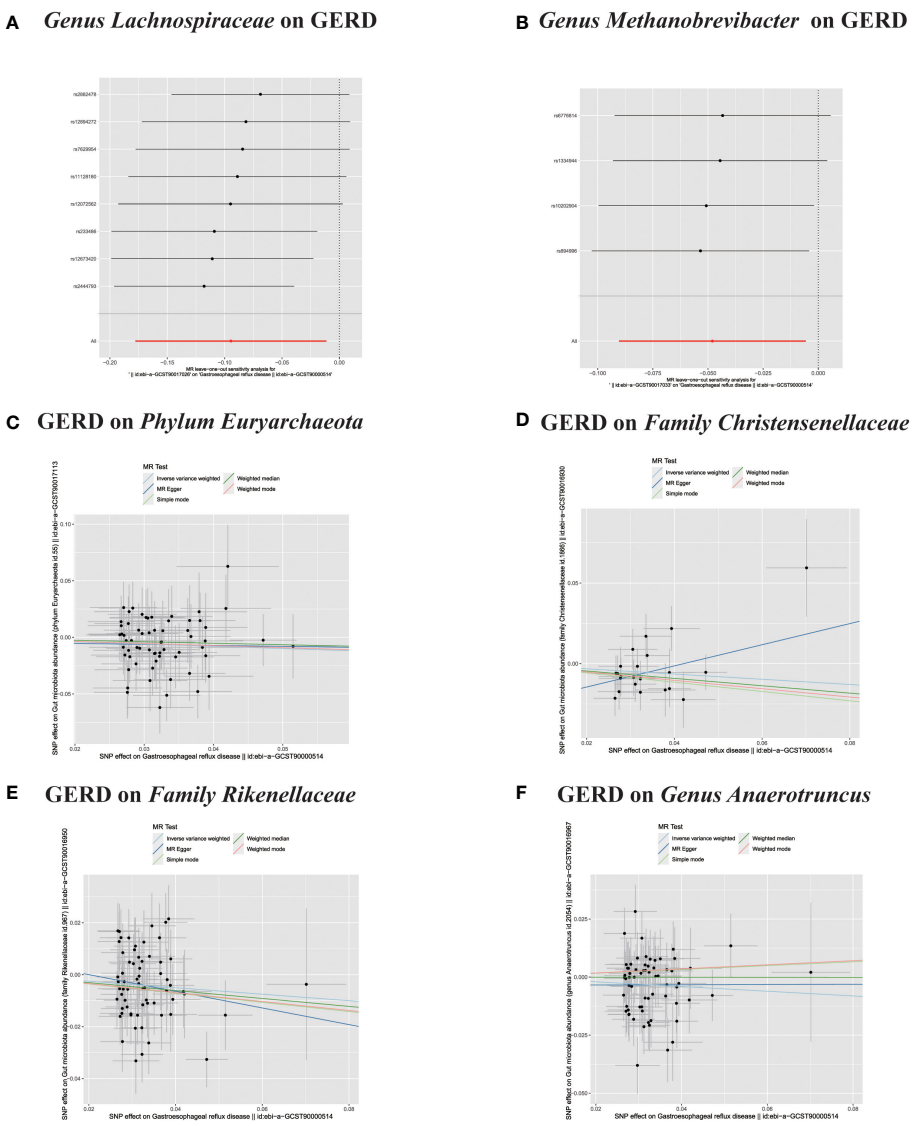

FIGURE 6
(A, B) Leave-one-out analysis for the impact of individual SNPs on the association between GM and GERD risk. **(C–F)** In reverse MR analysis, The scatter plots for association between GERD and gut microbiota.

microbial composition, termed intestinal dysbiosis, has been intricately linked to the etiology of numerous human pathologies. Innate lymphoid cells (ILCs), encompassing NK cells, ILC1s, ILC2s, ILC3s, and LTi cells, represent a subset of the innate immune system. Predominantly localized within the body's mucosal tissues, these cells have lately been the subject of significant academic scrutiny (43). Research has demonstrated a correlation between the presence of Clostridiales and a spectrum of esophageal pathologies, including esophagitis and BE. This association is hypothesized to influence the inflammatory processes of the esophageal mucosa and contribute to the development of intestinal metaplasia (44–46).

Recently, numerous research endeavors have delved into the association between gut microbiota and GERD. Ning L et al. documented a diminished prevalence of the *phylum Actinobacteria* in GERD patients, a result that is congruent with the findings of this study (47, 48). research indicated a substantial elevation in the levels of Proteobacteria and Bacteroidetes in pediatric subjects suffering from GERD. Concurrently, there was a notable decrease in the concentrations of Firmicutes and Actinobacteria (49). A Japanese research endeavor employed a distinctive method using quantitative 16S rRNA gene PCR to ascertain total bacterial quantities. The findings suggest that the relative proportions of taxa, including *Proteobacteria, Firmicutes,*

TABLE 2 Summary results of bidirectional MR (GERD on target Gut microbiome).

Exposure	Taxa	Outcome	Nsnp	Methods	Beta	SE	OR (95%CI)	*P* value	Heterogeneity		Horizontal pleiotrop	
									Cochran's Q	P value	Egger intercept P	MR-PRESSO P
GERD	Phylum	Euryarchaeota	64	Inverse variance weighted	-0.197	0.095	0.82 (0.68-0.99)	0.039	49.131	0.899	0.859	0.904
GERD	Family	Christensenellaceae	22	Inverse variance weighted	-0.161	0.080	0.85 (0.73-0.99)	0.045	22.259	0.384	0.052	0.398
GERD	Family	Rikenellaceae	65	Inverse variance weighted	-0.125	0.050	0.88 (0.80-0.97	0.012	81.301	0.071	0.527	0.072
GERD	Genus	Anaerotruncus	65	Inverse variance weighted	-0.101	0.046	0.90 (0.83-0.99	0.028	64.943	0.443	0.708	0.438
GERD	Genus	Christensenellaceae R 7group	65	Inverse variance weighted	-0.109	0.046	0.90 (0.82-0.98)	0.018	56.898	0.723	0.110	0.712
GERD	Genus	Collinsella	65	Inverse variance weighted	0.137	0.049	1.15 (1.04-1.26)	0.005	58.047	0.685	0.325	0.71
GERD	Genus	Eggerthella	65	Inverse variance weighted	0.219	0.082	1.24 (1.06-1.46)	0.007	57.356	0.708	0.147	0.706
GERD	Genus	Eubacterium rectale group	65	Inverse variance weighted	0.111	0.051	1.12 (1.01-1.24)	0.029	82.493	0.059	0.216	0.078
GERD	Genus	Eubacterium ventriosum group	65	Inverse variance weighted	0.111	0.050	1.12 (1.01-1.23)	0.026	70.367	0.273	0.391	0.28
GERD	Genus	Family XIII UCG001	65	Inverse variance weighted	0.109	0.054	1.12 (1.00-1.24)	0.046	70.735	0.262	0.395	0.28
GERD	Genus	Rikenellaceae RC9 gut group	64	Inverse variance weighted	-0.249	0.102	0.78 (0.64-0.95)	0.015	53.945	0.784	0.594	0.768
GERD	Genus	Ruminococcaceae NK4A214 group	65	Inverse variance weighted	-0.118	0.047	0.89 (0.81-0.98)	0.013	50.213	0.895	0.3660.-884GE-GERD-Genus-Rumino-cocca-ceae UC-G00565-Inverse variance weigh-ted-0.1080.-0460.90 (0.82-0.98) 0.01960-.1940.6-110.689-0.6	

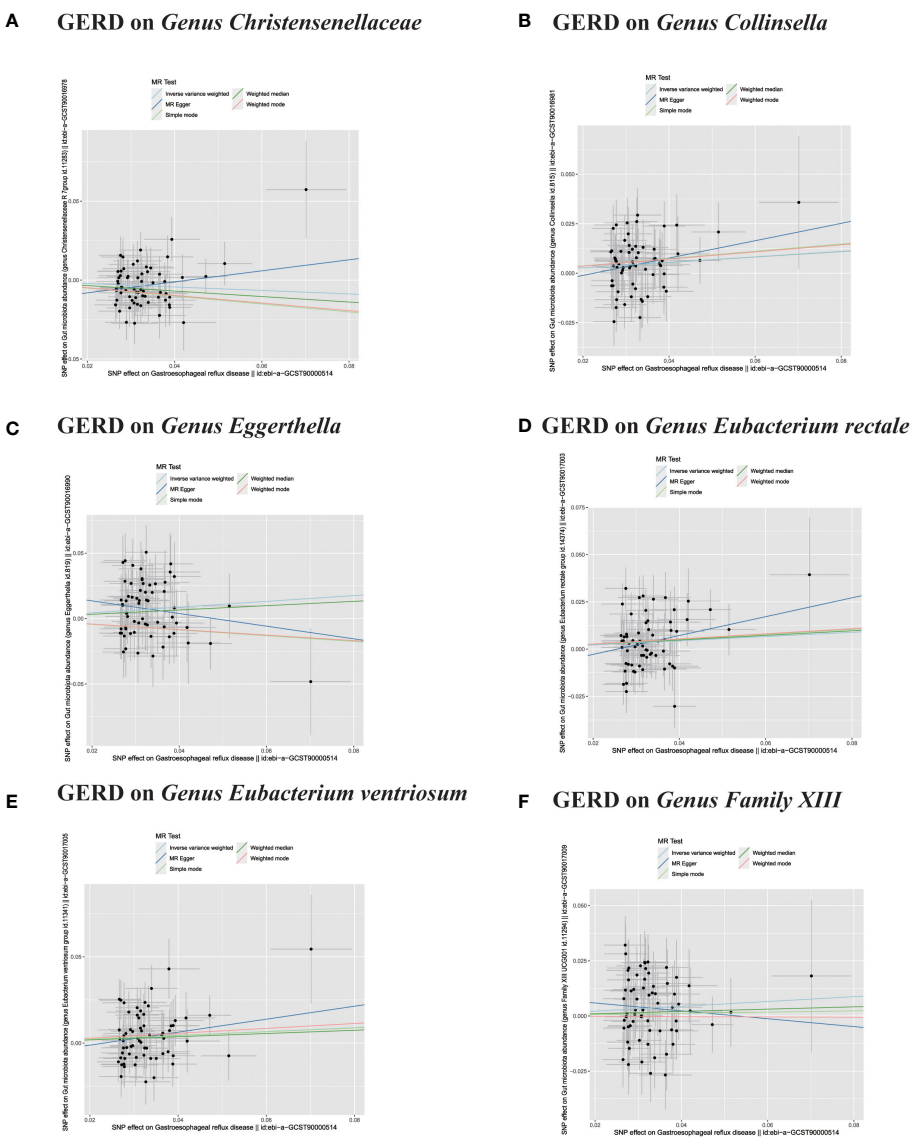

FIGURE 7
(A–F) In reverse MR analysis, The scatter plots for association between GERD and gut microbiota.

Bacteroidetes, Fusobacteria, and Actinobacteria, hold greater relevance to esophageal disorders than the absolute bacterial counts (47).

Our study initially demonstrated that the Family *Clostridiales Vadin BB60 group*, *Genus Methanobrevibacter*, and *Genus Lachnospiraceae UCG004* function as protective agents against GERD. These results underscore the putative roles of distinct gut microbiome entities in the pathogenesis of GERD, further accentuating the imperative for comprehensive studies to elucidate the foundational mechanisms and identify prospective therapeutic avenues. The hypothesis posits bacterial biofilm's role in GERD etiology (21). A recent investigation identified differential microbiota in NERD patients relative to control individuals and those with esophageal adenocarcinoma (EAC). Researchers employed 16S rRNA sequencing and mass spectrometry-based proteomics to profile the esophageal microbiota and the host mucosal proteome, respectively. An aggregate of 70 individuals spanning four patient categories (NERD, reflux esophagitis, Barrett's esophagus, and EAC) along with a control group were examined. The findings revealed a singular microbiota configuration in NERD, divergent from the control and other cohorts (50). Proton pump inhibitors (PPI) remain a

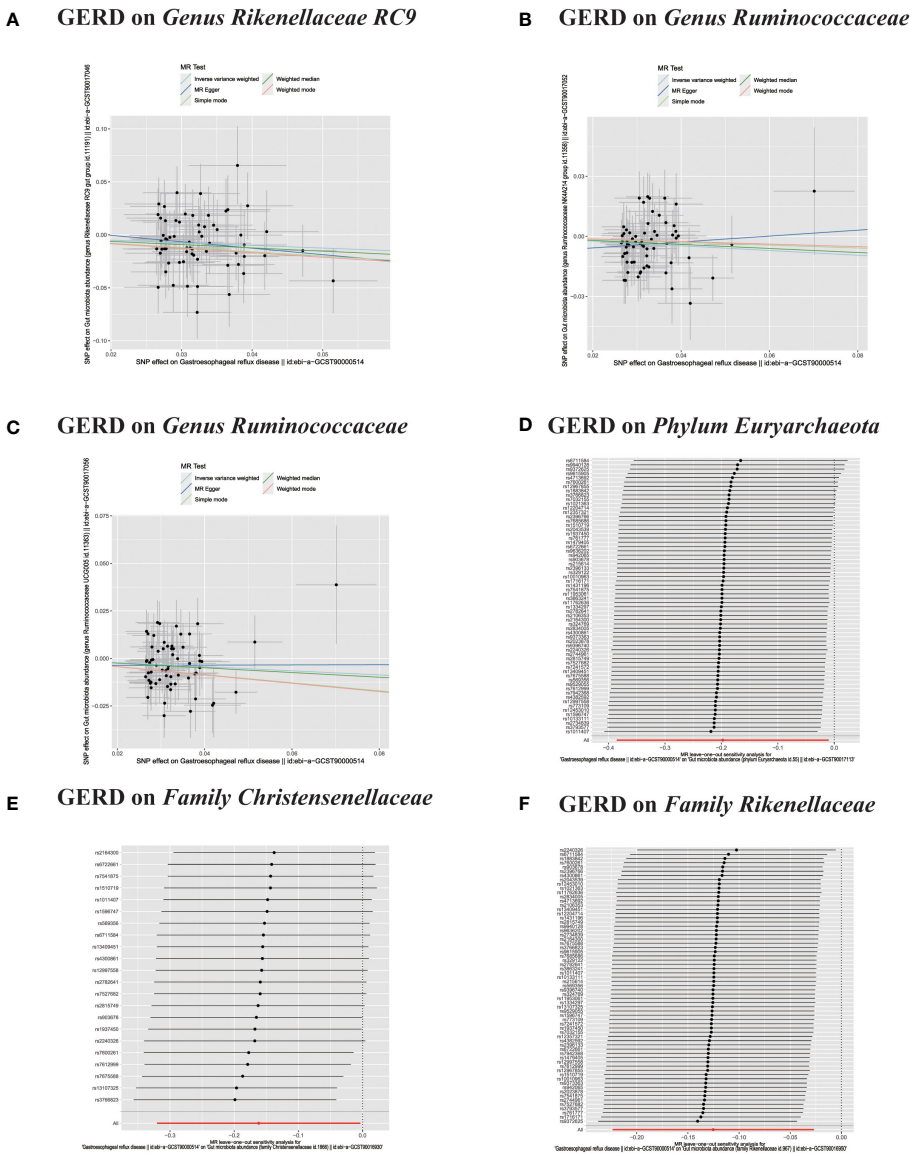

FIGURE 8
(A–C) In reverse MR analysis, The scatter plots for association between GERD and gut microbiota. **(D–F)** In reverse MR analysis, Plots for "leave-one-out" analysis for causal effect of GERD on gut microbiota risk;.

foundational component in the therapeutic approach to reflux disease. Modifications in the esophageal microbiome due to the diminished gastric acidity induced by PPI have been investigated in multiple research endeavors, illustrating their consequential impact on microbial community configurations (51–61). The gut microbiota comprises an extensive array of microorganisms residing in the human gastrointestinal tract, facilitating various physiological and biochemical processes for the host (62). Alterations in the composition of esophageal microbiota can be attributed to environmental influences. A diet rich in fats has been strongly correlated with localized mucosal inflammatory modifications in murine representations (63). The postulated mechanism for this advantage is the decelerated fermentation, resulting in enhanced luminal accessibility in contrast to conventional fiber-laden products. The preliminary investigation demonstrated notable beneficial impacts of sugarcane flour on alleviating GERD symptoms, necessitating a more expansive randomized controlled trial (64).Probiotics introduce bacterial strains via dietary supplementation, aiming to optimize the gut microbiota composition towards a more favorable equilibrium. Evaluations of probiotics encompassing Lactobacilli spp. and Bifidobacteria spp. have shown efficacy in alleviating GERD

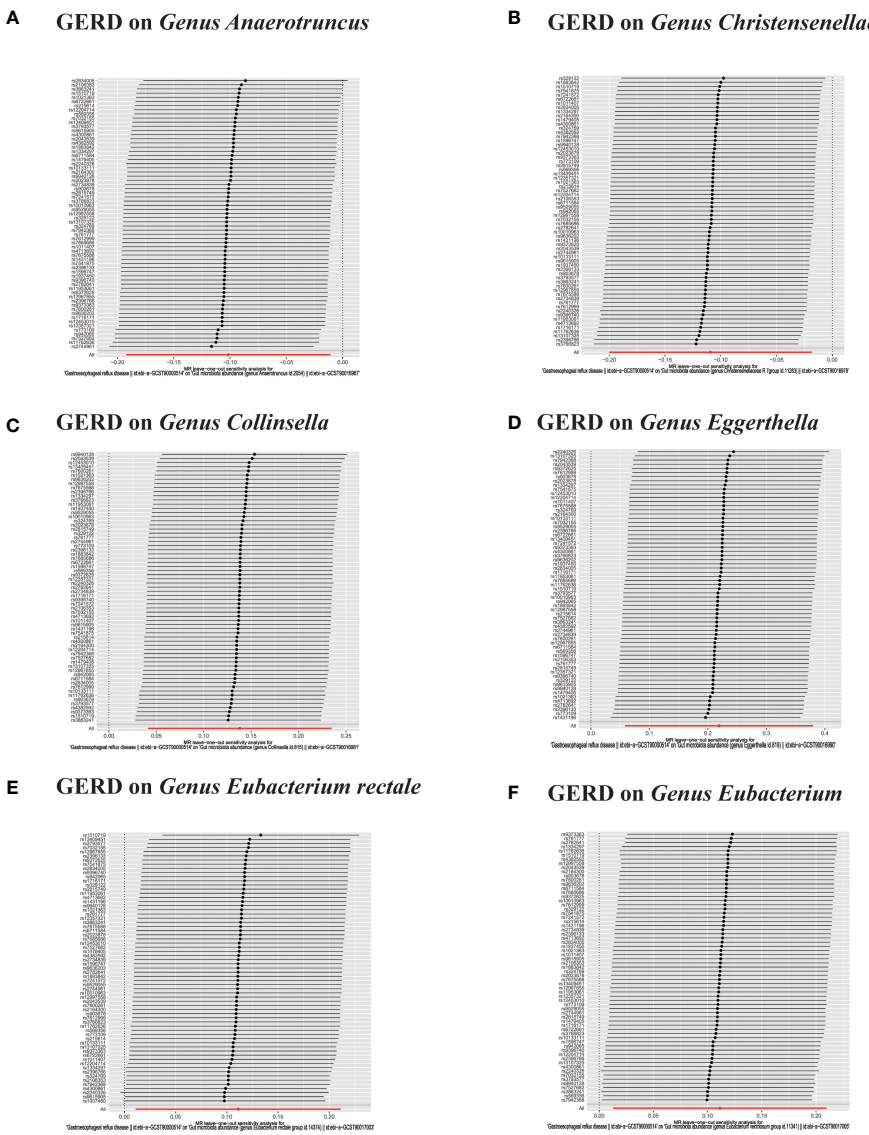

FIGURE 9
(A–F) In reverse MR analysis, Plots for "leave-one-out" analysis for causal effect of GERD on gut microbiota risk.

manifestations (65–68). This research seeks to determine a causal link between particular gut microbiota and GERD through MR analysis. Comprehending the relationship between gut microbial dysbiosis and the onset of GERD, as well as pinpointing the specific gut microbiota associated with GERD, can facilitate the proactive identification of individuals at elevated risk. This understanding permits the prompt initiation of targeted preventative measures and the tailoring of clinical interventions, which can mitigate symptoms such as regurgitation and heartburn. Furthermore, such approaches can enhance patients' overall well-being and curtail economic burden.

Our study possesses key strengths. Firstly, MR represents an analytical methodology employing genetic variants as IVs to elucidate the causal relationship between exposure and outcome. The MR framework mitigates unobserved confounders and counteracts reverse causation, which are prevalent in observational research. Second, we employed the most extensive GWAS pertaining to the gut microbiota currently available, though its sample size remains notably constrained (n = 14,306). Prospective GWAS investigations concerning the gut microbiota should endeavor to augment the sample size to conventional GWAS benchmarks (n > 100,000) to enhance statistical power and minimize potential

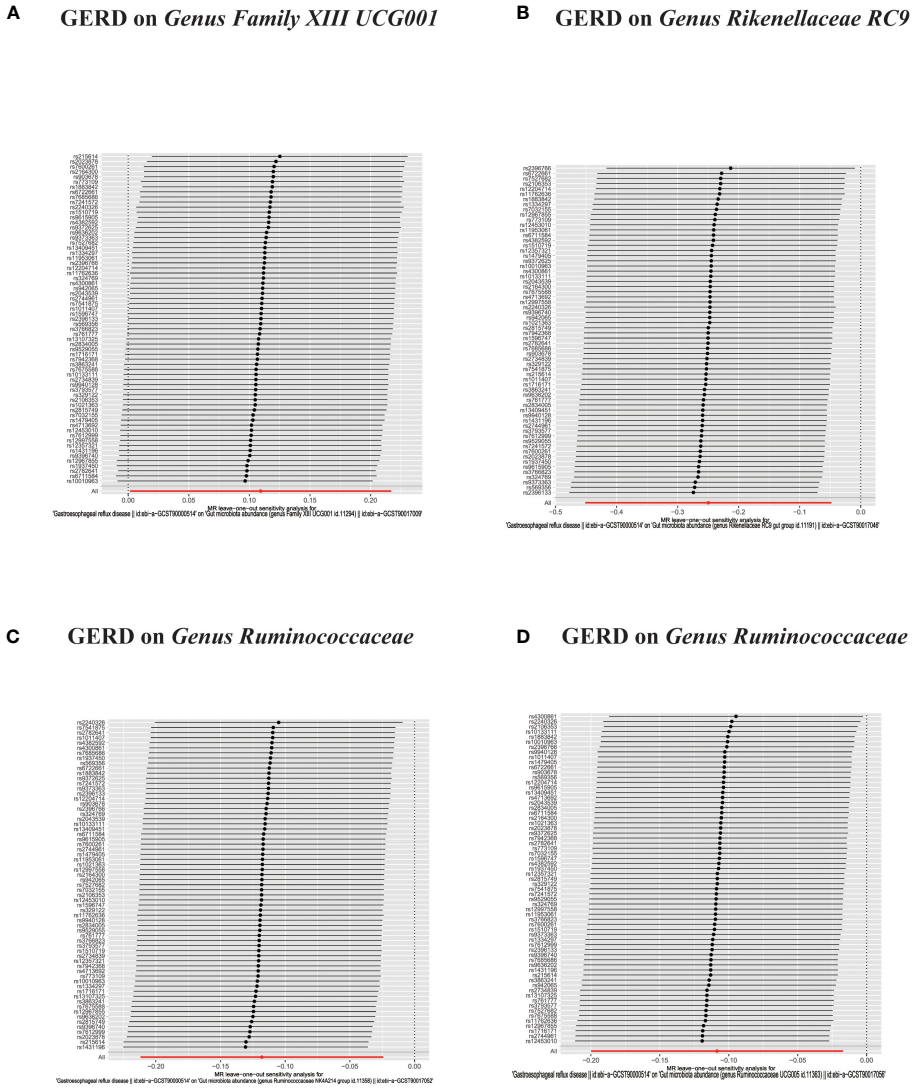

FIGURE 10
(A–D) In reverse MR analysis, Plots for "leave-one-out" analysis for causal effect of GERD on gut microbiota risk.

inaccuracies. Our research, admittedly, possesses certain limitations. First, A segmented analysis considering overarching determinants like age and gender was not feasible owing to the constraints inherent in the GWAS summary data. Second, we refrained from adjusting for multiple testing, as stringent corrections for multiple comparisons might overlook strains that have a causal association with GERD. Thirdly, the summary-level data from GWAS predominantly originate from European cohorts, constraining the universal applicability of our results.

In conclusion, while we have postulated a causal link between gut microbiota and GERD at the genetic dimension, the underlying biological pathways warrant further investigation. Our findings may serve as a foundational framework for delving into the mechanisms of specific gut microbiomes in individuals with GERD. In future clinical endeavors, it may be feasible to gauge the prevalence of gut microbiota in fecal samples as a prognostic tool for assessing GERD risk. Additionally, modulating the gut microbiota could serve as a preventive and therapeutic strategy for GERD.

Conclusion

This research identified certain microbial taxa as either protective or risk determinants for GERD. Such findings may offer valuable biomarkers for diagnostic purposes and potential therapeutic intervention points for GERD. Subsequent research endeavors ought to corroborate these results in human subjects and delve deeper into elucidating the underlying mechanisms.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by written informed consents were meticulously secured from all participating individuals. Concurrently, these investigations were granted the requisite endorsements from the pertinent ethical oversight bodies. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

KW: Conceptualization, Data curation, Investigation, Methodology, Software, Supervision, Validation, Visualization, Writing – original draft. SW: Conceptualization, Data curation, Investigation, Methodology, Software, Supervision, Validation, Writing – original draft. YC: Conceptualization, Formal analysis, Investigation, Software, Writing – original draft, Writing – review & editing, Visualization. XL: Conceptualization, Data curation, Investigation, Methodology, Software, Supervision, Validation, Writing – original draft. DW: Conceptualization, Data curation, Investigation, Software, Supervision, Writing – original draft. YZ: Conceptualization, Data curation, Formal analysis, Investigation, Project administration, Software, Supervision, Writing – review & editing. WP: Project administration, Supervision, Data curation, Methodology, Writing – original draft, Writing – review & editing. CZ: Conceptualization, Writing – original draft, Investigation, Data curation, Supervision. DZ: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1327503/full#supplementary-material

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Paraprobiotic derived from *Bacillus velezensis* GV1 improves immune response and gut microbiota composition in cyclophosphamide-treated immunosuppressed mice

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Paraprobiotics that benefit human health have the capacity to modulate innate and adaptive immune systems. In this study, we prepared the paraprobiotic from *Bacillus velezensis* GV1 using the heat-killing method and investigated its effects on immunity and gut microbiota *in vitro* and *in vivo*. The morphology of inactivated strain GV1 was observed using scanning electron microscopy. Treatment with GV1 promoted nitric oxide production and augmented cytokine (IL-6, IL-1β, and TNF-α) expression and secretion in RAW 264.7 macrophages. Moreover, the strain GV1 could alleviate cyclophosphamide monohydrate (CTX)-induced immunosuppression by reversing spleen damage and restoring the immune organ index, as well as by increasing the expression of immune-related cytokines (TNF-α, IL-1β, IFN-γ, and IL-2) in the spleen and thymus, respectively. Furthermore, GV1 treatment dramatically healed the CTX-damaged colon and regulated gut microbiota by increasing the relative abundance of beneficial bacterial families (*Lactobacillaceae*, *Akkermansiaceae*, and *Coriobacteriaceae*) and decreasing that of harmful bacterial families (*Desulfovibrionaceae*, *Erysipelotrichaceae*, and *Staphylococcaceae*). Thus, the heat-killed GV1 can be considered a potential immunoregulatory agent for use as a functional food or immune-enhancing medicine.

KEYWORDS

paraprobiotics, Bacillus velezensis GV1, immunoregulation, gut mirobiota, cyclophosphamide

1 Introduction

The immune system of an organism is responsible for protecting against pathogens and maintaining homeostasis for survival (1). Dysfunctional immune responses in the human body due to factors such as the environment, genetics, age, nutrition, and stress can result in immunodeficiency disorders (2). Recently, many immunopotentiation agents have been used to improve immune responses and enhance disease resistance. However, they frequently cause a variety of side effects, including gastrorrhagia, severe neurological lesions, anemia, and colic (3). Research has shown that the use of natural products from fungi, microorganisms, plants, and animals to regulate the immune system is safe and does not cause side effects (4). Therefore, natural products are potential sources of compounds that can improve the immune system without triggering unwanted responses.

Probiotics are known to provide numerous benefits to human health as living microorganisms. Particularly, they are recognized for their crucial features such as immune system enhancement, prevention of gastrointestinal infections, and protection against oxidative stress (5). However, along with these benefits, probiotics may induce side effects in specific population groups, and issues related to their viability, stability, and sensitivity to storage conditions are also associated (6, 7). Alternatives such as prebiotics, paraprobiotics, and postbiotics have been proposed to address these shortcomings of probiotics.

Paraprobiotics, also known as non-viable microbial cells or inactivated probiotics, have garnered recognition for their ability to confer health benefits when administered in suitable quantities. They offer safety advantages by addressing concerns related to viability, survival challenges, and safety considerations regarding microbial movement and infection (8). Recent research endeavors have been initiated to address the limitations associated with probiotics across various domains, including the food industry and therapeutic applications, through the application of paraprobiotics (9, 10). Studies have illustrated that paraprobiotics manifest anti-inflammatory effects, mitigating conditions like colitis, and contribute to enhanced skin moisturization, thereby preventing wrinkle formation (11–15). Numerous reports indicate that probiotics subjected to heat-killing, a potential method for creating paraprobiotics, have a significant impact on immunomodulation (12, 13). Furthermore, recent studies indicate that paraprobiotics manufactured based on this premise exert an influence on immune responses in macrophages and splenocytes (16, 17). Additionally, these investigations propose that peptidoglycan, lipoteichoic acid, and wall teichoic acid obtained from gram-positive microorganisms play a role in immune regulation (18–20). Consequently, these findings underscore the immunomodulatory efficacy of compositions derived from gram-positive paraprobiotics.

Bacillus velezensis is a gram-positive, spore-forming bacterium commonly found in soil, plant roots, and fermented foods (21). Spore-forming bacteria, widely employed in medical, veterinary, and more recently in the food industry, exhibit significant potential due to their high resistance and exceptional stability under processing conditions (22). Particularly within the realms of food and fermentation industries, the strain *B. velezensis* has gained notable recognition for its crucial role in ensuring safety and outcompeting rival microorganisms (23, 24). Although bacterial species producing heat-resistant spores are fortunately non-pathogenic, they can lead to food product spoilage (25). Hence, this study explores the immune-enhancing effects of paraprobiotics *Bacillus velezensis* GV1, with an emphasis on the safety of heat-treated strains, for potential applications in health functional foods and food industry, focusing on immune regulation and gut microbiota modulation.

2 Materials and methods

2.1 Materials

De Man, Rogosa and Sharpe (MRS) broth was obtained from Becton Dickinson & Company (B.D., New Jersey, USA). DMEM medium, penicillin-streptomycin (PS), and fetal bovine serum (FBS) were purchased from GenDEPOT (Katy, TX, USA). Macrogen (Seoul, Republic of Korea) designed all the primers. Live/Dead cell viability assay kits were provided by Thermo Fisher Scientific, USA. Eosin Y Alcoholic was obtained from BBC Biochemical (Mount Vernon, WA, USA). Cyclophosphamide monohydrate (CTX), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), and levamisole hydrochloride (LMS) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Bacterial strain and heat treatment

The strain GV1 was isolated from ginseng vinegar and identified by 16S rRNA sequencing using four primers (27F, 1492R, 518F, and 800R). The NCBI accession number (16S rRNA gene sequence) of GV1 was OP658964. Additionally, GV1 was deposited in the Korean Collection for Type Cultures (KCTC) under the accession number KCTC 15222BP. The strain was cultured in 1 L of MRS broth at 37°C for 24 h. After this incubation process, the bacterial cells were collected by centrifugation at 4000 rpm for 10 min. The bacterial cell pellet was washed thrice with phosphate-buffered saline (pH 7.4) and re-suspended such that the optical density of the suspension was OD_{600} 1.0. Heat treatment was conducted at 121°C for 15 min. Then, inactivated bacterial cells were freeze-dried for further experiments.

2.3 Scanning electron microscope

The strain GV1 was inoculated into MRS broth and cultured for 24 h at 37°C. Heat-killed GV1 was prepared as described above. Briefly, live and heat-killed bacterial cells were collected by centrifugation and pre-fixed with glutaraldehyde (2.5% *v*/*v*) for 2 h. Then, the samples were washed with 0.05 M sodium cacodylate buffer and treated with 1% osmium tetroxide for 1 h. After

dehydration using ethanol (in a stepwise elevation from 30% to 50%, 70%, 80%, 90%, and 100%), the samples were treated with hexamethyldisilazane and metallized using platinum. Observations were performed under a SIGMA Field-Emission Scanning Electron Microscope.

2.4 Cell culture and viability assay

RAW 264.7 cells were purchased from the Korean Cell Line Bank (KCLB, Korea). The macrophages were maintained in DMEM with 10% FBS and 1% PS inside a 5% CO_2 incubator at 37°C. After 90% confluency, the cells were seeded in 96-well plates (2×10^5 cells/mL). Then, the cells were treated with different concentrations of GV1 (0.5, 1, and 2 μg/mL) and a positive control, LPS (1 μg/mL), for 24 h. After removing the supernatant, MTT solution (100 μL at 0.5 mg/mL) was added and the plates were incubated for 3 h; then, DMSO was used to dissolve the formazan crystals. The absorbance at 560 nm was measured using a microplate reader (FilterMax F5, Molecular Devices, San Francisco, CA, USA).

2.5 Live/dead fluorescence assay

The cytotoxicity of GV1 toward RAW 264.7 cells was assessed using a Live/Dead staining kit (L-3224, Invitrogen, Carlsbad, CA, USA). The macrophages were sub-cultured in small cell culture dishes overnight and then incubated with GV1 (0.5, 1, and 2 μg/mL) and LPS (1 μg/mL). Following 24 h of treatment, calcein AM and ethidium homodimer-1 dyes were mixed with fresh media and added to the cells, which were thereafter incubated for 30 min in the dark. Then, the cells were observed using a Leica DMLS Clinical Microscope (Leica, Wetzlar, Germany).

2.6 Measurement of NO

After overnight incubation in 96-well plates (2×10^5 cells/mL), macrophages were treated with GV1 (0.5, 1, and 2 μg/mL) and LPS (1 μg/mL) for 24 h. The culture supernatants (100 μL) were transferred to new plates and 100 μL of Griess reagent was added for NO detection. The absorbance at 570 nm was measured using a microplate reader (FilterMax F5).

2.7 Quantitative real-time polymerase chain reaction

Total mRNA was extracted from RAW 264.7 cells and mouse organs in accordance with the TRIzol reagent kit instructions (Invitrogen). The amfiRivert cDNA Synthesis Platinum Enzyme Mix (GenDEPOT) was then used to reverse-transcribe total RNA. AmfiSure qGreen Q-PCR Master Mix (GenDEPOT) was used to perform qRT-PCR using 50 ng of cDNA in a 20 μL reaction volume. The sequences of primers are shown in Supplementary Table S1.

2.8 Enzyme-linked immunosorbent assay

For *in vitro* experiments, RAW 264.7 cells were seeded in 96-well plates and incubated with GV1 and LPS. The culture supernatants were collected to investigate the production of TNF-α, IL-1β, and IL-6 using an ELISA kit (R&D Systems, Minneapolis, MN, USA), based on the manufacturer's instructions.

For *in vivo* experiments, spleens of ICR mice in each treatment group were harvested and washed with PBS. Spleen tissues (100 mg) were homogenized and transferred to saline tubes. The tubes were centrifuged at 10000 rpm for 5 min and the supernatants were collected. The contents of TNF-α, IL-1β, IL-6 in the samples were determined using ELISA kits (R&D Systems).

2.9 Animal experiments

Male ICR mice (six weeks old) weighing 25 ± 1 g were obtained from OrientBio (Seongnam, Republic of Korea) and housed under stable condition (temperature: 23 ± 2°C, humidity: 50% ± 10%, light/dark cycle: 12 h). This study was approved by the Animal Care and Use Guidelines of Kyung Hee University (KHGASP-23-046). The mice were separated into 6 groups (8 mice per group): Control (CON) group (normal saline), only CTX-treated group, CTX + GV1 (5 mg/kg) group, CTX + GV1 (10 mg/kg) group, CTX + GV1 (20 mg/kg) group, and CTX + LMS (40 mg/kg) group (positive control). For immunosuppression, all groups of animals were injected with CTX (80 mg/kg) for 3 days before GV1 and LMS treatment, except the CON group, which received saline only. GV1 or levamisole hydrochloride (LMS) was orally administered to the mice once daily by gavage for a period of 20 days. Thereafter, the mice were sacrificed to obtain their spleens, guts, and thymus glands for further experiments. The spleens and thymus glands were weighed to calculate the organ index as follows:

$$Index = \frac{Weight\ \ of\ \ spleen\ \ or\ \ thymus\ \ (g)}{Body\ \ weght\ \ (g)}$$

2.10 Histopathological analysis of spleen and colon

Immediately after sampling, spleen and colon tissues were fixed with 10% formalin buffer solution and embedded in paraffin for hematoxylin and eosin (HE) staining. Then, the tissue sections were observed under a microscope.

2.11 High-throughput sequencing

The genomic DNA of mice feces in the CON, CTX, and GV1 (20 mg/kg) groups was extracted using an E.Z.N.A.® Soil DNA Kit (Omega Biotek, Norcross, GA, USA) following the manufacturer's protocol. The sequencing procedure was identical to that followed in our previous study (5).

2.12 Statistical analysis

Data were expressed as means ± standard deviations or standard errors for *in vitro* or *in vivo* experiments, respectively. All experiments were carried out in triplicate. Statistical comparisons between groups were conducted using Student's *t*-test and statistical significance was set at different levels ($p< 0.05$, $p< 0.01$, and $p< 0.001$). An analysis of variance (ANOVA) followed by Duncan's test was employed to evaluate the statistical significance between groups (SPSS 29.0). Different letters presented in tables and figures (a, b, c, d, e) were regarded as statistical significance ($p < 0.05$).

3 Results

3.1 Field-emission scanning electron microscope

Generally, the surface of heat-killed bacterial cells was rougher and uneven compared with that of viable cells. Figures 1A, B show representative FE-SEM images of both live and heat-treated GV1 (at 121°C for 15 min and, consequently, freeze-dried), respectively. There were obvious signs of damage on the surfaces of heat-treated bacterial cells, unlike the untreated cells, indicating that heat treatment inactivated GV1.

3.2 Cytotoxicity of GV1 toward RAW 264.7 cells

GV1 cytotoxicity toward RAW 264.7 cells was examined using an MTT assay and Live/Dead staining. The results (Live/Dead staining: Figure 2A; MTT assay: Figure 2B) revealed that treatment with GV1 (0.5, 1, and 2 μg/mL) had no impact on the viability of macrophages. However, LPS (1 μg/mL) exhibited a slight cytotoxic effect on RAW 264.7 cells.

3.3 Effect of GV1 on NO production and inducible *nitric oxide synthase* Expression in RAW 264.7 cells

NO exerts significant antimicrobial, anticancer, and immunomodulation effects but can also damage tissues (26). Hence, we examined the effect of GV1 on NO production in murine macrophages. As shown in Figure 2C, GV1 dose-

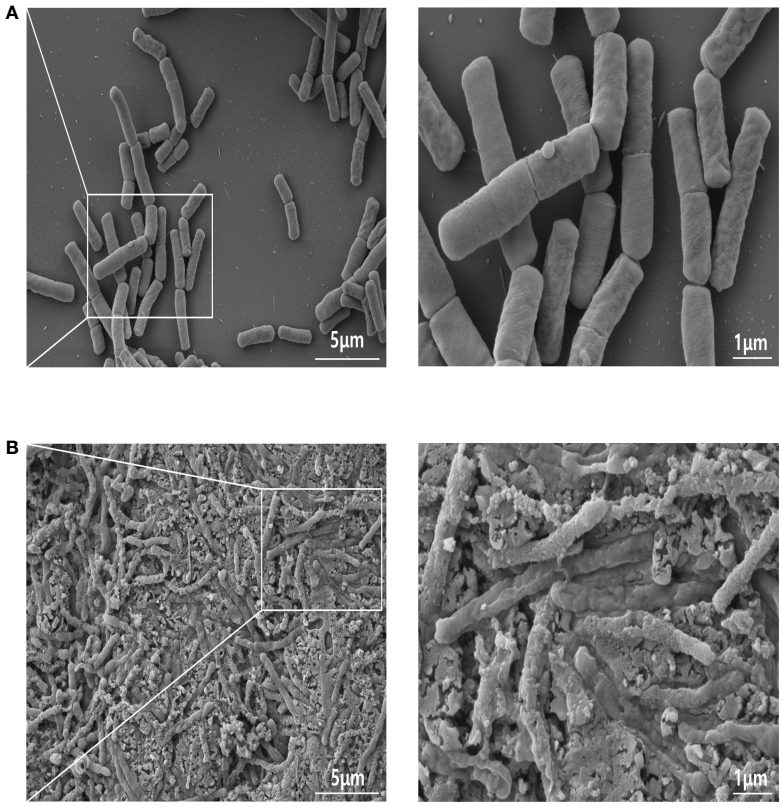

FIGURE 1
FE-SEM images of GV1: A comparative analysis of live and heat-killed morphology. **(A)** Live GV1; **(B)** Heat-Killed GV1. (10K X, Scale bar = 5μm; 30K X, Scale bar = 1μm).

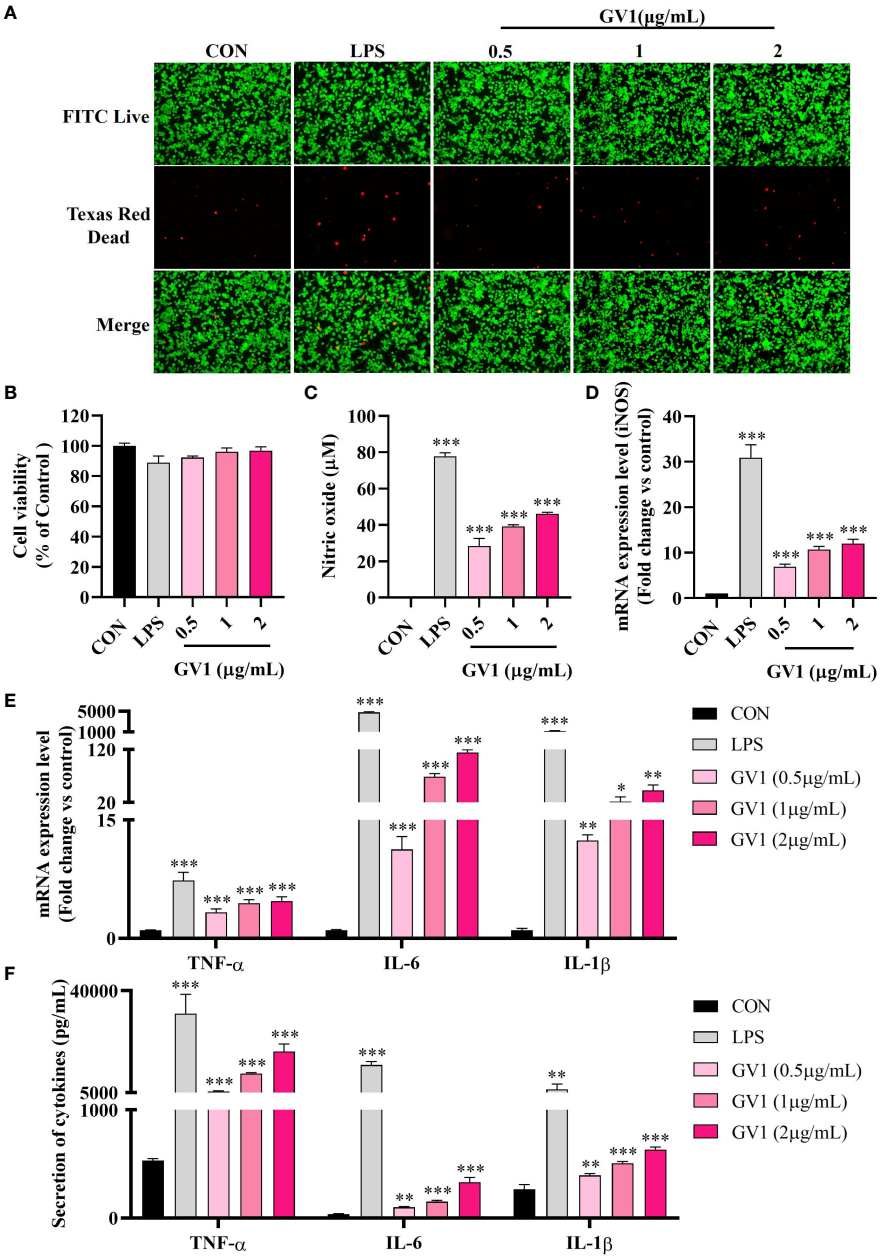

FIGURE 2
Macrophage responses to GV1: Cytotoxicity and immunity enhancement. **(A)** Live/Dead staining enhancement; **(B)** Cell viability; **(C)** NO production; Expression of mRNA **(D)** *iNOS*, **(E)** *TNF-α*, *IL-6*, and *IL-1β*; **(F)** Secretion of cytokines TNF-α, IL-6, and IL-1β. All data are presented as means ± S.D. **p*< 0.05, ***p*< 0.01, ****p*< 0.001 *vs*. CON group.

dependently enhanced NO production at 28.3 ± 4.2, 39.2 ± 0.9, and 46.1 ± 0.8 μM at concentration 0.5, 1, and 2 μg/mL, respectively. NO production was mediated by *iNOS*, which is also associated with immune responses (27). Cell stimulation by several agents such as LPS induces *iNOS* expression, thus increasing NO production (28). *iNOS* levels were determined using qRT-PCR (Figure 2D); GV1 significantly induced *iNOS* mRNA expression. These findings suggest that GV1 can stimulate RAW 264.7 cells by elevating the expression of *iNOS* and enhancing NO production.

3.4 Effect of GV1 on immune-related cytokines in RAW 264.7 cells

It is well known that macrophages modulate adaptive and innate immune systems by releasing immune-related cytokines such as TNF-α, IL-6, and IL-1β (29). Several studies have shown that LPS highly stimulates the expression of *TNF-α*, *IL-6*, and *IL-1β* mRNA (Figure 2E) (30, 31). Treatment with GV1 enhanced the expression levels of such cytokine mRNA in a dose-dependent

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manner (Figure 2E). ELISA analysis confirmed that GV1 markedly promoted TNF-α, IL-6 and IL-1β secretion (Figure 2F). These findings suggest that GV1 can increase the expression and secretion of immune-related cytokines in murine macrophages.

3.5 Effect of GV1 on body weight and immune-related organs in CTX-treated mice

In vitro experiments indicated that GV1 significantly enhances immunity. Therefore, we investigated the effects of GV1 on CTX-treated immunosuppressed mice. CTX, an alkylating cytotoxic drug, is effective in treating autoimmune disorders and cancer. However, long-term use of high doses of CTX can cause immunosuppression and intestinal problems such as viral infections and microflora disorders (32). Hence, CTX is frequently used to suppress immunity in mouse models. Body weight and immune organ indexes play important roles in the health of mice (33). LMS is a compound that improves immune responses, especially under immunocompromised conditions; hence, it was used as a positive control in this study (3). During the experimental period, body weight slightly increased in all the groups and there was no difference between groups (Figure 3A). As shown in Figure 3B, the spleen and thymus indexes of the CTX-induced group remarkably decreased compared with those of the control group. GV1 treatment restored the spleen and thymus indexes, and this recovery was greater than that in the LMS-

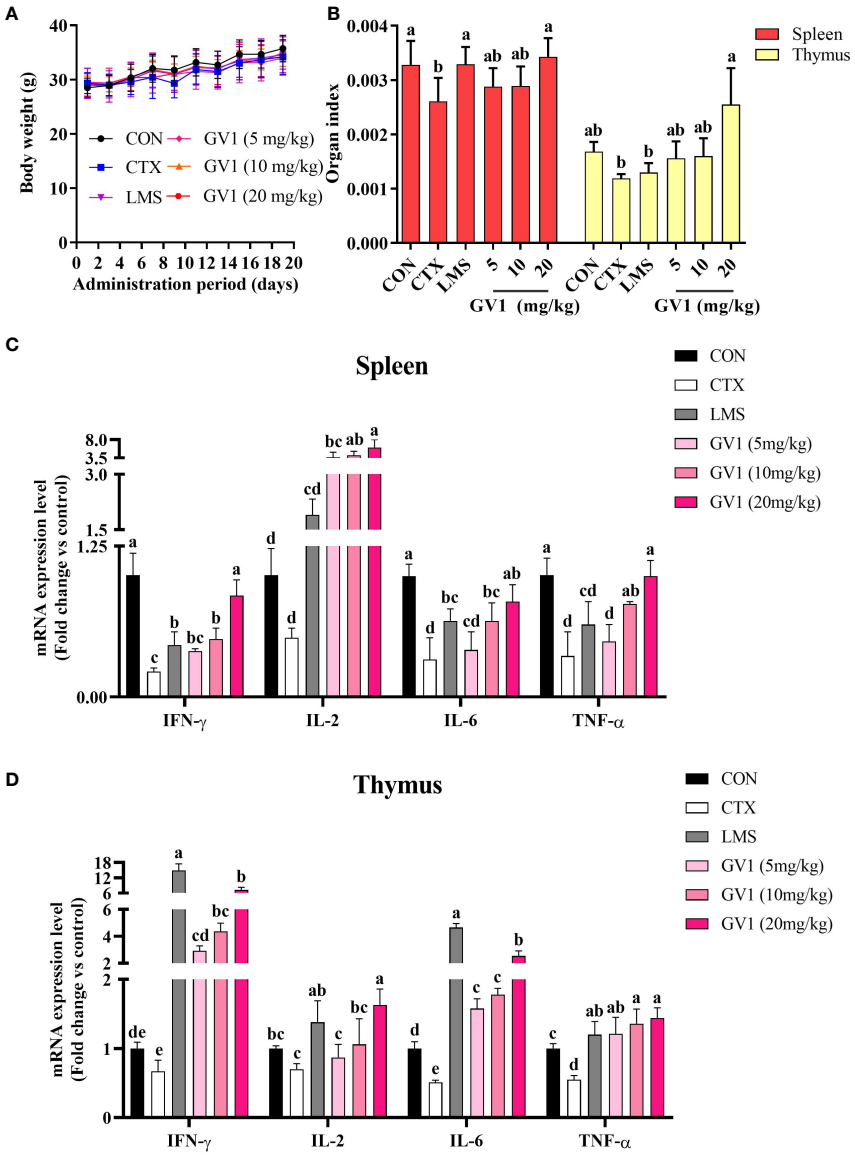

FIGURE 3
Effects of GV1 on body weight and immune-related organs of CTX-treated immunosuppressed mice. **(A)** Body weight; **(B)** Spleen and thymus indexes; **(C)** Expression of mRNA in spleen; **(D)** Expression of mRNA in thymus. All data are presented as means ± S.D. Adopt the Duncan analysis method. Different letter combinations (a–e) is significant ($p < 0.05$).

treated group. These data indicate that GV1 could reverse the immune organ atrophy induced by CTX.

Furthermore, we evaluated the effect of GV1 on the expression of mRNA of several immune-associated cytokines such as *IL-6*, *TNF-α*, *IFN-γ*, and *IL-2* in mouse spleen and thymus tissues, respectively. As shown in Figure 3C, the cytokine (*TNF-α*, *IL-1β*, *IFN-γ*, and *IL-2*) mRNA levels in the spleen tissue were dramatically suppressed in the CTX group compared with those in the control group. However, the GV1 groups exhibited dose-dependent improvements in cytokine expression, compared with that in the CTX group. Notably, the cytokine mRNA expression in the LMS group was significantly lower than that in the GV1 groups. Figure 3D illustrates that CTX remarkably reduced the cytokine (*TNF-α*, *IL-1β*, *IFN-γ*, and *IL-2*) expression levels in thymus tissues compared to those in the control group. In contrast, expression of cytokine mRNA in thymus tissues significantly increased in a dose-dependent manner after oral administration of GV1. These results suggest that GV1 greatly enhanced the expression of mRNA of immune-associated cytokines in spleen and thymus tissues of CTX-treated immunosuppressed mice.

3.6 Effect of GV1 on cytokine production and histopathological analysis of spleen in immunosuppressed mice

The spleen is an important immune organ that removes antigens from the blood and initiates innate and adaptive immune responses against pathogens (34). Therefore, we examined the effect of GV1 on cytokine production and conducted a histopathological analysis of the spleen in CTX-treated mice. As shown in Figure 4A, CTX treatment suppressed immunocyte action, leading to a decrease in the levels of immune-related cytokines (IL-1β, IL-6, and TNF-α) in spleen tissues of mice. GV1 dramatically increased the secretion of IL-1β, IL-6, and TNF-α in a dose-dependent manner. These findings suggest that GV1 could improve cytokine secretion in the spleen of immunosuppressed mice.

Next, the spleen histology was observed. Compared with those in the control group, the spleen cells in the CTX-treated group were sparse and irregularly arranged (Figure 4B). The HE stain histopathological images also showed clear necrotic areas devoid of cell structures and intercellular space dilatation. However, the number of such areas decreased in a dose-dependent manner in the

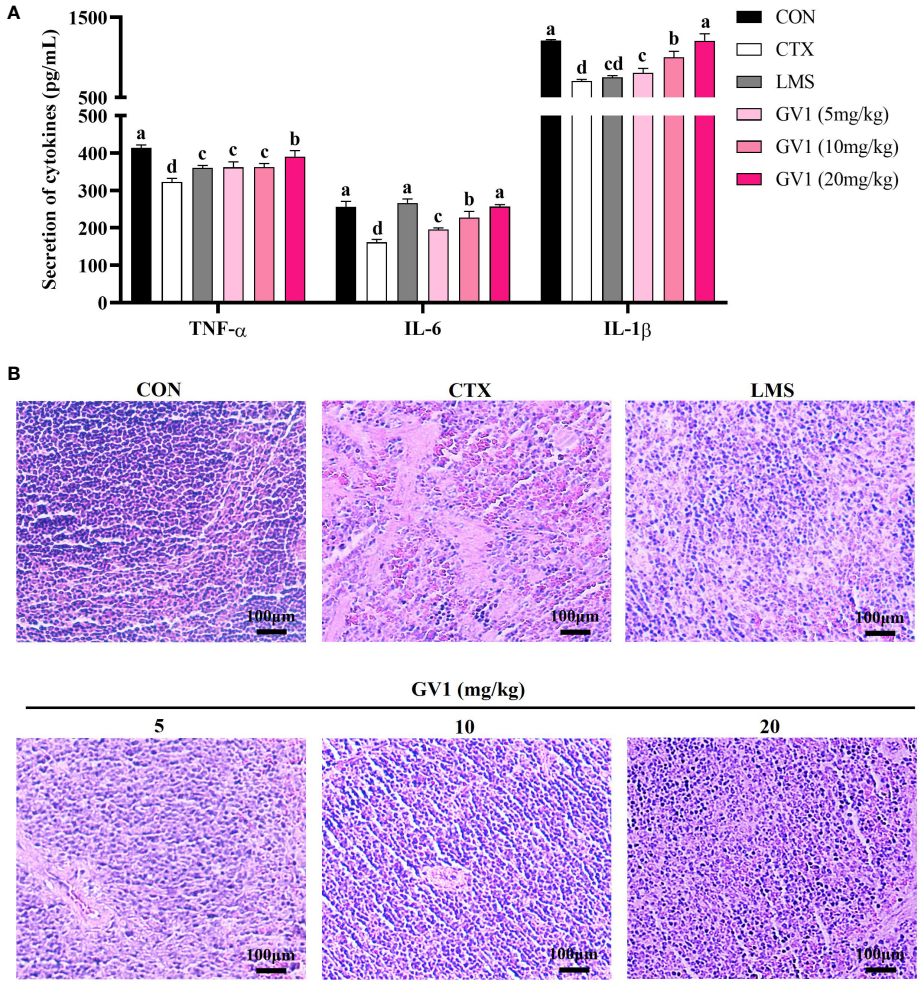

FIGURE 4
Immunomodulation effects of GV1 on spleen tissue in CTX-treated immunosuppressed mice. **(A)** Secretion of cytokines in spleen; **(B)** Histology of spleen. (100 X, Scale bar = 100μm). All data are presented as means ± S.D. Adopt the Duncan analysis method. Different letter combinations (a–d) is significant ($p < 0.05$).

GV1 treatment groups. In particular, at a concentration of 20 mg/kg GV1, the spleen tissues were tightly arranged and dense with clear nuclei as well as less interstitial spaces; thus, their state was better than that in the LMS group. These results demonstrate that GV1 can reverse CTX-induced damage in spleen tissues.

3.7 Effect of GV1 on colon histology in immunosuppressed mice

Clinical evidence has shown that CTX treatment can cause colon damage, which hinders gut immunity. Colon length was considerably lower in the CTX group than in the control group (Figure 5A); oral administration of GV1 reversed this decrease. At dosages of 10 and 20 mg/kg, GV1 dramatically increased colon length. HE-stained colorectal sections of CTX-treated mice indicated that the thickness of the epithelium had significantly reduced and that inflammatory cells had infiltrated the submucosa and mucosa (Figure 5B). In contrast, the GV1 groups exhibited remarkable protection against colonic crypt degradation and tissue inflammation. Interestingly, the colon tissue almost completely recovered upon GV1 treatment at a dosage of 10 mg/kg. Therefore, GV1 restored the length of the colon and reduced colonic damage in a mouse model of CTX-induced immunosuppression.

3.8 Effect of GV1 on gut microbiota in immunosuppressed mice

The modulatory effect of GV1 on gut microbiota was investigated *via* the high-throughput 16S rDNA sequencing of fecal samples. The Venn diagram in Figure 6A presents OTUs that were unique and common among three groups; there were a total of 965 OTUs in all the groups. There were 516 shared OTUs (55.8% of the total OTUs) among the groups. The CTX and GV1 groups showed 76 and 47 exclusive OTUs, respectively, whereas the CON group displayed 90 exclusive OTUs. In addition, the diversity and richness analysis, including metrics such as Ace, Chao1,

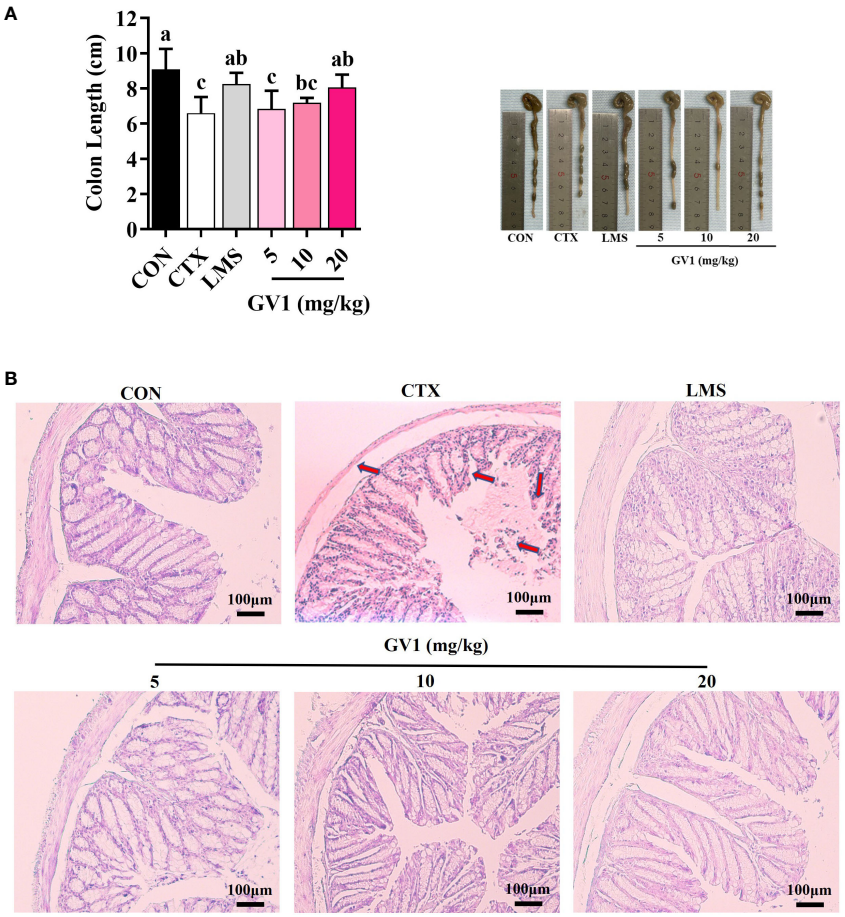

FIGURE 5
Protective effects of GV1 on colon in CTX-treated immunosuppressed mice. **(A)** Colon length; **(B)** Colon histology. (100 X, Scale bar = 100μm). All data are presented as means ± S.D. Adopt the Duncan analysis method. Different letter combinations (a–c) is significant ($p < 0.05$).

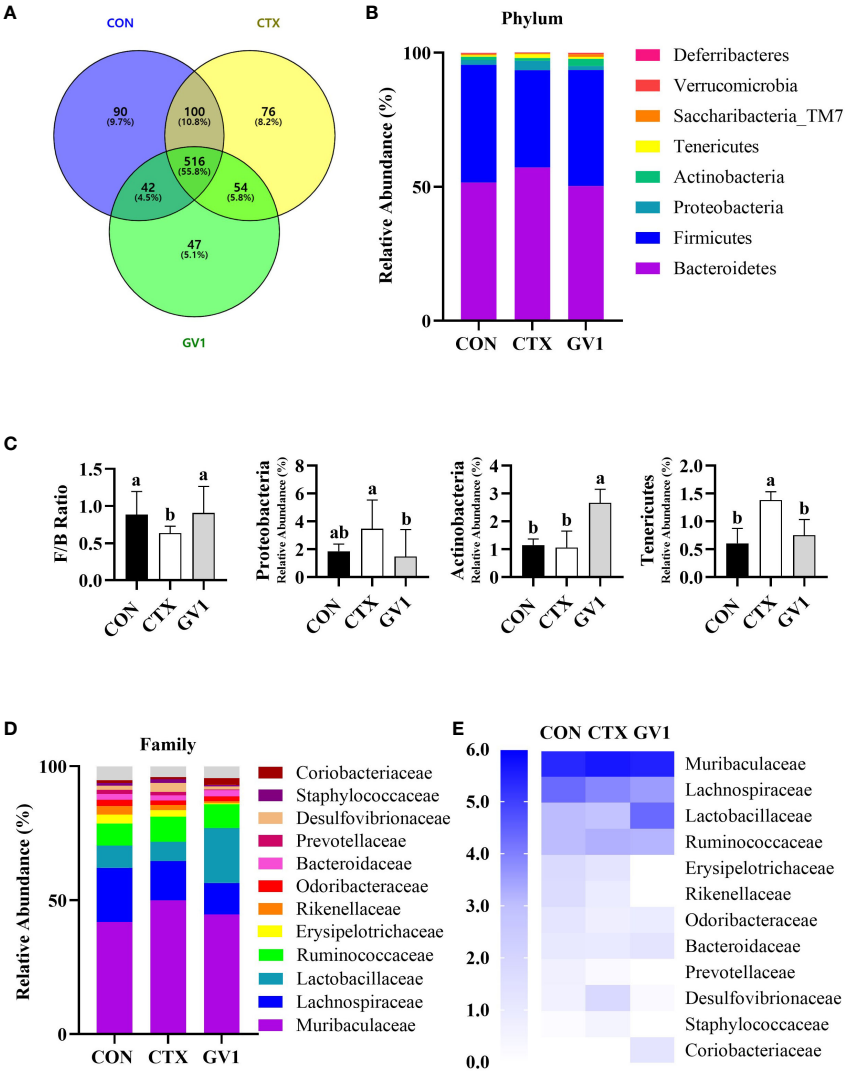

FIGURE 6
Modulation of gut microbiota composition by GV1 and comparative analysis phylum and family levels in CTX-treated immunosuppressed mice. **(A)** Venn diagram representation of OTUs in CON, CTX, and GV1 groups; **(B)** Relative abundances of gut microbiota in CON, CTX, and GV1 groups at the phylum level, presented as a stacked bar chart. **(C)** *Firmicutes/Bacteroidetes* ratio; Relative abundance of *Proteobacteria*, *Actinobacteria*, and *Tenericutes*; **(D)** Relative abundances of gut microbiota in CON, CTX, and GV1 groups at the family level, presented as a stacked bar chart; **(E)** Heatmap image of family level. All data are presented as means ± S.D. Adopt the Duncan analysis method. Different letter combinations (a, b) is significant ($p < 0.05$).

Shannon, and Simpson revealed that GV1 group exhibited lower values in comparison to the CON and CTX groups (Table 1).

We assessed the relative abundance of species at the phylum and family levels to identify specific taxa related to GV1. The intestines of mice mainly harbor *Firmicutes*, *Bacteroides*, *Proteobacteria*, *Actinobacteria*, and *Tenericutes* (Figure 6B). The differences in the relative abundance of these five major phyla were compared. CTX treatment led to a decreased *Firmicutes/Bacteroidetes* ratio and a significant increase in *Proteobacteria* and *Tenericutes* abundance, whereas GV1 treatment increased the abundance of *Actinobacteria*, resulting in a microbiota composition similar to that of the control group (Figure 6C). The gut microbiota varied at the family level, with the top 12 relative abundances being exhibited by *Muribaculaceae*, *Lachnospiraceae*,

TABLE 1 α-Diversity metrics of gut microbiota across study groups.

Group	ACE	Chao1	Shannon	Simpson
CON	661.7 ± 5.5	652.8 ± 4.86	4.4 ± 0.2	0.032 ± 0.006
CTX	650.0 ± 40.2	632.8 ± 38.2	4.3 ± 0.1	0.032 ± 0.004
GV1	601.1 ± 36.4	583.3 ± 31.8	4.0 ± 0.3	0.041 ± 0.017

Lactobacillaceae, *Ruminococcaceae*, *Erysipelotrichaceae*, *Rikenellaceae*, *Odoribacteraceae*, *Bacteroidaceae*, *Prevotellaceae*, *Desulfovibrionaceae*, *Staphylococcaceae*, and *Coriobacteriaceae* (Figure 6D). CTX altered the relative abundance of *Desulfovibrionaceae* and *Staphylococcaceae* compared with that in the control and GV1 groups. GV1 treatment significantly altered the relative abundances of *Lactobacillaceae*, *Bacteroidaceae*, and *Coriobacteriaceae* (Figure 6E).

LEfSe was utilized to identify taxa with significant differences in abundance. The linear discriminant analysis (LDA) is a statistical method widely used in multivariate analysis to find the linear combinations of features that best discriminate between different classes. The cladogram visually represents the evolutionary relationships or similarities between different groups, while the histogram provides a graphical representation of the distribution of LDA scores, shedding light on the significance and impact of each component's abundance in the context of differential effects (35). The LEfSe results of comparing the control and CTX treatment groups (Figure 7A) showed that CTX treatment promoted the relative abundance of *Muribaculaceae*, *Proteobacteria*, *Deltaproteobacteria*, *Desulfovibrionales*, *Desulfovibrionaceae*, *Ruminococcaceae*, *Tenericutes*, and *Mollicutes*. In contrast to the CTX treatment (Figure 7B), GV1 treatment suppressed the relative abundance of *Lachnospiraceae*, *Proteobacteria*, *Deltaproteobacteria*, *Desulfovibrionales*, *Desulfovibrionaceae*, *Erysipelotrichia*, *Erysipelotrichales*, *Erysipelotrichaceae*, *Rikenellaceae*, *Prevotellaceae*, *Bacillales*, *Staphylococcaceae*, *Tenericutes*, and *Mollicutes*. Moreover, GV1 treatment promoted the growth of *Bacilli*, *Lactobacillales*, *Lactobacillaceae*, *Actinobacteria*, *Coriobacteriia*, *Coriobacteriales*, *Coriobacteriaceae*, *Verrucomicrobia*, *Verrucomicrobiaceae*, *Verrucomicrobiales*, and *Akkermansiaceae*.

3.9 Spearman correlation between gut microbiota and host immune responses

Spearman correlation analysis was used to calculate the correlation coefficient between gut microbial families and immune response mediators. As depicted in Figure 8, immune

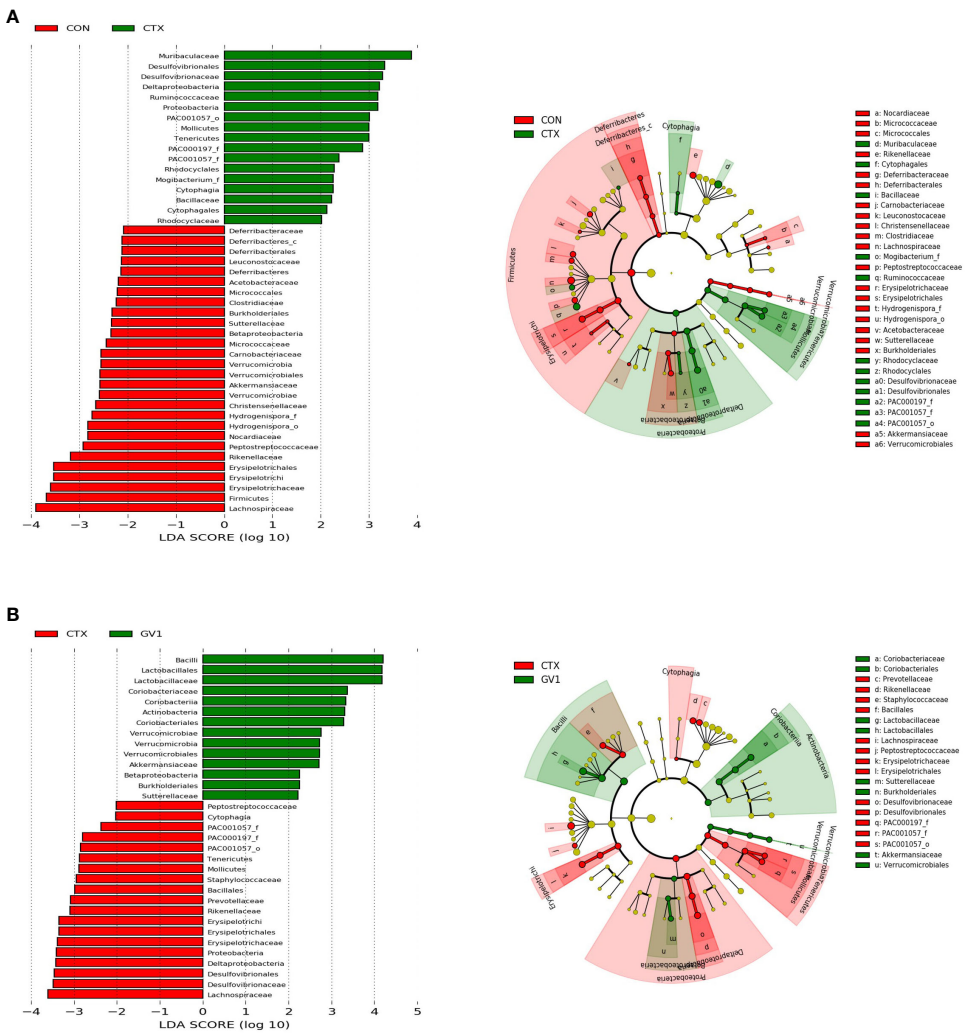

FIGURE 7
LEfSe analyses of fecal microbes *via* cladogram and histogram based on LDA score. **(A)** CON group *vs.* CTX group; **(B)** CTX group *vs.* GV1 group.

indicators such as immune-related cytokines (IFN-γ, TNF-α, IL-2, IL-6, and IL-1β) and immune organ indexes (spleen and thymus) had highly positive correlations with three types of host relative abundances, namely, those of *Akkermansiaceae*, *Coriobacteriaceae*, and *Lactobacillaceae* in the family level. In contrast, these indicators had strong negative correlations with the relative abundances of *Desulfovibrionaceae*, *Staphylococcaceae*, and *Prevotellaceae*.

4 Discussion

Paraprobiotics, also known as inactivated probiotics, have been prepared using several methods, such as heat-killing, sonication, and UV treatment, which not only inactivate microorganisms but also alter their cellular structure (13, 15, 36). The components of probiotic structures have been reported to play an important role in the regulation of immune responses (37). Live cells of *B. velezensis* have been studied extensively for their biological activity; however, the biological effect of inactive *B. velezensis* has not received much attention (38). Therefore, we studied the immunomodulatory effects of heat-killed *B. velezensis* GV1 *in vitro* and *in vivo* to identify the underlying contributing to the beneficial effects of paraprobiotics on immunomodulation.

Macrophages play a crucial role in the immune system by detecting and destroying pathogens. Upon activation, macrophages generate NO, accompanied by the increased expression of *iNOS* gene. NO serves as a signaling molecule that induces interactions among various immune cells to enhance the immune system's ability to respond to pathogens, inhibiting their replication and thereby, improving overall immune function (17). The increase in NO production and *iNOS* expression in RAW 264.7 macrophages treated with heat-killed GV1 suggests that GV1 enhances immunity (Figures 2A, B). Additionally, GV1 dose-dependently increased the expression and secretion of immune-related cytokines such as TNF-α, IL-6, and IL-1β. Macrophages have the ability to release cytokines, which bind to specific receptors on cells and initiate an immune response. The application of heat-treated *Levilactobacillus brevis* KU15159 has demonstrated the stimulation of immune responses which is evidenced in the upregulation of TNF-α, IL-6, IL-1β in RAW 264.7 cells (39). Therefore, we speculated that GV1 may induce immune responses by stimulating the secretion of NO and immune-related cytokines in RAW 264.7 macrophages.

To further characterize the immunity-enhancing effect of GV1, we studied its immunomodulatory activity in the CTX-treated immunosuppressed mouse model. The immune response is suppressed as evidenced by a decrease in the size of the spleen and thymus, along with a reduction in the expression and production of most immune-related cytokines, upon CTX treatment (40). The thymus primarily, oversees the development of T lymphocytes, and the spleen captures pathogens and activates and coordinates the response of immune cells. Therefore, the thymus and spleen play pivotal roles as major lymphoid organs in the immune system, contributing significantly to lymphocyte development and the

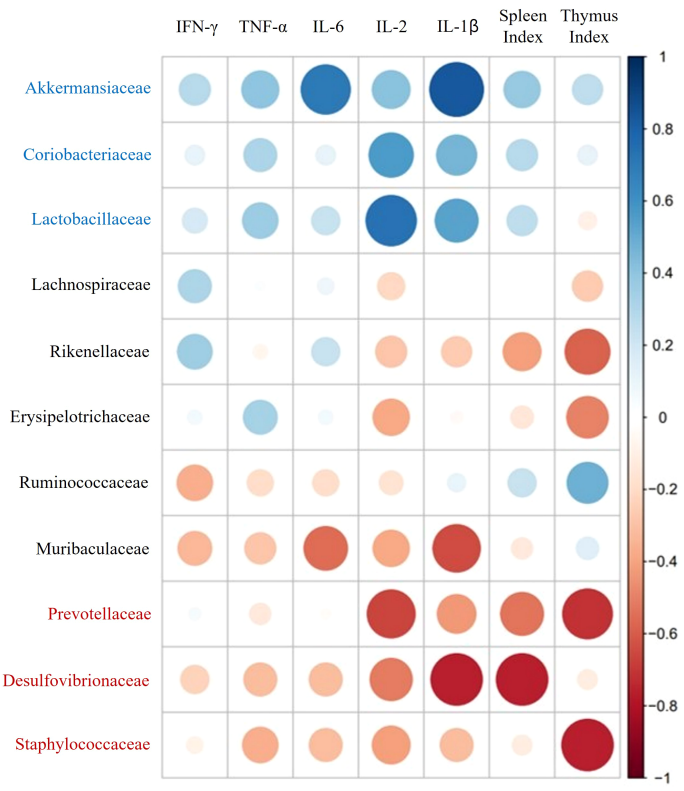

FIGURE 8
Spearman correlation analysis of immune-related mediators and key microbial communities.

orchestration of adaptive immune responses (41). As expected, the spleen and thymus indexes significantly decreased upon CTX administration. However, these decreases were reversed upon GV1 administration. Furthermore, GV1 dose-dependently enhanced the expression of *IL-6*, *TNF-α*, *IFN-γ*, and *IL-2* mRNA in the thymus and spleen tissues of immunodeficient mice. Notably, the expression of *IL-2* mRNA greatly increased in spleen tissues in the GV1 groups, compared with that in the LMS group. IL-2 is a multifunctional cytokine that increases NK cell lysis activity, promotes T and B cell proliferation, and activates Treg cells, thus impairing killer cell differentiation (42). These immune-related cells mainly reside in spleen tissues; therefore, the spleen is considered a key immune organ in the body (43). Hence, the protective effects of GV1 on the spleen in CTX-treated immunosuppressed mice were further investigated using ELISA assays and HE staining. Our data show that GV1 remarkably enhances IL- 6, TNF-α, and IL-1β production in the spleen of immunosuppressed mice. Histological results indicate that GV1 can repair CTX-induced spleen tissue damage. Similarly, in our previous study, *Curtobacterium proimmune* K3 lysate significantly stimulated the secretion of immune-related cytokines in the thymus and spleen, and reversed the atrophy of these organs in CTX-treated mice (33). Therefore, we conclude that GV1 can repair immune organ damage and promote the expression of immune-related cytokines to improve the immune system in immunosuppressed mice.

The intestine is a vital organ with key functions in digestion, nutrient absorption, and immunity. The intestinal ecosystem continuously interacts to optimize these functions and maintain the integrity of the gut (44). In conditions of immune suppression and inhibition, the immune system fails to activate adequately, leading to damage in the intestinal tract (45). In this study, we investigated the role of GV1 in improving colon damage caused by immune suppression. Treatment with GV1 demonstrated a protective effect on the colon by increasing its length and alleviating damage. This suggests that GV1 plays a role in ameliorating immune suppression-induced damage, restoring colon function, and enhancing the immune system's resilience against infections and other immune-related issues. The gut microbial community, especially in relation to intestinal mucosal immunity, plays a crucial role in regulating the host's innate and adaptive immune systems. Moreover, the efficacy of immunotherapy can vary depending on the composition of the gut microbiota (45). In this study, gut microbiota regulation by GV1 was investigated in CTX-treated mice. The number of OTUs, the diversity and richness index of the GV1 group slightly decreased compared to those of the normal and CTX groups, suggesting that GV1 can modulate the abundance of bacterial species. It is necessary to analyze the dominant gut microbiota at various taxonomic levels (phylum and family) in order to determine the overall differences and similarities between various groups. Our results indicate that *Bacteroidetes* and *Firmicutes* are the most abundant at the phylum level in fecal microbiota, which could be modulated by paraprobiotic GV1. The *Firmicutes/Bacteroidetes* (F/B) ratio is related to the maintenance of homeostasis, and an imbalance in this ratio can cause obesity or inflammatory bowel disease (46). In this study, CTX decreased the relative abundance of *Firmicutes* and increased that of *Bacteroidetes*, causing an imbalance in the F/B ratio. However, this ratio was balanced after oral administration of GV1, similar to the results of a prior study. Notably, GV1 reduced the relative abundance of *Proteobacteria*, which was promoted by CTX treatment. *Proteobacteria* is a major phylum of gram-negative bacteria that includes a large range of pathogenic organisms, including *Helicobacter pylori*, *Escherichia coli*, and *Salmonella* spp (47). Consistent with our results, *Lactobacillus plantarum* BF_15 has been reported to inhibit the growth of *Proteobacteria*, which was promoted by CTX treatment (48). GV1 administration reversed the CTX-induced reduction in gut microbiota diversity and richness possibly by inhibiting pathogenic bacteria (belonging to *Proteobacteria*). At the family level, the relative abundances of *Lactobacillaceae*, *Akkermansiaceae*, and *Coriobacteriaceae* in the GV1 group were higher than those in the CTX group. *Lactobacillaceae* have been reported to have an effect on the immune system and gastrointestinal microbial community of humans (49). *Akkermansiaceae* members exhibit probiotic properties and are inversely related to various diseases, including inflammation, diabetes, obesity, and metabolic disorders (50–53). *Coriobacteriaceae* family members perform important functions within organisms, such as modulating host glucose metabolism in the liver and regulating bile acid and lipid metabolism in the gut (54). In addition to enhancing the relative abundances of beneficial bacterial families, GV1 reduces the abundance of several families such as *Desulfovibrionaceae*, *Erysipelotrichaceae*, *Prevotellaceae*, and *Staphylococcaceae*, compared to that in CTX-treated immunosuppressed mice. Among these families, *Desulfovibrionaceae* are considered as harmful bacteria that can cause mucosal inflammation by inducing toxic hydrogen sulfide to secrete sulfated mucin (55). Kaakoush revealed that *Erysipelotrichaceae* are involved in gastrointestinal inflammatory disorders and that they are particularly found to be abundant in colorectal cancer patients (56). Based on the result (Figure 8), Spearman correlation analysis indicate that three types of key microorganisms (*Akkermansiaceae*, *Coriobacteriaceae*, and *Lactobacillaceae*) positively correlate with immune mediators such as immune-related cytokines and immune organ indexes, consistent with previous research (57). These data demonstrate that GV1 can promote immune responses in CTX-treated immunosuppressed mice by modulating gut microbiota dysbiosis.

This study delved into the entire process, from the production of paraprobiotics GV1 to its immunomodulatory effects, along with subsequent gut recovery and microbial community changes (Supplementary Figure S1). However, the predominant use of a mouse model as the experimental subject limits direct applicability to humans. Considering the diversity and complexity of the microbial community, additional diverse microbial community analyses and in-depth studies over time are required. Despite these limitations, GV1 demonstrated positive outcomes in enhancing immune function and regulating microbial community imbalance. This suggests promising potential for GV1 in the treatment and prevention of immune-related diseases and disorders. The research

providesinsights into the immunological and microbiological characteristics of paraprobiotics GV1, indicating its significance as fundamental data for future studies, particularly in the fields of the food industry and health-functional food applications.

5 Conclusion

This study focused on the immunomodulatory effects of paraprobiotics, specifically discussing the ability of the heat-treated form of *B. velezensis* GV1 to regulate the microbial community within the human body. GV1 robustly stimulated immune responses by enhancing the expression and secretion of inflammatory cytokines in RAW 264.7 macrophages. Furthermore, GV1 repaired damage to immune organs and increased the expression of immune-related cytokines in immunosuppressed mice induced by CTX. Additionally, GV1 promoted beneficial bacteria and suppressed harmful bacteria, restoring balance to the microbial community in the intestine. These findings indicate, for the first time, that paraprobiotics prepared from *B. velezensis* GV1 can act as a stimulant to enhance immune responses. Such discoveries could form the basis for developing paraprobiotics as functional foods or drugs aimed at improving the immune system. The effects of GV1 should be further investigated through clinical trials, exploring its potential for industrial use as an immunomodulatory agent.

Data availability statement

The datasets presented in this study can be publicly found in the online repository under the accession BioProject number PRJNA1073872 (http://www.ncbi.nlm.nih.gov/bioproject/1073872).

Ethics statement

The animal studies were approved by the Animal Care and Use Guidelines of Kyung Hee University (KHGASP-23-046). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

EEJ: Investigation, Writing – review & editing. H-JL: Methodology, Writing – original draft. MTHT: Formal analysis, Writing – review & editing. MHL: Data curation, Writing – review & editing. Y-JK: Supervision, Writing – review & editing.

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Conflict of interest

Y-JK is affiliated with the company KDBio Corporation.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1285063/full#supplementary-material

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The causality of gut microbiota on onset and progression of sepsis: a bi-directional Mendelian randomization analysis

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Background: Several observational studies have proposed a potential link between gut microbiota and the onset and progression of sepsis. Nevertheless, the causality of gut microbiota and sepsis remains debatable and warrants more comprehensive exploration.

Methods: We conducted a two-sample Mendelian randomization (MR) analysis to test the causality between gut microbiota and the onset and progression of sepsis. The genome-wide association study (GWAS) summary statistics for 196 bacterial traits were extracted from the MiBioGen consortium, whereas the GWAS summary statistics for sepsis and sepsis-related outcomes came from the UK Biobank. The inverse-variance weighted (IVW) approach was the primary method used to examine the causal association. To complement the IVW method, we utilized four additional MR methods. We performed a series of sensitivity analyses to examine the robustness of the causal estimates.

Results: We assessed the causality of 196 bacterial traits on sepsis and sepsis-related outcomes. Genus *Coprococcus2* [odds ratio (OR) 0.81, 95% confidence interval (CI) (0.69–0.94), p = 0.007] and genus *Dialister* (OR 0.85, 95% CI 0.74–0.97, p = 0.016) had a protective effect on sepsis, whereas genus *Ruminococcaceae UCG011* (OR 1.10, 95% CI 1.01–1.20, p = 0.024) increased the risk of sepsis. When it came to sepsis requiring critical care, genus *Anaerostipes* (OR 0.49, 95% CI 0.31–0.76, p = 0.002), genus *Coprococcus1* (OR 0.65, 95% CI 0.43–1.00, p = 0.049), and genus *Lachnospiraceae UCG004* (OR 0.51, 95% CI 0.34–0.77, p = 0.001) emerged as protective factors. Concerning 28-day mortality of sepsis, genus *Coprococcus1* (OR 0.67, 95% CI 0.48–0.94, p = 0.020), genus *Coprococcus2* (OR 0.48, 95% CI 0.27–0.86, p = 0.013), genus *Lachnospiraceae FCS020* (OR 0.70, 95% CI 0.52–0.95, p = 0.023), and genus *Victivallis* (OR 0.82, 95% CI 0.68–0.99, p = 0.042) presented a protective effect, whereas genus *Ruminococcus torques group* (OR 1.53, 95% CI 1.00–2.35, p = 0.049), genus *Sellimonas* (OR 1.25, 95% CI 1.04–1.50, p = 0.019), and genus *Terrisporobacter* (OR 1.43, 95% CI 1.02–2.02, p = 0.040) presented a harmful effect. Furthermore, genus *Coprococcus1* (OR 0.42, 95% CI 0.19–0.92, p = 0.031), genus *Coprococcus2* (OR 0.34, 95% CI 0.14–0.83, p = 0.018), and genus *Ruminiclostridium6* (OR 0.43, 95% CI 0.22–0.83, p = 0.012) were associated with a lower 28-day mortality of sepsis requiring critical care.

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Conclusion: This MR analysis unveiled a causality between the 21 bacterial traits and sepsis and sepsis-related outcomes. Our findings may help the development of novel microbiota-based therapeutics to decrease the morbidity and mortality of sepsis.

KEYWORDS

causal relationship, genetics, gut microbiota, Mendelian randomization, sepsis

1 Introduction

Sepsis, one of the oldest and most elusive syndromes in medicine (1), is a critical global public health issue and a leading cause of morbidity and mortality worldwide (2). With the aging of the population leading to suppressed immunity, advances in medical care including immune-modulating medications, and the impact of global warming, sepsis is predicted to become an increasingly prevalent concern (3). Sepsis currently accounts for nearly 26% of all global deaths, resulting in more than 20 deaths per minute (4). The pathogenesis of sepsis is still not fully understood. Sepsis can be caused by infections stemming from viruses, fungi, or parasites, and non-immune alterations are known to contribute to the imbalanced host response in sepsis (5). Recently, sepsis has been defined as a dysregulated host response to infection, resulting in life-threatening damage to organs and tissues (6, 7). Timely antibiotics and systemic supportive care are the standard treatment options, but effective therapies for sepsis remain elusive (8), resulting in persistently high incidence and mortality rates.

Trillions of symbiotic bacteria colonize the human intestine and are mainly composed of *Bacillota* and *Bacteroidota* (9–11); these bacteria are also called the second genome and play a crucial role in maintaining human health (12). It is widely accepted that various diseases such as obesity and diabetes are caused by dysbiosis of the gut microbiota (13–15). Moreover, the gut microbiota affects host susceptibility and responsiveness to sepsis through multiple pathways (16), and microbial dysbiosis has been recognized as a remarkable contributor to increased susceptibility to sepsis and subsequent organ dysfunction (17–19). In recent years, some observational studies (19–25) have suggested that the gut microbiota is associated with the onset and progression of sepsis. However, in traditional observational studies, the association between the gut microbiota and sepsis has been shown to be influenced by confounding factors such as antibiotic use and dietary habits, as well as reverse causality, which limits the inference of causality. To investigate the causal effect between the gut microbiota and sepsis, large-sample and high-quality randomized controlled trials (RCTs) are still needed for further validation. However, because of objective factors such as technology, cost, and research methods, there are significant limitations in identifying the types of strains associated with early diagnosis and prognosis.

Mendelian randomization (MR) analysis is a novel approach for inferring causal associations that provides an alternative to RCTs. This method utilizes single-nucleotide polymorphisms (SNPs) identified by genome-wide association studies (GWASs) as instrumental variables (IVs) to explore the causal association between exposure (e.g., the abundance of the genus *Dialister*) and outcome (e.g., sepsis) (26). Mendel's laws of inheritance dictate that parental alleles are randomly assigned to offspring, which is akin to random assignment in RCTs. Genetic variation, in theory, is not influenced by common confounding factors, such as the postnatal environment, and genetic variation precedes exposure and outcome, eliminating the issues of reverse causality and confounding factors. Large-scale GWAS data have provided a wealth of reliable genetic variation information for MR studies of the gut microbiota (26, 27), and many studies (28, 29) have utilized the two-sample MR method to investigate the causal associations between the gut microbiota and various diseases.

This study aimed to utilize summary statistics from the MiBioGen and UK Biobank consortiums and employ a two-sample MR approach to investigate the causal association between the gut microbiota and the onset and progression of sepsis.

2 Methods

2.1 Study design

The flow chart of this MR analysis is shown in Figure 1. This study utilized publicly available GWAS summary statistics for a two-sample MR analysis to assess the causal association between the gut microbiota and the onset and progression of sepsis. Our MR analysis relied on three assumptions (26): (1) the IVs are strongly associated with the exposure; (2) the IVs are unrelated to confounding factors that affect the exposure–outcome association; and (3) the IVs only affect the outcome through the exposure and not through any other pathways. Moreover, this study was reported according to the Strengthening the Reporting of Observational Studies in Epidemiology Using Mendelian Randomization guidelines (STROBE-MR, S1 Checklist) (30).

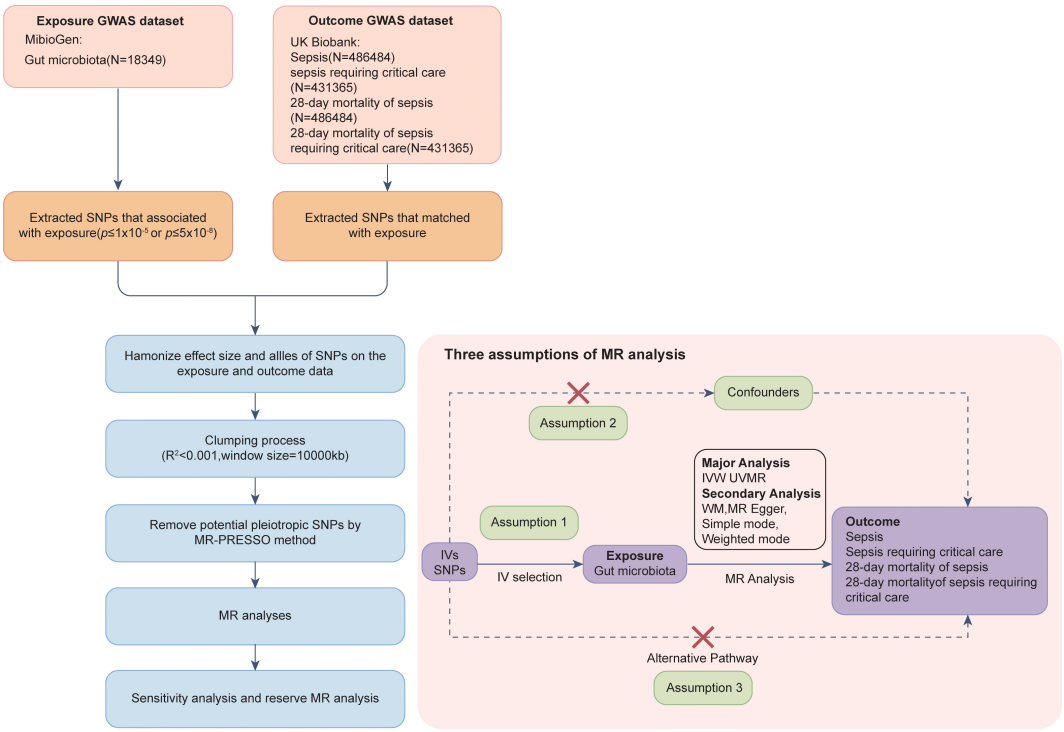

FIGURE 1
Synopsis of MR analysis procedures and major assumptions.

2.2 Exposure GWAS datasets

The genetic variation in the gut microbiota in this study was derived from a genome-wide meta-analysis conducted by the MiBioGen consortium (31), which represents the largest gut microbiota GWAS to date. This study identified genetic associations between gut microbial relative abundances and human host genes. In this study, genotyping data and 16S ribosomal RNA gene sequencing profiles from 18,340 participants across 24 cohorts in Europe, America, the Middle East, and East Asia were coordinated. Twenty cohorts included samples of single ancestry, 16 of which were of European ancestry, for a total of 13,266 participants. The baseline characteristics of the exposure population can be viewed in Supplementary Table S1. This multiethnic large-scale GWAS divided the gut microbiota into 211 taxa (131 genera, 35 families, 20 orders, 16 classes, and 9 phyla). Fifteen bacterial taxa (12 genera and 3 families) with unknown groups were excluded, with 196 bacterial taxa finally included in our MR analysis. Summary-level GWAS data of the gut microbiota are openly available at http://www.mibiogen.org/.

2.3 Outcome GWAS datasets

Summary-level GWAS statistics of sepsis, sepsis requiring critical care, and 28-day mortality of patients with sepsis and sepsis requiring critical care were obtained from the UK Biobank consortium with adjustment for sex and age. The UK Biobank is a large and publicly available biomedical database and research resource. Since 2006, blood, urine, and saliva samples and complete demographic, socioeconomic, lifestyle, and health information data have been collected from approximately 500,000 participants aged 40 to 69 years throughout the United Kingdom (32). All the participants in the case and control groups (both men and women) included in the UK Biobank are of European descent. The phenotype "sepsis, sepsis requiring critical care, 28-day mortality of sepsis, and 28-day mortality of sepsis requiring critical care" was applied in our research. Comprehensive information on the diagnostic criteria and recruitment methods used for participants in the UK Biobank consortium can be found in the original publications. The profiles of the GWAS datasets of the gut microbiota and sepsis and sepsis-related outcomes are available in Table 1.

2.3 Instrumental variables

SNPs strongly associated with each bacterial trait were selected as IVs in our MR analysis. To ensure the reliability and accuracy of the results regarding the causal association between the gut microbiota and the risk of sepsis and sepsis-related outcomes, we utilized the following selection criteria to choose IVs: (1) To improve the completeness of our results, SNPs associated with each gut microbial taxon at the genome-wide significance threshold ($p < 5\times10^{-8}$) and the locus-wide significance threshold ($p < 1\times10^{-5}$) were chosen as IVs (33). (2) Using the 1000 Genomes Project European sample data as the reference panel, this study conducted a clumping analysis ($r^2 < 0.001$, window size = 10,000 kilobases) to

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TABLE 1 Summary information of the datasets utilized in this MR analysis.

Trait	Consortium	Samples	Case	Control
Exposure				
Gut microbiota	MiBioGen	18,340	/	/
Outcome				
Sepsis	UK Biobank	486,484	11,643	474,841
Sepsis requiring critical care	UK Biobank	431,365	1,380	429,985
28-day mortality of sepsis	UK Biobank	486,484	1,896	484,588
28-day mortality of sepsis requiring critical care	UK Biobank	431,365	347	431,018

assess the linkage disequilibrium (LD) between the included SNPs and removed highly correlated SNPs to ensure that the included SNPs were independent of each other. (3) The exposure (gut microbiota) and outcome (sepsis and sepsis-related outcomes) data were harmonized, and palindromic SNPs with intermediate allele frequencies were removed. (4) The *F*-statistic for the IVs was calculated to evaluate potential bias due to weak IVs. An *F*-statistic > 10 was interpreted as an indication of negligible bias from weak IVs.

2.4 Statistical analysis

MR was conducted to analyze the causal relationships between the gut microbiota and sepsis and sepsis-related outcomes. The inverse-variance weighted (IVW) method was used as the primary method to identify potential causal associations, as it is regarded as the most powerful statistical method. A meta-analysis approach combined with the Wald estimates for each valid SNP was used to assess a total estimate of the effect of the exposure variables on outcome. For each bacterial trait of the gut microbiota, if the IVW method identified causality ($p < 0.05$), we performed the other four MR methods, MR–Egger, weighted median, simple mode, and weighted mode, to supplement the IVW results (34, 35). The MR–Egger method delivers unbiased estimates even when all chosen IVs exhibit pleiotropy, given that the Instrument Strength Independent of Direct Effect (InSIDE) assumption is satisfied (36). The weighted median method can still accurately estimate the causality effect even when less than 50% of the genetic variants violate the core assumptions of MR (34). Finally, we report the causal results as odds ratios (ORs) with 95% confidence intervals (95% CIs). The significance threshold was established at $p < 0.05$.

We considered an exposure–outcome pair to have a causal association only when all MR methods consistently identified the same direction of effect. To validate the robustness of the established causal associations, we conducted a series of sensitivity analyses. First, Cochran's IVW *Q* statistics were calculated to quantify the heterogeneity. A *Q*-value exceeding the total number of IVs reduced by one suggested the presence of heterogeneity and potentially invalid IVs. Similarly, *Q* statistics that yielded a *p*-value < 0.05 also indicated the existence of heterogeneity (37, 38). Second, we performed MR–Egger analysis to assess the confounding effects of directional pleiotropy. When the intercept of the MR–Egger was close to zero at a *p*-value > 0.05, we regarded directional pleiotropy as not significant. Third, to assess overall pleiotropy, Mendelian randomization pleiotropy residual sum and outlier (MR-PRESSO) analysis was performed (39). We reported the outcomes of the MR-PRESSO global test, and outlier-corrected ORs and confidence intervals (CIs) were calculated for outliers and horizontal pleiotropic SNPs. Finally, to detect pleiotropy caused by a single SNP, a leave-one-out analysis was also performed.

To investigate whether sepsis and sepsis-related outcomes had any causal influence on the identified significant gut microbiota, we also conducted reverse-direction MR analysis on bacteria with significant causal associations in forward-direction MR. The settings and methods were identical to those used for forward-direction MR.

All the statistical analyses were performed using R version 4.2.3 (R Foundation for Statistical Computing, Vienna, Austria, https://www.r-project.org/). MR analyses were performed using TwosampleMR (version 0.5.6) (26) and MR-PRESSO (version 1.0) (39).

3 Results

The details of the selected SNPs are shown in Supplementary Table S2 (i.e., SNPID, effect allele, other allele, beta, standard error, and *p*-value of exposure and outcome). Based on the selection criteria for IVs, we identified 196 traits of the gut microbiota at five biological levels (i.e., phylum, class, order, family, and genus) associated with sepsis and sepsis-related outcomes (Supplementary Table S3). As shown in Table 2, 8, 6, 12, and 9 bacterial traits were potentially causally associated with sepsis, sepsis requiring critical care, 28-day mortality from sepsis, and 28-day mortality from sepsis requiring critical care, respectively, according to the IVW MR analysis. Following the harmonization process, every pair of bacterial traits and sepsis and sepsis-related outcomes incorporated more than three SNPs. All of the *F*-statistics of the selected IVs in this research were greater than 10, suggesting that there was no weak instrument bias. It is important to acknowledge that the classifications of the gut microbiota have a considerable degree of overlap. Consequently, the SNPs included in the class and their corresponding order could coincide significantly (e.g., SNPs of the phylum *Lentisphaerae*, class *Lentisphaeria*, order *Victivallales*, and genus *Victivallis*). A heatmap was generated to visualize the causal association of bacterial traits identified in our MR analysis with sepsis, sepsis requiring critical care, and 28-day mortality of sepsis and sepsis requiring critical care (Figure 2).

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TABLE 2 MR results of causal effects between gut microbiota and sepsis and sepsis-related outcomes ($p < 1\times10^{-5}$).

Gut microbiota (exposure)	Method	nSNP	OR	95% CI	*p*-value	Egger intercept	Egger_intercept *p*-value	Cochrane *Q* statistic	Cochrane *Q* *p*-value	MR-PRESSO
Sepsis										
Class *Gammaproteobacteria*	IVW	6	1.37	1.08–1.73	0.010	−0.0009	0.979	7.1272	0.211	/
Class *Lentisphaeria*	IVW	8	0.86	0.78–0.94	0.002	0.0125	0.628	5.1588	0.641	/
Family *Clostridiaceae1*	IVW	10	1.21	1.04–1.40	0.011	−0.0242	0.168	5.6311	0.776	/
Genus *Coprococcus2*	IVW	8	0.81	0.69–0.94	0.007	0.0217	0.645	4.1443	0.763	/
Genus *Dialister*	IVW	11	0.85	0.74–0.97	0.016	−0.0092	0.658	4.7209	0.909	/
Genus *Ruminococcaceae UCG011*	IVW	8	1.10	1.01–1.20	0.024	−0.0036	0.909	6.1743	0.520	/
Order *Victivallales*	IVW	8	0.86	0.78–0.94	0.002	0.0125	0.628	5.1588	0.641	/
Phylum *Lentisphaerae*	IVW	9	0.89	0.80–0.99	0.035	0.0091	0.781	11.3614	0.182	/
Sepsis (critical care)										
Class *Lentisphaeria*	IVW	8	0.67	0.50–0.91	0.011	−0.0387	0.662	8.8974	0.260	/
Genus *Anaerostipes*	IVW	11	0.49	0.31–0.76	0.002	0.0328	0.507	7.7479	0.653	/
Genus *Coprococcus1*	IVW	11	0.65	0.43–1.00	0.049	0.0100	0.807	11.6969	0.306	/
Genus *Lachnospiraceae UCG004*	IVW	12	0.51	0.34–0.77	0.001	0.0406	0.467	11.9762	0.365	/
Order *Victivallales*	IVW	8	0.67	0.50–0.91	0.011	−0.0387	0.662	8.8974	0.260	/
Phylum *Lentisphaerae*	IVW	9	0.70	0.53–0.93	0.014	−0.0443	0.610	9.7340	0.284	/
Sepsis (28-day death)										
Class *Bacteroidia*	IVW	13	1.48	1.06–2.08	0.023	0.0187	0.533	7.9222	0.791	/
Class *Lentisphaeria*	IVW	8	0.68	0.53–0.87	0.002	−0.0070	0.921	7.7980	0.351	/
Genus *Coprococcus1*	IVW	11	0.67	0.48–0.94	0.020	0.0233	0.450	7.0174	0.724	/
Genus *Coprococcus2*	IVW	8	0.48	0.27–0.86	0.013	−0.0129	0.945	15.9305	0.026	/
Genus *Lachnospiraceae FCS020 group*	IVW	12	0.70	0.52–0.95	0.023	0.0395	0.202	8.8919	0.632	/
Genus *Ruminococcus torques group*	IVW	8	1.53	1.00–2.35	0.049	−0.0656	0.118	5.9762	0.543	/
Genus *Sellimonas*	IVW	9	1.25	1.04–1.50	0.019	0.0149	0.850	6.3185	0.612	/
Genus *Terrisporobacter*	IVW	5	1.43	1.02–2.02	0.040	0.0376	0.513	2.7535	0.600	/
Genus *Victivallis*	IVW	9	0.82	0.68–0.99	0.042	−0.0003	0.998	2.3065	0.970	/

(Continued)

TABLE 2 Continued

Gut microbiota (exposure)	Method	nSNP	OR	95% CI	*p*-value	Egger intercept	Egger_intercept *p*-value	Cochrane *Q* statistic	Cochrane *Q* *p*-value	MR-PRESSO
Sepsis (28-day death)										
Order *Bacteroidales*	IVW	13	1.48	1.06–2.08	0.023	0.0187	0.533	7.9222	0.791	/
Order *Victivallales*	IVW	8	0.68	0.53–0.87	0.002	−0.0070	0.921	7.7980	0.351	/
Phylum *Lentisphaerae*	IVW	9	0.72	0.56–0.93	0.012	−0.0131	0.866	10.7141	0.218	/
Sepsis (28-day death in critical care)										
Class *Bacteroidia*	IVW	13	2.43	1.10–5.37	0.029	−0.0163	0.817	7.8873	0.794	/
Class *Lentisphaeria*	IVW	8	0.54	0.30–0.95	0.034	0.0031	0.985	7.6435	0.365	/
Class *Mollicutes*	IVW	12	2.03	1.01–4.08	0.046	0.0251	0.809	7.0423	0.796	/
Genus *Coprococcus1*	IVW	11	0.42	0.19–0.92	0.031	−0.0236	0.743	2.0631	0.996	/
Genus *Coprococcus2*	IVW	8	0.34	0.14–0.83	0.018	−0.0311	0.908	3.6194	0.822	/
Genus *Ruminiclostridium6*	IVW	14	0.43	0.22–0.83	0.012	0.1002	0.197	12.9286	0.453	/
Order *Bacteroidales*	IVW	13	2.43	1.10–5.37	0.029	−0.0163	0.817	7.8873	0.794	/
Order *Victivallales*	IVW	8	0.54	0.30–0.95	0.034	0.0031	0.985	7.6435	0.365	/
Phylum *Tenericutes*	IVW	12	2.03	1.01–4.08	0.046	0.0251	0.809	7.0423	0.796	/

IVW, inverse-variance weighted method; nSNP, number of the SNP used as the IVs for the MR analyses; OR, odds ratio; CI, confidence interval.

3.1 MR analysis results (locus-wide significance, $p < 1\times10^{-5}$)

3.1.1 Causality of the gut microbiota on sepsis

We found that five bacterial traits (class *Lentisphaeria*: OR 0.86, 95% CI 0.78–0.94; genus *Coprococcus2*: OR 0.81, 95% CI 0.69–0.94; genus *Dialister*: OR 0.85, 95% CI 0.74–0.97; order *Victivallales*: OR 0.86, 95% CI 0.78–0.94; and phylum *Lentisphaerae*: OR 0.89, 95% CI 0.80–0.99) had a potential protective effect on sepsis, while three bacterial traits (class *Gammaproteobacteria*: OR 1.37, 95% CI 1.08–1.73; family *Clostridiaceae1*: OR 1.21, 95% CI 1.04–1.40; and genus *Ruminococcaceae UCG011*: OR 1.10, 95% CI 1.01–1.20) were causally associated with a greater risk of sepsis according to the IVW MR analysis. However, the weighted mode method revealed that five bacterial traits had a significant causal association with the risk of sepsis (class *Gammaproteobacteria*: OR 1.40, 95% CI 1.06–1.85; class *Lentisphaeria*: OR 0.85, 95% CI 0.75–0.98; order *Victivallales*: OR 0.85, 95% CI 0.75–0.97; phylum *Lentisphaerae*: OR 0.87, 95% CI 0.77–0.99; and genus *Dialister*: OR 0.83, 95% CI 0.70–1.00). Moreover, the results of MR–Egger regression showed that only the family *Clostridiaceae1* (OR 1.64, 95% CI 1.08–2.50) was significantly associated with the risk of sepsis. The comprehensive MR results of the causal associations between bacterial traits and sepsis are shown in Figure 3.

3.1.2 Causality of the gut microbiota on sepsis requiring critical care

This study also explored the causal effect of the gut microbiota on the risk of sepsis requiring critical care. All six bacterial traits (class *Lentisphaeria*: OR 0.67, 95% CI 0.50–0.91; genus *Anaerostipes*: OR 0.49, 95% CI 0.31–0.76; genus *Coprococcus1*: OR 0.65, 95% CI 0.43–1.00; genus *Lachnospiraceae UCG004*: OR 0.51, 95% CI 0.34–0.77; order *Victivallales*: OR 0.67, 95% CI 0.50–0.91; and phylum *Lentisphaerae*: OR 0.70, 95% CI 0.53–0.93) were significantly associated with a potential protective effect on sepsis requiring critical care in the primary IVW MR analysis. Moreover, the results of the weighted mode method demonstrated that the genus *Anaerostipes* (OR 0.46, 95% CI 0.25–0.84) and *Coprococcus1* (OR 0.55, 95% CI 0.31–0.95) were also associated with a lower risk of sepsis requiring critical care. The comprehensive MR results concerning the causal association between bacterial traits and sepsis requiring critical care are depicted in Figure 4.

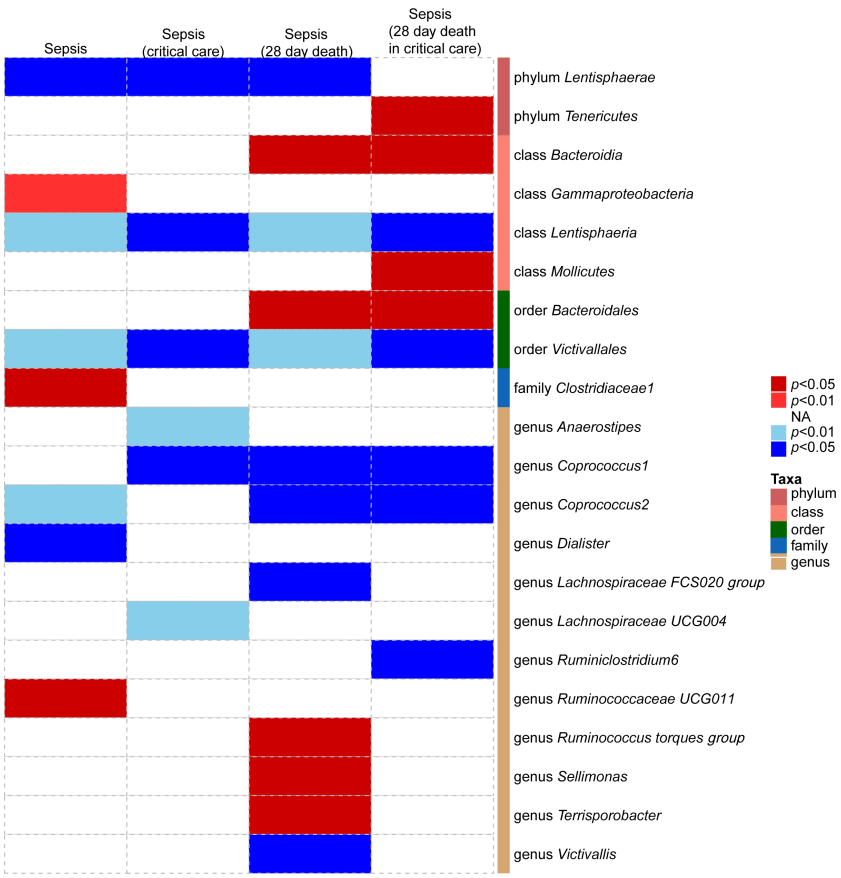

FIGURE 2
Heatmap of gut microbiota causally associated with sepsis, sepsis requiring critical care, 28-day mortality of sepsis, and 28-day mortality of sepsis requiring critical care identified by the IVW method. Red represents risk factors, whereas blue represents protective factors.

3.1.3 Causality of the gut microbiota on 28-day mortality from sepsis

Five bacterial traits (class *Bacteroidia*: OR 1.48, 95% CI 1.06–2.08; genus *Ruminococcus torques group*: OR 1.53, 95% CI 1.00–2.35; genus *Sellimonas*: OR 1.25, 95% CI 1.04–1.50; genus *Terrisporobacter*: OR 1.43, 95% CI 1.02–2.02; and order *Bacteroidales*: OR 1.48, 95% CI 1.06–2.08) were significantly associated with an increase in 28-day mortality from sepsis, while seven other bacterial traits (class *Lentisphaeria*: OR 0.68, 95% CI 0.53–0.87; genus *Coprococcus1*: OR 0.67, 95% CI 0.48–0.94; genus *Coprococcus2*: OR 0.48, 95% CI 0.27–0.86; genus *Lachnospiraceae FCS020 group*: OR 0.70, 95% CI 0.52–0.95; genus *Victivallis*: OR 0.82, 95% CI 0.68–0.99; order *Victivallales*: OR 0.68, 95% CI 0.53–0.87; and phylum *Lentisphaerae*: OR 0.72, 95% CI 0.56–0.93) were reported to be significantly associated with a lower risk of 28-day mortality from sepsis in the primary IVW MR analysis. Moreover, the weighted median method showed that the genus *Coprococcus2* had a significant protective effect on 28-day mortality from sepsis (OR 0.49, 95% CI 0.28–0.86), and the MR–Egger regression showed that the genus *Ruminococcus torques group* (OR 3.86, 95% CI 1.31–11.34) was associated with a greater risk of 28-day mortality from sepsis. The detailed results from the MR analysis showing the causal relationships between bacterial traits and 28-day mortality from sepsis are illustrated in Figure 5.

3.1.4 Causality of the gut microbiota on 28-day mortality from sepsis requiring critical care

The results of the primary IVW MR analysis showed that four bacterial traits (class *Bacteroidia*: OR 2.43, 95% CI 1.10–5.37; class *Mollicutes*: OR 2.03, 95% CI 1.01–4.08; order *Bacteroidales*: OR 2.43, 95% CI 1.10–5.37; and phylum *Tenericutes*: OR 2.03, 95% CI 1.01–4.08) were significantly associated with a greater risk of 28-day mortality from sepsis requiring critical care, and five bacterial traits (class *Lentisphaeria*: OR 0.54, 95% CI 0.30–0.95; genus *Coprococcus1*: OR 0.42, 95% CI 0.19–0.92; genus *Ruminiclostridium6*: OR 0.43, 95% CI 0.22–0.83; genus *Coprococcus2*: OR 0.34, 95% CI 0.14–0.83; and order *Victivallales*: OR 0.54, 95% CI 0.30–0.95) were causally associated with a lower risk of 28-day mortality from sepsis requiring critical care, suggesting a potential protective effect. Moreover, the estimates of the weighted median method showed that the class *Bacteroidia* was significantly associated with 28-day mortality from sepsis requiring critical care (OR 2.96, 95% CI 1.01–8.71). MR–Egger regression revealed that the genus *Ruminiclostridium6* was significantly associated with 28-day

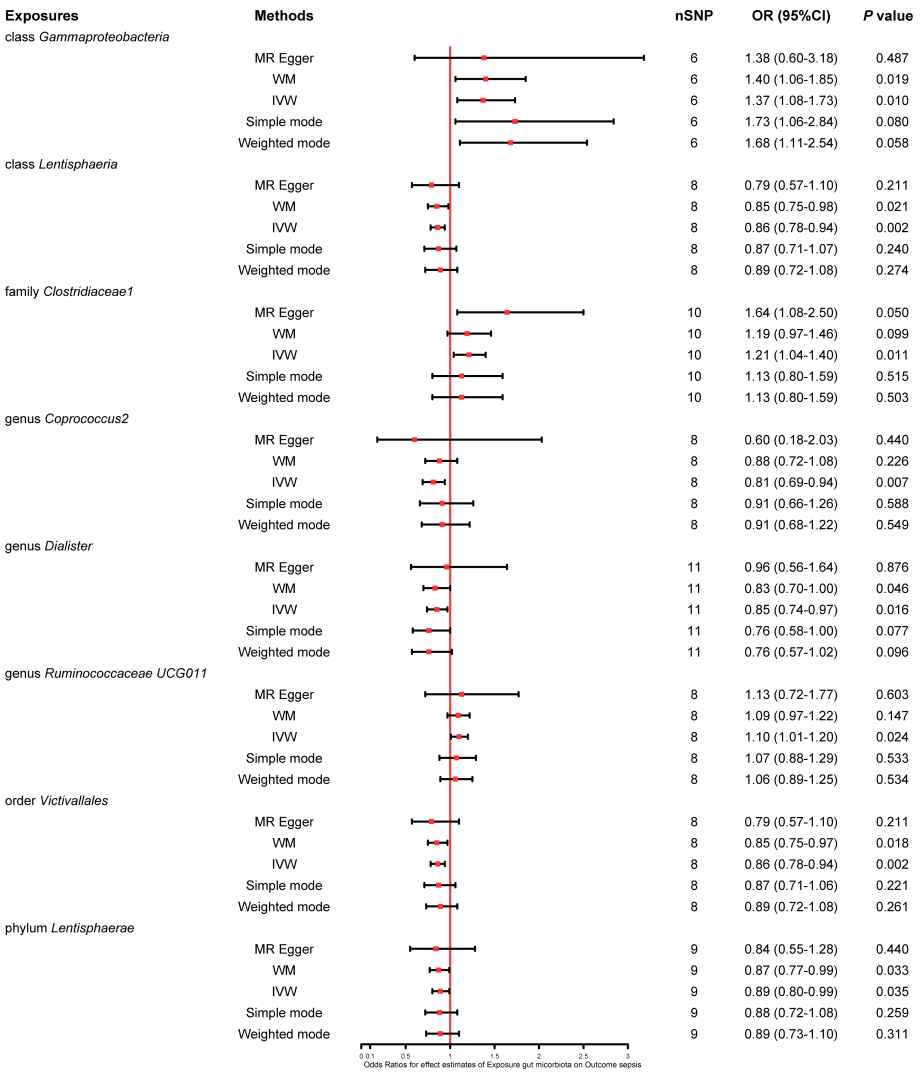

FIGURE 3
Forest plot of various MR results for eight bacterial traits causally associated with sepsis.

mortality from sepsis requiring critical care (OR 0.16, 95% CI 0.03–0.77). The extensive MR findings on the potential causal link between bacterial traits and 28-day mortality from sepsis requiring critical care are displayed in Figure 6.

3.1.5 Sensitivity analysis

The robustness of the MR analysis results was confirmed by scatter plots (Figures 7A–D) and leave-one-out plots (Figures 8A–D). According to the MR–Egger regression intercept methods, there was no evidence of horizontal pleiotropy for these 21 bacterial traits, with causal associations with sepsis and sepsis-related outcomes (Supplementary Table S4). Potentially significant heterogeneity was detected only for the association between 28-day mortality from sepsis and the genus *Coprococcus2* (Cochran's *Q* statistics = 15.93, p = 0.026) (Supplementary Table S4). Moreover, we found no significant heterogeneity ($p > 0.05$) according to Cochran's IVW *Q* statistics of the remaining 20 bacterial traits. Visual examination clearly revealed that the removal of any single IV did not significantly affect the overall results. Furthermore, MR-PRESSO tests showed the absence of outliers in the results (Supplementary Table S5).

3.2 Results of the MR analysis (locus-wide significance, $p < 5\times10^{-8}$)

In the MR analysis of the gut microbiota and its relationship with sepsis and sepsis-related outcomes, none of the five MR methods identified any significant causal associations. When a sensitivity analysis was conducted, no evidence of heterogeneity was found according to Cochrane's *Q* test. Furthermore, no horizontal pleiotropy was detected by either the MR–Egger intercept test or the MR-PRESSO global test, and no outliers were identified by the MR-PRESSO outlier test. The full results can be found in Supplementary Table S6.

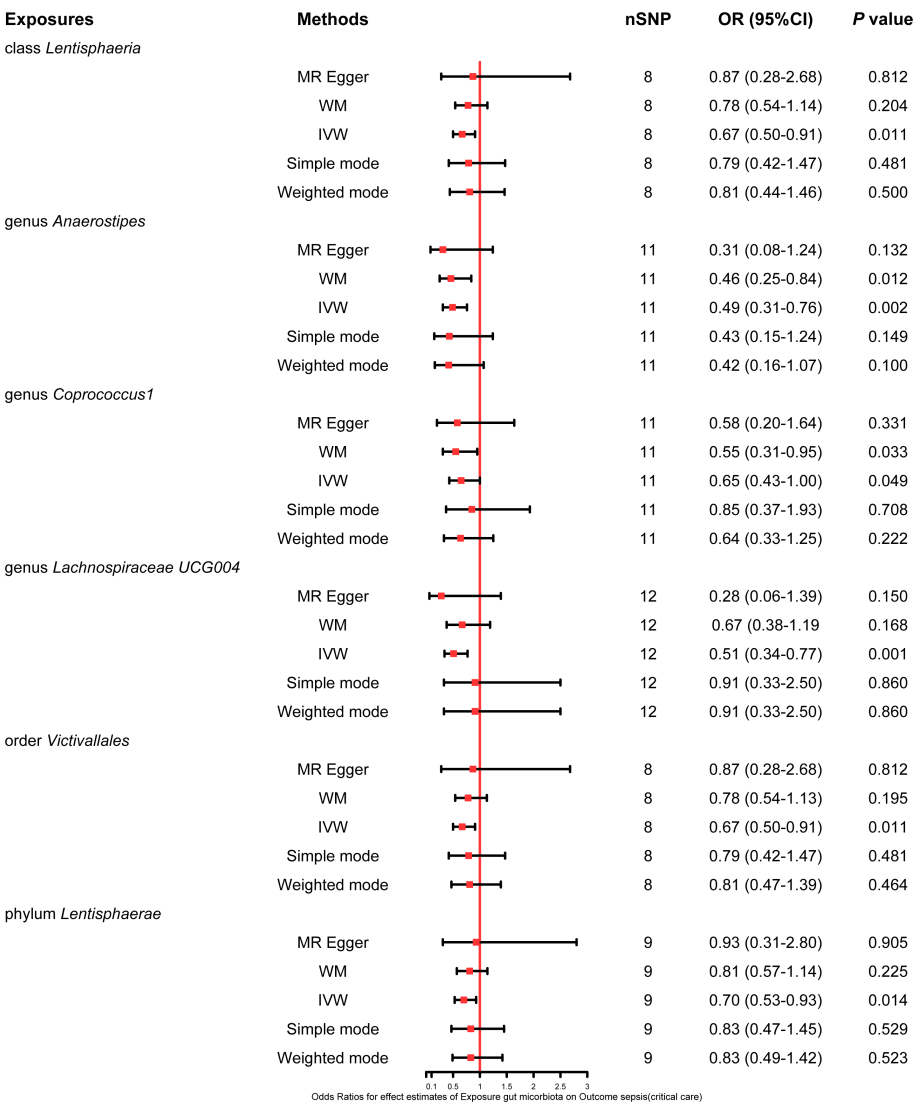

FIGURE 4
Forest plot of various MR results for six bacterial traits causally associated with sepsis requiring critical care.

3.3 Reverse-direction MR analyses

The reverse MR analysis results suggested that there is no causal effect of septic traits on bacterial traits (Supplementary Table S7).

4 Discussion

To our knowledge, this is the first MR analysis to comprehensively explore the causal effect of the gut microbiota on sepsis onset, progression, and mortality using publicly available genetic databases. In this study, MR analyses were performed on 196 bacterial traits to reveal the potential role of the gut microbiota in the onset and progression of sepsis. We found that 21 causal bacterial traits have a critical impact on the onset and progression of sepsis. Notably, two bacterial traits of the gut microbiota (*Victivallales* and *Lentisphaeria*) are the same, and therefore, we only report the results for *Victivallales*.

Muratsu et al. (40) noted an increase in *Ruminococcaceae* abundance during the subacute phase of sepsis in mice, suggesting a potential association between the presence of *Ruminococcaceae* and sepsis. However, Stoma et al. (41) reported a negative association between *Ruminococcaceae* and sepsis risk in a population study, which contrasts with our findings. Moreover, Zhang et al. (42) reported that the presence of *Ruminococcaceae* in rats was negatively associated with lipopolysaccharide (LPS)-binding protein (LBP) and proinflammatory factors, such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). Our research, for the first time, suggested that *Ruminococcaceae* may play a role in causing sepsis, potentially serving as a novel biomarker. Based on our findings, we propose that the effects of *Ruminococcaceae* on sepsis may depend on the specific species and strains. Burritt et al. (43) reported that the presence of *Gammaproteobacteria* in rats subjected to cecal ligation and puncture was positively associated with sepsis risk. *Gammaproteobacteria* has been shown to be positively associated

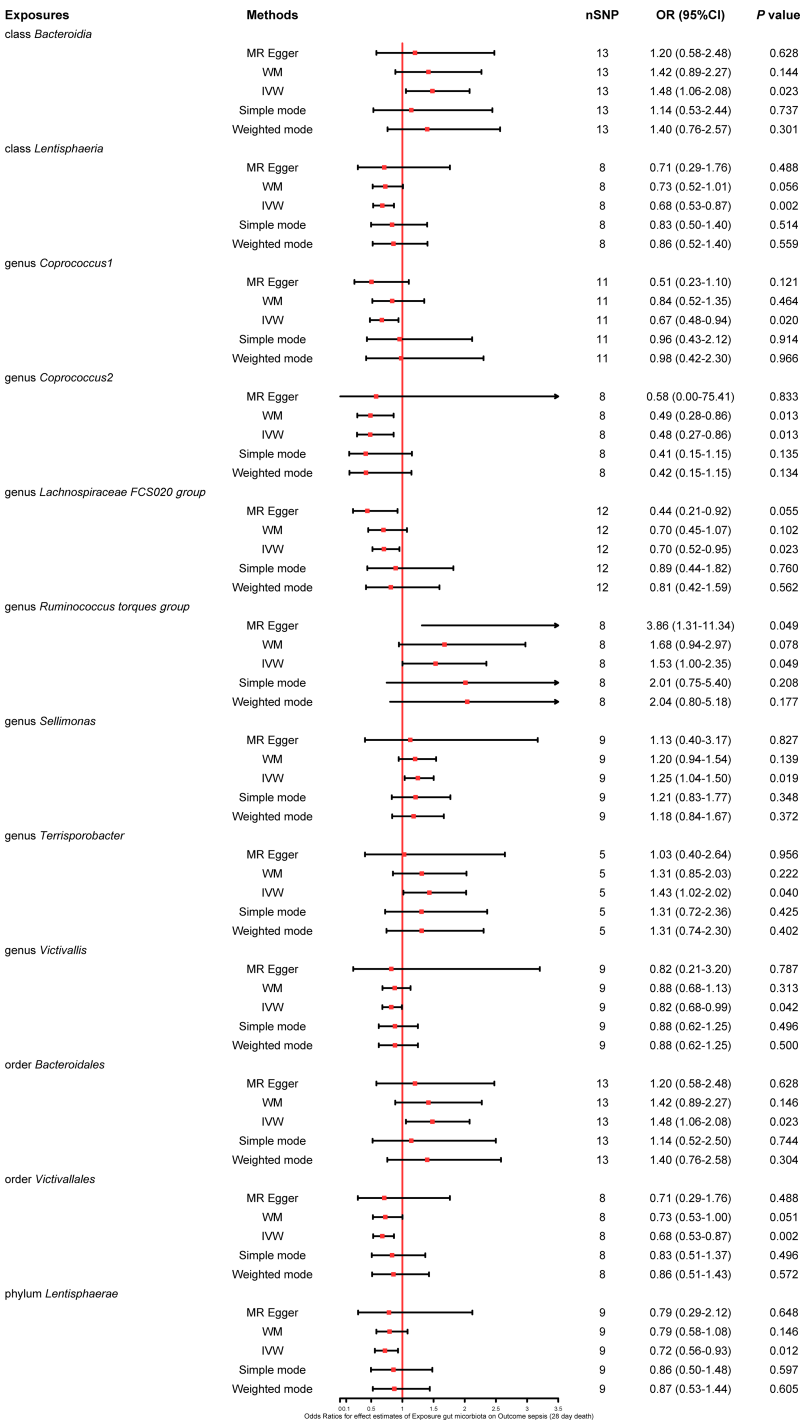

FIGURE 5
Forest plot of various MR results for 12 bacterial traits causally associated with 28-day mortality of sepsis.

with the pathways of severe LPS-related hyperinflammatory stress, which is a risk factor for sepsis in patients with decompensated cirrhosis (44). Based on our results, as with *Proteobacteria*, *Gammaproteobacteria* are considered to have proinflammatory properties (45–47), which increase the risk of sepsis. A study conducted by Arimatsu et al. (48) revealed that after mice were exposed to oral pathogens belonging to *Bacteroidales*, a significant positive association was observed between *Bacteroidales* and systemic inflammation. Furthermore, consistent with our findings, a positive association between *Bacteroidales* and the proinflammatory cytokine TNF-α was identified (48), with TNF-α known to be associated with the progression of sepsis (49). Consistent with our results, *Lachnospiraceae* has been shown to have health-promoting functions (50) and to play important roles in ulcerative colitis, diabetes, the immune response, and nutrient metabolism (51–55). Similarly, Peng et al. (56) reported a negative

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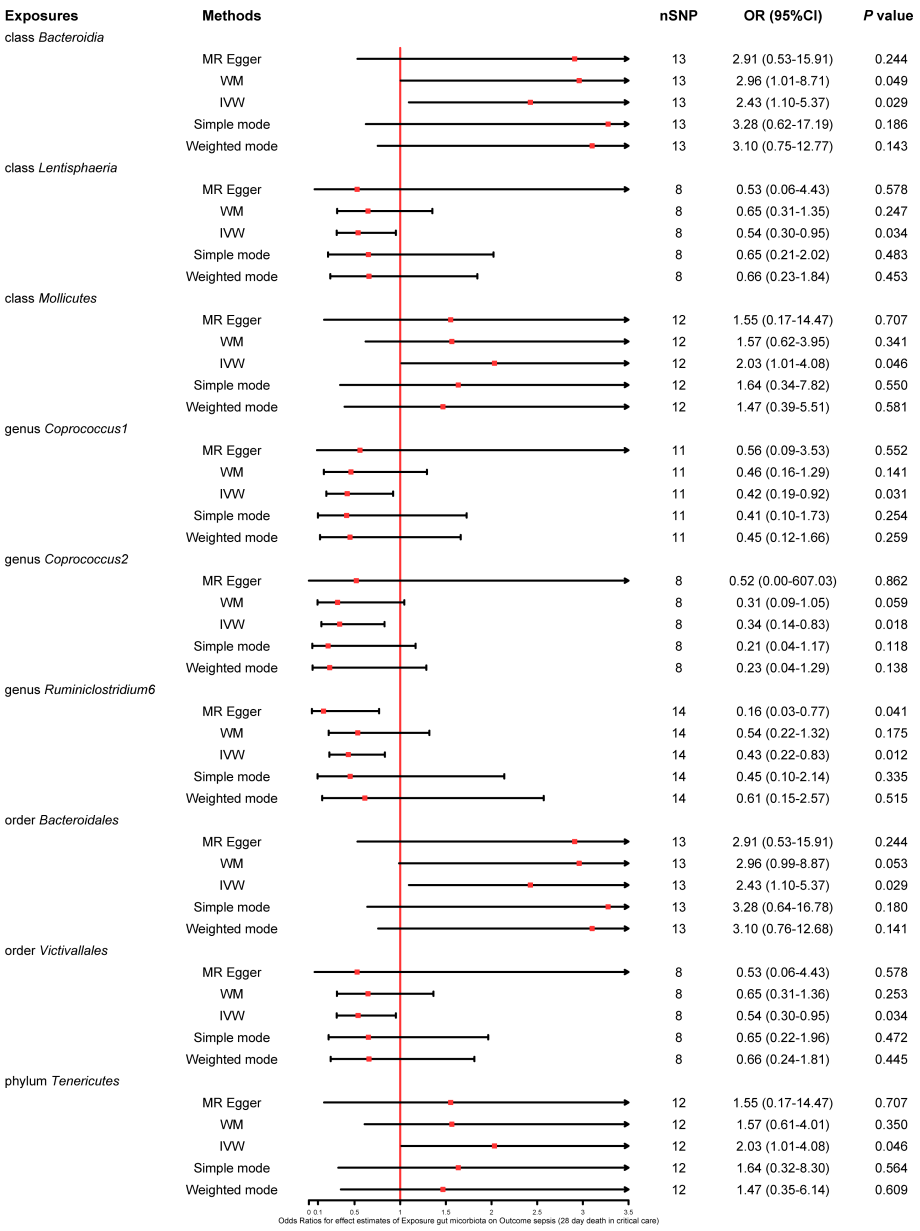

FIGURE 6
Forest plot of various MR results for nine bacterial traits causally associated with 28-day mortality of sepsis requiring critical care.

association between *Lachnospiraceae* and sepsis in the small intestines of mice. Moreover, Yu et al. (57) reported that *Lachnospiraceae* in septic mice fed a methyl diet was negatively associated with mortality, organ injury, and circulating levels of inflammatory mediators. Furthermore, Gai et al. (58) reported that the abundance of *Lachnospiraceae* in mice in the fecal microbiota transplantation (FMT) group was considerably greater than that in the control group, while septic mice in the FMT group exhibited reduced morbidity and mortality. There is a close relationship between the gut microbiota and the immune system (5, 59). IL-6 is a crucial cytokine involved in the innate immune response in sepsis, contributing to adverse outcomes in tandem with other pathophysiological processes (60–63). Moreover, animal models have shown that the elimination of proinflammatory cytokines such as TNF-α, IL-1b, IL-12, and IL-18 provides substantial protection against organ damage and mortality (49). There is now a consensus that the uncontrolled activity of proinflammatory cytokines contributes to sepsis-related injury.

Short-chain fatty acids (SCFAs), which primarily consist of acetic acid, propionic acid, and butyric acid, are the main end products of gut microbiota metabolism in the human body. This study identified a subset of the gut microbiota associated with the onset and progression of sepsis, which included SCFA-producing bacteria such as *Coprococcus* (64), *Dialister* (65), *Lachnospiraceae* (66), *Anaerostipes* (67), *Ruminococcaceae* (66), *Ruminococcus* (68), and *Ruminiclostridium* (69). Clinical and animal studies have shown that gut-derived SCFAs are associated with decreased sepsis risk and organ protection in patients with sepsis (70, 71).

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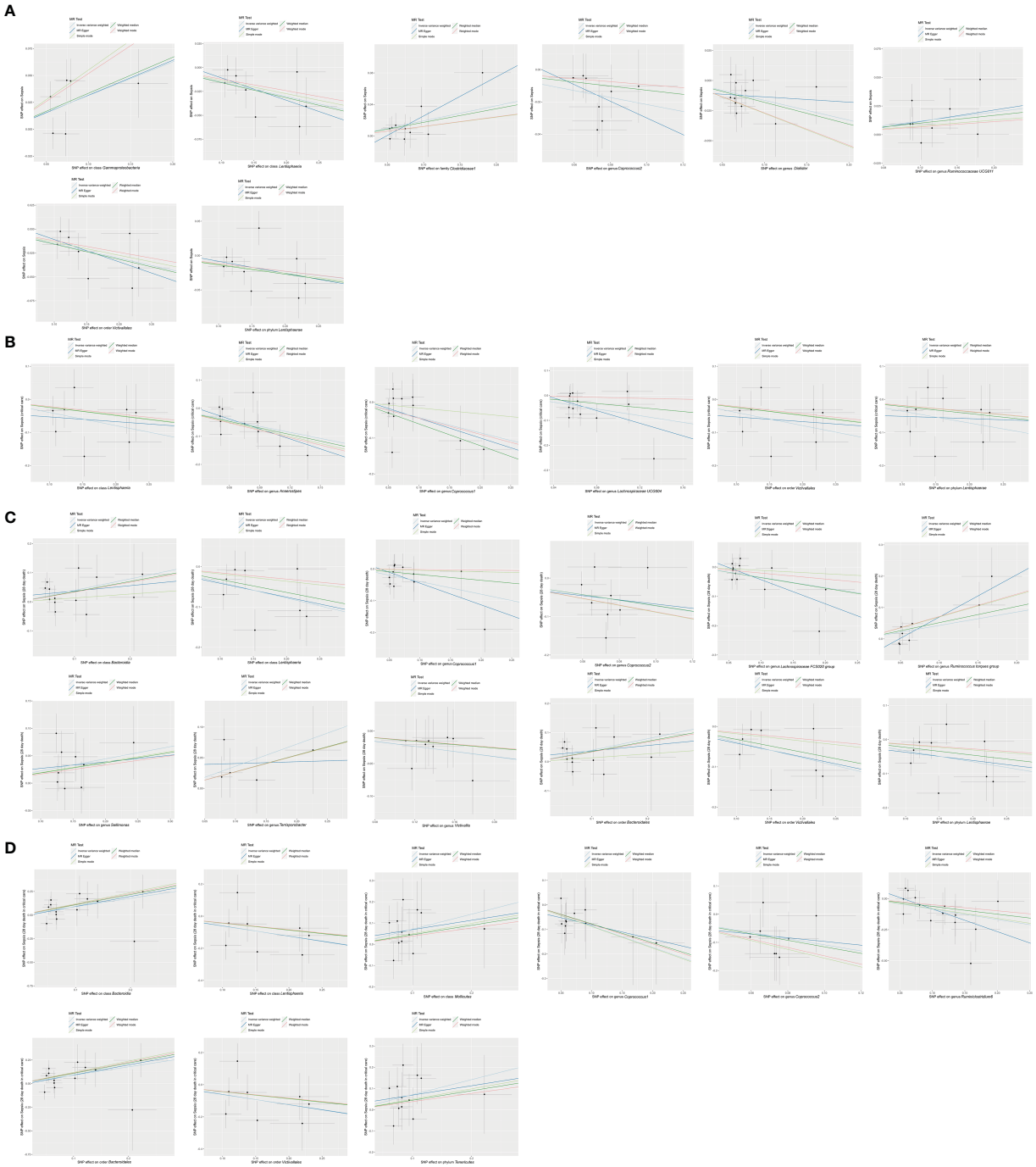

FIGURE 7
Scatter plot of MR results. **(A)** Scatter plot of genetic correlations of eight bacterial traits and sepsis using five MR methods. **(B)** Scatter plot of genetic correlations of six bacterial traits and sepsis requiring critical care using five MR methods. **(C)** Scatter plot of genetic correlations of 12 bacterial traits and 28-day mortality of sepsis using five MR methods. **(D)** scatter plot of genetic correlations of nine bacterial traits and 28-day mortality of sepsis requiring critical care using five MR methods.

Coprococcus, an SCFA-producing bacteria, was reported to decrease in patients with sepsis (72, 73), suggesting a negative association between *Coprococcus* and the risk of sepsis. Furthermore, previous mice experiments revealed that the presence of *Coprococcus* in septic mice pretreated with *Lactobacillus rhamnosus* GG was negatively associated with mortality (74, 75). Based on our findings, *Coprococcus* could be a protective factor against sepsis, suggesting a possible mechanism by which *Coprococcus* regulates the progression of sepsis by producing SCFAs. Furthermore, consistent with our findings, *Lachnospiraceae* has been shown to have the greatest contribution to intestinal protection through L-lysine fermentation to SCFAs, such as acetate and butyrate (76, 77). These substances play critical roles in maintaining immune balance and suppressing inflammation (78–80), thereby enhancing the

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FIGURE 8
Leave-one-out analysis for **(A)** 8 bacterial traits on sepsis, **(B)** 6 bacterial traits on sepsis requiring critical care, **(C)** 12 bacterial traits on 28-day mortality of sepsis, and **(D)** 9 bacterial traits on 28-day mortality of sepsis requiring critical care.

preventative and therapeutic efficacy against sepsis. Similarly, consistent with our results, *Ruminiclostridium*, a butyrate-producing bacteria, has been shown to be negatively associated with the proportion of inflammatory factors (81), which might reduce the inflammatory reaction and severity in sepsis.

Dysbiosis of the gut microbiota (an increase in pathogenic bacteria) may be a cause of bacterial sepsis (82). In the presence of a protective commensal microbiota, pathogenic bacteria in the gut of healthy hosts may not proliferate or cause disease, but the absence of a protective microbiota can lead to an overgrowth of pathogenic bacteria (83, 84). In a study by Hyoju et al. (85), mice were fed a high-fat or normal-fat diet, given broad-spectrum antibiotics, and then underwent partial hepatectomy. Compared to mice fed a normal diet, mice fed a high-fat diet had reduced microbial diversity in their gut microbiota, lower postoperative survival rates, an increase in multidrug-resistant Gram-negative bacteria, more intestinal bacterial spread, and higher mortality rates. Moreover, some large-scale observational studies on

patients have provided indirect evidence that disruption of the gut microbiota is likely to cause sepsis (18, 20, 21). Features of the gut microbiota in individuals with sepsis include diminished diversity; decreased relative abundance of taxa such as *Bacillota* and *Bacteroidetes*; decreased numbers of symbiotic bacteria such as *Faecalibacterium*, *Blautia*, and *Ruminococcus*; and excessive growth of potential pathogens, including *Enterobacter*, *Enterococcus*, and *Staphylococcus* (86–88). Similarly, significant alterations in the microbiota may be linked to the progression of sepsis (89). Research indicates that the gut microbiota plays a role and is a major risk factor for late-onset sepsis (90, 91). Furthermore, Du et al. (22) discovered that an imbalance in the gut microbiota is associated with increased mortality rates and that the gut microbiota can serve as a prognostic indicator for sepsis.

Maintaining a fine equilibrium between harmful pathogens and beneficial probiotics in the gut is crucial for preserving the function of the intestinal barrier (92). Hyoju et al. (85) reported that compared to mice fed a regular diet, mice fed a high-fat diet had decreased α-diversity of the gut microbiota, increased mortality rates, and more gut microbiota taxa from the intestine that spread throughout the body. Moreover, impairment of intestinal barrier function can increase the entry of LPS produced by the gut microbiota into the blood (92), triggering systemic inflammation. This reduces the host's ability to defend against infections, which may increase the risk of sepsis or further exacerbate immune dysregulation, ultimately leading to multiple organ failure. Some probiotics (such as *Lachnospiraceae*) have been proven to exhibit negative associations with intestinal permeability and plasma LPS levels (93). Furthermore, some SCFAs produced by probiotics, such as butyrate, are the main energy sources for intestinal epithelial cells. They participate in cell proliferation and differentiation, maintaining cellular homeostasis through anti-inflammatory and antioxidant effects (94, 95). In addition, SCFAs can influence the function of epithelial cells (70). Butyrate is known for both strengthening intestinal epithelial health and reinforcing barrier function (96) and is key for protecting against antigens such as endotoxins. Acetate shields mice from intestinal *Escherichia coli* translocation by influencing epithelial cell functions (97).

A leaky gut could be a cause or consequence of bacterial sepsis. Severe defects in the gut barrier can lead to the translocation of viable bacteria and bacteremia. This was shown in a study where mice with a leaky gut caused by dextran sulfate solution had higher levels of bacterial DNA in their blood (98). On the other hand, during sepsis, damage to the epithelial tight junctions in the intestines can contribute to the development of a leaky gut (99). In both scenarios, a leaky gut amplifies systemic inflammation through innate immune responses, particularly involving macrophages and neutrophils (100–102), which causes the onset and exacerbation of sepsis. *Bacteroidales* is significantly positively associated with the levels of endotoxin in the blood and significantly negatively associated with the gene expression of ileal tight junction proteins (48). Based on our results, we speculate that dysbiosis of the gut microbiota could enhance the translocation of *Bacteroidales* by increasing intestinal permeability and impairing the mucosal immune function of the gut, thereby exacerbating sepsis. Moreover, Palmieri et al. (103) reported that *Ruminococcus torques* degrades gastrointestinal mucin in patients with Crohn's disease, impairing the mucus barrier produced by intestinal epithelial cells (IECs). The mucus barrier separates intestinal immune cells from the microbial community, reducing intestinal permeability. Impaired intestinal permeability and mucosal immune function can lead to the translocation of pathogenic microorganisms, triggering the excessive production of inflammatory factors and ultimately causing or worsening sepsis (104). However, consistent with our results, *Lachnospiraceae* exhibits a protective effect, which has a negative association with intestinal permeability and plasma LPS levels (93) and prevents the excessive transfer of bacteria and toxins to extraintestinal organs, which further mitigates immune dysregulation in the body.

The gut microbiota and its metabolites activate the immune system through multiple pathways. By producing molecules with immunoregulatory and anti-inflammatory properties, such as SCFAs, indoles, and secondary bile acids, the gut microbiota modulates immune cells, including T cells, B cells, dendritic cells, and macrophages, thereby facilitating antigen presentation and immune modulation. Specifically, SCFAs enhance Th1 cell production of IL-10 via G protein-coupled receptor 43 (GPR43) (105) and stimulate IL-22 production by cluster of differentiation (CD)4^+ T cells and innate lymphoid cells through GPR41 and histone deacetylase inhibition (106). Secondary bile acids interact with Takeda G protein-coupled receptor 5 to reduce nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing-3 inflammasome activation and downregulate proinflammatory cytokine production in macrophages by inhibiting NF-κB signaling. They also suppress NF-κB-dependent inflammatory mediator expression in macrophages through interaction with the nuclear receptor farnesoid X receptor (107). Through polysaccharide A, *Bacteroides fragilis* induces T helper (Th1) cell development and promotes immune tolerance by interacting with Toll-like receptor 2 and T cells, inhibiting Th-17 differentiation, and enhancing regulatory T-cell activity (108). Immune cells recognize microbe-associated molecular patterns, such as LPS, peptidoglycan, and flagellin, and microbiota-derived metabolites that can translocate from the gut into the systemic circulation, thereby triggering immune responses (109, 110).

For other bacterial traits such as *Dialister*, *Victivallales*, *Lentisphaerae*, *Terrisporobacter*, and *Victivallis*, the mechanisms underlying their role in the onset and progression of sepsis remain unclear due to the lack of relevant research or the existence of greater controversy. Further exploration is needed to shed light on these aspects.

The gut microbiota can be regulated by several potential prevention and treatment strategies (111). First, potential pathogens can be eradicated through selective decontamination of the digestive tract. Second, beneficial bacteria or microbe-derived metabolites can be substituted using probiotics, prebiotics, or synbiotics. Finally, the gut microbiota can be partially replaced by FMT.

Our MR analysis revealed the protective effects of *Lentisphaerae*, *Victivallales*, *Lachnospiraceae*, *Victivallis*, *Ruminiclostridium*, *Dialister*, *Coprococcus*, and *Anaerostipes* and the harmful effects of *Tenericutes*, *Bacteroidia*, *Gammaproteobacteria*, *Mollicutes*, *Bacteroidales*,

Clostridiaceae, *Ruminococcaceae UCG 011*, *Terrisporobacter*, *Sellimonas*, and *Ruminococcus torques group* on sepsis. However, the effect of these bacterial traits in the gut microbiota on the onset and progression of sepsis has remained unclear until recently, which is limited by the current research.

Our MR study has several advantages. First, our study analyzed the causal effect of the gut microbiota on sepsis from the genus to the phylum level. This contributes to understanding the mechanisms and interactions between the gut microbiota and host immunity and facilitates the comprehensive assessment of the influence of various bacterial traits. Second, we performed MR analysis to explore the causal association between the gut microbiota and sepsis, effectively eliminating confounding factors and reverse causation, which may interfere with causal inference. Third, the genetic variants of the gut microbiota were sourced from the most extensive GWASs to date, which enhances the credibility of our findings.

Nonetheless, this MR study has limitations. First, although this study pinpointed causal associations from exposure to outcomes, it may not have accurately gauged the association's magnitude. Further research is needed to validate these findings. Second, the use of multiple statistical corrections could be overly stringent and conservative, which might lead to overlooking bacterial traits that could have a causal association with sepsis. Therefore, with biological plausibility in mind, we did not consider multiple testing results. Third, although the majority of the participants whose gut microbiota data were collected in our study were of European descent, a small amount of the microbiological data were from other races, which may have confounded our estimates to some extent. Fourth, we opted for a less strict threshold ($p < 1\times10^{-5}$) to perform horizontal pleiotropy examination and sensitivity analysis. Although this approach allowed us to identify a wider range of associations, it also increased the potential for detecting false positives. Increasing the sample size could increase the precision of the estimation of associations between the gut microbiota and sepsis. Finally, owing to the lack of individual data, we were unable to conduct further population stratification studies (e.g., gender) or explore possible differences in different populations.

5 Conclusion

In summary, the results of our study support the theory that the gut microbiota traits identified in this MR have a causal impact on the risk of sepsis, the risk of sepsis requiring critical care, and the 28-day mortality rate for sepsis and sepsis requiring critical care. This MR analysis could offer pioneering insights for the development of innovative prevention and treatment strategies against sepsis.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

Ethical approval was not required for the study involving humans in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements.

Author contributions

YG: Conceptualization, Data curation, Methodology, Visualization, Writing – original draft, Writing – review & editing. LL: Writing – review & editing, Data curation, Visualization. YC: Writing – review & editing, Data curation, Visualization. JZ: Visualization, Writing – review & editing, Data curation. XW: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1266579/full#supplementary-material

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The causal relationship between gut microbiota and nine infectious diseases: a two-sample Mendelian randomization analysis

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Background: Evidence from observational studies and clinical trials has associated gut microbiota with infectious diseases. However, the causal relationship between gut microbiota and infectious diseases remains unclear.

Methods: We identified gut microbiota based on phylum, class, order, family, and genus classifications, and obtained infectious disease datasets from the IEU OpenGWAS database. The two-sample Mendelian Randomization (MR) analysis was then performed to determine whether the gut microbiota were causally associated with different infectious diseases. In addition, we performed reverse MR analysis to test for causality.

Results: Herein, we characterized causal relationships between genetic predispositions in the gut microbiota and nine infectious diseases. Eight strong associations were found between genetic predisposition in the gut microbiota and infectious diseases. Specifically, the abundance of class *Coriobacteriia*, order *Coriobacteriales*, and family *Coriobacteriaceae* was found to be positively associated with the risk of lower respiratory tract infections (LRTIs). On the other hand, family *Acidaminococcaceae*, genus *Clostridiumsensustricto1*, and class *Bacilli* were positively associated with the risk of endocarditis, cellulitis, and osteomyelitis, respectively. We also discovered that the abundance of class *Lentisphaeria* and order *Victivallales* lowered the risk of sepsis.

Conclusion: Through MR analysis, we found that gut microbiota were causally associated with infectious diseases. This finding offers new insights into the microbe-mediated infection mechanisms for further clinical research.

KEYWORDS

gut microbiota, infectious diseases, causality, GWAS, Mendelian randomization

1 Introduction

Infections such as pneumonia and gastrointestinal infections are the most common infections in hospitalized patients (1). Statistically, these infections account for more than 20% of deaths globally, with 245,000 sepsis cases occurring in the United Kingdom (UK) alone annually (2, 3). Owing to antibiotic resistance, an aging population, and emerging pathogens, the infection-induced disease burden is expected to rise, making the identification of the factors that can modify these illnesses essential (4–6). Generally, severe bacterial infections are believed to be caused by the invasion of the blood and tissues by pathogenic microorganisms, resulting in tissue necrosis and even host death (7). Furthermore, with advancements in sepsis research in recent years, it has been found that uncontrolled infection may lead to dysregulation of the host's immune response. At the same time, excessive immune response results in the secretion of a multitude of cytokines, leading to organ dysfunction and, ultimately, host death (8–10). Therefore, effective prevention and treatment of serious infectious diseases has become critical.

In a healthy host, the gut microbiota regulate various homeostasis mechanisms, including immune function and gut barrier protection (11, 12). Mechanisms of gut microbiota leading to infectious diseases, including allowing the expansion of pathogenic gut bacteria, primes the immune system to produce a robust pro-inflammatory response, thus reducing the production of beneficial microbial products, such as short-chain fatty acids (13–15). Furthermore, gut microbiota interact with infectious diseases. On the one hand, susceptibility to infectious diseases may be aggravated by intestinal micro-ecological disorders. Under certain conditions, intestinal bacteria can directly invade peripheral blood through intestinal mucosa. They could also enter distant organs via the "gut–organ" axis, causing bacterial translocation and eliciting systemic inflammatory responses. Further illness progression can lead to organ dysfunction (16). On the other hand, severe infection could also cause alterations in the human intestinal microenvironment, resulting in the imbalance of intestinal flora and the release of inflammatory factors, damaging the intestinal mucosal barrier and further aggravating the disease (17). Although an increasing number of studies has associated gut microbiota with infectious diseases, the causal relationship between the two remains unclear.

In recent years, Mendelian randomization (MR) analysis, a statistical approach for investigating causal relationships, has been mainly applied to the causal inference of epidemiological diseases. Since alleles follow the random allocation principle, this impact is not affected by confounding factors and reverse causation in traditional epidemiological research (18). The publication of large-scale genome-wide association study (GWAS) data has resulted in the availability of a substantial number of reliable genetic variants for MR studies (19). As a result, this study analyzed the causal relationship between gut microbiota and infectious diseases through the MR analysis, providing useful insights into the clinical treatment of infectious diseases.

2 Materials and methods

2.1 Study population

As shown in Figure 1, we used a two-sample MR (TSMR) approach to characterize the causal relationship between the intestinal microbiome and infectious diseases and finally conducted quality control tests, including the heterogeneity and gene pleiotropy tests, to verify the reliability of the results.

The gut microbiota, which is investigated in the context of human genetics by MiBioGen, an international consortium, was the primary exposure factor for our study (20). Herein, the human gut microbiota GWAS data, encompassing 18,340 individuals from 24 population cohorts, was used. A total of 196 bacterial groups (including 9 phyla, 16 classes, 20 orders, 32 families, and 119 genera) were included after excluding 15 genera with no specific species names.

Our primary outcomes were various infectious diseases with GWAS datasets from the UK Biobank project (21), a prospective cohort study that collected deep genetic and phenotypic data on approximately 500,000 individuals across the UK. Each participant had a wealth of phenotypic and health-related information. Genome-wide genotype data were collected from all participants by linking health and medical records to provide follow-up information. Pneumonia, upper respiratory tract infections (URTIs), lower respiratory tract infections (LRTIs), endocarditis, urinary

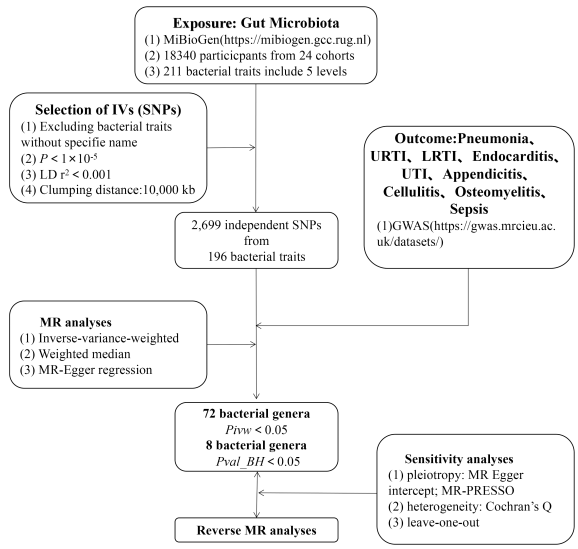

FIGURE 1
The study design of the present MR study of the associations of gut microbiota and sepsis. LD, linkage disequilibrium, which used to measure the correlations between SNPs; IVW, inverse-variance-weighted, the main analyses to evaluate the relationship between exposure and outcome; MR-PRESSO, Mendelian Randomization Pleiotropy RESidual Sum and Outlier, a method test the pleiotropic biases in the SNPs and correct the pleiotropic effects; MR, Mendelian randomization; SNPs, single-nucleotide polymorphisms, as instrumental variables for the exposures and outcomes.

tract infections (UTIs), appendicitis, cellulitis, osteomyelitis, and sepsis were among the infectious diseases evaluated. Information on exposure and outcome factor data is presented in Supplementary Table 1.

2.2 Single-nucleotide polymorphisms selection

Here, single-nucleotide polymorphisms (SNPs) significantly associated with the relative abundance of 196 gut microbiota were selected as available instrumental variables (IVs). According to previous research, including multiple IVs can enhance the interpretation of exposure variation and improve the accuracy and reliability of analysis results. As a result, to ensure the independence of the included SNPSs, this study selected IVs based on the results of association analysis (with $p < 1\times10^{-5}$ as the significance threshold), set the linkage disequilibrium criteria (with $R^2 < 0.001$) and genetic distance (with 10,000 kb), and excluded highly correlated SNPs (22). Finally, SNPs associated with the relative abundance of gut microbiota were projected into the GWAS data on infectious diseases and the corresponding statistical parameters were retrieved. To align the effect exposure and outcome values with the same effect allele, the data were unified based on the statistical parameters of the same site in the relative abundance of gut microbiota and GWAS results of infectious diseases.

2.3 Research design

When using SNPs as IVs in MR analysis, three key assumptions should be met to better estimate the causal effects: (1) The IVs must be closely related to exposure factors; (2) the IVs should not be related to confounding factors; and (3) the IVs should only affect the results through exposure and not by any other means.

2.4 Statistical analysis

In this study, Inverse variance weighted (IVW), MR-Egger, Weighted Median (WME), Simple Mode (SM), and Weighted Mode (WM) were used to estimate the causal effect. The IVW method presumes that all genetic variants are valid. The IVW approach employs the ratio method to calculate the causal effect size of individual IVs and obtains the total effect size by aggregating each estimate for weighted linear regression (23). The primary distinction between the MR-Egger and the IVW methods is that the former considers the existence of the intercept term in regression analysis (24). The WME approach takes advantage of all available genetic variants' intermediate effects. An estimate (25) was obtained by weighting the inverse variance of each SNP's correlation with the outcome. The SM and WM methods are modality-based approaches, and modality-based estimation models aggregate SNPs with similar causal effects and return the estimates of causal effects for most cluster SNPs. The influence of each SNP on the cluster was weighted by WM per the inverse variance of its resulting effect.

Given that the IVW approach is more efficient than the other four MR methods, it was used herein as the preferred causal effect estimation method. Additionally, the beta values obtained in the results were converted into odds ratios (OR), and the 95% confidence interval (CI) was calculated to better explain the results. To verify whether the results were "false positives" due to multiple tests, we used the Benjamini–Hochberg (BH) method under the false discovery rate (FDR) standard to correct the MR results for different classifications of gut microbiome (phyla, class, order, family, and genus); the calculation formula is $FDR(i) = p(i) *m/i$, specifically, all p-values are arranged in ascending order, where p-values are denoted as p, the serial number of p-values is denoted as i, and the total number of p-values is denoted as m (26). Using the F statistic to test IV strength, the association of effect estimates that test causation may be affected by weak instrumental bias. The F statistic is calculated as follows: $F = R^2 (N–K–1)/k (1–R^2)$, where R^2 = variance (per gut microbiome) interpreted by IV, and n = sample size. The R^2 is estimated from the minor allele frequency (MAF) and B-value using the following equation: $R^2 = 2 \times MAF \times (1–MAF) \times b2$ (27).

Additionally, we included sensitivity analysis, heterogeneity level test, and gene pleiotropy test in quality control to further test the stability and reliability of the results. For sensitivity analysis, the residual one method was used, and the combined effect value of the remaining SNPs was determined by sequentially deleting single SNP to evaluate the impact of each SNP on the results. The heterogeneity test was performed to assess the heterogeneity of SNPs. The SNP measurement error caused by experimental conditions and population analysis, among other factors, could lead to bias in estimating causal effects (28). Using the intercept term of the MR-Egger regression, the horizontal gene pleiotropy test assesses whether IVs affect outcomes by other means apart from exposure (29). Potentially abnormal SNPs were identified through the Mendelian Randomization Multi-Effect Residual and Outlier (MR-PRESSO) (30) and leave-one-out methods (31). Finally, we performed reverse MR to analyze whether there was a reverse causality between infectious diseases and meaningful gut microbiota. The MR Analysis and quality control for this study were analyzed using version 4.0.3 R and version 0.5.6 TwoSampleMR packages.

3 Results

3.1 TSMR analysis

The results of the 196 gut microbiota examined in relation to infectious disease are presented in Supplementary Table S2. The F-statistics for the gut flora ranged between 14.58 and 88.42 (all meeting the >10 threshold), implying that they are unlikely to be impacted by weak instrumental bias (Supplementary Table S3). Briefly, we identified 72 genera associated with infectious disease risk (Figure 2). However, after rigorous BH correction, only eight gut microbiota showed stability in their association with infectious diseases (Table 1).

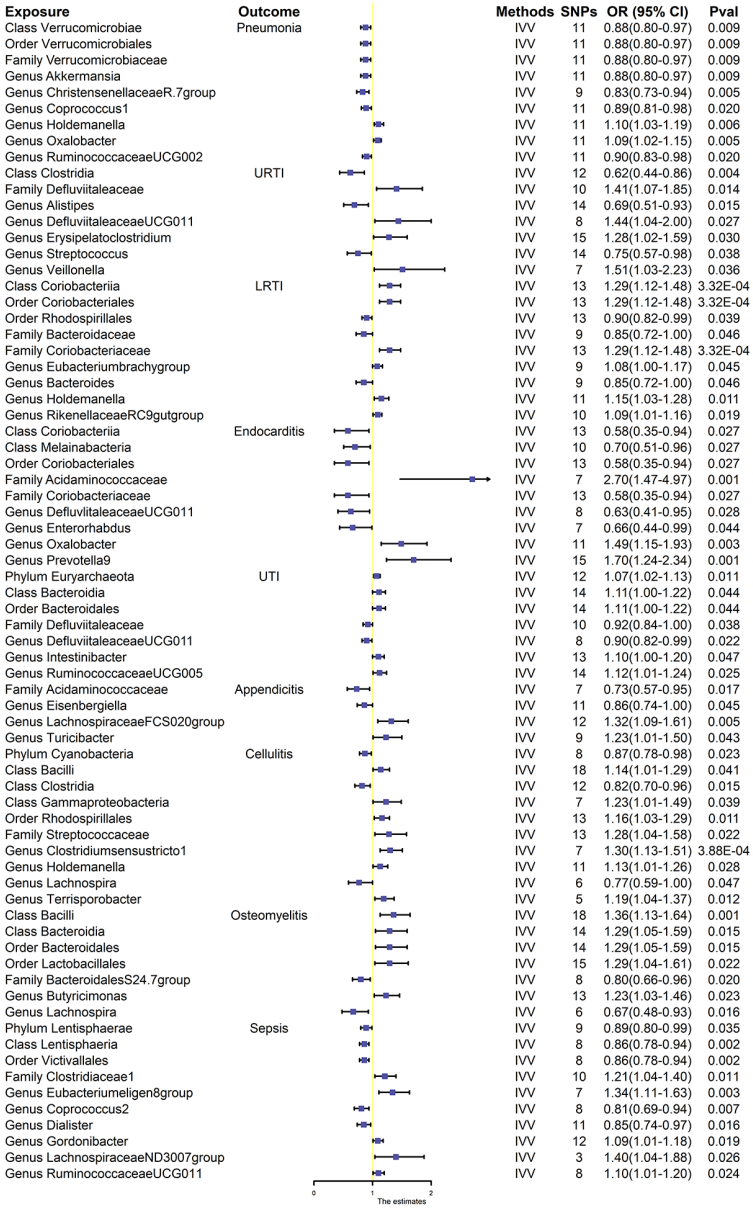

FIGURE 2
Effect estimates of the association between meaningful gut microbiota and infectious disease risk in IVW analysis. SNPs, single-nucleotide polymorphisms, as instrumental variables for the exposures and outcomes; OR, odds ratio; CI, confidence interval; URTI, upper respiratory tract infection; LRTI, lower respiratory tract infection; UTI, urinary tract infection.

3.2 Gut microbiota and pneumonia

Overall, nine gut microbiota were associated with the risk of respiratory infections in the primary MR analysis, suggesting that these gut microbiota may have an impact on the development of pneumonia. Among them, genus *Holdemanella* [OR:1.10, 95% confidence interval (CI): 1.03–1.19, p = 0.006] and genus *Oxalobacter* (OR: 1.09, 95% CI: 1.02–1.1.15, p = 0.005) were positively correlated with the risk of developing pneumonia. Class *Verrucomicrobiae* (OR: 0.88, 95% CI: 0.80–0.97, p = 0.009), order *Verrucomicrobiales* (OR: 0.88, 95% CI. 0.80–0.97, p = 0.009), family *Verrucomicrobiaceae* (OR: 0.88, 95% CI. 0.80–0.97, p = 0.009), genus *Akkermansi* (OR: 0.88, 95% CI: 0.80–0.97, p = 0.009), genus *ChristensenellaceaeR.7group* (OR: 0.83, 95% CI: 0.73–0.94, p = 0.005), genus *Coprococcus1* (OR: 0.89, 95% CI: 0.81–0.98, p = 0.020), and genus *RuminococcaceaeUCG002* (OR: 0.90, 95% CI: 0.83–0.98, p = 0.020) were negatively correlated with pneumonia (Figure 2). However, after BH correction, these genera were not associated with pneumonia.

3.3 Gut microbiota and URTI

In the primary MR analysis, seven gut microbiota were found to be associated with the risk of URTI. Among them, family *Defluviitaleaceae* (OR: 1.41, 95% CI:1.07–1.85, p = 0.014), genus

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TABLE 1 Effect estimates of the association between meaningful gut microbiota and infectious disease risk in MR analysis.

Gut microbiota	Outcome	SNPs	Methods	OR (95% CI)	*p*-value	p_{FDR}
Class *Coriobacteriia*	LRTI					
		13	MR-Egger	1.28 (0.74–2.22)	0.401	
		13	Weighted median	1.28 (1.05–1.55)	0.012	
		13	IVW	1.29 (1.12–1.48)	3.32E-04	0.005
		13	Simple mode	1.26 (0.91–1.73)	0.187	
		13	Weighted mode	1.26 (0.92–1.71)	0.176	
Order *Coriobacteriales*	LRTI					
		13	MR-Egger	1.28 (0.74–2.22)	0.401	
		13	Weighted median	1.28 (1.06–1.54)	0.010	
		13	IVW	1.29 (1.12–1.48)	3.32E-04	0.007
		13	Simple mode	1.26 (0.94–1.67)	0.147	
		13	Weighted mode	1.26 (0.92–1.71)	0.177	
Family *Coriobacteriaceae*	LRTI					
		13	MR-Egger	1.28 (0.74–2.22)	0.401	
		13	Weighted median	1.28 (1.07–1.53)	0.007	
		13	IVW	1.29 (1.12–1.48)	3.32E-04	0.011
		13	Simple mode	1.26 (0.93–1.69)	0.160	
		13	Weighted mode	1.26 (0.92–1.72)	0.184	
Family *Acidaminococcaceae*	Endocarditis					
		7	MR-Egger	0.73 (0.14–3.77)	0.719	
		7	Weighted median	1.67 (0.82–3.42)	0.159	
		7	IVW	2.70 (1.47–4.97)	0.001	0.045
		7	Simple mode	1.58 (0.61–4.05)	0.382	
		7	Weighted mode	1.60 (0.66–3.88)	0.341	
Genus *Clostridiumsensustricto1*	Cellulitis					
		7	MR-Egger	1.34 (0.96–1.87)	0.145	
		7	Weighted median	1.25 (1.01–1.54)	0.036	
		7	IVW	1.30 (1.13–1.51)	3.88E-04	0.046
		7	Simple mode	1.25 (0.94–1.65)	0.173	
		7	Weighted mode	1.24 (0.97–1.57)	0.132	
Class *Bacilli*	Osteomyelitis					
		18	MR-Egger	0.93 (0.57–1.53)	0.775	
		18	Weighted median	1.22 (0.93–1.61)	0.151	
		18	IVW	1.36 (1.13–1.64)	0.001	0.022
		18	Simple mode	2.02 (1.15–3.55)	0.025	
		18	Weighted mode	1.05 (0.68–1.64)	0.823	
Class *Lentisphaeria*	Sepsis					
		8	MR-Egger	0.79 (0.57–1.10)	0.211	
		8	Weighted median	0.85 (0.75–0.97)	0.016	

(Continued)

TABLE 1 Continued

Gut microbiota	Outcome	SNPs	Methods	OR (95% CI)	*p*-value	p_{FDR}
		8	IVW	0.86 (0.78–0.94)	0.002	0.026
		8	Simple mode	0.87 (0.71–1.07)	0.235	
		8	Weighted mode	0.89 (0.73–1.08)	0.273	
Order *Victivallales*	Sepsis					
		8	MR-Egger	0.79 (0.57–1.10)	0.211	
		8	Weighted median	0.85 (0.75–0.97)	0.015	
		8	IVW	0.86 (0.78–0.94)	0.002	0.033
		8	Simple mode	0.87 (0.71–1.08)	0.243	
		8	Weighted mode	0.89 (0.73–1.08)	0.266	

MR, Mendelian randomization; SNPs, number of single-nucleotide polymorphism. CI, confidence interval; OR, odds ratio; p_{FDR}, *p*-value was calculated by the Benjamini–Hochberg method; LRTI, lower respiratory tract infection; IVW, inverse variance weighted.

DefluviitaleaceaeUCG011 (OR: 1.44, 95% CI: 1.04–2.00, $p = 0.027$), genus *Erysipelatoclostridium* (OR: 1.28, 95% CI: 1.02–1.59, $p = 0.030$), and genus *Veillonella* (OR: 1.51, 95% CI: 1.03–2.23, $p = 0.036$) were positively associated with the risk of URTI, while class *Clostridia* (OR: 0.62, 95% CI: 0.44–0.86, $p = 0.004$), genus *Alistipes* (OR: 0.69, 95% CI: 0.51–0.93, $p = 0.015$), and genus *Streptococcus* (OR: 0.75, 95% CI: 0.57–0.98, $p = 0.038$) were negatively associated with the risk of URTI (Figure 2). None of these seven gut microbiota were associated with significance in URTI after BH correction.

3.4 Gut microbiota and LRTI

Nine gut microbiota were associated with the risk of LRTI (Figure 2). However, only three gut microbiota were associated with significance in LRTI after strict BH correction (Table 1). Specifically, we observed that the abundance of class *Coriobacteriia* (OR: 1.29, 95% CI: 1.12–1.48, $p_{FDR} = 0.005$), order *Coriobacteriales* (OR: 1.29, 95% CI: 1.12–1.48, $p_{FDR} = 0.007$), and family *Coriobacteriaceae* (OR: 1.29, 95% CI = 1.12–1.48, $p_{FDR} = 0.011$) were associated with a higher risk of LRTI.

In sensitivity analyses, the WME results were comparable to those of the IVW approach (OR: 1.28, 95% CI: 1.05–1.55, $p = 0.012$ for class *Coriobacteria*; OR: 1.28, 95% CI: 1.06–1.54, $p = 0.010$ for order *Coriobacteriales*; and OR: 1.28, 95% CI = 1.07–1.53, $p = 0.007$ for family *Coriobacteriaceae*), but with wider confidence intervals (Figure 3). Furthermore, the MR-Egger regression intercepts showed no evidence of pleiotropy of these gut microbiota with LRTI (intercept $p = 0.977$ for class *Coriobacteriia*; intercept $p = 0.977$ for order *Coriobacteriales*; and intercept $p = 0.977$ for family *Coriobacteriaceae*) (Table 2 and Supplementary Table S4). No outliers were detected in the MRPRESSO regression. Heterogeneity analysis confirmed the accuracy of the results (Table 2 and Supplementary Table S5). Data robustness was further validated by the leave-one-out results, showing a consistent positive association between gut flora and LRTI risk (Supplementary Table S6).

3.5 Gut microbiota and endocarditis

In the primary MR analysis, nine gut microbiota were associated with the risk of endocarditis (Figure 2). After BH correction, it was found that family *Acidaminococcaceae* abundance was positively associated with the risk of endocarditis (OR: 2.70, 95% CI: 1.47–4.97, $p_{FDR} = 0.045$) (Table 1).

In the sensitivity analysis, the WME method did not show statistical significance (OR: 1.67, 95% CI: 0.82–3.42, $p = 0.159$) (Figure 3). However, the MR-Egger regression intercept did not show evidence of multiplicity of family *Acidaminococcaceae* with endocarditis (Intercept $p = 0.159$) (Table 2 and Supplementary Table S4). MRPRESSO regression did not detect outliers, too. The results of heterogeneity analysis confirmed the accuracy of the results (Table 2 and Supplementary Table S5). The leave-one-out method further validated the data robustness (Supplementary Table S6).

3.6 Gut microbiota and UTI

Seven gut microbiota were confirmed to be associated with the risk of UTI after primary MR analysis. Among them, phylum *Euryarchaeota* (OR. 1.07, 95% CI: 1.02–1.13, $p = 0.011$), class *Bacteroidia* (OR: 1.11, 95% CI: 1.00–1.22, $p = 0.044$), order *Bacteroidales* (OR: 1.11, 95% CI: 1.00–1.22, $p = 0.044$), genus *Intestinibacter* (OR: 1.10, 95% CI: 1.00–1.20, $p = 0.047$), and genus *RuminococcaceaeUCG005* (OR: 1.12, 95% CI: 1.01–1.24, $p = 0.025$) were positively associated with the risk of UTI, while family *Defluviitaleaceae* (OR: 0.92, 95% CI: 0.84–1.00, $p = 0.038$) and genus *Defluviitaleaceae UCG011* (OR: 0.90, 95% CI: 0.82–0.99, $p = 0.022$) were negatively associated with the risk of UTI (Figure 2). No gut microbiota was causally associated with UTI after BH correction.

3.7 Gut microbiota and appendicitis

Primary MR analysis identified four gut microbiota associated with the risk of appendicitis. Among them, genus

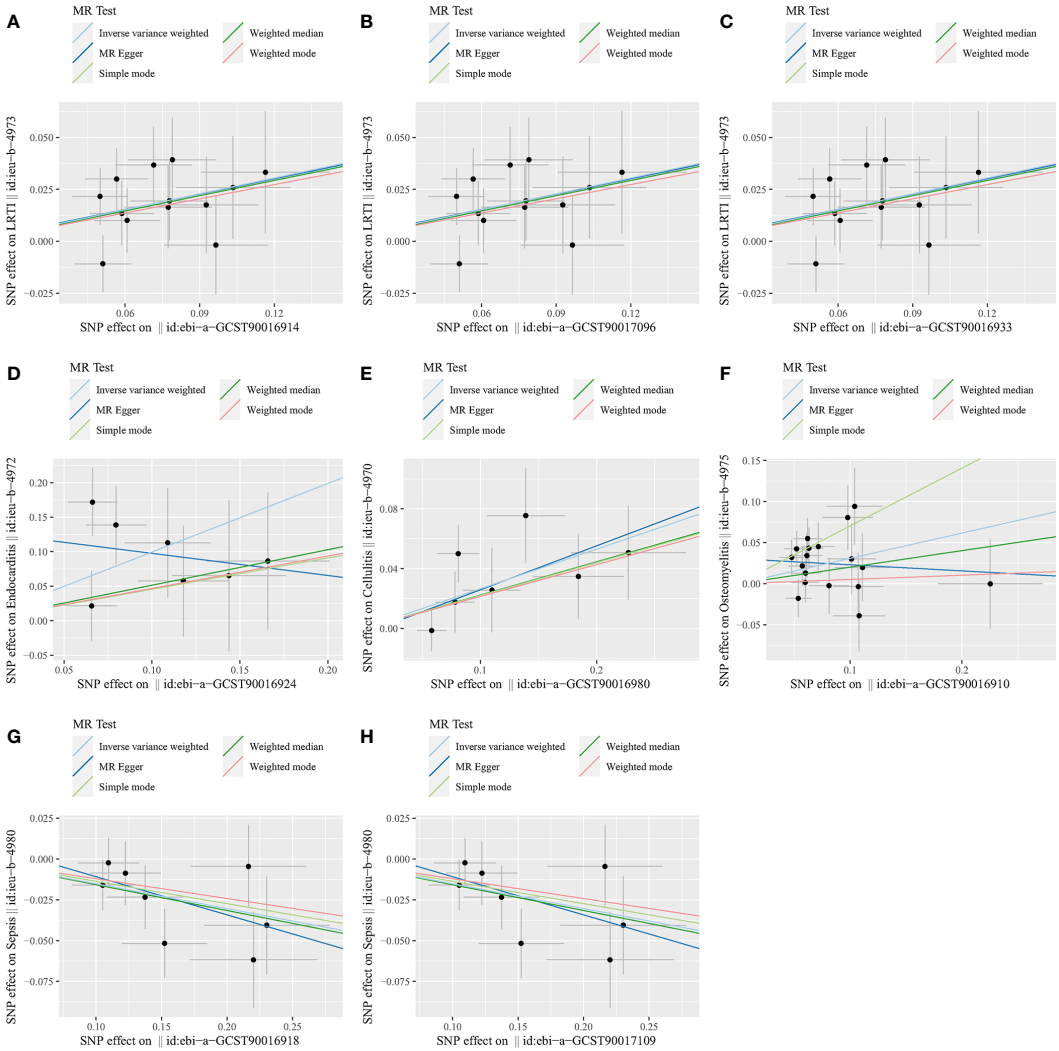

FIGURE 3
Scatter plots for the causal association between gut microbiota and infectious diseases. **(A)** Class *Coriobacteriia* and LRTI. **(B)** Order *Coriobacteriales* and LRTI. **(C)** Family *Coriobacteriaceae* and LRTI. **(D)** Family *Acidaminococcaceae* and endocarditis. **(E)** Genus *Clostridiumsensustricto1* and cellulitis. **(F)** Class *Bacilli* and osteomyelitis. **(G)** Class *Lentisphaeria* and sepsis. **(H)** Order *Victivallales* and sepsis. LRTI, lower respiratory tract infection.

LachnospiraceaeFCS020group (OR: 1.32, 95% CI:1.09–1.61, p = 0.005) and genus *Turicibactera* (OR: 1.23, 95% CI: 1.01–1.50, p = 0.043) were positively associated with the risk of developing appendicitis, while family *Acidaminococcaceae* (OR: 0.73, 95% CI: 0.57–0.95, p = 0.017) and genus *Eisenbergiella* (OR: 0.86, 95% CI: 0.74–1.00, p = 0.045) were negatively associated with the risk of developing appendicitis (Figure 2). No gut microbiota was causally associated with appendicitis after BH correction.

3.8 Gut microbiota and cellulitis

Although 10 gut microbiota were associated with the risk of cellulitis (Figure 2), only genus *Clostridiumsensustricto1* was positively associated with cellulitis after BH correction (OR: 1.30, 95% CI: 1.13–1.55, p_{FDR} = 0.046) (Table 1).

In sensitivity analyses, the WME method showed similar results to IVW (OR: 1.25, 95% CI: 1.01–1.54, p = 0.036) (Figure 3). The MR-Egger regression intercept did not show evidence of multiplicity of genus *Clostridiumsensustricto1* with cellulitis (Intercept p = 0.856) (Table 2 and Supplementary Table S3). MRPRESSO regression did not detect outliers. The results of heterogeneity analysis confirmed the accuracy of the results (Table 2 and Supplementary Table S5). Meanwhile, leave-one-out results further validated the data robustness (Supplementary Table S6).

3.9 Gut microbiota and osteomyelitis

Seven gut microbiota were associated with the risk of osteomyelitis (Figure 2). However, only class *Bacilliidae* was positively causally associated with osteomyelitis after BH correction (OR: 1.36, 95% CI: 1.13–1.64, p_{FDR} = 0.022) (Table 1).

In sensitivity analyses, the WME method showed similar results to IVW (OR: 1.22, 95% CI: 0.93–1.61, p = 0.151) (Figure 3). The MR-Egger regression intercept did not show evidence of

TABLE 2 Heterogeneity and sensitivity analysis between meaningful gut microbiota and infectious diseases.

Gut microbiota	Outcome	Methods	*Q*	*p*	Intercept	*p*	MR-PRESSO
Class *Coriobacteriia*	LRTI						
		IVW	7.998	0.785	0.001	0.977	0.927
		MR-Egger	7.997	0.714			
Order *Coriobacteriales*	LRTI						
		IVW	7.998	0.785	0.001	0.977	0.923
		MR-Egger	7.997	0.714			
Family *Coriobacteriaceae*	LRTI						
		IVW	7.998	0.785	0.001	0.977	0.929
		MR-Egger	7.997	0.714			
Family *Acidaminococcaceae*	Endocarditis						
		IVW	8.185	0.225	0.130	0.159	0.302
		MR-Egger	5.290	0.382			
Genus *Clostridium sensustricto1*	Cellulitis						
		IVW	5.574	0.473	-0.004	0.856	0.299
		MR-Egger	5.534	0.354			
Class *Bacilli*	Osteomyelitis						
		IVW	18.370	0.366	0.030	0.125	0.416
		MR-Egger	15.746	0.471			
Class *Lentisphaeria*	Sepsis						
		IVW	5.159	0.641	0.012	0.628	0.403
		MR-Egger	4.899	0.557			
Order *Victivallales*	Sepsis						
		IVW	5.159	0.641	0.012	0.628	0.394
		MR-Egger	4.899	0.557			

MR-PRESSO, Mendelian Randomization Pleiotropy RESidual Sum and Outlier; IVW, inverse variance weighted; LRTI, lower respiratory tract infection.

multiplicity of class *Bacilliidae* with cellulitis (Intercept $p = 0.125$) (Table 2 and Supplementary Table S3). The MRPRESSO regression did not detect outliers. The results of heterogeneity analysis confirmed the accuracy of the results (Table 2 and Supplementary Table S5). Meanwhile, leave-one-out results further validated the data robustness (Supplementary Table S6).

3.10 Gut microbiota and sepsis

We identified a total of 10 gut microbiota associated with sepsis (Figure 2); only 2 gut microbiota were associated with sepsis after BH correction (Table 1). Notably, class *Lentisphaeria* (OR: 0.86, 95% CI: 0.78–0.94, $p_{FDR} = 0.026$) and order *Victivallales* (OR: 0.86, 95% CI: 0.78–0.94, $p_{FDR} = 0.033$) abundance were negatively correlated with the risk of developing sepsis.

In the sensitivity analysis, the WME method showed similar results to IVW (OR: 0.85, 95% CI: 0.75–0.97, $p = 0.016$ for class *Lentisphaeria* and OR: 0.85, 95% CI: 0.75–0.97, $p = 0.015$ for order *Victivallales*) (Figure 3), and the MR-Egger regression intercept showed no evidence of pleiotropy (intercept $p = 0.125$ for class *Lentisphaeria* and intercept $p = 0.944$ for order *Victivallales*) (Supplementary Table S3). Heterogeneity analysis confirmed the accuracy of the results (Table 2 and Supplementary Table S5). Leave-one-out results verified data robustness (Supplementary Table S6).

3.11 Inverse MR analysis

In the reverse MR, infectious disease was used as an exposure factor, and gut microbiota, which has been associated with infectious disease, was the outcome factor. The IVW results did not support a causal relationship between infectious disease and altered gut microbiota (Supplementary Table 7).

4 Discussion

In this study, TSMR was used to investigate the causal relationship between the relative abundance of gut microbiota

and infectious diseases. It is currently believed that gut microbiota influences host metabolic health by producing a range of metabolites and molecules, including SCFA, bile acids, TMAO, and LPS. For instance, enterogenic SCFAs can affect the pulmonary immune environment in the respiratory system. Bacterial transmission, inflammation, and mortality increased when mice whose gut microbiota was disrupted by antibiotics developed pulmonary streptococcal infections. Furthermore, in mice with disrupted gut microbes, the alveolar macrophage metabolic pathway was upregulated, and the cellular response was altered, resulting in a reduced ability to phagocytize *S. pneumoniae*, causing a less pronounced immunomodulatory response (32). An imbalance of gut microbes can lead to damage to the intestinal wall, or "leaky gut." A large number of toxins and bacteria enter the bloodstream through intestinal leakage to specific organs and tissues, thus triggering a series of inflammatory immune responses. Acute appendicitis is an intestinal infectious illness. Pathogenic bacteria multiply and secrete endotoxins and exotoxins, damaging the mucosal epithelium, forming ulcers, and allowing bacterial entry into the muscle layer of the appendix via the ulcerative surface. Increased interstitial pressure in the appendix wall affects arterial blood flow, resulting in appendicular ischemia and, in severe cases, infarction and gangrene (33). Infective endocarditis refers to the inflammation of the inner lining of the heart valve or ventricle caused by direct infection by bacteria, fungi, and other microorganisms. Studies have shown that intestinal flora destroys the intestinal mucosal barrier, and *Enterococcus faecalis* are released into the blood to attach to the normal valve and cause endocarditis (34). The main pathogen of cellulitis is hemolytic streptococcus, which is caused by external invasion of subcutaneous tissue or caused by lymphatic and hematologic infection (35). The interaction between intestinal flora and susceptibility to recurrent urinary tract infections (rUTI) may promote intestinal colonization of uropathogenic *Escherichia coli* (UPEC) through intestinal flora dysregulation and increase the risk of bladder infection. Furthermore, intestinal flora has been reported as an instigator, and its imbalance may cause systemic inflammation, further worsening the inflammation and symptoms after bladder infection (36). Gut microbiota can release pro-inflammatory or anti-inflammatory mediators and cytokines to regulate systemic bone metabolism through blood circulation. Studies have shown that gut microbiota disturbances that upregulate pro-IL1βlevels indirectly affect osteomyelitis (37).The occurrence and development of sepsis are closely related to the imbalance of gut microbiota. The disturbance of gut microbiota can induce sepsis through the destruction of intestinal mucosal barrier function, mucosal immune function, and bacterial translocation. At the same time, sepsis can also aggravate the imbalance of intestinal flora, resulting in multiple organ dysfunction (38).

Our study identifies a causal link between gut microbiota and infectious diseases, particularly that the abundance of class *Coriobacteriia*, order *Coriobacteriales*, and family *Coriobacteriaceae* are positively associated with the risk of LRTI. *Coriobacteriia* can be found in the mouth, respiratory tract, gastrointestinal tract, and reproductive tract. In the gut, class *Coriobacteriia* performs important functions such as the conversion of bile salts and steroids and the activation of dietary polyphenols. However, they can also be regarded as pathological diseases. According to previous research, the abundance of class *Coriobacteriia* can increase the incidence of diseases such as allergic rhinitis and endometriosis (39, 40). Family *Acidaminococcaceae*, genus *Clostridiumsensustricto1*, and class *Bacilli* were positively related to the risk of endocarditis, cellulitis, and osteomyelitis, respectively. Family *Acidaminococcaceae* belongs to strictly anaerobic Gram-negative coccus. Amino acids, especially glutamate, are a major source of energy (41). Genus *Clostridiumsensustricto1* belongs to Gram-positive bacterium fusobacterium; in the case of hypoxia, fusobacterium causes serious infections including tetanus and gas gangrene (42). Class *Bacilli* can bind lipopolysaccharide (LPS) and neutralize endotoxin. Therefore, the microecological preparation prepared by *Bacilli* has played an important role in the treatment of intestinal flora disorders and *Candida* infection (43). However, *Bacillus* cereus strains usually cause local wound and eye infection and systemic diseases (44). At the same time, the increased abundance of class *Lentisphaeria* and order *Victivallales* decreased the risk of sepsis. Surprisingly, *Lentisphaerae* has been reported to be more abundant in cases of inflammatory bowel disease (45) and less abundant in patients with sepsis, which is consistent with our conclusions (46). Order *Victivallales* has important effects on human infection and immune development. Specifically, it was found to be positively associated with clinical response to anti-programmed cell death protein-1 (PD-1) immunotherapy in patients with advanced cancer (47). In this regard, we believe that these gut microbiota may play a role in the occurrence and development of infectious diseases by regulating immunity. Interestingly, the findings of the reverse MR study do not support a causal relationship between infectious diseases and changes in gut microbiota.

One of the strengths of this study is that it established a causal relationship between alterations in gut microbiota and infectious diseases, offering candidate gut microbiota for subsequent functional studies. However, the study also has limitations. First, it only used European population GWAS data for TSMR analysis, and the abundance of gut microbiota included herein is limited, GWAS data of other gut microbiota need to be obtained in the future, to explore the causal relationship between gut microbiota and infectious diseases more comprehensively. Second, we did not further validate these results with public or our own datasets. Third, although TSMR is an efficient method of causality analysis, animal tests should be conducted in the future to further verify whether there is a potential causal relationship between gut microbiota and infectious diseases. Fourth, there are few studies on these gut flora that have causal relationship with infectious diseases, and more extensive studies are needed to support our conclusions in the future. Fifth, the causal relationship between gut microbiota and infectious diseases is multifaceted, necessitating the exploration of the etiology and pathogenesis of infectious diseases from multiple perspectives.

In conclusion, we used TSMR to explore the causal relationship between gut microbiota and infectious diseases. The results showed that the abundance of class *Coriobacteriia*, order *Coriobacteriales*, and family *Coriobacteriaceae* was associated with LRTI risk; family *Acidaminococcaceae*, genus *Clostridiumsensustricto1*, and class *Bacilli* were found to be positively related to the risk of endocarditis, cellulitis,

and osteomyelitis, respectively. At the same time, the increased abundance of class *Lentisphaeria* and order *Victivallales* lowered the risk of sepsis. These findings elucidate the involvement of gut microbiota in the development of infectious diseases and offer a reference value for the treatment of infectious diseases.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

Since the data used are publicly available in the database, no additional ethical approval was needed in this case.

Author contributions

SW: Conceptualization, Data curation, Formal analysis, Visualization, Writing – original draft. FY: Investigation, Methodology, Resources, Writing – review & editing. WS: Software, Validation, Writing – original draft. RL: Methodology, Visualization, Writing – original draft. ZG: Formal Analysis, Writing – original draft. YW: Data curation, Writing – original draft. YZ: Conceptualization, Methodology, Writing – original draft. CS: Writing – original draft, Funding acquisition. DS: Conceptualization, Funding acquisition, Project administration, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1304973/full#supplementary-material

SUPPLEMENTARY TABLE 1
Information on exposure and outcome factors.

SUPPLEMENTARY TABLE 2
Effect estimates of the associations between 196 bacterial traits and risk of infectious diseases in MR analyses.

SUPPLEMENTARY TABLE 3
The F number and R^2 detect the intensity of the IVs between 196 bacterial traits and risk of infectious diseases in MR analyses.

SUPPLEMENTARY TABLE 4
MR-Egger regression analysis between 196 bacterial traits and risk of infectious diseases in MR analyses.

SUPPLEMENTARY TABLE 5
Testing for heterogeneity between 196 bacterial traits and risk of infectious diseases in MR analyses.

SUPPLEMENTARY TABLE 6
Leave one out between 196 bacterial traits and risk of infectious diseases in MR analyses.

SUPPLEMENTARY TABLE 7
Effect estimates of the associations between infectious diseases and risk of nine infectious diseases in the reverse MR analyses.

SUPPLEMENTARY TABLE 8
Code required during data processing.

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