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Epigenetics in the microbiome-host crosstalk: from mechanisms to therapeutics

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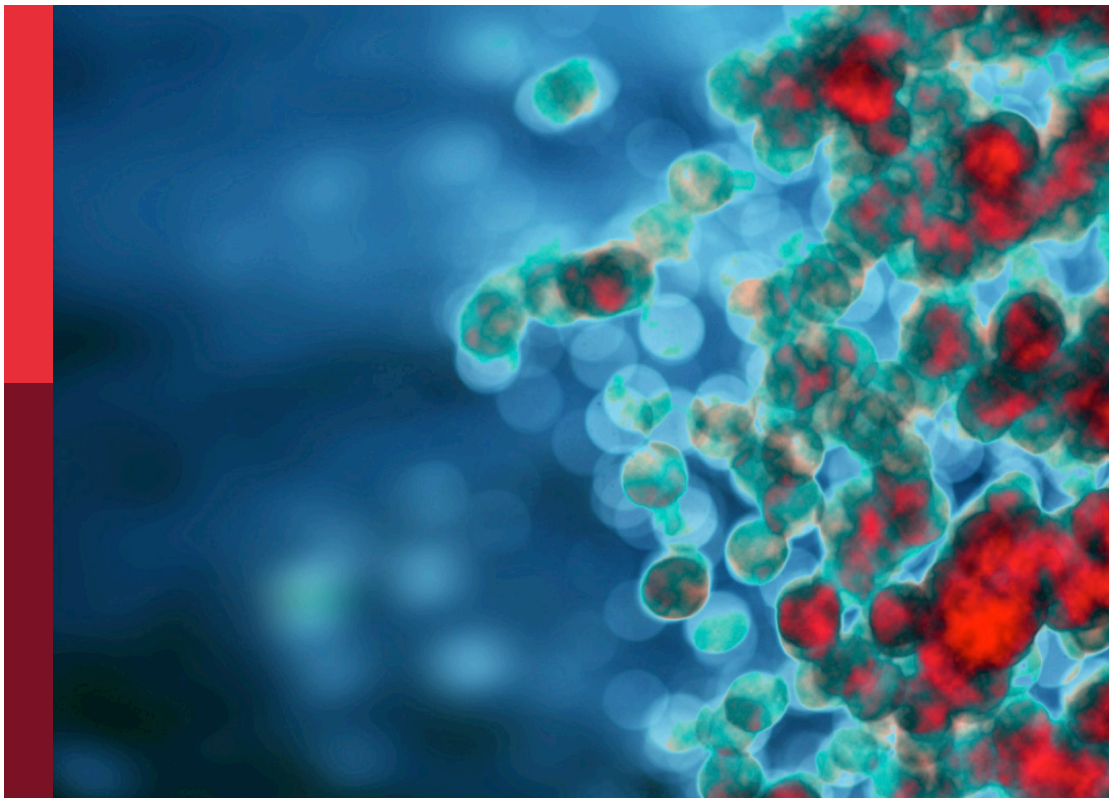

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Epigenetics in the microbiome-host crosstalk: from mechanisms to therapeutics

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Editorial: Epigenetics in the microbiome-host crosstalk: from mechanisms to therapeutics

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Editorial on the Research Topic
Epigenetics in the microbiome-host crosstalk: from mechanisms to therapeutics

In recent years, the study of the gut microbiota has gained significant interest due to its implications on the health status of the host and its capacity to influence host responses to environmental cues. The human digestive system harbors trillions of microorganisms that contribute to important functions in the host, including nutrient metabolism, intestinal permeability, and immune responses. The molecular mechanisms underlying these biological processes include epigenetic phenomena that may alter gene expression without modifying the DNA sequence, ultimately influencing the cell phenotype and the host physiology (1). Notably, the intestinal microbiota participates in the biosynthesis of substrates for DNA/histone methylation reactions as well as the regulation of epigenetically active enzymes and activation of cellular processes involving epigenetic pathways. Of note, emerging research has linked perturbation of these microbiota-epigenetic interactions with the onset and progression of immunological disorders, metabolic syndrome features, cancer, and neurological pathologies (2). This Research Topic includes nine original articles and one review that explore the gut microbiota-epigenetics connections involved in disease physiopathology, with potential implications for novel therapeutic interventions.

To analyze global research trends regarding intestinal microbiota and epigenetics, Tian and Chen performed a comprehensive bibliometric analysis to visualize the body of knowledge and research priorities in this field. They found that gut microbiota and epigenetics are closely related to pathologies such as breast and colorectal cancer, inflammatory bowel disease, and psychiatric disorders. Additionally, they underscored the potential of diet (probiotics) and a healthy lifestyle to regulate gut microbiota and reduce the burden of these diseases. Using a related bibliometric approach, Tang et al. systematically analyzed *Cryptococcus* species and their dynamism with the host immune system. They found that current research reveals intricate interactions between

Cryptococcus pathogenesis (mechanisms and complications) and host immunity, with implications in the development of immunotherapies and visualizing critical directions in this domain. Furthermore, Shi et al. identified *DRAM1*, *PSTPIP2*, and *UPP1* as differentially expressed genes in *Staphylococcus aureus* bloodstream infection using human and mouse samples and an integrative bioinformatics analysis, highlighting their potential as diagnostic biomarkers of this infection.

Mendelian randomization is increasingly used in human biology to assess the causal effects of modifiable risk factors on health outcomes by using genetic information from the host. In this Research Topic, Zhu et al. explored the causal association between microbiota and skin appendage disorders using Mendelian randomization. They found relevant causal relationships between genetic liability in the skin and gut microbiota with skin appendage disorders. Specifically, they identified several skin bacteria (*Staphylococcus, Streptococcus, and Propionibacterium*) as being positively associated, and *Bifidobacteria and Lactobacilli* as probiotics exerting a protective effect on this disorder. Similarly, Zhang et al. identified causal relationships between gut microbiota and premature rupture of membranes using a Mendelian randomization analysis. The results revealed that class *Mollicutes*, genus *Marvinbryantia*, genus *Ruminooccaceae UCG003* and phylum *Tenericutes* were associated with a reduced risk of premature rupture of membranes, while genus *Collinsella*, genus *Intestinibacter* and genus *Turicibacter* increased the risk for this condition. In addition, Chen et al. examined the potential causal connections between gut microbiota and rheumatic valve disease using a Mendelian randomization framework. They found *Lentisphaerae*, *Alphaproteobacteria*, and *Streptococcaceae* as having significant protective effects against rheumatic valve disease, whereas *Eubacterium eligens* and *Odoribacter* were identified as potential risk factors. These findings were mediated by specific immune cell traits and biomarkers. Moreover, Zhong et al. provided genetic evidence linking gut microbiota to colorectal cancer. Specifically, *Bifidobacterium kashiwanohense, GCA-900066755* sp*900066755, Geminocystis*, and *Saccharofermentanaceae* exhibited robust causal effects for this disease, mediated by specific circulating immune cells. Furthermore, Tian et al. reported an association between multiple bacterial genera and epigenetic clocks, which was mediated by inflammatory cytokines. Their study suggests a genetic relationship between gut microbiota and aging, providing new avenues for aging-related research and the development of new treatment modalities.

The role of the intestinal microbiota in regulating the immune system has attracted attention in inflammatory diseases. In this context, Qi et al. reviewed the complex interactions between gut microbiota homeostasis and immune regulation in rheumatoid arthritis pathogenesis. They highlighted that the imbalance in the composition and function of the gut microbiota (dysbiosis) may increase gut permeability, release pro-inflammatory molecules, and impair regulatory T cell function. These disruptions collectively contribute to immune dysregulation, ultimately driving the onset and progression of rheumatoid arthritis.

The microbiota–gut–brain axis, a bidirectional connection between the gut microbiota and the brain through the relevant pathways of the gut–brain axis, may play a role in the pathogenesis of neurological disorders, including epilepsy. In this regard, You et al. investigated relationships between the gut microbiota, the hypothalamus-pituitary-adrenal axis hormones, and the inflammatory cytokines in children with infantile spasms. The authors found that dysbiosis may be involved in the pathogenesis of infantile spasms and is related to the response to adrenocorticotropic hormone. Specifically, *Lachnospiraceae* and *Lachnospiracea_incertae_sedis* appear to be involved in disease onset, while *Sutterellaceae* may play a role in children's improved health.

Overall, the articles published on this Research Topic provide valuable insights into the relationship between microbiota and epigenetics in relation to host health. This knowledge enhances our understanding of inflammatory and neurological diseases and aids in identifying therapeutic targets that might be exploited through innovative intervention strategies. Future research in this field includes the analysis of different epigenetic mechanisms affected by alterations in the gut microbiota in highly prevalent metabolic diseases, such as obesity, type 2 diabetes, and fatty liver disease. Additionally, integrating other omics technologies, such as metabolomics, will strengthen the analysis of epigenetic regulation by metabolites from the gut microbiome in health and diseases. The role of environmental factors that modulate the microbiota and epigenome such as diet, exercise, sleep patterns, and emotions, should also be considered in this holistic vision.

Author contributions

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Global trends in *Cryptococcus* and its interactions with the host immune system: a bibliometric analysis

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Objectives: This manuscript undertakes a systematic examination of the research landscape concerning global *Cryptococcus* species and their dynamism with the host immune system spanning the past decade. It furnishes a detailed survey of leading knowledge institutions and critical focal points in this area, utilizing bibliometric analysis.

Methods: VOSviewer and CiteSpace software platforms were employed to systematically analyze and graphically depict the relevant literature indexed in the WoSCC database over the preceding ten years.

Results: In the interval between October 1, 2013, and October 1, 2023, a corpus of 795 publications was amassed. The primary research institutions involved in this study include Duke University, the University of Minnesota, and the University of Sydney. The leading trio of nations, in terms of publication volume, comprises the United States, China, and Brazil. Among the most prolific authors are Casadevall, Arturo; Wormley, Floyd L., Jr.; and Olszewski, Michal A., with the most highly cited author being Perfect, Jr. The most esteemed journal is Mbio, while Infection and Immunity commands the highest citation frequency, and the Journal of Clinical Microbiology boasts the most significant impact factor. Present research foci encompass the intricate interactions between *Cryptococcus* pathogenesis and host immunity, alongside immune mechanisms, complications, and immunotherapies.

Conclusion: This represents the first exhaustive scholarly review and bibliometric scrutiny of the evolving landscapes in *Cryptococcus* research and its interactions with the host immune system. The analyses delineated herein provide insights into prevailing research foci and trajectories, thus furnishing critical directions for subsequent inquiries in this domain.

KEYWORDS

***Cryptococcus* spp., cryptococcosis, host immune responses, bibliometric analysis, visualization techniques**

1 Introduction

Cryptococcus is an opportunistic pathogen responsible for deep-seated fungal infections with potential fatal outcomes. It utilizes a sophisticated immune evasion strategy that frequently compromises the host organism's immune system functionality. This yeast organism manifests in either spherical or elliptical morphologies, encased within a polysaccharide capsule. This yeast organism manifests in either spherical or elliptical morphologies, encased within a polysaccharide capsule. The capsule's principal components consist of glucuronic acid mannose polysaccharide (GXM) and galactoxyl mannose polysaccharide (GalXM), the latter also known as glucuronic acid galactoxyl mannose polysaccharide (GXMGal) (1–3). Notably, *C.neoformans* stands as the preeminent fungal pathogen endowed with a virulent capsule, regarded as its primary virulence determinant, not exclusive to fungi but also pervasive in bacteria. Studies have demonstrated that GXM and GalXM exhibit immunomodulatory properties, thereby bolstering fungal survival through facilitation of immune evasion from the host (4). Across various biological systems, GXM and GalXM have proven efficacious in inducing cellular apoptosis. For example, investigative findings suggest that GXM within the *Cryptococcus* capsule can prompt macrophage apoptosis via mechanisms entailing the Fas and Fas-L pathways (5, 6). Moreover, Pericolini et al. reported that GalXM is capable of precipitating apoptosis in human T cells through caspase-8 activation, thereby impeding the maturation of distinct T cell responses, with the resultant adverse effect being 50-fold more potent than the suppressive action of GXM (7). Additionally, by precipitating immune dysregulation, GalXM can also promote the depletion of particular B cell populations (8). Consequently, although GXM assumes a dominant role within the polysaccharide capsule, GalXM seems to exert a more substantial regulatory influence on the host cell immune response. Such discoveries considerably enhance our comprehension of the virulence factors utilized by *Cryptococcus* in its hostile invasion of the host. Typically, this yeast is primarily transmitted via the respiratory tract, with individuals frequently becoming infected through the inhalation of airborne propagules (9).The two preeminent species, *Cryptococcus neoformans (C. neoformans)* and *Cryptococcus gattii (C. gattii)*, are renowned for their proclivity to invade human hosts. *C. neoformans* exhibits a ubiquitous distribution across the globe, whereas *C. gattii* manifests a predilection for temperate, subtropical, and tropical climes (10–12). The incursion of *Cryptococcus* represents a significant health hazard, not solely to the immunocompromised—such as individuals undergoing corticosteroid therapy, those living with HIV, and patients presenting with antifungal drug resistance—but equally to immunocompetent hosts, who might unwittingly shelter latent infections. Typically, *C. neoformans* harbors an augmented affinity for assailing immunocompromised patients, while *C. gattii* is characterized by a comparatively subdued prevalence of infection, yet retains the capacity to afflict those with intact immune defenses (13, 14). At present, the therapeutic approach to cryptococcosis is predominantly centered around staged combination pharmacotherapy, with prevailing inclinations favoring the deployment of amphotericin B concomitant with flucytosine as the principal antifungal course of action (15). However, notwithstanding the administration of these mycotic therapeutics, patient mortality rates persistently reside above the 20% threshold. In addition to the hepatorenal toxicity linked with these pharmaceuticals, there is likewise an alarming escalation in antifungal drug resistance. Consequently, the therapeutic intervention and governance of cryptococcosis continue to pose a fundamentally arduous challenge, especially in the context of immunocompromised individuals (16). Immunosuppression constitutes a pivotal element in the etiology of cryptococcosis, with immune effector cells forming the cornerstone of the host's defense mechanism against this affliction. For those beset by *Cryptococcus* infection, it is imperative to not merely confront the malady itself, but also to fortify the host's immune constitution.

A survey of the extant literature reveals that the intricate symbiosis between fungal entities and the host immune system has captivated significant scholarly interest. Yet, heretofore, the scholarly community has not embarked on a bibliometric scrutiny of the corpus of research concerning *Cryptococcus* and its dynamic engagement with host immune mechanisms. Consequently, this investigation harnessed the Web of Science Core Collection (WoSCC) to amass an anthology of literature concerning *Cryptococcus* and its reciprocal engagement with host immunity spanning the preceding decade. Leveraging two preeminent visualization instruments, CiteSpace and VOSviewer, the study executed both quantitative and visual scrutinies. To decipher the contemporary landscape and progressive contours of international research on the dynamic interplay between *Cryptococcus* and host immunity, this inquiry endeavors to furnish an exhaustive exegesis of the field's evolutionary course. This encompasses pinpointing pivotal figures and ascertaining the prevailing state of research, in addition to forecasting imminent research trajectories and potentialities within this sphere.

2 Research methods and data sources

2.1 Research methods

Bibliometrics, a discipline markedly divergent from traditional narrative reviews, epitomizes a quantitative research paradigm underpinned by publication metrics, citation analyses, and textual data examination. It endeavors to delineate and elucidate the intricacies and progressive developments inherent to a scholarly discipline or research domain (17, 18). The yield of bibliometric inquiries extends beyond mere descriptive statistics to embrace the rigorous exploration of keywords, textual content, citation patterns, authorial contributions, institutional affiliations, and bibliographical references. Such explorations meticulously examine the frequency, interconnections, centrality, and clustering phenomena among authors and textual assemblages. Consequently, investigators habitually harness bibliometric methodologies to probe the evolutionary trajectories of a subject matter, discern publication inclinations, map authorial citation nexuses, and other constitutive elements (19).

VOSviewer accords primacy to the fabrication of visual representations, harnessing the potential of keywords, co-authorship dynamics, institutional collaborations, geopolitical distributions, and scholastic entities. It proffers an eclectic array of visual perspectives, encompassing Label Visualization, Density Visualization, Cluster Density Visualization, and Scatter Visualization. Within each graphical rendition, the magnitude of labels and circles serves as a visual corollary for their prominence within the designated field. The genesis of these visual depictions obviates the need for auxiliary computational tools, thereby underscoring the simplicity embodied in the mapping process and the aesthetic allure it provides (20).

CiteSpace leverages a synthesized compendium to dissect discrete modules, employing similitude algorithms to manifest graphical representations spanning a multitude of temporal dimensions. This facilitates the visualization of evolutionary trajectories and pivotal shifts within the scholarly domain (21, 22).

Although visualization tools may adeptly delineate evolutionary patterns within a scholarly arena, they might falter when it comes to apprehending the quintessential substance embedded in the literary corpus. Consequently, it is paramount to assimilate the foundational essence via conventional literary perusal techniques and to elucidate the overarching schema, progressive contour, and avant-garde vectors of the field through the aid of visualization tools. Such a methodology is indispensable for scrutinizing the evolutionary dynamics of *Cryptococcus* within the host immune system and for curating the most germane scholarly works for examination and distillation.

2.2 Data sources

The systematic retrieval methodology employed within the WoSCC encompassed the following stratagem: 1. The topical search was crafted utilizing the following algorithm: TS = [("*Cryptococcus*" OR "Cryptococcosis" OR "*Cryptococcus neoformans*") AND ("Host" OR "Immune")]. 2. The scholarly outputs were refined to encompass "Article" and "Review Article", with the stipulation of English as the language of publication. 3. The temporal parameters were demarcated from October 1, 2013, through to October 1, 2023. 4. The collation of data for this inquiry was executed on October 20, 2023, a measure instituted to obviate the inclination of bias that might emanate from the database's continual daily refreshes. Extraneous papers that did not align with the investigative theme of this study were systematically excised. The preliminary probe yielded 1589 manuscripts. Nevertheless, recognizing the propensity for redundancies and tangential materials to surface via the direct application of the search algorithm, a meticulous preprocessing was undertaken grounded on the aforementioned exclusionary criteria before any analytical endeavor. Upon rigorous scrutiny, a total of 795 valid scholarly references were distilled. The harvested bibliographic data were meticulously preserved as "full text records and references" in TXT format. The extracted corpus of literature included such elements as titles, names of authors, affiliations (inclusive of research establishments, academic institutions, hospitals), abstracts, periodical titles, dates of dissemination, and bibliographic references. Thereafter, the curated corpus of literature was meticulously transferred into a Microsoft Excel spreadsheet to facilitate subsequent analytical processes, as depicted in Figure 1.

3 Bibliometric analysis of the papers

3.1 Number of papers published

Fluctuations in the annual number of publications may signify shifts in research paradigms, intensity of scholarly inquiry, and evolving trends within the discipline. These 795 manuscripts originated from 3,495 authors across 63 nations and 956 institutions, featuring in 247 distinct journals and referencing 24,210 articles from 3,360 publications. Figure 2 illustrates the chronological dissemination of scholarly works related to *Cryptococcus* and host immune interactions. Collectively, the domain experienced a notable diminution in publication frequency in 2013, accompanied by marginally lower outputs in 2014 and 2016. Nonetheless, the volume of publications consistently surpassed 70 annually throughout the period extending from 2013 to 2023, achieving a zenith in 2020 and largely preserving equilibrium subsequently.

3.2 Institutions and countries

Table 1 delineates the premier 15 institutions, with Duke University at the vanguard of the global echelon through its contribution of 48 papers, succeeded by the University of Minnesota with a corpus of 33 papers, and the University of Sydney with 27 scholarly articles. It is salient to acknowledge that within the cadre of these preeminent 15 institutions, the preponderance originates from the United States (N=7), with Brazil (N=3), the United Kingdom (N=2), Australia (N=1), China (N=1), and Uganda (N=1) trailing. Following this, the formulation of a visual network illustration of institutional synergies (Figure 3) elucidated that Duke University sustains intimate collaborations with the University of Minnesota, Johns Hopkins University, Albert Einstein College of Medicine, University of Sydney, and Northeastern University. Furthermore, the Second Military Medical University engages in dynamic collaboration with Rutgers State University, NIAID, University of Sydney, and the Federal University of Rio de Janeiro.

To delineate the nations that have made the most substantial contributions to *Cryptococcus* and host immunity research over the decade spanning 2013–2023, this investigation analyzed the scholarly output of 63 countries. As demonstrated in Table 1, the United States stands at the forefront with a total of 391 scholarly works, amassing 9,867 citations with an average citation rate of 25.24, markedly outstripping China, which has produced 139 scholarly works yielding 1,695 citations at an average citation rate of 18.80, and Brazil, with 101 scholarly works accompanied by 1,894 citations at an average citation rate of 12.20. Subsequently, a graphical representation was created for countries with a minimum of three publications (refer to Figure 4), wherein the robustness of the interconnections between nodes was delineated by

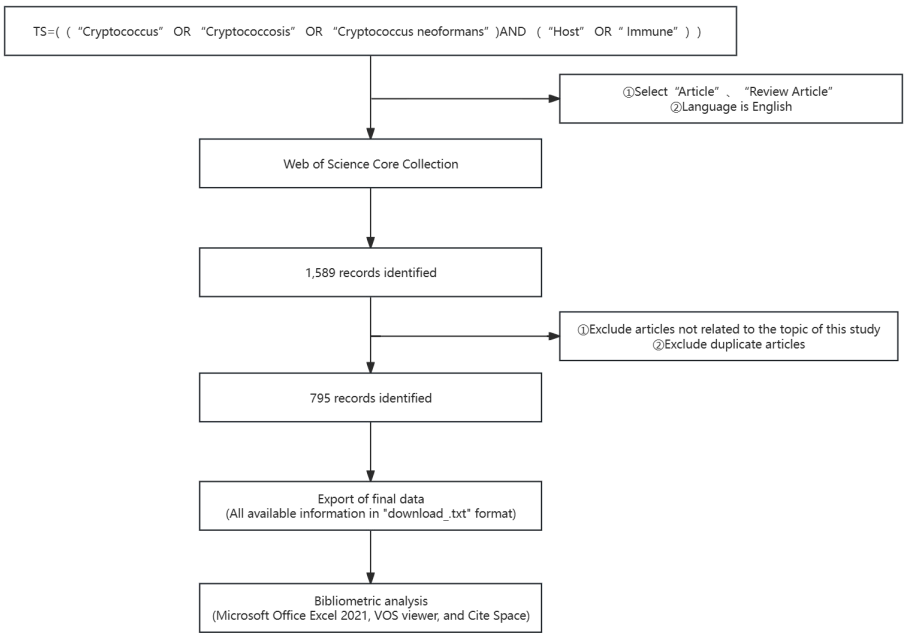
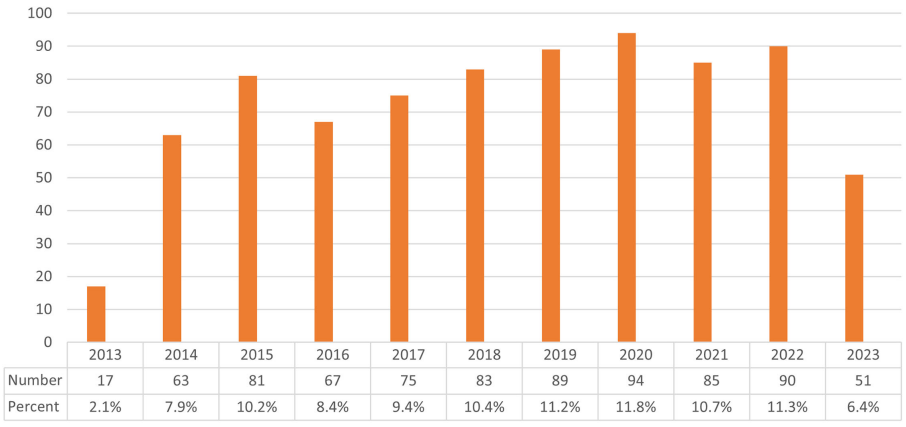


FIGURE 1
Illustrates the process of publication selection.

the links' thickness, signifying augmented collaboration in scholarly works among the respective nations. The chromatic differentiation of the nodes denoted distinct clusters. It is manifest that the distribution of nations contributing to this field's literature is highly disparate, with a pronounced 'top effect' wherein the majority of scholarly works are penned by academics from a select consortium of nations.

3.3 Author analysis

The frequency of citations garnered by academic papers stands as a quintessential barometer of their scholarly impact. Among the globally prolific scholars, a cadre of 3,495 researchers has rendered contributions to the corpus of knowledge concerning *Cryptococcus* and host immunity. Notably, each member of the distinguished coterie of the top 10 authors has disseminated in excess of 10 scholarly papers within this domain. Amongst these authors, a quartet has each amassed in excess of 300 citations. Most noteworthy are Perfect Jr. (N=347), Zaragoza, O. (N=336), Casadevall, A. (N=330), and Jarvis, J. N. (N=317), who spearhead the citation tally (refer to Table 2). Predicated on these data, we devised an intricate network graph of collaborations (see Figure 5A) for those authors who have disseminated a minimum of five scholarly works. A complement of 141 authors satisfied this criterion. Authors were segregated into distinct consortiums, with temporal dynamics accentuated via a chromatic coding schema. Each vertex within the graphical representation signifies an individual scholar, with the



	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023
Number	17	63	81	67	75	83	89	94	85	90	51
Percent	2.1%	7.9%	10.2%	8.4%	9.4%	10.4%	11.2%	11.8%	10.7%	11.3%	6.4%

FIGURE 2
Depicts the annual publication output related to *Cryptococcus* and host immunity research from 2013 to 2023.

TABLE 1 Top 15 countries and institutions in the field of *Cryptococcus* and host immunity research.

Rank	Country	Counts	Percent	Institution	Counts	Percent
1	USA	391	39.4%	Duke University (USA)	48	14.6%
2	China	139	14.0%	University of Minnesota (USA)	33	10.0%
3	Brazil	101	10.2%	University of Sydney (Australia)	27	8.2%
4	England	66	6.7%	Albert Einstein College of Medical (USA)	25	7.6%
5	Australia	48	4.8%	University of Texas-San Antonio (USA)	24	7.3%
6	Canada	40	4.0%	Universidade Federal do Rio de Janeiro (Brazil)	23	7.0%
7	Japan	37	3.7%	NIAID (USA)	23	7.0%
8	Germany	29	2.9%	University of Sao Paulo (Brazil)	19	5.8%
9	France	27	2.7%	University of Birmingham (UK)	19	5.8%
10	South Africa	26	2.6%	University Federal do Rio Grande do Sul (Brazil)	16	4.9%
11	Uganda	20	2.0%	Johns Hopkins Bloomberg school of public health (USA)	16	4.9%
12	India	20	2.0%	University of Washington (USA)	15	4.6%
13	South Korea	19	1.9%	Makerere University (Uganda)	15	4.6%
14	Thailand	15	1.5%	St Georges university of London (UK)	13	4.0%
15	Colombia	14	1.4%	Second Military Medical Universi (China)	13	4.0%

magnitude of the circle mirroring their scholarly output. The interlinking lines signify collaborative incidences amongst divergent authors. Casadevall, Arturo, Wormley, Floyd L. Jr., Olszewski, Michal A., Boulware, David R., and Perfect, John R. are denoted by the most prominent vertices within the graph, in recognition of their preeminent publication count in the pertinent discipline. For example, scholars such as Casadevall, Arturo, Perfect, John R., Williamson, Peter R., Liao, Wanqing, et al., exhibit a tightly interwoven collaborative matrix. Subsequent scrutiny divulged that upon imposing a minimum citation echelon of 20 for data filtration, 391 auteurs surmounted this benchmark, culminating in the genesis of a co-citation network graph (Figure 5B) predicated upon this dataset. For instance, a marked synergy is observed amongst a spectrum of co-cited luminaries, including Perfect, Jr., Rajasingham, R., Jarvis, Jn., and Park, bj.

3.4 Journal analysis

Commencing in 2014, there has been a consistent augmentation in scholarly articles addressing *Cryptococcus* and host immunity, signifying an escalating academic intrigue in this domain. This upward trajectory is projected to persist, with a sustained volume of publications anticipated through the culmination of 2023. These treatises have been disseminated across 247 disparate journals, with the foremost 15 periodicals enumerated in Table 3. The periodical

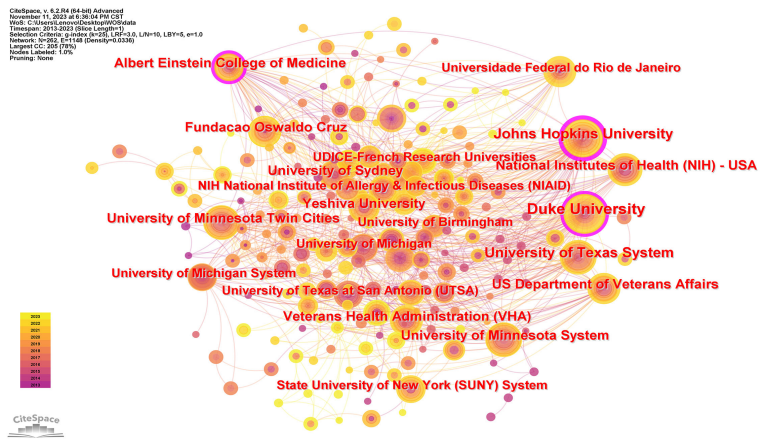


FIGURE 3
Visualization of the Institutions in the Field of *Cryptococcus* and Host Immunity Research.

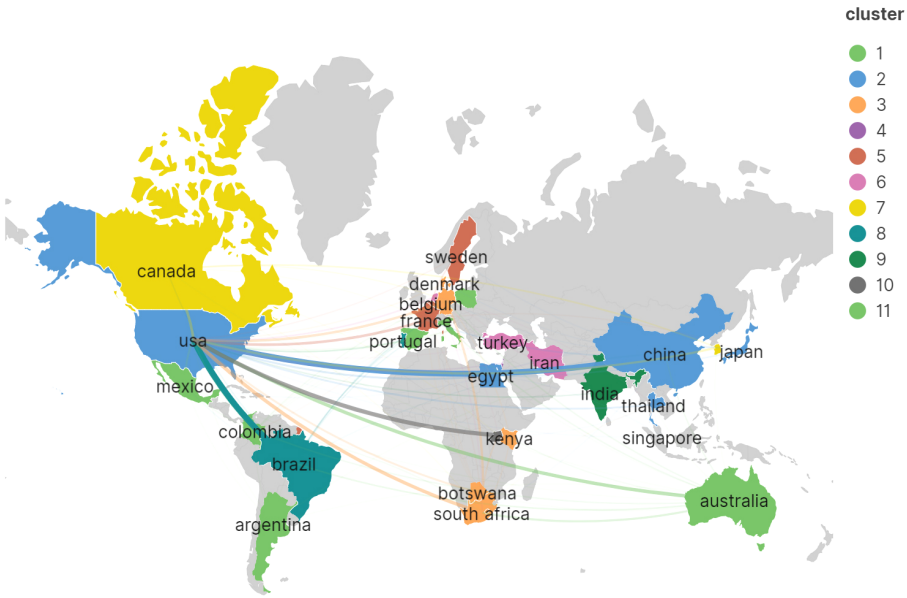


FIGURE 4
Visualization of the international network of collaboration in the research field of *Cryptococcus* and host immunity.

MBIO (N=52, 11%) has amassed a total of 1482 citations, with an average citation rate per article of 28.5, thereby securing its position at the apex. In close succession are the Journal of Fungi (N=46, 9.7%), Frontiers in Immunology (N=31, 6.6%), Infection and Immunity (N=27, 5.7%), and Frontiers in Cellular and Infection Microbiology (N=25, 5.3%). The journal with the most consequential impact factor is Nature Communications (IF=16.6).

As delineated in Table 4, it becomes manifest that within the cadre of the top 15 co-cited journals, a septenary has each amassed in excess of 1000 citations. Infection and Immunity (Co-Citations=4036) reigns supreme, succeeded by the Journal of Immunology (Co-Citations=2170), Clinical Infectious Diseases (Co-Citations=1890), Plos Pathogens (Co-Citations=1655), and Plos One (Co-Citations=1554). The periodical wielding the most distinguished impact factor is the Journal of Clinical Microbiology (IF=36.8).

TABLE 2 Top 10 authors and co-cited authors in *Cryptococcus* and host immune research from 2013 to 2023.

Rank	Authors	Count	Co-Cited Authors	Citations
1	Casadevall, arturo	29	Perfect, jr	347
2	Wormley, Floyd l.,Jr.	25	Zaragoza, o	336
3	Olszewski, michal a.	21	Casadevall, a	330
4	Boulware, david r.	18	Jarvis, jn	317
5	Perfect, john r.	17	Rajasingham, r	264
6	May, robin c.	16	Wozniak, kl	247
7	Nielsen, kirsten	16	Singh, n	201
8	Williamson, peter r.	16	Park, bj	198
9	Lin, xiaorong	15	Huffnagle, gb	181
10	Meya, david b.	15	O'meara, tr	179

An aggregate of 33 scholarly periodicals was discerned via VOSviewer, each boasting a minimum publication frequency of five scholarly articles, culminating in the construction of a journal network diagram (Figure 6A). It is noteworthy that dynamic citation interconnections emerged among such journals as Mbio, Journal of Fungi, and Frontiers in Immunology. Subsequently, a filtration criterion predicated on the minimal co-citation count (encompassing 154 journals) was instituted, resulting in the curation of 60 journals for the construction of a co-citation network diagram (Figure 6B). The bi-directional mapping overlay of periodicals elucidates the symbiotic relationships between the citing and co-citing entities, with the left delineating the citing periodicals and the right those being co-cited. As depicted in Figure 7, the orange trajectory denotes the primary citation conduit, signifying that research promulgated in the journal Molecular Biology Genetics is principally cited by treatises within the journal Molecular Biology Immunology. The verdant citation trajectory intimates that research emanating from Molecular Biology Genetics is habitually cited by the journal Medicine Medical Clinical.

3.5 Co-cited references and references bursts

In the decade spanning from October 1, 2013, to October 1, 2023, a total of 24,210 citations were interchanged pertaining to scholarly references that explore the interplay between *Cryptococcus* and host immune responses. Among the most prominent 10 co-cited works

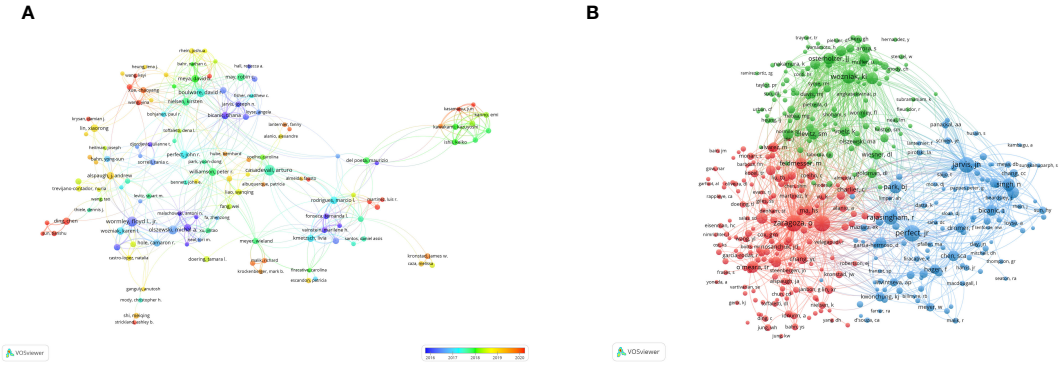


FIGURE 5
Visualization of authors in *Cryptococcus* and host immune research from 2013 to 2023 **(A)**. Visualization of co-cited authors in *Cryptococcus* and host immune research from 2013 to 2023 **(B)**.

(Table 5), each reference received no fewer than 60 co-citations, with a triumvirate of these works being co-cited in excess of 150 instances. Thereafter, works garnering 25 or greater co-citations were meticulously selected to fabricate the co-citation network diagram (Figure 8A), wherein the magnitude of the circles is proportionate to the citation frequency, thereby reflecting the scholarly significance of the works. For example, vigorous co-citation dynamics were observed among such notable works as "Rajasingham R, 2017, Lancet Infect Dis," "Perfect JR, 2010, Clin Infect Dis," and "Maziarz EK, 2016, Infect Dis Clin N Am." Figure 8B delineates ten discrete clusters elucidated via CiteSpace, encompassing: dendritic cell dynamics, nutritional imperatives, cryptococcal meningitis, intracellular signaling cascades, *C. neoformans* investigations, delta sgl1 gene influence, fungal-host interplay, pulmonary cryptococcosis, and phenotypic plasticity.

A burst citation denotes a reference that is frequently cited by scholars in a particular field within a specific timeframe. When a set of articles is repeatedly cited, it gives rise to the formation of a conceptual cluster (23). In the present study, CiteSpace has identified 20 references exhibiting strong burst citations. As depicted in Figure 9, the references are arranged according to burst sequence by their initial publication years, with each bar representing a year.

TABLE 3 Top 15 journals in the field of research related to *Cryptococcus* and host immune interactions.

Rank	Journal	IF	Q	Publications	IF	Citations	Average Citation / Publication
1	Mbio	6.4	Q1	52	6.4	1482	28.5
2	Journal of Fungi	4.7	Q2	46	4.7	725	15.8
3	Frontiers in Immunology	7.3	Q2	31	7.3	513	16.5
4	Infection and Immunity	3.1	Q2	27	3.1	596	22.1
5	Frontiers in Cellular and Infection Microbiology	5.7	Q2	25	5.7	319	12.8
6	Journal of Immunology	4.4	Q2	22	4.4	591	26.9
7	Plos One	3.7	Q3	19	3.7	287	15.1
8	Scientific Reports	4.6	Q3	19	4.6	397	20.9
9	Medical Mycology	2.9	Q3	18	2.9	223	12.4
10	Plos Pathogens	6.7	Q1	15	6.7	424	28.3
11	Frontiers in Microbiology	5.2	Q2	14	5.2	573	40.9
12	Fungal Genetics and Biology	3	Q3	13	3	440	33.8
13	Nature Communications	16.6	Q1	13	16.6	581	44.7
14	Mycoses	4.9	Q2	13	4.9	129	9.9
15	Open Forum Infectious Diseases	4.2	Q3	13	4.2	151	11.6

TABLE 4 Top 15 journals cited in the field of research related to *Cryptococcus* and host immune interactions.

Rank	Co-cited Journal	IF	Q	Co-Citations
1	Infection and Immunity	3.1	Q2	4036
2	Journal of Immunology	4.4	Q2	2170
3	Clinical Infectious Diseases	11.8	Q1	1890
4	Plos Pathog	6.7	Q1	1655
5	Plos One	3.7	Q3	1554
6	MBIO	6.4	Q1	1483
7	The Journal of Infectious Diseases	6.4	Q2	1009
8	Medical Mycology	2.9	Q3	899
9	Proceedings of the National Academy of Sciences of the United States of America	11.1	Q1	757
10	Journal of Biological Chemistry	4.8	Q2	684
11	Molecular Microbiology	3.6	Q2	651
12	Antimicrobial Agents and Chemotherapy	4.9	Q2	607
13	AIDS	3.8	Q2	602
14	Journal of Clinical Microbiology	36.8	Q1	560
15	Cellular Microbiology	3.4	Q2	535

The red lines signify a sudden burst of high-citation references in a particular year. The work entitled "Global burden of disease of HIV-associated cryptococcal meningitis: an updated analysis", penned by Radha Rajasingham et al (24),exhibits the most pronounced burst citation (intensity=30.14) and was sustained from 2019 to 2023. The study with the second-highest burst citation rate (intensity=19.9), authored by Benjamin J. Park et al. and appearing in AIDS (25), spanned from 2013 to 2014. Notable, as depicted in Table 6, the predominantly co-cited references both address the global burden of cryptococcal meningitis attributable to host immune deficiency-related diseases. The guideline ranking third in burst rate (strength=16.58), titled "Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the infectious diseases society of America," crafted by John R. Perfect et al. and featured in "Clinical Infectious Diseases," underwent a burst from 2013 to 2015 (26).This reference focuses primarily on the enhancement of treatment strategies for cryptococcosis, addressing factors such as host immunity, site of infection, antifungal drug toxicity, and underlying conditions, with the objective of updating efficacious management guidelines and amplifying patient diagnosis and treatment outcomes. Drawing upon these findings, one may deduce that the burst strength of these 20 references spans from 6.83 to 30.14, with durations extending from 2 to 6 years.

3.6 Hotspots and frontiers

Keywords distill the quintessence of scholarly works, providing a portal through which the scholarly corpus may be navigated. A critical analysis of keywords within a specific discipline can reveal the

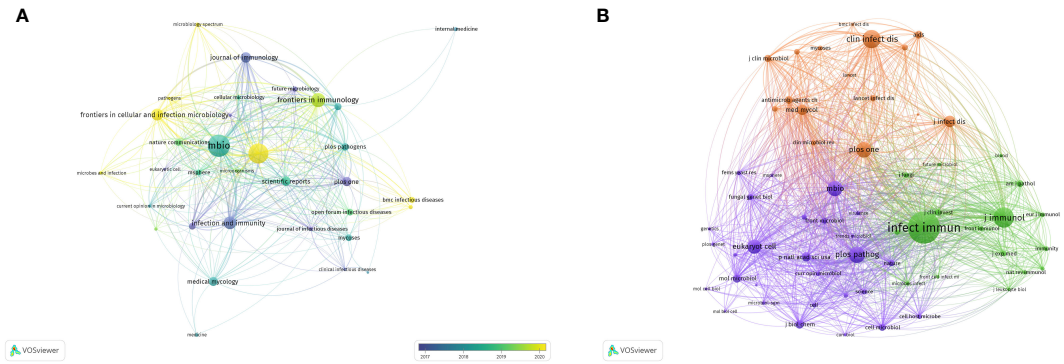


FIGURE 6
Visualization of Journal Publications in the Field of Research Related to *Cryptococcus* and Host Immune Interactions **(A)** and Visualization of Co-cited Journals **(B)**.

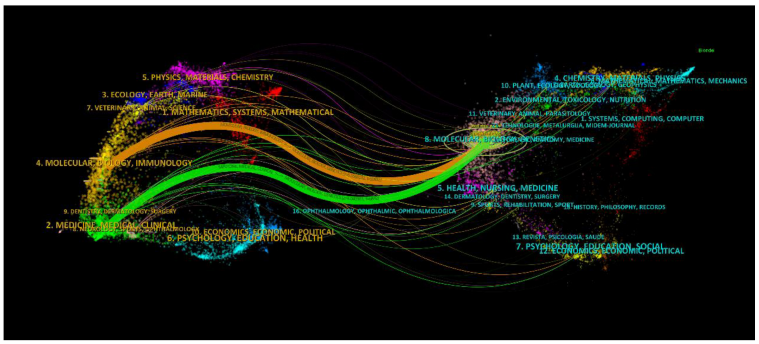


FIGURE 7
The superimposition of two graphs representing journals related to research on *Cryptococcus* and host immune interactions.

salient themes and prospective trajectories of interest within that domain. Of the 3,083 keywords identified, 98 exceed the delineated threshold upon employing VOSviewer with a stipulated minimum keyword occurrence of 15. The aggregation and computation of aggregate connectivity strength for these 98 keywords culminate in the graphical representation of keyword clusters (Figure 10A) and a corresponding density visualization (Figure 10B). Informed by the graphical depiction of keyword networks, three pronounced clusters have materialized, each symbolizing a distinct vector of inquiry: namely, the azure cluster (delving into the host immune defense mechanisms post *Cryptococcus* infection), the crimson cluster (probing into the pathogenesis, virulence, and immune evasion strategies of *Cryptococcus* infection), and the verdant cluster (examining the immunotherapeutic interventions and recuperation from *Cryptococcus* infection). This synthesis of findings is succinctly encapsulated in Table 7. The magnitude of the circles within the graph mirrors the prominence of the keywords, with more substantial circles denoting augmented significance, whilst the intricacy of the linkages between nodes signifies the prevalence of keyword co-occurrences. Through the scrutiny of the timeline graph, one may perceive the fluid progression of research focal points as denoted by the keywords spanning 2013 to 2023. This chronological dissection elucidates the ascent and wane of seminal keywords, mirroring the oscillations of scholarly pursuits within the discipline. Keywords of a homogenous cluster are arrayed along a horizontal trajectory, sequenced in temporal succession from left to right, signifying the continuum from historical to contemporary. The proliferation of keywords within a collective underscores the developmental magnitude and import of the cluster's contribution to scholarly advancements in the domain. Employing CiteSpace for keyword scrutiny, we devised a timeline graph to visually articulate the metamorphosis of research epicenters concerning the interplay between *Cryptococcus* and host immunity over the decade. The appraisal of the clustering delineation was executed via Modularity Q and Mean Silhouette metrics weighted for significance. The Silhouette coefficient, designated as S, functions as a metric for gauging homogeneity within a given cluster. An elevated S value is indicative of enhanced congruity amongst the cluster modules. A Q value in excess of 0.3 intimates a notable delineation of structure, whereas an S value surpassing 0.5 denotes a cogent clustering (27). In consonance with these thresholds, the keyword clustering module in this exposition exhibits a Q value of 0.3337 (>0.3) and an S value of 0.6644 (>0.5), thereby revealing a coherent clustering with a definitive architecture. Figure 11 exhibits a sextet of clusters, to wit: immune reconstitution inflammatory syndrome, virulence, dendritic cells, host defense, innate immunity, and *C.gattii*. Each cluster embodies a discrete consortium, designated as #0, #1, et cetera, with the more voluminous clusters subsuming an augmented congregation of members (28).

TABLE 5 The top 10 co-cited references on *Cryptococcus* and immunology research.

Rank	Co-cited reference	Citations
1	rajasingham r, 2017, lancet infect dis, v17, p873	217
2	park bj, 2009, aids, v23, p525	197
3	perfect jr, 2010, clin infect dis, v50, p291	167
4	charlier c, 2009, infect immun, v77, p120	85
5	zaragoza o, 2009, adv appl microbiol, v68, p133	77
6	o'meara tr, 2012, clin microbiol rev, v25, p387	74
7	eldmesser m, 2000, infect immun, v68, p4225	74
8	alvarez m, 2006, curr biol, v16, p2161	70
9	kwon-chung kj, 2014, csh perspect med, v4,p123	68
10	zaragoza o, 2010, plos pathog, v6,p232	67

4 Discussion

4.1 General information

The corpus of literature was harvested from the Web of Science, with subsequent visual dissection of the publications undertaken via CiteSpace and VOSviewer. Ensuingly, the scholarly findings incorporated an examination of publication trajectories, authorial contributions, institutional affiliations, terminological foci,

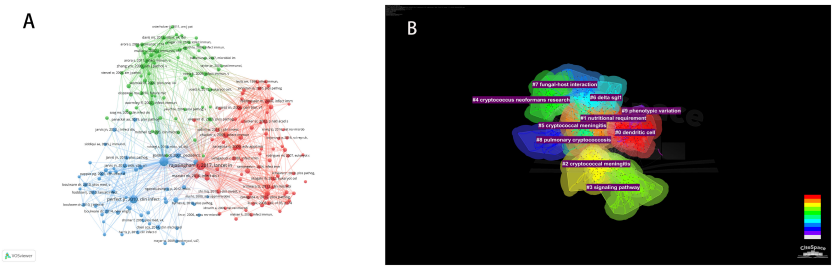


FIGURE 8
Visualization **(A)** of co-cited references on *Cryptococcus* and immunology research. Cluster network diagram **(B)** of co-cited references.

geographic provenance, periodical distributions, and bibliographic interconnections. In 2013, the domain of inquiry was in its embryonic phase. Yet, in the period extending from 2014 to 2022, there ensued a marked augmentation in scholarly outputs, with the annual production uniformly surpassing 60 treatises. The zenith of publication frequency was attained in 2020, and it is forecasted that the volume of treatises will persist in its ascension in the years succeeding 2023.

In the landscape of contemporary scientific inquiry, the host immune response has surfaced as a pivotal element in the study of cryptococcal pathogenesis. A synthesis of data from a multitude of nations and academic bodies delineates the United States, China, and Brazil as the principal nations propelling investigation in this sphere, nurturing intimate collaborative networks. Collectively, inter-nation collaboration manifests robustly across the preponderance of countries, conversely, a select assemblage finds itself within embryonic stages of scholarly pursuit, exhibiting a tempered zeal for joint scholarly ventures. A modicum of cooperation harbors the potential to propel the maturation of this investigative field and surmount scholarly impediments with heightened efficacy. Duke University (USA, N=48) distinguishes itself as the preeminent institution in publication frequency, indicative of its seminal and significant engagement within this realm of research. The hierarchical registry of periodicals manifests that MBIO (N=52), Journal of Fungi (N=46), and Frontiers in Immunology (N=31) stand as paragons of publication output in this academic field, with Infection and Immunity (Co-Citations=4,036) holding the distinction of being the most assiduously cited journal within the collective citation nexus. Additionally, the Journal of Clinical Microbiology lays claim to the most eminent impact factor, standing at 36.8.

Arturo Casadevall, of the Johns Hopkins Bloomberg School of Public Health in the United States, has ascended as the most distinguished author within this research territory (N=29). In collaboration with Floyd L. Wormley Jr., their scholarly pursuits encompass the intricacies of host immune defense mechanisms amidst cryptococcal infection, the nuances of host-pathogen interplay with *Cryptococcus*, the nature of inflammatory responses, the complexity of virulence determinants, and the pursuit of anti-cryptococcal therapeutic strategies. Their research endeavors penetrate the elucidation of underlying mechanisms at molecular, cellular, tissue, and organ levels of damage, thereby augmenting the comprehensive grasp of cryptococcal pathogenesis

Top 20 References with the Strongest Citation Bursts

References	Year	Strength	Begin	End	2013 - 2023
Park BJ, 2009, AIDS, V23, P525, DOI 10.1097/QAD.0b013e328322ffac, DOI	2009	19.9	2013	2014	
Perfect JR, 2010, CLIN INFECT DIS, V50, P291, DOI 10.1086/649858, DOI	2010	16.58	2013	2015	
Wozniak KL, 2011, PLOS ONE, V6, P0, DOI 10.1371/journal.pone.0017204, DOI	2011	8.46	2013	2015	
Kronstad JW, 2011, NAT REV MICROBIOL, V9, P193, DOI 10.1038/nrmicro2522, DOI	2011	7.22	2013	2016	
Jarvis JN, 2012, AIDS, V26, P1105, DOI 10.1097/QAD.0b013e3283536a93, DOI	2012	8.68	2014	2017	
Arora S, 2011, INFECT IMMUN, V79, P1915, DOI 10.1128/IAI.01270-10, DOI	2011	7.34	2014	2016	
Hardison SE, 2010, AM J PATHOL, V176, P774, DOI 10.2353/ajpath.2010.090634, DOI	2010	7.06	2014	2015	
Hardison SE, 2012, J IMMUNOL, V189, P4060, DOI 10.4049/jimmunol.1103455, DOI	2012	6.95	2014	2016	
Day JN, 2013, NEW ENGL J MED, V368, P1291	2013	7.19	2015	2017	
Jarvis JN, 2015, PLOS PATHOG, V11, P0, DOI 10.1371/journal.ppat.1004754, DOI	2015	7.01	2016	2019	
Santiago-Tirado FH, 2017, MBIO, V8, P0, DOI 10.1128/mBio.02183-16, DOI	2017	7.55	2018	2023	
Rajasingham R, 2017, LANCET INFECT DIS, V17, P873	2017	30.14	2019	2023	
Dambuza IM, 2018, PLOS PATHOG, V14, P0, DOI 10.1371/journal.ppat.1006978, DOI	2018	9.51	2019	2023	
Hommel B, 2018, PLOS PATHOG, V14, P0, DOI 10.1371/journal.ppat.1006982, DOI	2018	8.96	2019	2023	
Trevijano-Contador N, 2018, PLOS PATHOG, V14, P0, DOI 10.1371/journal.ppat.1007007, DOI	2018	8.06	2019	2023	
Kwon-Chung KJ, 2017, MSPHERE, V2, P0, DOI 10.1128/mSphere.00357-16, DOI	2017	7.76	2019	2023	
Williamson PR, 2017, NAT REV NEUROL, V13, P13, DOI 10.1038/nrneurol.2016.167, DOI	2017	7.17	2019	2023	
Maziarz EK, 2016, INFECT DIS CLIN N AM, V30, P179, DOI 10.1016/j.idc.2015.10.006, DOI	2016	8.72	2020	2021	
Zaragoza O, 2019, VIRULENCE, V10, P490, DOI 10.1080/21505594.2019.1614383, DOI	2019	8.55	2020	2023	
Casadevall A, 2019, VIRULENCE, V10, P822, DOI 10.1080/21505594.2018.1431087, DOI	2019	6.83	2020	2023	

FIGURE 9
The top 20 most frequently cited references.

TABLE 6 The primary research themes of the 15 cited references.

Rank	Strength	Title	Journal	Author	Year
1	19.9	Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS.	AIDS	Benjamin J Park	2009
2	16.58	Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the infectious diseases society of america.	Clin Infect Dis	John R Perfect	2010
3	8.46	Role of IL-17A on resolution of pulmonary C. neoformans infection.	PLoS One	Karen L Wozniak	2011
4	7.22	Expanding fungal pathogenesis: Cryptococcus breaks out of the opportunistic box.	Nat Rew Microbiol	James W Kronstad	2011
5	8.68	Adjunctive interferon-γ immunotherapy for the treatment of HIV-associated cryptococcal meningitis: a randomized controlled trial.	AIDS	Joseph N Jarvis	2012
6	7.34	Effect of cytokine interplay on macrophage polarization during chronic pulmonary infection with Cryptococcus neoformans.	Infect Immun	Shikha Arora	2011
7	7.19	Combination antifungal therapy for cryptococcal meningitis.	N Engl J Med	Jeremy N Day	2013
8	7.55	Trojan Horse Transit Contributes to Blood-Brain Barrier Crossing of a Eukaryotic Pathogen.	mBio	Felipe H Santiago-Tirado	2017
9	30.14	Global burden of disease of HIV-associated cryptococcal meningitis: an updated analysis.	Lancet Infect Dis	Radha Rajasingham	2017
10	9.51	The Cryptococcus neoformans Titan cell is an inducible and regulated morphotype underlying pathogenesis.	PLoS Pathog	Lvy M Dambuzza	2018
11	8.96	Titan cells formation in Cryptococcus neoformans is finely tuned by environmental conditions and modulated by positive and negative genetic regulators.	PLoS Pathog	Benjamin Hommel	2018
12	8.06	Cryptococcus neoformans can form titan-like cells in vitro in response to multiple signals.	PLoS Pathog	Nuria Trevijano-Contador	2018
13	7.76	The Case for Adopting the "Species Complex" Nomenclature for the Etiologic Agents of Cryptococcosis.	mSphere	Kyung J Kwon-Chung	2017
14	8.72	Cryptococcosis.	Infect Dis Clin North Am	Eileen K Maziarz	2016
15	8.55	Basic principles of the virulence of Cryptococcus.	Virulence	Oscar Zaragoza	2019

(29, 30). They hypothesize that in the incipient phase of cryptococcal infection, host-activated macrophages excrete extracellular vesicles (EVs) that act as pivotal 'priming' signals, prompting the polarization of naïve macrophages toward a pro-inflammatory phenotype and potentiating macrophage microbicidal prowess. Exploiting this salient macrophage characteristic harbors potential for the genesis of innovative immunotherapeutic modalities (31, 32). The preponderance of the top 10 references concentrates on facets of host immunity, inflammatory processes, virulence determinants, and therapeutic interventions. This focal point arguably emanates from the predilection of cryptococcal infection toward individuals with compromised immune systems, with its insidious infiltration of the blood-brain barrier culminating in cryptococcal meningitis. Furthermore, this mycotic adversary not only exhibits adaptability to the host milieu but also masterminds immune subversion,

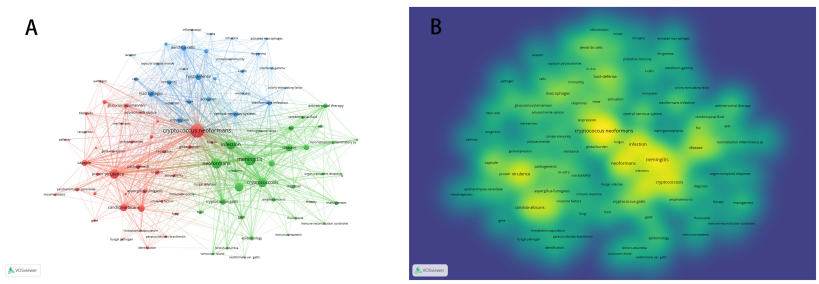


FIGURE 10
Illustrates the visualization of keyword clustering **(A)** and keyword density **(B)** in the context of research on *Cryptococcus* and host immunity.

TABLE 7 Illustrates the three major clusters of keywords to *Cryptococcus* and host immune responses.

Cluster	Color	Key words
1		Activated macrophages、activation、alveolar macrophages、capsular polysaccharide、cells、central-nervous-system、colony-stimulating factor、dendritic cells、expression、host-defense、IFN-gamma、immune-response、immunity、in-vivo、inflammation、innate、interferon-gamma、macrophages、mice、monocytes、neoformans infection、protective immunity、Pulmonary infection、receptor、responses、t-cells、TNF-α
2		aspergillus-fumigatus、candida-albicans、capsule、cryptococcus neoformans、cryptococcus-neoformans、extracellular vesicles、fungal infection、fungal pathogen、fungal pathogenesis、fungi、fungus、gene、gene-expression、global burden、glucuronoxylomannan、histoplasma-capsulatum、identification、immune response、in-vitro、innate immunity、mechanisms、melanin、morphogenesis、paracoccidioides-brasiliensis、pathogen、pathogenesis、pathway、phagocytosis、polysaccharide 、polysaccharide capsule、protein、recognition、resistance、saccharomyces-cerevisiae、susceptibility、titan cells、virulence、virulence factors、yeast
3		Aids、amphotericin-b、antiretroviral therapy、british-columbia、cerebrospinal-fluid、cryptococcal meningitis、cryptococcosis、cryptococcus、cryptococcus gattii、diagnosis、disease、epidemiology、fluconazole、gattii、hiv、host、immune reconstitution inflammatory syndrome、immune reconstitution syndrome、immunocompetent、infection、infections、lateral flow assay、management、meningitis、meningoencephalitis、neoformans、neoformans var. gattii、organ transplant recipients、pulmonary cryptococcosis、reconstitution inflammatory syndrome、therapy、vancouver-island

persistently engineering virulence factors to besiege the host (33–35).Hence, it remains of utmost significance to refine the modulation of the host's immune defenses as a strategy for the immunotherapy of those at heightened vulnerability.

4.2 Hotspots and frontiers

Examination of high-frequency keywords can illuminate the research dynamics and emerging trends in a particular field of study. Based on keyword clustering, three principal domains have been identified to ascertain the distribution and trajectory of hotspots in the research area of *Cryptococcus* and host immunity.

4.2.1 The pathogenesis of *Cryptococcus* infection

The scarlet module delineates the pathogenesis of *Cryptococcus* infection. *Cryptococcus* infection constitutes a widespread, invasive fungal infection with a global distribution, whereby virulence factors are produced during the course of infection, permeating the host and ultimately wreaking havoc on the human host (15). Contemporary investigations have elucidated that common pathogenic factors of *Cryptococcus* include adaptation to the host environment, immune evasion, and virulence factors (36). It is broadly recognized that the virulence factors of *Cryptococcus* are crucial in pathogenesis, including polysaccharide capsule, melanin, cell wall integrity, and temperature-dependent variations within the host. Nonetheless, in subsequent years, novel virulence factors have captured the attention of researchers, such as the atypical titan cells, which are deemed the

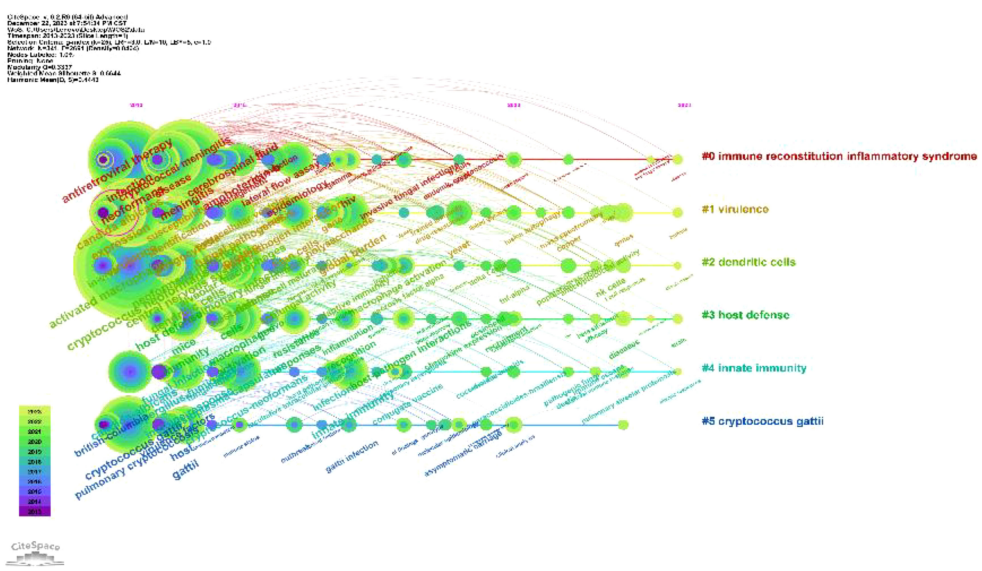


FIGURE 11
Depicts a timeline of keywords in the field of *Cryptococcus* and immune responses.

optimal cellular manifestation of *Cryptococcus*, eliciting deleterious adaptive immune responses in the host and enhancing the pathogen's survivability within the host (37–39). Similar to other fungi, *Cryptococcus* possesses the capacity to adapt and proliferate profusely under 37°C conditions, thereby precipitating the activation of pertinent signaling pathways. Strategic targeting of these virulence factors' key loci may provide an avenue for the inhibition of *Cryptococcus* in subsequent treatments.

4.2.2 The interaction between *Cryptococcus* infection and host immunity

The schematics penetrate the labyrinthine interplay between *Cryptococcus* and host immunity. Customarily, subsequent to invasion, *Cryptococcus* assumes a yeast form and enters a dormancy within the lungs of immunocompromised individuals. At the onset, the host's immune mechanisms erect a defense against these fungi; however, should their virulence exceed the host's tolerance, it can precipitate latent infection and even propagate to the central nervous system, resulting in fatal outcomes (40). Following invasion, the host's initial barricade against the pathogen *Cryptococcus* is constituted by phagocytic cells, including dendritic cells, neutrophils, and alveolar macrophages (41). *Cryptococcus* possesses the capacity to orchestrate the host's immune response to suppress inflammation, thereby circumventing phagocytic clearance and securing access to the central nervous system. Fascinatingly, research has disclosed that even subsumed by phagocytic cells, *Cryptococcus* can thrive prodigiously within these cells, and even prompt host cell lysis (42–44).Cytokines constitute diminutive molecules that facilitate interactions between various types of immune cells, frequently assuming a pivotal role in the defense against *Cryptococcus* infection. The eradication of *Cryptococcus* infection necessitates a Th1 immune response, and these protective cytokines can elicit the host Th1 immune response and amplify its efficacy. For instance, IFN-γ, TNF-α, IL-2, and IL-12 each fulfill a guardian role in forestalling *Cryptococcus* infection (45, 46). The precocious activation of TNF-α assists in the activation of dendritic cells through the classical pathway, sustaining an equilibrium of Th1/Th2 cytokines during *Cryptococcus* infection and thus diminishing host impairment (47, 48). In summation, comprehensive insight into the symbiosis between *Cryptococcus* infection and host immunity may yield advantages in circumventing immune evasion and the propagation mechanisms of *Cryptococcus*.

4.2.3 Complications of *Cryptococcus* infection

The verdant module encapsulates the intricate morbidities associated with *Cryptococcus* infection. *Cryptococcus* infection primarily presents within the pulmonary system and may thereafter diffuse to the central nervous system, with the gravest consequence entailing the onset of cryptococcal meningitis. It represents the most common etiology of adult meningitis and a significant cause of mortality, particularly among individuals living with HIV/AIDS. The quantitative depletion and functional impairment of CD4+ T lymphocytes in the context of HIV infection predispose individuals to severe immunosuppression, rendering them incapable of effectively clearing *Cryptococcus* infection. This underscores the pivotal role of T cells in orchestrating host-mediated immune responses. The effective management of this disease has become a focal point of interest. Antiretroviral therapy (ART) has emerged as a potent strategy for restoring cellular immunity in individuals afflicted with HIV/AIDS. Following the administration of ART, although there is a rapid restoration of cellular immunity in individuals with HIV/AIDS-associated *Cryptococcus* infection, there is a propensity for the emergence of immune reconstitution inflammatory syndrome (IRIS). This phenomenon is characterized by a pronounced inflammatory response within the central nervous system, correlated with a heightened mortality rate among affected individuals (49, 50). Cryptococcal IRIS induces an aberrant inflammatory cascade that eventuates in host-mediated neuropathology (51). Experimental *in vivo* and *in vitro* inquiries posited that the pathogenesis of this affliction may originate from the hyperactivation of CD4+ T cells, engendering a cellular immune response that precipitates the proliferation of a myriad of inflammatory mediators within the central nervous system, including TNF-α, IFN-γ, and IL-6 (52, 53). Furthermore, the established therapeutic protocol for *Cryptococcus* infection comprises a triad of amphotericin B, flucytosine, and fluconazole. However, the pervasive incidence of complications in individuals with *Cryptococcus* infection renders the conventional treatment modalities suboptimal. Consequently, substantial scientific scrutiny has been directed towards immunotherapeutic interventions to augment the immune function of afflicted individuals, especially those deficient in CD4+ T cells. Investigations have highlighted the critical function of CD4+ T cell-mediated Th1 immune responses in forestalling cryptococcosis within animal models. For instance, the exogenous administration of cytokine IFN-γ concurrent with *Cryptococcus* therapy has demonstrated efficacy in augmenting fungal clearance from the cerebrospinal fluid (54, 55). In summation, the dynamic interaction between *Cryptococcus* and the host immune responses modulates the clinical course of *Cryptococcus* infection diseases. This necessitates an enhanced comprehension of the pathogen-host immune interface and the identification of more effective immunotherapeutic strategies to combat *Cryptococcus* infection.

4.3 Limitation

To guarantee the integrity of the bibliometric analysis, this investigation selected the WoSCC as the source for literature procurement. Nevertheless, owing to the rigorous standards and conventions prescribed by bibliometric analysis tools for statistical data, the study confined its data collection exclusively to journal articles indexed within the WoSCC. Despite endeavors to ameliorate this limitation, the study intrinsically grapples with potential omissions of articles within the database, which could engender a partial analysis of the data. Moreover, the quantitative dissection of data intrinsically incorporates subjective elements. Additionally, the temporal correlation with citation metrics suggests that newly published articles may accrue fewer citations relative to their predecessors, thereby rendering bibliometric indicators insufficient for assessing the merit of individual scholarly works. The article analysis was executed utilizing VOSviewer and CiteSpace, both prominent tools extensively applied across a multitude of

academic evaluations, thereby providing indispensable perspectives for investigators within the pertinent discipline.

5 Conclusion

In this instance, we harnessed VOSviewer and CiteSpace for the bibliometric interrogation, assessing the contributions of nations, institutions, scholars, publications, and thematic concentrations. The ascending trajectory of annual scholarly output signals the burgeoning global scholarly interest in this domain. Beyond the robust collaborative nexus between the United States, Brazil, and China, there exists potential for enhancement in the cooperative endeavors amongst other nations. This analysis elucidates that the prevalent research foci are centered predominantly around the pathogenesis of *Cryptococcus*, the host immune response, immunological mechanisms, complications, and strategies for immunotherapy. In summation, this inquiry offers an invaluable compendium for academicians engaged in this sphere. Despite the complex pathogenesis of *Cryptococcus*, it is compelling to posit that the vigorous inquiry into the interplay between *Cryptococcus* and the host immune system will persist in bestowing considerable scholarly worth and propitious applications towards the identification of therapeutic targets.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.webofscience.com/wos/woscc/summary/250318c0-253d-40f2-9755-506e08c996dc-c690f18e/relevance/1.

Ethics statement

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

Author contributions

ST: Conceptualization, Data curation, Investigation, Software, Visualization, Writing – original draft, Formal analysis, Methodology, Project administration, Writing – review & editing. RH: Data curation, Methodology, Writing – original draft. XL: Formal analysis, Writing – original draft. HH: Data curation, Writing – original draft. YT: Formal analysis, Writing – original draft. TJ: Formal analysis, Writing – original draft. ZL: Formal analysis, Writing – original draft. XJL: Funding acquisition, Resources, Supervision, Writing – review & editing. YX: Funding acquisition, Resources, 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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Global research progress of gut microbiota and epigenetics: bibliometrics and visualized analysis

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Background: Gut microbiota is an important factor affecting host health. With the further study of the mechanism of gut microbiota, significant progress has been made in the study of the link between gut microbiota and epigenetics. This study visualizes the body of knowledge and research priorities between the gut microbiota and epigenetics through bibliometrics.

Methods: Publications related to gut microbiota and epigenetics were searched in the Web of Science Core Collection (WoSCC) database. Vosviewer 1.6.17 and CiteSpace 6.1.R2 were used for bibliometric analysis.

Results: WoSCC includes 460 articles from 71 countries. The number of publications on gut microbiota and epigenetics has increased each year since 2011. The USA, PEOPLES R CHINA, and ITALY are at the center of this field of research. The University of California System, Harvard University, and the University of London are the main research institutions. Li, X, Yu, Q, Zhang, S X are the top authors in this research field. We found that current research hotspots and frontiers include short-chain fatty acids (SCFA) play an important role in gut microbiota and epigenetic mechanisms, gut microbiota and epigenetics play an important role in host obesity, diet, and metabolism. Gut microbiota and epigenetics are closely related to colorectal cancer, breast cancer, and inflammatory bowel disease. At the same time, we found that gut microbiota regulates epigenetics through the gut-brain axis and has an impact on psychiatric diseases. Therefore, probiotics can regulate gut microbiota, improve lifestyle, and reduce the occurrence and development of diseases.

Conclusion: This is the first comprehensive and in-depth bibliometric study of trends and developments in the field of gut microbiota and epigenetics research. This study helps to guide the direction of research scholars in their current field of study.

KEYWORDS

gut microbiota, epigenetics, bibliometrics, mechanism, host diseases, gut-brain axis

1 Introduction

Trillions of species of symbiotic microbes persist in the gastrointestinal tract, collectively known as the gut microbiota, and they are important factors affecting host health and disease (1). The human body and the microbiome are in a state of dynamic balance, and the microorganisms in the gut participate in many physiological functions of the human body, such as fermentation-related food components, vitamin synthesis, and maintenance of intestinal homeostasis (2). In recent years, with the deepening of the study of gut microbiota, it has been found that microbial signals can calibrate the transcriptional program of host cells through epigenetic modification without changing the underlying genetic code. DNA modification, histone modification, and regulation of non-coding RNA are forms of epigenetic changes to which the microbiome is sensitive (3). Studies have found that epigenetics is a key mechanism to regulate the development of host intestinal homeostasis and metabolic disorders. Epigenetic regulation of microbial communities can be influenced by host diet, antibiotic use, infection, etc (4, 5). The effects of microbial metabolites on host health can be achieved by inducing epigenetic modifications, altering DNA methylation, and microRNAs expression (6).

With the in-depth study of the mechanism of gut microbiota, Research on gut microbiota and epigenetics has attracted more and more attention. However, this research area has not been thoroughly dissected using bibliometrics analysis. Bibliometrics analysis allows for quantitative analysis of literature in the field of study, using mathematical and statistical knowledge (7). Bibliometrics analysis can reflect the hot spots, emphases, and frontiers of the research field (8). In order to better grasp the knowledge of this research field, this study focuses on the hot spots, emphases, and trends of gut microbiota and epigenetics research.

2 Methods

2.1 Literature resources

We searched literature data related to the research field in the Web of Science Core Collection (WoSCC), a multidisciplinary and comprehensive database with a complete citation network (9). The search strategy is presented in Supplementary Material, which uses a combination of subject and free words for gut microbiota and epigenetics. The time for a literature search is no limit. The document type is set to Article or Review. The last step is to export and store all the retrieved documents as text files for further bibliometric research. On March 15, 2024, two researchers conducted an independent search of literature data. The complete retrieval process is shown in Figure 1.

2.2 Literature analysis

We used CiteSpace.6.1.R2, Vosviewer1.6.17, and Microsoft Office Excel 2010 for data analysis and management. Microsoft Office Excel 2010 software can manage data, tally annual publications, and create related tables. In addition, CiteSpace 6.1.R2 creates a visual map that provides a detailed summary analysis of annual publications by number, country, institution, author, keyword, and highly cited article. Vosviewer1.6.17 visualizes highly co-cited literature and co-occurrence of authors. The specific parameter Settings and results of CiteSpace are the same as those of previous Settings (8). Nodes can represent countries and institutions.

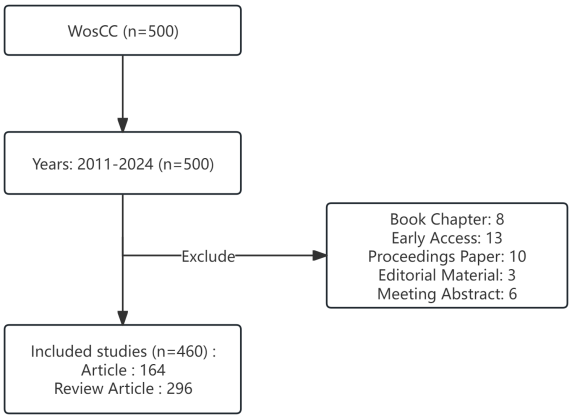

FIGURE 1
Flow diagram of the included articles.

3 Results

3.1 Analysis of annual publications and trends in publications

Until March 15, 2024, a total of 500 articles have been published in this field, including 164 articles and 296 review articles. Trends in a particular field of research can be measured by annual publications. The analysis shows that the number of papers in this field has increased year by year, from 4 papers in 2011 to a peak in 2022 and 2023 (n=85 papers) (Figure 2). This indicates that the field is receiving increasing attention from researchers. In addition, the growth trend model shown in Figure 2 [coefficient of determination (R^2) = 0.5203] shows a positive correlation between publication year and publication, which means that the number of annual publications in the field will continue to rise.

3.2 Analysis of the trend of countries, institutions, and authors

Articles were published in 71 countries/regions. The 71 nodes and 336 links represent countries and cooperation between countries in Figure 3. The more a country has published in that area of study, the larger the nodes shown in the graph. If the centrality is greater than 0.1, the purple circle will appear outside the corresponding node on the network map. Table 1 lists the top 10 countries in terms of the number of published papers and their centrality. The United States published the most papers (168 publications, 32.81%), followed by

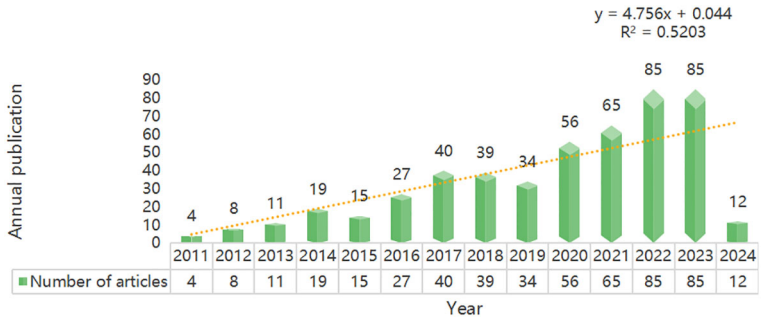

FIGURE 2
Published trend chart concerning gut microbiota and epigenetics.

China (77 publications, 15.04%) and Italy (54 publications, 10.55%), all of which are priority countries for gut microbiota and epigenetics research. Cooperation among countries is positively correlated with centrality. The results show that the United States (0.43), Italy (0.19), the People's Republic of China (0.18), the United Kingdom (0.16) and India (0.14) are the five countries with the highest centrality.

299 institutions contributed to the field of research. Figure 4 shows the collaboration between institutions, which includes 299 nodes and 693 connections. From Table 1, We found that the top five universities with the highest number of published papers are the University of California System (17 publications, 16.83%), Harvard University (14 publications, 13.86%), the University of London (11 publications, 10.89%), and Baylor College of Science Medicine (10 publications, 9.90%), CIBER-Centro de Investigacion Biomedica en Red (9 publications, 8.91%). The University of California System (0.27), University of London (0.23), Harvard University (0.18), CIBER - Centre for Biomedical Research (0.15), and Karolinska Institutet (0.11) are the top five institutions with the most centricity, representing the most collaboration. The world's top universities and institutions have made outstanding contributions to the development of the field.

As shown in Figure 5, 293 authors have published papers on gut microbiota and epigenetics. Table 1 lists the five authors with the highest number of published articles. Four authors, Li, X, Yu, Q, Zhang, S X, He, P F, contributed the most to the number of articles (4 publications per person, 21.05%), followed by Dinan, Timothy G (3 publications, 15.79%). These five authors play important roles in the field of gut microbiota and epigenetics research. The centrality of all authors is 0, indicating that the cooperation between authors still needs to be strengthened.

3.3 Analysis of co-cited references

Co-cited references are those cited collectively by researchers. Through the analysis of co-cited references, VOSviewer visualizes the co-cited references, highlighting common research areas between gut microbiota and epigenetics. According to

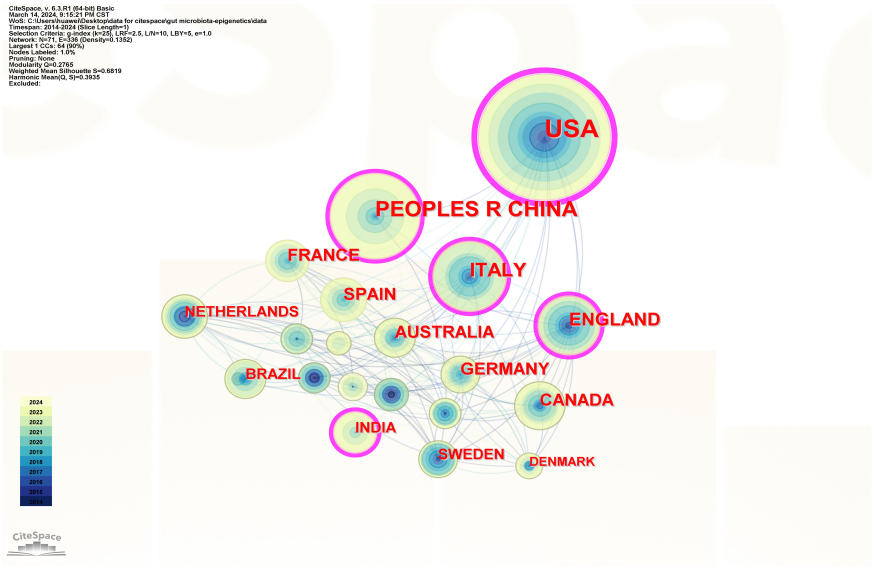

FIGURE 3
Country/region collaboration network of research on gut microbiota and epigenetics. Created with CiteSpace.

TABLE 1 Countries/regions, institutions, and authors ranked by publications and centrality.

Item	Rank	Name	Publications	Name	Centrality
Countries/ Regions	1	USA	168 (32.81%)	USA	0.43
	2	PEOPLES R CHINA	77 (15.04%)	ITALY	0.19
	3	ITALY	54 (10.55%)	PEOPLES R CHINA	0.18
	4	ENGLAND	40 (7.81%)	ENGLAND	0.16
	5	CANADA	33 (6.45%)	INDIA	0.14
	6	FRANCE	32 (6.25%)	AUSTRALIA	0.10
	7	SPAIN	31 (6.05%)	SPAIN	0.09
	8	AUSTRALIA	30 (5.86%)	SWEDEN	0.09
	9	GERMANY	27 (5.27%)	FRANCE	0.08
	10	NETHERLANDS	20 (3.91%)	NETHERLANDS	0.06
Institutions	1	University of California System	17 (16.83%)	University of California System	0.27
	2	Harvard University	14 (13.86%)	University of London	0.23
	3	University of London	11 (10.89%)	Harvard University	0.18
	4	Baylor College of Medicine	10 (9.90%)	CIBER - Centro de Investigacion Biomedica en Red	0.15
	5	CIBER - Centro de Investigacion Biomedica en Red	9 (8.91%)	Karolinska Institutet	0.11
	6	Harvard Medical School	8 (7.92%)	Brigham & Women's Hospital	0.10
	7	University System of Ohio	8 (7.92%)	University of Arizona	0.10
	8	Karolinska Institutet	8 (7.92%)	University College Cork	0.09
	9	INRAE	8 (7.92%)	Centre National de la Recherche Scientifique (CNRS)	0.08
	10	Centre National de la Recherche Scientifique (CNRS)	8 (7.92%)	Helmholtz Association	0.08
Authors	1	Li, X	4 (21.05%)	Li, X	0.00
	2	Yu, Q	4 (21.05%)	Yu, Q	0.00
	3	Zhang, S X	4 (21.05%)	Zhang, S X	0.00
	4	He, P F	4 (21.05%)	He, P F	0.00
	5	Dinan, Timothy G	3 (15.79%)	Dinan, Timothy G	0.00

VOSviewer's results, a total of 49,507 references were cited in this research area. When the number of citations is reduced to 18, 37 references remain. From Figure 6, we can find that the co-cited references are divided into four clusters, corresponding to the four colors in the visualization diagram. The red cluster mainly shows the epigenetic regulation of host metabolism by intestinal microbes, including the epigenetic regulation of host obesity by gut microbiota (10), the interaction between diet and intestinal microbes mediates the epigenetic inheritance of host tissues or diseases (11, 12), and the epigenetic regulation between gut microbiota and host metabolism (13, 14). The literature on green clusters mainly introduces the research on the types and functions of gut microbiota and gene sequencing (15–17). Blue clusters of literature mainly focus on the basic studies on the regulation of intestinal inflammation and immune response by gut microbiota through derivative substances such as butyrate and receptor GPR43 (18–20). The literature in the yellow cluster mainly focuses on the link between diet and gut microbiota, including the key role of short-chain fatty acids (SFCAs) (21–23).

Table 2 lists the top 10 cited literature, most of which are from the world's top journals, such as Nature, Science, etc. Therefore, the research on gut microbiota and epigenetics is the current research hotspot and frontier of the scientific community. "Diet-Microbiota Interactions Mediate Global Epigenetic Programming in Multiple Hosts Tissues "is the most widely cited paper in 2016 published in Molecular Cell (12). Among them, Krautkramer et al. proposed that microbial regulation of protein acetylation and methylation in host tissues through diet, as well as short-chain fatty acids fermented by gut microbes, can promote transcriptional responses to host epigenetic programming. In addition, it can be found from the

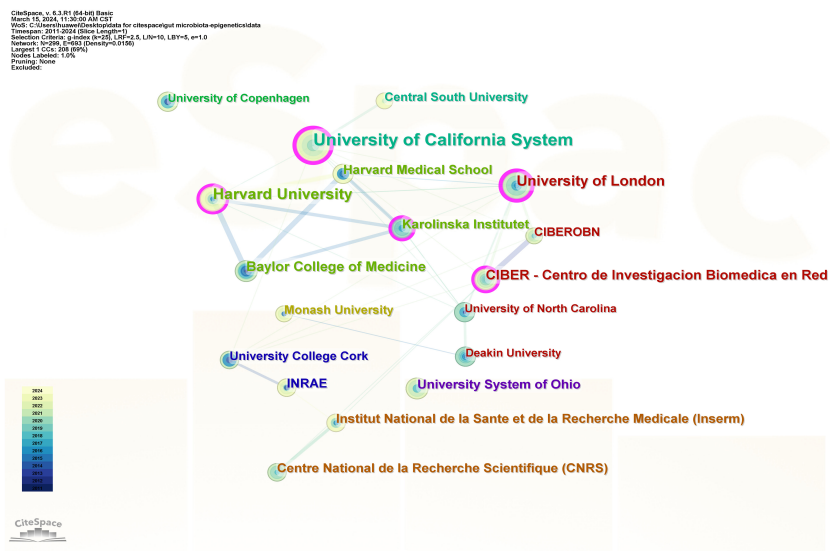

FIGURE 4
Institution' collaboration network of research on gut microbiota and epigenetics. Created with CiteSpace.

top 10 most-cited papers that most of the cited papers come from high-quality journals such as Nature and Science, which indicates the cutting-edge and innovative nature of this research field. Secondly, most studies in the cited literature focus on how diet and obesity act on the epigenetic inheritance of multiple tissues through gut microbiota, and the regulation of inflammatory immunity by gut microbiota derivatives.

The analysis of the top ten cited literature focused on the mechanism between the gut microbiota and epigenetics. In the first ten cited articles, Kimberly A Krautkramer found that short-chain fatty acid (SCFA), a

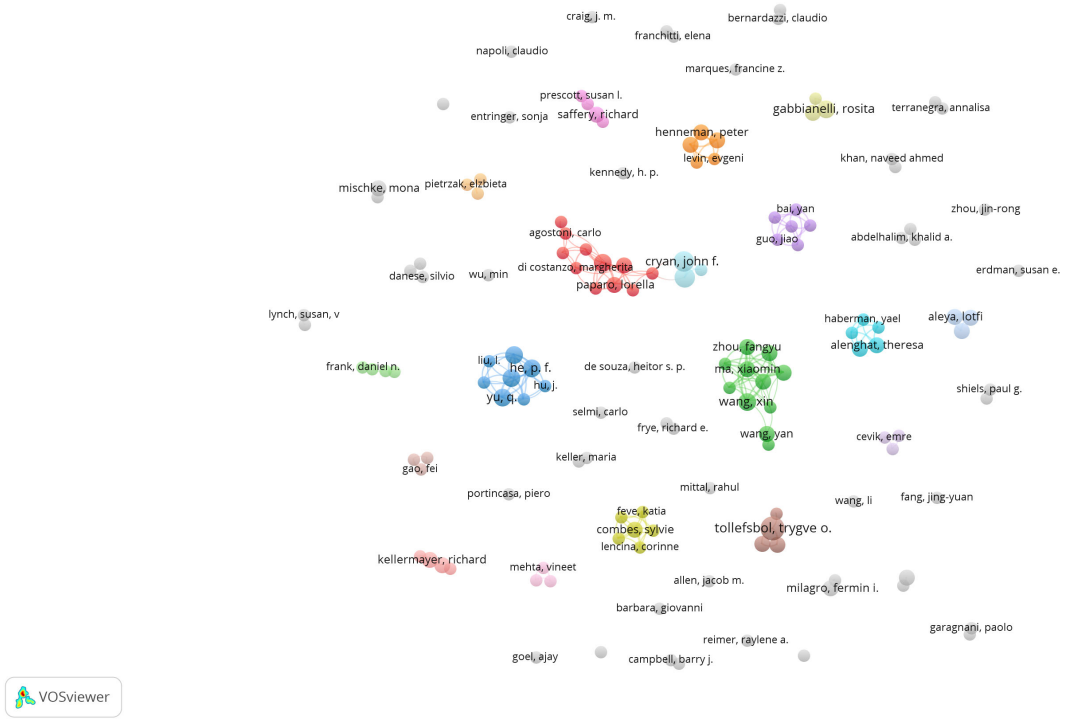

FIGURE 5
Authors' collaboration network of research on gut microbiota and epigenetics. Created with VOSviewer.

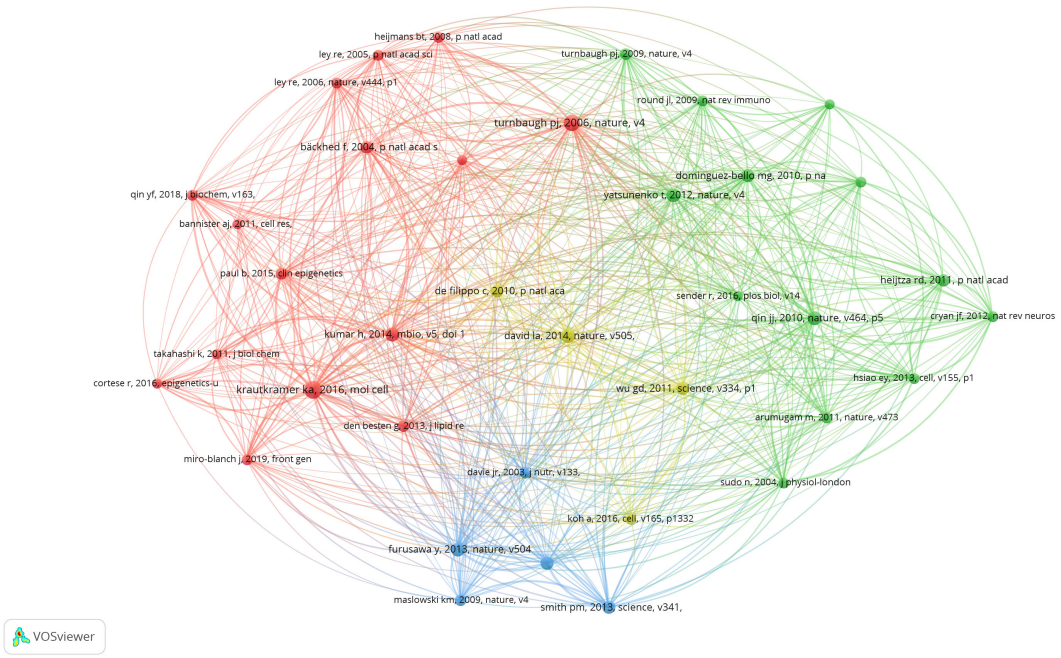

FIGURE 6
Visualization of a clustering map of co-cited references. Created with VOSviewer.

major derivative of the gut microbiota, is able to influence host-related epigenetic phenotypes and is sensitive to host diet (10). Himanshu Kumar's study found that the gut microbiota, as an epigenetic regulator, in the group dominated by Firmicutes, genes with differential methylation promoters are associated with disease risk, mainly associated with cardiovascular disease, especially with lipid metabolism, obesity, and inflammatory response (29). Patrick M Smith's study found that short-chain fatty acids, the fermentation products of intestinal microbiota, can regulate Regulatory T cells (Tregs) and thus regulate intestinal inflammation (20). Yukihiro Furusawa's study found that differentiation of colonic regulatory T cells is induced by butyrate derived from the gut microbiota to improve intestinal inflammation and immune response (19).

3.4 Analysis of highly cited literature

Table 3 shows the top 10 highly cited literature on gut microbiota and epigenetics, most of them come from the world's top journals and represent the forefront of scientific development. The most cited article is titled "Diet-Microbiota Interactions Mediate Global Epigenetic Programming in Multiple Host Tissues " (12) indicates that gut mediates the epigenetic state of host tissues and changes in chromatin status to the host and that SCFA influences host epigenetic programming. At the same time, the mechanism research of gut flora and epigenetics also ranked in the top 10.

TABLE 2 Top 10 highly co-cited references.

Item	Rank	Title	Citation	Year
Highly co-cited references	1	Diet-Microbiota Interactions Mediate Global Epigenetic Programming in Multiple Host Tissues	24	2016
	2	An obesity-associated gut microbiome with increased capacity for energy harvest	25	2006
	3	Diet rapidly and reproducibly alters the human gut microbiome	26	2014
	4	Gut microbiota as an epigenetic regulator: pilot study based on whole-genome methylation analysis	38	2014
	5	Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells	27	2013
	6	A human gut microbial gene catalogue established by metagenomic sequencing	27	2010
	7	Human gut microbiome viewed across age and geography	28	2012
	8	The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis	30	2013
	9	Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation	31	2013
	10	Linking long-term dietary patterns with gut microbial enterotypes	31	2011

TABLE 3 Top 10 highly cited references.

Item	Rank	Title	Citation	Year
Highly cited references	1	Diet-Microbiota Interactions Mediate Global Epigenetic Programming in Multiple Host Tissues	30	2016
	2	Diet rapidly and reproducibly alters the human gut microbiome	18	2014
	3	Epigenetic Regulation at the Interplay Between Gut Microbiota and Host Metabolism	18	2019
	4	Crosstalk between the microbiome and epigenome: messages from bugs	16	2018
	5	Gut microbiota as an epigenetic regulator: pilot study based on whole-genome methylation analysis	15	2014
	6	Epigenetic regulation by gut microbiota	15	2022
	7	Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation	13	2013
	8	Revised Estimates for the Number of Human and Bacteria Cells in the Body	13	2016
	9	Microbiota derived short chain fatty acids promote histone crotonylation in the colon through histone deacetylases	12	2018
	10	The Epigenetic Connection Between the Gut Microbiome in Obesity and Diabetes	12	2020

3.5 Analysis of keywords co-occurrence, clustering, burst

Keyword co-occurrence gives us an idea of the topic and scope of the research field (Figure 7). The top 20 keywords in the co-occurrence rate and centrality of gut microbiota and epigenetics from 2011 to 2024 are shown in Table 4. "gut microbiota" is the keyword of occurrence frequency, followed by "DNA methylation" and "chain fatty acids". And more importantly, "gut microbiome", "gene expression", "intestinal microbiota", "expression", "oxidative stress", and "colorectal" Keywords such as "cancer" are used more than 30 times, revealing the current research focus and topics in this field. Centrality is positively correlated with the degree of connection between keywords. In Table 4, "gut microbiota" is the main intestinal microbiota, followed by "gut microbiome", "dna methylation", "association", and "intestinal microbiota". These keywords still focus on the link between gut microbiota and epigenetics and the relationship with DNA methylation.

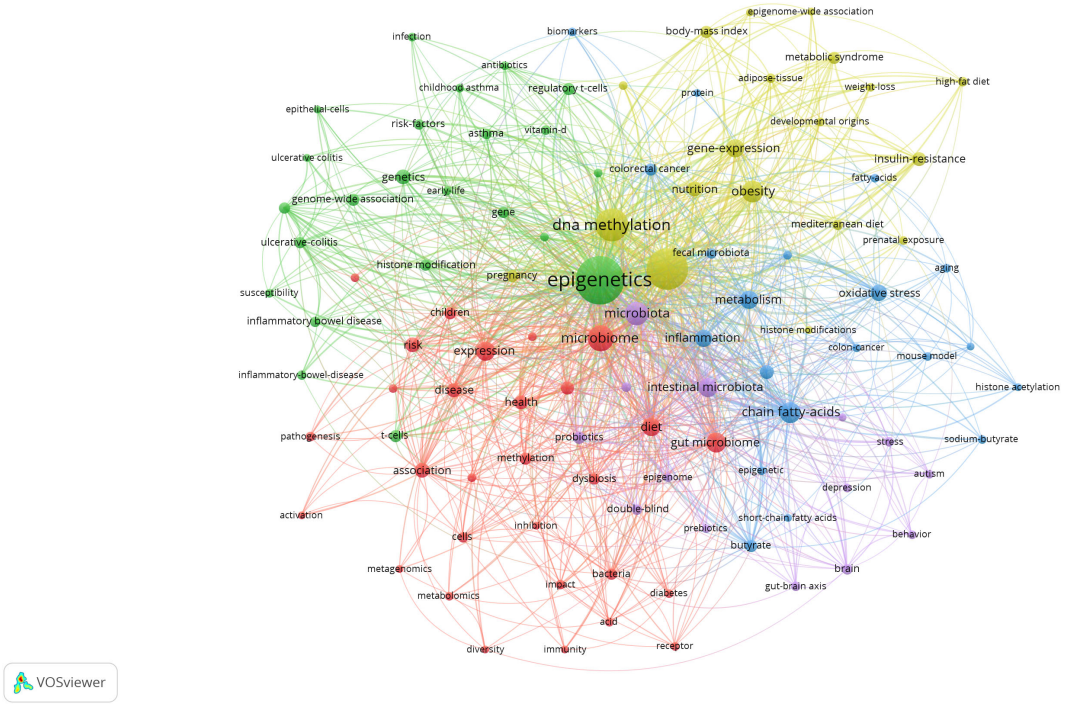

FIGURE 7
Keyword co-occurrence map of gut microbiota and epigenetics. Created with VOSviewer.

TABLE 4 Top 20 keywords in terms of frequency and centrality.

Rank	Keyword	Frequency	Keyword	Centrality
1	gut microbiota	247	gut microbiota	0.23
2	dna methylation	148	gut microbiome	0.23
3	chain fatty acids	58	dna methylation	0.21
4	gut microbiome	55	association	0.14
5	gene expression	51	intestinal microbiota	0.12
6	intestinal microbiota	51	chain fatty acids	0.11
7	expression	48	gene expression	0.11
8	oxidative stress	33	expression	0.10
9	colorectal cancer	30	colorectal cancer	0.08
10	epigenetics	29	inflammatory bowel disease	0.08
11	association	28	obesity	0.08
12	insulin resistance	25	regulatory t cells	0.07
13	risk	23	health	0.06
14	metabolism	22	inflammation	0.06
15	inflammatory bowel disease	22	mechanisms	0.06
16	health	21	bacteria	0.06
17	inflammation	20	epigenetics	0.05
18	mechanisms	19	insulin resistance	0.05
19	obesity	19	risk	0.05
20	body mass index	19	body mass index	0.04

To understand the research frontiers of gut microbiota and epigenetics since 2011, CiteSpace was used to cluster keywords for gut microbiota and epigenetics. Nine clusters are shown in Table 5, Figures 8 and 9. In general, when Silhouette is greater than 0.5, the clustering effect is reasonable (8). Cluster #0 is labeled "inflammatory bowel disease", followed by Cluster #1 "Precision nutrition", Cluster #2 "Noncommunicable diseases", Cluster #3 "Gut microbiota", Cluster #4 "Allergy development", Cluster #5 "Machine learning", Cluster #6 "Breast cancer", Cluster #7 "Psychiatric disorder", Cluster #8 "Programmable epigenome", representing the forefront of research since 2017.

Keyword bursts sum up the sudden growth of research content over a period of time, which may indicate future trends in research. Figure 10 shows the top 25 items with the highest burst intensity in this research subject. The red line in the graph indicates the length of time the keyword bursts. As we observe from the chart, the keyword themes gradually changed from "intestinal microbiota", "long noncoding RNAs", "childhood asthma", "genome-wide association" to the current "breast cancer ", "weight loss", "sodium butyrate", "protein" and "microbiota". This suggests that the correlation between the gut microbiota and epigenetic effects on cancer, metabolism, and mechanisms is the main focus of this research now and in the future.

4 Discussion

4.1 General information discussion

This study collected all WoSCC data related to the research field to identify research hotspots and frontiers. The number of publications each year has been steadily increasing. With 168 publications, the United States produced the most publications, followed by China and Italy. Because of its very strong economic strength and beneficial policy and scientific support, the United States is the largest country in this field of research. At the same time, although China is a developing country, it has an important position in the field of gut microbiota and epigenetics research.

In specific research areas, collaborations between authors, institutions, and countries can be evaluated using bibliometrics (32). Centrality represents the closeness of cooperation. The top five countries with the highest centrality are the United States, Italy, China, the United Kingdom, and India, meaning that these countries can actively cooperate with different countries. Collaboration between institutions shows that the University of California System, the University of London, Harvard University, CIBER - Centro de Investigacion Biomedica en Red, and Karolinska Institutet cooperate most closely and have the highest central

TABLE 5 Keyword cluster analysis.

Cluster	Size	Silhouette	Mean year	Label (LLR)	Other keywords
0	52	0.572	2017	inflammatory bowel disease	inflammatory bowel disease; gut microbiome; inflammatory bowel diseases; new insight; epigenetic modification; gut microbiota; strain t2; mitochondrial dna; microbial influence; pathogenic role
1	45	0.716	2018	Precision nutrition	gut microbiome; precision nutrition; tackling atherosclerosis; predicting response; current method | gut microbiota; epigenetic regulation; epigenetic mechanism; dna methylation; early life
2	38	0.672	2018	Noncommunicable diseases	developmental origin; noncommunicable diseases; disease concept; cohort profile; neurotypical lymphoblastoid cell line; gut microbiota; gut microbiome; asthma development; recent advance; dna methylation
3	37	0.668	2015	Gut microbiota	gut microbiota; epigenetic regulation; epigenetic factor; gut microbiome; allergic diseases; gut-brain axis; molecular mechanism; functional food; aberrant dna methylation profile; multiple sclerosis patient
4	34	0.741	2015	Allergy development	gut-brain axis; allergy development; emerging role; molecular mechanism; colorectal cancer; gut metabolite; dietary prevention; epigenetic effect; gut maturation; early life
5	31	0.724	2018	Machine learning	brain disorder; multi-omics data analysis; microbiome-mediated epigenetic regulation; machine learning; future direction; wide perspective; glial cell; endocrine cross-talk; review article; gastrointestinal tract
6	28	0.778	2020	Breast cancer	colorectal cancer; breast cancer; short-chain fatty acid; redox homeostasis; host dna methylation change; multi-omics era; intracranial hemorrhage management; central nervous system; practical application; precision oncology
7	18	0.86	2017	Psychiatric disorder	psychiatric disorder; severe mental illness; predictive role; targeting aggression; metabolomic marker; marked methylation change; intestinal gene; preterm neonate; perinatal period; human milk
8	9	0.97	2014	Programmable epigenome	Adult disease; programmable epigenome; peroxisome proliferator-activated receptor; zinc finger protein; connecting homocysteine;intraocular inflammation; new paradigm; inflammatory bowel diseases; adult disease; human health

position. Li, X, Yu, Q, and Zhang, S X have published the most papers in this field. However, the centrality of all authors is 0, indicating that there is no cooperation among authors, and cooperation among authors in the field needs to be strengthened. Cooperation among authors requires cooperation in related research fields and policy support from governments. We believe that close cooperation between States, institutions, and authors will help to achieve great progress in this area.

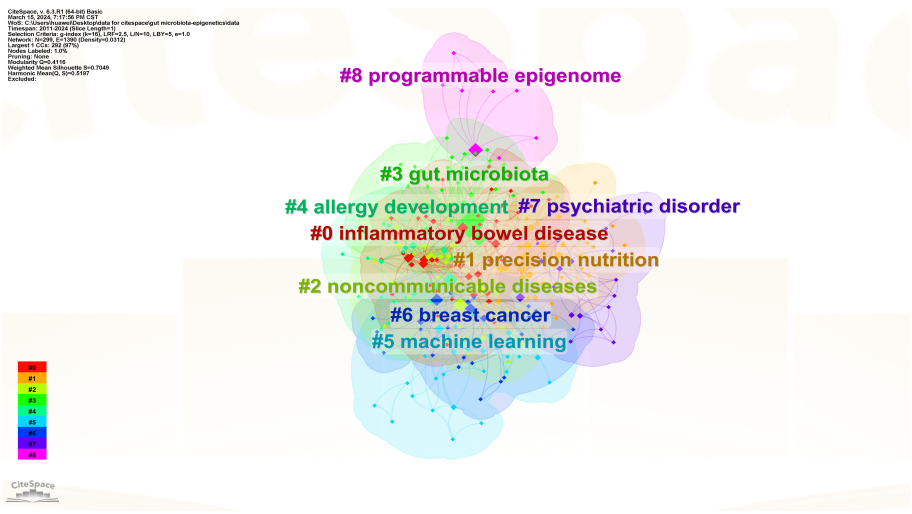

FIGURE 8
Keyword cluster map of gut microbiota and epigenetics. Created with CiteSpace.

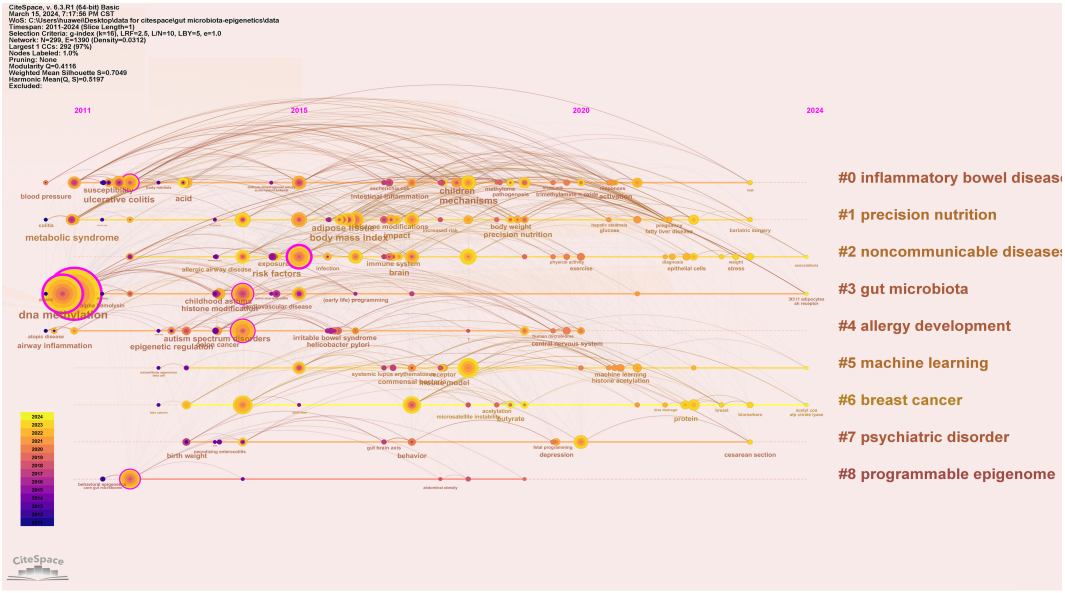

FIGURE 9
Keyword timeline map of gut microbiota and epigenetics. Created with CiteSpace.

Top 25 Keywords with the Strongest Citation Bursts

Keywords	Year	Strength	Begin	End	2014 - 2024
intestinal microbiota	2014	3.05	2014	2016	
long noncoding rnas	2014	1.99	2014	2017	
childhood asthma	2014	1.95	2014	2016	
genome wide association	2015	2.91	2015	2016	
food allergy	2015	2.46	2015	2016	
inflammatory bowel disease	2015	2.33	2015	2018	
cardiovascular disease	2015	2.14	2015	2017	
metabolic syndrome	2016	2.57	2016	2017	
adipose tissue	2016	2.21	2016	2019	
helicobacter pylori	2016	2.03	2016	2019	
early life	2016	2.01	2016	2018	
crohns disease	2017	2.45	2017	2018	
intestinal inflammation	2017	2.13	2017	2018	
promoter methylation	2017	1.84	2017	2019	
ulcerative colitis	2017	1.75	2017	2018	
psychiatric disorders	2017	1.69	2017	2020	
children	2018	1.85	2018	2021	
cells	2021	2.54	2021	2022	
gut-brain axis	2021	2.5	2021	2022	
mediterranean diet	2018	2.18	2021	2022	
breast cancer	2021	1.97	2021	2024	
weight loss	2022	3.46	2022	2024	
sodium butyrate	2022	3.11	2022	2024	
protein	2022	2.42	2022	2024	
microbiota	2022	2.07	2022	2024	

FIGURE 10
Top 25 keywords with the strongest citation bursts.

4.2 Research focus and hotspot

Bibliometrics analysis can reflect the hot spots and frontiers of this research field. Based on multiple analyses of references and keywords, we found that the hot spots and trends of gut microbiota and epigenetics are related to host metabolism and mechanisms, including obesity, diet, DNA methylation, and the role of SFCAs. In addition, through keyword burst analysis and keyword clustering, it can be seen that scholars have conducted more comprehensive and in-depth research on gut microbiota and epigenetics, and have begun to study the impact of this field on host diseases, such as cancer, inflammatory bowel disease, and mental disorders, as well as research on gut-brain axis theory.

4.3 Regulatory mechanisms between gut microbiota and epigenetics

A growing body of evidence supports the interaction of gut microbiota with epigenetic processes. Epigenetic modifications affect host health and disease development by altering the cell's transcriptional machinery to reprogram the host genome (33). Through bibliometrics analysis, we can learn that the current mechanism between gut microbiota and epigenetics is mainly related to SCFAs, so we will discuss this in detail. The fermentation of complex carbohydrates or starches involves a number of pathways associated with microorganisms (34, 35). After the initial fermentation of carbohydrates in the small intestine, the microbiome ferments it into SFCAs, in which butyrate, propionate, and acetate account for the largest proportion (31). SCFAs can reduce the activity of deacetylase and play an important role in modifying gene expression (36). In one study, SCFAs revealed microbially relevant chromatin modification states and transcriptional reactions, including the regulation of histone acetylation and methylation (12). In addition, propionate and butyrate can promote adipocyte differentiation, which may partially inhibit the effect of histone deacetylase activity (37). SCFAs produced by Akkermansia muciniphila in the mouse ileum can be involved in the expression of histone deacetylase, transcription factors, cellular lipid metabolism, and satiety genes (30). All the above experiments indicate that SCFAs produced by gut microbiota through fermentation have an important influence on host epigenetics.

4.4 Effects of interactions between gut microbiota and epigenetic on host metabolism

Based on the results of the bibliometrics analysis, we found that the role of gut microbiota and epigenetics may play an important role in host diet, obesity, and metabolism. The complex interplay between epigenetics, gut microbiota, and diet has important implications for host obesity risk and host metabolic syndrome (6). The study found that the microbial diversity and abundance of obese patients were decreased, the proportion of Bacteroides and Lactobacillus was different, and the methylation levels of FFAR3 gene (FFAR3) and TLR genes TLR4 and TLR2 were decreased. There was a correlation between BMI and methylation of FFAR3 and TLR genes TLR4 and TLR2 (28). In addition, deep sequencing DNA methylation revealed a clear association between gut microbiota and epigenetics (29). One study confirmed that fecal micro-RNA (miRNA) is an important component of the gut microbiome (27). miRNA can mediate bidirectional host-microbial interaction (38). These studies provide insights into the relationship between gut microbes and metabolism-related epigenetics. Based on relevant literature data, further discovery of dietary approaches for beneficial bacterial populations and epigenetic changes in energy homeostasis may have important implications for obesity and metabolism-related clinical manifestations.

4.5 Effects of interactions between gut microbiota and epigenetics on host disease

Based on the results of the bibliometrics analysis, we found that the role between gut microbiota and epigenetics may play an important role in inflammatory bowel disease, cancers (colorectal cancer and breast cancer), and psychiatric disorders Research evidence suggests that intestinal microbiota disturbances and alterations in carcinogenic and tumor suppressor genes can cause colorectal cancer (39). The gut microbiota ferments dietary residues, providing energy for the microbiota and ultimately releasing short-chain fatty acids, including butyrate. Butyrate inhibits inflammation and cancer by affecting immunity, gene expression, and epigenetic regulation (40). It was found that microbial fermentation products and activated phytochemicals (such as butyrate and polyphenols) can prevent tumor transformation by inhibiting epigenetic mechanisms such as histone deacetylase (26, 41, 42). The ERα gene and BRCA1 gene, which are strongly associated with breast cancer, have been observed in epigenetic programming (43). The production of butyrate by the gut flora has been shown to activate epigenetic genes in cancer cells such as p21 and BAK (44). However, although gut bacteria can facilitate, epigenetic reprogramming, and contribute to the tumor process, microbiome epigenetic induction of tumor formation has not been proven. Further experiments are needed to confirm this.

Many factors, such as genetic, environmental, intestinal microbiota and immune abnormalities, are related to the occurrence of IBD (45). Genome-wide association studies of IBD identified more than 200 genetic risk loci for IBD, providing important evidence for the role of microorganisms in the pathogenesis of IBD (46). The gut microbiota may regulate epigenetic mechanisms by regulating multiple micronutrients and food components, which may increase the risk of IBD (47).

Multiple evidence suggests that mental illness is related to gut flora and interacts with each other through the gut-brain axis (25, 48, 49). The bidirectional connection between the gut and the brain is called the gut-brain axis. The microbiome is an important part of the triangular conversation (50). Gut microbiota regulates brain

function by stimulating neuronal responses or secreting metabolites associated with nerves (51). The gut-brain axis may be involved in the transmission of vagus nerve and hormone signals (52). The gut-brain axis may influence brain functions such as cognition and learning, so targeting a patient's specific gut flora may reduce symptoms of neurodegenerative diseases (53). The modes of epigenetic regulation include DNA methylation, post-transcriptional histone modification, and gene expression regulation of non-coding RNA (54). DNA methylation is closely related to neurological diseases (24). Gut microbiota can secrete synthetic folic acid, vitamin B12, and choline to produce methyl donors (6-methyltetrahydrofolate) and to form S-adenosine methionine (SAM), which is the main methyl donor in DNA methylation (55). Choline is not only an important nutrient for the brain but also promotes SAM production and is a key methyl donor for DNA and histone methylation (56). The hypothalamic-pituitary-adrenal axis (HPA) is an important communication pathway in the gut-brain axis (51). The normal operation of the HPA axis requires the presence of GR (ligand-activated transcription factor). Studies have found that individuals with genetic abnormalities of the GR gene in the brain are associated with bipolar disorder and schizophrenia (57). Epigenetic modifications do not change the DNA sequence, so the DNA sequence is stable for a long time. The microbiome is capable of modifying the host epigenome via the gut-brain axis and causing visible behavioral or phenotypic changes in the host. Although epigenetic modification changes are more lasting, they are not permanent, so it is possible to restore the gut microbiota and make lifestyle changes (such as sleep, diet, exercise, etc.) through supplementation with probiotics and probiotics, which have important implications for the improvement of conditions such as diabetes, obesity, neurodegenerative diseases, and depression (57).

5 Advantages and limitations of research

Visual analysis of bibliometrics can comprehensively display the key points, hot spots, and frontiers of the current research field, and provide researchers with reference research directions. However, there are some limitations to our study. First, we did not search all the databases, which may have led to the omission of literature. In addition, we failed to ensure that every piece of literature fully met the requirements of the study. Finally, the quality of the retrieved articles cannot be completely guaranteed, which will affect the rigor of the analysis.

6 Conclusion

This study evaluated and visualized relevant publications on gut microbiota and epigenetics using bibliometrics and visualization analysis. The number of research publications in the field of gut microbiota and epigenetics is increasing every year. The country with the highest number of articles is the United States. The University of California System and Li, X are among the most influential institutions and authors in the field. In addition, our study provides a comprehensive analysis of the research hotspots and research directions of gut microbiota and epigenetics. Based on the bibliometric analysis of gut microbiota and epigenetics, we found that short-chain fatty acids are an important component of the mechanism between gut microbiota and epigenetics. The interaction between gut microbiota and epigenetics play an important role in host obesity, diet, and metabolism. Gut microbiota and epigenetics are closely related to colorectal cancer, breast cancer, and inflammatory bowel disease. At the same time, we found that gut microbiota regulates epigenetics through the gut-brain axis and has an impact on psychiatric diseases. Therefore, probiotics can regulate gut microbiota, improve lifestyle, and reduce the occurrence and development of 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 author.

Author contributions

ST: Conceptualization, Data curation, Methodology, Software, Supervision, Visualization, Writing – original draft. MC: Conceptualization, Data curation, Methodology, Software, 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.2024.1412640/full#supplementary-material

SUPPLEMENTARY MATERIAL

Retrieval strategies for gut microbiota and epigenetics.

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Exploring gut microbiota's role in rheumatic valve disease: insights from a Mendelian randomization study and mediation analysis

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Background: Investigating the relationship between gut microbiota and Rheumatic Valve Disease (RVD) is crucial for understanding the disease's etiology and developing effective interventions. Our study adopts a novel approach to examine the potential causal connections between these factors.

Methods: Utilizing a two-sample Mendelian Randomization (MR) framework, we incorporated a multi-variable MR (MVMR) strategy to assess the mediatory mechanisms involved. This approach involved analyzing data from the MiBioGen consortium for gut microbiota and the FinnGen for RVD, among other sources. Instrumental variables (IVs) were carefully selected based on rigorous MR principles, and statistical analysis was conducted using bidirectional two-sample MR, such as inverse variance-weighted (IVW), weighted median, MR-Egger regression and MR Steiger Test methods. The MR-PRESSO strategy was employed for outlier detection, and MVMR was used to untangle the complex relationships between multiple microbiota and RVD.

Results: Our analysis highlighted several gut microbiota classes and families with potential protective effects against RVD, including *Lentisphaerae, Alphaproteobacteria*, and *Streptococcaceae*. In contrast, certain genera, such as *Eubacterium eligens* and *Odoribacter*, were identified as potential risk factors. The MVMR analysis revealed significant mediation effects of various immune cell traits and biomarkers, such as $CD4^{-}CD8^{-}$ T cells, CD3 on Terminally Differentiated CD8+ T cell and Pentraxin-related protein PTX, elucidating the complex pathways linking gut microbiota to RVD.

Conclusion: This study underscores the intricate and potentially causal relationship between gut microbiota and RVD, mediated through a range of immune and hormonal factors. The use of MVMR in our methodological approach provides a more comprehensive understanding of these interactions, highlighting the gut microbiota's potential as therapeutic targets in RVD management. Our findings pave the way for further research to explore these complex relationships and develop targeted interventions for RVD.

KEYWORDS

gut microbiota, mediation analysis, Mendelian randomization, rheumatic valve disease, immune cell, estradiol

Introduction

Rheumatic Valve Disease (RVD), a chronic heart condition often originating from rheumatic fever, is a significant global health challenge, particularly in regions with limited healthcare resources (1). RVD is primarily believed to result from an autoimmune response triggered by antigenic mimicry. This mimicry occurs between certain surface proteins of group A streptococci and human cell surface antigens, particularly in genetically predisposed individuals (2). The bacterial proteins resemble elements of human cardiac myosin, including the N-acetyl glucosamine carbohydrate epitope and spiral M protein (3). This resemblance activates $CD4^+$ T cells, B cells, and macrophages, which mistakenly target the body's own cells (4). Acute rheumatic fever, affecting approximately 0.3–3% of individuals infected with group A streptococci, is characterized by transient inflammatory tissue damage, usually resolving within weeks to months. Initial valvular involvement is often minimal to moderate. However, permanent valvular damage and chronic rheumatic disease develop in about 30–45% of these patients. Chronic rheumatic disease is marked by continuous heart tissue inflammation even in the absence of bacterial presence (5).

The risk of developing acute rheumatic fever or long-term Rheumatic Heart Disease (RHD) hinges on three key factors: the specific bacterial strain, the genetic predisposition of the host, and abnormal immune responses of the host (6, 7). In this context, the role of the gut microbiota, the diverse community of microorganisms residing in the human gastrointestinal tract, has emerged as a subject of interest. Recent studies have begun to shed light on the potential influence of gut microbiota on cardiovascular health. For instance, research has indicated that specific microbial compositions can contribute to systemic inflammation, a known risk factor for various cardiovascular diseases (8). Shi and colleagues' study on RHD patients revealed altered gut and oral microbiota, potentially influencing RHD pathogenesis (9). The study found increased levels of *Bifidobacterium* and Eubacterium, and decreased levels of *Faecalibacterum* and *Bacteroides* in RHD patients compared to controls, suggesting these changes might worsen the disease by affecting the mitral valves.

This study employs Mendelian randomization (MR) to explore the causal relationship between gut microbiota composition and the risk of RVD. MR analysis uses genetic variants from Genome Wide Association Study (GWAS) as instrumental variables to assess causality, thus reducing the biases inherent in traditional observational studies (10).

It relies on the principle that these genetic variants are randomly inherited, thus acting like a natural randomized controlled trial (11). This randomness helps to avoid confounding factors that usually affect observational studies. If a genetic variant linked to a risk factor is also associated with a health outcome, it suggests a causal relationship between the risk factor and the outcome. MR provides a more robust approach to infer causality compared to traditional epidemiological methods. By leveraging genetic markers associated with microbiota profiles, this approach aims to determine whether alterations in gut microbiota are a contributing factor in RVD pathogenesis or simply an association.

Through this Mendelian randomization analysis, our objective is to provide robust, causal evidence of the role played by gut microbiota in the development of RVD. The study extends beyond analyzing microbiota composition by also exploring potential mediators that could influence the relationship between gut microbiota and RVD. These mediators include immune cells, cardiovascular proteins, and Estradiol. By examining these factors, the research aims to understand how they might interact with gut microbiota to affect the development and progression of RVD, providing a more comprehensive view of the disease's pathogenesis.

Method

Study design

Our investigation employed a two-sample MR approach to explore the potential causal connections between gut microbiota and RVD. We adopted a multi-variable MR strategy to enhance our understanding of the mediatory mechanisms involved. The design and progression of our study are detailed in Figure 1.

Data sources

For gut microbiota data, we utilized the extensive dataset from the MiBioGen consortium (12), comprising genome-wide genotypes and 16S fecal microbiome data from 18,340 participants across 24 cohorts. This dataset, primarily from European-descent cohorts, has undergone rigorous adjustments

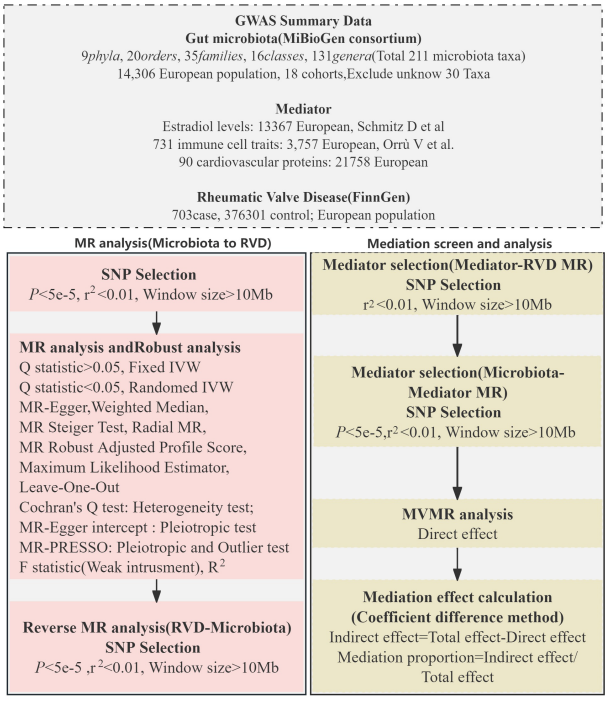

FIGURE 1
Mendelian randomization flowchart.

for sex, age, and genetic principal components. Quality control measures were implemented, though external factors like diet and medication were not considered in our analysis. Details can be found in Supplementary Table 1. For RVD data, we extracted information from the FinnGen R9 GWAS, which includes 703 cases and 376,301 controls (13).Additionally, datasets from Orrù et al. (14), Folkersen et al. (15), and Schmitz et al. (16) provided insights into immune cell traits, cardiovascular proteins, and Estradiol levels, respectively. All participants in these studies are of European descent. Further information and data link is available in Supplementary Table 1.

Single nucleotide polymorphisms selection

Our Single nucleotide polymorphisms (SNPs) selection adhered to three key MR principles (Figure 2) (17): a) Independence assumption: SNPs should be robustly associated with the exposure or mediator without confounding factors; b) SNPs should have a strong link with the exposure or mediator; c) Exclusion restriction assumption: SNPs should affect the outcome only through the exposure (mediator).

We selected SNPs with genome-wide significance ($P < 5 \times 10^{-8}$) related to exposures initially, then we relaxed the threshold to 5×10^{-5} due to a small number of SNPs under $P < 5 \times 10^{-8}$, $P < 5 \times 10^{-7}$ and $P < 5 \times 10^{-6}$. For the reverse analysis focusing on RVD, we selected SNPs with a significance level of $P < 5 \times 10^{-5}$. In the mediation analysis, SNP selection was further refined by adjusting the *P*-value threshold based on the number of SNPs involved, ensuring a more precise and tailored approach to identifying potential mediators in the relationship between gut microbiota and RVD. Linkage disequilibrium (18) clumping was used to refine SNP selection ($r^2 < 0.01$, window size > 10,000 kb), as detailed in Supplementary Table 2. We ensured the reliability of these genetic instruments by calculating the F statistic, $F = R^2 \times [(N - 1 - k)/k] \times (1 - R^2)$, where R^2 represents the variance in the exposure explained by the selected SNPs, N is the sample size, and k denotes the number of SNPs used as instrumental variables. For single SNP, We use $Beta^2_{exposure}/SE^2_{exposure}$ to calculate the F statistic (19). F value of SNP above 10 indicating sufficient strength to avoid weak instrument bias (20). All of SNP selected can be found in Supplementary Table 2.

Statistical analysis strategy

To investigate the relationships between gut microbiota and RVD, we employed a robust two-sample MR framework. And explore potential mediation including immune cell traits, cardiovascular proteins, and Estradiol levels.

Primary Methodology: Inverse Variance-Weighted (IVW) Method (21): This method aggregates estimates from individual genetic variants to produce a summary causal estimate, assuming all used genetic instruments are valid.

Sensitivity Analysis Methods: Weighted Median (WM) and Simple Median (22): These methods are employed to ensure reliability of our estimates even if some instruments are invalid, with the WM method requiring that at least 50% of instruments are valid. MR-Egger Regression (23): Enables the detection of directional pleiotropy by providing an estimate of the intercept from the regression analysis, which indicates the presence of pleiotropic effects. MR Steiger Test (24): Conducted to ensure the correct direction of causality in genetic associations used in our MR analyses. MR Robust Adjusted Profile Score (MR RAPS) (25): Applied to adjust for pleiotropic effects that may bias the results, enhancing the robustness of causal estimates. Maximum Likelihood Estimator (MLE) (26): This method was used to maximize the statistical efficiency and provide unbiased estimates under the assumption model. Radial MR (27): Offers further investigation into the influence of individual SNPs, ensuring that our causal estimates are not disproportionately affected by any single genetic variant.

Outlier Detection and Heterogeneity Assessment: Leave-One-Out Analysis (28): To assess the impact of each individual genetic variant on the overall MR estimate, we employed the leave-one-out method. This sensitivity analysis involves recalculating the MR estimates repeatedly, each time excluding one genetic variant at a time. This method helps identify whether any specific SNP disproportionately influences the results, thereby ensuring the robustness and stability of our findings. MR-PRESSO (29):

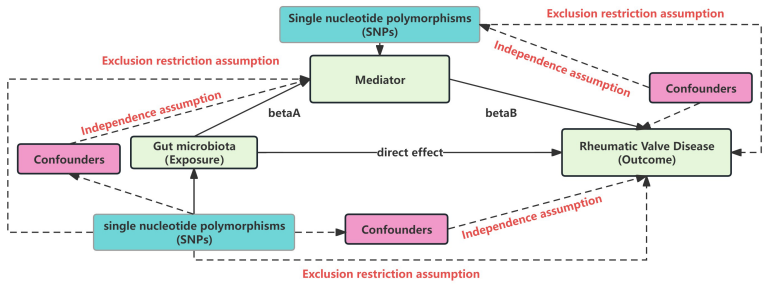

FIGURE 2
Mendelian randomization core assumption. This Figure represent the MR and mediation assumption, including three core assumption: Independence assumption: SNPs should be robustly associated with the exposure or mediator without confounding factors; SNPs should have a strong link with the exposure or mediator; Exclusion restriction assumption: SNPs should affect the outcome only through the exposure (mediator).

Identifies and adjusts for outliers in the genetic instruments to refine the validity of our instrumental variable analysis. Cochran's Q Test (30): Detects heterogeneity among the estimates provided by different SNPs. We applied a random-effects IVW model when significant heterogeneity was detected.

Multiple Testing Correction: Benjamini-Hochberg Method (FDR) (31): To control the false discovery rate given the multiple comparisons inherent in our analysis, we set a significance threshold of $P < 0.1$. We also noted taxa achieving nominal significance ($P < 0.05$) but not meeting the FDR-adjusted significance threshold ($P_{FDR} > 0.1$) as potentially causal associations.

Software Utilization: All statistical analyses were conducted using R software (version 4.3.1), and the results were visualized through Python-based plotting libraries to ensure clarity and precision.

Result

MR analysis between the gut microbiota and RVD

Our study's Mendelian randomization analysis reveal the relationship between gut microbiota and RVD (Figure 3), encompassing a broad range of microbiota. We found that the *phylum*, *class Lentisphaerae* (OR 0.39, *P*=0.008; OR 0.78, *P*=0.006), *classes Alphaproteobacteria* (OR 0.76, *P*=0.025), as well as the *family Clostridiales vadin BB60* (OR 0.74, *P*=0.007), exhibited potential protective effects against RVD. The *family Streptococcaceae* (OR 0.64, *P*=0.002) also showed a similar protective trend. The *genus Eubacterium oxidoreducens* (OR 0.71, *P*=0.002), *genus Roseburia* (OR 0.71, *P*=0.019), and *order Victivallales* (OR 0.78, *P*=0.006) were associated with a reduced risk of RVD.

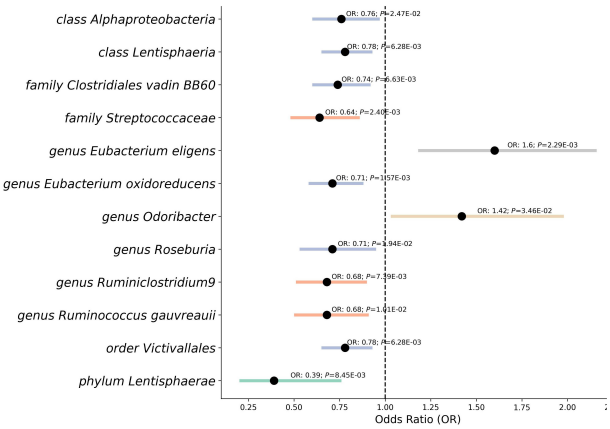

FIGURE 3
Mendelian randomization analysis between gut microbiota and the risk of RVD. This forest plot represents a Mendelian randomization analysis exploring the association between different classifications of gut microbiota and the risk of Rheumatic Valve Disease (RVD). Each point indicates the Odds Ratio (OR), showing the strength and direction of the association. Horizontal lines represent Confidence Intervals, providing a range for the true OR. Points to the left of the vertical line (OR < 1) suggest a protective effect against RVD, while points to the right (OR > 1) indicate a potential risk. *P*-value denote the statistical significance of each association.

In contrast, the *genus Eubacterium eligens* (OR 1.6, *P*=0.002) and the *genus Odoribacter* (OR 1.42, *P*=0.035) presented as potential risk factors, indicating an increased likelihood of RVD. Furthermore, our analysis pointed to the *genus Ruminococcus gauvreauii* (OR 0.68, *P*=0.010), *genus Ruminiclostridium9* (OR 0.68, *P*=0.007) suggested protective associations.

After using the MR Steiger Test, we found that no SNPs violated the causal relationship from gut microbiota to RVD. With F-statistics consistently between 14 and 1092 for each SNP, the strength of the genetic instruments used was affirmed. While most of the associations were backed by sensitivity tests like MR Egger, Weighted Median, Simple median, Maximum likelihood, Radial, Raps (Supplementary Table 3), and no outliers or pleiotropy were detected by MR-PRESSO analysis (Supplementary Table 4). During the leave-one-out sensitivity analysis (Supplementary Table 5), we identified that the *genus Ruminococcus1*, *class Bacilli* had some SNP outlier, and Radial, Raps analysis can not support their significant casual relationship with RVD, which may potentially distort the causal estimate. Then we used LDtrait (32) found 100 unique SNPs (Supplementary Table 6) with significant associations with other traits, raising the possibility of pleiotropy or confounding. We reanalyzed our data after removing the 100 SNPs and results of this reanalysis are presented in Supplementary Table 7. The associations between *genus Ruminococcus* and *class Bacilli* with RVD were also no longer significant. After careful consideration, we have decided to exclude these two exposure. The *phylum Lentisphaerae* did display pleiotropy, leading us to adopt the MR-Egger method as the main result for this taxa. These results post-FDR adjustment revealed that several GM taxas remain significant: *Class Lentisphaeria*: P_{FDR} = 0.094; *Family Clostridiales vadin BB60*: P_{FDR} = 0.099; *Family Streptococcaceae*: P_{FDR} = 0.072; *Phylum Lentisphaerae*: P_{FDR} = 0.084. Other results are considered as potentially causal associations.

Furthermore, our reverse MR analysis indicated a no significant association between RVD and these 12 microbiotas (Figure 4, Supplementary Table 8).

Mediators selection

In our Mendelian randomization study, we first examined the potential mediators including immune cell traits, cardiovascular proteins, and Estradiol levels to ascertain their effect on RVD (Figure 5, Supplementary Table 9). The analysis revealed several mediators with significant associations: $CD4^{-}CD8^{-}$ T cell Absolute Count, CD25 on $IgD^{+}CD24^{+}$ B cells, CD3 on Terminally Differentiated $CD8^{+}$ T cells, CD45RA on resting CD4 regulatory T cells, Pentraxin-related protein PTX3 levels, and Estradiol levels all showed varying degrees of association with RVD, with odds ratios ranging from 0.91 to 1.23 and *P* from 0.0292 to 0.0451. After removing confounding SNPs, The associations between Estradiol levels and RVD were no longer significant(Supplementary Table 9).

Subsequent analysis delved into how gut microbiota influences these mediators (Figure 6 and Supplementary Table 10). Notably,

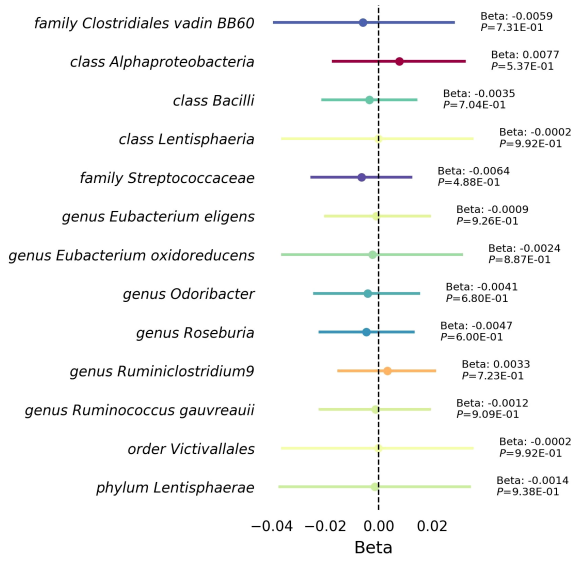

FIGURE 4
Mendelian randomization analysis between RVD gut microbiota. This forest plot illustrates a statistical analysis investigating the relationship between Rheumatic Valve Disease (RVD) and gut microbiota. Each horizontal line represents a different microbial classification, with the central dot indicating the beta coefficient (effect size). The span of each line shows the 95% confidence interval, indicating the range within which the true effect size is likely to fall. A beta coefficient to the right of the zero line (positive value) suggests a potential increase microbiota abundance with RVD, while a coefficient to the left (negative value) indicates a potential decrease microbiota abundance with RVD. The annotations to the right of each line provide the beta coefficient and *P*-value, with the *P*-value indicating the statistical significance of the association.

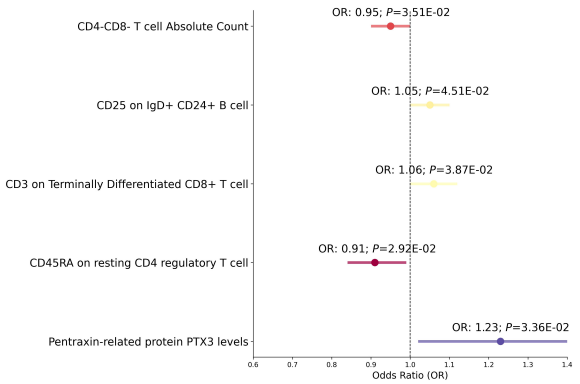

FIGURE 5
Mediation analysis between Mediator and RVD. This forest plot illustrates a statistical analysis investigating the relationship between various immunological markers and hormone levels with the risk of RVD. Each horizontal line represents the odds ratio (OR) for RVD risk. An OR below 1 suggests a potential protective effect, while an OR above 1 indicates a potential increased risk. The span of each line shows the 95% confidence interval, indicating the range within which the true effect size is likely to fall. The annotations above each line provide the OR and *P*-value, with the P-value indicating the statistical significance of the association.

the *class Lentisphaeria* was positively associated with CD25++ CD45RA+ CD4 not regulatory T cell %CD4+ T cell and $CD4^-$ $CD8^-$ T cell Absolute Count. *Genus Roseburia* increased CD45RA expression on resting CD4 regulatory T cells. Similarly, the *order Victivallales* showed positive associations with CD25++ CD45RA+ CD4 not regulatory T cell %CD4+ T cell and $CD4^-CD8^-$ T cell counts. In contrast, *genus Eubacterium oxidoreducens* and *genus Ruminiclostridium9* demonstrated an inverse relationship with CD3 on Terminally Differentiated CD8+ T cell and PTX3 levels respectively.

Mediation effect

The multivariable Mendelian randomization analysis in our study elucidated the intricate mediating role of immune cells and biomarkers in the association between gut microbiota classes and RVD (Table 1). *Class Lentisphaeria*, as well as the *order Victivallales*, showed notable mediation effects through immune cell proportions and counts, with mediation effects of 18.17%. The *genus Eubacterium oxidoreducens* displayed a direct influence on RVD, moderated by CD3 on Terminally Differentiated $CD8^+$ T cells, respectively, with mediation effects above 11%. Interestingly, *genus Roseburia* and *genus Ruminiclostridium9* also emerged as significant players, with mediation effects of 13.86% and 37.57% through CD45RA on resting CD4 regulatory T cells and Pentraxin-related protein PTX3 levels, respectively.

Discussion

In the discussion of our findings from the Mendelian randomization analysis, we reflect on the complex interactions between gut microbiota and RVD. Our study identifies 12 taxa (include 1 *phylum*, 1 *order*, 2 *class*, 2 *family*, 6 *genus*) within the gut microbiome that appear to exert a protective effect against RVD, such as the *class Lentisphaeria* and the *family Clostridiales vadin BB60*. These associations are supported by robust statistical analyses, with F-statistics indicating strong instrument strength and sensitivity analyses confirming the reliability of our findings.

The selection of mediators such as immune cell types and cardiovascular proteins revealed significant associations with RVD, suggesting that the effects of the gut microbiota on RVD may be mediated through these biological pathways. Our multivariable MR analysis confirmed the mediating roles of these factors, with certain microbiota classes showing substantial mediation effects, such as the *genus Ruminiclostridium9*'s impact on PTX3 levels.

These findings underscore the potential for gut microbiota to affect the immune system and inflammatory processes, contributing to the development or exacerbation of RVD. The mediation effects observed, ranging from 11% to over 37%, indicate that a significant portion of the microbiota's influence on RVD may be channeled through these immune and inflammatory mediators.

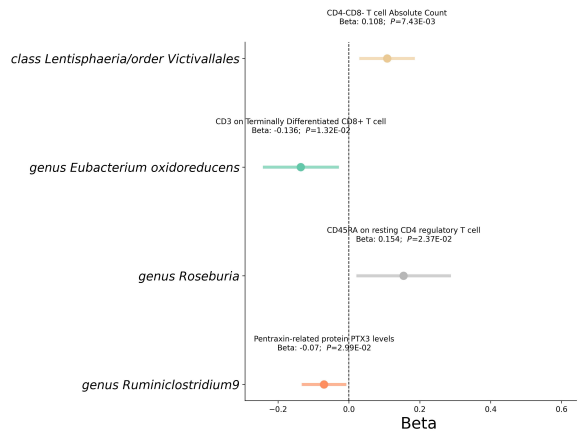

FIGURE 6
Mediation analysis between gut microbiota and Mediator. This forest plot illustrates a statistical analysis investigating the relationship between gut microbiota and mediator. Each horizontal line represents the effect of microbial classification on mediator, with the central dot indicating the beta coefficient (effect size). The span of each line shows the 95% confidence interval, indicating the range within which the true effect size is likely to fall. A beta coefficient to the right of the zero line (positive value) suggests a potential increase in RVD risk with that mediator, while a coefficient to the left (negative value) indicates a potential decrease in risk. The annotations above each line provide the beta coefficient and *P*-value, with the P-value indicating the statistical significance of the association.

The intestinal microbiota, a complex and diverse community of microorganisms, plays a crucial role in human health. This ecosystem contains over 1,500 different species of bacteria, predominantly from the *phyla Bacteroidetes* and *Firmicutes*, which together make up about 90% of the gut microbial community (33). These microorganisms are essential for various bodily functions, including digestion and immune system support.

One of the key roles of the gut microbiota is to protect the host against pathogens (34). This is achieved through various mechanisms, including colonizing mucosal surfaces and producing microbial metabolites. These metabolites can inhibit the growth of harmful bacteria and contribute to the overall health of the host. Apart from the gut, the second most complex microbial community in the human body is found in the oral cavity (35). This community is not only important for oral health but also has a significant impact on systemic health. The metabolites produced by the gut microbiota, including SCFA like acetate and butyrate, amino acid derivatives such as indole, bioactive gases, bile acid transformations, polyamines, vitamins, bioactive peptides, and endocannabinoids, play essential roles in maintaining physiological homeostasis (36). These metabolites impact various health outcomes, influencing metabolic processes, immune function, and gut-brain communication.

Within the human gut microbiome, the *class Lentisphaeria* and the *order Victivallales*, part of the *Lentisphaerae phylum*, are primarily represented by the species *Victivallis vadensis* (37). Notably, *Victivallis vadensis* is known for its unique production of acetate and formate (38). Our study associates these microbiota with a reduced risk of RVD. The mediation analysis suggests a possible mechanism: these microbes might increase the count of $CD4^-CD8^-$ (double-negative, DN) T cells, which are known to have both innate and adaptive immune functions, distinct from conventional $CD4^+$ and $CD8^+$ T cells. DN T cells, despite comprising only 3–5% of T lymphocytes in peripheral blood, have demonstrated their potential in regulating immune responses. This includes suppressing activated T cells, B cells, and dendritic cells in various mouse models (39–42). The increase in DN T cells, potentially influenced by acetate and formate from *Lentisphaeria* and *Victivallales*, could contribute to the observed reduced risk of RVD. Supporting this, research by Park et al. (43) emphasizes acetate's significant role in T lymphocyte proliferation, modulated by various cytokines and immune factors. Acetate is particularly effective in enhancing the population of IL-10 producing T lymphocytes, which are crucial for their anti-inflammatory properties. This effect of acetate aligns with our findings, suggesting that the metabolites produced by *Lentisphaeria* and *Victivallales*, particularly acetate, may contribute to the modulation of immune responses, thereby impacting the risk and progression of inflammatory conditions like RVD.

Butyrate production in the gut, crucial for colon health, is primarily facilitated by specific bacterial species within the *Ruminococcus*, *Faecalibacterium*, *Eubacterium*, and *Roseburia genera*. These bacteria play a key role in synthesizing butyrate, a vital SCFA in the gastrointestinal tract (44, 45). Butyrate is particularly influential in T cell differentiation and function, notably enhancing the presence and activity of regulatory T cells (Tregs) in the gut (46). Tregs are crucial for maintaining immune tolerance and preventing autoimmune disorders (47). In the context of RVD, our study finds a correlation between the presence of certain gut microbiota and a decreased risk of RVD. Specifically, *genera* such as *Ruminiclostridium9*, *Ruminococcus gauvreauii*, and *Eubacterium oxidoreducens* show this association. Our mediation

TABLE 1 Multivariable Mendelian randomization analysis among Gut microbiota, Mediator and RVD.

Exposure	Mediator	Outcome	Total effect	Direct effect	Mediation effect
class Lentisphaeria/ order Victivallales	CD4-CD8- T cell Absolute Count	RVD	-0.247	-0.202	18.17%
genus Eubacterium oxidoreducens	CD3 on Terminally Differentiated CD8+ T cell	RVD	-0.338	-0.300	11.23%
genus Roseburia	CD45RA on resting CD4 regulatory T cell	RVD	-0.342	-0.295	13.86%
genus Ruminiclostridium9	Pentraxin-related protein PTX3 levels	RVD	-0.384	-0.240	37.57%

analysis further suggests that *Eubacterium* can reduce the expression of CD3 on terminally differentiated $CD8^+$ T cells. Given that CD3 is integral to the T-cell receptor (TCR) complex and essential for TCR signaling and T cell activation (48), this reduction could influence the immune response related to RVD.

Additionally, our analysis indicates that *Ruminiclostridium9* can lower levels of pentraxin PTX3. PTX3, part of the long pentraxin subfamily and inducible by IL-1 or TNF, plays a role in infection defense, tissue repair, and managing inflammation in cancer (49, 50). It's also implicated in various cardiovascular diseases, including heart failure, atherosclerosis, acute coronary syndrome, peripheral vascular diseases, and rheumatic mitral valve stenosis (51–54). PTX3's involvement extends to the breakdown of fibrin-rich deposits at injury sites and the subsequent collagen deposition (55). This data suggests a multifaceted influence of the gut microbiota on RVD risk, where specific bacterial genera not only affect the butyrate levels and thus Treg function but also modulate key immune mediators like CD3 and PTX3, which are critical in cardiovascular health and immune response regulation.

RVD is primarily characterized by an autoimmune response involving $CD4^+$ T helper cells, which play a central role in orchestrating the immune response (56). These cells activate other immune cells, such as B cells and macrophages, potentially leading to inflammation and subsequent damage to the heart valves (57, 58). Within the human $CD4^+$ T cell population, there are two distinct subpopulations with different phenotypes and functions: $CD45RA^+$ resting regulatory T cells (rTreg cells) and $CD45RA^-$ activated regulatory T cells (aTreg cells). $CD45RA^+$ rTreg cells, upon stimulation, differentiate into $CD45RA^-$ aTreg cells and proliferate (59). Given the rapid turnover of aTreg cells and the robust proliferative ability of rTreg cells upon activation, therapeutic strategies aimed at expanding Treg cells ex vivo should prioritize rTreg cells, as supported by existing research. Our mediation analysis reveals that the *genus Roseburia* increases the levels of CD45RA in resting CD4 regulatory T cells. This finding is particularly significant for managing long-term inflammation in RVD. By potentially enhancing the rTreg cell population, *Roseburia* may contribute to a more controlled and balanced immune response, thereby mitigating the progression and severity of inflammation in RVD. This underscores the importance of understanding gut microbiota's influence on specific immune cell subsets, especially in the context of autoimmune diseases like RVD.

Our study interestingly suggests that the *Streptococcaceae family* may play a role in reducing the risk of RVD. This observation aligns with the absence of *Streptococcus hemolyticus*, a pathogenic bacterium associated with RVD, in the normal human gut microbiome. Our GWAS data from the European population, which did not report RVD patients, supports the hypothesis that beneficial members of the *Streptococcaceae family*, such as *Streptococcus thermophilus*, commonly found in the human gut (60), might contribute to this protective effect against RVD.

Our study distinguishes itself through its thorough and multi-faceted approach, meticulously examining the links between gut microbiota and RVD. Our methodology's strength lies in the use of diverse, rigorous analytical techniques including the weighted median, MR-Egger, IVW method, MR Steiger Test, MR RAPS and Leave-One-Out, all of which contribute to the reliability of our findings. Additionally, the implementation of the MR-PRESSO strategy enhances the validity of our results by identifying and correcting for potential outliers, thus reducing bias. A notable aspect of our research is the in-depth investigation of specific genera within the gut microbiota and their correlation with RVD. Although some correlations became statistically insignificant after adjusting for multiple tests, we maintain a focus on uncovering as many potential associations as possible, accepting the risk of encountering false positives. These findings offer valuable insights into possible biological interactions. Another strength of our study is the homogeneity of our sample population, which predominantly consists of individuals of European descent. This uniformity helps to minimize variations due to population differences, adding another layer of consistency to our research.

However, our research does have certain limitations. The most significant of these is the reliance on data from European populations, which could introduce biases and limit the generalizability of our results to other ethnic groups. Additionally, the absence of individual-level data limited our ability to delve into more complex relationships, possibly leading to an oversight of non-linear associations between the gut microbiota, immune cell traits, and RVD. Therefore, specific patterns of association, like U-shaped or J-shaped relationships, might not have been fully captured in our study.

Overall, our study adds to the growing body of evidence that gut microbiota are intricately linked to systemic diseases such as RVD. It points to the importance of understanding the microbiome's role in disease mechanisms, which could pave the way for novel therapeutic strategies that target the microbiome to modulate disease risk and progression.

Future research

Future research should pivot towards a multifaceted approach. This includes expanding the scope to include a broader range of ethnic groups for a more comprehensive understanding of microbiota variations across different demographics. A deeper analysis of how specific bacteria and their metabolites, such as acetate and butyrate, influence immune responses is crucial. Investigating therapeutic interventions like diet modifications or probiotics can reveal how changes in the gut microbiome impact RVD risk and progression. Personalized microbiome-based treatment strategies, tailored to individual microbiome compositions, could be a breakthrough in RVD prevention and management. Long-term studies are essential to track gut microbiota changes over time and their correlation with the development of RVD. Employing advanced sequencing and data analysis techniques will uncover novel microbial species and complex interactions within the microbiome. Lastly, public health education focusing on the importance of gut health and its broader impact on diseases like RVD is vital. Such a comprehensive and integrated research approach will pave the way for more effective strategies in understanding and managing RVD.

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 data utilized for this study are accessible in the public data, have received ethical approval, and were collected with informed consent from the participants.

Author contributions

XC: Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. GH: Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. DN: Writing – review & editing, Validation, Supervision, Software, Methodology, Formal analysis. DW: Writing – review & editing, Validation, Supervision, Resources, Funding acquisition.

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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.2024.1362753/full#supplementary-material

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Genetic association of the gut microbiota with epigenetic clocks mediated by inflammatory cytokines: a Mendelian randomization analysis

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Background: A new aging biomarker epigenetic clock has been developed. There exists a close link between aging and gut microbiota, which may be mediated by inflammatory cytokines. However, the relationship between the epigenetic clock, gut microbiota, and the mediating substances is unclear.

Methods: Two large genome-wide association meta-analyses were analyzed by two-sample Mendelian randomization. The results between gut microbiota and epigenetic clock were investigated using the four methods (Inverse variance weighted, MR-Egger, weighted median, MR-PRESSO). Genetic correlation was measured by Linked disequilibrium score regression (LDSC). The correctness of the study direction was checked by the Steiger test. Cochran's Q statistic and MR-Egger intercept were used as sensitivity analyses of the study. The two-step method was used to examine the mediating role of inflammatory cytokines. We use the Benjamini-Hochberg correction method to correct the P value.

Results: After FDR correction, multiple bacterial genera were significantly or suggestively associated with four epigenetic clocks (GrimAge, HannumAge, IEAA, PhenoAge). And we detected several inflammatory factors acting as mediators of gut microbiota and epigenetic clocks.

Conclusion: This study provides genetic evidence for a positive and negative link between gut microbiota and aging risk. We hope that by elucidating the genetic relationship and potential mechanisms between aging and gut microbiota, we will provide new avenues for continuing aging-related research and treatment.

KEYWORDS

gut microbiota, epigenetic clocks, inflammatory cytokines, Mendelian randomization analysis, mediation

Introduction

Aging has been an area of particular concern to humans throughout history. One of the characteristics of aging is epigenetic aging (1). Because most clinical biomarkers are inadequate to represent the underlying mechanisms of aging, it has been difficult to identify molecular targets for interventions for human health longevity (2). Recently, research has shown that the "epigenetic clock", which is a biomarker of aging found at specific cytosine-phospho-guanine (CpG) sites, can provide accurate age estimates for any tissue or organ throughout the human life course (3). The emergence of epigenetic clocks may help solve many long-standing questions, such as the central question of aging, "How do we get old?".

The epigenetic clock acts as a heritable indicator of the DNA of biological aging by capturing the unique characteristics of epigenetic aging based on different CpG sites (4). HannumAge (5) and Horvath (6) clocks constitute the inaugural generation of epigenetic clocks, predicting chronological age utilizing DNA methylation data. These methodologies have been extensively applied across blood samples and 51 distinct human tissue and cell types. HannumAge delineated 71 age-associated CpG sites within blood samples (6), whereas HorvathAge ascertained 353 age-related CpG sites across various human tissues and cell types, with adjustments made for blood cell counts (6). Intrinsic Epigenetic Age Acceleration (IEAA) as a derivative of Horvath was developed after the removal of blood cell composition estimates (7). A second representative epigenetic clock, PhenoAge (Levine et al., 2018) and GrimAge (7), predicts associated morbidity and mortality by combining some information about risk and age (e.g. smoking, plasma protein levels, white blood cell counts). PhenoAge included data on 9 clinical biomarkers associated with mortality and 513 CpGs (8). GrimAge included data on seven plasma proteins and 1030 CpGs associated with smoking (7). The second generation of representative genetic clocks can measure the incidence of various diseases and is better at predicting mortality than the first generation (8, 9). GrimAge outperforms PhenoAge and first-generation epigenetic clocks in predicting the time of death (10, 11).

At present, multiple studies has proved that gut microbiota occupies an important position in the aging process (12–14). Dysregulation of gut microbiota is implicated in the modulation of immune and inflammatory responses during the aging process and is associated with the onset of numerous age-related diseases, both intestinal and systemic (13). Interestingly, from the perspective of interactions between gut microbes, inflammatory mediators, and the immune system, the regulation of gut microbiota may help promote both physiological and non-pathological aging processes and may be a potential target for aging interventions (12). However, the genetic relationship and mechanisms of gut microbiota and aging are unclear, and no researchers have explored the causal relationship between gut microbiota and aging from the perspective of epigenetic clocks. Therefore, we use Mendelian randomization (MR) as a novel method that can be used to study genetic associations and causality between the gut microbiota and the epigenetic clock.

MR is a statistical method to assess the causal relationship between the genetic variation associated with exposure and the outcome (15). Compared with traditional observation methods, MR is less affected by residual confounding and reverse causation (16). In the MR Analysis, we are not only interested in the link between epigenetic clocks and gut microbiota but also in the mechanism of how exposure affects the outcome. Mediation MR Analyses can attempt to determine the causal pathways by which exposure affects outcomes and their relative importance. Mediating MR Analysis can identify factors mediating between exposure and outcome, and interventions on these mediating factors can mitigate or enhance the impact of exposure on outcome (17).

Consequently, we conducted a two-sample MR Analysis to investigate the association between gut microbiota and the epigenetic clock. Additionally, a mediation MR Analysis was employed to elucidate the mechanistic role of inflammatory cytokines in the relationship between gut microbiota and the epigenetic clock.

Methods

Research description

Figures 1, 2 illustrate the MR research description. Two-sample Mendelian randomization analysis was performed to analyze the link between gut microbiota and the epigenetic clock. Instrumental variables independent of confounding factors such as sex and age were used in the MR Analysis to simulate the random assignment of progeny single nucleotide polymorphisms (SNPs) in randomized controlled trials (RCTS). In addition, the MR design must satisfy three assumptions: (i) genetic tools are correlated with exposure; (ii) genetic tools are independent of potential confounding factors; (iii) Genetic instrumental variables affect results only through exposure. We then used a two-step method mediated MR Analysis to analyze the mediating role of inflammatory cytokines between gut microbiota and the epigenetic clock.

Exposure data source

Gut microbiota genetic variation data comes from the MiBioGen Consortium (https://mibiogen.gcc.rug.nl/), which is by far the largest gut microbiota genome-wide meta-analysis (18). 18340 individuals were included to analyze the composition of microorganisms in the variable regions of 16S rRNA genes V4, V3-V4, and V1-V2. By mapping microbiota quantitative trait loci (mbQTL), the relationship between host genetic variation and bacterial species abundance in gut microbiota was identified. 131 genera with an average abundance greater than 1% were identified (of which 12 were unknown). Therefore, 119 genera were included in this study for MR Analysis. The instrumental variables (IVs) of gut microbiota were chosen as follows (1): Significant SNPs at the genome-wide level ($P < 1\times10–5$) (19); (2) SNP aggregation using PLINK algorithm ($r^2 = 0.001$, window size = 10mB); (3) Palindromic SNPs will be removed (20).

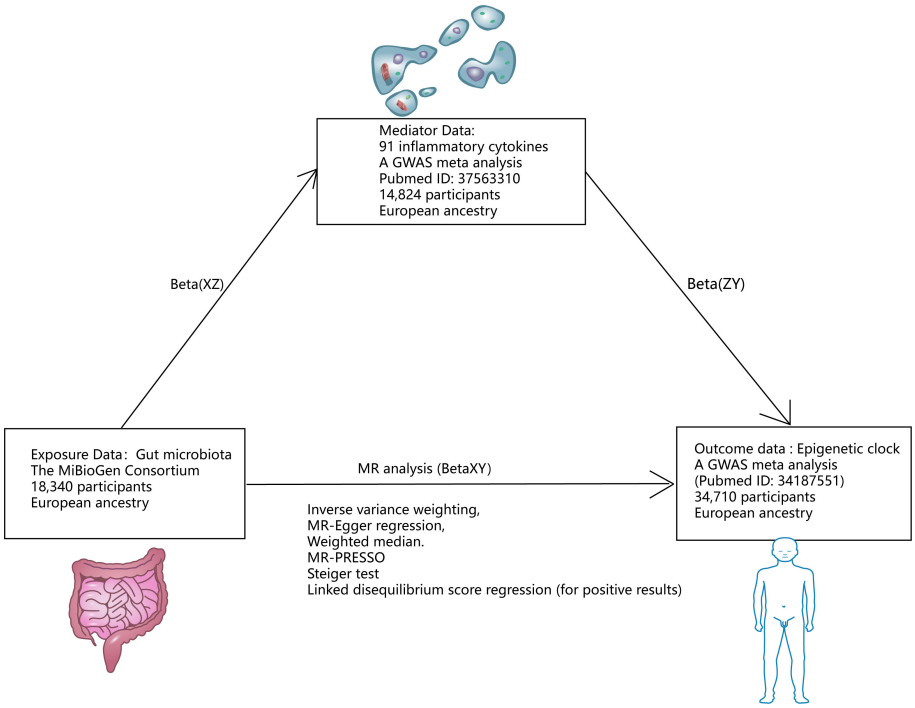

FIGURE 1
Research design. *Mediating effect =Beta(XZ) x Beta(ZY); Direct effect =Beta(XY)-Beta(XZ) x Beta(ZY).

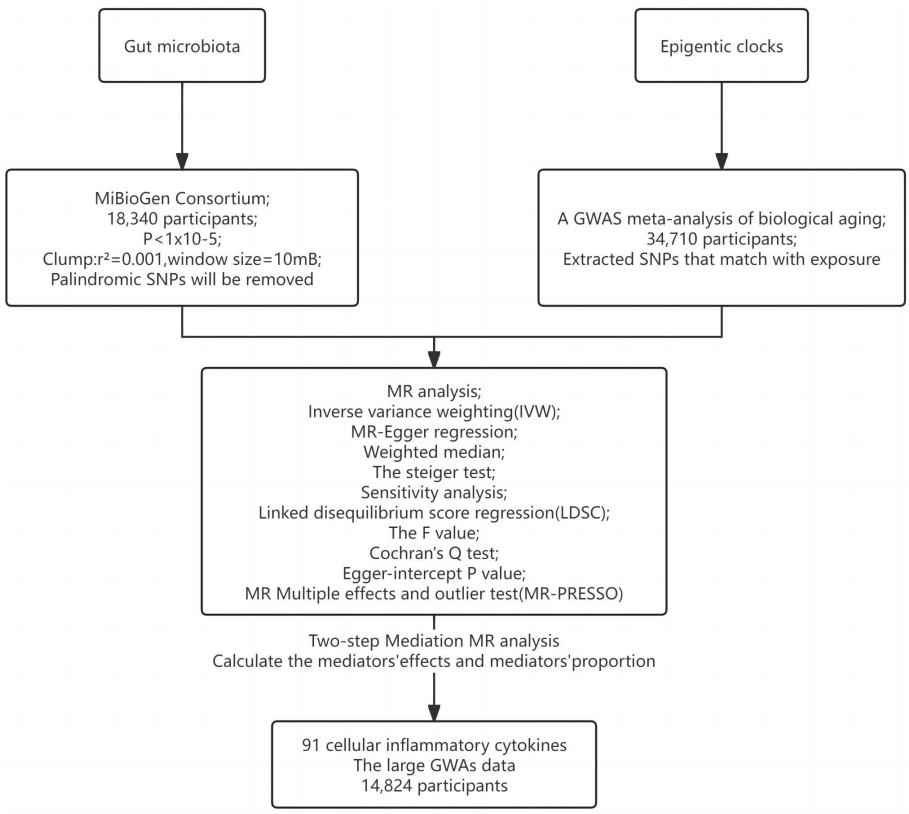

FIGURE 2
Study flow chart.

Outcome data source

Genetic associations of epigenetic clocks (HannumAge, IEAA, PhenoAge, and GrimAge) in 34,710 European participants were derived from a recent GWAS meta-analysis of biological aging (21). Of the 28 subjects of European descent in the study, women participated in 57.3% of the studies. Horvath epigenetic age calculator software (https://dnamage.genetics.ucla.edu) was used in the study or independent script age-adjusted estimate of DNA methylation HannumAge, IEAA, PhenoAge, GrimAge. Abnormal samples of clock methylation estimates that differ by +/-5 standard deviations from the mean will be excluded. Quality control and interpolation procedures were systematically applied across each study. For each cohort, the GWAS summary statistics underwent refinement through adjustments for sex and genetic principal components employing an additive linear model. Then, the data of different races were analyzed by METAL software using the inverse variance fixed-effect scheme (22). Summary statistics were processed and coordinated for each cohort study using the R software package EasyQC (23).

Mediator data source

Data on the genetic variation of 91 cellular inflammatory cytokines were obtained from the latest large GWAS data, published in August 2023 (24). The investigation quantified 91 inflammatory cytokines across 14,824 subjects and conducted a genome-wide protein quantitative Trait Locus (pQTL) analysis utilizing the Olink Target platform. This was subsequently followed by a meta-analysis of the collected data. These data were combined with disease GWASs to represent the impact of disease-associated variants. MR And mediation analyses are used to identify proteins that are causally linked to the cause of immune-mediated disease.

Statistical analysis

First, a two-sample MR Analysis was performed for 4 epigenetic clocks and gut microbiota. The random effects inverse variance weighting (IVW) was used as the main analysis result. The F-value was used to measure the potency of instrumental variables (IVs) to test whether this study might violate the first MR Hypothesis (25). Cochran's Q test was used to quantify the heterogeneity of IVs (26). Horizontal pleiotropy may violate the third MR Hypothesis. We used the MR-Egger regression (27), weighted median (28) method, and MR Multiple effects and outlier test (MR-PRESSO) (29) to test and attempt to correct possible violations of the second and third MR Assumptions. In the weighted regression model, MR-Egger realizes directional pleiotropy by intercept. A value where the intercept term significantly deviates from zero suggests the existence of horizontal pleiotropy (27). The weighted median method sorts the MR Estimates obtained using each IV and then weights the reciprocal of its variance. Individual MR Estimates are provided by median results (27). The weighted median assumes that at least half of the tools are valid and do not require any pleiotropy to affect the intermediate phenotype (30). The SNP results from MR-PRESSO exposure were regressed and the square of the residual was used to identify outlier SNPS that may have pleiotropic effects (29). At the same time, we consider the reverse causality between the gut microbiota and the epigenetic clock, so we use the Steiger test to ensure that our directionality is accurate and that $P < 0.05$ is significant (31). We employed linkage disequilibrium score regression (LDSC) (available at https://github.com/bulik/ldsc) to evaluate the genetic correlation between Mendelian Randomization (MR) positive outcomes for gut microbiota and epigenetic clocks (32). LDSC represents a robust methodology for the analysis of genetic correlations across complex diseases or traits. It is capable of differentiating between genuine polygenic signals and confounding biases, such as population stratification, among others. If the genetic association is statistically significant as well as by LDSC analysis, we can be sure of a causal association between the two genetic phenotypes (33). When negative genetic particles are present in the sample, the LDSC will not be able to produce results (34). Because LDSC only considers genetic correlations, causation cannot be judged (35). Therefore, when the results of LDSC are inconsistent with the analysis result of MR Analysis, we focus on the analysis result of MR Analysis.

In order to explore the mechanism of positive gut microbiota and epigenetic clock outcomes, we used two-step mediated MR To explore the mediated association of 91 inflammatory cytokines between positive gut microbiota and epigenetic clock. We then screened for mediating inflammatory cytokines associated with positive gut microbiota and epigenetic clocks based on the following criteria (1): There is a genetic association between the epigenetic clock and gut microbiota. (2) There is a genetic association between the mediating inflammatory cytokines and gut microbiota, and the effect of education on mediating should be one-way, because if there is a bidirectional relationship between the two, the effectiveness of mediation analysis may be affected (36). (3) There is a genetic association between the epigenetic clock and inflammatory cytokines and the epigenetic clock. The detailed selection of mediators, as well as the calculation of mediators' effect and mediators' proportion are shown in Figure 1.

R (version 4.3.1), TwoSampleMR (0.5.5), Mendelian Randomization (0.5.0), MR-PRESSO, and LDSC software packages (37–39) were used for all analyses. The False Discovery Rate (FDR) method was used to correct P values, according to the Benjamin and Hochberg (BH) method. When $q < 0.1$, the results were significant. While $P < 0.05$ but $q > 0.1$ was considered suggestive of causality.

Results

Detailed information regarding the selected instrumental variables (IVs) is presented in Supplementary Table S1 of the Supplementary Material. The F-statistic for each IV exceeds 10, signifying the absence of weak instrumental variables within this study. The positive MR Results are shown in Tables 1, 2 and

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TABLE 1 Genetic association of gut microbiota and epigenetic clock.

Outcome	Exposure	Methods	P-value	OR(95%CI)	q-value	LDSC rg_p
GrimAge	*Ruminococcusgnavus_group*	MR Egger	0.1577	0.48 (0.18–1.23)	1.0000	0.04
		Weighted median	0.4655	0.91 (0.69–1.18)	1.0000	
		Inverse variance weighted	0.0002	0.78 (0.64–0.97)	0.0261	
GrimAge	*Dorea*	MR Egger	0.0842	2.79 (1.01–7.72)	1.0000	
		Weighted median	0.0221	1.77 (1.09–2.89)	1.0000	
		Inverse variance weighted	0.0117	1.60 (1.11–2.32)	0.4635	
GrimAge	*Eisenbergiella*	MR Egger	0.6698	1.44 (0.28–7.31)	1.0000	0.28
		Weighted median	0.0285	1.39 (1.04–1.87)	0.8476	
		Inverse variance weighted	0.0286	1.26 (1.02–1.56)	0.8511	
GrimAge	*Lactococcus*	MR Egger	0.7944	1.16 (0.39–3.43)	1.0000	
		Weighted median	0.0002	1.56 (1.17–2.08)	0.0269	
		Inverse variance weighted	0.0002	1.44 (1.14–1.83)	0.0137	
GrimAge	*Prevotella7*	MR Egger	0.7160	0.82 (0.29–2.33)	1.0000	0.53
		Weighted median	0.0588	0.80 (0.64–1.01)	0.9998	
		Inverse variance weighted	0.0432	0.84 (0.71–0.99)	1.0000	
GrimAge	*Ruminococcaceae-UCG-010*	MR Egger	0.5421	1.72 (0.35–8.50)	1.0000	
		Weighted median	0.0640	1.69 (0.97–2.93)	0.9522	
		Inverse variance weighted	0.0486	1.54 (1.00–2.37)	0.8259	
GrimAge	*Victivallis*	MR Egger	0.3352	1.91 (0.55–6.59)	1.0000	
		Weighted median	0.1126	1.19 (0.96–1.47)	1.0000	
		Inverse variance weighted	0.0456	1.19 (1.00–1.40)	0.9049	
HannumAge	*Haemophilus*	MR Egger	0.0756	1.83 (1.04–3.23)	1.0000	0.29
		Weighted median	0.0052	1.59 (1.15–2.20)	0.6187	
		Inverse variance weighted	0.0004	1.43 (1.12–1.83)	0.0462	
HannumAge	*Ruminococcaceae-UCG-004*	MR Egger	0.5565	0.60 (0.12–3.04)	1.0000	0.43

(Continued)

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TABLE 1 Continued

Outcome	Exposure	Methods	P-value	OR(95%CI)	q-value	LDSC rg_p
		Weighted median	0.1456	0.76 (0.52–1.10)	1.0000	
		Inverse variance weighted	0.0456	0.76 (0.57–0.99)	1.0000	
HannumAge	*Sellimonas*	MR Egger	0.6098	1.31 (0.49–3.51)	1.0000	0.92
		Weighted median	0.1749	1.18 (0.93–1.49)	1.0000	
		Inverse variance weighted	0.0478	1.19 (1.00–1.41)	1.0000	
HannumAge	*Senegalimassilia*	MR Egger	0.2463	2.28 (0.74–7.05)	1.0000	
		Weighted median	0.1435	1.36 (0.90–2.06)	1.0000	
		Inverse variance weighted	0.0211	1.47 (1.06–2.05)	1.0000	
IEEA	*Coprococcus1*	MR Egger	0.2771	1.63 (0.71–3.76)	0.9992	0.72
		Weighted median	0.0346	1.60 (1.03–2.46)	1.0000	
		Inverse variance weighted	0.0320	1.42 (1.03–1.95)	0.7625	
IEEA	*Howardella*	MR Egger	0.2814	1.64 (0.71–3.79)	0.9848	
		Weighted median	0.0313	1.33 (1.03–1.73)	1.0000	
		Inverse variance weighted	0.0240	1.23 (1.03–1.48)	0.9526	
IEEA	*Peptococcus*	MR Egger	0.6249	1.23 (0.55–2.76)	0.9785	0.04
		Weighted median	0.0315	1.35 (1.03–1.78)	1.0000	
		Inverse variance weighted	0.0320	1.24 (1.02–1.52)	0.6356	
IEEA	*Subdoligranulum*	MR Egger	0.0452	3.08 (1.19–7.94)	1.0000	0.10
		Weighted median	0.1246	1.48 (0.90–2.43)	1.0000	
		Inverse variance weighted	0.0253	1.56 (1.06–2.29)	0.7518	
IEEA	*Veillonella*	MR Egger	0.5832	2.81 (0.09–88.38)	0.9774	0.72
		Weighted median	0.1737	1.41 (0.86–2.33)	1.0000	
		Inverse variance weighted	0.0172	1.61 (1.09–2.39)	1.0000	
PhenoAge	*Ruminococcustorques_group*	MR Egger	0.4172	0.56 (0.15–2.09)	0.9547	
			0.0346	0.49 (0.26–0.95)	0.8229	

(Continued)

TABLE 1 Continued

Outcome	Exposure	Methods	P-value	OR(95%CI)	q-value	LDSC rg_p
		Weighted median				
		Inverse variance weighted	0.0249	0.58 (0.36–0.93)	0.7419	
PhenoAge	*Dorea*	MR Egger	0.2490	2.27 (0.62–8.29)	1.0000	
		Weighted median	0.1570	1.58 (0.84–3.00)	1.0000	
		Inverse variance weighted	0.0279	1.69 (1.06–2.69)	0.6647	
PhenoAge	*Lachnospiraceae-UCG-001*	MR Egger	0.2736	2.32 (0.55–9.72)	1.0000	
		Weighted median	0.0641	1.46 (0.98–2.18)	1.0000	
		Inverse variance weighted	0.0370	1.41 (1.02–1.93)	0.6286	
PhenoAge	*Lachnospiraceae-UCG-008*	MR Egger	0.1223	3.62 (0.83–15.84)	1.0000	
		Weighted median	0.0326	1.54 (1.04–2.30)	0.9684	
		Inverse variance weighted	0.0004	1.51 (1.14–2.00)	0.0512	
PhenoAge	*Lactobacillus*	MR Egger	0.0259	0.27 (0.11–0.69)	1.0000	
		Weighted median	0.0715	0.69 (0.47–1.03)	1.0000	
		Inverse variance weighted	0.0379	0.71 (0.51–0.98)	0.5643	
PhenoAge	*Tyzzerella3*	MR Egger	0.4171	1.71 (0.49–5.95)	0.9732	
		Weighted median	0.0571	1.36 (0.99–1.88)	1.0000	
		Inverse variance weighted	0.0005	1.39 (1.10–1.75)	0.0299	

Figure 3. All MR Results are shown in Figure 4 and Supplementary Materials.

The results of gut microbiota and GrimAge

GrimAge has a significant causality with *Ruminococcusgnavus_group* (P = 0.002, Odds Ratio(OR)= 0.78, 95% Confidence Interval(CI) = 0.64–0.97, q = 0.065, rg_p_{LDSC} = 0.043), *Lactococcus* (P = 0.0002, OR = 1.44, 95%CI = 1.14–1.83, q = 0.014).

GrimAge shows a suggestive causality with *Dorea* (P = 0.012, OR = 1.6, 95%CI = 1.11–2.32, q = 0.463), *Eisenbergiella* (P = 0.029, OR = 1.26, 95%CI = 1.02–1.56, q = 0.851, rg_p_{LDSC} = 0.278), *Prevotella7* (P = 0.043, OR = 0.84, 95%CI = 0.71–0.99, q = 1, rg_p_{LDSC} = 0.526), *Ruminococcaceae-UCG-010* (P = 0.049, OR = 1.54, 95%CI = 1.00–2.37, q = 0.826), and *Victivallis* (P = 0.046, OR = 1.19, 95%CI = 1.00–1.40, q = 0.905).

The results of gut microbiota and HannumAge

HannumAge had a significant causality with *Haemophilus* (P = 0.0004, OR = 1.43, 95%CI = 1.12–1.83, q = 0.046, rg_p_{LDSC} = 0.29).

HannumAge had a suggestive causality with *Ruminococcaceae-UCG-004* (P = 0.046, OR = 0.76, 95%CI = 0.57–0.99, q = 1, rg_p_{LDSC} = 0.435), *Sellimonas* (P = 0.048, OR = 1.19, 95%CI = 1.00–1.41, q = 1,

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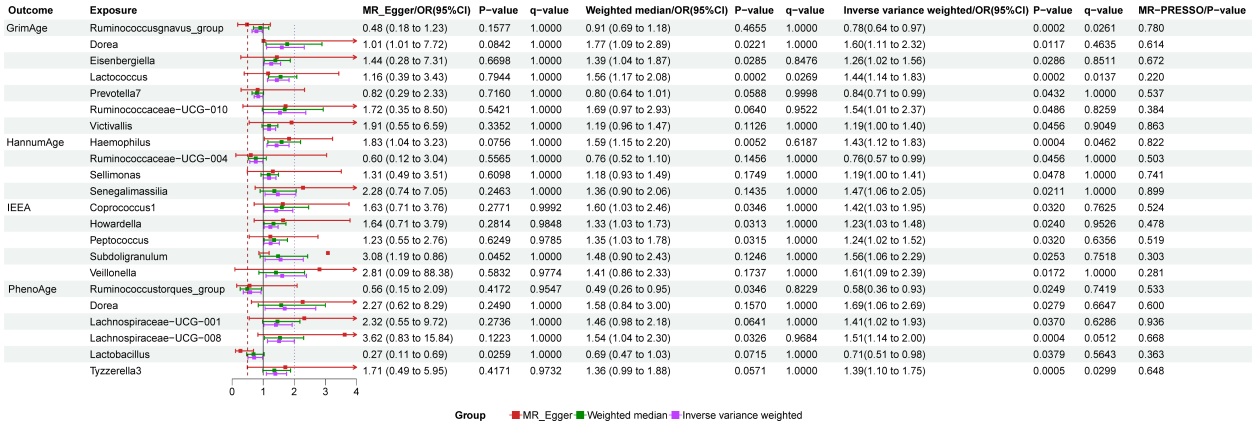
TABLE 2 Sensitivity analysis of the results of Mendelian randomization of gut microbiota and epigenetic clock.

Outcome	Exposure	MR PRESSO Global_test P value	Cochran's Q P value	Egger-intercept P value	Steiger
GrimAge	*Ruminococcusgnavus_group*	0.78	0.75	0.32	2.48842E-41
GrimAge	*Dorea*	0.614	0.59	0.29	9.20712E-26
GrimAge	*Eisenbergiella*	0.672	0.64	0.88	3.432E-37
GrimAge	*Lactococcus*	0.22	0.17	0.70	1.79718E-31
GrimAge	*Prevotella7*	0.537	0.50	0.96	7.74447E-42
GrimAge	*Ruminococcaceae-UCG-010*	0.384	0.36	0.89	3.73886E-16
GrimAge	*Victivallis*	0.863	0.85	0.47	3.51908E-40
HannumAge	*Haemophilus*	0.822	0.81	0.38	9.94033E-36
HannumAge	*Ruminococcaceae-UCG-004*	0.503	0.48	0.79	6.47868E-29
HannumAge	*Sellimonas*	0.741	0.74	0.85	5.16753E-37
HannumAge	*Senegalimassilia*	0.899	0.89	0.48	1.2944E-16
IEEA	*Coprococcus1*	0.524	0.46	0.73	8.15555E-34
IEEA	*Howardella*	0.478	0.44	0.52	5.58563E-38
IEEA	*Peptococcus*	0.519	0.47	0.98	5.95127E-43
IEEA	*Subdoligranulum*	0.303	0.27	0.16	1.93328E-29
IEEA	*Veillonella*	0.281	0.23	0.76	3.23535E-18
PhenoAge	*Ruminococcustorques_group*	0.533	0.48	0.96	6.06165E-28
PhenoAge	*Dorea*	0.6	0.58	0.64	3.04292E-26
PhenoAge	*Lachnospiraceae-UCG-001*	0.936	0.92	0.50	4.76372E-40
PhenoAge	*Lachnospiraceae-UCG-008*	0.668	0.64	0.27	2.09483E-37
PhenoAge	*Lactobacillus*	0.363	0.31	0.07	2.37984E-33
PhenoAge	*Tyzzerella3*	0.648	0.62	0.75	4.61924E-48

rg_p_{LDSC} = 0.917), *Senegalimassilia* (P = 0.021, OR = 1.47, 95%CI = 1.06–2.05, q = 1).

The results of gut microbiota and IEAA

IEEA had a suggestive causality with *Coprococcus1* (P = 0.032, OR = 1.42, 95%CI = 1.03–1.95, q = 0.762, rg_p_{LDSC} = 0.72), *Howardella* (P = 0.024, OR = 1.54, 95%CI = 1.00–2.37, q = 0.953), *Peptococcus* (P = 0.03, OR = 1.24, 95%CI = 1.02–1.52, q = 0.636, rg_p_{LDSC} = 0.041), *Subdoligranulum* (P = 0.025, OR = 1.56, 95%CI = 1.06–2.29, q = 0.752, rg_p_{LDSC} = 0.099), *Veillonella* (P = 0.017, OR = 1.61, 95%CI = 1.09–2.39, q = 1, rg_p_{LDSC} = 0.718).



Outcome	Exposure	MR_Egger/OR(95%CI)	P-value	q-value	Weighted median/OR(95%CI)	P-value	q-value	Inverse variance weighted/OR(95%CI)	P-value	q-value	MR-PRESSO/P-value
GrimAge	Ruminococcusgnavus_group	0.48 (0.18 to 1.23)	0.1577	1.0000	0.91 (0.69 to 1.18)	0.4655	1.0000	0.78(0.64 to 0.97)	0.0002	0.0261	0.780
	Dorea	1.01 (1.01 to 7.72)	0.0842	1.0000	1.77 (1.09 to 2.89)	0.0221	1.0000	1.60(1.11 to 2.32)	0.0117	0.4635	0.614
	Eisenbergiella	1.44 (0.28 to 7.31)	0.6698	1.0000	1.39 (1.04 to 1.87)	0.0285	0.8476	1.26(1.02 to 1.56)	0.0286	0.8511	0.672
	Lactococcus	1.16 (0.39 to 3.43)	0.7944	1.0000	1.56 (1.17 to 2.08)	0.0002	0.0269	1.44(1.14 to 1.83)	0.0002	0.0137	0.220
	Prevotella7	0.82 (0.29 to 2.33)	0.7160	1.0000	0.80 (0.64 to 1.01)	0.0588	0.9998	0.84(0.71 to 0.99)	0.0432	1.0000	0.537
	Ruminococcaceae-UCG-010	1.72 (0.35 to 8.50)	0.5421	1.0000	1.69 (0.97 to 2.93)	0.0640	0.9522	1.54(1.01 to 2.37)	0.0486	0.8259	0.384
	Victivallis	1.91 (0.55 to 6.59)	0.3352	1.0000	1.19 (0.96 to 1.47)	0.1126	1.0000	1.19(1.00 to 1.40)	0.0456	0.9049	0.863
HannumAge	Haemophilus	1.83 (1.04 to 3.23)	0.0756	1.0000	1.59 (1.15 to 2.20)	0.0052	0.6187	1.43(1.12 to 1.83)	0.0004	0.0462	0.822
	Ruminococcaceae-UCG-004	0.60 (0.12 to 3.04)	0.5565	1.0000	0.76 (0.52 to 1.10)	0.1456	1.0000	0.76(0.57 to 0.99)	0.0456	1.0000	0.503
	Sellimonas	1.31 (0.49 to 3.51)	0.6098	1.0000	1.18 (0.93 to 1.49)	0.1749	1.0000	1.19(1.00 to 1.41)	0.0478	1.0000	0.741
	Senegalimassilia	2.28 (0.74 to 7.05)	0.2463	1.0000	1.36 (0.90 to 2.06)	0.1435	1.0000	1.47(1.06 to 2.05)	0.0211	1.0000	0.899
IEEA	Coprococcus1	1.63 (0.71 to 3.76)	0.2771	0.9992	1.60 (1.03 to 2.46)	0.0346	1.0000	1.42(1.03 to 1.95)	0.0320	0.7625	0.524
	Howardella	1.64 (0.71 to 3.79)	0.2814	0.9848	1.33 (1.03 to 1.73)	0.0313	1.0000	1.23(1.03 to 1.48)	0.0240	0.9526	0.478
	Peptococcus	1.23 (0.55 to 2.76)	0.6249	0.9785	1.35 (1.03 to 1.78)	0.0315	1.0000	1.24(1.02 to 1.52)	0.0320	0.6356	0.519
	Subdoligranulum	3.08 (1.19 to 0.86)	0.0452	1.0000	1.48 (0.90 to 2.43)	0.1246	1.0000	1.56(1.06 to 2.29)	0.0253	0.7518	0.303
	Veillonella	2.81 (0.09 to 88.38)	0.5832	0.9774	1.41 (0.86 to 2.33)	0.1737	1.0000	1.61(1.09 to 2.39)	0.0172	1.0000	0.281
PhenoAge	Ruminococcustorques_group	0.56 (0.15 to 2.09)	0.4172	0.9547	0.49 (0.26 to 0.95)	0.0346	0.8229	0.58(0.36 to 0.93)	0.0249	0.7419	0.533
	Dorea	2.27 (0.62 to 8.29)	0.2490	1.0000	1.58 (0.84 to 3.00)	0.1570	1.0000	1.69(1.06 to 2.69)	0.0279	0.6647	0.600
	Lachnospiraceae-UCG-001	2.32 (0.55 to 9.72)	0.2736	1.0000	1.46 (0.98 to 2.18)	0.0641	1.0000	1.41(1.02 to 1.93)	0.0370	0.6286	0.936
	Lachnospiraceae-UCG-008	3.62 (0.83 to 15.84)	0.1223	1.0000	1.54 (1.04 to 2.30)	0.0326	0.9684	1.51(1.14 to 2.00)	0.0004	0.0512	0.668
	Lactobacillus	0.27 (0.11 to 0.69)	0.0259	1.0000	0.69 (0.47 to 1.03)	0.0715	1.0000	0.71(0.51 to 0.98)	0.0379	0.5643	0.363
	Tyzzerella3	1.71 (0.49 to 5.95)	0.4171	0.9732	1.36 (0.99 to 1.88)	0.0571	1.0000	1.39(1.10 to 1.75)	0.0005	0.0299	0.648

FIGURE 3
Forest map of gut microbiota and epigenetic clock positive results.

The results of gut microbiota and PhenoAge

PhenoAge had a significant causality with *Lachnospiraceae-UCG-008* (P = 0.0004, OR = 1.51, 95%CI = 1.14–2.00, q = 0.051), *Tyzzerella3* (P = 0.0005, OR = 1.39, 95%CI = 1.10–1.75, q = 0.03).

PhenoAge had a suggestive causality with *Ruminococcustorques_group* (P = 0.025, OR = 0.58, 95%CI = 0.36–0.93, q = 0.742), *Dorea* (P = 0.028, OR = 1.69, 95%CI = 1.06–2.69, q = 0.665), *Lachnospiraceae-UCG-001* (P = 0.037, OR = 1.41, 95%CI = 1.02–1.93, q = 0.629), *Lactobacillus* (P = 0.038, OR = 0.71, 95%CI = 0.51- 0.98, q = 0.564).

Sensitivity analysis

IVW, MR-Egger, and weighted median methods show the same causal estimates of direction. There are no outliers in the MR-PRESSO method, and the MR Egger intercept test (P < 0.05) indicates that horizontal pleiotropy does not exist in MR research. Cochran's Q test (P < 0.05) found no heterogeneity among instrumental variables. Steiger test (P < 0.05) indicated that the direction of MR Analysis was correct and there was no reverse causality.

Mediation MR Analysis

We used formulas to calculate the direct and mediated effects of inflammatory factors between the gut microbiota and the epigenetic clock (Mediating effect =Beta(XZ) x Beta(ZY); Direct effect = Beta (XY) - Beta (XZ) x Beta (ZY). Among the 91 inflammatory factors, our study found that 4 inflammatory factors met the screening criteria, so mediation analysis was included and the mediation effect and mediation ratio of inflammatory factors were calculated. Beta-nerve growth factor plays a mediating role in *Howardella* and IEAA (mediator effect: -4.08%, direct effect: 25.1%). Oncostatin-M plays a mediating role in *Ruminococcaceae-UCG-010* and GrimAge (mediator effect: -7.71%, direct effect: 59.92%). Interleukin-12 subunit B plays a mediating role in *Prevotella7* and GrimAge (mediator effect: -0.43%, direct effect: -16.96%). C-C motif chemokine 25 plays a mediating role in *Lachnospiraceae-UCG-008* and PhenoAge (mediator effect: -0.35%, direct effect: 41.5%).

Discussion

In recent years, population aging has posed a global challenge, resulting in increased burdens on national healthcare systems, so we need to explore how to slow down aging and extend life (40). By MR Analysis of four kinds of epigenetic clocks with aging characteristics, genetic correlation with gut microbiota was found. In addition, further mediated MR Analysis identified the inflammatory cytokine pathways that contribute to aging in the gut microbiota. Gut microbiota is associated with aging, providing potential targets for new interventions to promote healthy aging

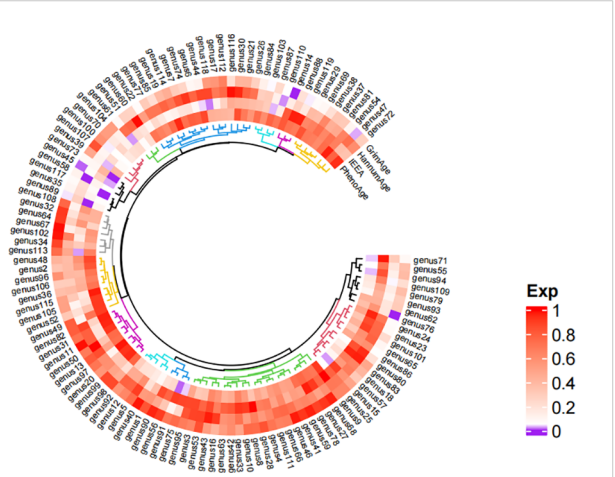


FIGURE 4
Heat map of the results of Mendelian randomized analysis of gut microbiota and epigenetic clock. *Purple represents positive results, and white and red represent negative results. The comparison table of gut microbiota is in the Supplementary Material.

(41). The results of LDSC regression analysis showed that there were suggestive genetic correlations between some epigenetic clock and gut microbiota.

Potential causal link between epigenetic clock and gut microbiota

Studies have shown that the periodicity and activity of epigenetic clock genes are significantly associated with changes in age (42). Biological aging may be related to the richness and diversity of gut microbiota (12, 13, 43). The results of previous studies are consistent with our MR Analysis in which we found that multiple gut bacteria genera have genetic associations with epigenetic clocks. Higher biological age and lower physical fitness were significantly associated with increased *Dorea* abundance (44). Observational study results have shown a significant increase in *Salmonella* and *Haemophilus* in older individuals (45, 46). *Coprococcus 1* and *Ruminococcus* were found to have the strongest association with age-related phenotypes (47). The relative abundance of *Peptococcus* increased with age (48). *Subdoligranulum* is positively associated with lipopolysaccharide (LPS) biosynthesis and short-chain fatty acid (SCFA) degradation pathways that accelerate epigenetic clock aging (49). An MR Analysis revealed a genetic link between *Veillonella* and longevity (50). At the same time, studies have found that *Lactobacillus* can reduce age-related diseases and regulate the imbalance of gut microbiota (51). The results of MR are different from those of previous studies, which show that the use of *Lactococcus*, and *Lachnospiraceae* can delay aging (52, 53). Due to the few literatures and the influence of confounding factors, this result still needs to be discussed. Interestingly, we also found gut microbiota associated with aging that had not been previously reported, including *Eisenbergiella, Prevotella7, Victivallis, Howardella, Senegalimassilia, and Tyzzerella*. The discovery of these gut microbiota can provide thinking for future scientific research work.

The reduced diversity and abundance of the gut microbiota may be the main reason for the effect of the gut microbiota on the epigenetic clock. It has been found in the literature that the diversity of gut microbiota and the abundance of butyricogenes decreased in the elderly (54–56). The lower bacterial diversity in the elderly showed that Bacteroidetes and Firmicutes still dominated, but the relative proportion of Firmicutes subgroups changed (57). Reducing the pH value of the gut through propionate and butyrate can effectively prevent the overgrowth of pathogens such as *Escherichia coli*, stimulate the growth of beneficial bacteria, and play a regulatory role in the intestinal microbiome (58). However, in the intestinal microbial environment of the elderly, the number of several butyrate-producing gut microbiota is relatively small (such as *Ruminococcus*, etc.). This may lead to the reproduction of intestinal pathogens and the inhibition of beneficial bacteria in the intestine, becoming an important reason for the acceleration of the epigenetic clock.

Inflammatory cytokines act as mediators of gut microbiota and epigenetic clock

The study found that specific epigenetic features in the DNA of gut microbes in human feces, particularly those associated with inflammation, are strongly associated with disease (59). In our study, we found some possible inflammatory cytokine pathways in the gut microbiota associated with the epigenetic clock. The gastrointestinal tract (GI) and central nervous system (CNS) are constantly confronted with complex human environments. As a result, a complex network of cells, including immune cells and neuronal cells, are able to coordinate local and systemic inflammatory responses (60). Nerve Growth Factor (NGF) modulates the survival, proliferation, and differentiation of neuronal cells within both the peripheral and central nervous systems (61). Some studies have shown that gut microbes can influence levels of NGF in the brain, which in turn affects neurodevelopment and cognitive function (62, 63). Recent studies have shown that the gut-brain axis is able to regulate inflammation and immune responses, thereby influencing the aging process (60, 64). We found that nerve growth factor plays a potential mediating role between gut microbiota and epigenetic clock, and thus may advance the study of the role of gut-brain axis theory in aging. Nerve growth factors regulated by gut microbiota may have potential benefits against neurodegenerative diseases during aging, as these factors are able to protect neurons and slow cognitive decline (65). As a member of the interleukin-6 cytokine family, Oncostatin M (OSM) plays a significant role in inflammation, autoimmune and cancer (66). Specific gut microbes may prompt host cells to restrain Oncostatin-M, which in turn affects inflammatory pathways and immune regulation, mechanisms that may be associated with the aging process, influencing the epigenetic clock by regulating the inflammatory response (67). Interleukin-12 (IL-12) is indispensable in cellular immunity and is considered an effective drug to enhance the anti-tumor immune response. Gut microbiota can influence IL-12B expression through its metabolites or by activating immune cells in the intestinal mucosa. Newly discovered evidence suggests that IL-12B is a key cytokine that enables T helper cells (Th1 and Th17) to differentiate and function (68). Most Th17 and Th1 are present in the gastrointestinal tract and play an important homeostasis role, while positive responses to the flora are thought to be related to inflammation and pathogenesis (69). This effect may indirectly affect the aging process and epigenetic clock by affecting inflammatory states. We found that gut microbiota may control the development of cancer through OSM and IL-12, thus slowing down the effects of aging. C-C motif chemokine 25 (CCL25) is a chemokine that is mainly expressed in the small intestine and plays an important role in attracting immune cells such as T cells to the intestine (70). The composition and function of gut microbiota can influence the intestinal immune environment, including CCL25 expression (71). By regulating the activity of immune cells in the gut, the gut microbiota may indirectly influence the levels of immune regulation and inflammation associated with aging, thereby affecting the

epigenetic clock. CCL25 is also involved in the expression of liver inflammatory genes (72). Our findings may be able to control liver inflammation by regulating gut microbiota, thereby delaying aging.

The gut microbiota plays a crucial role in the inflammatory process in the human body (12). In older mice, *Lactobacillus* has been shown to enhance the tight junction of the intestinal barrier, reduce the expression of pro-inflammatory cytokines, and inhibit the activation of NF-kB (73). SCFAs are seen as a central point of connection between the host and the gut microbiota (74). SCFAs can reduce the production of inflammatory factors to achieve immune regulation (75). SCFAs can regulate intestinal transport time, play a role in insulin response, and are closely associated with metabolic diseases (76). SCFAs are an important regulator of microglia integrity in the central system, which is particularly important in older adults and may lead to cognitive decline (77). In addition, there is research evidence that compounds from the gut microbiota can activate macrophages through the blood, putting them into a pro-inflammatory state that leads to atherosclerosis. This may lead to the development of cardiovascular disease (78). The diseases listed above are closely related to human aging, which speeds up the epigenetic clock.

Our study has several advantages: The use of MR Analysis excludes other factors and assesses the genetic association between the epigenetic clock and gut microbiota from a genetic perspective. At the same time, we used LDSC to evaluate the causal link, making the results more reliable. We also used the Steiger test to prove the correctness of the directionality of our study. In addition, in the MR Analysis, we use the F-number to guarantee the strength of the IVs. The MR-PRESSO and MR-Egger regression intercepts can test the horizontal pleiotropy of the study to avoid result bias. European populations were used for exposure and results, avoiding population stratification of results. We used a two-step mediation to determine the role of relevant inflammatory cytokines between gut microbiota and the epigenetic clock.

However, there are limitations to the study. Genus is the lowest classification level in the gut microbiota data, so we were unable to further explore the relationship between exposure and outcome at the species level. Due to the need for the number of SNPs in the sensitivity analysis and horizontal pleiotropy test of this study, our investigation did not achieve the conventional GWAS significance threshold, which is typically set at $P < 5 \times 10^{-8}$. So we use FDR correction to limit the possibility of positive errors. We only investigated the effect of inflammatory factors as mediators on the epigenetic clock, in fact, the mediators that affect the epigenetic clock may be diverse, such as BMI. Due to the interference of demographic stratification, we analyzed GWAS data from European populations, so the findings may not be applicable to other ethnic groups or populations (79).

Conclusion

In summary, this two-sample MR Study found a causal relationship between the gut microbiota and the epigenetic clock. Further experimental studies are needed to elucidate the mechanisms by which gut microbiota contribute to the epigenetic clock.

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

ST: Writing – original draft, Writing – review & editing, Methodology, Formal analysis, Visualization, Resources, Conceptualization. XL: Methodology, Writing – review & editing, Visualization. SC: Methodology, Writing – review & editing. YW: Data curation, Writing – review & editing, Visualization. MC: Data curation, Writing – review & editing, Investigation.

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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.2024.1339722/full#supplementary-material

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Causal associations between gut microbiota and premature rupture of membranes: a two-sample Mendelian randomization study

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Background: Previous study has indicated a potential link between gut microbiota and maternal pregnancy outcomes. However, the causal relationship between gut microbiota and premature rupture of membranes (PROM) remains a topic of ongoing debate.

Methods: A two-sample Mendelian Randomization (MR) study was used to investigate the relationship between gut microbiota and PROM. Genetic data on gut microbiota was obtained from the MiBioGen consortium's largest genome-wide association study (GWAS) (n=14,306). Genetic data on PROM (3011 cases and 104247 controls) were sourced from publicly available GWAS data from the Finnish National Biobank FinnGen consortium. Various methods including Inverse variance weighted (IVW), MR-Egger, simple mode, weighted median, and weighted mode were utilized to assess the causal relationship by calculating the odd ratio (OR) value and confidence interval (CI). Sensitivity analyses for quality control were performed using MR-Egger intercept tests, Cochran's Q tests, and leave-one-out analyses.

Results: The IVW method revealed that *class Mollicutes* (IVW, OR=0.773, 95%CI: 0.61-0.981, *pval* = 0.034), *genus Marvinbryantia* (IVW, OR=00.736, 95%CI: 0.555-0.977, *pval* = 0.034), *genus Ruminooccaceae UCG003* (IVW, OR=0.734, 95%CI: 0.568-0.947, *pval* = 0.017) and *phylum Tenericutes* (IVW, OR=0.773, 95%CI: 0.566-1.067, *pval* = 0.034) were associated with a reduced risk of PROM, while *genus Collinsella* (IVW, OR=1.444, 95%CI: 1.028-2.026, *pval* = 0.034), *genus Intestinibacter* (IVW, OR=1.304, 95%CI: 1.047-1.623, *pval* = 0.018) and *genus Turicibacter* (IVW, OR=1.282, 95%CI: 1.02-1.611, *pval* = 0.033) increased the risk of PROM. Based on the other four supplementary methods, six gut microbiota may have a potential effect on PROM. Due to the presence of pleiotropy (*pval*=0.045), *genus Lachnoclostridium* should be ruled out. No evidence of horizontal pleiotropy or heterogeneity was found in other microbiota (*pval* >0.05).

Conclusions: In this study, we have discovered a causal relationship between the presence of specific probiotics and pathogens in the host and the risk of PROM. The identification of specific gut microbiota associated with PROM through MR studies offers a novel approach to diagnosing and treating this condition, thereby providing a new strategy for clinically preventing PROM.

KEYWORDS

gut microbiota, premature rupture of membranes, genetic variable, Mendelian randomization, causality

Introduction

Premature rupture of membranes (PROM) is a prevalent perinatal complication, with an incidence rate of approximately 7% to 8% (1, 2). PROM can result in severe fetal complications such as placental abruption, umbilical cord compression, respiratory distress syndrome, preterm birth, and cerebral damage (3–5). Mothers with PROM face increased risks of intra-amniotic infections, placental abruption, cord prolapse, sepsis, and even death, posing significant threats to both maternal and neonatal health (6, 7). While factors such as infection, inflammation, immunity, oxidative stress, and nutrient metabolism are implicated in the pathogenesis of PROM, reliable early diagnostic indicators and effective preventive measures remain lacking (8, 9).

Gut microbiota, a complex community within the digestive tract, plays a crucial role in nutrient digestion and absorption during energy metabolism. It also maintains physiological functions and regulates various pathological processes in the body (10–12). Numerous studies suggest that gut microbiota plays a significant role in maternal and fetal health, undergoing changes during pregnancy, disruption of maternal gut microbiota during gestation can alter offspring microbiota and immunity (13–16). For example, *Bacteroides fragilis* (*B. fragilis*) dominates the gut microbiomes of individuals with intrahepatic cholestasis of pregnancy (ICP). Through its bile salt hydrolase (BSH) activity, B. fragilis aggravates ICP by inhibiting FXR signaling, thereby disrupting bile acid metabolism (17). In overweight and obese pregnant women at 16 weeks gestation, the abundance of butyrate-producing bacteria and butyrate production in the gut microbiota are significantly negatively associated with blood pressure and with plasminogen activator inhibitor-1 levels. Increasing butyrate-producing capacity may contribute to the maintenance of blood pressure in obese pregnant women (18). The study on PROM has revealed that being infected with *Helicobacter pylori* is a risk factor for PROM (19). Furthermore, it is widely believed that infection is the primary cause of PROM, which has led to an oversight of the crucial role played by gut microbiota. The current links between gut microbiota and PROM are primarily derived from observational studies, which may be influenced by confounding factors, such as lifestyle, age, and environment (20, 21). Hence, these conditions limit the inference of causality between gut microbiota and PROM, highlighting the need for further research to elucidate their relationship.

Mendelian randomization (MR) has been widely utilized to estimate the causal association between exposure and outcome by using genetic variants as instrumental variables (IVs) (22). Genetic variants are randomly inherited from parents to offspring, making them more independent. This characteristic effectively assists MR in mitigating bias from reverse causality and confounding factors (23, 24). Large-scale genome-wide association studies (GWAS) on gut microbiota and PROM provide an opportunity for MR analysis with greatly improved statistical power.

This study aims to evaluate the potential causal association between gut microbiota and PROM using GWAS summary statistics from the FinnGen and MiBioGen consortiums through two-sample MR analysis. Our findings may identify specific pathogenic microbiota and offer new insights for early prediction and intervention in PROM.

Materials and methods

Study design and data sources

In the investigation, we followed the guidelines established in the STROBE-MR Statement (Guidelines for strengthening the reporting of MR studies) for reporting observational studies in epidemiology (25).

MR statistical analysis utilizes genetic instrumental variables (single nucleotide polymorphisms, SNPs) to infer the relationship between exposure and outcome based on three key assumptions: (1) a strong correlation between instrumental variables and exposure factors, (2) no correlation between instrumental variables and confounding factors, (3) the sole association of instrumental variables with outcomes through exposure (26, 27). The flowchart of this MR study has been shown in Figure 1.

Genetic data on gut microbiota was obtained from a large-scale GWAS meta-analysis conducted by the MiBioGen consortium (www.mibiogen.org), which aimed to study the influence of human genes on intestinal flora at the whole genome level and

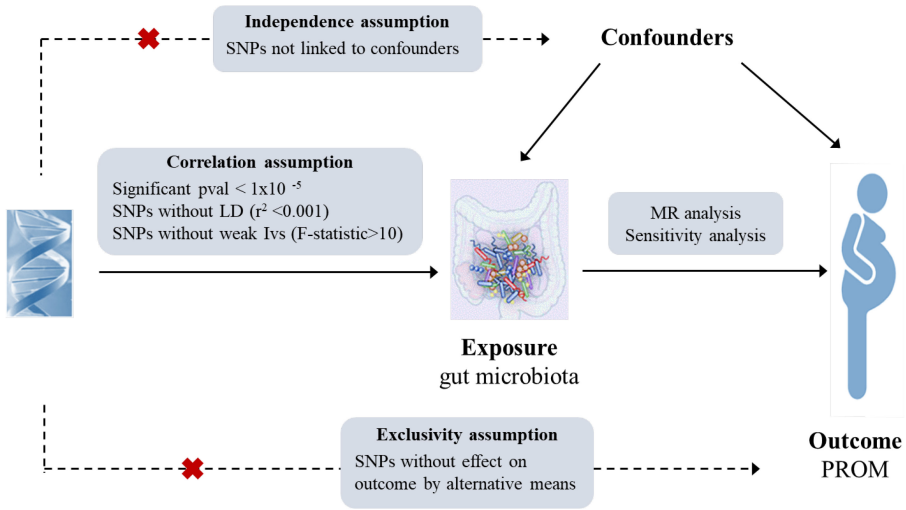

FIGURE 1
Flow chart of study design.

included 18340 participants from 24 cohorts, most of whom had European ancestry (Canada, Netherlands, Sweden, United States, United Kingdom, Belgium, Denmark). Bacteria contain three kinds of rRNA sequences, namely 23S, 16S and 5S. Among them, 16S rRNA is the most commonly used molecular clock in bacterial systematics because of its moderate number of nucleotides, large amount of information, high stability, and easy extraction and analysis. Variable regions (V4, V3-V4, V1-V2) of the 16S rRNA gene were used to profile the composition of gut microbiota, and microbiota quantitative trait loci (mbQTL) mapping was performed to identify the host genetic variants in the relative abundance of microbial taxa. A total of 210 taxa (9 phyla, 16 classes, 20 orders, 31 families, 119 genera, 3 unknown families, and 12 unknown genera) were extracted for analysis in this study. Further details on the gut microbiota can be found in the original study or the website (http://mibiogen.gcc.rug.nl) (28). GWAS datasets on PROM were extracted from the IEU OpenGWAS project and derived from the FinnGen consortium (http://www.finngen.fi/en), including 3011 cases and 104247 controls (19). More details (endpoint definition, mean age, and other longitudinal metrics) can be found in the FinnGen database.

All relevant data sources are publicly available. Ethical approval and participant consent were obtained in the original studies included in the GWAS, thus further ethical clearance for this study was not required.

Instrumental variable selection

First, SNPs closely associated with gut microbiota (significant threshold $p < 1.0\times10^{-5}$, genetic distance = 10000 kb, $r^2 < 0.001$) were screened to ensure the correlation assumption and test the effect of linkage disequilibrium (LD) and the independence of IVs (29). IVs with the F-statistics < 10 were excluded to mitigate weal instrumental bias (30). The formula of the F-statistics calculation is as follows: $F= R^2 \times (n-1-k)/[(1-R^2) \times k]$, where R^2 represents the portion of exposure variance explained by the IVs, k represents the number of IVs, n is the sample size. Following the above steps, the remaining SNPs were used for MR analysis (31).

Data analysis

The data analyses were conducted by the "TwoSampleMR" package in R4.2.3. Five methods, including inverse variance weighted (IVW), MR Egger, simple mode, weighted mode, and weighted median, were employed to assess the causal association between gut microbiota and PROM. The IVW method can combine with the Wald ratio of each SNP to obtain the total effect of gut microbiota on PROM when SNP fully conforms to the three principles of MR study. Significant results obtained through the IVW method ($p < 0.05$) can be deemed credible in the absence of pleiotropy and heterogeneity, even if other methods yield non-significant findings (32). The MR-Egger method is used to evaluate potential pleiotropic effects of IVs. MR-Egger intercept analysis can better explain why this potential pleiotropy exists. If the intercept significantly deviates from zero ($p < 0.05$), it indicates the presence of horizontal pleiotropy associated with the IVs (33). This suggests that the outcome may be influenced by factors other than exposure. When the pleiotropic effect is unrelated to its genetic association with the exposure, the slope of the MR-Egger regression still offers a valid MR estimate, even in the presence of horizontal pleiotropy.

The simple mode, weighted mode, and weighted median are used as complementary methods. The simple model serves as a robust method for evaluating causal relationships between genes and phenotypes, effectively addressing potential biases. In contrast, the weighted model calculates SNP effect estimates using weights and identifies the SNP with the greatest weighted effect as the final estimate. The weighted median method takes into account the weights (inverse of standard error, SE) of IVs and calculates the median of MR-related evaluation (34).

In the sensitivity analysis, Cochran's Q statistics with Q and p-value were used to quantify the heterogeneity in IVs, A *pval* > 0.05 indicates the absence of heterogeneity (32). Horizontal pleiotropy was evaluated using MR Egger intercept analysis, with *pval* > 0.05 indicating no pleiotropy. Outlier analysis was conducted to ascertain the presence of influential SNPs through the leave-one-out method.

For a more rigorous interpretation of causality, we employed the Bonferroni method to examine the p-value for various classifications of gut microbiotas. The results were as follows: genus p = 3.82×10^{-4} (0.05/131), family p =1.47×10^{-3} (0.05/34), order p = 2.50×10^{-3} (0.05/20), class p = 3.13×10^{-3} (0.05/16), and phylum p = 5.56×10^{-3} (0.05/9).

Results

Instrumental variable selection

After undergoing a series of rigorous quality control procedures for IV screening, a total of 2722 independent SNPs from 210 gut microbiotas were extracted in the analysis (Supplementary Table 1), with statistical significance at *pval*<1.0×10^{-5}, kb=10000, r2<0.001. All IVs exhibited F-statistics > 10, indicating robust IV effects and alleviating concerns of weak IV bias. Additionally, to mitigate potential confounding effects on causal inferences, PhenoScanner was utilized for screening, resulting in no exclusions of SNPs. Therefore, the genetic IVs should be deemed valid for use in this MR analysis.

MR analysis

Two-sample MR analysis results

After conducting MR analysis, we generated a heatmap using IVW method to screen 193 gut microbiotas (Figure 2). This approach provides a more intuitive representation of the gut microbiota that play a significant role in PROM, as indicated by their p value and OR value. Based on the significance levels (*pval*<0.05) obtained from any of the five methods (IVW, MR Egger, simple mode, weighted mode and weighted median), a forest plot was generated, and 14 gut microbiotas (including *class Mollicutes*, *family Actinomycetaceae*, *genus Collinsella*, *genus Dorea*, *genus Family XIII AD3011 group*, *genus Intestinibacter*,

FIGURE 2
All results of IVW between gut microbiota and PROM.

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genus Lachnoclostridium, *genus Marvinbryantia*, *genus Ruminococcaceae UCG003*, *genus Ruminococcaceae UCG010*, *genus Turicibacter*, *order Actinomycetales*, *phylum Actinobacteria*, *phylum Tenericutes*) were identified as potentially related to PROM, excluding undefined microbiotas (Figure 3; Supplementary Table 2). The scatter plots had been shown in Figure 4. IVW estimates suggested that the *class Mollicutes* (IVW, OR=0.773, 95% CI: 0.61-0.981, *pval* = 0.034), *genus Marvinbryantia* (OR=00.736, 95%CI: 0.555-0.977, *pval* = 0.034), *genus Ruminooccaceae UCG003* (OR=0.734, 95%CI: 0.568-0.947, *pval* = 0.017) and *phylum Tenericutes* (OR=0.773, 95%CI: 0.566-1.067, *pval* = 0.034) were associated with a reduced risk of PROM and demonstrated protective effects. Conversely, the *genus Collinsella* (OR=1.444, 95%CI: 1.028-2.026, *pval* = 0.034), *genus Intestinibacter* (OR=1.304, 95%CI: 1.047-1.623, *pval* = 0.018) and *genus Turicibacter* (OR=1.282, 95%CI: 1.02-1.611, *pval* = 0.033) were associated with an increased risk of PROM showed pathological effects.

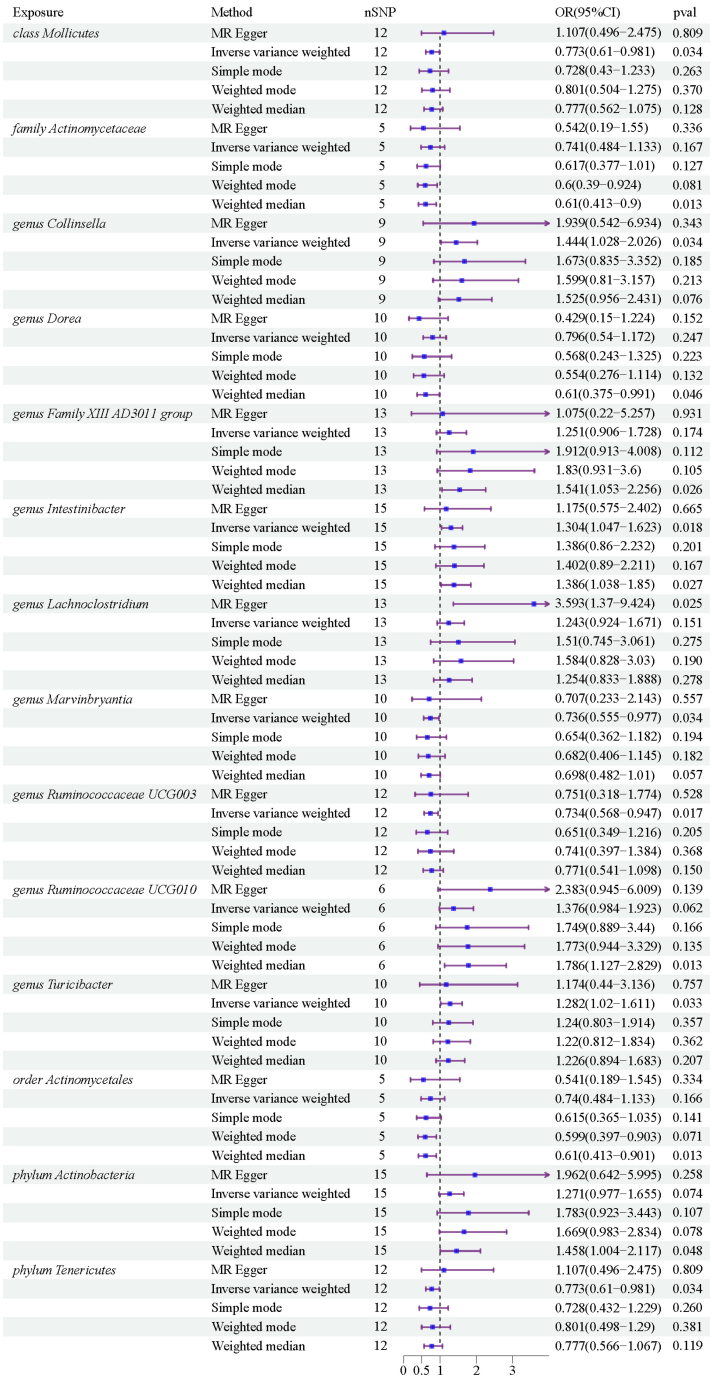

FIGURE 3
Forest plots of MR results for 14 gut microbiotas on PROM.

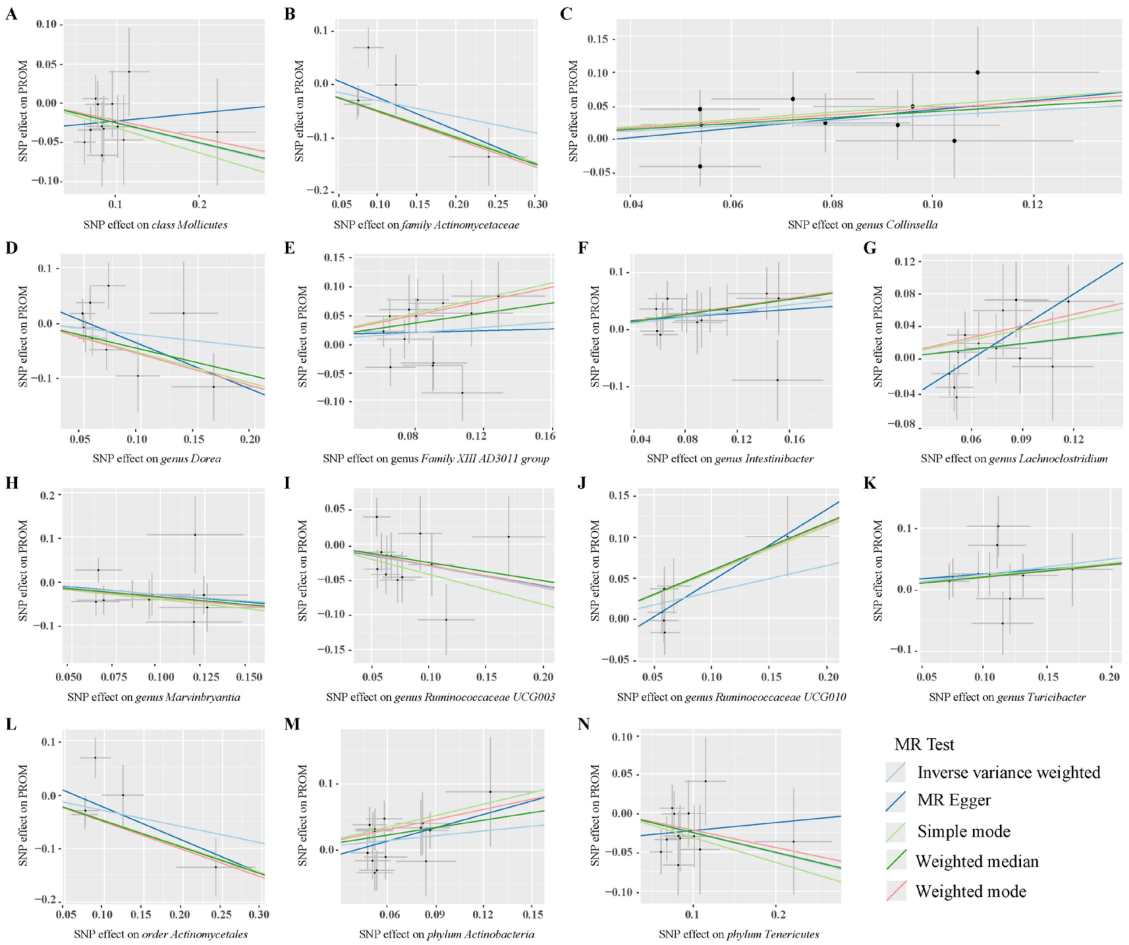

FIGURE 4
Scatter plots of MR analysis on the causal relationship between 14 gut microbiotas and PROM. **(A–N)** represents different gut microbiotas, respectively.

Although the IVW method did not support the causal associations of other six gut microbiotas, Weighted median estimates revealed that several microbial taxa exhibited potential associations with PROM. Specifically, *family Actinomycetaceae* (OR=0.61, 95%CI: 0.413-0.9, *pval* = 0.013), *genus Dorea* (OR=0.61, 95%CI: 0.375-0.991, *pval* = 0.046), *genus Family XIII AD3001 group* (OR=1.541, 95%CI: 1.053-2.256, *pval*= 0.026), *genus Ruminococcaceae UCG010* (OR=1.786, 95%CI: 1.127-2.829, *pval*=0.013), *order Actinomycetales* (OR=0.61, 95%CI: 0.413-0.901, *pval*=0.013) and *phylum Actinobacteria* (OR=1.458, 95%CI: 1.004-2.117, *pval*=0.048) were found to have a suggestive association with PROM. Additionally, among these 14 gut microbiotas, MR Egger estimate of *genus Lachnoclostridium* (OR=3.593, 95%CI: 1.37-9.424, *pval*=0.02) showed a suggestive relationship with PROM as well, however, it is important to note that there was evidence of horizontal pleiotropy (*pval*=0.045).

Sensitivity analysis

Sensitivity analyses were conducted to assess the robustness of the results. IVW and MR Egger in Cochran's Q test showed no significant heterogeneity in the IVs associated with PROM (Figure 5 and Supplementary Table 3). Additionally, MR Egger intercept analysis detected horizontal pleiotropy only in *genus Lachnoclostridium* (*pval*=0.045), while no pleiotropy was found in the other 13 gut microbiotas (*pval*>0.05). The detailed results were showed in Supplementary Table 4.

Leave-one-out sensitivity analyses indicated that removing specific SNPs did not alter the causal inference outcomes, suggesting no individual IVs were solely responsible for the associations (Figure 6). Collectively, these findings indicated that there was no significant bias attributable to individual gut microbiota SNPs on PROM.

Discussion

This MR study provides compelling evidence for the causal relationship between specific gut microbiota and PROM, identifying bacteria that either decrease or increase the risk. By utilizing extensive GWAS summary data from the MiBioGen consortium for gut microbiota and the FinnGen consortium for PROM, we have pinpointed specific gut bacteria that either decrease

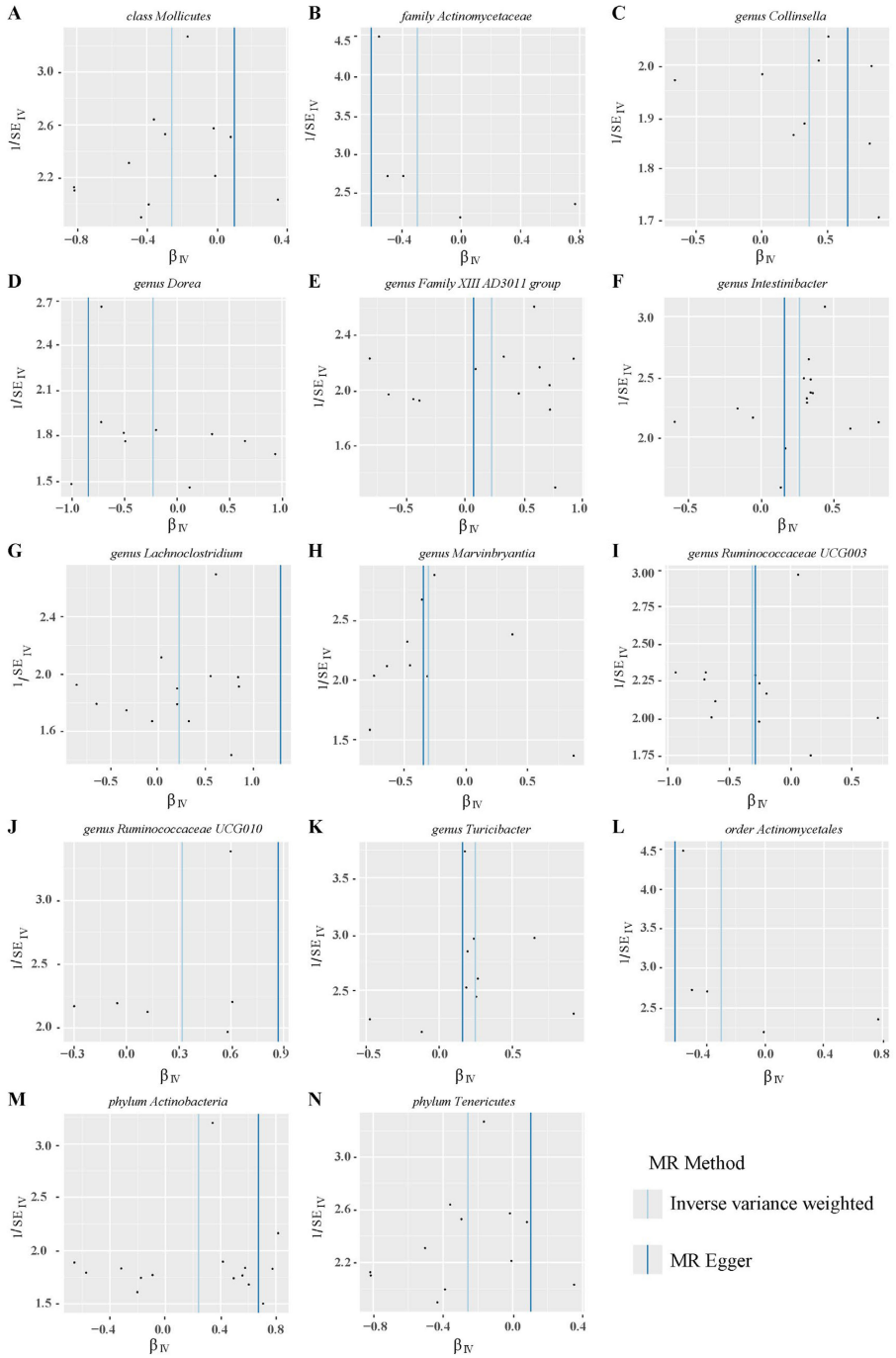

FIGURE 5
Funnel plots of heterogeneity analysis on 14 gut microbiotas and PROM. **(A–N)** represents different gut microbiotas, respectively.

or increase the risk of PROM. These findings are consistent with existing literature, highlighting the intricate relationship between gut microbiota and pregnancy outcomes. To ensure the robustness of our findings, we employed multiple MR methods, including inverse variance weighted (IVW), MR-Egger, simple mode, weighted median, and weighted mode approaches. Sensitivity analyses such as MR-Egger intercept tests, Cochran's Q tests, and leave-one-out analyses were performed to detect and correct for pleiotropy and heterogeneity. These methods help ensure that our results are not confounded by other factors. For example, *genus Lachnoclostridium* was excluded due to evidence of pleiotropy (*pval*=0.045), highlighting the importance of rigorous quality control in MR studies. These findings not only enhance our understanding of the link between gut microbiota and PROM, but also pave the way for new therapeutic strategies and personalized medicine in managing pregnancy complications.

Several studies have revealed the complex relationship between gut microbiota and adverse pregnancy outcomes and complications

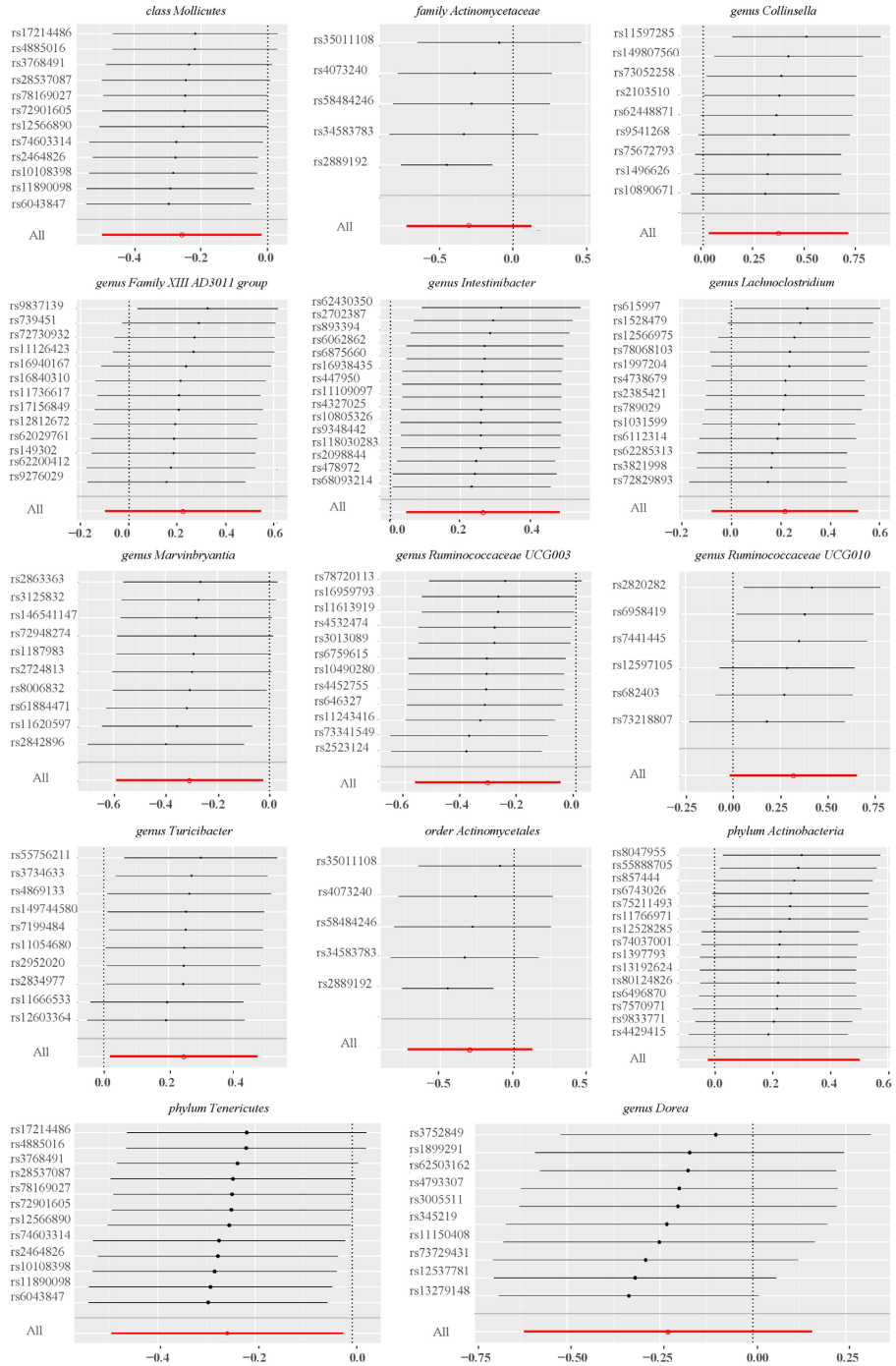

FIGURE 6
Leave-one-out plots of sensitivity analysis on 14 gut microbiotas and PROM.

(17, 19, 35–37). This study identified 147 SNPs linked to 14 gut microbiotas associated with PROM. It was found that Mollicutes (IVW, OR=0.773, 95%CI: 0.61-0.981, *pval* = 0.034) and *Tenericutes* (IVW, OR=0.773, 95%CI: 0.566-1.067, *pval* = 0.034) were protective factors against PROM. *Mollicutes*, which include species like Mycoplasma and Ureaplasma, are known for their unique immunomodulatory properties (38–40). *Mycoplasma* and *Ureaplasma* can modulate the immune system by reducing pro-inflammatory cytokines, which may help maintain the integrity of fetal membranes and reduce PROM risk (41–44). This finding is consistent with previous studies indicating that *Mollicutes* can influence immune responses and protect against membrane rupture (45). Similarly, *Marvinbryantia* (IVW, OR=0.736, 95%CI: 0.555-0.977, *pval* = 0.034) and *Ruminococcaceae* UCG003 (IVW, OR=0.734, 95%CI: 0.568-0.947, *pval* = 0.017) also demonstrated protective effects on PROM. *Marvinbryantia* is associated with anti-inflammatory properties as it produces metabolites that have been shown to reduce inflammation (46, 47). *Ruminococcaceae UCG003*

plays a crucial role in fermenting dietary fibers into short-chain fatty acids (SCFAs) like butyrate. Butyrate enhances gut barrier function and has systemic anti-inflammatory effects, which likely contribute to the strengthening of fetal membranes and reducing the risk of PROM (48, 49).

On the contrary, our study has identified specific gut microbiotas that are associated with an increased risk of PROM. *Collinsella* (IVW, OR=1.444, 95%CI: 1.028-2.026, *pval* = 0.034) was significantly associated with a higher risk. *Collinsella* has been linked to systemic inflammation and metabolic disorders, both of which can compromise gut barrier integrity (50–52). Elevated levels of *Collinsella* can disrupt gut barrier function and promote inflammatory pathways, weakening fetal membranes and increasing the risk of PROM. Additionally, *Intestinibacter* (IVW, OR=1.304, 95%CI: 1.047-1.623, *pval* = 0.018) and *Turicibacter* (IVW, OR=1.282, 95%CI: 1.02-1.611, *pval* = 0.033) were also found to be associated with an increased risk. *Intestinibacter* is associated with inflammatory conditions and has been shown to exacerbate inflammation and disrupt gut barrier function leading to weakened fetal membranes and ultimately contributing to PROM (53). *Turicibacter* has been known to influence immune responses and promote pro-inflammatory cytokines, further compromising membrane integrity thus increasing the likelihood of PROM (54, 55).

The identification of specific gut microbiota associated with PROM has significant clinical implications. These microbiotas may affect pregnancy health through various mechanisms such as metabolites, endocrine, inflammation, or immune system (41, 56, 57). The changes of gut microbiota through dietary interventions, probiotics, or prebiotics could be a viable strategy to prevent PROM. Increasing the abundance of protective bacteria such as *Marvinbryantia* and *Ruminococcaceae UCG003* through probiotic and prebiotic supplements could help maintain membrane integrity. Additionally, dietary interventions aimed at reducing harmful bacteria like *Collinsella* and *Intestinibacter* could also be beneficial in preventing PROM.

MR analysis was performed to ascertain the causal relationship between gut microbiota and PROM, effectively mitigating the influence of confounding factors. However, our study has several limitations, which could affect the interpretation of the results. Firstly, summary statistics from the public database rather than raw data were used in this MR analysis, which prevented us from performing subgroup analyses such as term PROM and preterm PROM. Secondly, the population of this study mainly focused on European ancestry, raising the possibility that the findings may not be fully applicable to other racial groups. Thirdly, due to the moderate sample size of the gut microbiota, we did not perform reverse MR analysis as it may be prone to potential instrumental biases in the findings. Fourthly, because 16S rRNA sequencing only allowed for taxonomic classification at the genus level, we were unable to investigate more specific species levels between gut microbiota and PROM. Finally, based on previous microbiota studies, we selected a relaxed p-value threshold (*pval* $<1.0\times10^{-5}$) to screen genetic instruments, which may lead to weak bias. To address this issue, we calculated the instrument strength and excluded the F statistics <10 as a conventional cutoff to mitigate potential bias effects.

Future research should focus on diverse population, detailed subgroup analysis, larger sample size, longitudinal studies tracking the gut microbiome composition during pregnancy to strengthen the important relationship between microbiotas and PROM. And the precise biological mechanisms also make us better explore the PROM's therapeutic targets.

Conclusion

In conclusion, this study provides strong evidence for the causal relationship between specific gut microbiota and the risk of PROM. By identifying both protective and pathogenic bacteria, our findings open new avenues for preventive strategies and therapeutic interventions, and aim at improving maternal and fetal health condition. Further research will be essential to refine these strategies and gain a comprehensive understanding of the complex interactions between gut microbiota and pregnancy.

Data availability statement

Existing datasets are available in a publicly accessible repository: Publicly available datasets were analyzed in this study. This data can be found here: (https://gwas.mrcieu.ac.uk/).

Author contributions

LZ: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Funding acquisition. QL: Formal analysis, Methodology, Writing – original draft. JH: Data curation, Software, Visualization, Writing – original draft. QZ: Data curation, Investigation, Software, Writing – original draft. HZ: Data curation, Investigation, Software, Writing – original draft. XZ: Investigation, Software, Visualization, Writing – original draft. YS: Conceptualization, Supervision, Writing – review & editing, Funding acquisition. CL: Conceptualization, Supervision, Writing – review & editing, Funding acquisition.

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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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Causal roles of skin and gut microbiota in skin appendage disorders suggested by genetic study

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Objectives: There is evidence from observational studies that human microbiota is linked to skin appendage Disorders (SADs). Nevertheless, the causal association between microbiota and SADs is yet to be fully clarified.

Methods: A comprehensive two-sample Mendelian randomization (MR) was first performed to determine the causal effect of skin and gut microbiota on SADs. A total of 294 skin taxa and 211 gut taxa based on phylum, class, order, family, genus, and ASV level information were identified. Summary data of SADs and eight subtypes (acne vulgaris, hidradenitis suppurativa, alopecia areata, rogenic alopecia, rosacea, rhinophyma, seborrhoeic dermatitis, and pilonidal cyst) were obtained from the FinnGen consortium. We performed bidirectional MR to determine whether the skin and gut microbiota are causally associated with multiple SADs. Furthermore, sensitivity analysis was conducted to examine horizontal pleiotropy and heterogeneity.

Results: A total of 65 and 161 causal relationships between genetic liability in the skin and gut microbiota with SADs were identified, respectively. Among these, we separately found 5 and 11 strong causal associations that passed Bonferroni correction in the skin and gut microbiota with SADs. Several skin bacteria, such as *Staphylococcus*, *Streptococcus*, and *Propionibacterium*, were considered associated with multiple SADs. As gut probiotics, *Bifidobacteria* and *Lactobacilli* were associated with a protective effect on SAD risk. There was no significant heterogeneity in instrumental variables or horizontal pleiotropy.

Conclusions: Our MR analysis unveiled bidirectional causal relationships between SADs and the gut and skin microbiota, and had the potential to offer novel perspectives on the mechanistic of microbiota-facilitated dermatosis.

KEYWORDS

skin microbiota, gut microbiota, skin appendage disorders, Mendelian randomization, causal inference

Introduction

As the largest organ of the body, the skin functions as our primary barrier against external threats. However, it also serves as a diverse environment for a multitude of microorganisms, whose interactions significantly contribute to the skin's overall health, immune response, and disease development (1). Skin appendages, including sweat glands, hair follicles, sebaceous glands, and arrector pili muscles, are found in the dermis and adipose tissue. The surface and appendages of the skin are colonized by the skin microbiota, whose composition is contingent upon the microenvironment (2). Disturbances in the balance of the skin microflora, known as dysbiosis, have been recognized as contributing factors in various dermatological conditions, especially skin appendage disorders (SADs) (3, 4).

Meanwhile, the gut, home to the densest microbial population within the human body, exerts effects that extend beyond its confines. Gut microorganisms are primarily recognized for their roles in metabolic processes and immune system development (5). Interestingly, their influence transcends the gut, affecting various physiological systems, including the skin (6). This influential link between the gut and skin, termed the gut-skin axis, is emerging as an integral component in skin health and disease (6, 7). Despite these advances, the concurrent role of gut and skin microbiota in SADs remains an intriguingly uncharted area of research. Additionally, without experimental methods that rely on cultivated isolates, it is challenging to determine causality due to the close relationship between the microbiota and its host (8, 9).

Mendelian randomization (MR) is a powerful epidemiological technique that indicates causal associations by utilizing genetic variations (10). MR is inherently not confounding since environmental and self-adapted variables have no effect on genetic differences, as these are randomly allocated at conception. Moreover, this approach can circumvent the issue of reverse causality, as germline genotypes remain unaltered by physiological disturbances resulting from disease. Our study embarks on an exploration using comprehensive bidirectional MR to unravel potential causal relationships between the skin and gut microbiota and multiple SADs (acne vulgaris, hidradenitis suppurativa, alopecia areata, androgenic alopecia, rosacea, rhinophyma, seborrheic dermatitis, and pilonidal cyst). We aim to offer insights into the possible involvement of these microbial communities in the pathogenesis and progression of SADs, and pave the way for microbiome-oriented therapeutic strategies.

Methods

Data sources

Genetic variations of skin microbiota were derived from the GWAS conducted by Moitinho-Silva et al. (11). A sum of 1656 skin samples was acquired from individuals within two German cohorts, KORA FF4 (n = 635) and PopGen (n=1021). The samples were collected from three skin microenvironments, including moist skin (antecubital fossa in both cohorts), dry skin (dorsal and volar forearm in PopGen), and sebaceous skin (forehead in PopGen and retroauricular fold in KORA FF4). Microbial community patterns were obtained through the 16 S rRNA gene. Amplicon sequence variants (ASVs) and taxonomic groups from genus to phylum level were utilized in the GWAS. In total, 294 taxa in both cohorts were included in the analysis (14 phyla, 22 classes, 24 orders, 30 families, 54 genera, and 150 ASVs).

SNPs associated with the composition of the gut microbiota were selected as instrumental variables (IVs) within a GWAS database belonging to the MiBio-Gen consortium (12). This large-scale multi-ethnic GWAS integrated 16S rRNA gene sequencing data from 18,340 individuals across 24 cohorts to investigate the link between human autosomal genetic variants and the intestinal microbiota. There were 211 taxa in all, including 9 phyla, 16 classes, 20 orders, 35 families, and 131 genera.

GWAS summary data for SADs (377,277 individuals), acne vulgaris (AV) (363,927 individuals), hidradenitis suppurativa (HS) (362,071 individuals), alopecia areata (AA) (361,822 individuals), androgenic alopecia (AGA) (201,214 individuals), rosacea (ROS) (363,350 individuals), rhinophyma (RPH) (361,275 individuals), seborrhoeic dermatitis (SD) (339,277 individuals), and pilonidal cyst (PC) (358,708 individuals) were acquired from the R9 release of FinnGen consortium (13). Comprehensive information regarding the encompassed cohorts, genotypic data, endpoint specifications, and association testing can be accessed through the FinnGen webpage.

Instrumental variable selection

We applied the following criteria to select the instrumental variables (IVs): (1) potential IVs were identified as single nucleotide polymorphisms (SNPs) at the locus-wide significance threshold ($P < 1.0\times10^{-5}$) that was widely utilized in the previous MR studies (14–17); (2) a linkage disequilibrium parameter (R^2) of SNP was set at 0.01, with a genetic distance of 10,000 kb; (3) A minor allele frequency (MAF) of less than 0.01 was used to eliminate SNPs; (4) palindromic SNPs were discarded to guarantee that the allelic effects of SNPs during the harmonization process; and (5) IVs with an F statistic <10 were excluded.

Mendelian randomization analysis

The MR study was structured as depicted in Figure 1. We applied five MR methods for features with multiple IVs: inverse-variance weighted (IVW) (18), weighted median (19), MR-Egger regression (20), simple mode (21), and weighted mode (22). The IVW method has been shown to have more power than the others under some conditions (22); hence, we mainly used the IVW method for the results, and the other four methods as supplements. For a more stringent interpretation of the causal relationship, we performed a Bonferroni correction, based on the number of bacteria within each level. For skin microbiota, the

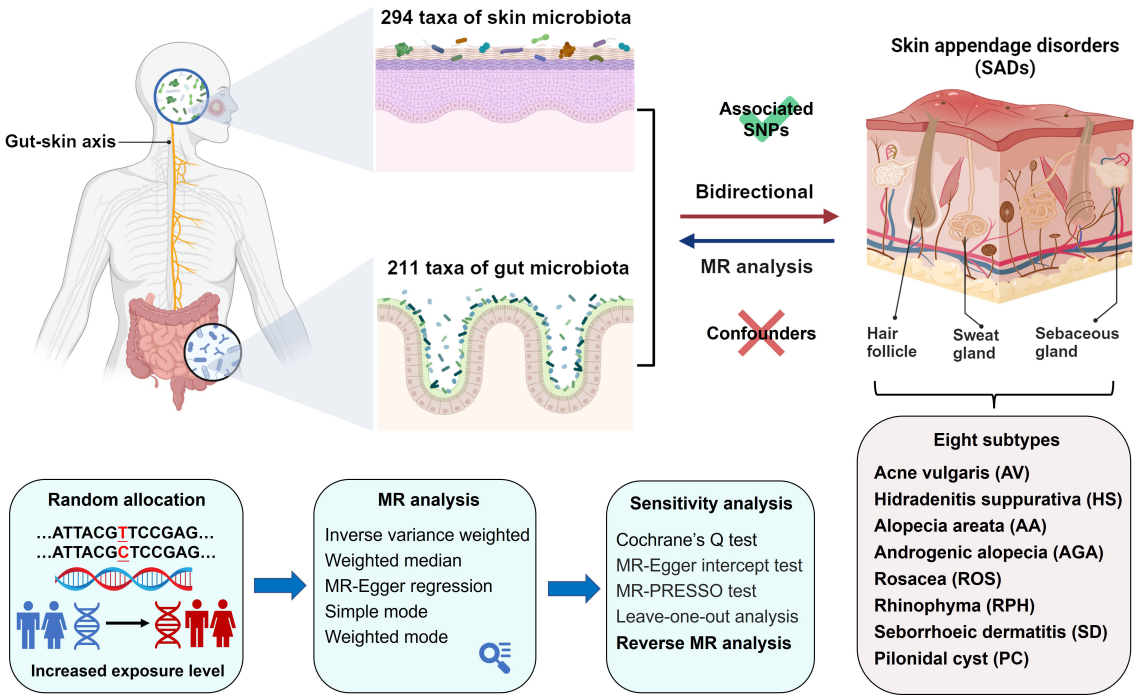

FIGURE 1
Study design and flowchart.

threshold significance was set as follows: phylum $P = 7.14 \times 10^{-3}$ (0.05/7), class $P = 4.55 \times 10^{-3}$ (0.05/11), order $P = 4.17 \times 10^{-3}$ (0.05/12), family $P = 3.33 \times 10^{-3}$ (0.05/15), genus $P = 1.85 \times 10^{-3}$ (0.05/27), ASV $P = 6.67 \times 10^{-4}$ (0.05/75). For gut microbiota, the threshold significance was set as follows: phylum $P = 5.56 \times 10^{-3}$ (0.05/9), class $P = 3.13 \times 10^{-3}$ (0.05/16), order $P = 2.50 \times 10^{-3}$ (0.05/20), family $P = 1.43 \times 10^{-3}$ (0.05/35), genus $P = 3.82 \times 10^{-3}$ (0.05/131). *P*-values falling within the range between 0.05 and the corrected value were regarded as nominal significance with potential causal effects. To investigate whether SADs exerted any causal influence on the identified skin and gut microbiota, we also conducted a reverse MR analysis. The methodologies and settings employed were in line with those of the forward MR. Two-sample MR (version 0.5.6) and MRPRESSO (version 1.0) packages with R software (version 4.2.2) were used. The MR study was conducted in accordance with STROBE-MR guidelines (23, 24).

Sensitivity analysis

To evaluate the heterogeneity of instrumental variables, we employed Cochran's Q statistics (25). Additionally, a leave-one-out (LOO) analysis was conducted to assess the influence of individual SNPs on the overall causal estimate (26). By systematically excluding each SNP from the analysis, we evaluated the robustness of our results. Consistent results across LOO analyses enhance confidence in the causal inference, indicating that the findings are not driven by specific SNPs (26). We also used MR-Egger intercept tests and MR-PRESSO to verify the existence of horizontal pleiotropy. MR-PRESSO performs a global test to detect the presence of horizontal pleiotropy by comparing the observed distribution of SNP-exposure and SNP-outcome associations with their expected distribution under no pleiotropy (27). A significance ($P < 0.05$) indicates a substantial influence of pleiotropic SNPs on the original causal estimate. In MR-Egger regression, the intercept term provides an estimate of the average pleiotropic effect across all SNPs (28). A non-zero intercept indicates the presence of directional pleiotropy. To further confirm if the observed causalities were skewed due to reversed causation, the Steiger directionality test was applied (29). If the SNPs explain more variance in the exposure than in the outcome, it supports the correct direction of causality (27). Confirming the direction of effect reduces the risk of reverse causation bias, where the outcome could mistakenly appear to cause the exposure (27). Given the distinctiveness of skin microbiota across different microenvironments (moist, dry, and sebaceous areas) in KORA FF4 and PopGen cohorts, meta-analyses were carried out by combining data sets originating from the same microenvironment. Statistical analyses were applied using the META (version 6.5.0) package.

Results

SNP selection

Following the quality control procedures, a total of 838 SNPs and 1031 SNPs in the forward MR analysis were selected as IVs from skin and gut microbiota, respectively (Supplementary Tables S1, S2). In the reverse MR analysis, a total of 3316 SNPs and 3259 SNPs were severally selected from SADs as IVs for skin and gut microbiota (Supplementary Tables S3, S4). The F statistics of the

remaining IVs were all greater than 10, which suggests that there was less chance of weak instrument bias affecting the estimates. Detailed information on the major IVs in the MR analysis that passed the Bonferroni correction between SADs and skin and gut microbiota was displayed in Supplementary Table S5.

Causal associations of skin and gut microbiota and SADs

In the forward MR analysis, 115 causal associations were found between SADs and skin microbiota of the KORA FF4 and PopGen cohorts (Supplementary Table S6). After the microenvironment-based meta-analysis of two cohorts, 30 causal associations remained significant ($P < 0.05$) in the moist, dry, or sebaceous skin (Figures 2, 3). As for the gut, 83 causal associations were found between SADs and gut microbiota (Figures 4, 5). Details about the causalities between skin and gut microbiota and multiple SADs are shown below.

SADs

For the skin microbiota, class *Alphaproteobacteria* and genus *Streptococcus* were associated with an inducing effect on total SAD risk. Order *Actinomycetales* and phylum *Proteobacteria* were associated with a protective effect on total SAD risk (Supplementary Table S8).

For the gut microbiota, order *Bifidobacteriales* (OR = 0.86, 95% CI = 0.77 - 0.95, $P = 2.55 \times 10^{-3}$, IVW) and phylum *Actinobacteria* (OR = 0.85, 95% CI = 0.76 - 0.94, $P = 2.82 \times 10^{-3}$, IVW) displayed strong causal associations with a decreased risk of SADs. Order *MollicutesRF9*, family *Lachnospiraceae*, and genus *Allisonella* were

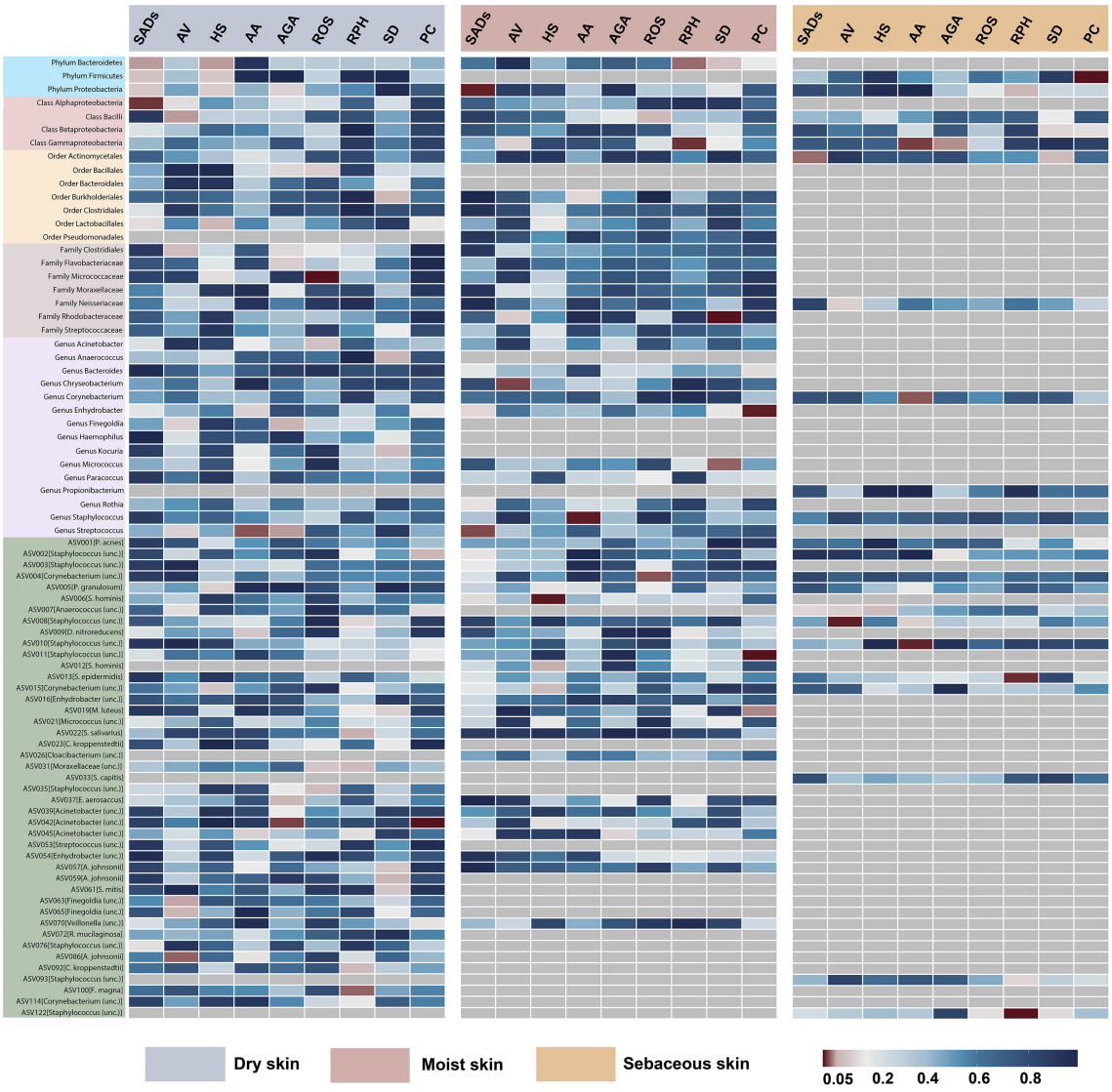

FIGURE 2
Heatmap showing causal associations between skin microbiota and SADs. SADs, skin appendage Disorders; AV, acne vulgaris; HS, hidradenitis suppurativa; AA, alopecia areata; AGA, androgenic alopecia; ROS, rosacea; RPH, rhinophyma; SD, seborrhoeic dermatitis; and PC, pilonidal cyst.

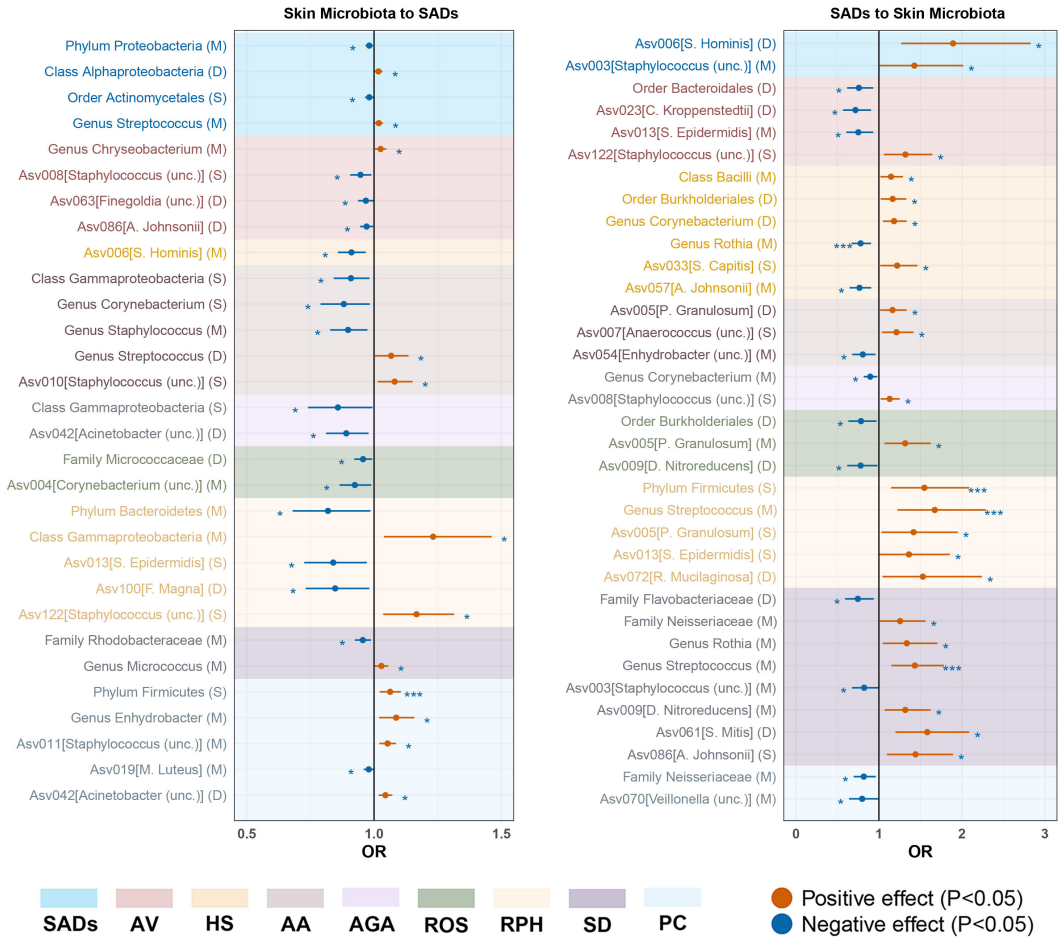

FIGURE 3
Forest plot of significant bidirectional causalities between skin microbiota and SADs. M, moist; D, dry; S, sebaceous; SADs, skin appendage Disorders; AV, acne vulgaris; HS, hidradenitis suppurativa; AA, alopecia areata; AGA, androgenic alopecia; ROS, rosacea; RPH, rhinophyma; SD, seborrhoeic dermatitis; and PC, pilonidal cyst. * and *** represent nominal causalities and strong causal associations, respectively.

associated with an inducing effect on SADs. Ten bacterial taxa, namely, order *Enterobacteriales* and *Lactobacillales*, class *Actinobacteria*, family *Bifidobacteriaceae* and *Enterobacteriaceae*, and genus *Bifidobacterium*, *Butyrivibrio*, *Clostridiuminnocuumgroup*, *Eubacteriumcoprostanoligenesgroup*, and *Haemophilus*, were associated with a protective effect on SADs risk (Supplementary Table S10).

Acne vulgaris

For the skin microbiota, ASV008 [*Staphylococcus* (unc.)], ASV063 [*Finegoldia* (unc.)] and ASV086 [*A. johnsonii*] were associated with an inducing effect on AV risk. Genus *Chryseobacterium* was associated with a protective effect on AV risk (Supplementary Table S8).

For the gut microbiota, class *Actinobacteria* (OR = 0.76, 95% CI = 0.64 - 0.92, $P = 4.21 \times 10^{-3}$, IVW), and family *Bifidobacteriaceae* (OR = 0.70, 95% CI = 0.56 - 0.87, $P = 1.08 \times 10^{-3}$, IVW) displayed strong causal associations with a decreased risk of AV. Genus *Allisonella* (OR = 1.39, 95% CI = 1.16 - 1.67, $P = 3.81 \times 10^{-3}$, IVW) showed a strong causal association with an increased risk of AV. Five bacterial taxa, namely, family *Bacteroidaceae*, *Clostridiaceae1*, *Porphyromonadaceae*, and genus *Bacteroides* and *Victivallis*, were associated with an inducing effect on AV risk. Six bacterial taxa, namely, phylum *Actinobacteria*, order *Lactobacillales*, family *Lactobacillaceae*, and genus *Bifidobacterium*, *Fusicatenibacter*, and *Lactobacillus* were associated with a protective effect on AV risk (Supplementary Table S10).

Hidradenitis suppurativa

For the skin microbiota, ASV006 [*S. hominis*] was associated with a protective effect on the risk of HS (Supplementary Table S8).

For the gut microbiota, phylum *Lentisphaerae* and family *Prevotellaceae* were associated with an inducing effect on HS risk. Genus *Bifidobacterium*, *Eubacteriumfissicatenagroup*, and *Fusicatenibacter* were associated with a protective effect on HS risk (Supplementary Table S10).

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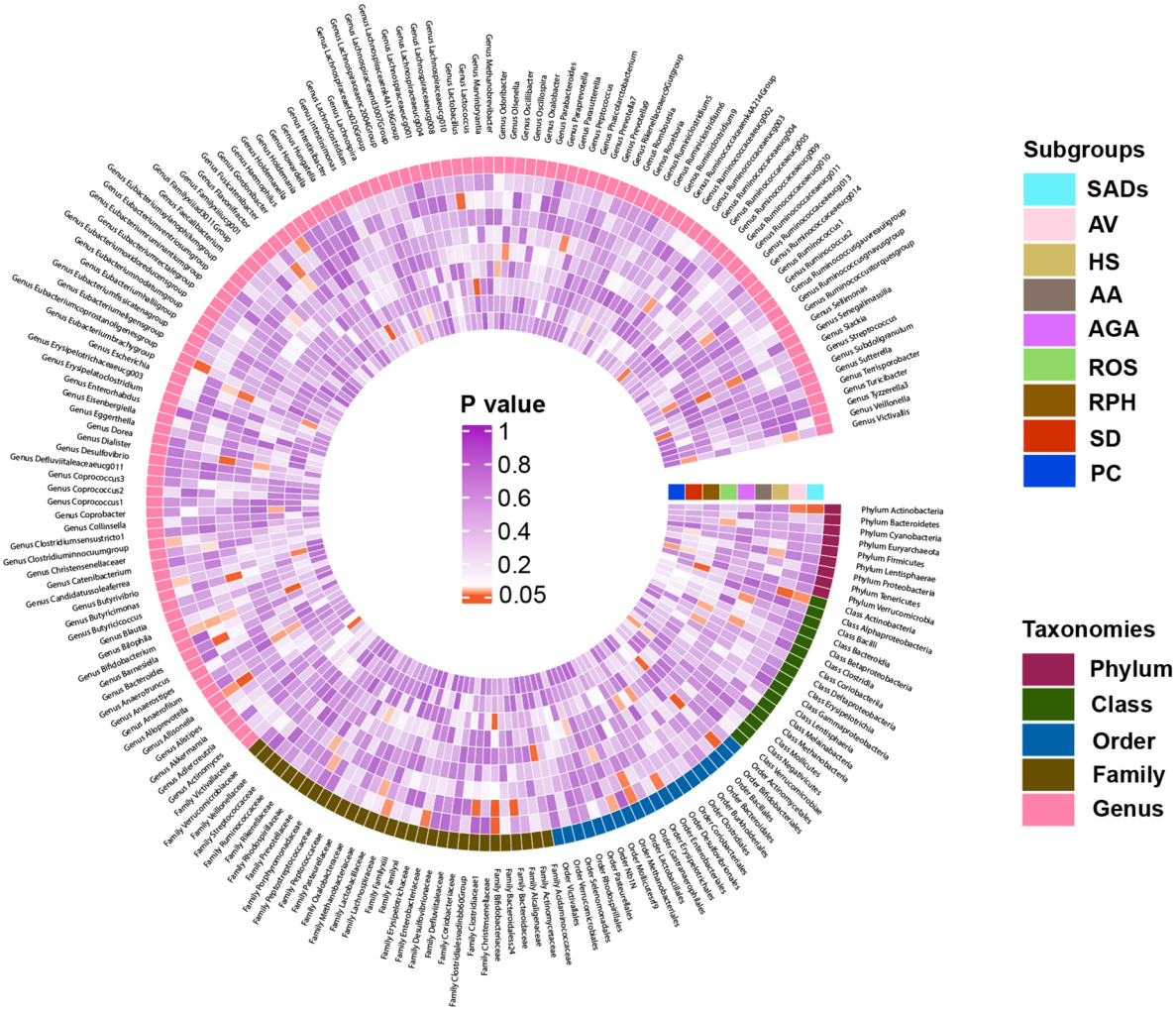

FIGURE 4
Heatmap showing causal associations between gut microbiota and SADs. SADs, skin appendage Disorders; AV, acne vulgaris; HS, hidradenitis suppurativa; AA, alopecia areata; AGA, androgenic alopecia; ROS, rosacea; RPH, rhinophyma; SD, seborrhoeic dermatitis; and PC, pilonidal cyst.

Alopecia areata

For the skin microbiota, ASV010 [*Staphylococcus* (unc.)] and genus *streptococcus* were associated with an inducing effect on AA risk. Class *Gammaproteobacteria*, genus *Corynebacterium*, and *Staphylococcus* were associated with a protective effect on AA risk (Supplementary Table S8).

For the gut microbiota, order *Lactobacillales* (OR = 0.38, 95% CI = 0.22 - 0.65, $P = 4.07 \times 10^{-4}$, IVW) showed a strong causal association with a decreased risk of AA. Six bacterial taxa, namely, order *MollicutesRF9*, genus *Olsenella*, *RuminococcaceaeUCG004*, *Eubacteriumnodatumgroup*, *Faecalibacterium*, and *Peptococcus*, were associated with an inducing effect on AA risk. Eight bacterial taxa, namely, class *Bacilli* and *Clostridia*, family *Acidaminococcaceae*, and genus *Anaerofilum*, *Butyricimonas*, *Dialister*, *Ruminococcus2*, and *Slackia*, were associated with a protective effect on AA risk (Supplementary Table S10).

Androgenic alopecia

For the skin microbiota, ASV042 [*Acinetobacter* (unc.)] and class *Gammaproteobacteria* were associated with a protective effect on AGA risk (Supplementary Table S8).

For the gut microbiota, genus *Olsenella* and *Ruminococcaceae UCG004* were associated with an inducing effect on AGA risk. Family *Acidaminococcaceae* and genus *Anaerofilum* were associated with a protective effect on AGA risk (Supplementary Table S10).

Rosacea

For the skin microbiota, ASV004 [*Corynebacterium* (unc.)] and family *Micrococcaceae* were associated with an inducing effect on ROS risk (Supplementary Table S8).

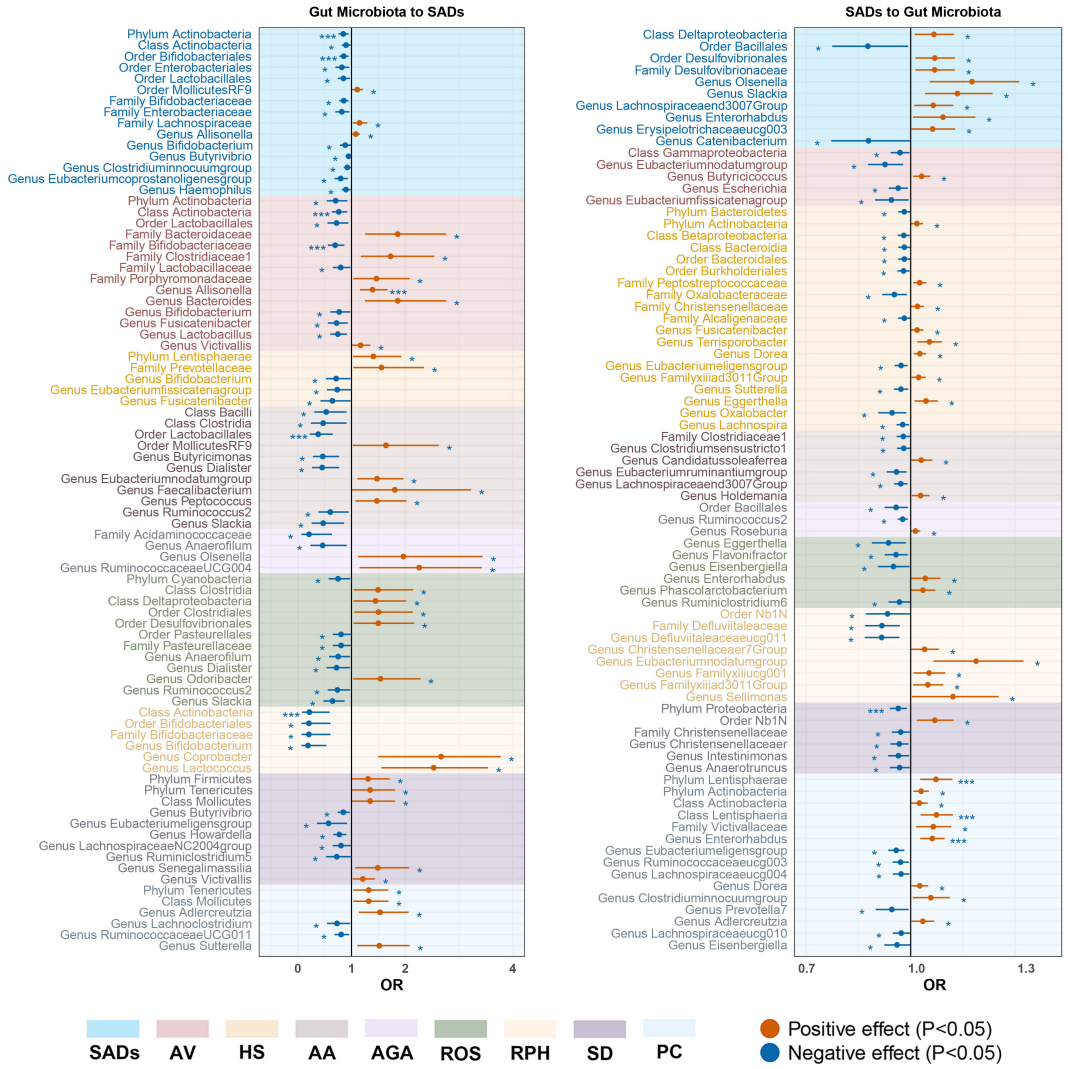

FIGURE 5
Forest plot of significant bidirectional causalities between gut microbiota and SADs. SADs, skin appendage Disorders; AV, acne vulgaris; HS, hidradenitis suppurativa; AA, alopecia areata; AGA, androgenic alopecia; ROS, rosacea; RPH, rhinophyma; SD, seborrhoeic dermatitis; and PC, pilonidal cyst. * and *** represent nominal causalities and strong causal associations, respectively.

For the gut microbiota, five bacterial taxa, namely, class *Clostridia* and *Deltaproteobacteria*, order *Clostridiales* and *Desulfovibrionales*, and genus *Odoribacter*, were associated with an inducing effect on ROS risk. Phylum *Cyanobacteria*, order *Pasteurellales*, Family *Pasteurellaceae*, and genus *Anaerofilum*, *Dialister*, *Ruminococcus2*, and *Slackia*, were associated with a protective effect on ROS risk (Supplementary Table S10).

For the gut microbiota, class *Actinobacteria* (OR = 0.22, 95% CI = 0.08 - 0.59, $P = 2.93 \times 10^{-3}$, IVW) exhibited a strong causal association with a decreased risk of RPH. Genus *Coprobacter* and *Lactococcus* were associated with an inducing effect on RPH risk. Order *Bifidobacteriales*, family *Bifidobacteriaceae*, and genus *Bifidobacterium* were associated with a protective effect on RPH risk (Supplementary Table S10).

Rhinophyma

For the skin microbiota, ASV122 [*Staphylococcus* (unc.)] and class *gammaproteobacteria* were associated with an inducing effect on RPH risk. Phylum *Bacteroidetes*, ASV013 [*S. epidermidis*] and ASV100 [*F. magna*] were associated with a protective effect on RPH risk (Supplementary Table S8).

Seborrhoeic dermatitis

For the skin microbiota, the genus *Micrococcus* was associated with an inducing effect on SD risk. Family *Rhodobacteraceae* was associated with a protective effect on SD risk (Supplementary Table S8).

For the gut microbiota, five bacterial taxa, namely, phylum *Tenericutes* and *Firmicutes*, class *Mollicutes*, and genus

Senegalimassilia and *Victivallis*, were associated with an inducing effect on SD risk. Genus *Butyrivibrio*, *Eubacteriumeligensgroup*, *Howardella*, *LachnospiraceaeNC2004group*, and *Ruminiclostridium5* were associated with a protective effect on SD risk (Supplementary Table S10).

Pilonidal cyst

For the skin microbiota, phylum *Firmicutes* (OR = 0.22, 95% CI = 0.08 - 0.59, $P = 2.93 \times 10^{-3}$, IVW) displayed a strong causal association with an increased risk of PC. ASV011[*Staphylococcus* (unc.)], ASV042 [*Acinetobacter* (unc.)] and genus *Enhydrobacter* were associated with an inducing effect on PC risk. ASV019[*M. luteus*] was associated with a protective effect on PC risk (Supplementary Table S8).

For the gut microbiota, phylum *Tenericutes*, class *Mollicutes*, genus *Adlercreutzia*, and *Sutterella* were associated with an inducing effect on PC risk. Genus *Lachnoclostridium* and *RuminococcaceaeUCG011* were associated with a protective effect on PC risk (Supplementary Table S10).

Reverse MR analysis

In the reverse MR analysis, 119 causal associations were found between SADs and skin microbiota of the KORA FF4 and PopGen cohorts (Supplementary Table S7). After the microenvironment-based meta-analysis of two cohorts, 35 causal associations remained significant ($P < 0.05$) in the moist, dry, or sebaceous skin (Supplementary Table S9). Among the causalities, HS displayed a strong causal association with a decreased abundance of genus *Rothia* (OR = 0.78, 95% CI = 0.67 - 0.91, $P = 1.10 \times 10^{-3}$, IVW). RPA showed strong causal associations with an increased abundance of phylum *Firmicutes* (OR = 1.55, 95% CI = 1.14 - 2.08, $P = 4.20 \times 10^{-3}$, IVW) and genus *Streptococcus* (OR = 1.67, 95% CI = 1.22 - 2.29, $P = 1.20 \times 10^{-3}$, IVW). SD exhibited a strong causal association with an increased abundance of genus *Streptococcus* (OR = 1.43, 95% CI = 1.15 - 1.78, $P = 1.30 \times 10^{-3}$, IVW).

As for the gut, 78 causal associations were found between SADs and gut microbiota (Supplementary Table S11). Among the causalities, PC showed strong causal associations with an increased abundance of phylum *Lentisphaerae* (OR = 1.07, 95% CI = 1.03 - 1.12, $P = 1.40 \times 10^{-3}$, IVW), class *Lentisphaeria* (OR = 1.07, 95% CI = 1.03 - 1.12, $P = 1.10 \times 10^{-3}$, IVW) and genus *Enterorhabdus* (OR = 1.06, 95% CI = 1.03 - 1.10, $P = 3.50 \times 10^{-4}$, IVW). SD demonstrated a strong causal relationship with a drop abundance in the phylum *Proteobacteria* (OR = 0.96, 95% CI = 0.94 - 0.99, $P = 4.60 \times 10^{-3}$, IVW).

Sensitivity analysis

The causal estimates for magnitude and direction remained consistent across the weighted median, MR-Egger, weighted mode, and simple mode methods (Figure 6). The results of the LOO analysis indicated that no single SNP disproportionately influenced the overall causal estimate. No horizontal pleiotropy of the IVs was detected, as evidenced by the MR-PRESSO global test ($P > 0.05$) and MR-Egger regression ($P > 0.05$). Moreover, the Cochrane *Q* statistics indicated no significant heterogeneity ($P > 0.05$). The Steiger directionality test implied that the causalities identified were free of reverse causality bias ($P < 0.05$) (Supplementary Table S12-15).

Discussion

The results of this study provide a robust foundation for understanding the intricate relationships between the skin and gut microbiota and SADs. A total of 65 and 161 causal relationships between genetic liability in the skin and gut microbiota with SADs were identified, respectively. Among these, we separately found 5 and 11 strong causal associations that passed Bonferroni correction in the skin and gut microbiota with SADs. Several skin bacteria, such as *Staphylococcus*, *Streptococcus*, and *Propionibacterium*, were considered associated with multiple SADs. As gut probiotics, *Bifidobacteria* and *Lactobacilli* were associated with a protective effect on SAD risk. Our findings indicated the crucial role that gut and skin microbiota play in the host-microbiota interaction of skin diseases, supporting the notion that adjusting the host-microbe balance is crucial for the prevention and therapy of SADs.

Commensal bacteria are crucial for maintaining the immune system. Numerous studies have investigated the gut microbiome concerning various illnesses, including its effects on the skin (5). Skin bacteria, although less numerous than those found in the gastrointestinal tract, have comparable roles in immunological modulation and disease development (9). It is increasingly evident that both the cutaneous and gut microbiomes profoundly impact human health, particularly in the context of SADs.

P. acnes is recognized as the main disease-associated bacterium in the case of AV (30, 31). Research comparing the skin of acne sufferers and healthy individuals found that while the relative abundances of *P. acnes* species were similar, significant differences were observed at the strain level between the two cohorts (32). Certain strains had a strong connection to acne, whereas other strains were more prevalent in healthy skin. Interestingly, microbiome research has shown that AV is more closely associated with the virulence of specific *P. acnes* strains rather than the total quantity of *P. acnes* (31). Dysbiosis in AV is indicated by a reduction in the percentage of *P. acnes* strains RT6 and a notable increase in the percentages of *P. acnes* strains RT4, RT5, RT7, RT8, RT9, and RT10 (33). Moreover, a competitive relationship has been observed between *S. epidermidis* and *C. acnes* (34, 35). *S. epidermidis* promotes glycerol fermentation and releases succinic acid, which prevents *C. acnes* from proliferating (34). Conversely, *P. acnes* maintains the acidic environment of the pilosebaceous follicle, hydrolyzes sebaceous triglycerides, and produces propionic acid to inhibit *S. epidermidis* proliferation (35). Our research demonstrated that ASV001 [*P. acnes*] at sebaceous skin sites was positively correlated with AV in the PopGen cohort, whereas AV was negatively correlated with ASV013 [*S. epidermidis*] at moist skin sites in the KORA FF4 cohort. Although these associations lost significance in the meta-analysis, they potentially illustrate the role of *P. acnes* in promoting acne and inhibiting the colonization of *S. epidermidis* in acne patients.

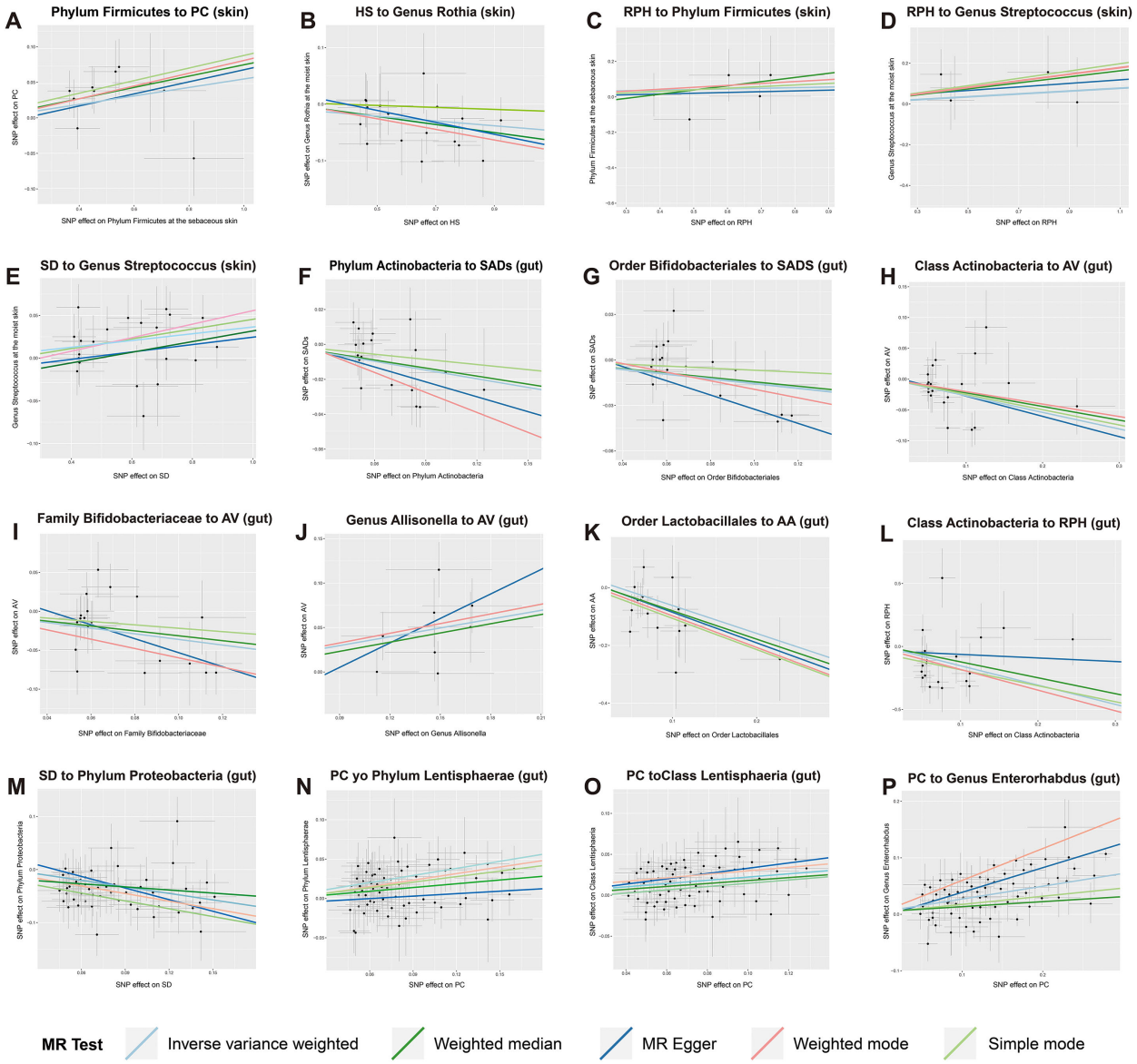

FIGURE 6
Scatter plots of strong causal associations between skin and gut microbiota and SADs. **(A–E)** the causal associations between skin microbiota and multiple SADs; **(F–P)** the causal associations between gut microbiota and multiple SADs. SADs, skin appendage Disorders; AV, acne vulgaris; SD, seborrhoeic dermatitis; HS, hidradenitis suppurativa; AA, alopecia areata; RPH, rhinophyma; and PC, pilonidal cyst.

Bifidobacteria and *Lactobacilli*, the commensal microorganisms inhabiting the gastrointestinal tract, have garnered interest for their therapeutic potential as probiotics in the amelioration of inflammatory dermatological conditions, including AV (34, 36). Their therapeutic actions are purportedly mediated through the modulation of systemic oxidative stress levels, the regulation of cytokine production, and the attenuation of inflammatory biomarkers (34). Clinical investigations have revealed that the administration of a composite probiotic formulation containing *Lactobacillus acidophilus*, *Lactobacillus delbrueckii bulgaricus*, and *Bifidobacterium bifidum* may rival the efficacy of conventional antibiotic therapy, such as minocycline, in acne management (37). An observed lesion reduction of 67% after a 12-week therapeutic regimen, coupled with a reduced incidence of adverse effects, substantiates this claim (37). The oral administration of various *Lactobacillus* species has been shown to reduce the total lesion count by 56%-67%, decrease sebum content by 81%, and improve the Investigators Global Assessment in 80% of patients (38). The MR outcomes of our research are consistent with the findings of previous studies. *Bifidobacteria* exhibited a significant causal association with inhibiting AV, and *Lactobacilli* were also regarded as potential protective factors for AV. Notably, the genus *Allisonella* showed a significant causal effect on facilitating AV, which may be due to its unique biochemical capability to produce histamine, a compound associated with gut inflammation, immune response, and allergic reactions (39).

Microbial changes in patients with AA revealed a lower abundance of *S. epidermidis* and over-colonization with *P. acnes*;

however, it remains unclear if these alterations are the cause or effect of the illness (35). In this study, AA was positively correlated with ASV005 [*P. granulosum*] at moist skin sites, while the genus *Staphylococcus* at moist skin sites displayed a potential inhibitory effect on AA. Recent research has also connected AA to gut dysbiosis in addition to skin microbiota alterations. The role of the gut microbiome in the pathogenesis of AA is supported by cases of long-term hair growth following fecal microbiota transplants (40). However, some detected variations in gut flora in AA patients were not statistically significant (41). Following the Bonferroni adjustment, no apparent causal relationships between AA and the gut microbiota were found according to the reverse MR analysis, which aligns with previous studies. However, this MR study indicated that the order *Lactobacillales* displayed a significant causal association with inhibiting AA. Mechanistic studies have shown that *Lactobacillales*, as an important probiotic group, may influence AA through the modulation of immune function (36). *Lactobacillales* can regulate the body's immune response via various mechanisms, such as promoting the production of regulatory T cells and reducing the release of pro-inflammatory cytokines (42). This immunomodulatory action may contribute to the suppression of the autoimmune response underlying AA.

There is growing evidence that a dysbiotic skin microbiome is associated with HS (43); however, the exact causal link between changes in the skin microbiome and the onset of the disease is still unknown. It was found that the bacterial community on the skin surface of HS patients was significantly altered, primarily characterized by a significant decrease in *Staphylococcus epidermidis* and *Staphylococcus hominis* in the axilla, gluteal cleft, and groin areas of HS patients (44). *Propionibacterium* was also observed to be more abundant in controls than in HS patients (44). The MR outcomes indicated that ASV006 [*S. hominis*] at moist skin sites was regarded as a potential protective factor for HS, and HS could induce the growth of ASV033 [*S. capitis*] at sebaceous skin sites. However, no causal association was found between *Propionibacterium* and HS, suggesting that this genus may not have a direct impact on the etiology of the illness. This dysbiosis of the skin microbiome may be related to chronic inflammation and a hypoxic environment in the HS focal areas. There are several comorbid conditions linked to HS. Notably, patients with HS have up to eight times higher rates of inflammatory bowel disease compared to the general population, with Crohn's disease outpacing ulcerative colitis in frequency (45). An altered gut microbiota may be a factor in the development of HS since it has been linked to several pathophysiologies, including immune dysregulation (46). In this study, the genera *Bifidobacterium*, *Eubacterium fissicatenagroup*, and *Fusicatenibacter* exhibited potential causal effects on inhibiting HS, while the family *Prevotellaceae* and phylum *Lentisphaerae* showed potential causal effects on promoting HS. However, the specific mechanisms and modes of intervention for these causalities need to be further explored.

ROS, RPH, and SD are skin conditions associated with microbiological and immunological dysbiosis of the skin environment, as well as with *Demodex mites* and *Malassezia fungus* (47–49). Microbiota-associated alterations in the skin and small intestine have been concurrently noted in these diseases. Since ROS is exacerbated or triggered by emotional stress, the brain may play a role in the gut-brain-skin axis (47, 48). Unfortunately, conflicting findings have been reported in microbial research conducted on ROS patients at both the skin and gut levels (50). Some bacteria thought to be connected to ROS include *S. epidermidis*, *Helicobacter pylori*, *Chlamydophila pneumoniae*, and *Bacillus oleronius* (50). Our MR results indicated that ASV013 [*S. epidermidis*] at sebaceous skin sites could reduce the incidence of RPH, which is often considered a late complication of ROS. It has been suggested that ROS may be improved by taking oral probiotics such as *Lactobacillus salivarius* and *Bifidobacterium*, which was also observed in the MR results (36). The family *Bifidobacteriaceae* and the genus *Bifidobacterium* exhibited potential causal associations with inhibiting RPH. Additionally, it was reported that *Acinetobacter*, *Staphylococcus*, and *Streptococcus* predominated in lesional skin when examining the bacterial microbiota in 24 individuals with SD (3). This phenomenon was also reflected in the reverse MR analysis, which showed an increase in the genus *Streptococcus* and ASV086 [*A. johnsonii*]. Furthermore, it was shown that the phylum *Firmicutes* significantly contributed to the development of PC. Nevertheless, the connection between PC and the skin and gut microbiota has not been well studied, and further research is still required.

The intricate relationships between the skin and gut microbiota and SADs can be explained through several biological mechanisms. Firstly, microbes can significantly influence the host immune system. For instance, *Staphylococcus aureus* is known to produce superantigens and other virulence factors that hyperactivate the immune system, leading to chronic inflammation and tissue damage observed in conditions such as atopic dermatitis and hidradenitis suppurativa (51). These virulence factors can trigger the release of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α, contributing to the inflammatory milieu characteristic of these disorders (52). Conversely, beneficial gut microbes like *Bifidobacteria* and *Lactobacilli* play a protective role by modulating systemic immune responses. These probiotics can enhance the production of anti-inflammatory cytokines, such as IL-10, and promote the differentiation of regulatory T cells, which help maintain immune homeostasis and prevent excessive inflammation (53). Secondly, microbial metabolites, such as short-chain fatty acids (SCFAs), play a crucial role in maintaining skin health. SCFAs like butyrate, acetate, and propionate, produced by gut microbiota during the fermentation of dietary fibers, have potent anti-inflammatory properties and can strengthen gut barrier function (54). These metabolites can enter the bloodstream and exert systemic effects, including on the skin. For example, SCFAs have been shown to enhance the differentiation of keratinocytes and promote wound healing, which could be beneficial for conditions like eczema and psoriasis (55). Moreover, the integrity of epithelial barriers in both the gut and skin is vital for preventing pathogen invasion and maintaining overall health. In the gut, harmful bacteria can disrupt tight junction proteins, leading to increased intestinal permeability, also known as "leaky gut," which allows endotoxins to enter the bloodstream and trigger systemic inflammation (56). This systemic inflammation can adversely affect skin health, exacerbating conditions like acne and psoriasis (57).

Although the conservativeness of the Bonferroni correction method may lead to false negatives (58), the stringent control provided by the Bonferroni correction is essential for the integrity of our findings, thereby enhancing the reliability of causal inferences in genetic epidemiology (27, 28, 59). Many causal correlations in this study were regarded as nominally significant due to failing the Bonferroni corrected test. We speculate that the contribution of a single microbiome to illness may not be as substantial as initially estimated. Rather, the illnesses may be caused and coordinated by multiple bacteria. These bacteria with nominal causalities may also be involved in skin and intestinal-related SADs. The investigation of the human microbiota in SADs is presently ongoing, and the precise role of the microbiome in the pathophysiology of SADs remains to be thoroughly studied. Understanding the causality of the interplay between various microbiotas and SADs can aid in comprehending the intricate crosstalk between the skin and gut, and offer guidance for future targeted multi-flora medication development.

To the best of our knowledge, this is the first MR study to comprehensively examine the causal effect between skin and gut microbiota and SADs. A bidirectional, two-sample MR design following STROBE-MR guidelines was employed to eliminate the potential for reverse causation and confounding factors. Exposure and outcome summary data were separately acquired from German and Finnish populations to ensure nonoverlapping data sets and avoid bias. A microenvironment-based meta-analysis of skin microbiota was conducted to enhance the statistical power of the results. However, several limitations of our study should be noted. First, we included SNPs that met the locus-wide significance level (1×10^{-5}), as the SNPs identified using the genome-wide significance threshold (5×10^{-8}) were insufficient for sensitivity analysis and horizontal pleiotropy detection. Second, some taxa of skin microbiota at the ASV level lacked species-level annotations, possibly due to uncertain matches to the Ribosomal Database Project (RDP) database. Additionally, most 16S rRNA sequencing of human microbiota has focused on species composition. Recent research has indicated that distinct strains of microorganisms can exert significantly different effects on the host, even within the same species (60). Variations at the strain level have not been well studied and remain an area of interest for microbiota research. Third, there is limited knowledge of the causal link between SADs and non-bacterial components of the microbiota, such as fungi, archaea, and viruses. Therefore, future research using more advanced sequencing technology should be conducted to further elucidate the effects of skin and gut microbiota on SADs.

In summary, our study provided comprehensive evidence for the causal roles of skin and gut microbiota in SADs through the application of bidirectional MR analysis. This novel approach allowed us to establish strong causal links between specific microbial taxa and various SADs, underscoring the intricate interplay between microbial communities and skin health. The identification of specific skin and gut microbiota that influence the risk of SADs offers promising avenues for the development of microbiome-based diagnostic and therapeutic strategies. Future studies involving genomic, transcriptomic, and metabolomic analyses should focus on elucidating the underlying mechanisms of how specific microbes influence SADs and modulate skin health.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

YZ: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. WL: Writing – original draft, Methodology, Formal analysis, Data curation. MW: Writing – original draft, Visualization, Software, Resources. XW: Writing – original draft, Visualization, Software, Resources. SW: Writing – review & editing, Validation, Conceptualization.

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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.2024.1427276/full#supplementary-material

ADDITIONAL FILE 2
STROBE-MR checklist of suggested items for Mendelian randomization studies.

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The gut homeostasis-immune system axis: novel insights into rheumatoid arthritis pathogenesis and treatment

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Rheumatoid arthritis is a widely prevalent autoimmune bone disease that imposes a significant burden on global healthcare systems due to its increasing incidence. In recent years, attention has focused on the interaction between gut homeostasis and the immune system, particularly in relation to bone health. Dysbiosis, which refers to an imbalance in the composition and function of the gut microbiota, has been shown to drive immune dysregulation through mechanisms such as the release of pro-inflammatory metabolites, increased gut permeability, and impaired regulatory T cell function. These factors collectively contribute to immune system imbalance, promoting the onset and progression of Rheumatoid arthritis. Dysbiosis induces both local and systemic inflammatory responses, activating key pro-inflammatory cytokines such as tumor necrosis factor-alpha, Interleukin-6, and Interleukin-17, which exacerbate joint inflammation and damage. Investigating the complex interactions between gut homeostasis and immune regulation in the context of Rheumatoid arthritis pathogenesis holds promise for identifying new therapeutic targets, revealing novel mechanisms of disease progression, and offering innovative strategies for clinical treatment.

KEYWORDS

gut homeostasis, immune system, rheumatoid arthritis, pathogenesis, novel therapeutic approaches

1 Introduction

Rheumatoid arthritis (RA) is a multifaceted, chronic autoimmune disorder characterized by persistent inflammation across multiple joints, leading to joint damage and functional impairment. This condition is typified by the production of autoantibodies, chronic synovitis, and sustained inflammation (1). Affecting approximately 1% of the global population, RA exhibits a higher prevalence among females (2). Hallmark manifestations include widespread joint swelling, tenderness, and systemic inflammation

driven by autoantibodies (2). The inflammatory pathways in RA are marked by alterations in the T helper 1 cell profile, which result in an imbalance between anti-inflammatory and pro-inflammatory cytokines (2). Autoantibodies linked to RA, such as rheumatoid factor, anti-citrullinated protein antibodies, and anti-carbamylated antibodies, can be detected in serum long before the clinical manifestation of the disease (3, 4). Although joint involvement is a hallmark of RA. Affected joint spaces exhibit the release of cytokines such as Interleukin-1, Interleukin-6, and tumor necrosis factor-alpha, accompanied by reduced levels of Interleukin-11, Interleukin-13, and Interleukin-10 (IL-10) (5). However, the pathogenesis of RA may involve extra-articular sites, including the lungs, periodontal tissues, gut microbiota, or citrullination processes in adipose tissue. This may also explain why some anti-citrullinated protein antibody-positive patients with joint pain present with normal synovial tissue (6).

In recent years, microbial factors have garnered significant attention for their association with the pathogenesis of RA. Patients with RA exhibit notable dysbiosis of the oral microbiome, which can be partially restored through RA treatment, with the extent of recovery closely correlated with the patient's therapeutic response (7). Epidemiological studies reveal that periodontitis, a chronic infectious oral disease, is highly prevalent among RA patients and is strongly linked to the disease. *Porphyromonas gingivalis*, a key pathogen in periodontitis, has been implicated in the development of RA due to its ability to synthesize bacterial peptidylarginine deiminase (7). This enzyme induces citrullination of α- and β-fibrin chains within the synovium, generating autoantigens that promote the production of anti-citrullinated protein antibodies (7). These anti-citrullinated protein antibodies form immune complexes with citrullinated proteins, which bind to fragment crystallizable region and complement component 5a receptors on immune and inflammatory cells, triggering a complex cascade of immune responses and the release of inflammatory mediators, ultimately resulting in synovial inflammation and the onset of RA (8).

The gut microbiota plays a pivotal role in modulating host immune responses and is intricately linked to the development of several autoimmune conditions (2). The complex interplay between gut health and immune system homeostasis may be a crucial determinant in the etiology and progression of RA. Regulation of gut and immune system homeostasis is vital for overall health. Gut homeostasis involves the composition, function, and stability of the gut microbiome, as well as the integrity of both physical and immune barriers (9). The gut microbiota, often considered an invisible organ, plays a crucial role in maintaining intestinal barrier function, nutrient absorption, and overall immune and metabolic balance (10, 11). One of its primary metabolic functions is aiding the digestion of indigestible food residues, such as polysaccharides, oligosaccharides, and unabsorbed sugars and alcohols (12). The intestinal barrier consists of multiple layers: an outer mucus layer, a symbiotic gut microbiota, defense proteins like antimicrobial peptides and secretory immunoglobulin A; a middle layer of intestinal epithelial cells; and an inner layer of innate and adaptive immune cells (13, 14). The mucosal immune system helps protect the body by activating and regulating effector cells. When this immune system is disrupted, pathogenic flora can escape the intestinal tract (15). Evidence suggests that gut microbiome dysbiosis is a critical environmental factor in RA pathogenesis and may provoke abnormal immune responses (16, 17). Gut microbiota dysbiosis increases intestinal mucosal permeability, elevating endotoxin levels in the bloodstream and disrupting the balance of downstream metabolites that regulate systemic inflammation. These disruptions can initiate chronic systemic inflammatory responses, potentially influencing the onset and progression of osteoarthritis (18). Although the role of the gut-immune homeostatic axis in RA has been explored, its intricate regulatory network is not yet fully understood. Further research into how the gut microbiota and its metabolites affect RA is valuable for understanding RA pathogenesis and developing novel treatment strategies.

2 Interactions between gut homeostasis and the immune system

Gut microbes are essential for sustaining immune system balance. Changes in the gut microbiota and their metabolites can trigger inflammatory disorders related to the immune system, while a compromised intestinal barrier increases permeability and stimulates immune responses (19). The gastrointestinal tract houses the majority of the body's immune cells and constantly interacts with the gut microbiota, influencing their function and characteristics. The gut microbiota's delicate equilibrium in fostering either commensal or symbiotic relationships enables continuous bidirectional communication with the host immune system (20). System is shown in Figure 1.

2.1 Interactions between the gut microbiome and the immune system

The interactions between gut microbes and the host immune system are complex and context-dependent. The critical role of the gut microbiome in immune system development and response has been well established (21, 22). The microbiota is essential for regulating immune cell activities and inflammatory cytokines, thereby normalizing immune responses (23). Various gut microbes modulate immune responses differently. For example, segmented filamentous bacteria (SFB) promote the development of intestinal helper T cells 17 (Th17), and the SFB-dependent Th17 response inhibits bacterial translocation in mice with constitutively activated myosin light chain kinase (24). SFB enhances autoimmune responses in a context-dependent manner while strengthening intestinal barrier integrity. *Clostridium*, on the other hand, induces regulatory T cells (Treg) (25, 26).

Abbreviations: RA, Rheumatoid arthritis; SFB, Segmented filamentous bacteria; Th17, Helper T cells 17; Treg, Regulatory T cells; IL-10, Interleukin-10; LPS, Lipopolysaccharide; LGS, leaky gut syndrome; PAD, peptidyl-arginine deiminase; TJ, tight junction.

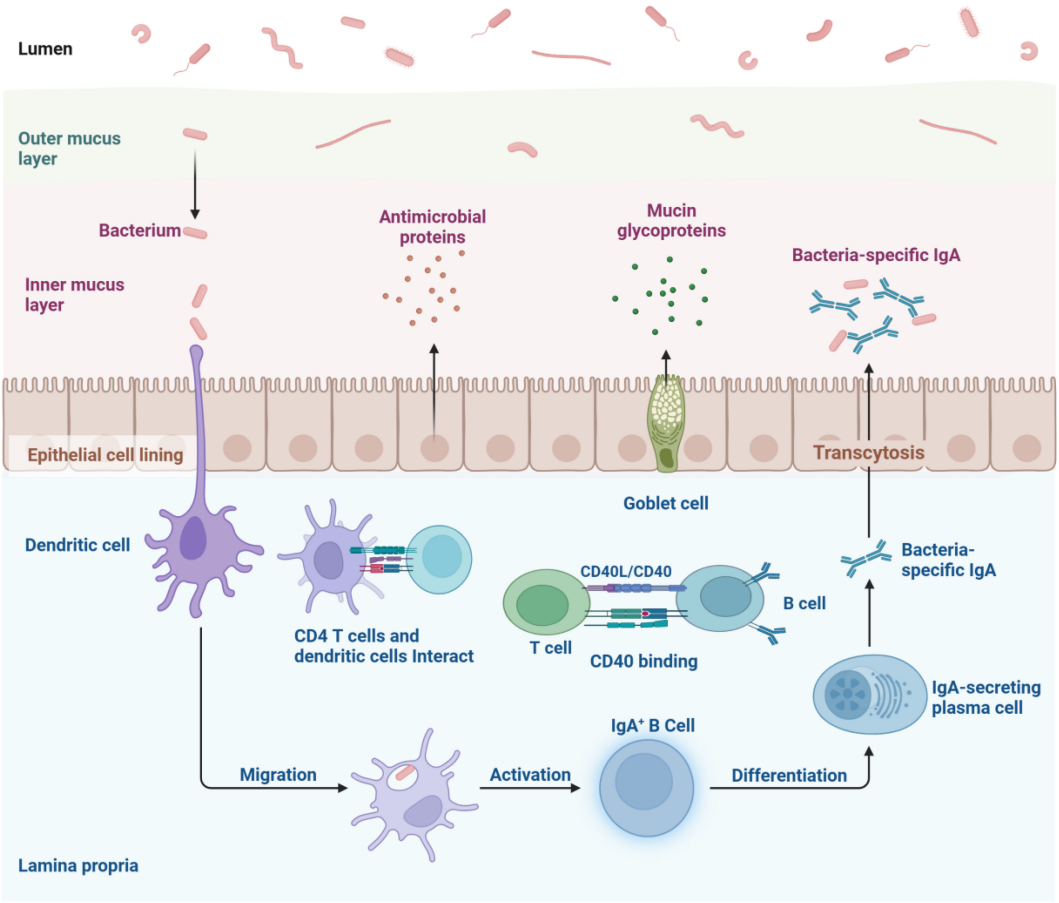

FIGURE 1
The intestinal mucosal immune network represents a intricate system comprising innate, adaptive, and immunoglobulin A-mediated immune mechanisms. Epithelial cells and dendritic cells recognize antigens via pattern recognition receptors and present them to T and B lymphocytes within lymphoid tissues, triggering adaptive immune responses. Simultaneously, they activate signaling cascades, stimulate the production of cytokines and antimicrobial peptides, and mount acute inflammatory reactions to eliminate offending agents. B cells differentiate into plasma cells that secrete immunoglobulin A, which is then transported into the gut lumen, where it neutralizes antigens and pathogens through its binding capabilities.

Additionally, distinct populations of Th17 cells exist in the gut, with their development largely influenced by different microorganisms. SFB-induced Th17 cells maintain homeostasis, whereas Th17 cells induced by Citrobacter are pro-inflammatory (27). Different bacterial species trigger distinct systemic immune responses. Probiotics like *Lactobacillus* and *Bifidobacterium* promote immune regulation by increasing regulatory T cell numbers, thereby maintaining immune tolerance and suppressing inflammatory responses. In contrast, harmful bacteria, such as those in the *Enterobacteriaceae* family, activate the Nuclear Factor kappa-light-chain-enhancer of Activated B cells signaling pathway and promote the release of pro-inflammatory cytokines, leading to systemic inflammation. Clinical studies have shown that elevated levels of *Prevotella* and *Veillonella* are closely associated with the development of autoimmune diseases. These bacteria exacerbate conditions like rheumatoid arthritis and inflammatory bowel disease by disrupting the gut barrier and facilitating the translocation of pathogens and their metabolites (28). Furthermore, during an inflammatory response, the intestinal microbiota can become disturbed, leading to an increase in harmful bacteria and a decrease in beneficial ones, which may exacerbate inflammation. For instance, the expansion of Pseudomonas aeruginosa causes intestinal inflammation and disrupts epithelial barrier integrity, allowing bacterial antigens to enter systemic circulation and activate immune responses at distant sites (29). Dysbiosis can result in the overactivation of T-helper 1 cells and Th17 cells, leading to excessive production of pro-inflammatory cytokines such as tumor necrosis factor, interleukin-6, and interleukin-17. This triggers systemic inflammation and immune dysregulation. Such abnormal cellular immune responses are pivotal in the pathogenesis and progression of autoimmune diseases like rheumatoid arthritis (19).

2.2 Gut microbial metabolite interactions with the immune system

The gut microbiota generates a variety of metabolites, including SCFAs, amines, polyamines, vitamins, and other small molecules. These metabolites are transported through the bloodstream to

tissues and organs throughout the body, where they influence immune responses and regulate inflammatory processes. Metabolites derived from gut microbiota are crucial in modulating the development and function of both adaptive and innate immune cells. IL-10 produced by effector T cells is a key self-regulatory mechanism that maintains immune balance (30). Short-chain fatty acids enhance IL-10 production in T-helper 1 Cells cells through a GPR43-dependent pathway and inhibit histone deacetylase histone deacetylase activation during T helper 1 and Th17 differentiation (22). Interleukin-22, a member of the IL-10 family, is vital for preventing intestinal inflammation, with CD4+ T cells being a major source of Interleukin-22 during chronic inflammation. Short-chain fatty acids stimulate Interleukin-22 production in CD4+ T cells via the G protein-coupled receptor 41 pathway and reduce histone deacetylase activity (22). Butyrate-treated dendritic cells support Treg differentiation and inhibit T-helper 1 Cells differentiation by increasing the expression of immunosuppressive enzymes such as indoleamine 2,3-dioxygenase 1 and aldehyde dehydrogenase 1 family member A2, through an SLC5A8-dependent mechanism (31). Microbial tryptophan metabolites, including indole and its derivatives, interact with aryl hydrocarbon receptors and influence B cell development, differentiation, cytokine production, and regulation through aryl hydrocarbon receptors signaling (32–34). Lipopolysaccharide (LPS), a component of Gram-negative bacteria in the gut, These metabolites are able to enter the bloodstream, initiates a cell-factor cascade that contributes to T cell-mediated inflammatory processes (35). Additionally, bile acids and their metabolites modulate immune responses by regulating signaling pathways and balancing Th17 and Treg cells (36).

2.3 Interactions between the gut barrier and the immune system

The gut barrier, which includes intestinal epithelial cells, tight junction proteins, and mucus layers (37). The integrity of the gut barrier is vital for preventing pathogen invasion and maintaining immune homeostasis. When this barrier is compromised, undigested food particles, pathogens, and toxins can penetrate into the bloodstream, triggering systemic immune responses and promoting chronic inflammation (38). Dysfunction of the gut barrier has been associated with various immune-mediated diseases, including rheumatoid arthritis and inflammatory bowel disease (39). Gut-resident immune cells, such as dendritic cells, goblet cells, and T cells, are essential for regulating both local and systemic immune responses by detecting and responding to gut microbes and their metabolites (40). Disruption of the gut barrier can cause apoptosis of intestinal epithelial cells and create a pro-inflammatory environment, which includes the activation of autoreactive Th17 cells and other helper T cells (19). Pro-inflammatory cytokines like tumor necrosis factor-alpha and interferon-gamma can damage tight junctions (41–43), whereas immunosuppressive cytokines such as interleukin-10 and transforming growth factor-beta help to preserve their integrity (44). A weakened mucosal immune defense reduces Immunoglobulin A secretion or causes Immunoglobulin A dysfunction, while dysbiosis activates B cells, leading to abnormal levels of pro-inflammatory immunoglobulins like Immunoglobulin G. This can elevate rheumatoid arthritis-specific antibodies, further promoting systemic autoimmune responses (29, 45).

3 Impact of the gut homeostasis-immune system axis on rheumatoid arthritis

RA is complex and multifactorial (46, 47). The human body is home to over 100 trillion microbes, predominantly residing in the gut (48). Advances in bacterial DNA sequencing technology have elucidated the relationship between gut bacteria and RA, underscoring the significant role of the gut microbiota in the disease (29, 47). Research has shown notable differences in the gut microbiome between RA patients and healthy individuals, and these differences can affect RA manifestations (49, 50). The effect of intestinal homeostasis on rheumatoid arthritis is shown in Figure 2. Pro-inflammatory gut pathogens reshape the immune landscape by overactivating the innate immune system, which subsequently drives abnormal activation of the adaptive immune system, inducing RA. The gut microbiota composition also modulates sex hormone levels, influencing RA risk. Hormonal deficiencies increase intestinal permeability, elevating Th17 cells, nuclear factor kappa-light-chain-enhancer of activated B cells, Interleukin-17, and tumor necrosis factor-αlevels in peripheral blood, which promotes bone resorption. Gut homeostasis imbalance is closely linked to RA pathogenesis through several mechanisms, including immune modulation by microbial metabolites and local inflammation initiated by bacterial components in synovial tissue (51).

3.1 Gut microbiome dysbiosis regulates inflammatory responses and influences rheumatoid arthritis

In healthy individuals, the gut microbiota is characterized by high diversity and stability, creating a dynamically balanced ecosystem crucial for maintaining gut homeostasis and immune balance (46). Increasing research focuses on the interaction of the gut microbiome in RA patients. The gut microbiota of RA patients undergoes significant alterations, characterized by a decrease in beneficial bacteria and an increase in pathogenic species. This dysbiosis is closely associated with immune system imbalance, driving inflammatory responses and exacerbating RA severity. Studies have demonstrated that in RA patients, significant upregulation is observed in bacterial genera such as Klebsiella, *Enterobacteriaceae*, *Eggerthella*, and *Flavobacteriaceae*, while genera like *Clostridium*, *Blautia*, and *Enterococcus* are downregulated (28). The proportion of Firmicutes is notably reduced in the RA gut, whereas Bacteroidetes levels are relatively

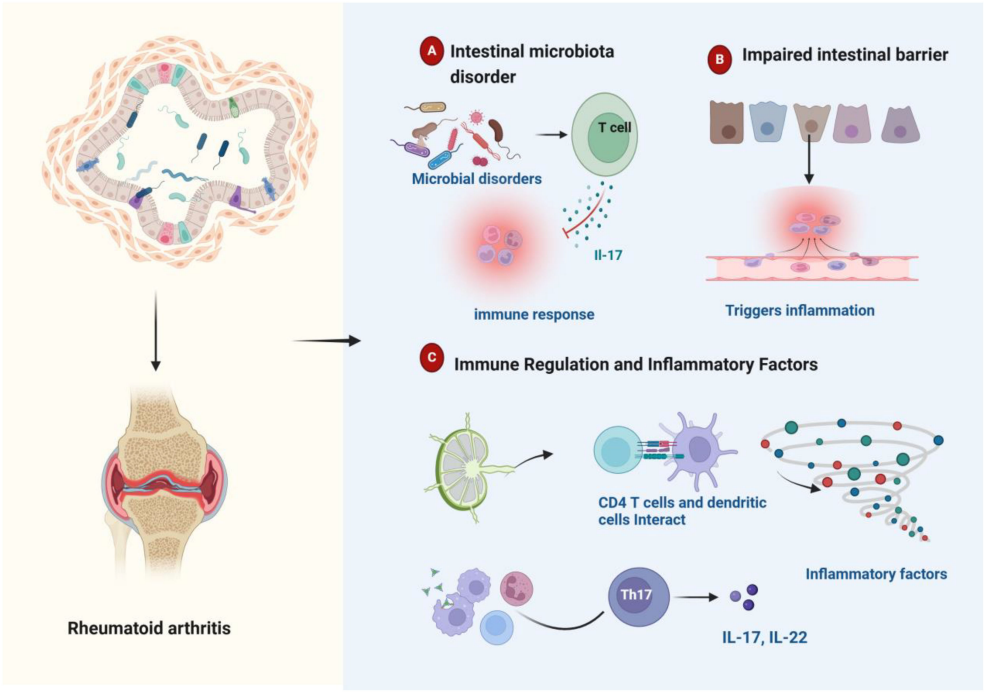

FIGURE 2
Schematic representation of intestinal homeostasis influencing rheumatoid arthritis pathogenesis. **(A)** Dysbiosis of the gut microbiome can trigger an immune response. **(B)** Damage to the gut barrier can trigger inflammation. **(C)** Immune cells residing in the gut play a regulatory role in modulating inflammatory cytokine production. Gut microbial imbalance is associated with aberrant inflammatory cytokine profiles and dysregulated immune responses, which collectively contribute to the development and progression of rheumatoid arthritis.

increased (28). Firmicutes members, including lactobacilli and bifidobacteria, exert anti-inflammatory effects and modulate immune responses. Conversely, some Bacteroidetes produce toxins and metabolites that exacerbate inflammation and RA. In treatment-naive, new-onset RA patients, *Prevotella* is significantly enriched and shows gene-cluster rearrangement; its 27-kDa protein stimulates a T helper 1 response in 42% of these patients (52, 53). *Lactobacillus* casei has been shown to significantly reduce the expression of interferon-gamma, tumor necrosis factor-alpha, and interleukin-1 beta, thereby preventing joint damage (54). Analysis of stool metabolites in RA patients reveals higher levels of glycerophospholipids, benzene and its derivatives, and cholesterol, while sphingolipids and tryptophan downstream metabolites are found at lower levels (28). In a collagen-induced arthritis mouse model, etanercept significantly decreased the abundance of *Escherichia/Shigella* while increasing the relative proportions of *Tannerella*, *Lactobacillus*, and *Clostridium* XIVa. Furthermore, certain natural compounds, such as clematichinenoside, have shown promise in improving RA-related gut dysbiosis and have been proven effective in reducing arthritis symptoms (55).

3.2 Impaired intestinal barrier and autoimmune activation

The intestinal barrier, consisting of epithelial cells, tight junction proteins, and mucus layers, primarily functions to prevent pathogens and harmful substances from entering the bloodstream (56, 57). This multilayered barrier is crucial for maintaining gut homeostasis and systemic immune responses. The integrity of the gut barrier is essential for maintaining health. LPS is widely used as a biomarker to assess gut barrier dysfunction, directly indicating the translocation of endotoxins into the bloodstream. Other markers, such as D-lactate and L-lactate, produced by gut bacteria, indirectly reflect changes in gut permeability (58). Barrier proteins, such as zonulin and adhesion molecules, are crucial for maintaining tight junctions, and their circulating levels reflect the functional status of the barrier. When the gut mucosal barrier is impaired, the disruption of tight junctions increases permeability, allowing undigested food particles, toxins, and pathogens to cross the compromised barrier and enter the bloodstream. Impairment of the intestinal barrier leads to tight junction breakdown, increased intestinal permeability, and the translocation of undigested food particles, toxins, and pathogens into the bloodstream, a condition known as leaky gut syndrome (LGS) (59). When these substances enter the bloodstream, they are identified by the immune system as “non-self,” triggering an immune response that produces autoantibodies and inflammatory mediators. LGS initiates inflammatory responses in both the gut and peripheral tissues (60, 61) and is associated with autoimmune disorders such as type 1 diabetes mellitus, multiple sclerosis, rheumatoid arthritis, and celiac disease (62–64). LGS can induce the release of pro-inflammatory cytokines, including tumor necrosis factor-alpha, Interleukin-1β, and Interleukin-6, by activating dendritic and goblet cells. These cytokines can elicit local inflammation and affect the immune system systemically, leading

to widespread inflammation and perpetuation (38). Additionally, LGS can enhance autoantigen exposure, stimulate autoimmune responses, and produce autoantibodies, thereby triggering and exacerbating RA.

3.3 Immune regulation and inflammatory mediators

The innate immune cells present in the gut-associated lymphoid tissue serve as the primary line of defense against foreign substances in the gastrointestinal tract. Dysbiosis of the gut microbiota can lead to inappropriate activation of these innate immune cells, resulting in an increased production of pro-inflammatory cytokines such as interleukin-12, interleukin-23, and type I interferon, coupled with a concomitant reduction in anti-inflammatory cytokines like transforming growth factor-β and interleukin-10 produced by gut-resident leukocytes (39). In the context of autoimmunity, adaptive lymphocytes, particularly T and B cells, play a pivotal role, with their aberrant activation contributing to the development of RA. Pro-inflammatory antigens originating from the gut can trigger maladaptive responses in the immune system by altering the immune milieu through excessive activation of innate immunity (19). Microbial antigens presented by dendritic cells and goblet cells can activate CD4+ T cells, leading to the differentiation of inflammatory T cell subsets. Notably, Th17 cells, a pro-inflammatory subset of CD4+ T cells, are characterized by their production of interleukin-17 (65). Conversely, CD4+ T cells can also differentiate into Tregs, which aid in suppressing Th17 responses (66, 67). An increased Th17/Treg ratio has been strongly implicated in RA, with this balance being closely regulated by the gut microbiota and its metabolites (39, 68). Furthermore, microbial antigens can stimulate follicular helper T cells, promoting the activation of B lymphocytes and subsequent production of pathogenic autoantibodies that may contribute to the pathogenesis of RA (39). Gut microbiota dysbiosis can trigger the migration of autoreactive cells to the joints, leading to local joint inflammation (69). These autoreactive cells activate macrophages, promoting the production of inflammatory cytokines. Additionally, cytokines such as tumor necrosis factor-α, Interleukin-6, and Interleukin-1 induce fibroblasts to secrete matrix metalloproteinases and receptor activator of nuclear factor κB ligand, further mediating bone and cartilage destruction and driving the progression of RA (70). These intricate interactions underscore the significant role of the gut microbiota in modulating systemic inflammatory responses and suggest that gut dysbiosis, inflammatory cytokines, and immune responses are interconnected factors influencing the development of RA (40).

4 Role of the gut-immune system axis in the pathogenesis of rheumatoid arthritis

RA may be intricately linked to how disruptions in gut homeostasis influence immune function through metabolites produced by the gut microbiota (28, 71). Immune T and B cells in the intestinal mucosa have specific phenotypes and functions that are modulated by the microbiota (72). For instance, bacterial peptidoglycan components found in the synovial tissue of RA patients may contribute to inflammation within the joint microenvironment (53, 73). Recent research indicates that changes in gut microbiota composition in RA patients are a crucial factor in initiating abnormal systemic immune responses (38, 74, 75). The gut-immune system axis in the pathogenesis of rheumatoid arthritis shown in Figure 3.

4.1 Gut microbiota - immune system - rheumatoid arthritis

The mechanisms underlying the association between gut microbiome disturbances and RA have been extensively studied. Imbalance in the intestinal microbiota can impact the host immune system and its functions. The gut microbiota can activate antigen-presenting cells, such as dendritic cells, which alters cytokine production and antigen-presenting processes, interferes with T cell differentiation and function, and modulates the host immune response (76). Gut microbes interact with pattern recognition receptors, key innate immune receptors that detect pathogen-associated molecular patterns, including Toll-like receptors (50). Additionally, gut microbes can promote peptide citrullination through the enzymatic action of peptidyl-arginine deiminase (PAD). The human intestinal epithelium is a primary source of citrullinated peptides, and PAD activity is present in the human gut, with some gut microbes encoding functional microbial PADs (77). Consequently, the intestine can act as a source of citrullinated peptides and other mucosal surface antigens. Citrullination of peptides mediated by *Porphyromonas gingivalis*-expressed PAD is a significant factor in linking periodontitis with increased susceptibility to RA (50, 78). For instance, *Prevotella*-derived Pc-p27 can induce a Th1-mediated immune response by binding to human leukocyte antigen DR molecules in RA patients (2). This association is further supported by immunoglobulin A antibody responses to Pc-p27 in patients with acute and chronic RA, which are linked to the production of Th17 cell factors and anti-citrullinated protein antibodies (53). Moreover, gut microbes influence the host immune system by regulating T cell differentiation and disrupting the balance between Th17 and Treg cells. In a mouse model of RA, specific changes in the gut microbiota may enhance the pathogenic role of Th17 cells while diminishing the inhibitory effects of Tregs thereby promoting Th17-mediated mucosal inflammation (50).

4.2 Gut metabolites - immune system - rheumatoid arthritis

When Gram-negative bacteria overgrow, they produce excessive amounts of LPS. Dysfunctional gut microbiota can disrupt gut barrier function, allowing pro-inflammatory substances like LPS to enter the circulatory system through the compromised barrier, thereby triggering systemic inflammation (79). Elevated LPS is recognized by Toll-like receptor 4, which activates the Nuclear Factor kappa-

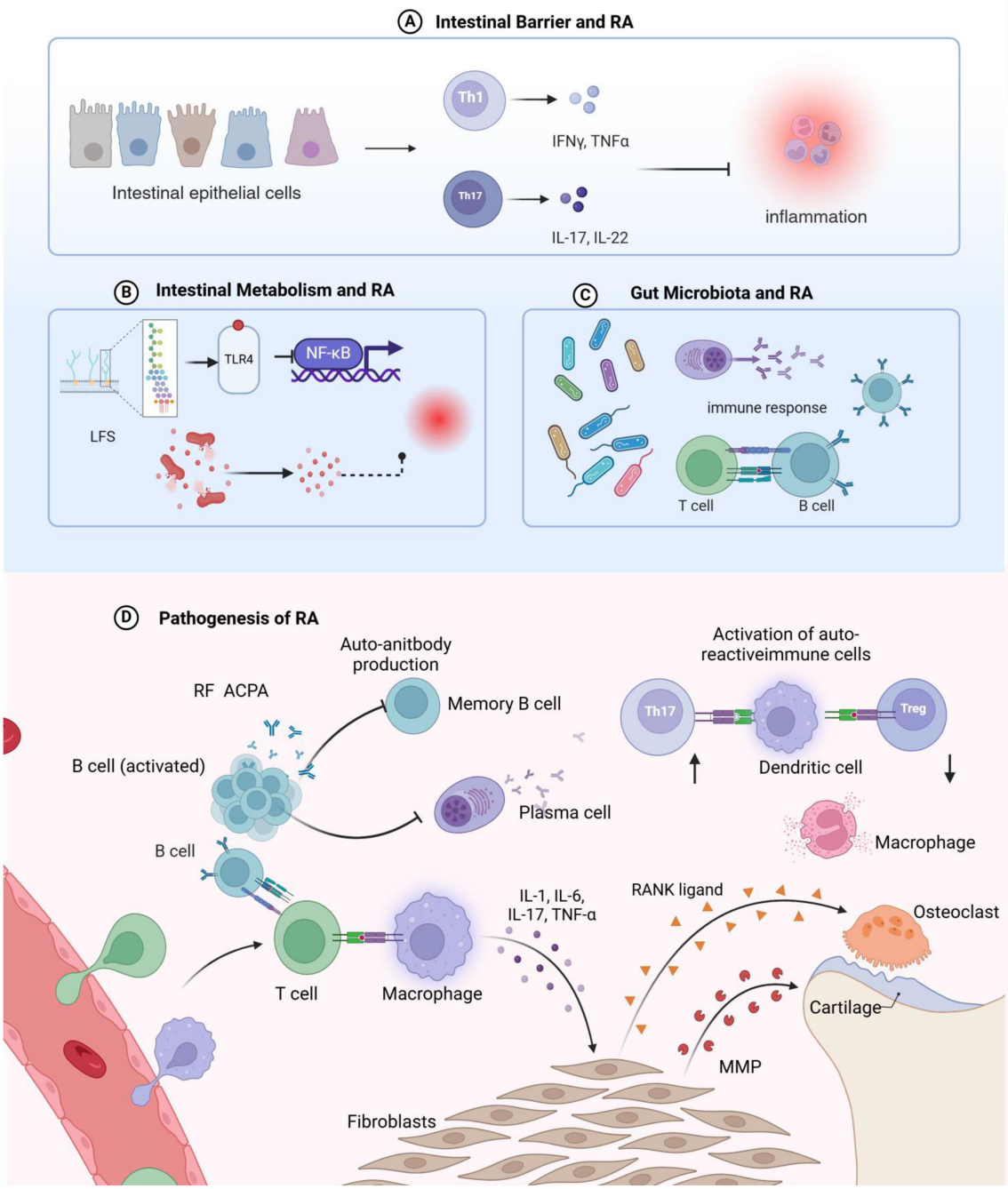

FIGURE 3
Schematic representation of the mechanisms by which intestinal homeostasis regulates rheumatoid arthritis pathogenesis. **(A)** Disruption of the intestinal barrier leads to increased intestinal permeability, induces T cell-mediated inflammatory responses, and facilitates the migration of autoreactive T cells from the gut to the joints, triggering rheumatoid arthritis in the articular tissues. **(B)** Metabolites derived from the intestinal microbiota can promote systemic inflammation; lipopolysaccharides from intestinal bacteria translocate into the circulation, are recognized by Toll-like receptors, and stimulate the Nuclear Factor kappa-light-chain-enhancer of activated B cells signaling pathway, thereby exacerbating inflammatory processes and contributing to rheumatoid arthritis development. **(C)** Dysbiosis of the intestinal microbiome can perturb the host immune system and its functions, interfere with T cell differentiation, and dysregulate host immune responses. **(D)** Rheumatoid arthritis arises from the convergence of multiple inflammatory pathways, ultimately leading to an imbalanced immune system and perpetuating the chronic inflammatory state.

light-chain-enhancer of activated B cells signaling pathway and initiates inflammation (80). Additionally, LPS can activate the complement alternative pathway, which plays a critical role in the development of arthritis, potentially contributing to its progression (81). *Bacteroides* fragilis stimulates T helper 1 responses during early colonization by producing polysaccharide A. It may also contribute to RA pathogenesis by increasing intestinal permeability, reducing tight junction (TJ) protein expression, and affecting epithelial production of interleukin-17A (50, 82). Research shows that SCFAs, such as butyrate, can cross the gut-blood barrier and enter systemic

circulation, regulating the function of distant joints and immune cells. In RA patients, SCFA production is reduced, leading to impaired Treg cell function. Lower butyrate levels in the bloodstream are associated with increased inflammatory markers, such as C-reactive protein and interleukin-6, which enhance pro-inflammatory responses and accelerate RA progression. This underscores the direct influence of gut metabolite balance on inflammation. Additionally, fecal analyses reveal a significant reduction in butyrate-producing microbiota in RA patients, further supporting the link between gut microbial metabolism and RA progression (83, 84). Thus, imbalances in gut-derived metabolites directly affect the onset and development of RA through multiple pathways.

4.3 Intestinal epithelial barrier - immune system - rheumatoid arthritis

Destruction of the intestinal barrier leads to leakage of intestinal contents, creation of a pro-inflammatory environment, and the activation and infiltration of autoreactive Th17 and other effector T cells (38). Our data indicate that gut barrier dysfunction occurs prior to the clinical onset of arthritis in mouse models (85). Human studies also reveal elevated serum markers associated with impaired gut barrier function before RA onset, which correlates with a higher risk of developing RA later (85). Disruption of the gut barrier allows bacterial components to translocate across the intestinal wall and enter the bloodstream, triggering a robust immune response and inducing the release of pro-inflammatory cytokines, such as tumor necrosis factor-α and interleukin-6 (86). These pro-inflammatory mediators are key drivers of RA pathogenesis, promoting synovial inflammation and tissue destruction.

The TJ between intestinal epithelial cells is crucial for maintaining intestinal barrier integrity. Zonulin, a regulatory protein secreted by intestinal epithelial cells in response to dietary or microbial stimuli, modulates TJ function and intestinal epithelial permeability (87). Zonulin affects the expression of TJ proteins such as Zonula Occludens-1, occludin, and claudin-1 (88). In mouse models, elevated zonulin levels lead to the degradation of essential TJ proteins like Zonula Occludens-1 and an increase in claudin-2 and claudin-15. This combination disrupts TJ integrity, causing significant barrier dysfunction and increased intestinal permeability (89). This disruption not only initiates T-cell-mediated intestinal mucosal inflammation but also facilitates the migration of autoreactive T cells, including T-helper 1 Cells and Th17 cells, from the gut to the joints, thereby triggering RA.

5 A novel perspective on rheumatoid arthritis treatment targeting the gut-immune axis

Changes in gut microbiota composition and diversity can affect the onset and progression of RA by influencing the immune system (85). Interventions targeting the gut microbiome in RA patients aim to reshape microbiota composition and diversity, thereby alleviating autoimmune inflammatory responses. Recent studies in rat models have explored the efficacy and safety of strategies like probiotic supplementation and gut barrier stabilization in the management of RA (90).

5.1 Development of biomarkers and diagnostic tools - prevention before disease onset

The development of biomarkers and diagnostic tools is essential for the clinical study of RA. Gut microbiota composition and serum cytokine levels are key biomarkers for early diagnosis, monitoring disease activity, and evaluating treatment responses. The gut microbiota in RA patients differs significantly from that in healthy individuals, and these differences could serve as potential markers for early diagnosis (91). Notably, a significant increase in the proportion of Bacteroidetes and a decrease in Firmicutes are characteristic changes in RA (92). Early diagnosis of RA is critical for slowing joint damage. In early-stage RA patients, *Prevotella* abundance increases, while *Bacteroides* decreases (52). Transplanting gut microbiota from early RA patients into germ-free sakuragi mouse model mice induces severe arthritis (93). During active RA, Haemophilus decreases, whereas *Lactobacillus* salivarius increases (94). Furthermore, the relative abundance of *Collinsella* and *Akkermansia* is higher in patients with active RA compared to those in remission (95). These findings suggest that gut microbiota alterations significantly influence RA severity, and shifts in microbial composition could serve as important biomarkers for RA diagnosis. Additionally, variations in serum levels of pro- and anti-inflammatory cytokines can reflect disease activity and treatment efficacy. For instance, elevated levels of tumor necrosis factor-alpha and Interleukin-6 are often associated with increased disease activity, while reduced levels of Interleukin-10 indicate weakened anti-inflammatory mechanisms (19, 96). Comprehensive analysis of gut microbiota and serum cytokine changes can lead to the development of novel diagnostic tools based on the gut-immune axis, supporting early diagnosis and personalized treatment of RA. Recent advancements, such as high-throughput sequencing and metabolomics, enable precise analysis of gut microbiota composition and its metabolites, offering in-depth information on biomarkers. Integrating multi-omics data with machine learning algorithms can enhance diagnostic accuracy and sensitivity. The adoption of these advanced technologies will advance diagnostic tools for RA and strengthen clinical practice.

5.2 Treatment of rheumatoid arthritis targeting intestinal homeostasis

RA is a chronic, multisystem autoimmune disease characterized by inflammation in peripheral joints. The primary goals of RA treatment are to alleviate pain and swelling and to control disease progression. Methotrexate is commonly used as the first-line treatment for RA; however, individual sensitivity and tolerance to methotrexate can vary widely. Additionally, methotrexate may accumulate in the kidneys, liver, and pleural and peritoneal

effusions, with significant variability in its clearance rate. The human gut microbiota and its enzymatic products influence the bioavailability, clinical efficacy, and toxicity of many drugs through both direct and indirect mechanisms. Moreover, various drugs and active compounds achieve therapeutic effects by normalizing gut microbiota composition, thereby regulating immune cell function (19). An emerging therapeutic approach involves targeting intestinal homeostasis to manage RA, and preliminary clinical studies have shown promising results. Regulating the intestinal microbiota and improving the balance of intestinal homeostasis and immune function offer potential therapeutic benefits for RA.

5.2.1 Microbiome modulators

Probiotics directly modulate the immune system by downregulating Toll-like Receptor expression, thereby reducing inflammation (55). They also regulate antigen-presenting cells. Kwon et al. further demonstrated that probiotics induce a Treg immune response in experimental RA models, significantly promoting the differentiation of T cells into forkhead box P3-expressing Tregs, which play a critical role in regulating and suppressing the inflammatory cascade (97). Additionally, probiotics produce gut metabolites, such as short-chain fatty acids, which have antibacterial and anti-inflammatory properties and can alleviate some symptoms of rheumatoid arthritis (55). Probiotic strains such as *Lactobacillus* and *Bifidobacterium* are known to produce beneficial short-chain fatty acids and contribute to maintaining intestinal mucosal homeostasis (98). Hatakka et al. evaluated the impact of *Lactobacillus* rhamnosus supplementation in patients with stable RA who were not receiving disease-modifying antirheumatic drugs. Although no significant differences were observed in inflammatory markers or clinical disease status compared to the placebo group, patients receiving the probiotic supplementation reported subjective improvements in health (99). In a study conducted RA patients underwent an eight-week probiotic regimen comprising *Lactobacillus* acidophilus, *Lactobacillus* casei, and *Bifidobacterium* bifidum. Compared to the control group, this intervention group exhibited enhanced disease activity scores 28 and reduced levels of high-sensitivity C-reactive protein, suggesting a favorable impact on clinical disease markers (100, 101). Furthermore, a compound probiotic capsule containing *Lactobacillus* casei LC-11, *Lactobacillus* acidophilus LA-14, Lactococcus lactis LL-23, *Bifidobacterium* lactis BL-04, and *Bifidobacterium* bifidum BB-06 also demonstrated therapeutic potential in patients with active RA (102).

Commensal bacteria play a pivotal role in regulating epithelial barrier function. While dysregulated LPS induction can trImmunoglobulin Ger inflammatory responses, microbial byproducts such as butyrate and short-chain fatty acids contribute to maintaining TJ integrity (38). Given the involvement of commensal microbiota in the development of LPS-related autoimmune diseases, interventions targeting the microbiota are emerging as novel strategies for preventing or treating such conditions (103, 104). Secondary bile acids, notably lithocholic acid, have exhibited anti-inflammatory effects in collagen-induced arthritis models, indicating their potential therapeutic role in RA (105). Additionally, propionate derived from *Bacteroides* fragilis may offer an alternative or complementary approach to current RA therapies (106). The dietary indole derivative indole-3-carbinol has been demonstrated to mitigate adjuvant-induced arthritis (107). Sinomenine, a compound that enhances specific gut microbiota-derived indole tryptophan metabolites, activates the aryl hydrocarbon receptor aryl hydrocarbon receptors, and modulates the Nuclear Factor kappa-light-chain-enhancer of activated B cells and mitogen-activated protein kinase pathways, can alleviate RA-associated inflammation and immune responses (108).

5.2.2 Maintenance of intestinal barrier integrity

The zonulin family of peptides is a key regulator of intestinal tight junctions, highly expressed in autoimmune-prone mice and humans, and can predict the transition from autoimmunity to inflammatory arthritis. Elevated serum zonulin levels are strongly associated with gut barrier leakage, malnutrition, and inflammation. Studies suggest that restoring gut barrier function in the pre-arthritis phase using butyrate or type 1 cannabinoid receptor agonists can effectively inhibit the progression of arthritis (85). Treatments aimed at improving intestinal barrier function can help alleviate arthritis symptoms. For instance, microbial metabolites like butyrate are known to regulate epithelial tight junction protein expression effectively and restore intestinal barrier function, highlighting their role in mitigating the impact of microbial dysregulation on barrier integrity (109). Butyrate treatment has been shown to not only restore epithelial barrier function and gastrointestinal permeability to fluorescein Isothiocyanate-dextran but also significantly reduce the incidence of arthritis (109). In preclinical arthritis models, specific inhibition of the zonulin peptide by the antagonist larazotide acetate decreased the development of arthritis by nearly 50% and reduced intestinal permeability, thus preventing immune cell migration from the gut to the joints (85). Therapeutic interventions targeting zonulin can restore tight junction structure, improve barrier function, and partially protect joints from inflammatory damage (110). Early intervention in autoimmune diseases such as rheumatoid arthritis can improve long-term outcomes and increase remission rates. Thus, targeting zonulin and intestinal barrier function early in the disease provides a novel therapeutic strategy for modulating autoimmune development (79). The expression of hypoxia-inducible factor-1αin intestinal epithelial cells is crucial for maintaining barrier integrity. By inhibiting necroptosis in intestinal epithelial cells, hypoxia-inducible factor-1αhelps preserve the intestinal epithelial barrier and is identified as a key regulatory factor in RA treatment (111). There is a potential mechanistic link between disrupted intestinal barrier function and arthritic autoimmune reactions (69, 85, 112, 113). Therefore, reinforcing or restoring intestinal barrier integrity is considered a promising strategy for RA treatment.

6 Conclusions

RA is a complex autoimmune disease involving multiple factors, with the gut homeostasis-immune system axis being particularly crucial. The bidirectional regulation between gut homeostasis and the immune system forms a regulatory network that significantly affects RA. RA is closely associated with gut dysbiosis, characterized by an increase in harmful bacteria and a decrease in beneficial

bacteria. Gut homeostasis interacts with the immune system to influence RA progression. The gut microbiota and its metabolites regulate immune cells through multiple pathways, affecting immune responses. Gut microbes participate in antigen activation and regulate tight junction proteins to modulate gut mucosal permeability, thereby influencing RA development. Excessive production of metabolites like LPS can damage the gut barrier, promoting inflammation and accelerating RA progression. SCFAs and beneficial bacteria play essential roles in maintaining gut homeostasis and exert anti-inflammatory effects, but their levels are reduced in RA patients. Gut microbiota composition analysis has become a valuable tool for predicting RA susceptibility and controlling disease progression. By integrating gut microbiota data with serum cytokine profiles, new diagnostic approaches based on the gut-immune axis can be developed, offering crucial support for early diagnosis and personalized treatment of RA. Probiotics can induce Treg immune responses, promoting the differentiation of T cells into forkhead box P3-expressing Tregs. Moreover, SCFAs and other metabolites possess antibacterial and anti-inflammatory properties, helping to alleviate RA symptoms.

Therapeutic strategies targeting the gut microbiome have shown potential in modulating the immune system, offering new avenues for RA treatment. However, several limitations remain: the exact mechanisms by which specific microbiota or their metabolites influence RA pathogenesis through immune modulation are not fully understood; the molecular processes linking dysbiosis or intestinal barrier disruption to systemic inflammation and RA need further elucidation. The impact of intestinal homeostasis on arthritis and its underlying mechanisms requires more detailed investigation. The clinical efficacy and safety of RA treatments that focus on regulating intestinal homeostasis need to be thoroughly evaluated and validated. Future research should include additional clinical studies to confirm the effects and mechanisms of gut microbiota regulation in RA prevention and treatment. Investigating the action mechanisms of specific probiotics or metabolites, developing novel microbiome-targeted therapies, and integrating traditional drug treatments with lifestyle interventions could enhance the comprehensive management of RA. Further exploration of drug metabolism in RA and the interplay between gut homeostasis and the immune system is also needed. Advancing our understanding of strategies to regulate intestinal homeostasis may offer new insights and therapeutic targets, improving patient outcomes and quality of life.

Author contributions

PQ: Investigation, Writing – original draft, Writing – review & editing. XC: Writing – review & editing. JT: Writing – review & editing. KZ: Writing – review & editing. ZQ: Writing – review & editing. ML: Writing – review & editing. XX: Funding acquisition, Methodology, Writing – original draft, Writing – review & editing.

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Conflict of interest

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Study on the gut microbiota, HPA, and cytokine levels in infantile spasms

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Objective: The mechanisms driving the progression of infantile spasms are not well understood. We aimed to investigate the changes and correlations of the gut microbiota, the hypothalamus–pituitary–adrenal (HPA) axis hormones, and the inflammatory cytokines in children with infantile spasms before and after treatment in order to provide a reference for future pathogenesis research.

Methods: Children with infantile spasms who were admitted to our hospital were recruited into the case group. The case group was divided into the pre-treatment group (group A, n = 14), the 2 weeks after treatment group (group B), and the 1 month after treatment group (group C). On the other hand, healthy children with the same sex ratio as the case group were recruited into the control group (group D, n = 14). Three stool and blood samples were collected before treatment, 2 weeks after treatment, and 1 month after treatment. The serum samples were analyzed using cytometric bead array (CBA), enzyme-linked immunosorbent assay (ELISA), and chemiluminescent immunoassay (CLIA) to measure the levels of HPA axis hormones and inflammatory cytokines. The collected stool samples were sequenced using 16S rDNA.

Results: The pre-treatment group demonstrated elevated levels of corticotropin-releasing hormone (CRH), interleukin 2 (IL-2), IL-4, IL-6, and IL-17α, which decreased with treatment. The level of CRH was lower in the effective group than that in the ineffective group. *Sutterellaceae* was lower in the pre-treatment group than that in the control group. *Lachnospiracea_incertae_sedis* was positively associated with CRH concentration ($p < 0.05$). After treatment, *Sutterellaceae* was negatively associated with IL-2 and TNF-α ($p < 0.05$).

Conclusion: This study found that imbalance of the gut microbiota may be involved in the pathogenesis of infantile spasms and is related to the response to adrenocorticotropic hormone (ACTH). *Lachnospiraceae* and *Lachnospiracea_incertae_sedis* might be involved in the disease onset. *Sutterellaceae* might have a link to children's improved health.

KEYWORDS

gut microbiota, infantile spasm, inflammatory cytokines, HPA axis hormones, correlations

1 Introduction

Infantile spasms (IS), also known as West syndrome, is a refractory epilepsy syndrome characterized by clustered nodding, hugging spasm episodes, a hypsarrhythmic pattern in the electroencephalogram (EEG), and neurodevelopmental delay (1). Recently, IS has been reclassified into infantile epileptic spasms syndrome (IESS) to encompass those patients who do not fully meet the criteria for West syndrome (2). This condition primarily affects infants, with an incidence of approximately 2 to 5 per 10,000, predominantly in male infants, and peaks between the ages of 4 and 7 months (3). According to the most recent American Expert Consensus in 2010, adrenocorticotropic hormone (ACTH) and vigabatrin are the most commonly used first-line medications for this condition (4). Other therapeutic approaches, such as glucocorticoids, topiramate, pyridoxine, and a ketogenic diet, are often utilized as initial treatments in resistant or relapsed cases that do not respond to first-line medications (5), although these are generally less effective. It is generally acknowledged that the stress mechanism and the neuroinflammatory mechanism, caused by the disturbance of the hypothalamus–pituitary–adrenal (HPA) axis, are implicated in the pathogenesis of IS (6, 7).

In recent years, there have been increasing studies on the microbiota–gut–brain axis (MGBA) (8, 9), whose mechanism of action involves the gut microbiota being bidirectionally connected to the brain through the relevant pathways of the gut–brain axis (10) and may play a role in the pathogenesis of epilepsy. These pathways include the neuroendocrine (HPA axis) (11), the vagus nerve, the intestinal immune, the neurotransmitter, and the neuromodulator pathways (12, 13). This study focused on how the neuroendocrine and inflammatory response pathways of the gut microbiota affect IS.

The mechanisms driving the progression of IS are not well understood. Therefore, we aimed to investigate the changes and correlations of the gut microbiota, the HPA axis hormones, and the inflammatory cytokines in children with IS before and after treatment in order to provide a reference for future pathogenesis research.

2 Materials and methods

2.1 Subjects

Children with IS who were hospitalized in the Department of Neurology at our hospital and treated with ACTH from February 2021 to December 2021 were recruited into the case group. All children met the IS diagnostic criteria of the 2010 US Expert Consensus (4, 7). The inclusion criteria for the case group were as follows: 1) children diagnosed for the first time and not previously treated with antiepileptic drugs; 2) those who met the diagnostic criteria for IS established by the 2010 US Expert Consensus; and 3) those aged between 1 and 12 months. The exclusion criteria were: 1) severe malnutrition or excess nutrition; 2) history of digestive diseases and infections in the past 2 weeks with antibiotic treatment; 3) those immunocompromised and those who took hormones or immunosuppressants before diagnosis; 4) those with neurological disorders other than intracranial hemorrhage, stroke, and other epilepsy; and 5) patients with hematological malignancies (such as leukemia) that can cause impaired immunity or intracranial tumors that can disrupt hormone secretion or cause secondary epilepsy. The exclusion criteria after 2 weeks and 1 month of treatment were: 1) not receiving ACTH therapy during treatment; 2) with digestive diseases after enrollment; 3) using antibiotics and probiotics after enrollment; and 4) with neurological disorders other than intracranial hemorrhage, stroke, and other epilepsy. The pre-treatment group was defined as group A, the 2 weeks after treatment group was defined as group B, and the 1 month after treatment group was defined as group C. After 2 weeks of treatment, children with basic remission or with more than 50% decrease of daily seizure occurrence were classified as the effective group according to the clinical relief after using ACTH in the case group. Patients with less than a 50% reduction in daily seizure occurrence, or with little or no remission, were classified as the ineffective group (8).

Healthy children aged 1–12 months were recruited as a blank control group (group D) for the collection of single fecal and blood samples. The technology roadmap is shown in Supplementary Figure S1. This study has been reviewed by the ethics committee with number HCHLL-2020-53.

2.2 Methods

2.2.1 Sample and general information collection

Blood samples: The case group had a first blood collection before treatment, a second blood collection 2 weeks after ACTH treatment, followed by a third collection 1 month after the administration of ACTH or other medications. The samples were then centrifuged after collection, with the upper serum retained and frozen in a refrigerator at −80°C for testing.

Stool specimen: Three stool samples were collected before, 2 weeks after, and 1 month after treatment. All fecal samples were collected and immediately frozen in ice boxes before transportation to the laboratory within 30 min and then stored at −80°C.

Basic information: The following data were collected: name, gender, age, date of birth, weight, height, gestational age, birth weight, birth method, birth condition and feeding method, clinical data, type of diagnosis, pattern of seizure, frequency of seizures, duration of each seizure, time of the first onset, and EEG results.

Follow-up of treatment effect: Data on the current status of medication, the clinical efficacy (seizure frequency), and EEG changes were collected.

Drug use: After the diagnosis of IS, all children in the case group were treated with ACTH intravenous fluids and completed a 2-week course of treatment in the first and second weeks of treatment. During weeks 3–4 of treatment, three children continued ACTH therapy, while the remaining children received oral prednisone and other antiepileptic drugs.

2.2.2 Detection of HPA axis hormones and inflammatory cytokines

Cortisol hormone (COR) was measured using a chemiluminescent immunoassay (CLIA). The enzyme-linked immunosorbent assay (ELISA) method was used to measure ACTH and corticotropin-releasing hormone (CRH). The cytometric bead array (CBA) method was used to detect seven cytokines (i.e., IL-2, IL-4, IL-6, IL-10, IL-17α, TNF-α, and IFN-γ).

2.2.3 DNA extraction and high-throughput 16S rDNA gene sequencing

16S rDNA amplicon sequencing was performed by Genesky Biotechnologies Inc. (Shanghai, 201315, China). Briefly, total genomic DNA was extracted using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. The integrity of genomic DNA was examined through agarose gel electrophoresis, while its concentration and purity were determined using the NanoDrop 2000 and Qubit 3.0 spectrophotometer. The V4–V5 hypervariable regions of the 16S rDNA gene were amplified with the primers 515F (5'-GTGCCAGCMGCCGCGG-3') and 907R (5'-CCGTCAATTCMTTTR AGTTT-3') and then sequenced using the Illumina MiSeq 6000 platform.

2.2.4 Gut microbial analysis

The raw read sequences were further filtered to remove adapter sequences, primers, and low-quality reads to improve the accuracy of the subsequent analysis.

Alpha diversity was evaluated using abundance and diversity indices. Principal coordinates analysis (PCoA) of beta diversity (based on the Bray–Curtis distance) based on the operational taxonomic unit (OTU) abundance table was performed to evaluate the community composition and structure of the gut microbiota. Linear discriminant analysis effect size (LEfSe) was used to identify the species that are most likely to explain differences between groups with the linear discriminant analysis (LDA) histogram and the cladogram. Metastats analysis compared the samples between groups at different taxonomy levels, which determined the species with significant differences at each taxonomic level. A heat map for Spearman's rank-sum correlation coefficient was used to evaluate the correlation between the gut microbiota of each group and the levels of inflammatory cytokines and HPA axis hormones.

2.2.5 Statistical analysis

The experimental data were analyzed using SPSS (Version 25) and R software (Version 4.2.3). The measurement data, when normally distributed, were expressed as the mean ± standard deviation ($X \pm S$), with an independent-samples t-test and analysis of variance used to compare differences among groups; otherwise, data were expressed as the median and interquartile range [M (P_{25}–P_{75})], with the Mann–Whitney test and the Kruskal–Wallis test used to compare differences among groups. *Post-hoc* multiple tests were used for pairwise comparisons between groups. Categorical data were expressed as percentages, and the comparison differences between two groups were evaluated with the chi-square test. The correlation between the gut microbiota and the levels of inflammatory cytokines and HPA axis hormones was evaluated using Spearman's rank-sum correlation coefficient. A p-value <0.05 was considered as statistically significant.

3 Results

3.1 Data analysis between the pre-treatment group and the control group

3.1.1 Clinical characteristics of subjects

In this study, 19 children were recruited as cases based on the inclusion criteria, samples from whom were collected before treatment and 2 weeks and 1 month after treatment. During the collection process, two cases were not treated with ACTH, one case was excluded due to infection and antibiotic use during hospitalization, and two cases were lost during follow-up 1 month after treatment. Finally, 14 children with IS were included into the case group. There were 9 children out of the 14 in the case group who had more than three seizures per day, while four children had more than five seizures. Four children had each seizure lasting more than 1 min. There were five children with more than 10 times nodding and/or hugging spasms. There were no significant differences in the general data such as age, gestational age, birth weight, and mode of delivery between the pre-treatment group and the control group ($p > 0.05$), as shown in Table 1.

3.1.2 Comparison of the HPA axis hormone and inflammatory cytokine levels between the two groups

The analysis revealed that the levels of CRH, IL-2, IL-4, IL-6, and IL-17α in the pre-treatment group were significantly higher than those in the control group ($p < 0.05$) (Table 2).

3.1.3 Analysis of the gut microbiota differences between the two groups

There were no significant differences in the alpha diversity values between the pre-treatment group and the control group ($p > 0.05$). The PCoA found that the gut microbiota composition of the pre-treatment group was highly similar to that of the control group (Figures 1A, B).

The results of LEfSe showed that six bacteria were enriched in the control group and eight bacteria were enriched in the pre-treatment group (Figures 1C, D).

Metastats analysis was conducted to determine the species with significant differences at the phylum, genus, and family levels. No significant differences were found in the major gut microbiota at the phylum level (Figure 1E). However, at the genus and family levels, *Proteus*, *Gemmiger*, and *Morganella* increased, while *Sutterella* and

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TABLE 1 Clinical characteristics of the subjects in the pre-treatment and control groups.

		Pre-treatment group (n = 14)	Control group (n = 14)	p
Age (months)		6.64 ± 3.15	7.21 ± 2.72	0.61
Gender	Male	7	7	1
	Female	7	7	
Gestational age (weeks)	Mature	14	13	1
	Premature	0	1	
Birth weight (g)	≥2,500	12	13	0.60
	<2,500	2	1	
Delivery	Cesarean section	7	4	0.44
	Normal delivery	7	10	
Feeding	Breastfeeding	6	5	0.79
	Mixed feeding	4	3	
	Formula milk	4	6	
Intrauterine distress	Yes	1	1	1
	No	13	13	
Seizure frequency	≤5/day	10	0	
	>5/day	4	0	
Seizure duration (min)	≤1	10	0	
	>1	4	0	

Sutterellaceae decreased significantly in the pre-treatment group ($p < 0.05$) (Figures 1F, G).

3.2 Data analysis of the case group before treatment and 2 weeks and 1 month after treatment

3.2.1 Comparison of the HPA axis hormone and inflammatory cytokine levels among the three groups

The levels of the HPA axis hormones and inflammatory cytokines in the case group decreased after drug therapy. Compared with the pre-treatment group, the levels of CRH and ACTH after 2 weeks of ACTH treatment decreased significantly ($p < 0.05$). After 1 month of treatment with ACTH and other drugs, the levels of CRH, ACTH, and COR decreased significantly compared with those in the pre-treatment group ($p < 0.05$). After 1 month of treatment with ACTH and other drugs, the levels of IL-2, IL-4, IL-6, and IL-17α decreased significantly compared with those before treatment ($p < 0.05$) (Table 3).

3.2.2 Analysis of the gut microbiota differences among the three groups

There were no differences in the alpha and beta diversity among the three groups (Figures 2A, B).

The results of LEfSe showed that three bacteria were enriched in the pre-treatment group and three bacteria were enriched in the 2 weeks after treatment group. There was no abundant gut microbiota in the 1 month after treatment group (Figures 2C, D).

Metastats analysis showed that *Lactobacillus* and *Lactobacillaceae* decreased with the extension of the treatment time, and the relative abundance of these two bacteria in the 1 month after treatment group

TABLE 2 Comparison of the levels of the hypothalamus–pituitary–adrenal (HPA) axis hormones and inflammatory cytokines between the pre-treatment and control groups.

	Pre-treatment group (n = 14)	Control group (n = 14)	t/z value	p
CRH (ng/ml)	31.50 ± 4.42	19.76 ± 4.05	53.718	0.000**
ACTH (pg/ml)	43.018 (37.4–46.3)	45.172 (44.0–48.2)	−1.149	0.251
COR (g/dl)	12.056 (5.6–18.7)	8.323 (6.5–10.8)	−0.827	0.408
IL-2 (pg/ml)	2.610 (2.375–4.513)	2.045 (1.005–2.738)	−2.183	0.029*
IL-4 (pg/ml)	3.855 (3.003–5.268)	2.370 (1.943–3.153)	−2.918	0.004**
IL-6 (pg/ml)	5.060 (4.353–9.838)	3.620 (2.780–6.393)	−2.184	0.029*
IL-10 (pg/ml)	5.025 (4.160–6.393)	4.180 (3.428–6.385)	−1.264	0.206
IL-17α (pg/ml)	14.565 (10.115–17.925)	8.835 (5.630–10.605)	−2.964	0.003**
TNF-α (pg/ml)	4.400 (3.213–6.245)	3.085 (1.555–5.683)	−1.701	0.089
IFN-γ (pg/ml)	2.180 (0.925–2.698)	1.285 (0.925–1.908)	−1.057	0.29

CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone; COR, cortisol hormone.
*$p < 0.05$; **$p < 0.01$.

FIGURE 1
Comparison of the gut microbiota between the pre-treatment group (group A) and the control group (group D). **(A, B)** Comparison of the alpha diversity **(A)** and the beta diversity **(B)** between the two groups. **(C, D)** Linear discriminant analysis (LDA) value distribution and the cladogram of the linear discriminant analysis effect size (LEfSe) between the two groups. **(E–G)** Metastats analysis at the phylum **(E)**, family **(F)**, and genus **(G)** levels between the two groups.

was significantly lower than that in the other two groups ($p < 0.05$). At the same time, *Lachnospiraceae* decreased with the extension of the treatment time. However, no significant difference was found ($p < 0.05$) (Figures 2E, F, 3).

3.3 Data analysis between the effective and ineffective groups in the 2 weeks after treatment group

3.3.1 Comparison of the HPA axis hormone and inflammatory cytokine levels between the two groups

The level of IFN-γ was significantly higher and that of CRH was significantly lower in the effective group compared with the ineffective group (Table 4).

3.3.2 Analysis of the gut microbiota differences between the two groups

There were no differences in the alpha and beta diversity between the two groups.

The results of LEfSe revealed three bacteria enriched in the effective group and 10 bacteria enriched in the ineffective group.

Metastats analysis showed that *Alistipes* and *Rikenellaceae* were significantly higher and that *Megamonas*, *Faecalibacterium*, and *Ruminococcus* were significantly lower in the effective group than those in the ineffective group ($p < 0.05$) (Figure 4).

3.4 Correlation analysis between the gut microbiota and the HPA axis hormone and inflammatory cytokine levels

We used the heat map of the Spearman's rank-sum correlation coefficients to determine the correlation between the gut microbiota and the HPA axis hormone and inflammatory cytokine levels. In the pre-treatment group, *Lachnospiracea_incertae_sedis* was positively associated with CRH. *Lactobacillaceae* and *Lactobacillus* were positively associated with IL-2 and IL-4. *Alistipes* and *Rikenellaceae* were negatively correlated with IL-17a and IFN-γ in the 2 weeks after treatment group. In the 1 month after treatment group, *Sutterellaceae* was positively associated with CRH and negatively associated with IL-2 and TNF-α. *Alistipes* and *Rikenellaceae* were negatively associated with IL-6 and IFN-α (Figure 5A).

Subsequently, we compared the correlation between the differences in the gut microbiota and the differences in the levels of HPA axis hormones and inflammatory cytokines. It was found that *Lachnospiracea_incertae_sedis* was positively associated with CRH and COR. *Alistipes* and *Rikenellaceae* were negatively associated with IL-10 (the relative abundance of *Alistipes* and *Rikenellaceae* in group B was higher than that in group C, while the level of IL-10 in group B was lower than that in group C) (Figure 5B).

In the effective group, *Ruminococcaceae* was positively associated with IL-2, IL-4, and IFN-γ. *Lactobacillaceae* was positively associated with ACTH (Figure 5C).

TABLE 3 Comparison of levels of the hypothalamus–pituitary–adrenal (HPA) axis hormones and inflammatory cytokines in the case group before treatment and 2 weeks and 1 month after treatment.

	Pre-treatment group (n = 14)	2 weeks after treatment group (n = 14)	1 month after treatment group (n = 14)	t/z value	p
CRH (ng/ml)	32.466 (28.919–34.368)[a,c]	22.376 (17.999–26.193)[a]	18.465 (15.943–20.335)[c]	26.359	0.000**
ACTH (pg/ml)	43.018 (37.398–46.273)[a,c]	32.181 (31.005–37.954)[a]	27.226 (23.710–32.948)[c]	19.752	0.000**
COR (μg/dl)	12.056 (5.645–18.727)[c]	8.475 (7.393–10.470)[b]	4.854 (1.168–7.362)[b,c]	10.366	0.006**
IL-2 (pg/ml)	2.610 (2.375–4.513)[c]	1.770 (0.797–3.212)	1.510 (0.902–2.715)[c]	8.099	0.017*
IL-4 (pg/ml)	3.855 (3.002–5.268)[c]	3.035 (1.295–4.772)	1.605 (0.893–3.200)[c]	10.236	0.006**
IL-6 (pg/ml)	5.060 (4.353–9.838)[c]	3.875 (2.367–5.093)	3.160 (1.442–4.232)[c]	11.38	0.003**
IL-10 (pg/ml)	5.025 (4.160–6.393)	3.150 (1.640–4.377)	3.845 (1.440–6.450)	5.546	0.062
IL-17α (pg/ml)	14.565 (10.115–17.925)[c]	10.470 (3.667–15.970)	6.720 (3.515–11.190)[c]	8.049	0.018*
TNF-α (pg/ml)	4.400 (3.212–6.245)	4.150 (1.340–5.152)	2.420 (0.368–5.232)	4.411	0.11
IFN-γ (pg/ml)	1.903 ± 0.899	1.422 ± 0.811	1.214 ± 0.897	2.312	0.113

CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone; COR, cortisol hormone.
The three groups were compared in pairs (see below) and post-hoc multiple tests were used.
*$p < 0.05$; **$p < 0.01$.
[a]Comparison of the pre-treatment group and the 2 weeks after treatment group ($p < 0.05$).
[b]Comparison of the 2 weeks after treatment group and the 1 month after treatment group ($p < 0.05$).
[c]Comparison of the pre-treatment group and the 1 month after treatment group ($p < 0.05$).

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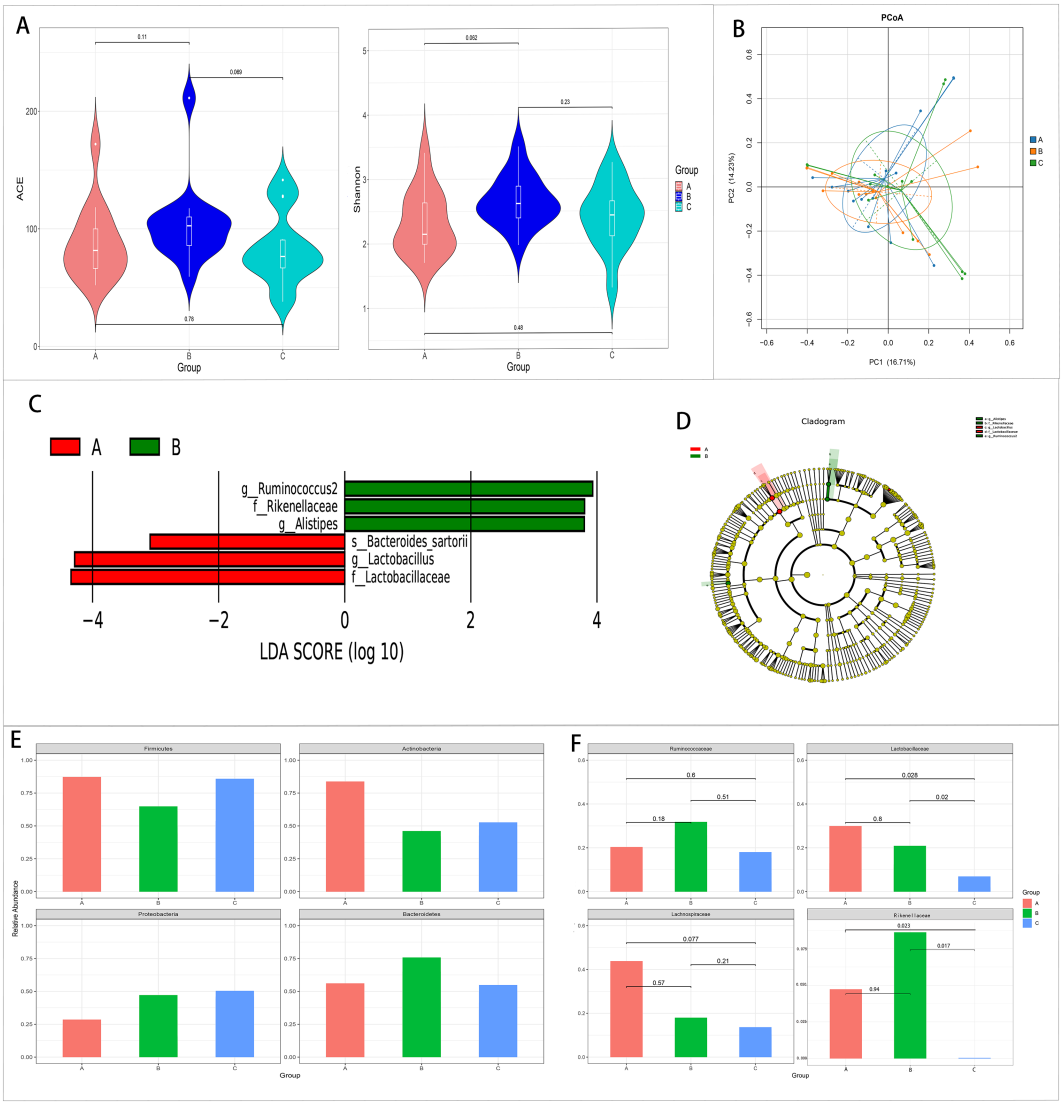

FIGURE 2
Comparison of the gut microbiota among the pre-treatment (group A), 2 weeks after treatment (group B), and 1 month after treatment (group C) groups in the case group. **(A, B)** Comparison of the alpha diversity **(A)** and the beta diversity **(B)** among the three groups. **(C, D)** Linear discriminant analysis (LDA) value distribution and the cladogram of the linear discriminant analysis effect size (LEfSe) among the three groups. **(E, F)** Metastats analysis at the phylum **(E)** and family **(F)** levels among the three groups.

4 Discussion

In this study, it was found that the levels of IL-2, IL-4, IL-6, and IL-17α in the pre-treatment group were considerably greater than those in the control group, indicating that the level of inflammatory cytokines in infants with IS increased. After disruption of the central nervous system in infants with spasm, damage of the endothelial cells in the brain tissue causes the release of a variety of inflammatory factors. Based on pathological research, the inflammatory regulators and receptors have been found to be greatly enhanced in brain tissue specimens from patients with epilepsy, viral encephalitis, and other neurological injuries, as well as in the cerebral cortex and limbic tissues of rats with epilepsy. For instance, inflammatory factors such as IL-2 and IL-6 in serum showed a substantial increase (14, 15). IL-2, a γc family cytokine, is primarily secreted by T cells, promoting lymphocyte proliferation, inducing natural killer (NK) cells, and participating in immune regulation (16). In the central nervous system, IL-6 possesses a range of biological actions, which can regulate the functions of nerve cells (17). Studies have indicated that IL-6 is overexpressed in the brain tissue of patients with epilepsy and is released into circulation through cerebral blood vessels. The level of IL-6 was found to be much higher in patients with recurrent generalized tonic–clonic seizures compared with those with single seizures (18). IL-17α is an important inflammatory factor that can stimulate macrophages, endothelial cells, fibroblasts, and epithelial cells to produce a variety of inflammatory factors, promoting the incidence and development of neurological diseases (19). Related studies have reported that the level of IL-17α was higher in the cerebrospinal fluid of children with acute seizures, which is consistent with our findings (20).

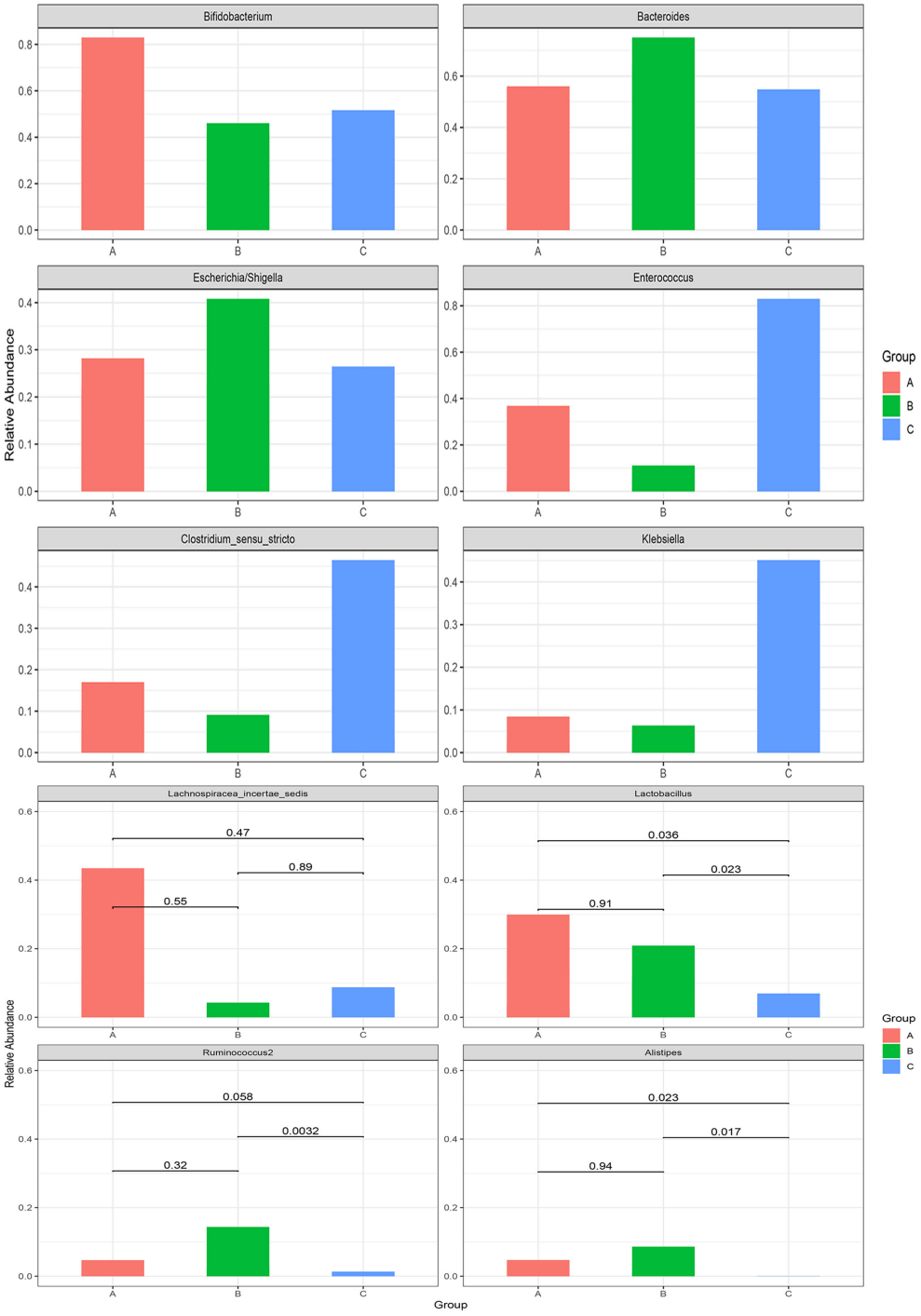

FIGURE 3
Metastats analysis at the genus level among the pre-treatment group (group A), the 2 weeks after treatment group (group B), and the 1 month after treatment group (group C) in the case group.

This study discovered that the CRH level in the pre-treatment group was much greater than that in the control group, and the levels of CRH, ACTH, and COR on the HPA axis decreased following ACTH therapy. Previous studies have shown that the concentrations of CRH can increase in response to certain conditions such as external stress and can trigger IS by interacting with brain-specific cortical targets (21). ACTH, an anti-epileptic drug, is currently derived from corticotropin extracted from exogenous bovine, horse, and other animals. Its mechanism may involve negative feedback to inhibit CRH secretion in the HPA axis, thereby temporarily suppressing the secretion of endogenous ACTH, resulting in a decreased ACTH concentration after treatment (22). COR is a part of the HPA axis, regulated by superiors. Most of the children in this study received prednisone oral therapy following ACTH administration at the end of 2 weeks of treatment. Prednisone, acting as COR, could regulate the

TABLE 4 Comparison of the levels of the hypothalamus–pituitary–adrenal (HPA) axis hormones and inflammatory cytokines between the effective and ineffective groups.

	Effective group (n = 9)	Ineffective group (n = 5)	t/z value	p
CRH (ng/ml)	21.16 ± 3.86	25.86 ± 3.02	5.489	0.037*
ACTH (pg/ml)	33.07 ± 3.85	36.85 ± 5.05	2.492	0.14
COR (μg/dl)	9.222 (6.4–10.6)	7.728 (7.6–14.7)	−0.067	0.947
IL-2 (pg/ml)	2.08 ± 1.83	1.81 ± 1.86	0.065	0.803
IL-4 (pg/ml)	3.56 ± 2.76	2.25 ± 1.23	0.997	0.338
IL-6 (pg/ml)	3.980 (2.9–5.2)	2.420 (1.9–11.1)	−0.6	0.549
IL-10 (pg/ml)	3.75 ± 2.40	2.09 ± 1.01	2.109	0.172
IL-17α (pg/ml)	13.810 (4.5–15.2)	4.090 (1.8–10.6)	−1.133	0.257
TNF-α (pg/ml)	3.75 ± 2.73	2.54 ± 1.82	0.771	0.397
IFN-γ (pg/ml)	1.390 (1.2–2.4)	0.590 (0.4–0.8)	−2.467	0.014*

CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone; COR, cortisol hormone.
*$p < 0.05$.

upstream CRH and ACTH levels with negative feedback, leading to a decrease in the COR concentration after 1 month of treatment. According to previous studies, patients with epilepsy had abnormal immune function, which affected the endocrine system through the "neuroendocrine–immune network" and promoted cortisol release (23).

Interestingly, the levels of IL-2, IL-4, IL-6, and IL-17α decreased significantly after treatment, which further explained the effect of cranial nerve injury and inflammatory response on the pathological changes of IS. Previous studies have demonstrated that ACTH exerts a regulatory effect on the immune-mediated inflammatory responses (24). This mechanism might account for the reduction in the levels of inflammatory cytokines in the case group following treatment. Given the incomplete understanding of the specific pathogenesis of IS, additional research is needed to elucidate the precise mechanism of ACTH in the treatment of IS.

There were no significant differences in the gut microbiota diversity across all groups in this study, suggesting that the gut microbiota of patients were similar to those of normal infants. Moreover, the treatment and efficacy had little effect on the diversity of the gut microbiota, which is consistent with the findings of a study on infantile spasmodic gut microbiota (9).

Compared with the control group, *Sutterellaceae* and *Sutterella* decreased significantly in the pre-treatment group. *Sutterella* belongs to *Sutterellaceae*, and animal model studies have shown that *Sutterellaceae* are typical intestinal dominant bacteria whose variations in abundance are related to the function of the intestinal mucosal barrier (25). These findings showed that a reduction in *Sutterellaceae* is associated with disease severity.

When comparing the conditions before and after treatment in the case group, *Lachnospiraceae* was found to decrease with the extension of the treatment time. After 2 weeks of treatment, *Lachnospiracea_incertae_sedis* decreased compared with that before treatment. However, there was no significant difference. *Lachnospiracea_incertae_sedis*, which belongs to *Lachnospiraceae*, are anaerobic bacteria known to promote glucose fermentation and to produce lactic and formic acids, impacting intestinal permeability and stimulating intestinal chromaffin cell synthesis and the release of serotonin (5-HT) (26). 5-HT, a neurotransmitter, influences the excitability/inhibitory balance of the cerebral cortex and subcortical regions and is involved in various physiological and pathological processes in the brain. Studies have shown that 5-HT could affect the onset and progression of epilepsy by influencing the response to ACTH therapy in infants with IS (27). Furthermore, other research found that the relative abundance of *Lachnospiracea_incertae_sedis* in patients with Parkinson's disease increased, while it decreased significantly in patients who responded to treatment (28). This supports the notion that, as therapy continued and the condition improved, the dominant bacteria of the gut microbiota in children with IS gradually shifted toward those of a normal human gut microbiota.

After 2 weeks of treatment in the case group, *Alistipes* and *Rikenellaceae* increased in the effective group, while *Megamonas*, *Faecalibacterium*, *Ruminococcus*, and *Romboutsia* increased in the ineffective group. *Ruminococcus* belongs to *Firmicutes*, and its abundance increased in the gut microbiota of drug-resistant epilepsy patients (29). *Ruminococcus* and other uncommon bacteria could participate in the occurrence of epilepsy by regulating the adenosine triphosphate binding cassette transporters (29). *Ruminococcus* has been shown to be positively correlated with glutamate and to be negatively correlated with 5-HT in animal studies (30). In addition, *Ruminococcus* is associated with lower levels of *N*-acetylaspartic acid in patients with epilepsy, which is a neuronal health marker (31). Therefore, *Ruminococcus* is thought to influence drug susceptibility to epilepsy by regulating certain neurotransmitters.

This study found that *Lachnospiracea_incertae_disedis* was positively associated with CRH. *Lachnospiracea_incertae_disedis* is significantly higher in children with autism spectrum disorder, and studies have shown that autism is associated with disorders of the HPA axis caused by maternal stress and trauma during pregnancy (32). These might suggest that *Lachnospiracea_incertae_disedis* could

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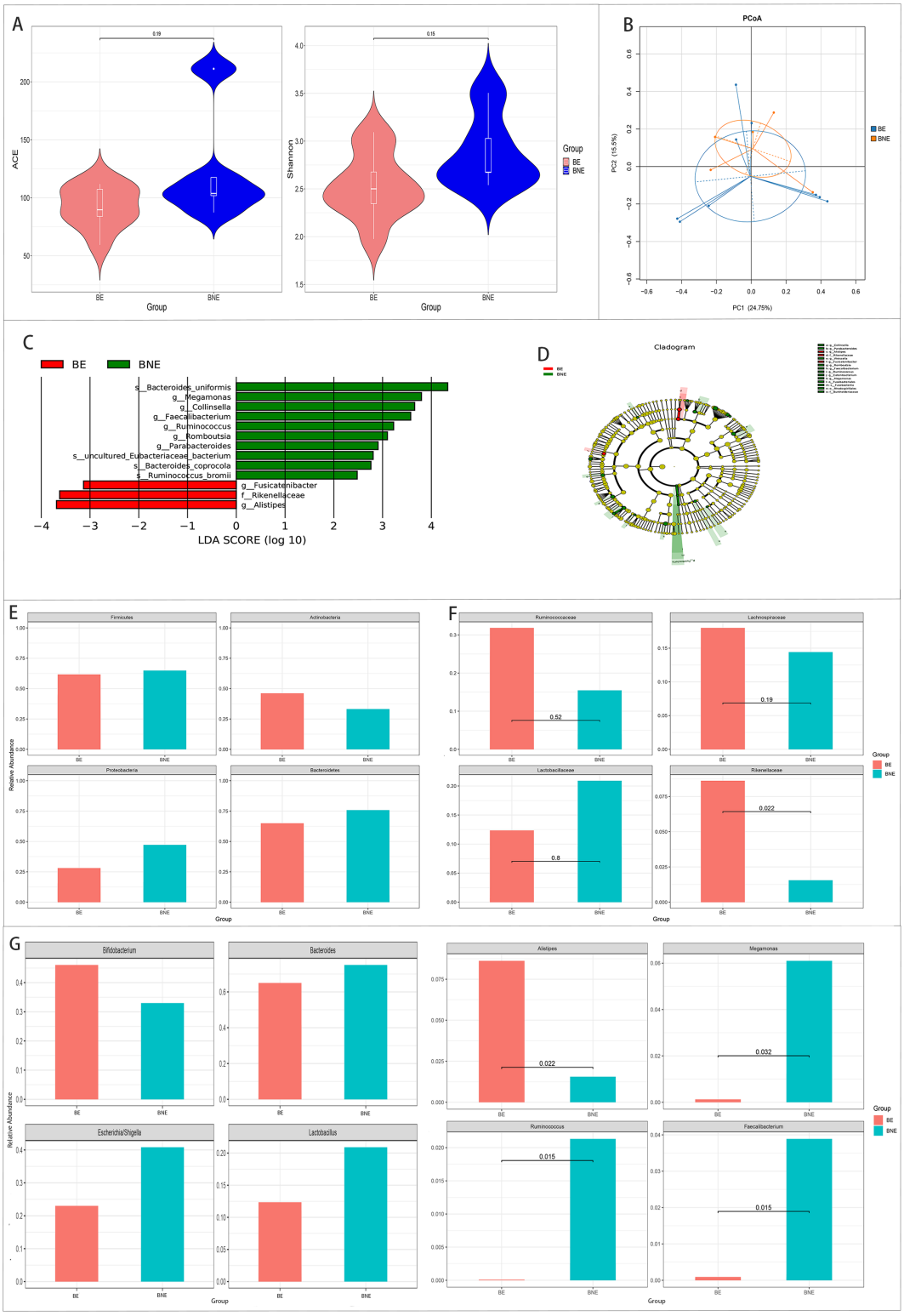

FIGURE 4
Comparison of the gut microbiota between the effective (BE) and ineffective (BNE) groups in the 2 weeks after treatment group. **(A, B)** Comparison of the alpha diversity **(A)** and the beta diversity **(B)** between the two groups. **(C, D)** Linear discriminant analysis (LDA) value distribution and the cladogram of the linear discriminant analysis effect size (LEfSe) between the two groups. **(E–G)** Metastats analysis at the phylum **(E)**, family **(F)**, and genus **(G)** levels between the two groups.

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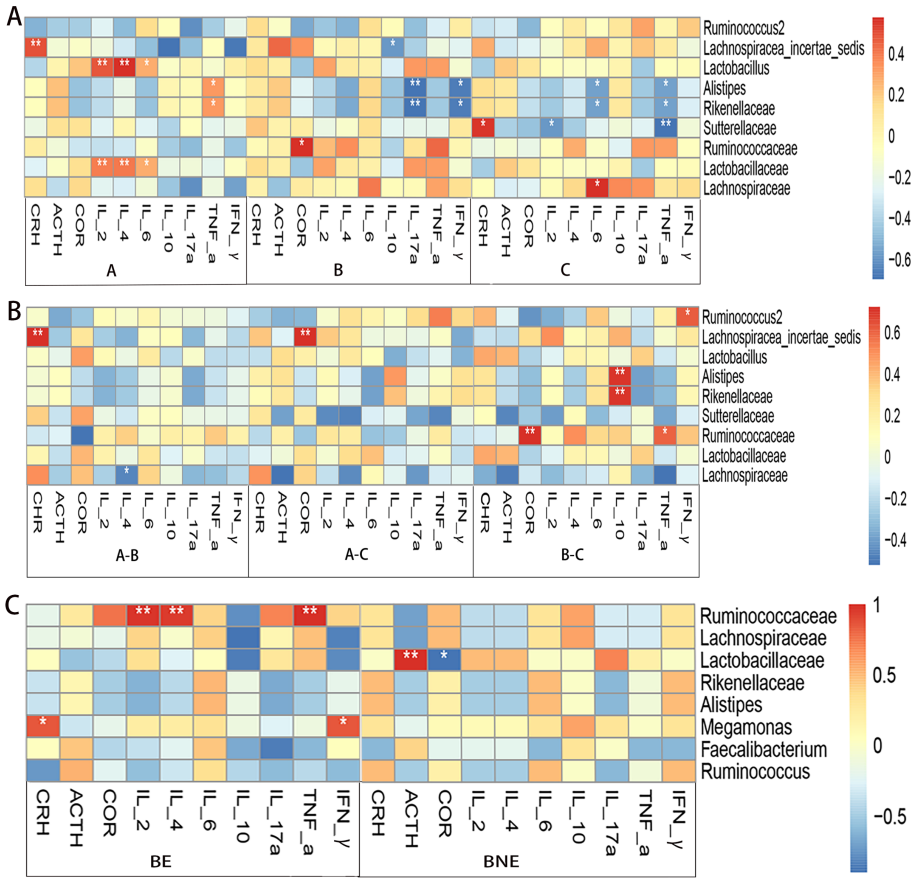

FIGURE 5
Correlation analysis between the gut microbiota and the levels of hypothalamus–pituitary–adrenal (HPA) axis hormones and inflammatory cytokines. **(A)** Correlation analysis of the pre-treatment (group A), 2 weeks after treatment (group B), and 1 month after treatment (group C) groups in the case group. **(B)** Correlation analysis between the differences in the gut microbiota with differences in the levels of HPA axis hormones and inflammatory cytokines in the case group. **(C)** Correlation analysis of the effective (*BE*) and ineffective (*BNE*) groups $*p < 0.05$; $**p < 0.01$.

influence the occurrence of neurological diseases through the HPA pathway. *Ruminococcaceae* was positively associated with IL-2, IL-4, and IFN-γ. This is consistent with the increase in the relative abundance of *Ruminococcus* in the ineffective group.

It was also discovered that, after treatment, *Sutterellaceae* was positively associated with CRH and negatively associated with IL-2 and TNF-α. *Sutterella* has been shown to be negatively associated with inflammatory cytokines (IL-12, IL-13, and IFN-γ) in a clinical cohort study (33), which is consistent with this study. The CRH levels increased and *Sutterellaceae* decreased, which did not match the expected results. This could be related to factors such as the experimental design, the subjects' age, and the sample acquisition time, among others, which made it difficult to cross-compare some experiments.

In summary, the MGBA plays an important role in IS. In a study on the association between IS and the gut microbiota, researchers found that *Lactobacillus*, *Roseburia*, and *Lachnospira* were lower in the IS group than those in the healthy group. Compared with that in the ACTH-NR (no response) group, *Bifidobacterium* was higher in the ACTH-response group (34). Studies have shown that probiotic supplementation could significantly reduce the frequency and the severity of seizures (35, 36). In addition, research found that the ratio of *Bacteroidota-to-Firmicutes* increased in epileptic (Epi) rats compared with non-epileptic (No-Epi) and sham control rats (37). These are consistent with the findings of this study, where the gut microbiota was involved in the development of IS. Specific gut microbiota could be used as a potential therapeutic target for IS, and disease control might be achieved by restoring the gut microbiota.

This study has certain limitations. Firstly, the sample size was small, which should be expanded in future studies to improve the accuracy of the results. Secondly, in this study, 16S rDNA sequencing was used for the gut microbiota, which could only ensure the accuracy of the microflora at the genus level and above. Therefore, future research needs to use metagenomic sequencing technology to improve the breadth and accuracy of sequencing. Thirdly, the follow-up time of the study was short, and follow-up should be up to 3 and 6 months and 1 year after treatment. Data on the improvement of the children, the serological samples at the time of review, and the stool results should be collected in order to track the changes in the relationship of the HPA hormones, inflammatory cytokines, and gut microbiota. Finally, we studied the relationship of the HPA axis hormones, inflammatory cytokines, and gut microbiota; however, the various ways how the gut microbiota

affects the development of IS, as well as the in-depth mechanism of this effect, have not been studied. Therefore, future studies need to conduct animal and cell experiments to explore how the gut microbiota affects the pathogenesis of IS.

5 Conclusion

The gut microbiota of children with IS differed from that of healthy children. *Lachnospiraceae* and *Lachnospiracea_incertae_disedis* might be associated with the disease onset. *Sutterellaceae* might be linked to children's improved health. In addition, certain gut microbiota might affect the levels of some HPA axis hormones or inflammatory cytokines in IS.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found https://www.ncbi.nlm.nih.gov/sra/PRJNA1145298, PRJNA1145298.

Ethics statement

The studies involving humans were approved by the Ethics Committee of Hunan Children's Hospital (Changsha, China. No. HCHLL-2020-53). 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.

Author contributions

JJY: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal Analysis, Data curation, Conceptualization. LL: Writing – original draft, Visualization, Software, Methodology, Data curation, Conceptualization. XFP: Writing – review & editing, Software, Methodology. LWW: Writing – review & editing, Resources, Project administration. LHT: Writing – review & editing, Supervision, Resources, Project administration. CCZ: Writing – review & editing, Software, Data curation. SWF: Writing – review & editing, Software. ZHX: Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. JQ: Writing – review & editing, Validation, Supervision, Software, Resources, Project administration, Conceptualization.

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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.2024.1442677/full#supplementary-material

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Identification and validation of diagnostic biomarkers and immune cell abundance characteristics in *Staphylococcus aureus* bloodstream infection by integrative bioinformatics analysis

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Staphylococcus aureus (*S. aureus*) is an opportunistic pathogen that could cause life-threatening bloodstream infections. The objective of this study was to identify potential diagnostic biomarkers of *S. aureus* bloodstream infection. Gene expression dataset GSE33341 was optimized as the discovery dataset, which contained samples from human and mice. GSE65088 dataset was utilized as a validation dataset. First, after overlapping the differentially expressed genes (DEGs) in *S. aureus* infection samples from GSE33341-human and GSE33341-mice samples, we detected 63 overlapping genes. Subsequently, the hub genes including DRAM1, PSTPIP2, and UPP1 were identified via three machine-learning algorithms: random forest, support vector machine-recursive feature elimination, and least absolute shrinkage and selection operator. Additionally, the receiver operating characteristic curve was leveraged to verify the efficacy of the hub genes. DRAM1 (AUC=1), PSTPIP2 (AUC=1), and UPP1 (AUC=1) were investigated and demonstrated significant expression differences (all $P < 0.05$) and diagnostic efficacy in the training and validation datasets. Furthermore, the relationship between the diagnostic markers and the abundance of immune cells was assessed using cell-type identification by estimating relative subsets of RNA transcripts (CIBERSORT). These three diagnostic indicators also correlated with multiple immune cells to varying degrees. The expression of DRAM1 was significantly positively correlated with B cell naive and mast cell activation, and negatively correlated with NK cells and $CD4/CD8^+$ T cells. The expression of PSTPIP2 was significantly positively correlated with macrophage M0, macrophage M1, B cell naive, and dendritic cell activation, while the expression of PSTPIP2 was negatively correlated with NK cells and $CD4/CD8^+$ T cells. Significant negative correlations between UPP1 expression and T cell CD4 memory rest and neutrophils were also observed. Finally, we established a mouse model of *S. aureus* bloodstream infection and collected the blood samples for RNA-Seq analysis and RT-qPCR experiments. The analysis results

in RNA-Seq and RT-qPCR experiments further confirmed the significant expression differences (all $P < 0.05$) of these three genes. Overall, three candidate hub genes (DRAM1, PSTPIP2, and UPP1) were identified initially for *S. aureus* bloodstream infection diagnosis. Our study could provide potential diagnostic biomarkers for *S. aureus* bloodstream infection patients.

KEYWORDS

***Staphylococcus aureus*, bloodstream infection, machine-learning, biomarkers, immune cell abundance**

Introduction

The opportunistic pathogenic bacterium *Staphylococcus aureus* has successfully adapted to the human body's environmental conditions (1). It causes a spectrum of infections in communities and hospitals, ranging from skin and soft tissue infections to life-threatening bloodstream infections (2). A critical feature of bloodstream infections by *S. aureus* is the coordinated and timely expression of virulence factors and other relevant genes by the pathogen. Due to its prevalence, *S. aureus* ranks among the leading pathogens causing bloodstream infections (3). The *S. aureus* bloodstream infections are characterized by high mortality rates (ranging from 20% to 50%), frequent recurrence (5-10%), and sustained injury in over one-third of survivors (3–5). Over the past three decades, the incidence rate of *S. aureus* bloodstream infection has been increasing in developed countries (3, 4), but remains a significant but often overlooked issue in developing countries (6). Therefore, there is an urgent need to identify biomarkers for the diagnosis of *S. aureus* bloodstream infection.

During infections, *S. aureus* could trigger inflammatory responses, including the secretion of cytokines and chemokines that recruit leukocytes to the area of infection. These recruited neutrophils, monocytes, macrophages, NK cells, Dendritic cells (DCs), and CD4/$CD8^+$ T cells play crucial roles in both the direct killing of bacteria and the indirect control of infection, such as contributing to the cytokine milieu, clearing damaged cells, and presenting antigen to initiate adaptive immunity (7). Hence, discovering new immunological biomarkers was important not only for the diagnosis but also for the application of immunotherapy in *S. aureus* bloodstream infection.

High-throughput sequencing was a valuable method for investigating changes in disease gene expression and distinguishing possible disease-related genes for new diagnostic and therapeutic biomarkers (8). Gene expression levels serve as essential indicators for diagnosing various disorders, including *S. aureus* bloodstream infection (9). Machine learning method assists in assessing high-dimensional transcriptome data and identifying biologically significant genes (10).

In this study, we integrated multiple high-throughput sequencing datasets of *S. aureus* bloodstream infections and employed machine learning algorithms for the first time to identify three characteristic genes associated with these infections, distinguishing our work from previous studies (8, 11–13). In addition, using a mouse model of *S. aureus* bloodstream infection, we validated the diagnostic value of these three genes through RNA-Seq and RT-qPCR experiments. Additionally, we investigated the relationship between diagnostic markers and immune cell abundance to acquire a more in-depth understanding of the molecular immune mechanisms underlying *S. aureus* bloodstream infections. Our study may offer potential diagnostic biomarkers and select potential candidates receiving immunotherapy for patients with *S. aureus* bloodstream infection.

Methods

Public gene expression datasets

Accessing the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/), which is a public collection of high-throughput gene expression data, chips, and microarrays, was how the information was collected. We searched the GEO database with the keywords "*Staphylococcus aureus*" [MeSH Terms] AND "Bloodstream infection"[All Fields]. None of the included samples were associated with any other diseases. The sample size of both the pediatric sepsis group and the normal group was greater than 10. Finally, GSE33341 (14) was utilized as the discovery dataset, which contained samples from human and mice. Another dataset GSE65088 (15) was applied as a validation dataset.

Identification of the differentially expressed genes

The Wilcoxon test was utilized to identify differentially expressed genes (DEGs) between the *S. aureus* bloodstream infection group and the control group. A volcano plot was generated to visualize the differential expression of DEGs. A P value < 0.05 and $|\log2FC| > 1$ were considered to be the cutoffs for DEGs.

Evaluation of functional enrichment

Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were conducted via the "clusterProfiler" (16) package in R to explore possible biological features of DEGs. Gene set enrichment analysis (GSEA) (17) was also used to investigate the enrichment pathways via the "clusterProfiler" package in R.

Screening and validation of diagnostic markers

Firstly, we intersected the up-regulated genes in the human *S. aureus* infection group and the mice *S. aureus* infection group in GSE33341 to obtain genes associated with *S. aureus* bloodstream infection. Subsequently, these genes were further screened using three machine-learning algorithms, random forests (RF), support vector machine-recursive feature elimination (SVM-RFE), and least absolute shrinkage and selection operator (LASSO) logistic regression, to identify robust biomarkers for *S. aureus* bloodstream infection. The "randomForest" R package in R was used to implement the random forest technique with 100 trees generated for each datapoint, and genes with top MeanDecreaseAccuracy were screened out (18). LASSO logistic regression investigation was conducted with the R package "glmnet", and minimal lambda was considered optimal. In our study, the selection of optimization parameters was cross-verified by a factor of 10, and the partial likelihood deviation met the minimum criteria (19). The DEGs were also determined by applying a support vector machine recursive feature elimination (SVM-RFE) algorithm based on a nonlinear SVM using R package "kernlab", "e1071", and "caret" (20). It was evaluated based on the study of receiver operating characteristic (ROC) curves, and the area under the curve (AUC) was calculated to assess the predictive capability of these markers. The GSE65088 dataset was enrolled to validate the predictive power of these biomarkers.

Assessment of immune cell abundance

Immune cell abundance was assessed by computing the differential abundances of 22 immune cells using the CIBERSORT (21) algorithm. The correlation between gene expression and immune cells were assessed using Pearson's correlation coefficients. Correlation plots were plotted using the "ggpubr" R package.

Construction of *S. aureus* bloodstream infection

Methicillin-sensitive *Staphylococcus aureus* (MSSA) Newman strain was grown for 16 h on TSB medium at 37°C. Overnight cultures were centrifuged at 2683g (RCF) for 5 min at room temperature and adjusted to a concentration of 2×10^9 CFU/mL using phosphate-buffered saline (PBS). Next, injected into female Balb/c mice via the tail vein with 100 µL PBS containing 2×10^8 CFU bacterial cells suspended. At 8 h.p.i., anesthetized the mice with 2,2,2-tribromoethanol (5 mg/25 g). Used surgical scissors to remove mouse whiskers, then clamped the eyeball with tweezers and quickly removed it, allowing blood to flow from the eye socket into the EP tube. The blood sample was immediately placed in liquid nitrogen and maintained at −80°C until RNA extraction.

RNA-Seq and data processing

After the blood samples were collected from mice infected with *S. aureus*, the RNA for RNA-Seq samples were immediately mixed with Trizol Reagent (Ambion®) and then sent to Shanghai Personal Biotechnology Cp. Ltd for the subsequent RNA transcriptome sequencing work. Following library preparation and pooling of different samples, the samples were subjected to Illumina sequencing. Commonly, the RNA-Seq use PE150 (paired-end 150nt) sequencing. Raw data (raw reads) of FASTQ format were first processed through in-house perl scripts. In this step, clean data (clean reads) were obtained by removing the following reads: (1) reads with adapter; (2) reads with more than 3 N; (3) reads with more than 20% nucleotides with Qphred<=5; At the same time, Q20, Q30 and GC content of the clean data were calculated. Then, map the clean reads to the silva database to remove the rRNA. All the downstream analyses were based on clean data without rRNA. Paired-end clean reads were aligned to the reference genome using Hisat2 (22). Featurecount (23) was used to count the reads numbers mapped to each gene.

RNA extraction and real-time polymerase chain reaction

The total RNA of blood was isolated by Trizol Reagent (24) and then was reverse transcribed into cDNA using the PrimeScript RT reagent kit with gDNA Eraser (Takara). Real-time quantitative PCR (RT-qPCR) was performed using TB GreenTM Premix Ex TaqTM II (Takara) on QuantStudioTM 5 Real-Time PCR System (Applied Biosystems). RNA expression levels of DRAM1, PSTPIP2, and UPP1 genes unified to GAPDH were calculated by the formula $2^{-\Delta\Delta Ct}$. All primers used in this study were listed in Supplementary Table 1. Each reaction was performed trice.

Statistical analysis

R version 4.2.2 was utilized for all statistical analyses and graphics except for RT-qPCR results which were analyzed by GraphPad Prism 8 (GraphPad Software Inc. San Diego, CA, USA). Statistical significance was determined by a two-tailed test with a P value of less than 0.05. **P < 0.01, ****P < 0.0001.

Results

Screening of DEGs in *S. aureus* bloodstream infection

The clinical characteristics of the two groups of samples are presented in Supplementary Table 2. Figure 1 displays the study design of this research. The human blood samples in GSE33341, consisting of 31 *S. aureus* infection samples and 43 control samples, were applied to obtain 482 DEGs. Following the identification of DEGs, heatmap (Figure 2A) and volcano plots (Figure 2B) were drawn to present these findings.

Functional enrichment analysis of DEGs

Functional analysis was performed to gain a more thorough understanding of the biological functions of these DEGs. GO enrichment analysis showed that up-regulated DEGs were related to positive regulation of cytokine production and activation of the immune response (Figure 3A, left). Down-regulated DEGs were enriched in mononuclear cell differentiation, lymphocyte differentiation, and immune response-regulating signaling pathway (Figure 3A, right). Likewise, KEGG analysis for up-regulated DEGs was associated with Prion disease, Parkinson disease, and NOD-like receptor signaling pathway (Figure 3B, left). KEGG analysis for down-regulated DEGs was enriched in hematopoietic cell lineage, Th1 and Th2 cell differentiation, and Th17 cell differentiation (Figure 3B, right).

Screening and validation of diagnostic markers

The human blood samples in GSE33341, consisting of 31 *S. aureus* infection samples and 43 control samples, and the mice blood samples in GSE33341, including 10 *S. aureus* infection samples and 21 control samples, were exploited for analyzing the up-regulated genes separately. There were 482 up-regulated DEGs in the GSE33341-human samples and 305 up-regulated DEGs in the GSE33341-mice samples. A Venn plot was drawn to present the up-regulated genes intersected by GSE33341-human and GSE33341-mice samples (Figure 4A). Then we adopted three machine-learning algorithms to identify feature genes: Random Forest selected the top 10 genes (Figure 4B); SVM-RFE screened 4 genes (Figure 4C) and LASSO regression analysis was utilized to select 3 predicted genes from among the statistically significant univariate variables (Figure 4D). The three algorithms finally identified DRAM1, PSTPIP2, and UPP1 as the diagnostic markers (Figure 5A).

In the GSE33341, these three genes not only were highly expressed in the *S. aureus* infection group but also presented with good discriminative power between *S. aureus* and the control group (Figure 5B; Supplementary Figure 1). ROC curves for DRAM1, PSTPIP2, and UPP1 also highlighted them as potential diagnostic biomarkers (Figure 5C).

Meanwhile, we also acquired another independent cohort with *S. aureus* infection to validate the above findings. In GSE65088, the significantly high expression for DRAM1, PSTPIP2, and UPP1 in *S. aureus* group was also observed (Supplementary Figure 2A), along

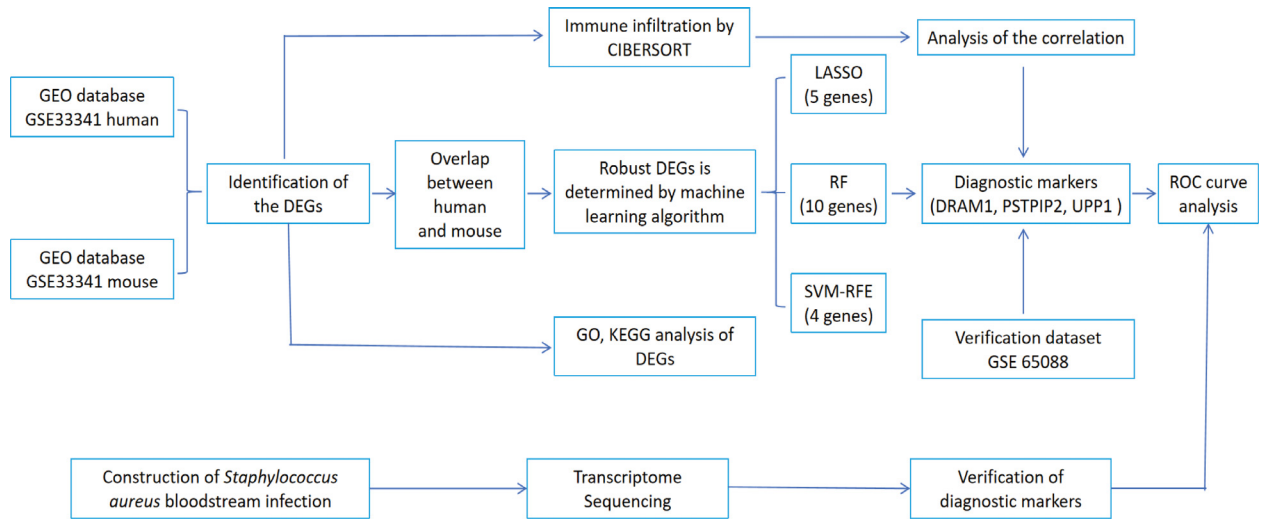

FIGURE 1
The flowchart depicting the investigation procedure. GEO, gene expression omnibus; GSEA, gene set enrichment analysis; CIBERSORT, cell-type identification by estimating relative subsets of RNA transcripts; DEGs, differentially expressed genes; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; LASSO, Least absolute shrinkage and selection operator; RF, random forest; SVM-RFE, support vector machine-recursive feature elimination; ROC, receiver operating characteristic curve.

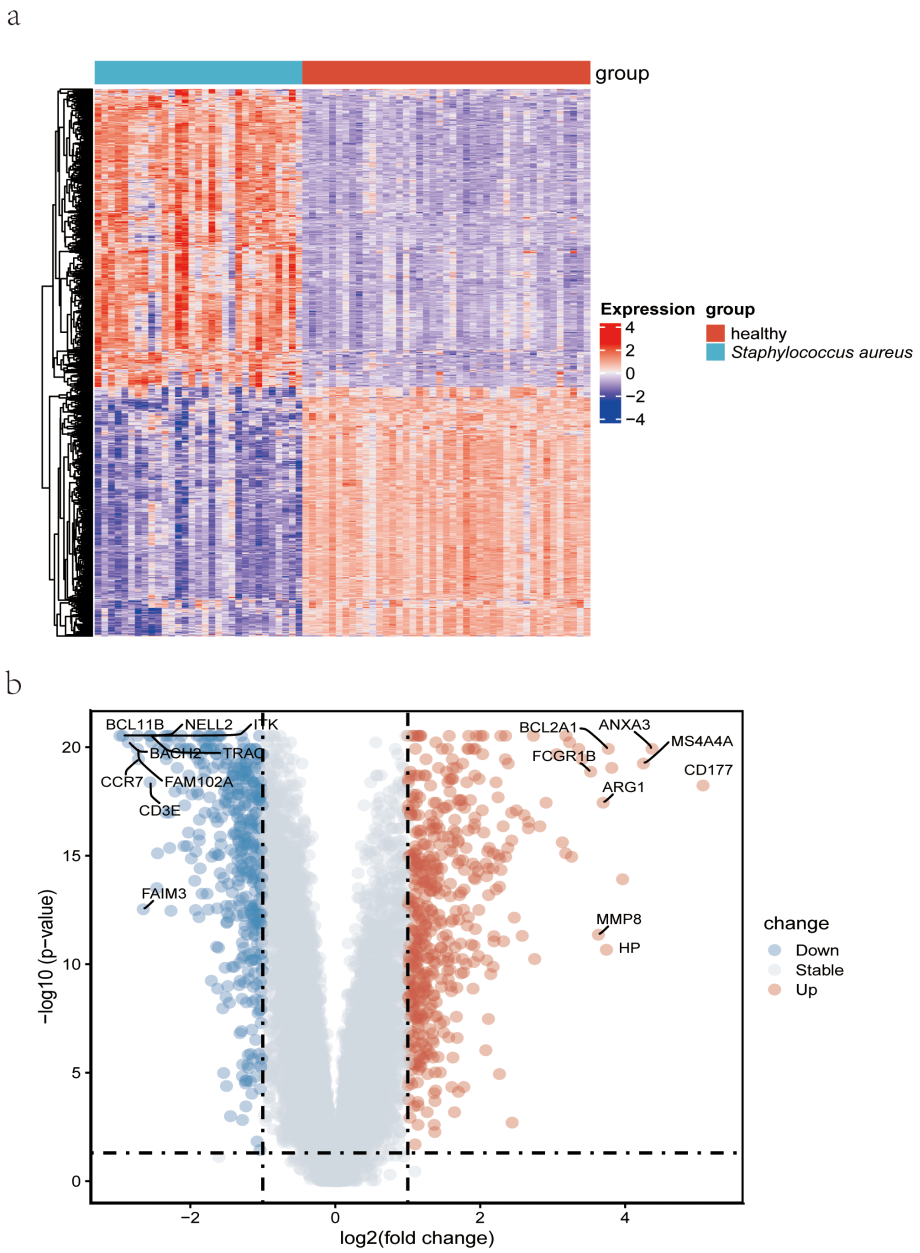

FIGURE 2
Detection of differentially expressed genes from datasets GSE33341 on *S. aureus* patients. **(A)** A heatmap comparing the genes that were differentially expressed in *S. aureus* bloodstream patients and control patients; **(B)** Volcano plot of the 482 DEGs. DEGs, differentially expressed genes; FC, fold-change.

with high AUC values (Supplementary Figure 2B), which indicates that the biological markers had high predictive value accuracy.

Association of biomarkers with immune cells abundance

CIBERSORT algorithm was utilized to evaluate the immune cell abundance. Based on a correlation analysis, we assessed the relationship between immune cells and three diagnostic biomarkers. We found DRAM1 was significantly positively associated with B cell naive and Mast cell activated. However, the expression of DRAM1 was negatively associated with NK cells and CD4/CD8$^+$ T cells (Figure 6A). Meanwhile, PSTPIP2 was notably positively correlated with Macrophages M0, Macrophages M1, B cell naive, and Dendritic cells activated, the expression of PSTPIP2 was negatively associated with NK cells and CD4/CD8$^+$ T cells (Figure 6B). Furthermore, UPP1 was remarkably negatively related to T cells CD4 memory resting and Neutrophils (Figure 6C). The above results of analysis suggested there was a potential connection between these three biomarkers and a wide variety of immune cells. Immunological prophylaxis and therapy for *S. aureus* are attractive goals. Our findings provided reasonable application of these markers for screening potential patients with *S. aureus* bloodstream infection for immunotherapy.

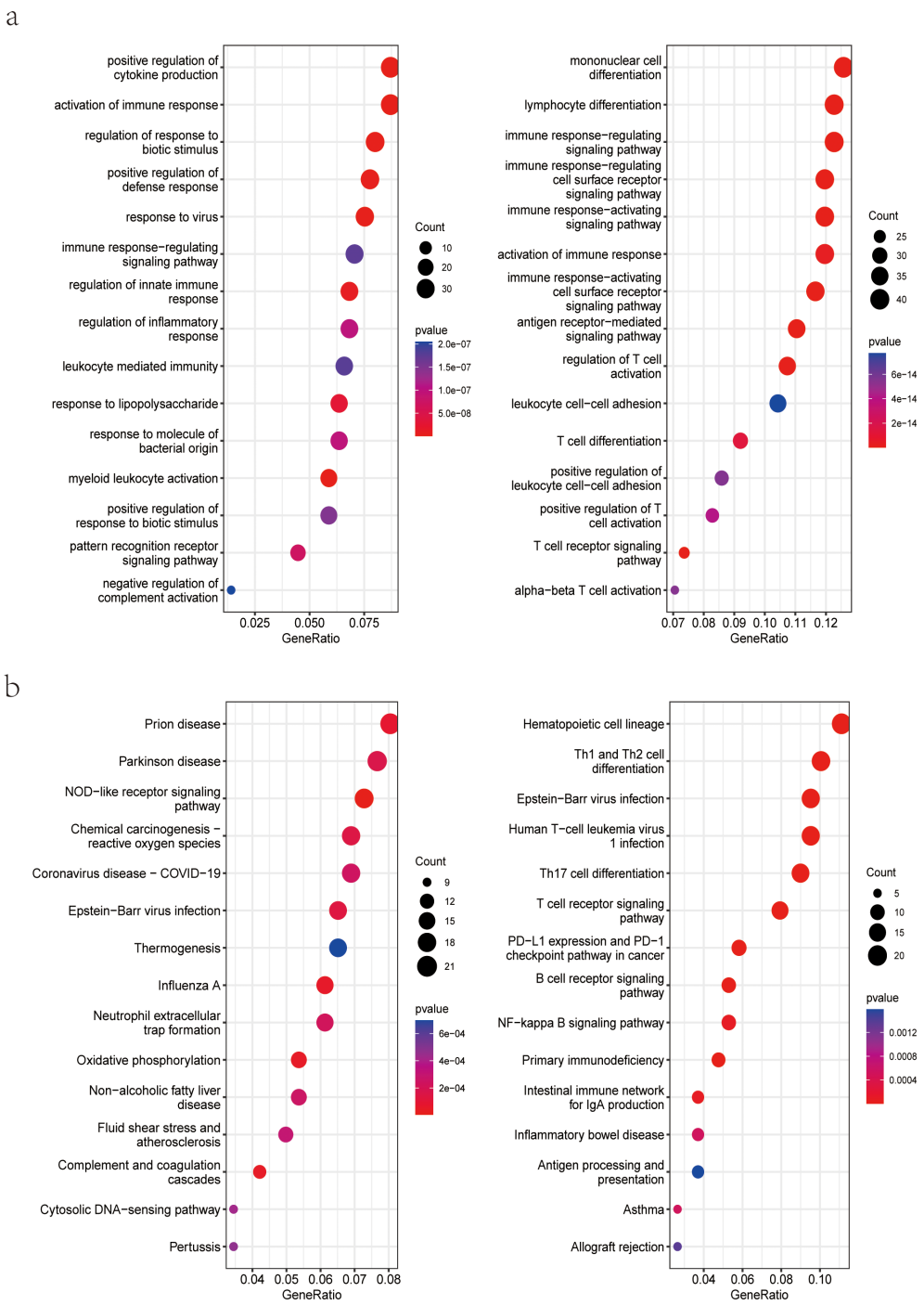

FIGURE 3
Functional enrichment analysis of DEGs. **(A)** GO enrichment analysis of upregulated (left) and downregulated (right) genes in the dataset GSE33341 human group; **(B)** KEGG pathway enrichment analysis of upregulated (left) and downregulated (right) genes in the dataset GSE33341 human group. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Validation of diagnostic markers using RNA-Seq and RT-qPCR for *S. aureus* bloodstream infection mice models

To further confirm the diagnostic value of these three genes, we constructed a mice model of *S. aureus* bloodstream infection and collected the blood of mice for RNA-Seq analysis and RT-qPCR experiments. The reason we chose the *S. aureus* Newman strain was that it is a hypervirulent stain that has been widely applied in the various models of *S. aureus*. We isolated the total RNA of the blood and synthesized the cDNA. RNA-Seq analysis results confirmed the above results (Figure 7A), with ROC results showing the high diagnostic value for these three genes (Figure 7B). RT-qPCR results further validated that the RNA expression levels of

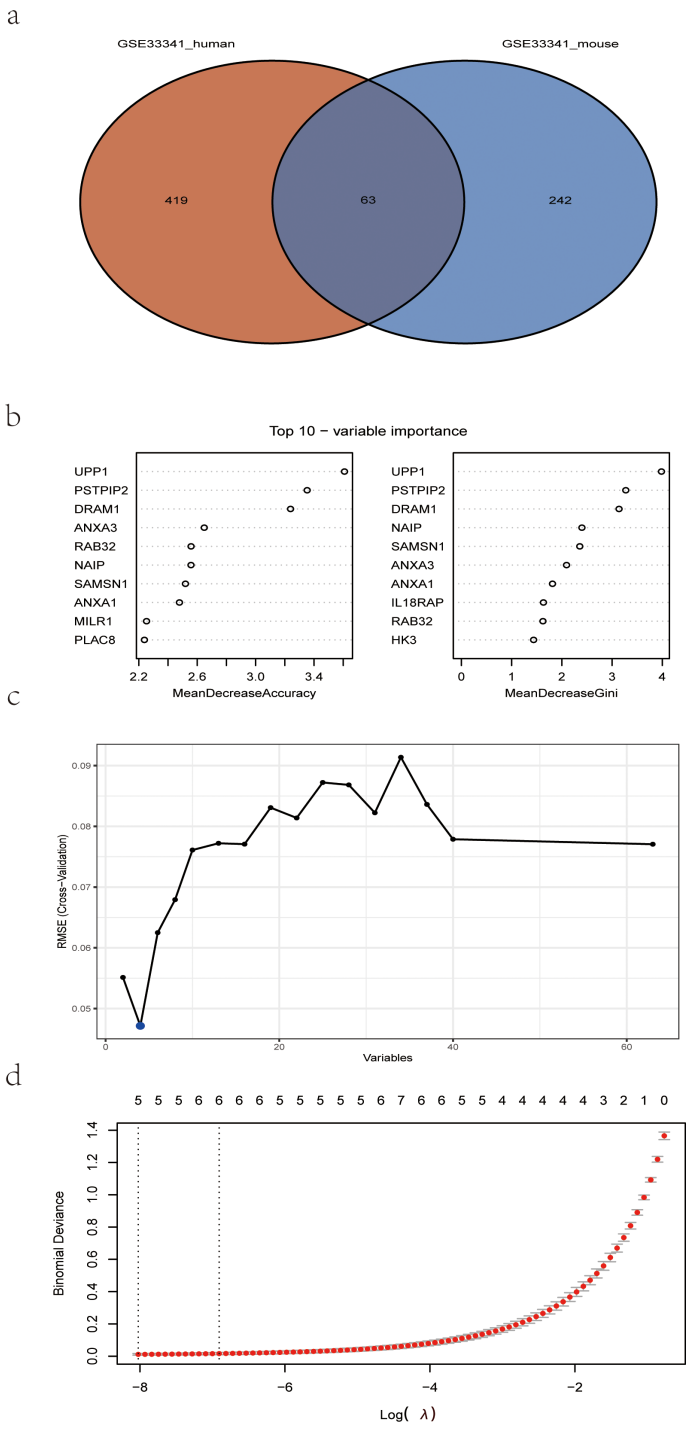

FIGURE 4
Detection of diagnostic markers using a thorough method. **(A)** Venn diagram of upregulated genes of human *S. aureus* infection versus mice group in GSE33341; **(B)** based on RF algorithm to screen biomarkers; **(C)** Based on SVM-RFE to screen biomarkers; **(D)** LASSO logistic regression algorithm to screen diagnostic markers. RF, random forest; SVM-RFE, support vector machine-recursive feature elimination; LASSO, least absolute shrinkage, and selection operator.

DRAM1, PSTPIP2, and UPP1 genes were significantly increased in the *S. aureus* Newman tread group compared to the control group (Figure 7C). Meanwhile, these three genes were significantly higher in *S. aureus* group in the combined mouse dataset including GSE33341 mice and blood infection mouse model sequencing data (Figure 7D).

Discussion

The opportunistic pathogen *S. aureus* adapted to human hosts, could result in fatal bloodstream infection (25). It represented a heterogeneous clinical entity with a high risk of metastatic complications and a high in-hospital mortality rate of 20% to

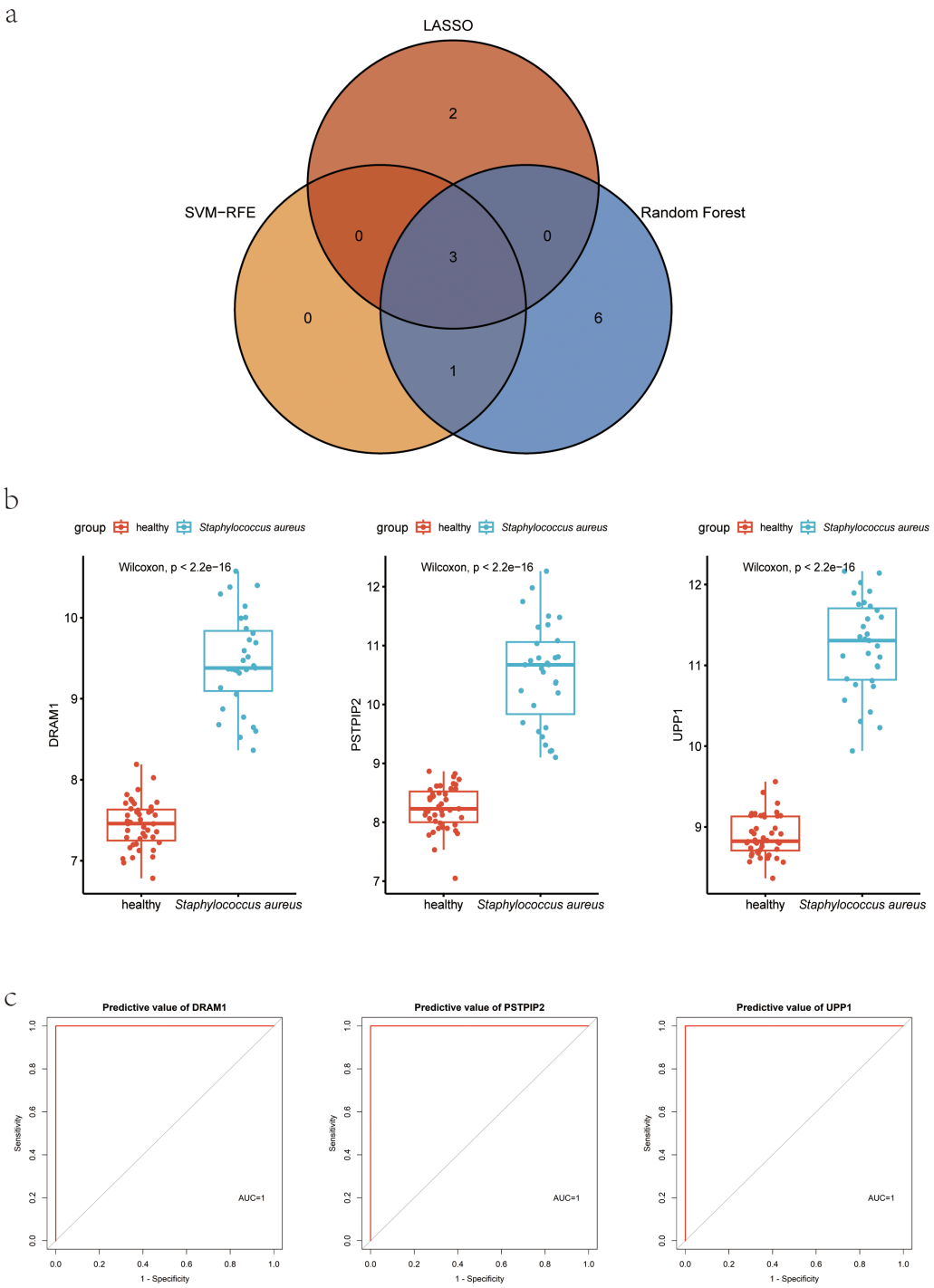

FIGURE 5
Hub genes for *S. aureus* blood infection diagnosis. **(A)** Venn diagram showed the intersection of diagnostic markers obtained by the three algorithms; **(B)** Boxplot showed the expression of hub genes between the *S. aureus* infection group and control group in discovery dataset GSE33341 human group; **(C)** The ROC curve of the diagnostic efficacy verification between the *S. aureus* infection group and control group in discovery dataset GSE33341 human group.

30%. Optimized diagnostic and therapeutic approaches can improve patients' outcomes (26). The Agr quorum-sensing system, one of the earliest regulators discovered to be involved in *S. aureus* bloodstream infections, is essential for the secretion of numerous toxins and other soluble virulence factors (27). Another virulence regulatory system closely related to *S. aureus* bloodstream infections is the two-component ArlRS system, and its downstream effector, the global regulator MgrA (27). Under the background of *S. aureus* bloodstream infections, this cascade reaction was shown to regulate plasma aggregation, adhesion, and interactions with endothelium (28, 29). Furthermore, the ArlRS-MgrA cascade regulates the expression of several immune evasion genes to

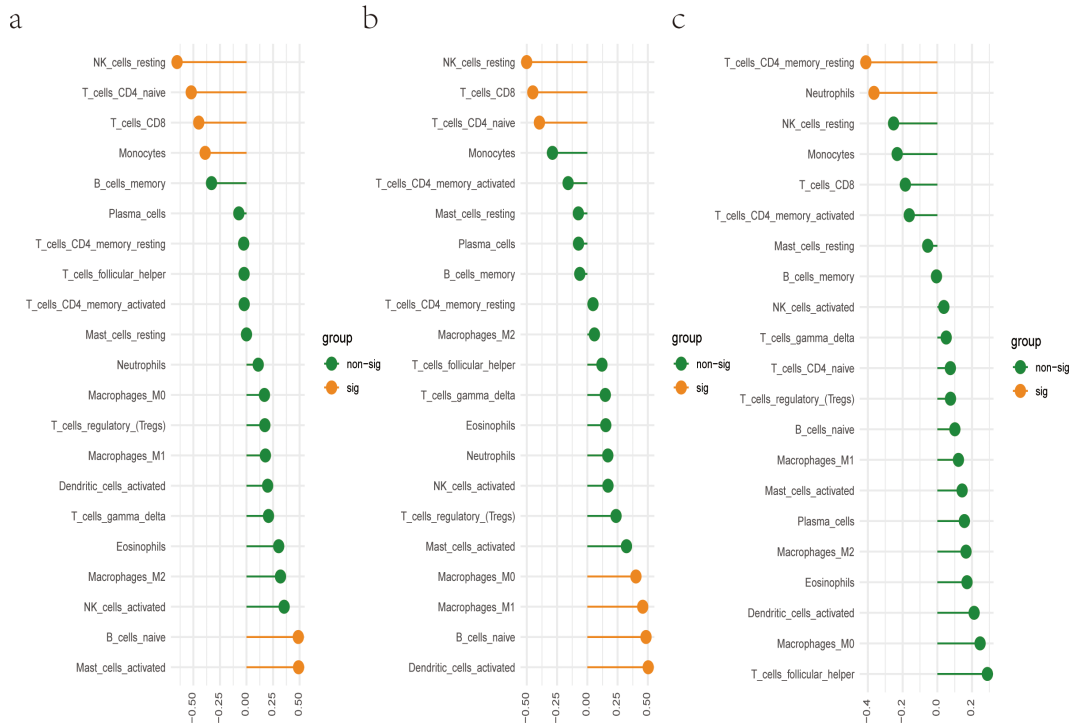

FIGURE 6
There is a correlation between hub genes and immune cells. **(A)** Correlation between DRAM1 and immune cells; **(B)** Correlation between PSTPIP2 and immune cells; **(C)** Correlation between UPP1 and immune cells.

evade host defense (30–32). These findings underscore *S. aureus's* ability to cause bloodstream infection by expressing a series of virulence genes, emphasizing the urgency of finding biomarkers for bloodstream infection of *S. aureus* for early diagnosis and treatment.

In recent years, extensive studies have attempted to discover diagnostic biomarkers for *S. aureus* bloodstream infections. Erin et al. found that *S. aureus* induced a muted host response in human blood that blunts the recruitment of neutrophils to promote the survival of pathogens during invasive infection (33). Sun et al. constructed a predictive model for sepsis in children with *S. aureus* bloodstream infections, which could guide clinicians in optimizing the treatment plan according to these risk factors and drug sensitivity results for minimizing unnecessary invasive procedures (34). Rachel et al. found that manipulation of autophagy in phagocytes facilitated *S. aureus* bloodstream infection (35). Sinead et al. carried out a prospective study in 61 patients with *S. aureus* bloodstream infection and revealed that IL-6 might be an early inflammatory marker of complicated *S. aureus* bloodstream infection (36). However, these diagnostic biomarkers more or less suffer from some limitations. Identifying new diagnostic markers for *S. aureus* bloodstream infection was urgently needed.

In this study, we attempted to identify new diagnostic biomarkers for *S. aureus* bloodstream infection. First, we identified the up-regulated genes in the *S. aureus* infection group common to the GSE33341-human and GSE33341-mouse datasets. Subsequently, the hub genes including DRAM1, UPP1, and PSTPIP2 were certificated by the use of three machine-learning algorithms. Further, we verified the findings by another dataset GSE65088, and developed a mice model of *S. aureus* bloodstream infection to collect the blood of mice for RNA-Seq analysis and RT-qPCR experiments. The receiver operating characteristic curve was employed to verify the efficacy of the hub genes. To summarize, our results suggest that DRAM1, UPP1, and PSTPIP2 were potential *S. aureus* bloodstream infection diagnostic indicators.

Currently, many studies have focused on the three genes mentioned above in *S. aureus* or other bacterial infections. DNA damage-regulated autophagy modulator 1 (DRAM1) is a stress-inducible regulator of autophagy and cell death (37). Xie et al. confirmed that DRAM1 could independently promote the zebrafish host defense against *Mycobacterium marinum* (38), and its role in facilitating Lysosomal Delivery of *Mycobacterium marinum* in murine RAW264.7 macrophages (39), suggesting DRAM1 is a host resistance factor against intracellular mycobacterial infection. Similarly, Zhang et al. demonstrated that deficiency in DRAM1 exacerbated pyroptotic cell death of Mycobacteria-infected macrophages (40). Han et al. found that DRAM1 expression was up-regulated in the *S. aureus*-treated bovine mammary epithelial cells and triggered the production of autophagosome (41). This appeared to coincide with our results since we demonstrated that DRAM1 was highly expressed in the blood of mice infected with *S. aureus*. Sun et al. proved upregulation of DRAM1 was involved in

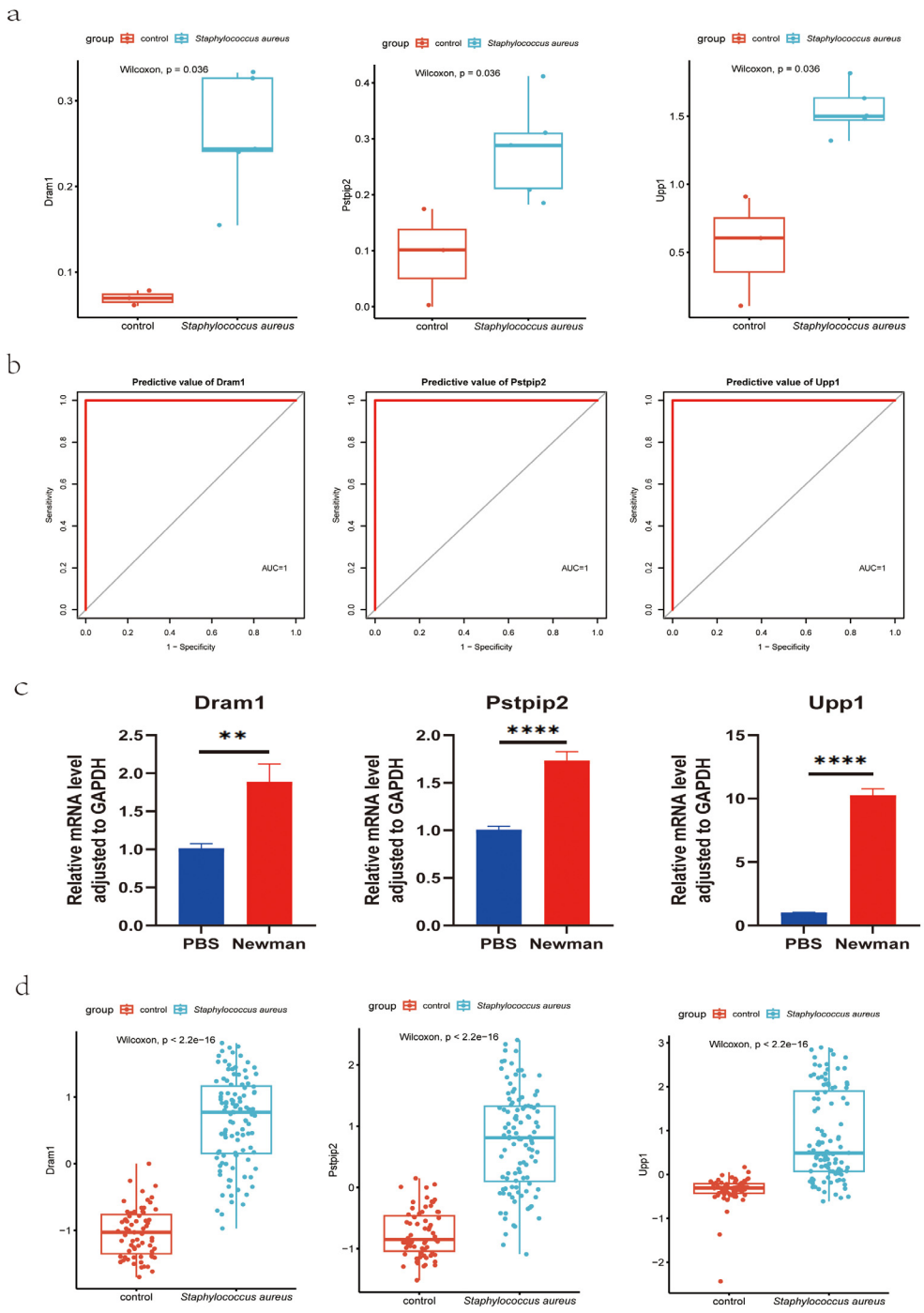

FIGURE 7
Validation of hub genes for *S. aureus* blood infection diagnosis. **(A)** Validation of diagnostic markers using RNA-Seq; **(B)** The ROC curve of the diagnostic efficacy verification in RNA-Seq analysis; **(C)** Validation of diagnostic markers using RT-qPCR; **P < 0.01 and ****P < 0.0001. d Validation of diagnostic markers via the combined mouse sequencing data including GSE33341 mice and blood infection mouse model sequencing data. AUC, area under the curve; ROC, receiver operating characteristic curve.

regulating autophagy and glycolysis in C10_ULK1 cells in response to both *Escherichia coli* (*E. coli*) infection and E. coli sepsis (42). Uridine phosphorylase 1 (UPP1) encodes uridine phosphorylase, a key enzyme that participates in the regulation of intracellular uridine homeostasis and the metabolism of pyrimidine ribonucleosides (43). Fan et al. revealed that UPP1 emerged with remarkable diagnostic value in pediatric septic shock and was involved in immune cell infiltration (44). Similarly, Lai et al. analyzed GEO datasets and found that UPP1 was upregulated in the sepsis group, and confirmed this finding by establishing a sepsis-

induced acute lung injury model (45). Our research further expanded the diagnostic value of UPP1 and indicated its likelihood as a diagnostic biomarker. Proline-serine-threonine phosphatase Interacting Protein 2 (PSTPIP2), also known as macrophage F-actin–associated and tyrosine-phosphorylated protein (MAYP), is a Fes CIP4 homology domain (FCH) and Bin/Amphiphysin/Rvs (BAR; F-BAR) protein, predominantly expressed in the myeloid lineage (46). Johnny et al. uncovered that PSTPIP2 was highly expressed in the confirmed bacterial infection patients, correlating with infection status (47). Chen et al. validated the high expression of PSTPIP2 in patients infected with *E. coli* by analyzing GEO datasets and ex-vivo human blood models (48).

Additionally, the relationship between these diagnostic markers and infiltrating immune cells was further studied. The changes in various immune cell infiltration may be relevant to the occurrence and progression of *S. aureus* bloodstream infection (33, 49). NK cells are pivotal in the first line of defense of the human immune system (50, 51). They mediated some immune responses during anti-tumor and various viral infections and were the "natural barrier" in the human immune system (52). Under the stimulation of LPS and so on, dormant macrophages (M0) could induce polarization into M1 type macrophages, secreting a large number of pro-inflammatory factors, including IL-1, IL-6, and TNF-α, to promote inflammation, bacterial killing, and phagocytosis (53). M2 macrophages were mainly activated by IL-4 inflammatory factors and inhibit M1 macrophages by secreting anti-inflammatory cytokines such as IL10 (54). Neutrophils are one of the important cells in the immune system, with various functions, including chemotactic, regulatory, phagocytic, degranulation, and bactericidal effects (55, 56). Dendritic cells could uptake, process, and present antigens, and were initiators of adaptive immune responses (57, 58). $CD4^+$ T cells mainly recognized foreign antigens presented by antigen-presenting cells (APCs) and generated responses (59). $CD8^+$ T cells cloud secrete cytokines including TNF-α, IFN-γ, and the production and release of cytotoxic particles to defend against intracellular viruses and bacteria (60). In our research, DRAM1 expression was significantly positively correlated with B cell naive and mast cell activation, and negatively correlated with NK cells and $CD4^+$/$CD8^+$ T cells. PSTPIP2 expression was significantly positively correlated with macrophage M0, macrophage M1, B cells naive, and dendritic cells, while negatively correlated with NK cells and CD4/$CD8^+$ T cells. UPP1 expression showed significant negative correlations with T cell CD4 memory rest and neutrophils. Li et al. (61) associated PD-1/PD-L1 signaling with the immunosuppressive state in *S. aureus* osteomyelitis, suggesting potential novel therapies combining PD-1/PD-L1 blockade with antibiotics for the treatment of *S. aureus* osteomyelitis. Therefore, our proposed diagnostic biomarkers may also be used to select potential patients with *S. aureus* bloodstream infection for the utilization of immunotherapy.

However, this study also had some limitations. Despite having reported the diagnostic value of these three markers in *S. aureus* bloodstream infections, unfortunately, we have not yet collected blood samples from patients with *S. aureus* bloodstream infection, which is a limitation of our study. Additionally, the *S. aureus* bloodstream infection-related molecular mechanisms should be investigated further by constructing animal models and cellular experiments. Meanwhile, the differences in immune response between mice and humans might affect the translational relevance of our findings.

Here, our study initially identified that DRAM1, PSTPIP2, and UPP1 were potential diagnostic indicators for *S. aureus* bloodstream infection. Furthermore, these three diagnostic genes also correlate with multiple immune cells to varying degrees and may be used for the *S. aureus* selection of potential patients for the utilization of immunotherapy.

Data availability statement

The raw data has been uploaded to the NCBI database (accession number: PRJNA1171428).

Ethics statement

This study was approved by the Ethics Committee of Shanghai Pulmonary Hospital, School of Medicine, Tongji University, Shanghai, China. All animal experiments were carried out under the guidelines approved by the Ethics Committee of the Shanghai Pulmonary Hospital of Tongji University School, Tongji University, Shanghai (Project number: K22-183Y).

Author contributions

JS: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. LS: Data curation, Writing – original draft. YX: Data curation, Writing – original draft. CW: Data curation, Writing – original draft. BW: Data curation, Writing – original draft. PZ: Methodology, Writing – original draft. JZ: Investigation, Writing – original draft. WH: Project administration, Writing – original draft. RH: Formal analysis, Writing – original draft. FY: Funding acquisition, Writing – review & editing. HW: 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1450782/full#supplementary-material

SUPPLEMENTARY TABLE 1
Primer pairs used in RT-PCRs.

SUPPLEMENTARY TABLE 2
Information on microarray datasets obtained from GEO.

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Gene prediction of immune cells association between gut microbiota and colorectal cancer: a Mendelian randomization study

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Background: An increasing number of studies have revealed that gut microbiota influences the development and progression of Colorectal cancer (CRC). However, whether a causal relationship exists between the two remains unclear, and the role of immune cells in this context is not well understood.

Objective: To elucidate the causal relationship between gut microbiota and CRC and to explore the potential mediating role of circulating immune cells.

Materials and methods: To analyze the causal relationship between gut microbiota and CRC, we employed a univariable Mendelian randomization (UVMR) approach. Subsequently, a two-step multivariable Mendelian randomization (MVMR) to assess the potential mediating role of circulating immune cells. Primarily, applied the Inverse-Variance Weighted method to evaluate the causal relationship between exposure and outcome. To ensure the robustness of the results linking gut microbiota and CRC, we validated the findings using Robust Inverse-Variance Weighted, Penalized Inverse-Variance Weighted, and Penalized Robust Inverse-Variance Weighted methods. Additionally, we employed MR-Egger Intercept to mitigate the influence of horizontal pleiotropy. MR-PRESSO was used to detect and correct outliers by excluding anomalous instrumental variables. Finally, we supplemented our analysis with methods such as Bayesian Weighted Mendelian Randomization (BWMR), Maximum-Likelihood, Lasso, Debiased Inverse Variance Weighted, and Contamination Mixture to establish a robust and compelling causal relationship.

Results: After accounting for reverse causality, horizontal pleiotropy, and various methodological corrections, *Bifidobacterium kashiwanohense*, *GCA-900066755 sp900066755*, *Geminocystis*, and Saccharofermentanaceae exhibited strong and robust causal effects on CRC. Specifically, CD40 on monocytes (2.82%) and CD45 on $CD33^{+}HLA\text{-}DR{+}CD14^{-}$ cells (12.87%) mediated the causal relationship between *Bifidobacterium kashiwanohense* and CRC risk. Furthermore, CD45 on $CD33^{-}HLA\text{-}DR^{+}$ (3.94%) mediated the causal relationship between *GCA-900066755 sp900066755* and CRC risk. Additionally, terminally differentiated $CD4^{+}$T cells (11.55%) mediated the causal relationship between *Geminocystis* and CRC risk. Lastly, CD40 on monocytes

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(2.35%), central memory $CD4^+$T cells (5.76%), and CD28 on $CD28^+CD45RA^+CD8^+$T cells (5.00%) mediated the causal relationship between Saccharofermentanaceae and CRC risk.

Conclusion: Our mediation MR analysis provides genetic evidence suggesting that circulating immune cells may mediate the causal relationship between gut microbiota and CRC. The identified associations and mediation effects offer new insights into potential therapeutic avenues for CRC.

KEYWORDS

colorectal cancer, gut microbiota, immune cells, Mendelian randomization, mediation analysis

1 Introduction

Colorectal cancer (CRC) is a malignant tumor originating from the epithelial cells of the colon or rectal mucosa. According to the Global Cancer Statistics 2020, there were 1,931,590 new cases and 935,173 deaths from CRC worldwide in 2020, making it the third most common cancer globally (1, 2). Moreover, with significant lifestyle changes, the incidence of CRC is increasing annually and is also showing a trend towards affecting younger (3–6). It is the leading cause of cancer-related deaths in men under 50 and the second leading cause in women of the same age group (7). Consequently, CRC poses a severe threat to human health and has become a pressing public health issue. CRC is initiated through the interaction of genetic alterations, including proto-oncogene activation, tumor suppressor gene inactivation, chromosomal instability, microsatellite instability, and epigenetic changes, with environmental factors such as high-fat and high-carbohydrate diets, unhealthy lifestyles, smoking, alcohol consumption, and physical inactivity (8). The specific mechanisms underlying CRC development and progression are not fully understood, and research on effective treatment strategies remains limited. Therefore, investigating the pathogenesis of CRC and seeking effective therapeutic approaches are of paramount importance for reducing its incidence and mortality rates.

The human gut microbiota comprises approximately 10^{13} to 10^{14} microorganisms, with a genomic content that is roughly 100 times greater than that of the human genome (9–11). Hence, it is often referred to as the "second genome" of humans (12). These microorganisms interact with host cells through various mechanisms, including metabolic processes and immune responses (9). Changes in lifestyle factors such as diet, smoking, and physical activity can lead to dysbiosis of the gut microbiota, which has been associated with gastrointestinal diseases, certain neurological disorders, respiratory diseases, metabolic diseases, and cardiovascular diseases, including gastric and CRC (13). For example, there are significant differences in the abundance of gut microbiota between CRC patients and healthy individuals. In CRC tissues, higher levels of *Escherichia coli*, *Bacteroides fragilis*, *Enterococcus faecalis*, *Streptococcus gallolyticus*, and *Peptostreptococcus* species have been detected, while *Ruminococcus*, *Faecalibacterium*, and *Bifidobacterium* are notably reduced (14–18). Furthermore, during the different stages of colorectal tumor development, such as multiple polypoid adenomas and intramucosal carcinoma, both the microbiome and metabolome exhibit significant changes. Notably, the relative abundance of *Fusobacterium nucleatum* significantly increases as intramucosal carcinoma progresses to more advanced stages (19).

The role of the gut microbiota in CRC is now well-recognized, with gut microbiota and their metabolites being critical factors influencing the intestinal immune system (20). *Bacteroides fragilis*, for instance, can rapidly induce the progression of adenomatous polyps to colitis and colon tumors in mice, accompanied by a marked downregulation of effector T cell responses and an upregulation of Treg responses (21). Furthermore, metabolites influenced by the gut microbiota, such as tryptophan, bile acids, and short-chain fatty acids (SCFAs), may also impact the development of CRC through the modulation of immune responses (22–24). The gut microbiota breaks down carbohydrates to produce SCFAs, primarily acetate, propionate, and butyrate. Butyrate can enhance the activity of cytotoxic $CD8^+$T cells through metabolic and epigenetic reprogramming, increasing the expression of antitumor molecules such as CD25, IFN-γ, and TNF-α (25). Additionally, the gut microbiota can stimulate CRC cells to produce various chemokines, thereby activating immune responses and promoting the accumulation of cytotoxic T lymphocytes, Th1 helper T cells, and Th17 cells producing interleukin-17 within tumor tissues (26). However, the causal relationship between gut microbiota and colorectal carcinogenesis remains inadequately defined, and the mechanisms by which immune cells mediate interactions between CRC and gut microbiota are complex. Therefore, a comprehensive investigation into the interplay among gut microbiota, immune cells, and CRC is urgently needed to

enhance our understanding of CRC pathogenesis. Clarifying these interactions is essential for identifying potential therapeutic targets, which could play a critical role in developing more effective strategies for CRC treatment.

Mendelian Randomization (MR) is a method used to investigate the causal relationships between risk factors and outcomes by employing genetic variations as instrumental variables (IVs) instead of directly measuring the risk factors themselves (27). This approach allows for the assessment of causal relationships between exposure factors and outcomes. Unlike traditional observational methods, Mendelian randomization (MR) analysis is less vulnerable to reverse causation and confounding factors. This is because genetic variations are randomly assigned at conception, thereby rendering them independent of environmental influences. In this study, we utilized two-sample univariate MR (UVMR) and multivariate MR (MVMR) based mediation analysis to determine the causal relationship between gut microbiota and CRC, and to explore the potential mediating role of immune cells in this process. When selecting single nucleotide polymorphisms (SNPs) to be used as IVs, three criteria must be met: 1) Each IV must be significantly associated with the exposure. 2) Each IV should influence the outcome only through the exposure, without reverse causation. 3) Each IV should not be affected by confounding factors, thereby minimizing bias due to linkage disequilibrium (LD) (28).

2 Materials and methods

2.1 Ethical considerations

For this study, we have provided a comprehensive Strengthening the Reporting of Observational Studies in Epidemiology using Mendelian Randomization (STROBE-MR) statement. The detailed content can be found in the STROBE-MR checklist (29).

2.2 Study design

Figure 1 illustrates the MR study: First, we conducted a forward UVMR analysis to investigate the relationship between gut microbiota as the exposure and CRC as the outcome. To validate the robustness of the results, we employed various methods, including Inverse-Variance Weighted (IVW), Robust Inverse-Variance Weighted, Penalized Inverse-Variance Weighted, and Penalized Robust Inverse-Variance Weighted. Additionally, we used MR-Egger Intercept, Penalized MR-Egger Intercept, Robust MR-Egger Intercept, and Penalized Robust MR-Egger Intercept to mitigate the influence of horizontal pleiotropy. Finally, MR-PRESSO was applied to detect and correct for outliers by

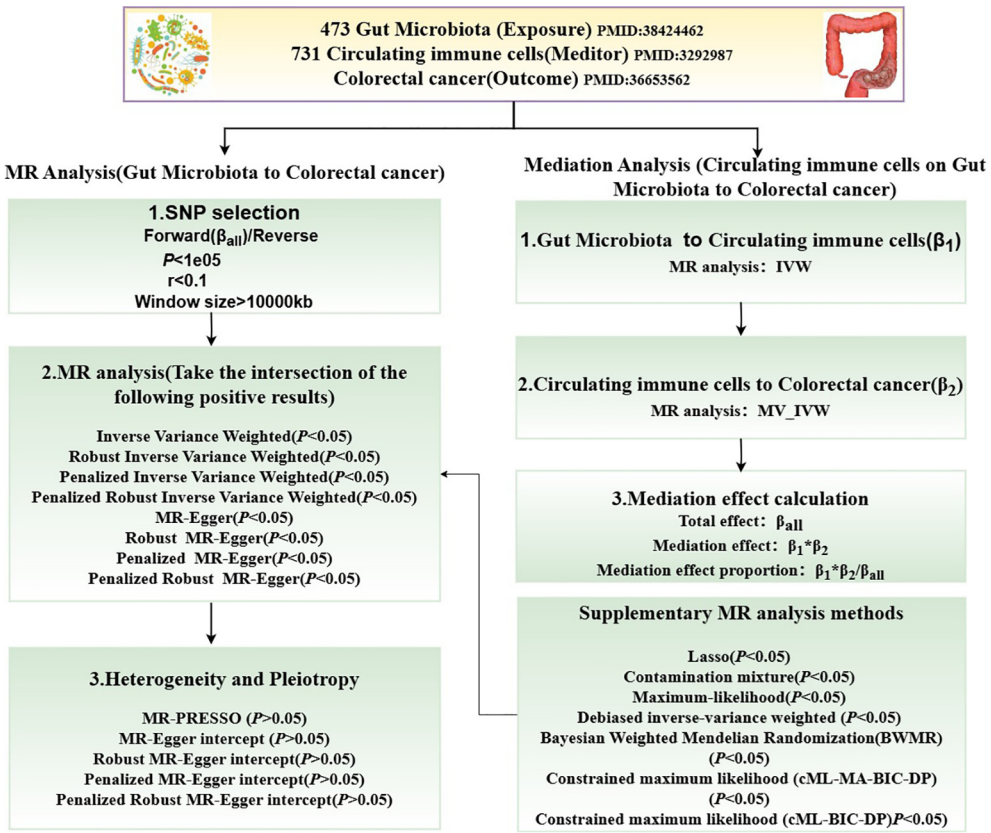

FIGURE 1
Flowchart of the MR study.

removing anomalous IVs, resulting in the most robust gut microbiota findings. Furthermore, we supplemented our analysis with Constrained Maximum Likelihood (cML-MA-BIC-DP), Constrained Maximum Likelihood (cML-BIC-DP), Bayesian Weighted Mendelian Randomization (BWMR), Maximum Likelihood, Lasso, Debiased Inverse-Variance Weighted, and Contamination Mixture methods to obtain strong and robust causal relationships.

The two-step method based on MVMR (30) was employed to investigate the genetically predicted overall effect of gut microbiota on CRC risk mediated by immune cells. In the first step of this two-step approach, a conventional UVMR analysis was conducted between gut microbiota and 731 types of immune cells, yielding BETA1 (P<0.05). In the second step, the identified positive immune cells and gut microbiota were then analyzed using MVMR in relation to CRC, resulting in BETA2 (P<0.05). The total effect obtained from the UVMR analysis of gut microbiota on CRC was designated as BETA. The mediation effect was calculated as BETA1*BETA2, the direct effect was determined by BETA-BETA1*BETA2, and the proportion of the mediation effect was represented as BETA1*BETA2/BETA.

2.3 Data sources

2.3.1 Data source for CRC

The GWAS data for CRC was obtained from the FinnGen database (31),accessible at https://storage.googleapis.com/finngen-public-data-r10/summary_stats/finngen_R10_C3_COLORECTAL_EXALLC.gz.g. The total sample size of this study comprised 321,040 individuals, including 6,847 CRC cases and 314,193 controls. Among the patients, 2,798 were female and 4,049 were male (Table 1). The 15-year absolute risk of mortality for CRC patients was 0.01 (Table 2).

2.3.2 Data source for gut microbiota

The GWAS data for gut microbiota was sourced from the study conducted by Youwen Qin et al. (32). This study included a large cohort of 5,959 genotyped individuals. Through multivariate analysis, linear logistic regression models, and Akaike information criterion multidimensional analysis, revealed the complex interactions between host genes, gut microbiota, and diet, as well as their impact on health.

TABLE 1 Key figures of colorectal cancer samples.

items	Key figures		
	All	Female	Male
Number of individuals	6847	2798	4049
Unadjusted prevalence (%)	1.66	1.22	2.23
Mean age at first event (years)	66.67	64.33	68.29

TABLE 2 Mortality of colorectal cancer samples.

Follow-up	Absolute risk	HR [95% CI]	p	N
1998–2019	0.1	4.64 (3.65, 5.90)	4.5E-36	2361
15 years	0.01	1.53 (1.24, 1.87)	5.00E-05	574

2.3.3 Data source for immune cells

The GWAS data for circulating immune cells used in this study was obtained from the Catalog GWAS database. The study sample consisted of 3,757 individuals from Sardinia, ranging in age from 18 to 102 years. Approximately 22 million genetic variants were analyzed, with a particular focus on their effects on 731 immune cell traits (33).

2.4 IVs selection

When investigating the relationship between gut microbiota as the exposure factor and CRC as the outcome, we imposed specific requirements on the IVs to ensure the stability of the study data and the accuracy of the results. Therefore, the IVs must meet the following criteria: (a)The IVs associated with gut microbiota must have a genome-wide significance threshold of $P<1\times10^{-5}$ (34). (b) To satisfy the conditions for MR analysis, we performed LD analysis based on the European 1000 Genomes Project, requiring IVs to have R^2 <0.001 and LD=10000kb. (c) To prevent the influence of alleles on the causal relationship between gut microbiota and CRC, we evaluated the strength of the genetic variants used as IVs with F-statistics. Variants with F-statistics ≤10 were considered weak IVs, potentially leading to biased analysis results. Conversely, F-statistics >10 indicated robust instrumental variables, thus IVs with F-statistics less than 10 were excluded (35). Additionally, in reverse MR analysis, the IVs for CRC had to meet the following criteria: $P<5\times10^{-8}$, R^2<0.001, LD=10000kb, and F-statistics greater than 10 (IVs for gut microbiota are detailed in Supplementary Material S2, and IVs for CRC are detailed in Supplementary Material S1).

When performing two-step mediation MR and MVMR analyses, we established the following criteria for immune cells as IVs: $P<1\times10^{-5}$, R^2<0.001, and LD of 10000kb between loci. Additionally, IVs with F-statistics less than 10 were excluded from the analysis (details of the IVs for immune cells are provided in Supplementary Material S3).

2.5 Statistical analysis

We obtained the necessary data from publicly available Catalog GWAS and FinnGen databases to conduct Bidirectional MR analysis, investigating the causal relationship between gut microbiota and CRC. Subsequently, we employed a two-step mediation MR analysis to explore the total genetic predictive role of immune cells in the impact of gut microbiota on CRC

risk. During the MR analysis, we primarily utilized R (version 4.2.3), complemented by the "Two Sample MR" R package (version 0.5.7) (36)、"Mendelian Randomization" R package (version 0.10.0), and "BWMR" R package (version 0.1.1) (37). The R^2 statistic was used to quantify the proportion of phenotype variance explained by SNPs, calculated as $R^2 = \frac{2*BETA^2*EAF*(1-EAF)}{2*BETA^2*EAF*(1-EAF)+SE^2*2*Sample\ \ size*EAF*(1-EAF)}$. To assess the strength of IVs, we computed the F-statistic using the formula $F = \frac{R^2*(Sample\ \ size-1-k)}{(1-R^2)*k}$, where R^2 represents the proportion of phenotype variance explained by SNPs and k is the number of SNPs included in the instrument (38). A threshold F-statistic greater than 10 is typically considered statistically significant, indicating an unbiased causal relationship (39).

In the UVMR analysis, we first employed the IVW method to validate the efficacy of all IVs and calculate the weighted overall effect based on the *P*-value (40). To ensure the robust conclusions, we utilized three methods to mitigate bias in causal analysis: 1) The Robust Inverse-Variance Weighted method to reduce sensitivity to outliers and strong pleiotropic IVs. 2) The Penalized Inverse-Variance Weighted method to adjust for outliers or inconsistent effect estimates, thus achieving more reliable causal estimates. 3) The Penalized Robust Inverse-Variance Weighted method to adjust for outliers and inconsistent effect estimates while minimizing the impact of pleiotropic IVs, thereby providing stricter and more robust causal estimates. Subsequently, we introduced the *P*-value of the MR-Egger intercept to detect the presence of directional pleiotropy (41). If $P>0.05$, it indicates no significant directional pleiotropy, which enhances the reliability of the causal effect estimate. Additionally, to verify the reliability of the causal effects in our conclusions, we employed four methods to exclude the interference of horizontal pleiotropy: 1) The MR-Egger Intercept to detect directional pleiotropy in IVs, assessing whether the mean pleiotropic effect differs from zero. 2) The Penalized MR-Egger Intercept, which incorporates a penalty term to reduce the influence of pleiotropic IVs. 3) The Robust MR-Egger Intercept, which applies robustness adjustments to the MR-Egger Intercept method to mitigate the impact of outliers and strong pleiotropic IVs. 4) The Penalized Robust MR-Egger Intercept, which combines robustness and pleiotropy penalties to improve the accuracy and robustness of causal estimates through dual mechanisms. Lastly, we used the MR-PRESSO test to identify and correct outliers by excluding anomalous IVs (42). The results are deemed more reliable when the effect size from IVW is consistent with that from sensitivity analyses and $P<0.05$. Additionally, we conducted supplementary MR analyses using various methods, including BWMR, Maximum-Likelihood, Debiased Inverse-Variance Weighted, Contamination Mixture, and MR-Egger. The Contamination Mixture MR analysis, although not removing outliers, assumes that the effective IVs represent the largest subset of all IVs, providing a more precise causal effect than IVW (43). The Maximum-Likelihood MR analysis method is applicable to both related and unrelated genetic variants, employing a random effects model to analyze existing heterogeneity when the fixed effects model in IVW is incorrect and the causal effects of different variables exhibit significant heterogeneity (44). In the presence of unavoidable weak IVs, we used the Debiased Inverse-Variance Weighted method for MR analysis, which is robust to many weak IVs without the need for screening (45). The MR-Egger method assesses directional pleiotropy, causal effect testing, and causal effect estimation, providing consistent causal estimates under weaker assumptions (46). BWMR considers the uncertainty caused by weak effects due to polygenicity and addresses violations of MR assumptions caused by polygenicity through Bayesian weighted detection of outliers (37).

In the two-step mediation MR analysis, the first step involves conducting a UVMR analysis between the most robust gut microbiota and immune cells to derive BETA1. Subsequently, in the second step, we perform a MVMR analysis between the positively identified mediators (immune cells) from the first step and the most robust gut microbiota to obtain BETA2. At this point, the UVMR analysis of gut microbiota and CRC provides the total effect BETA. The mediation effect was calculated as BETA1*BETA2, the direct effect as BETA–BETA1*BETA2, and the proportion of the mediation effect as BETA1*BETA2/BETA. In the second step of the two-step MVMR, we utilize the multivariable Inverse-Variance Weighted method to validate the efficacy of all IVs and generate the weighted overall effect by assessing the significance of the *P*-values (40).

3 Results

3.1 Causal effects of gut microbiota on CRC

Initial IVW analysis identified 34 gut microbiota taxa with causal effects on CRC. To validate the robustness of these results, employed the Robust Inverse-Variance Weighted, Penalized Inverse-Variance Weighted, and Penalized Robust Inverse-Variance Weighted methods (Figure 2; Supplementary Material S4). Furthermore, to address the potential interference of horizontal pleiotropy, we utilized the MR-Egger Intercept, Penalized MR-Egger Intercept, Robust MR-Egger Intercept, and Penalized Robust MR-Egger Intercept methods (Supplementary Material S4). The MR-PRESSO test was subsequently applied to detect and correct outliers by removing anomalous instrumental variables (IVs), thereby ensuring the reliability of the gut microbiota findings (Supplementary Material S5). In addition, we conducted supplementary analyses using Constrained Maximum Likelihood (cML-MA-BIC-DP and cML-BIC-DP), BWMR, Maximum-Likelihood, Lasso, Debiased Inverse-Variance Weighted, and Contamination Mixture methods, yielding robust gut microbiota associations (Figure 3; Supplementary Material S4, S6). Notably, *Bifidobacterium kashiwanohense*, *GCA-900066755 sp900066755*, *Geminocystis*, and Saccharofermentanaceae exhibited strong and robust causal effects on CRC (Figure 4). Specifically, *Bifidobacterium kashiwanohense* and Saccharofermentanaceae were negatively correlated with CRC (OR<1), while *GCA-900066755 sp900066755* and *Geminocystis* were positively correlated with CRC (OR>1).

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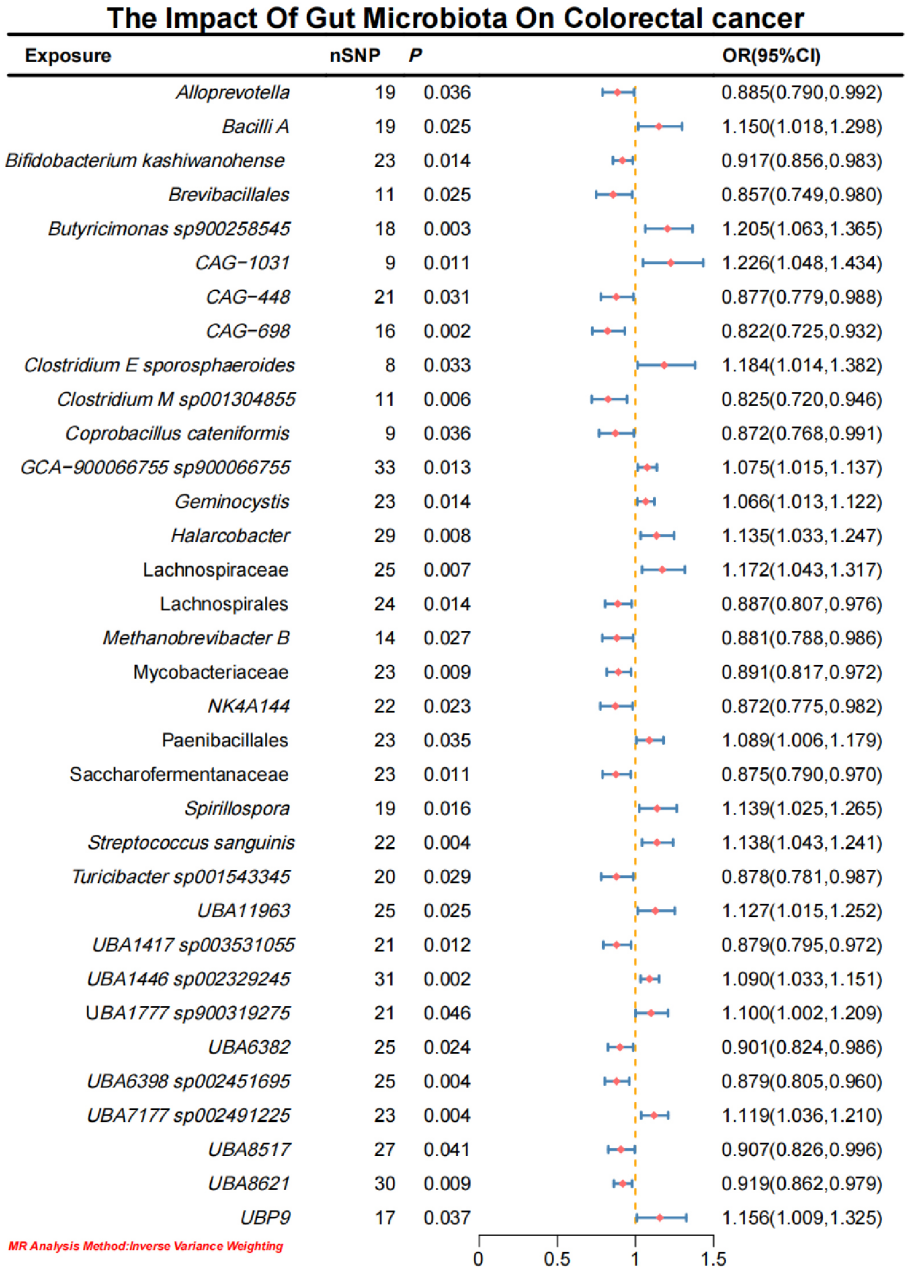

FIGURE 2
Forest plot of the causal effect of Gut microbiota on CRC.

3.2 Causal effects of CRC on gut microbiota

Initial IVW analysis indicated a reverse causal relationship between CRC and seven gut microbiota taxa: *Brevibacillales*, *CAG-245*, Caloranaerobacteraceae, *Caloranaerobacter*, *Comamonas*, *Dokdonella*, and Endozoicomonadaceae (Figure 5; Supplementary Material S7). The robustness of these findings was confirmed using Robust Inverse-Variance Weighted, Penalized Inverse-Variance Weighted, and Penalized Robust Inverse-Variance Weighted methods. Additionally, we employed MR-Egger Intercept, Penalized MR-Egger Intercept, Robust MR-Egger Intercept, and Penalized Robust MR-Egger Intercept to reduce horizontal pleiotropy. The MR-PRESSO test was used to detect and correct outliers by removing anomalous IVs, resulting in robust gut microbiota findings (Figure 6; Supplementary Material S7, S8). After employing novel MR methods, including Maximum-Likelihood, Lasso, Contamination Mixture, Debiased Inverse-Variance Weighted, Constrained Maximum Likelihood (cML-MA-BIC-DP and cML-BIC-DP), and BWMR, we identified *Brevibacillales* and *Comamonas* as having a robust reverse causal relationship with CRC. It can be inferred that the four gut microbiota taxa previously identified to positively contribute to CRC

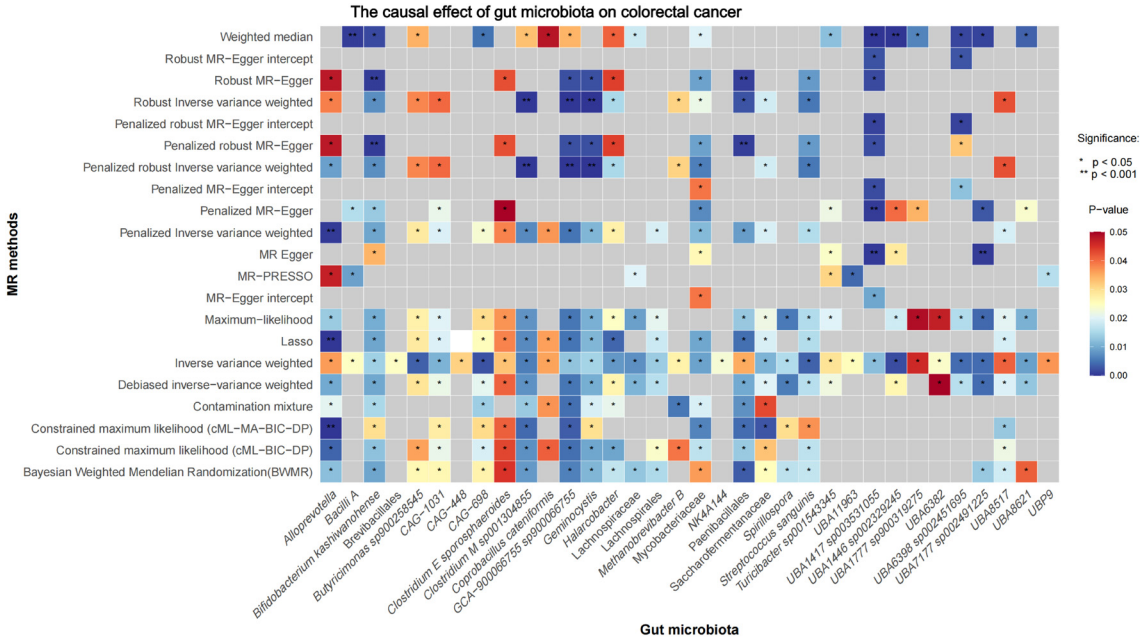

FIGURE 3
Heatmap of the Causal Effect of Gut microbiota on CRC (gray boxes represent P>0.05).

did not demonstrate inverse causal relationships (Supplementary Material S7, S9).

3.3 Causal effects of gut microbiota on immune cells

The aforementioned gut microbiota were subjected to UVMR analysis with 731 immune cells, revealing a causal relationship between the identified positive gut microbiota and six immune cell. Specifically, *Bifidobacterium kashiwanohense* demonstrated a positive causal effect with CD40 on monocytes and CD45 on $CD33^+$ HLA-DR + $CD14^-$ (OR > 1). *GCA-900066755 sp900066755* exhibited a positive causal effect with CD45 on $CD33^-$HLA-DR^+ (OR> 1). *Geminocystis* showed a negative causal effect with terminally differentiated $CD4^+$ T cells (OR<1). Saccharofermentanaceae displayed a positive causal effect with CD40 on monocytes, central memory $CD4^+$T cells, and

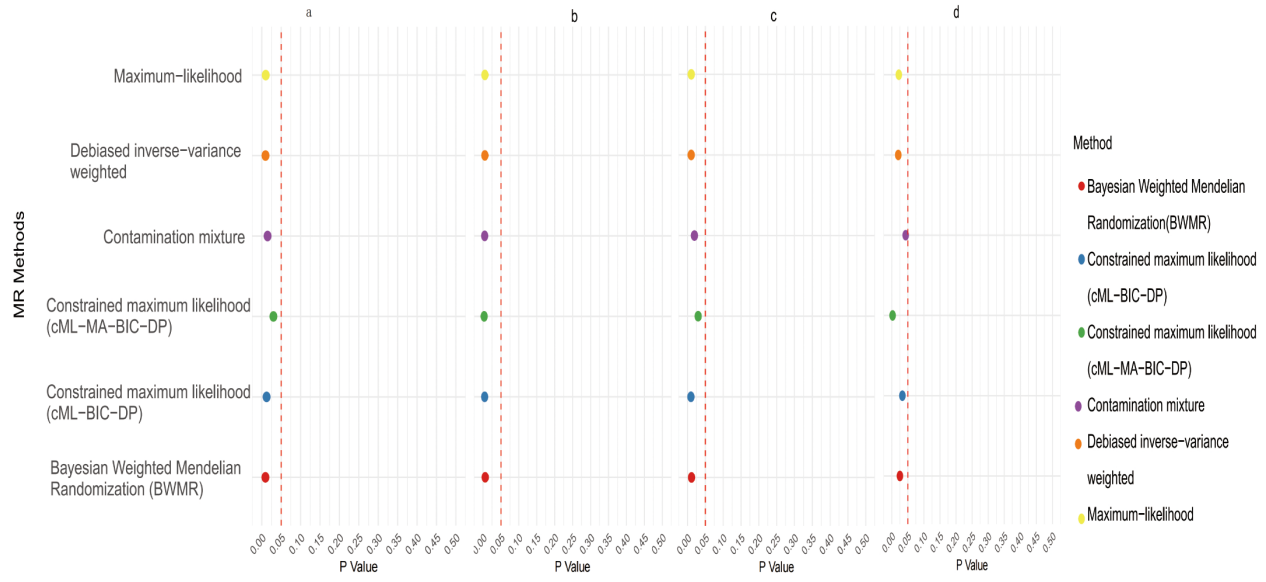

FIGURE 4
Scatter plot of the causal effect of four positive Gut microbiota on CRC (a. *Bifidobacterium kashiwanohense*; b. *GCA−900066755 sp900066755*; c. *Geminocystis*; d. Saccharofermentanaceae).

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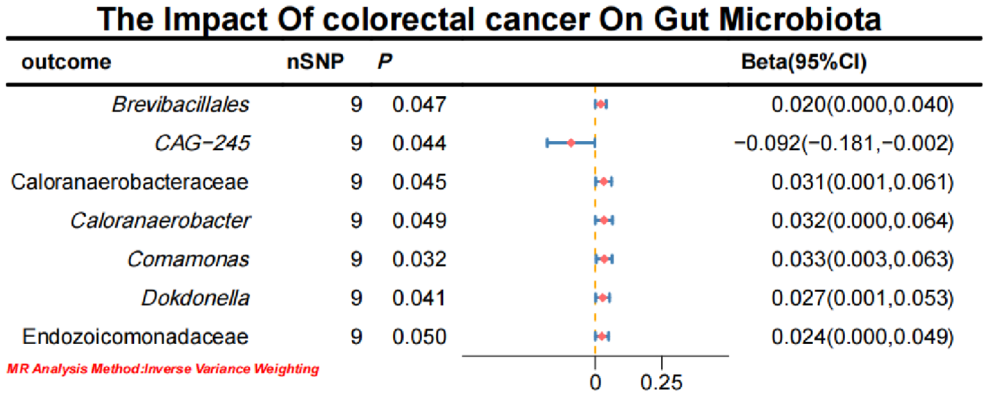

FIGURE 5
Forest plot of the causal effect of CRC on Gut microbiota.

CD28 on $CD28^+CD45RA^+CD8^+$T cells (OR>1) (Figure 7; Supplementary Material S10).

3.4 Causal effects of immune cells on CRC

In the MR analysis of immune cells on CRC, the IVW results indicated that CD40 on monocytes, CD45 on $CD33^+$HLA-DR $+CD14^-$, terminally differentiated $CD4^+$T cells, central memory $CD4^+$T cells, and CD28 on $CD28^+CD45RA^+CD8^+$T cells have a negative causal effect on CRC. Conversely, CD45 on $CD33^-$HLA-DR^+ shows a positive causal effect on the occurrence of CRC (Figure 8; Supplementary Material S11).

3.5 Mediation effects of immune cells on genetic predictors of gut microbiota and CRC

To elucidate the underlying mechanisms of CRC development, we performed mediation MR analyses to pinpoint the causal pathways between gut microbiota and CRC, mediated by immune cells. The mediation MR analysis yielded the following results: when *Bifidobacterium kashiwanohense* served as a protective factor against CRC, CD40 on monocytes (2.82%) and CD45 on $CD33^+$HLA-DR+$CD14^-$ (12.87%) mediated its genetic predictive effect on CRC risk. Conversely, when *GCA-900066755 sp900066755* acted as a risk factor for CRC, CD45 on $CD33^-$ $HLA\text{-}DR^+$ (3.94%) mediated its genetic predictive effect on CRC risk. Furthermore, in the scenario where *Geminocystis* was a risk factor for CRC, terminally differentiated $CD4^+$ T cells (11.55%) mediated its genetic predictive effect on CRC risk. Finally, when Saccharofermentanaceae acted as a protective factor against CRC, CD40 on monocytes (2.35%), central memory $CD4^+$T cells (5.76%), and CD28 on $CD28^+CD45RA^+CD8^+$T cells (5.00%) mediated its genetic predictive effect on CRC risk (Table 3; Supplementary Material S12).

4 Discussion

This study investigates the causal relationship between gut microbiota, circulating immune cells, and CRC using large-scale genetic data and MR analysis. Rigorous inclusion criteria and sensitivity analyses were applied, supplemented by methods such as Constrained Maximum Likelihood, BWMR, and others, to ensure robustness and independence from confounding factors. UVMR analysis indicated that *Bifidobacterium kashiwanohense* and Saccharofermentanaceae were negatively associated (OR<1), whereas *GCA-900066755 sp900066755* and *Geminocystis* showed positive associations (OR>1) with CRC risk. MVMR mediation analysis identified specific immune cell types potentially driving pathways: CD40 on monocytes, CD45 on $CD33^+$HLA-DR+$CD14^-$ for *Bifidobacterium kashiwanohense*, CD45 on $CD33^-HLA\text{-}DR^+$ for *GCA-900066755 sp900066755*, Terminally Differentiated $CD4^+$T cell for *Geminocystis*, CD40 on monocytes, Central Memory $CD4^+$T cell, CD28 on $CD28^+CD45RA^+CD8^+$T cell for Saccharofermentanaceae and CRC. Collectively, these findings underscore the association between gut microbiota and CRC and highlight the mediating role of immune cells in this process.

Bifidobacterium are beneficial intestinal microorganisms found extensively in the digestive tracts and luminal environments of humans and animals (47), and have the ability to immune regulation, maintenance of intestinal barrier integrity, and inhibition of pathogenic microorganism growth (48, 49).*Bifidobacterium kashiwanohense*, a species within the *Bifidobacterium* genus (50), was shown in our MR analysis to decrease in abundance as CRC progresses. This finding aligns with prior studies, which reported a significantly lower abundance of *Bifidobacterium* in the feces of CRC patients compared to healthy controls and a markedly reduced presence in tumor tissues relative to adjacent normal mucosa (51). Additionally, it has been found that the number of Tregs in the colon is increased in germ-free mice after *Bifidobacterium bifidum* PRI1 fixation, a phenomenon mediated by the upregulation of regulatory factors and DC mRNA expression of CD86 and CD40 costimulatory molecules (52). Further MR analysis revealed that the abundance of

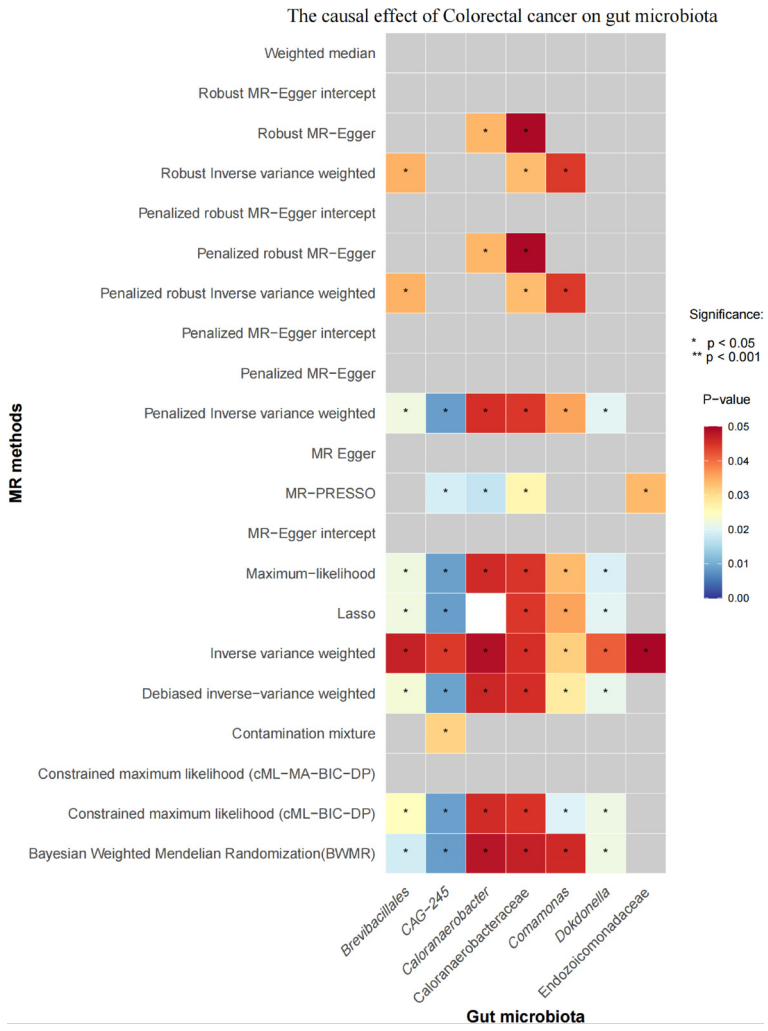

FIGURE 6
Heatmap of the Causal Effect of CRC on Gut microbiota (gray boxes represent *P*>0.05.

Bifidobacterium kashiwanohense is positively associated with CD40 on monocytes and CD45 on $CD33^{+}HLA\text{-}DR{+}CD14^{-}$ cells, both of which are inversely associated with CRC risk. CD40 is a key immunomodulatory molecule widely expressed on immune cells such as monocytes/macrophages, B-lymphocytes, and dendritic cells, and its activation is critical for initiating and regulating immune responses (53). Binding of CD40 to its ligand, CD40L, promotes maturation and activation of immune cells and enhances cytokine production, which modulates immune responses (54), and in turn induces cancer cells to undergo extensive apoptosis while preserving normal cells (55). Elevated circulating levels of sCD40, a natural antagonist of the mCD40-CD40L complex, may serve as a biomarker for the risk of liver metastasis in CRC (56). CD45, a pan-leukocyte antigen, is widely expressed on all hematopoietic cells and plays a crucial role in the development and activation of immune cells (57). Studies have shown that increased infiltration of $CD45^{+}$ immune cells within primary tumors is strongly associated with higher survival rates in CRC patients (58), which is consistent with our MR findings. Consequently, our MR analysis suggests that *Bifidobacterium kashiwanohense* may mitigate CRC risk by upregulating CD40 on monocytes and CD45 on $CD33^{+}HLA\text{-}DR{+}CD14^{-}$ expression. Previous research has highlighted the potential roles of *Bifidobacterium bifidum* PRI1 in CRC patients, our study further underscores the potential of *Bifidobacterium kashiwanohense* in CRC prevention and treatment, emphasizing the critical role of gut microbiota in cancer immunoregulation.

Geminocystis, a genus within Cyanobacteriota, was predicted by our MR analysis to increase in abundance with the progression of CRC. Although no direct association between *Geminocystis* and CRC has been reported in the literature, prior studies have shown that the cyanotoxin microcystin-leucine arginine (MC-LR) produced by cyanobacteria, may activate the Wnt/β-catenin pathway via the PI3K/Akt pathway, thereby promoting CRC cell proliferation (59). Additionally, members of the Cyanobacteriota phylum have been implicated not only in CRC pathogenesis but also exhibit significant abundance differences in colorectal adenomas, a precancerous condition (60). Further MR analysis revealed an inverse causal relationship between Terminally Differentiated $CD4^{+}$T cells and CRC, as well as between *Geminocystis* and Terminally Differentiated $CD4^{+}$T cells. $CD4^{+}$T

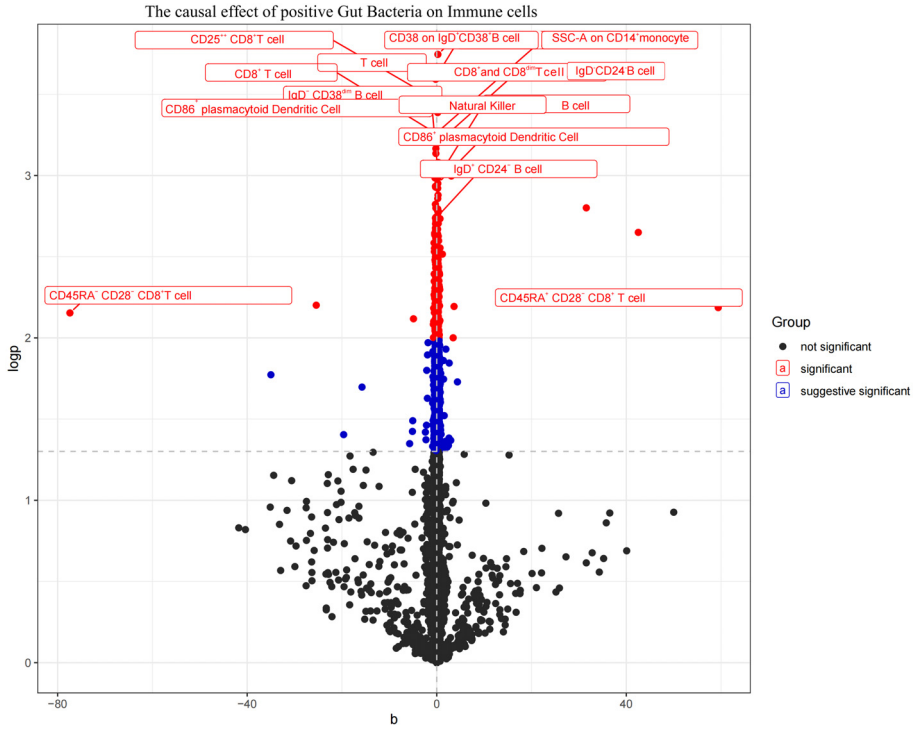

FIGURE 7
Volcano plot of the causal effect of positive Gut microbiota on Immune cells (black points represent *P*>0.05, red points represent *P*<0.05, blue points represent *P*<0.001).

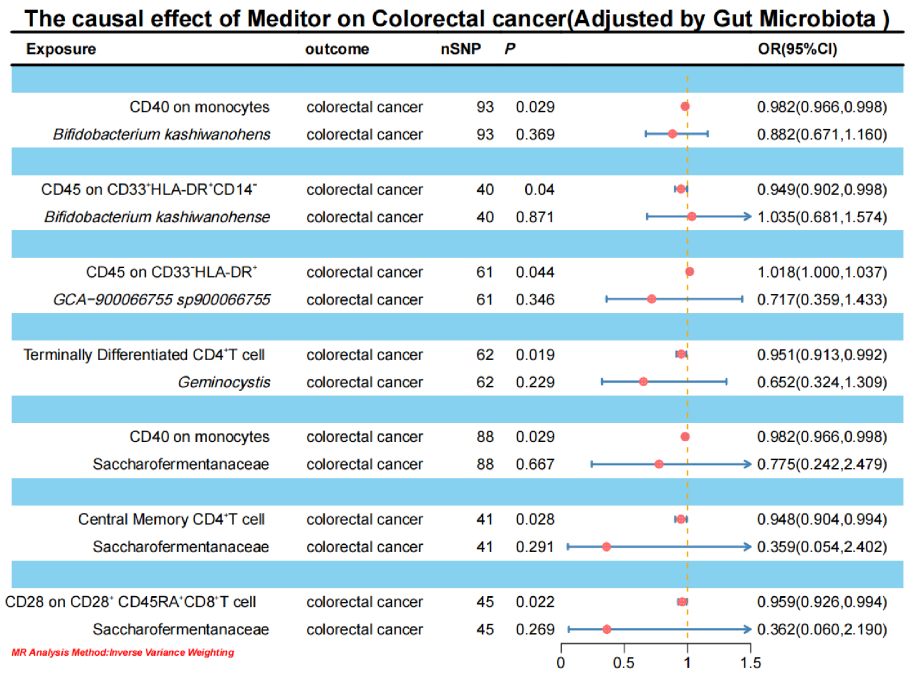

FIGURE 8
Forest plot of the causal effect of positive mediators on CRC.

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TABLE 3 Immune cells mediate genetically predicted mediating effects of positive gut bacteria and CRC Table.

Exposure	Meditor	Outcome	Log OR (SE) per 1 SD higher exposure, *P* value			Proportion of effect mediated
			Exposure-Outcome	Exposure-Meditor	Meditor-Outcome	
Bifidobacterium kashiwanohense	CD40 on monocytes	colorectal cancer	-0.087(0.035),0.014	0.133 (0.052),0.010	-0.018 (0.008),0.029	2.82%
Bifidobacterium kashiwanohense	CD45 on $CD33^{+}$ HLA-DR + $CD14^{-}$		-0.087(0.035),0.014	0.211 (0.078),0.007	-0.053 (0.026),0.040	12.87%
GCA-900066755 sp900066755	CD45 on $CD33^{-}$ $HLA\text{-}DR^{+}$		0.072(0.029),0.012	0.155 (0.077),0.045	0.018(0.009),0.044	3.94%
Geminocystis	Terminally Differentiated $CD4^{+}$ T cell		0.064(0.026),0.014	-0.148 (0.056),0.008	-0.050 (0.021),0.019	11.55%
Saccharofermentanaceae	CD40 on monocytes		-0.133(0.052),0.011	0.174 (0.073),0.017	-0.018 (0.008),0.029	2.35%
Saccharofermentanaceae	Central Memory $CD4^{+}$ T cell		-0.133(0.052),0.011	0.144 (0.068),0.035	-0.054 (0.024),0.028	5.76%
Saccharofermentanaceae	CD28 on $CD28^{+}$ $CD45RA^{+}$ $CD8^{+}$ T cell		-0.133(0.052),0.011	0.160 (0.073),0.029	-0.042 (0.018),0.022	5.00%

cells are critical components of the immune system, orchestrating both innate and adaptive immune responses against pathogens and tumors through various mechanisms (61). Research has demonstrated that $CD4^{+}$T cells exert antitumor effects by producing cytotoxic molecules, including granzymes and perforins (62). Concurrently, a marked reduction in the absolute counts of T cells and their subsets has been observed in CRC patients (63). Thus, integrated MR results suggest that *Geminocystis* may heighten CRC risk by reducing the expression of Terminally Differentiated $CD4^{+}$T cells. However, the precise mechanisms through which *Geminocystis* influences CRC risk via Terminally Differentiated $CD4^{+}$T cells necessitate further investigation.

The phylum Firmicutes consists of microorganisms predominantly found in the human gut, playing a critical role in maintaining intestinal health, facilitating gut functionality, and modulating the host immune system (64). Saccharofermentanaceae is a family of the phylum Firmicutes, and our MR analysis showed that Saccharofermentanaceae was negatively associated with the risk of CRC. Existing research also supports our findings, demonstrating significant reductions in the genera *Bifidobacterium*, *Lactobacillus*, *Clostridium*, and the phylum Firmicutes in tumor tissues of colorectal cancer patients compared to adjacent normal tissues (65). Similarly, a decreased abundance of gram-positive bacterial phyla was observed in the AOM/DSS-induced CRC mouse model (66). Gut microbiota influence host health by engaging in metabolic pathways, regulating gene expression, and producing bioactive compounds, such as SCFAs, amines, secondary bile acids, and vitamins (67), with SCFAs exerting beneficial effects on gut epithelial cells and the immune system (68, 69). SCFAs produced by the phylum Firmicutes can directly act on intestinal epithelial cells and immune cells to improve the immune microenvironment (70). Further MR analyses revealed that Saccharofermentanaceae exhibited a positive causal association with CD40 on monocytes, Central Memory $CD4^{+}$T cells, and CD28 expression on $CD28^{+}CD45RA^{+}CD8^{+}$T cells, while showing a negative causal association with CRC risk. It has been found that the phylum Firmicutes, *Actinobacillus*, *Aspergillus*, *Mycobacterium*, and *Verrucomicrobium* are associated with enhancing the clinical response to immune checkpoint blockade (71). For instance, a clinical trial demonstrated that fecal microbiota transplantation combined with anti-PD-1 therapy improved immunotherapy efficacy in certain anti-PD-1-resistant melanoma patients, possibly through increased gut microbiota, $CD8^{+}$T cell activation, and decreased IL-8 inflammation (72). $CD8^{+}$T cells are crucial immune surveillance cells in the body, which are responsible for recognizing and eliminating tumor cells. However, the process of cancer development is often accompanied by the depletion of $CD8^{+}$T cells, which leads to the inhibition of their killing function, allowing cancer cells to escape and promote disease progression (73). Enhancing $CD8^{+}$T cell functionality remains a vital strategy to improve CRC immunotherapy outcomes, and our findings support this approach. Therefore, synthesizing the MR results we hypothesized that Saccharofermentanaceae may reduce the risk of CRC by increasing the expression of CD40 on monocytes, Central Memory $CD4^{+}$T cells, and CD28 on $CD28^{+}CD45RA^{+}CD8^{+}$T cells. Prior research also indicates that butyrate, a metabolite of *Roseburia intestinalis* (within the Firmicutes family Lachnospiraceae), can impede CRC progression by enhancing $CD8^{+}$T cell function through TLR5 dependent NF-κB signalin (74), indirectly supporting our hypothesis. It is noteworthy that different families or genus within Firmicutes exhibit functional diversity, which may influence their role in CRC. Initial analysis using the IVW method showed an inverse association between *Clostridium M sp001304855* (family Lachnospiraceae) and CRC, whereas *Clostridium E sporosphaeroides* (family Acutalibacteraceae) demonstrated a positive association. The consistency of these associations across multiple validation methods reinforces the reliability of these findings. This study underscores the complexity and diversity of gut microbiota in CRC, and as sequencing technologies advance,

identifying more bacterial families or genera and understanding their functional distinctions will facilitate the development of disease prevention, early screening, and personalized therapeutic strategies.

GCA-900066755 sp900066755, a member of the Lachnospiraceae family, was found to be positively associated with the risk of CRC according to our MR analysis. In contrast, previous studies suggest a suppressive role of Lachnospiraceae in CRC onset and progression (75). At the family level, Lachnospiraceae and Fusobacteriaceae were more abundant in CRC patients with high immune scores compared to those with low scores. *In vitro* experiments validated an inverse relationship between Lachnospiraceae abundance and CRC cell line proliferation (76). Further MR analysis showed *GCA-900066755 sp900066755* had a positive causal effect on CD45 on $CD33^{-}$HLA-DR^{+}, while CD45 on $CD33^{-}$HLA-DR^{+} had a positive causal effect with the risk of CRC. Therefore, we hypothesize *GCA-900066755 sp900066755* elevates CRC risk by upregulating CD45 on $CD33^{-}$ HLA-DR^{+}. However, discrepancies with existing studies may stem from variable metabolite levels produced by *GCA-900066755 sp900066755* or Lachnospiraceae across individuals and time scales. Metabolites from specific gut microbiota may have varied effects on CRC stages, highlighting the need for further research into their nuanced roles.

This study investigated the causal relationship between gut microbiota and CRC using MR design, and assessed the mediating effects of immune cells in this association. Nevertheless, our study has several limitations. Firstly, the study predominantly involved participants of European ancestry. Genetic backgrounds, environmental exposures, and lifestyles vary among different ethnic groups, which may affect the interactions among gut microbiota, immune cells, and CRC, limiting the generalizability of our findings to other populations. Secondly, our analysis used generalized CRC data lacking specific subgroup details such as cancer staging and segment characteristics. Due to the varied pathophysiological characteristics of CRC across different stages and locations, differences in gut microbiota composition and immune cell levels may be substantial. Therefore, accurately evaluating these differences across different stages and locations of CRC poses a challenge. Furthermore, fundamentally, the intricate relationship between specific gut microbiota exposures, immune cell mediators, and CRC is complex. However, our study, based solely on large-scale data analysis, lacks direct biological validation. Future studies could focus on three key directions. First, additional basic and clinical experiments are needed to validate the efficacy of these gut microbiota in CRC prevention and to clarify their potential immune regulatory mechanisms. Second, to further elucidate the relationship between these gut bacteria and CRC, large-scale epidemiological studies could examine associations between exposure to these microbiota and CRC incidence, focusing on specific microbial metabolites or immune markers. Such biomarkers, if stable and sensitive in blood or fecal samples, may offer a promising approach for non-invasive CRC screening, providing earlier detection and intervention opportunities for high-risk individuals. Finally, considering the immune-activating or immunomodulatory properties of certain gut microbiota, we hypothesize that these bacteria may exert anti-tumor effects through distinct immune pathways, warranting investigation into their potential synergistic effects with immune checkpoint inhibitors. This synergy could enhance immunotherapy outcomes for CRC patients and pave the way for novel combination therapies. While this study highlights novel research avenues for CRC prevention and treatment, the clinical efficacy of these findings requires further investigation, particularly regarding safety, effectiveness, and delivery methods, to ensure the feasibility and applicability of microbial interventions in CRC prevention and therapy.

5 Conclusion

In conclusion, this study is the first to evaluate the causal relationship between gut microbiota, immune cells, and CRC, emphasizing the mediating role of immune cells in this process. The identified gut microbiota and immune phenotypes have potential as biomarkers, providing new insights for developing therapeutic strategies against CRC.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Ethics statement

All individual participants in the study provided informed consent.

Author contributions

YZ: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. GC: Conceptualization, Data curation, Formal analysis, Writing – review & editing. MC: Conceptualization, Data curation, Formal analysis, Writing – review & editing. JC: Supervision, Writing – review & editing. QT: Supervision, Writing – review & editing. ZX: Conceptualization, Formal analysis, Funding acquisition, Project administration, 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.2025.1460936/full#supplementary-material

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