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Gut microbiota and immunity in health and disease: dysbiosis and eubiosis's effects on the human body

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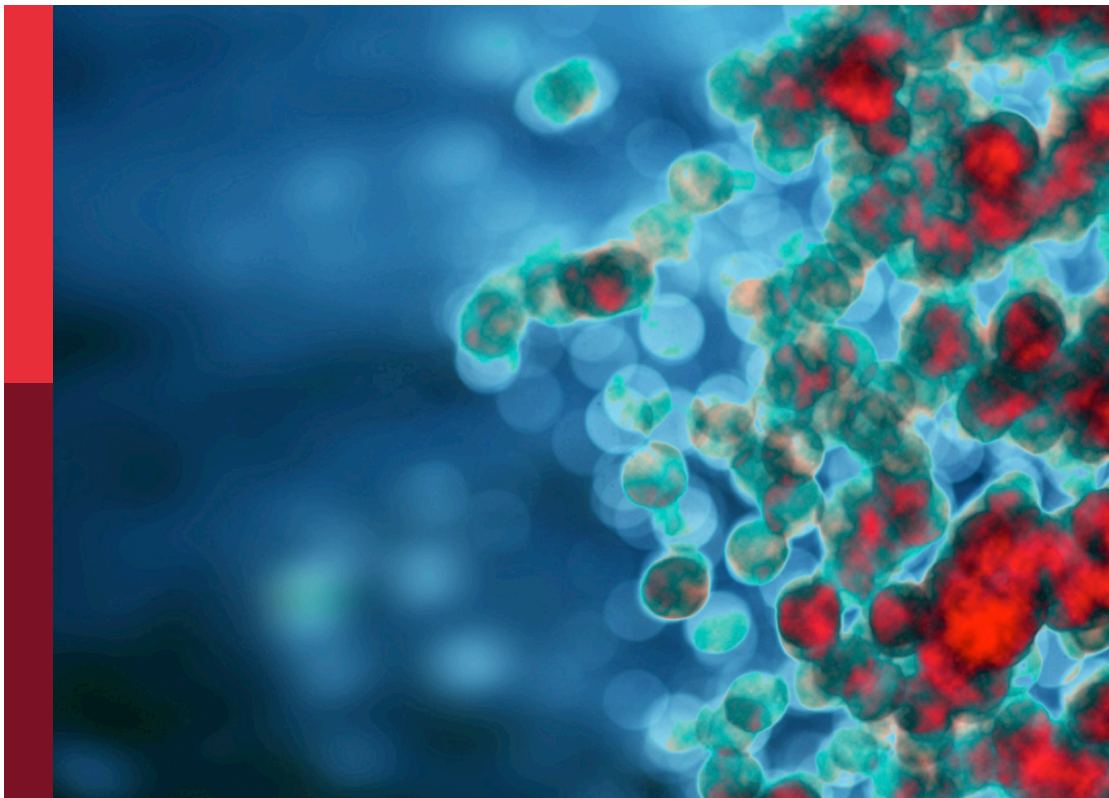

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Gut microbiota and immunity in health and disease: dysbiosis and eubiosis's effects on the human body

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Editorial: Gut microbiota and immunity in health and disease: dysbiosis and eubiosis's effects on the human body

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Editorial on the Research Topic
Gut microbiota and immunity in health and disease: dysbiosis and eubiosis's effects on the human body

The relationship between the gut microbiota and its human host is a complex and dynamic communication. Various host factors, such as genetics, immune function, age, gender, lifestyle (including pregnancy, delivery mode, nutrition, social behavior, and stress), body mass index (BMI), disease duration, and medical treatments, directly shape the composition of the gut microbiome (1–6).

When the gut microbiota is in balance—a state known as eubiotics—it supports the host's health by producing beneficial microbial metabolites. On the other hand, an imbalance (dysbiosis), characterized by a dominance of harmful microbes and a lack of beneficial ones, can disrupt homeostasis and lead to various health problems (1, 7–9).

In recent years, research has intensified on the intricate connections between gut microbiota, eubiotics, and their impacts on human health and disease. To advance this growing field, this Research Topic of *Frontiers in Immunology* launched a dedicated Research Topic. We are proud to present a Research Topic of 11 impactful publications contributed by 69 researchers from around the globe.

In an experimental study conducted by Micek et al. in Poland, the researchers explored whether there is an association between the consumption of polyphenols, lignans, and herbal sterols and the presence of immune-stimulating microbiota, such as *Escherichia coli* and *Enterococcus* spp. The study included 95 non-obese participants aged 25–45 years, comprising 22 women and 73 men. The findings demonstrated a significant correlation between higher intake of these phytochemical compounds and a reduced risk of COVID-19 infection. The enhancement of gut microbiota likely mediated this effect. However, the authors recommended further research to confirm and expand upon these observations.

Another investigation, led by Chen et al., used a bidirectional two-sample Mendelian randomization approach to examine the causal relationship between nicotine dependence and gut microbiota composition. This study analyzed genome-wide association study (GWAS) data from 38,602 former smokers of African-American and European descent with varying levels of nicotine dependence. The findings suggested that the gut microbiome plays a role in nicotine metabolism and may influence disease progression associated with nicotine dependence.

These studies highlight the pivotal role of gut microbiota in modulating health outcomes and underscore the need for continued exploration in this dynamic research area.

In the review by Luo et al., the authors aimed to investigate the correlation between intestinal microbiota, vitamin A metabolism, and the retinoic acid (RA) signaling pathway in connection with bladder cancer. This review suggests intestinal microbiota may influence bladder tumorigenesis through the RA signaling pathway. Overall, the interaction between gut microbiota and RA exhibits synergistic anti-tumor effects.

Su et al. investigated the causal relationship between gut microbiota and six lung diseases: asthma, chronic bronchitis, chronic obstructive pulmonary disease (COPD), interstitial lung disease (ILD), lower respiratory tract infection (LRTI), and pulmonary arterial hypertension (PAH). The results revealed a correlation between the causality of gut microbiota and these lung diseases. Specifically, individual bacterial families may either increase or decrease the risk of developing lung diseases.

Li et al. conducted a Mendelian randomization study to investigate the causal relationship between gut microbiota composition, plasma metabolome, peripheral immune and blood cells, inflammatory cytokines, and obesity. Given that obesity is a metabolic and chronic inflammatory disease influenced by environmental and genetic factors, the researchers aimed to identify potential causal links between these factors. Among different correlations, the authors reported a pathway analysis that revealed 12 obesity-related metabolic pathways, particularly D-arginine, D-ornithine, linoleic acid, and glycerophospholipid metabolism, which were closely related to obesity.

Petakh et al. set out to explore how posttraumatic stress disorder (PTSD) affects gut microbiota and inflammatory biomarkers. By analyzing 15 studies, they uncovered significant shifts in the gut microbiota's composition and diversity in people with PTSD. Interestingly, certain bacterial species seemed to play a role in these changes. However, when it came to inflammatory biomarkers, they didn't find any notable differences between those with PTSD and those without it.

Masad et al. took a closer look at the effects of Manuka honey (MH) on colorectal cancer (CRC). Their research showed that MH, when taken orally, could trigger the interferon (IFN) signaling pathway through Toll-like Receptors (TLRs). This was observed in both BALB/c and C57BL/6 mouse models of CRC. Beyond that, MH seemed to reshape the tumor environment by boosting inflammatory cytokines and chemokines that regulate the immune response. The honey also influences gut microbiota, reducing harmful bacteria and enhancing its anti-tumor effects.

Warren et al. examined the microbiota-gut-brain-immune axis and its role in neuroinflammatory diseases. They argued that advancing global gut microbiome research and personalized healthcare means providing low- and middle-income countries (LMICs) with training, fostering collaboration, ensuring ethical engagement, and using standardized, multi-omics approaches.

In Warren et al.'s second review, chronic stress, mental health issues, and immune dysfunction were explored as links to the microbiota-gut-brain axis. They reviewed evidence-based prevention strategies and potential therapeutic targets.

Hong et al. tackled the link between juvenile idiopathic arthritis and uveitis with gut microbiota using Mendelian randomization. Their findings suggested a direct relationship between changes in gut bacteria and the development of these conditions, offering new insight into their underlying causes.

Finally, Nenciarini et al. studied how *Saccharomyces cerevisiae* and *Lactobacillus* spp. interact to influence the immune system. Using strains from kefir, probiotics, and stool samples from a Crohn's disease patient, they discovered that co-cultures of these microbes could activate immune cells and promote a tolerant immune response. These findings point out the potential of using microbial interactions to fine-tune immunity.

All in all: "Tell me what you eat, and I will tell you what you are."

(https://courier.unesco.org/en/articles/tell-me-what-you-eat-and-ill-tell-you-who-you-are).

Author contributions

VD: Supervision, Validation, Writing – review & editing. SM: Supervision, Validation, Writing – review & editing. PB: Conceptualization, Supervision, Validation, Writing – original draft, Writing – review & editing.

Conflict of interest

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

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Association of dietary intake of polyphenols, lignans, and phytosterols with immune-stimulating microbiota and COVID-19 risk in a group of Polish men and women

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Objectives: Devastating consequences of COVID-19 disease enhanced the role of promoting prevention-focused practices. Among targeted efforts, diet is regarded as one of the potential factors which can affect immune function and optimal nutrition is postulated as the method of augmentation of people's viral resistance. As epidemiological evidence is scarce, the present study aimed to explore the association between dietary intake of total polyphenols, lignans and plant sterols and the abundance of immunomodulatory gut microbiota such as Enterococcus spp. and *Escherichia coli* and the risk of developing COVID-19 disease.

Methods: Demographic data, dietary habits, physical activity as well as the composition of body and gut microbiota were analyzed in a sample of 95 young healthy individuals. Dietary polyphenol, lignan and plant sterol intakes have been retrieved based on the amount of food consumed by the participants, the phytochemical content was assessed in laboratory analysis and using available databases.

Results: For all investigated polyphenols and phytosterols, except campesterol, every unit increase in the tertile of intake category was associated with a decrease in the odds of contracting COVID-19. The risk reduction ranged from several dozen percent to 70 %, depending on the individual plant-based chemical, and after controlling for basic covariates it was statistically significant for secoisolariciresinol (OR = 0.28, 95% CI: 0.11–0.61), total phytosterols (OR = 0.47, 95% CI: 0.22–0.95) and for stigmasterols (OR = 0.34, 95% CI: 0.14–0.72). We found an inverse association between increased β-sitosterol intake and phytosterols in total and the occurrence of *Escherichia coli* in stool samples outside reference values, with 72% (OR = 0.28, 95% CI: 0.08–0.86) and 66% (OR = 0.34, 95% CI: 0.10–1.08) reduced odds of abnormal level of bacteria for the highest compared with the lowest tertile of phytochemical consumption. Additionally, there was a

trend of more frequent presence of Enterococcus spp. at relevant level in people with a higher intake of lariciresinol.

Conclusion: The beneficial effects of polyphenols and phytosterols should be emphasized and these plant-based compounds should be regarded in the context of their utility as antiviral agents preventing influenza-type infections.

KEYWORDS

polyphenols, lignans, plant-sterols, phytochemicals, SARS-CoV-2, gut microbiota, nutrition

1. Introduction

The coronavirus 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and contributing to high morbidity and mortality in the last 3 years all over the world was a global challenge. The lack of satisfactory treatment against COVID-19, including therapeutic regimens or vaccines, and the urgent need of fighting the dangerous pathogen forced people to reach for alternatives. Until now, there is a concern about remedies that can stop the spread of microorganisms. Modifiable risk factors such as a proper diet abundant in vitamins and minerals, as well as in other constituents strengthening the natural immune system may be of primary importance in preventing influenza-type illnesses and minimizing their symptoms. A good nutritional status of individuals is mandatory to defeat the viruses and even might be treated as a measure of resilience toward pathogens such as SARS-CoV-2 (1). Various dietary components may shape the immune responses in different ways, among others, by determining the gut microbial composition. Specifically, the antioxidant constituents and anti-inflammatory agents of diet such as polyphenols and phytosterols have been shown to possess antiviral and immune-boosting properties. Notably, the evidence on health benefits of phytochemicals toward diseases underlined by oxidative stress and subclinical inflammation, including certain types of cancer (2, 3) and cardiovascular diseases (4, 5), has increased over the last few years. The research has demonstrated that dietary polyphenols can affect dendritic cells, increase the proliferation of B cells and T cells and might alter the phenotype of macrophages thus having an immunomodulatory effect (6). Phytosterols comprise many active compounds which determine their physiological functions, notably they have therapeutic potential against oxidative stress, gut dysbiosis, and inflammation (7). Plant-based diet rich in phytochemicals can help in lipid metabolism regulation counteracting virus entry into the cell and virus propagation (8). Therefore, there is growing evidence for recommending plant-based diets as an alternative effective and safe strategy which can prevent infections, although the research in a group of middle-aged, non-obese adults without comorbidities is limited. Moreover, SARS-CoV-2 as well as flu infections are easily disseminated in this group of subjects and also might pose a threat to them. While the elderly and those with underlying health conditions are at higher risk for severe complications from influenza-type and pneumonia-type diseases, young people can also experience dangerous health consequences as a result of these illnesses. The current burden of the disease highlighted the need for targeted efforts to decrease susceptibility to infectious illnesses, which in the European region during the 2022–2023 flu season were widespread and very severe. Although previous research explored how certain nutritional factors may affect COVID-19 infection *via* modulation of immune system (9, 10), to the best of our knowledge there is no study that investigated the potential of individual lignans and phytosterols in interacting with the immune system from the gastrointestinal tract, and in affecting viral infections, including SARS-CoV-2. Therefore, on the example of COVID-19, we aimed to perform the study examining the association of dietary intake of polyphenols and plant sterols with the abundance of immunomodulatory gut microbiota such as Enterococcus spp. and *Escherichia coli* and with the risk of contraction of the disease among physically active, non-obese early adults and early middle aged subjects without comorbidities. A better understanding of the protective dietary factors may help disseminate the strategies to counteract a variety of forthcoming viral infections.

2. Materials and methods

2.1. Study design, participants, and data collection

The present study was conducted in Poland and was designed to examine nutritional habits, physical activity and gastrointestinal microbiota of healthy young adults. The recruitment process was established through posting advertisements on social media and was further amplified by promotion requests, allowing to transmit the questionnaire to someone else. Details of the study have been described previously (11). Briefly, enrollment and data collection commenced in 2020. For each sex the separate arm of the study with the same research design was organized. The dates of examinations were between July 2020 and December 2020 for men and between October and November 2020 for women. Respondents were instructed not to change their daily routine, including eating habits and physical exercise patterns. During 1-week follow-up, participants were tracking their physical activity, total energy expenditure (TEE) and sleep duration using a Polar M430 watch and were keeping dietary records. After this time, stool samples were collected for gut microbiome testing, and core elements of anthropometry and body composition were recorded as well as participants were asked to complete a socio-demographic questionnaire. The diagnostics of the gastrointestinal microbiota was performed in the laboratory using KyberKompactPro test. The inclusion criteria covered: age between 25 and 45 years, body mass index (BMI) in the healthy weight range (18.5–24.9 kg/m^2) or

overweight range (25–29.9 kg/m^2) and not having chronic diseases. Body weight and body composition were measured using Tanita's Bioelectrical Impedance Analysis technology.

To check the hypothesis that a plant-based diet could be protective against the development of infections, we re-contacted all study participants in June 2021 and interviewed them regarding the prevalence and course of COVID-19 since the beginning of the pandemic. The questions included the information about duration of the illness, hospitalization and symptoms, and vaccination against the disease. Out of 104 individuals invited, nine were excluded because they did not have irrefutably confirmed diagnosis whether they had contracted COVID-19 ($n = 4$) or were vaccinated in too short interval of time since the examination ($n = 5$).

Finally, 95 persons were included in analyses among whom 24 had confirmed diagnosis of COVID-19 disease based on: positive PCR test results ($n = 8$), positive antibody test results ($n = 7$), typical COVID-19 symptoms, including loss of smell and taste ($n = 9$). The study was conducted in accordance with the Declaration of Helsinki for medical research and obtained positive approval from the Bioethics Committee of Jagiellonian University (No. 1072.6120.5.2020 and 1072.6120.202.2019). Following a careful explanation of research conditions and procedures, an informed consent was signed by all subjects before they participated in the study.

2.2. Dietary assessment and other measurements

Based on the data collected in 7-day diaries, the nutritional value of foods was determined using the Dieta 6.0 program developed by the National Food and Nutrition Institute in Poland and including information on total fat and individual fatty acids, protein and individual amino acids, carbohydrates, cholesterol, fiber, vitamins and minerals. The method of determination of polyphenols and phytosterols content in foods was previously described in detail (12–14). Dietary polyphenol and plant sterol intakes were calculated according to the amount of various kinds of foods and dishes consumed by the participants combined with their phytochemical content. Total polyphenol content was assessed mostly in laboratory analysis based on 367 foods and dishes consumed typically in Poland and taking into consideration the degree of processing with the division to uncooked/raw products and products submitted to culinary treatments (13). Additionally, the available databases were searched to retrieve the mean content of lignans (3 data sources, primary Dutch lignan database) and plant sterols (13 data sources, e.g., British database of Food Composition, the USDA database) in all foods, as well as total polyphenols (Phenol-Explorer database) for a very small number of foods not subjected to laboratory analysis (12–14). Product-specific macro-, micronutrient, polyphenol, lignan and plant sterol intake was obtained as a result of the multiplication of their content in food by the daily consumption of each food. Finally, these values were summed across all foods which the individual subject consumed. To reduce extraneous variation and eliminate noncausal association with disease due to confounding generated by the correlation of total energy intake with both nutrients intake and the disease risk, daily consumption of each phytochemical was additionally adjusted for total energy intake using the residual method (15). The categorization of phytochemical intakes was based on tertile distribution. Regarding covariates used in the analysis, total energy intake [kcal], BMI [kg/m^2], age [years], and physical activity [hours per day] (logarithmically transformed) were incorporated into the models as continuous variables. Based on the body fat (BF) percentage and cutoff points by age and sex suggested by Gallagher et al. (16), the subjects were divided into the following groups: underfat, normal fat and overfat. Alcohol consumption was categorized as (i) none or moderate when consuming less than 5 g ethanol per day for women and less than 10 g/d for men, and (ii) regular otherwise. Sex, diet and smoking status were dichotomized as male/female, traditional/vegetarian and current/other, respectively.

2.3. Statistical analysis

Categorical variables were depicted by absolute numbers and percentages whereas continuous features were described using means and standard deviations. Background characteristics of individuals were made for different tertile categories of total lignan and total phytosterol intake, and subsequently were compared across premade groups. To reduce the right skewness of daily phytochemical intakes and physical activity, logarithmic transformation (using base 2) was adopted. Significant improvements in the shape of their distribution to forms closely resembling the Gaussian curve were noted for all variables. Therefore, Log 2 transformed phytochemical intakes were compared between participants who have contracted and who have not contracted COVID-19 disease applying Student *T*-test. Differences between tertile categories of consumptions in univariable analyses were checked with Chi-square or Fisher exact test in the case of categorical variables, and with ANOVA in the case of continuous variables (after checking normality assumption and homogeneity of variance). The odds ratios (ORs) and 95% confidence intervals (CIs) were retrieved from multivariable logistic regression models after controlling for (i) age, total energy intake (model 1); (ii) additionally for sex, diet, body fat and smoking status (model 2); and finally for (iii) covariates in model 2 and BMI, physical activity and alcohol consumption (model 3). Different levels of adjustment allowed for verification whether associations were independent of the aforementioned variables, ensuring the robustness and stability of the findings. The logistic regression analysis was modeled by introducing exposures as: (i) three-level categorical variables with lowest tertiles as referent categories, (ii) as continuous variables (logarithmically transformed) to show the effects associated with a double increment of intake (per 1 unit increase in log 2), and additionally as (iii) score variables which were constructed by coding tertile groups with 1, 2 and 3 and treated as numerical. *p*-values from two-sided tests were reported under a significance level of 0.05. R software (Development Core Team, Vienna, Austria, version 4.0.4) was used for all the statistical analysis. Additionally, the *post hoc* power analysis based on the multiple logistic regression was performed applying G*Power tool (version 3.1). We verified the hypothesis that increasing total polyphenols intake for 1 unit in log2 scale significantly changes the chance of having contracted COVID-19. We set: 95 subjects, OR = 0.20, probability of COVID-19 incidence when adequate amounts of phytochemicals were consumed: 0.16, variability in the main exposure that is accounted for by other covariates: 0.25. By default, two-tailed test and probability of type I error at a level 0.05

have been maintained. We obtained a highly satisfactory power equal to 0.995.

3. Results

3.1. Baseline characteristics of participants

The study was conducted on 95 persons, 73 men (76.8%) and 22 women (23.2%), aged 25–45 years (mean = 34.66, SD = 5.76), every fourth of whom ($n = 24$) have contracted COVID-19. Participants from various tertiles of lignans and phytosterols did not differ in age, sex, marital status, BMI categories, body fat, smoking status, physical activity, total energy intake, total energy expenditure and sleep duration. However, there were significantly more vegetarians and regular alcohol drinkers in the highest category of phytosterols consumers compared with others. Detailed characteristics of examined adults are presented in Table 1.

3.2. Relationship of lignan and phytosterol intake with COVID-19 contraction

Intake of total polyphenols, and total and major groups of lignans and phytosterols by categories of people who have contracted and who have not contracted COVID-19 was shown in Figure 1; Supplementary Table S1. Habitual consumption of total polyphenols, secoisolariciresinol, total phytosterols, stigmasterol and β-sitosterol was significantly lower among those who fell ill with COVID-19.

To better visualize the relationship between a phytochemical-rich diet and the prevalence of the disease, percentages of cases across tertiles of exposures, as well as odds ratios for 1 category increase in tertile of

TABLE 1 Baseline characteristics of the study participants ($N = 95$).

	Total lignan intake			Total phytosterol intake		
Variable	T1 ($N = 32$)	T2 ($N = 31$)	T3 ($N = 32$)	T1 ($N = 32$)	T2 ($N = 31$)	T3 ($N = 32$)
Age [years], mean (sd)	34.53 (6.15)	33.68 (5.68)	35.75 (5.42)	35.81 (6.49)	34.61 (5.44)	33.56 (5.22)
Sex, *n* (%)						
Male	24 (75.00)	26 (83.87)	23 (71.88)	24 (75.00)	23 (74.19)	26 (81.25)
Female	8 (25.00)	5 (16.13)	9 (28.13)	8 (25.00)	8 (25.81)	6 (18.75)
Marital status, *n* (%)						
Single or divorced	18 (56.25)	16 (51.61)	14 (43.75)	13 (40.63)	16 (51.61)	19 (59.38)
Married or cohabiting	14 (43.75)	15 (48.39)	18 (56.25)	19 (59.38)	15 (48.39)	13 (40.63)
BMI category, *n* (%)						
Normal	20 (62.50)	22 (70.97)	24 (75.00)	22 (68.75)	20 (64.52)	24 (75.00)
Overweight	12 (37.50)	9 (29.03)	8 (25.00)	10 (31.25)	11 (35.48)	8 (25.00)
Diet, *n* (%)						
Traditional	22 (68.75)	16 (51.61)	16 (50.00)	23 (71.88)	23 (74.19)	8 (25.00)***
Vegetarian	10 (31.25)	15 (48.39)	16 (50.00)	9 (28.13)	8 (25.81)	24 (75.00)
Body fat, *n* (%)						
Under fat	2 (6.25)	4 (12.90)	4 (12.50)	4 (12.50)	3 (9.68)	3 (9.38)
Normal	21 (65.63)	25 (80.65)	23 (71.88)	20 (62.50)	23 (74.19)	26 (81.25)
Overfat	9 (28.13)	2 (6.45)	5 (15.63)	8 (25.00)	5 (16.13)	3 (9.38)
Smoking, *n* (%)						
No	28 (87.50)	27 (87.10)	28 (87.50)	28 (87.50)	28 (90.32)	27 (84.38)
Yes	4 (12.50)	4 (12.90)	4 (12.50)	4 (12.50)	3 (9.68)	5 (15.63)
Alcohol, *n* (%)						
None or moderate	21 (65.63)	21 (67.74)	18 (56.25)	23 (71.88)	23 (74.19)	14 (43.75)*
Regular	11 (34.38)	10 (32.26)	14 (43.75)	9 (28.13)	8 (25.81)	18 (56.25)
Energy intake [kcal], mean(sd)	2,261 (575)	2,221 (503)	2,174 (394)	2,133 (445)	2,364 (611)	2,164 (379)
BMI [kg/m^2], mean (sd)	23.65 (2.69)	23.43 (2.60)	22.93 (2.69)	23.65 (2.46)	23.50 (2.89)	22.86 (2.60)
Log Physical Activity [h/d], mean (sd)	0.58 (0.14)	0.56 (0.15)	0.58 (0.19)	0.54 (0.12)	0.61 (0.17)	0.56 (0.18)
TEE [kcal], mean (sd)	2,529 (461)	2,590 (385)	2,523 (491)	2,486 (410)	2,622 (466)	2,536 (461)
Sleep duration [h], mean (sd)	7.44 (0.70)	7.44 (0.91)	7.44 (0.86)	7.44 (0.84)	7.44 (0.84)	7.44 (0.84)

Results are expressed as *n* (%), or mean (sd), T1, T2, T3, tertile groups; TEE, total energy expenditure; BMI, body mass index, mean, *p* values are based on Chi-squared test of independence or ANOVA test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

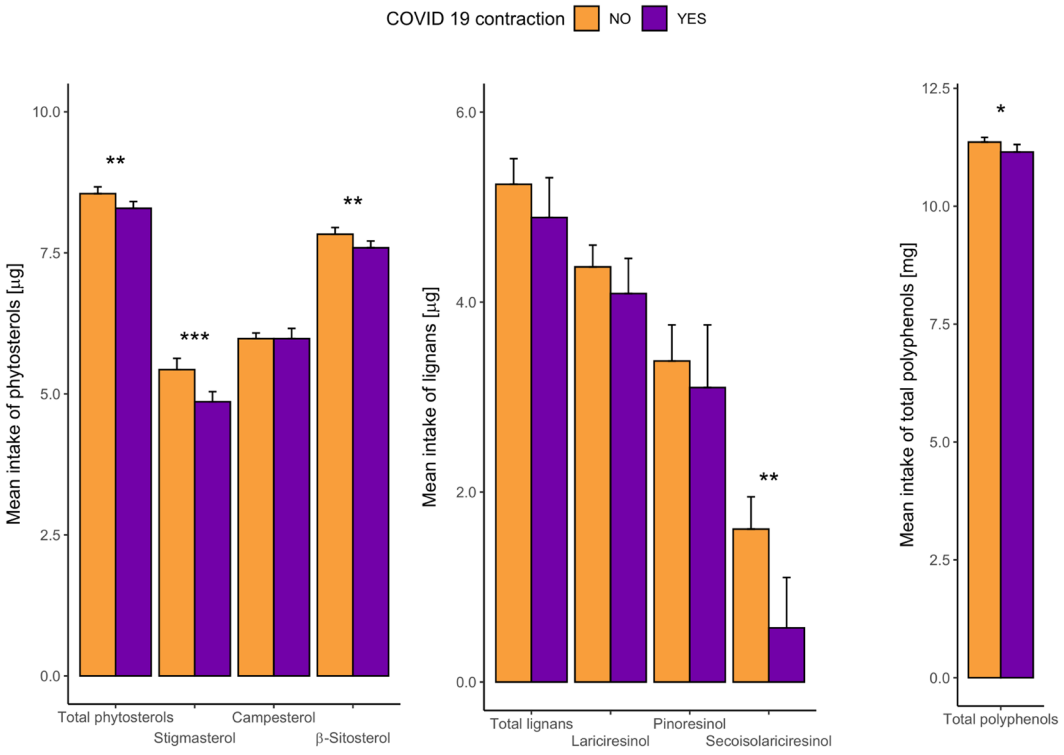

FIGURE 1
Comparison of distribution of energy adjusted logarithmically transformed daily consumption of specific phytochemicals between respondents who have contracted and who have not contracted COVID-19. For better visualization particular phytochemical intake were expressed in different units. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ for Student *T*-test analysis ($N = 95$).

TABLE 2 Association between phytochemical intake and COVID-19 contraction – comparison of distribution of risk of disease by tertiles of consumptions and crude logistic regression analysis for continuous exposure level ($N = 95$).

Phytochemicals	Phytochemical-specific tertiles			OR (95% CI)	
	T1 ($N = 32$)	T2 ($N = 31$)	T3 ($N = 32$)	Per 1 category of tertile increase	Per 1 unit increase in Log2
	COVID-19 contraction, *n* (%)				
Total polyphenols [mg]	11 (34.4)	8 (25.8)	5 (15.6)	0.60 (0.33–1.08)	0.29 (0.09–0.92)*
Total lignans [µg]	11 (34.4)	6 (19.4)	7 (21.9)	0.72 (0.40–1.27)	0.74 (0.47–1.15)
Lariciresinol [µg]	10 (31.3)	7 (22.6)	7 (21.9)	0.78 (0.44–1.38)	0.74 (0.44–1.23)
Matairesinol [ng]	9 (28.1)	12 (38.7)	3 (9.4)*	0.60 (0.33–1.08)	0.90 (0.70–1.16)
Pinoresinol [µg]	8 (25.0)	9 (29.0)	7 (21.9)	0.92 (0.52–1.62)	0.90 (0.67–1.20)
Secoisolariciresinol [µg]	15 (46.9)	6 (19.4)	3 (9.4)**	0.33 (0.17–0.64)**	0.50 (0.31–0.81)**
Total phytosterols [mg]	12 (37.5)	9 (29.0)	3 (9.4)*	0.45 (0.24–0.84)*	0.21 (0.05–0.84)*
Stigmasterol [mg]	14 (43.8)	7 (22.6)	3 (9.4)**	0.37 (0.19–0.70)**	0.26 (0.11–0.64)**
Campesterol [mg]	8 (25.0)	10 (32.3)	6 (18.8)	0.85 (0.48–1.49)	1.03 (0.35–3.03)
β-sitosterol [mg]	12 (37.5)	8 (25.8)	4 (12.5)	0.50 (0.27–0.92)*	0.23 (0.06–0.89)*

Results are expressed as *n* (%), or OR (95% CI), OR, odds ratio; CI, confidence interval; T1, T2, T3, tertile groups; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ from Chi-squared test or logistic regression analysis.

intake and for doubling intake were depicted in Table 2. In univariable analysis, both chi-squared test and logistic regression models have shown that higher consumption of secoisolariciresinol, total phytosterols and stigmasterol was associated with lower risk of COVID-19. Additionally, compared with lower tertiles, a significantly lower frequency of contraction of COVID-19 was noted in the highest tertile of consumption of matairesinol and decreased chance of the illness was observed for people with greater intake of total polyphenols and β-sitosterol (Table 2).

The negative associations between COVID-19 prevalence and dietary intake of secoisolariciresinol, total phytosterols and stigmasterol were confirmed in multivariable analysis, showing very stable and robust results after controlling for different sets of potential

confounders. In fully adjusted models, independently of age, total energy intake, sex, diet, smoking status, BF, BMI, physical activity and alcohol consumption, the diet richest in specific phytochemical diminished the odds of the occurrence of COVID-19 about 90, 84, and 88% compared with the diet poorest in these compounds (OR = 0.10, 95% CI: 0.02–0.46 for secoisolariciresinol, OR = 0.16, 95% CI: 0.03–0.76 for total phytosterols and OR = 0.12, 95% CI: 0.02–0.54 for stigmasterol, Table 3; Figure 2). These relations were also reflected by a decline in the risk of the disease with each movement to a higher category of tertile intake (OR = 0.28, 95% CI: 0.11–0.61 for secoisolariciresinol, OR = 0.47, 95% CI: 0.22–0.95 for phytosterols and OR = 0.34, 95% CI: 0.14–0.72 for stigmasterols) and with doubling of intake (OR = 0.47, 95% CI: 0.24–0.79 for secoisolariciresinol, OR = 0.23, 95% CI: 0.04–1.01 (marginally significant) for phytosterols and OR = 0.29, 95% CI: 0.09–0.72 for stigmasterol, Table 3). Moreover, the evidence of protective effect against COVID-19 contraction was found for total polyphenols, matairesinol and β-sitosterol, in most cases with significant or marginally significant results and a reduction in the odds of the disease ranging from 70 to 84% in analysis comparing extreme categories of intake, from 44 to 51% in analysis reflecting change category of tertile to one level higher and, except matairesinol, from 74 to 80% when doubling intake, although wide confidence intervals were observed (Table 3; Figure 2). No univocal patterns of trends could have been found concerning total lignans, two individual lignan groups, namely lariciresinol and pinoresinol and one individual phytosterol group, namely campesterol; despite a general tendency of decreasing the risk of COVID-19 observed with a higher intake, no result reached statistical significance (Table 3).

3.3. Relationship of lignan and phytosterol intake with the immunostimulatory microbiota *Escherichia coli* and Enterococcus spp. as well as with immunomodulatory profile of the diet assessed by POLA index

The frequencies and odds ratios of the occurrence of abnormal amounts of each of the two strains of immune-stimulating microbiota (below 10^6 CFU/g in feces) within groups of people with different consumption of phytochemicals was shown in Figures 3, 4. Generally, a decreasing trend in the occurrence of abnormal values of Enterococcus spp. was noted across tertile categories of total polyphenols and all types of lignans except secoisolariciresinol. In the case of *Escherichia coli* a tendency of analogical trends was observed between premade groups of phytosterols. However, the result for Enterococcus spp. was statistically significant only for lariciresinol ($p < 0.05$) and on the boundary of significance only for total lignans ($p < 0.1$), showing lowering the odds of abnormal values about 42 and 38%, respectively, with each increment of tertile category. The largest disparities in the frequency of prevalence of aberrant values of strains of *Escherichia coli* were found between tertiles of β-sitosterols intake. Independently of age, sex, diet, and total energy intake, persons in the third tertile of β-sitosterols compared with the first tertile had 72% reduced odds of having an abnormal level of bacteria compared with those from the first tertile, despite a very wide confidence interval (OR = 0.28, 95% CI: 0.08–0.86). No other statistically significant results were observed (Figures 3, 4; Supplementary Table S2).

Moreover, there was a positive association between a higher intake of lignans (however nonsignificant) and phytosterols and more beneficial immunomodulatory profile of the diet assessed by the POLA index which has been previously shown to be negatively related to the risk of COVID-19 (Supplementary Table S3) (17).

4. Discussion

The present study examined the relationship between phytochemical consumption, gut microbiota and the risk of COVID-19 disease among non-obese 25-45-year-old subjects without comorbidities. The results showed that higher intake of total polyphenols, specific lignans such as secoisolariciresinol and matairesinol, as well as total phytosterols and some subclasses: stigmasterols and β-sitosterols was associated with a lower risk of COVID-19.

There are currently no human studies available evaluating the effectiveness of higher dietary polyphenol, lignan and phytosterol intake in reducing COVID-19 risk. However, the antiviral efficacy of polyphenols including lignans and plant sterols has been confirmed against SARS-CoV, MERS-CoV, Ebola virus, HIV, influenza virus and other viruses causing respiratory tract infections (18–21). Recent studies have also demonstrated their beneficial effects against the SARS-CoV-2 virus (18, 22–24) resulting from their ability to bind to peak protein sites on the ACE2 receptor used by SARS-CoV-2 to infect cells (25, 26), regulate ACE2 expression and also interfere with SARS-CoV-2 replication by inhibiting the virus protease or inhibiting SARS-CoV-2 RNA-dependent RNA polymerase (RdRp) (27, 28). The antiviral activity of some of them is comparable to or stronger than pharmacological agents (naringenin, vs. remdesivir (29), citrus flavonoids and polyphenols from Curcuma spp. vs. lopinavir and nafamostat (30)).

Although diet alone is not sufficient for the prevention of any virus infection, adequate nutrition is viewed as one of the best complementary approaches for controlling many types of infections, including SARS-CoV-2. Optimal dietary intake of macro- and micronutrients and other bioactive constituents may affect the immune system and thus strengthen the protection against influenza-like illnesses such as COVID-19 disease (1, 11, 17, 31). Unfortunately, the pandemic has had a significant impact on nutritional habits, yet dietary changes have manifested differently and to various extents among men and women as well as in people with distinct socioeconomic status (32–34). For example, improvements in line with a Mediterranean diet which is proven to help boost immunity, were associated with higher education, wealth, skilled manual occupations and male sex (33). Nevertheless, the transition to the new habits during the pandemic, primarily, was associated with negative eating behaviors.

It has been reported that consumptions of polyphenols and phytosterols such as β-sitosterol, campesterol, and stigmasterol, are beneficial for the human immune system and health due to their antioxidant, anti-inflammatory and cholesterol-lowering activity (7, 35, 36). Also, lignans that include secoisolariciresinol and matairesinol are among the most promising immunotherapeutics (37) that exhibit antioxidant and anti-inflammatory activity (38). The immune system is critical for the clearance of a variety of infections. In the case of COVID-19, induced cytokine release

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TABLE 3 Association between phytochemical intake and COVID-19 contraction.

Phytochemicals	T1 (*N* = 31)	T2 (*N* = 31)	T3 (*N* = 32)	Per 1 category of tertile increase	Per 1 unit increase in Log2
Total polyphenols [mg]					
Model 1	1 (ref.)	0.78 (0.24–2.52)	0.28 (0.07–0.96)*	0.55 (0.29–1.00)	0.22 (0.06–0.74)*
Model 2	1 (ref.)	0.70 (0.20–2.41)	0.30 (0.07–1.09)	0.56 (0.28–1.06)	0.21 (0.05–0.76)*
Model 3	1 (ref.)	0.71 (0.20–2.45)	0.30 (0.07–1.13)	0.56 (0.27–1.07)	0.20 (0.05–0.77)*
Total lignans [μg]					
Model 1	1 (ref.)	0.44 (0.12–1.47)	0.43 (0.12–1.36)	0.64 (0.34–1.17)	0.70 (0.43–1.10)
Model 2	1 (ref.)	0.44 (0.11–1.63)	0.47 (0.12–1.62)	0.68 (0.35–1.29)	0.73 (0.44–1.17)
Model 3	1 (ref.)	0.40 (0.09–1.55)	0.48 (0.13–1.67)	0.69 (0.35–1.30)	0.74 (0.44–1.17)
Lariciresinol [μg]					
Model 1	1 (ref.)	0.62 (0.18–2.03)	0.52 (0.15–1.71)	0.72 (0.39–1.31)	0.66 (0.37–1.11)
Model 2	1 (ref.)	0.68 (0.19–2.38)	0.58 (0.16–2.00)	0.76 (0.40–1.42)	0.70 (0.38–1.20)
Model 3	1 (ref.)	0.64 (0.17–2.38)	0.59 (0.16–2.06)	0.76 (0.40–1.44)	0.69 (0.37–1.20)
Matairesinol [ng]					
Model 1	1 (ref.)	1.41 (0.46–4.43)	0.24 (0.05–0.97)*	0.56 (0.29–1.04)	0.89 (0.67–1.17)
Model 2	1 (ref.)	1.68 (0.47–6.20)	0.20 (0.03–0.97)*	0.51 (0.24–1.03)	0.90 (0.65–1.21)
Model 3	1 (ref.)	1.85 (0.51–7.09)	0.16 (0.02–0.81)*	0.49 (0.23–1.01)	0.88 (0.63–1.21)
Pinoresinol [μg]					
Model 1	1 (ref.)	1.29 (0.39–4.34)	0.76 (0.22–2.59)	0.88 (0.48–1.58)	0.88 (0.65–1.18)
Model 2	1 (ref.)	1.66 (0.46–6.25)	0.80 (0.21–2.98)	0.89 (0.47–1.67)	0.90 (0.66–1.22)
Model 3	1 (ref.)	1.66 (0.44–6.62)	0.81 (0.21–3.03)	0.88 (0.46–1.67)	0.90 (0.65–1.22)
Secoisolariciresinol [μg]					
Model 1	1 (ref.)	0.18 (0.05–0.61)**	0.09 (0.02–0.37)**	0.28 (0.12–0.57)**	0.47 (0.26–0.75)**
Model 2	1 (ref.)	0.18 (0.05–0.63)*	0.11 (0.02–0.46)**	0.29 (0.12–0.62)**	0.48 (0.26–0.79)**
Model 3	1 (ref.)	0.15 (0.03–0.57)**	0.10 (0.02–0.46)**	0.28 (0.11–0.61)**	0.47 (0.24–0.79)*
Total phytosterols [mg]					
Model 1	1 (ref.)	0.88 (0.28–2.74)	0.19 (0.04–0.73)*	0.49 (0.25–0.90)*	0.25 (0.05–0.88)*
Model 2	1 (ref.)	0.81 (0.25–2.61)	0.16 (0.03–0.72)*	0.47 (0.22–0.92)*	0.23 (0.04–0.99)*
Model 3	1 (ref.)	0.82 (0.24–2.70)	0.16 (0.03–0.76)*	0.47 (0.22–0.95)*	0.23 (0.04–1.01)
Stigmasterol [mg]					
Model 1	1 (ref.)	0.43 (0.13–1.36)	0.14 (0.03–0.54)**	0.39 (0.19–0.74)**	0.30 (0.11–0.69)**
Model 2	1 (ref.)	0.32 (0.09–1.11)	0.13 (0.02–0.56)*	0.35 (0.16–0.73)**	0.29 (0.10–0.71)*
Model 3	1 (ref.)	0.31 (0.07–1.12)	0.12 (0.02–0.54)**	0.34 (0.14–0.72)**	0.29 (0.09–0.72)*
Campesterol [mg]					
Model 1	1 (ref.)	1.87 (0.58–6.37)	0.68 (0.19–2.41)	0.85 (0.46–1.52)	1.20 (0.38–3.64)
Model 2	1 (ref.)	1.63 (0.48–5.73)	0.60 (0.16–2.19)	0.80 (0.42–1.47)	1.13 (0.34–3.63)
Model 3	1 (ref.)	1.71 (0.49–6.33)	0.65 (0.16–2.55)	0.83 (0.42–1.58)	1.19 (0.35–3.88)
β-sitosterol [mg]					
Model 1	1 (ref.)	0.59 (0.18–1.85)	0.23 (0.05–0.81)*	0.49 (0.25–0.91)*	0.24 (0.05–0.87)*
Model 2	1 (ref.)	0.52 (0.15–1.72)	0.24 (0.05–0.93)*	0.49 (0.24–0.96)*	0.24 (0.04–1.02)
Model 3	1 (ref.)	0.52 (0.14–1.78)	0.24 (0.05–0.97)*	0.49 (0.23–0.98)*	0.24 (0.04–1.02)

Results of multiple logistic regression analysis for tertiles of consumption and for continuous exposure ($N = 95$). Results are presented as ORs (95% CIs), T1, T2, T3, tertile groups, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ from multiple logistic regression analysis, Model 1, adjusted for age and total energy intake; Model 2, adjusted for variables in model 1 and sex, diet, smoking status, BF; Model 2, adjusted for variables in model 2 and for BMI, physical activity and alcohol consumption ($N = 95$).

syndrome is suggested to play a pivotal role in the pathology of the disease. Therefore, there is a significant concern regarding the search for methods of treatment that focus on prevention of cytokine storm in ill patients. Such an activity is demonstrated by polyphenols and phytosterols, which, through their effects on macrophages, inhibit the secretion of the pro-inflammatory

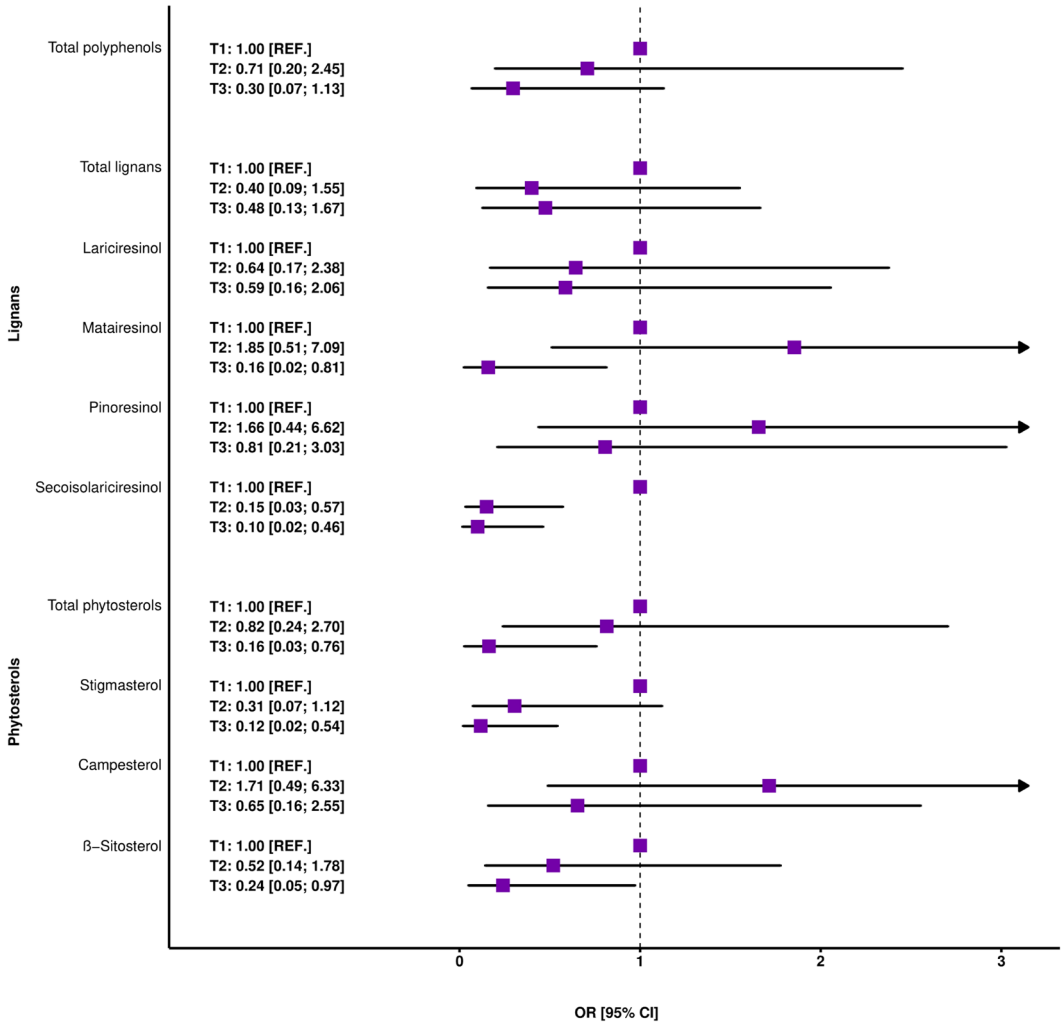

FIGURE 2
Odds ratios of COVID-19 contraction across specific-phytochemical tertiles. The results of fully adjusted models after controlling for age, total energy intake, sex, diet, smoking status, BF, BMI, physical activity, and alcohol consumption ($N = 95$).

mediators interleukin-1-beta (IL-1ß), IL-2, IL-6, γ-interferon (IFN-γ), and tumor necrosis factor α (TNF-α) reducing inflammation caused by hyperactivation of cytokines (39–41). Anti-inflammatory and preventive effects against diseases associated with immune dysregulation are shown, for example, by polyphenols in red wine (they raise interleukin IL-21 levels and reduce the release of IL-1β and IL-6) (42) or polyphenols from green tea, pomegranate, grape seed, mango (6).

The high capacity to reduce oxidative stress and overproduction of reactive oxygen species (ROS) exacerbates the anti-inflammatory effects of polyphenols and phytosterols (43–45). Polyphenols can also enhance resistance to foreign pathogens through other inflammation-related pathways like activation of T regulatory cells (Treg), which can suppress cytotoxic T cell function (6, 46), affecting dendritic cells, increasing B and T lymphocyte proliferation and inducing apoptosis (6). On the other hand, some phytosterol compounds, in addition to their ability to attenuate the inflammatory response in lipopolysaccharide-induced macrophage models (47), may also exert antiproliferative effects (β-sitosterol) (48).

Both polyphenols and phytosterols reduce cholesterol levels in the cell membrane or destabilize the structure of lipid rafts, which are the main docking sites for COVID-19 entry and genome release (49). Cholesterol is also essential for the replication and infectivity of enveloped viral particles, and influences the molecular and cellular events of immune cells and subsequent biological responses through many other mechanisms (50). Plant sterols have similar chemical structure to cholesterol, and esters of both compounds can be seen as the rivals to each other due to their competition for hydrolysis by enzymes in the gastrointestinal tract. As phytosterols are more lipophilic, they can contribute to the reduction of micellar solubility of cholesterol in the intestine and are capable of eliciting the reduction of the cholesterol absorption by enterocytes (36). Phytosterols (sitosterol and campesterol) can activate the bile acid excretion pathway and accelerate cholesterol metabolism (51–53), reduce cholesterol synthesis and disrupt cholesterol homeostasis (stigmasterol) (54, 55). A therapy with a polyphenol composition resulted in a significant increase in HDL fraction cholesterol (56), low levels of which were associated with a more severe course of

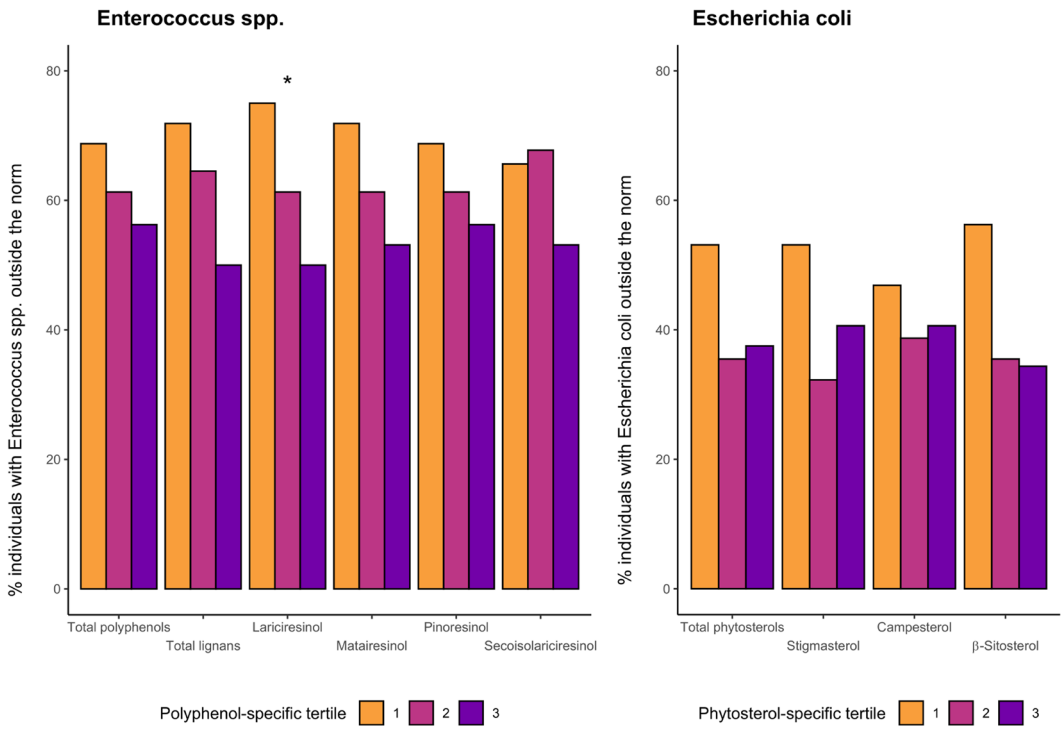

FIGURE 3
Comparison of percentage of people in whom the amount of bacteria Enterococcus spp. and *Escherichia coli* was outside the norm across specific-polyphenol tertiles, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ for logistic regression analysis testing decreasing trend between tertiles after controlling for age, sex, diet, and total energy intake ($N = 95$).

COVID-19 (57). The cardioprotective effects of these compounds (58, 59) may also improve the course of COVID-19 in patients with cardiovascular disease. They also have anti-diabetic effects (43, 60, 61) and may reduce obesity (62).

Numerous studies confirm that a diet high in plant-based products containing, among others, phytosterols naturally present in the cell membranes of lipid-rich plants (nuts, seeds, legumes, olive oil (63)), is associated with a lower risk of infection and a milder course of COVID-19 (64, 65). Some dietary patterns including products abundant in polyphenols and phytosterols have been also shown to be competent to antagonize inflammation by many pathways. The Mediterranean diet characterized by a high amount of antioxidant vitamins and phytochemicals has been reported to reduce oxidative stress, block pro-inflammatory cytokines, suppress inflammatory and increase antioxidant gene expression, as well as activate transcription factors that counteract chronic inflammation (66, 67). In the large cohort of the MOLI-SANI study, a Mediterranean eating pattern as well as specifically flavonoid and lignan intakes measured by Polyphenol Antioxidant Content (PAC) score, have been proven to be related to novel cellular biomarkers of low-grade inflammation such as platelet, leucocyte counts and granulocyte:lymphocyte ratio (68). Regarding the possible effect of other dietary, clinical and environmental factors, the flavonoid and lignan content of diet explained a relatively high proportion of the variation of INFLA score evaluating the synergetic effect of inflammatory biomarkers in both men and women (66).

Interestingly, in our study among the subjects who consumed the most phytosterols there were regular alcohol drinkers. This was related to their consumption of beer, which was a source of a significant amount of these compounds (69, 70). Although the amount of phytosterols in beer is considered too low for a health effect, some wheat beers with a high yeast content, or with the addition of whole grain, may contain higher levels of ergosterol or sitosterol (70). The benefits of alcohol consumption remain controversial concerning both the type of alcohol and the drinking pattern, and it is difficult to recommend alcohol as a source of phytosterols.

Our study shows that while habitual intake of the analyzed compounds did not protect against COVID-19, higher intake of total polyphenols, specific lignans such as secoisolariciresinol, total phytosterols and some subclasses: stigmasterol and β-sitosterol was associated with lower risk of COVID-19. Also in other studies, high doses of polyphenols (71) and a high intake of β-sitosterol (72) have been shown to possess a protective effect against COVID-19. In an *in silico* computational study secoisolariciresinol was found to be more effective against non-structural proteins (nsp10 and nsp16) of SARS-CoV-2 than remdesivir and more significant than lopinavir (73). There are no results to date on the effect of an increased matairesinol intake in COVID-19. A diet with a significant amount of matairesinol compared to a typical northern Italian diet resulted in reduced vascular inflammation and endothelial dysfunction (74). Matairesinol attenuated sepsis-induced brain damage (45). However, the beneficial role of matairesinol in reducing the frequency of COVID-19 contractions was demonstrated here for the first time.

Various factors influence the incidence and course of COVID-19. COVID-19 infection has been shown to have the lowest mortality rate among children with a log-linear increase among the elderly (75),

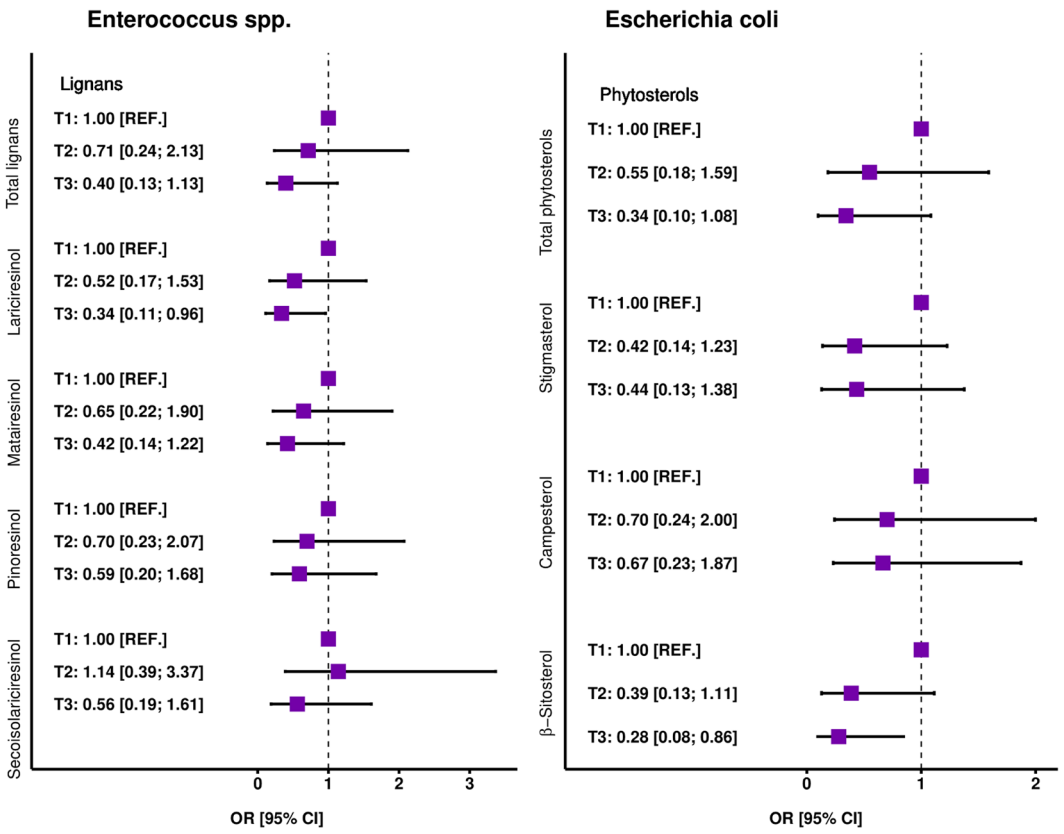

FIGURE 4
Odds ratios of bacteria Enterococcus spp. and *Escherichia coli* outside the norm across specific-polyphenol tertiles. Multiple logistic regression after controlling for age, sex, diet, and total energy intake ($N = 95$).

sexual dimorphism with a tendency toward greater severity and mortality among men (76), a worse prognosis and higher mortality among those who are obese (77) or consume large amounts of alcohol (78). In contrast, people with a balanced diet, who were physically active and did not use stimulants were at significantly lower risk of COVID-19 infection (11). However, our study showed that regardless of age, total energy intake, sex, diet, smoking status, BF, BMI, physical activity and alcohol consumption, a diet richest in secoisolariciresinol, total phytosterols and stigmasterol reduced the odds of COVID-19 by 90, 84 and 88% respectively, and a diet richest in total polyphenols, matairesinol and β-sitosterol showed a protective effect against COVID-19 contraction with reduced odds of the disease ranging from 70 to 84% in analysis comparing extreme categories of intake. These relationships were also reflected in a decrease in the risk of disease with each shift to a higher category of tertile intake and with doubling of intake for most of them. The implication is that sufficiently high dietary intake of these phytochemicals may affect the infection rate, course and mortality caused by SARS-CoV-2 more strongly than the other factors. Their efficacy against COVID-19 when consumed with the diet was independent of low bioavailability (79, 80), environmental factors and enzymatic activity in the gastrointestinal tract, which are indicated as factors that potentially reduce the concentration of polyphenols and even cause partial or complete loss of their bioactivity (79). Thus, it appears that the pharmacological and molecular effects of dietary phytochemicals may be quite different from those of single compounds, due to complex complementary, additive or synergistic interactions between polyphenols and/or other classes of phytochemicals.

A growing body of evidence in the literature suggests a link between intestinal dysbiosis and a variety of illnesses and their courses (42, 81), including COVID-19 disease severity (82, 83). The gut microbiome and its composition is highly important for human organism not only due to its participation in digestion, absorption and metabolism, but also due to its modulating activity in the immune responses. Some strains of commensal bacteria species such as *Escherichia coli* and Enterococcus spp. are involved in the production of antibodies, maturation of B lymphocytes and maintaining the balance of Th1/Th2 lymphocytes by activating the cytokine network. Enterococcus bacteria, stimulate plasmocytes in the intestinal epithelium to synthesize secretory IgA, and non-pathogenic *Escherichia coli* strains activate gut-associated lymphoid tissue (GALT) cells to synthesize antimicrobial factors and mature dendritic cells (84).

Polyphenols and phytosterols are among the dietary components suggested in the literature to help maintain intestinal homeostasis (6, 7). Absorbed polyphenols interact with the immune system from the gastrointestinal tract and thus contribute to the prevention of some immune diseases (42, 46). Immune cells express polyphenol receptors enabling the activation of signaling pathways to initiate an immune response (46). It has been documented that SARS-CoV-2-induced dysbiosis of the gut microbiota might be modified by the prebiotic effects of polyphenols (85).

In vitro and *in vivo* animal as well as human studies have shown that an adequate diet rich in phytochemicals can promote the growth of beneficial microflora in the gut and suppress pathogenic bacteria (86). Considering the symbiotic relationship of *Escherichia coli* and Enterococcus spp. with the host's immune system we checked the aforementioned hypotheses for these microbes. In our study we observed a positive association of β-sitosterol intake and phytosterols in total with the occurrence of normal amounts of *Escherichia coli* in stool samples. Additionally, there was a trend of more frequent presence of Enterococcus spp. at the relevant level, i.e., $>10^6$ CFU/g, in people with a higher intake of lariciresinol.

These findings are in agreement with the results of other studies. Lariciresinol isolated from Rubia philippinensis showed a reduction in bacterial cell viability and had antimicrobial activity against pathogenic strains of *Escherichia coli* (87). Feeding animals with high doses of stigmasterol (88) or β-sitosterol (48) resulted in the alleviation of intestinal dysbiosis. The consequence was increased cholesterol and coprostanol excretion, as well as decreased hepatic esterified cholesterol (7). Consumption of polyphenols from red wine and dealcoholized red wine significantly increased the number of Enterococcus groups and the concentration of Enterococcus in feces (89, 90). Since dysbiosis of the intestinal microflora is associated with the development of many noncommunicable diseases, including cardiovascular disease, obesity and neurodegenerative diseases, a beneficial interaction with polyphenolic compounds could potentially provide health benefits. With that said, the ability of dietary polyphenols to produce clinical effects may attribute, at least in part, to a bidirectional relationship with the gut microbiota. Polyphenols can influence the composition of the gut microbiota and gut bacteria metabolize polyphenols into bioactive compounds that confer clinical benefits (61).

Although we have made efforts to minimize the probability of bias during conducting this research, our study is not free from certain limitations. Firstly, as it is common in observational studies, we cannot rule out the possibility of residual confounding by unmeasured dietary variables or other factors. Notwithstanding, for counteracting such a threat, the study participation was constrained by some exclusion criteria and we adjusted the intake of all phytochemicals to total energy intake. At the same time, we conducted several types of analyses to check the stability of the results, controlling constructed models to different sets of covariates. Secondly, some lignans, especially matairesinol, were consumed by participants in small amounts and, therefore, the results for them should be interpreted with caution. Thirdly, e.g., due to seasonal variation in the accessibility of fresh fruits and vegetables in Poland, participants could change their dietary patterns during follow-up and might not maintain their eating habits recorded in diaries. However, since a pandemic wave came around the time of the examination, the time of collecting information about dietary practices seems to be optimal to test whether the nutritional status of the body could shape to a great extent the immune responses against SARS-CoV-2. Finally, mostly due to difficulties associated with escalating lockdown restrictions, and consequently shortening duration of the second arm of the study, the final distribution of sex was in favor of men. Additionally, the online recruitment may have led to over- or under-representation of the target population.

Even though COVID-19 vaccines are the main course of action to curb the development of the SARS-CoV-2 pandemic, additional efforts are being made to mitigate the pathological effects of COVID-19 and other viral respiratory diseases.

The antiviral effects of phytochemicals, combined with well-established antioxidant, anti-inflammatory and anti-cholesterol activities, have proven to be effective in the prevention and treatment of COVID-19 and may provide an alternative or adjuvant solution to drug treatment. Especially since they show comparable effects and fewer side effects than pharmaceutical preparations.

Despite possible drug interactions, increasing the supply of phytosterols and polyphenols including lignans in the diet appears to be one of the simplest and safest methods of counteracting the infection and supporting the treatment of COVID-19. In patients infected with SARS-CoV-2, the use of an appropriate dietary model may prove more effective than the use of single purified compounds including dietary supplements.

5. Conclusion

The results of the current study support the hypothesis that a diet rich in phytosterols and polyphenols, including lignans, can help to reduce the risk of COVID-19 contraction. Furthermore, the findings suggest that high consumption of their several representatives, namely β-sitosterol and lariciresinol, may be positively associated with the presence at a relevant level of some strains of commensal bacteria species such as *Escherichia coli* and Enterococcus spp. which support immune system. Various phytochemicals can have a differential effect on gut microbiome and influenza-like diseases. Therefore, further research is needed to explore these outcomes in relation to the bioavailability of specific phytosterols and lignans.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the Bioethics Committee of Jagiellonian University No. 1072.6120.5.2020 and 1072.6120.202.2019. The patients/participants provided their written informed consent to participate in this study.

Author contributions

AM, PJ, IB, and JG contributed to conception and design of the study. AM, IB, and JG wrote the first draft of the manuscript. AM performed the statistical analysis. PJ, AW, and AMW investigation. AM, IB, PJ, KK, AW, AMW, JP, and JG wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1241016/full#supplementary-material

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Insights into vitamin A in bladder cancer, lack of attention to gut microbiota?

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Vitamin A has long been associated with bladder cancer, and many exogenous vitamin A supplements, vitamin A derivatives, and synthetic drugs have been investigated over the years. However, the effectiveness of these strategies in clinical practice has not met expectations, and they have not been widely adopted. Recent medical research on intestinal flora has revealed that bladder cancer patients exhibit reduced serum vitamin A levels and an imbalance of gut microbiota. In light of the close relationship between gut microbiota and vitamin A, one can speculate that a complex regulatory mechanism exists between the two in the development and occurrence of bladder cancer. As such, further exploration of their interaction in bladder cancer may help guide the use of vitamin A for preventive purposes. During the course of this review, attention is paid to the influence of intestinal microbiota on the vitamin A metabolism and the RA signaling pathway, as well as the mutual promotion relationships between them in the prevention of bladder cancer, In addition, it emphasizes the importance of intestinal microbiota for bladder cancer prevention and treatment.

KEYWORDS

vitamin A, retinoic acid, gut microbiota, lipopolysaccharides, bladder cancer

1 Introduction

There has been an increase in the incidence of bladder cancer in recent years, particularly among women. It affects the urinary system and can be fatal. In 2020, it was reported to account for 573,278 new cases and 212,536 deaths worldwide, ranking ninth and 13th in terms of incidence and mortality among malignant tumors, respectively (1). Bladder cancer includes a variety of pathological types, including urothelial carcinoma, squamous cell carcinoma and adenocarcinoma, among which urothelial carcinoma is the most common pathological type (2). In reality, approximately 75% of patients with bladder cancer are afflicted with non-muscle invasive bladder cancers (NMIBC), which can have varying levels of risk for recurrence and progression. Generally, the 5-year survival rate for NMIBC exceeds 90%, contributing to the high incidence and low mortality rates of bladder

cancer. Nonetheless, most NMIBC patients require long-term surveillance and preventive interventions, such as cystoscopy, which significantly impact their quality of life and impose a financial burden (3, 4). Therefore, chemoprophylaxis and other strategies to reduce postoperative bladder cancer recurrence have been widely employed in clinical practice, with retinoic acid (RA) being the most commonly used chemoprophylaxis drug. RA possesses remarkable anti-tumor properties. As early as 1990, it was discovered that RA could arrest hematopoietic cell cycle and induce cell differentiation into hematopoietic terminal cells, leading to its application in treating acute promyelocytic leukemia (5). Other cancer types, such as thyroid and prostate cancer, have also been shown to respond to RA's anti-tumor effects, including inhibition of cell proliferation and induction of cell differentiation (6, 7). Vitamin A has been shown to prevent and treat bladder cancer in numerous studies conducted over the past 50 years. A review of these studies on vitamin A and bladder cancer is presented in Table 1. Researchers have confirmed that patients with bladder cancer have lower levels of serum vitamin A than healthy people, as shown in a substantial body of research. In addition, low vitamin A levels are increasingly regarded as risk factors for bladder cancer. Several *in vivo* experiments on vitamin A are summarized in Table 2. Almost all studies have shown positive results, mainly manifested as vitamin A can inhibit apoptosis, reduce tumor size, and inhibit the progression of bladder cancer. However, although *in vivo* experiments showed consistent promising results, the results of several clinical trials of vitamin A supplementation were not as expected (19–21). As a result, enhancing the efficiency of vitamin A in bladder cancer prevention and treatment would be an important research endeavor.

Intestinal microbiomes are collections of microorganisms found in the gastrointestinal tract. Advancements in technologies such as 16S rRNA sequencing have revealed the significant role of gut microbiota in non-infectious diseases, particularly in tumor diseases. It is becoming increasingly clear that gut microbiota influences immunity and inflammation in intricate ways, implying its complex involvement in tumor occurrence and development (27). In addition, studies have reported enhanced anti-cancer effects associated with gut microbiota (28). Consequently, there is increasing attention on the role of gut flora in cancer. In a case-control study, bladder cancer patients' gut microbiota was compared to that of healthy individuals. According

TABLE 1 Clinical trials investigation on vitamin A and bladder cancer.

Country	Study period	Age (years)	Case/ subjects	Pathology	Main Findings	Reference
Sweden	1985 to 1987	40 to 74	418/929	urothelial carcinoma	Vitamin A supplement plays a certain preventive effect on urothelial carcinoma.	(8)
Eypt	1957 to 1965	Not mentioned	70/144	Not mentioned	Vitamin A levels were significantly lower in bladder cancer patients with squamous cell carcinoma than in normal individuals	(9)
Japan	1990 to 2007	>40	42/1666	urothelial carcinoma	High serum carotene levels reduce the risk of bladder cancer	(10)
USA	1957 to 65	40 to 89	-/8606	Not mentioned	Dietary vitamin A is associated with a reduced risk of squamous epithelial carcinoma	(11)
Turkey	Not mentioned	40 to 79	23/91	urothelial carcinoma	Compared to the control group, patients had significantly lower serum vitamin A levels	(12)
USA	2001 to 2004	30 to 79	1418/2589	urothelial carcinoma	Elevated plasma carotene levels significantly reduce the risk of bladder cancer	(13)
USA	1993 to 2007	45 to 75	581/185885	Not mentioned	Women with high vitamin A and carotene intake have a lower risk of bladder cancer.	(14)
USA	1971 to 1995	52 to 71	111/222	urothelial carcinoma	No significant correlation between serum carotene levels and bladder cancer risk after adjustment with smoking.	(15)
Japan	1971 to 1975	Not mentioned	27/6800	Not mentioned	Serum vitamin A levels were not associated with bladder cancer risk	(16)
Belgium	1999 to 2004	Not mentioned	178/540	urothelial carcinoma	Retinol intake was not significantly associated with bladder cancer.	(17)
Netherlands	1981 to 1989	55 to 69	569/3692	Not mentioned	There was no association between bladder cancer and dietary or supplemental intake of vitamin A and most carotenoids	(18)
USA	1981 to 1989	Not mentioned	335/11580	Not mentioned	Supplementing with β-carotene and vitamin A did not reduce bladder cancer risk significantly	(19)
USA	1980 to 2000	30 to 55	237/88796	Not mentioned	Vitamin A and carotene intake were not associated with bladder cancer risk.	(20)
USA	2000 to 2007	50 to 76	330/77050	urothelial carcinoma	Supplementation of carotene and retinol cannot effectively prevent the occurrence of urothelial carcinoma.	(21)

TABLE 2 *In vivo* studies investigating the effects of vitamin A in animal models of bladder cancer.

In Vivo Model–Carcinogen	Species	Outcome	Reference
MNNG	Rat	Bladder cancer incidence induced by MNNG was higher in rats with low vitamin A diet	(22)
FANFT	Rat	Vitamin A deficiency accelerated the carcinogenic efficiency of FANFT, but high vitamin A did not significantly inhibit FANFT-induced bladder cancer	(23)
BBN	Rat	Hyperretinemia inhibited the incidence of BBN-induced transitional cell carcinoma and neoplasms of the bladder	(24)
BBN	Rat	Vitamin A diet could reduce the progression of early bladder cancer by reducing BBN-induced urothelial atypia.	(25)
BBN	Rat	Vitamin A supplementation reduce the incidence of tumor and tumor size.	(26)
BBN	Mouse	Vitamin A treatment reduce the urothelial atypia and apoptosis in early bladder cancer.	(25)

MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; FANFT, N-[4-(5-nitro-2-furyl)-2-thiazolyl] formanmide; BBN, N-butyl-N-(4-hydroxybutyl) nitrosamine.

to the findings, patients with bladder cancer showed a significant reduction in the abundance of specific bacteria in their gut. Additionally, this work used real-time qPCR to analyze the differences among 12 major Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria bacteria. The findings demonstrated that the numbers of domain Bacteria, Clostridium cluster XI and Prevotella in patients were significantly lower than those in healthy group (29). Some studies (30–32) have achieved positive results through the application of intestinal probiotics such as Bifidobacterium pseudolongum, Lactobacillus johnsonii and Lactobacillus rhamnosus preparation in the prevention and treatment of bladder cancer. We have observed that the abundance of some of these probiotics such as Lactobacillus in the intestine is closely related to RA, either promoting or inhibiting (33, 34). So, the relationship between RA, gut flora, and bladder cancer is very subtle, and it is therefore necessary to further explore the interaction between these three factors in order to gain a deeper understanding.

2 Vitamin A metabolism and its role in bladder cancer

2.1 Absorption, transport and metabolism of vitamin A

The human body lacks the ability to directly synthesize RA, so we primarily obtain it from our diet. It is possible to absorb preformed vitamin A directly from animal foods, such as liver or fish, in the form of retinol, retinal, RA, and retinyl esters, which can be directly absorbed into the bloodstream by the gut and stored in the liver (35). However, for our bodies, food sources rich in β-carotene are the primary source of vitamin A. In the gastrointestinal tract, β-carotene is broken down and released, subsequently converted into retinal and retinol with the help of β-carotene oxygenase (BCO) (36). It then binds to retinol-binding protein 4 (RBP4) and is transported through the bloodstream to the liver for further metabolism. In addition, retinoids and β-carotenes can be directly absorbed from food, packaged as chylomicrons, and enter the bloodstream through the lymphatic system (37) (Figure 1).

Target cells take up retinol that binds RBP4 from the blood through retinoic acid 6 (STRA6). Alternatively, retinol and β-carotenes from chylomicrons are taken up by target cells through lipoprotein-specific receptors (38). After entering the target cells, β-carotenes are converted to retinol by BCO. However, retinol has poor water solubility, so to enhance its transportation within cells, it binds with cellular retinol-binding proteins (CRABPs), which helps it exert its metabolic activity more effectively (39). Then, retinol is converted into RA through the action of retinol dehydrogenases (RDHs) and aldehyde dehydrogenases (ALDHs) (37, 40). The enzyme lecithin retinol acyltransferase (LRAT) finally converts RA and retinol into esterified products (36). RA, being the most active molecule among retinoids and the primary component of metabolically active vitamin A, activates the RA signaling pathway, which controls cell proliferation, differentiation, and apoptosis (41). It can be oxidized into non-biologically active compounds by the enzyme cytochrome P450 (CYP26) or transported to the nucleus by binding with CRABP or fatty acid binding protein 5 (FABP5) to activate the RA signaling pathway and exert its biological activity (42, 43).

RARs are classified as members of the steroid/thyroid hormone nuclear receptor superfamily, and RXRs are their indispensable eheterodimerization partners, all of which exist in the form of three para-homologs (RARα, RARβ, and RARγ as well as RXRα, RXRβ, and RXRγ) (44). There are more than 500 genes currently dependent on RA signaling, and activation of different isomers can lead to different biological effects (45–47). After entering the nucleus through CRABP, RA binds to RAR-RXR heterodimers and affects gene expression, which can be described as a molecular switch (48). RARs attach to the co-repressors NcoR1 and NcoR2 when RA is absent, and the co-repressors serve as bridges to connect a polymer complex with histone deacetylase activity (49). The complex has the ability to remove the acetyl group from the end of the histone to retain the chromatin's condensation state and prevent the target gene from being transcribed. Conversely, co-repressors are dissociated from the RAR-RXR heterodimers and replaced by the co-activators such as nuclear receptor co-activator (NcoA1, NcoA2, and NcoA3) when RA binds to RAR. These co-activators may acetylate lysine residues in histone H3 and H4 or act as a platform to let other proteins or complexes on DNA change

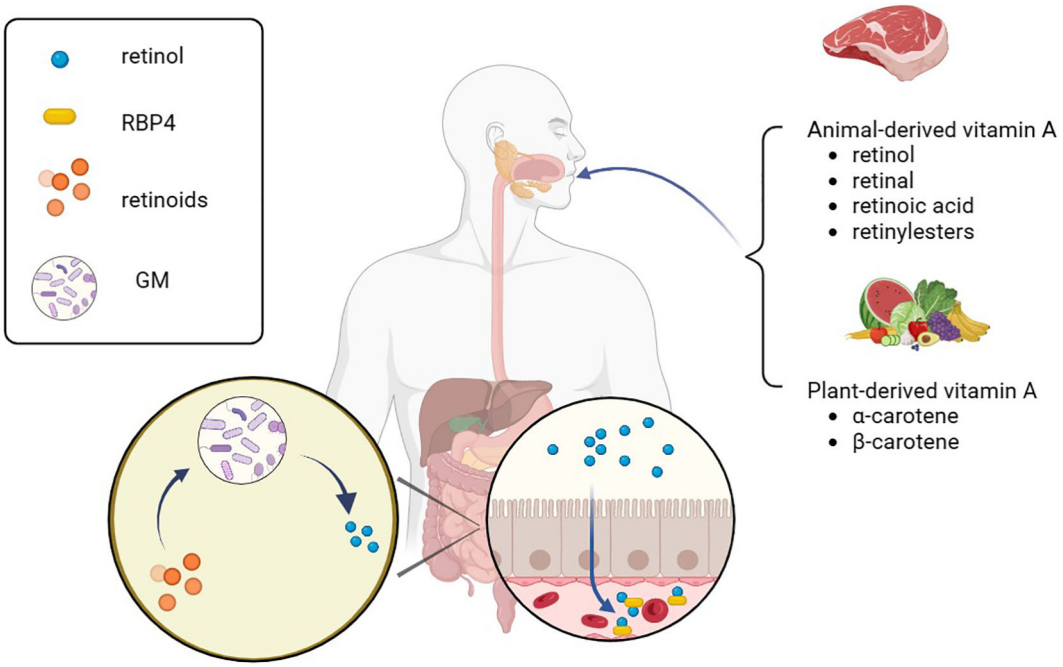

FIGURE 1
Vitamin A absorption. Various animal-derived vitamin A is first converted into retinol in the gastrointestinal tract, then absorbed into the blood through the intestine, combined with RBP4 for transport in the blood, and finally transported to the liver for storage. A part of phytogenic vitamin A can be converted into retinol by β-carotene oxygenase and absorbed, and the other part can be integrated into chylomicron with retinol and enter the blood circulation through lymphatic reflux. RBP4, retinol-binding protein 4; GM, gut microbiota.

dynamically and rebuild nucleosomes (50). Finally, RA triggered modification of the chromatin, activation of the transcription machinery, and transcription of the target gene (Figure 2). Furthermore, it has been reported that after being transported to the nucleus through FABP5, RA can also bind to the peroxisome proliferator activating receptor (PPARβ/δ) to regulates the expression of genes that control cell proliferation, metabolism, and other vital functions (51, 52). But this conclusion is still controversial.

2.2 The effect of RA signaling pathway in bladder cancer

It has been demonstrated that RA induces the differentiation of mouse embryonic stem cells (ESC) into urinary tract epithelial cells in a vitro environment (53). Gandhi et al. (54) emphasized the crucial role of RA in maintaining adult urothelial homeostasis and confirmed the involvement of the RA signaling pathway in urothelial specification, homeostasis, and regeneration. Their study suggested that RA synthesized in stromal compartments acts as a critical regulator of urothelial maintenance through Wnt, Bmp, and Shh signaling. Moreover, RA also plays a significant role in tumor invasion and migration. A study (55) revealed that RA effectively inhibits the expression of matrix metalloproteinase-13 (MMP-13) mRNA, which is known to promote tumor invasion and metastasis by enhancing extracellular matrix degradation during tumor growth. Based on Wang et al. (56) experiments, he demonstrated that synthetic RA 4-HPR increases E-cadherin expression, as well as increased cell adhesion, promoting its translocation to the nucleus and inducing epithelioid cell transformation while reducing cell invasion capability. Additionally, RA can reverse epithelial-mesenchymal transition (EMT) and inhibit the invasion and migration of bladder cancer cells.

Table 3 summarizes the *in vitro* study results of retinoids in bladder cancer cell lines. The RA plays a significant role in the proliferation, differentiation, migration, and invasion of bladder cancer cells, which makes it a key player in the disease's development and progression. As a result, low serum levels of carotene and retinol are often seen among bladder cancer patients (9, 10, 12). In light of the important role of the retinoid signaling pathway in bladder cancer, restoring retinoid function could be a potential therapeutic option to prevent and treat bladder cancer. The different signal transduction pathways in retinoids have been shown to interfere with cell cycle progression in a variety of human cancer cells, particularly by regulating cyclins, CDKs, and cell cycle inhibitors (63, 64). Wang et al. (65) co-cultured RA with bladder cancer cells EJ and found that RA could significantly inhibit the growth of bladder cancer cells and reduce the expression of mutant P53 in cells. Zou et al. (62) evaluated the effects of three types of retinoids, namely all-trans-retinoic acid (ATRA), N-4-hydroxyphenyl-retinamide (4-HPR), and 6-[3-(1-adamantyl)-4 hydroxyphenyl]-2-naphthalene carboxylic acid (CD437), on the growth, apoptosis, cell cycle, and receptor expression of bladder cancer cells. They found that cells exposed to all three retinoids

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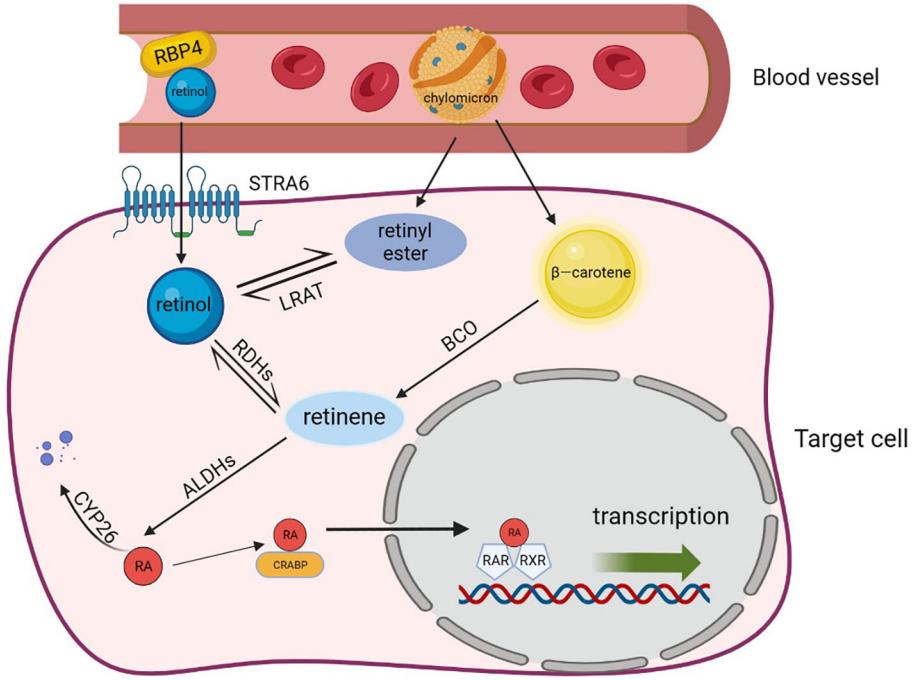

FIGURE 2
Retinoic acid metabolism. Retinol, β-carotene and chylomicron enter target cells through specific receptors and are transformed into the most active RA after a multi-step enzymatic reaction. RA binds different protein transporters and then interacts with different receptors. It also binds to CRABP and is transported to the RAR/RXR dimer to activate transcription, which can regulate the expression of genes such as cell proliferation and metabolism. In addition, RAs that are not transported to the nucleus are eventually degraded by CYP26 and lose functional activity. RBP4, retinol-binding protein 4; STRA6, stimulated by retinoic acid gene 6 protein; LRAT, lecithin retinol acyltransferase; RDHs, retinol dehydrogenases; ALDHs, aldehyde dehydrogenases; RA, retinoic acid; FAB5, fatty acid binding protein; CRABP, cellular retinol-binding proteins; PPAR, peroxisome proliferator activating receptor; RAR, retinoic acid receptor; CYP26, cytochrome P450 family 26.

exhibited varying levels of apoptosis, G1 cell cycle arrest, and growth inhibition. Numerous animal experiments have also evaluated the chemoprophylaxis and treatment effects of RA on animal models of bladder cancer. Among the carcinogens, N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN) is commonly used, as it is closely related to certain carcinogens found in tobacco smoke and exhibits remarkable bladder specificity (66, 67). Several studies (26, 68) have demonstrated that RA can significantly reduce urothelial

TABLE 3 The effects of retinoids in bladder cancer cell lines.

Retinoids	Application	Molecular and phenotypic effects	Reference
ATRA	RT112	Inhibition of epidermal growth factor-induced cell proliferation.	(57)
	HT-1376	Inhibits cell proliferation by inhibiting the activity of related transcription factors	(58)
	T24	Inhibition of cellular retinol-binding protein-II expression Direct inhibition peroxisome proliferator-activated receptor PPARβ/δ	(59)
4-HPR	T24	Promote the expression of E-cadherin and promote the transfer of β-catenin from the nucleus to the cytoplasm	(56)
	RT4 UM-UC-9/10/14	Inhibition of cell growth and the induction of apoptosis	(60)
13-cis-RA	NHU	inhibition of squamous metaplasia and reverting to basal phenotype	(61)
ATRA CD437 4-HPR	RT4 T24 UM-UC-2/3/6/10/13/14	Induction of apoptosis and G1 cell cycle arrest, and the inhibition of cell growth	(62)
ATRA 9-cis-RA 13-cis-RA	RT4 T24	Inhibit the expression of matrix metalloproteinases	(55)

ATRA, all-trans retinoic acid; 4-HPR, N-(4-Hydroxyphenyl)-retinamide or fenretinide; 9-cis-RA, 9-cis-retinoic acid; 13-cis-RA, 13-cis-retinoic acid.

atypia and apoptosis, decrease the incidence of urothelial carcinoma, and effectively inhibit BBN-induced urothelial carcinoma.

Taken together, the RA signaling pathway is implicated in developing and progressing bladder cancer, and the use of exogenous RA supplementation may prove to be an effective method of preventing bladder cancer occurrence as well as postoperative recurrence. However, the current clinical use of RA supplementation is limited due to variations in the expression and distribution of PPAR and RXR subtypes in the human urothelium and the potential toxic effects of vitamin A (69, 70). In addition, pharmacological applications of RA also have limitations, such as short half-life, poor water solubility, sensitivity to light, heat and oxidants, and rapid degradation during digestion, leading to low bioavailability and bioaccessibility (71, 72). Therefore, addressing these challenges is crucial for improving the effectiveness of RA supplementation.

3 Promoting effect of gut microbiota on RA pathway

Therefore, it is evident that vitamin A undergoes a complex series of pathways encompassing absorption, metabolism, RA production, and subsequent activation of the RA signaling pathway. Any disruption in these processes can potentially impact the efficacy of vitamin A. Consequently, this review provides a comprehensive overview of the influence exerted by intestinal flora on these intricate pathways.

3.1 Promotes RA absorption through bile acids

As mentioned above, various forms of vitamin A precursors are present in different foods, and in the small intestine, they are absorbed mostly at the proximal part. β-carotene from plant-based foods is absorbed by small intestinal epithelial cells through passive diffusion after forming micelles with bile acids and dietary fats (73). However, animal-derived retinyl esters must be converted to retinol by retinyl ester hydrolases (REHs) before being absorbed by intestinal cells, and they are not directly absorbed by the intestine as retinol esters in the intestinal lumen (74). The primary enzymes involved in retinol ester hydrolysis in the intestinal lumen include pancreatic triglyceride lipase (PTL), carboxyl ester lipase (CEL) and the intestinal brush border membrane enzyme phospholipase B (PLB) (75), among which PTL is the most important REH in the intestinal cavity (76). It has been shown that bile acid sequestrants can lower serum levels of total carotenoids in humans, and bile acids can enhance the activities of PTL and CEL enzymes, promoting the absorption of retinol and its derivatives from animal-derived foods. In addition to absorption by intestinal epithelial cells, retinoids and β-carotenes can also be incorporated into chylomicrons along with triglycerides, cholesterol esters, phospholipids, cholesterol, and proteins in the Golgi apparatus of intestinal epithelial cells. These chylomicrons are then transported into the lymphatic circulation and subsequently re-enter the bloodstream through the lymph system. This process is also influenced by bile acids, as impaired chylomicron excretion has been observed in the absence of bile acids (59). In a mouse model of chylomicron retention disease, severely impaired fats and vitamins A and E absorption were observed, along with significantly reduced growth rates (77). Therefore, it is essential for the digestion, absorption, and dissolution of fat-soluble vitamin A from food that the concentration of bile acids in the small intestine is high.

Cholesterol is the raw material used to synthesize bile acids, which are a class of cholenoic acids. By passing through the tubule membrane of the gallbladder, they are synthesized in the liver and secreted into bile. The duodenum releases cholecystokinin after eating, stimulating the contraction of the gallbladder, which releases bile acids into the intestinal cavity for digestion (78). Approximately 95% of bile acids are then reabsorbed into the ileum and return to the liver through the portal vein, where they are once again secreted into bile (79). However, a portion of bile acids (approximately 200 to 800 mg per day in humans) escapes reabsorption in the gut and reaches the colon, where they are further metabolized by the gut flora, resulting in the production of secondary bile acids with increased hydrophilicity (80, 81).

Bile acids are metabolized by the gut microbiota, leading to secondary bile acids after three modifications are completed: uncoupling, 7α-dehydroxylation, and differential isomerism. An enzyme known as bile salt hydrolase (BSH) works on bile acids conjugated with glycine or taurine, which is required to perform 7α-dehydroxylation. Song et al. (82) investigated individuals from 11 groups across six continents and reported that the classification and identification of intestinal bacteria BSHs differed in taxonomy and abundance of BSHs in the human intestinal microbiome. The decoupled free bile acids were further transformed into secondary bile acids by 7α-dehydroxylation by microorganisms. Michael et al. (83) analyzed the signaling pathway of cholic acid dehydroxylation and found that certain strains, such as Bacteroides, Clostridium, Escherichia, Eubacterium and Lactobacillus, with the core bai gene cluster could induce dehydroxylation. In addition, the molecular modification of bile acids by intestinal bacteria also includes differential isomerization, which is the main process to enrich the diversity of intestinal bile acids.

It can thus be concluded that gut flora can influence bile acid pool size and bile acid composition in secondary forms, as confirmed in a study by Swann et al. (84), who observed that bile acid diversity was significantly reduced in sterile or antibiotic-treated rats while taurine-binding bile acid abundance was significantly increased. Further comprehensive studies (85) on gut microbiota, bile acid and vitamin A metabolism revealed that remodeling or alteration of gut microbiota resulted in lower bile acid levels, consequently reducing the absorption of vitamin A. Moreover, these studies support the hypothesis that the entire gut flora has a role in vitamin A metabolism.

3.2 Gut microbiota affects RA content by influencing the content of Ra-related enzymes

The content of RA is not only influenced by intestinal absorption but is also closely related to the levels and activities of RA synthesizing

and degrading enzymes, such as ALDHs and CYP26. ALDHs are primarily found in the liver and intestines and comprise three main subtypes, namely ALDH1A1, ALDH1A2 and ALDH1A3, among which ALDH1A1 is the most abundant (37). The main function of ALDH1A1 is to participate in the second step of retinol oxidation, which oxidizes the retinol transported into cells into RA. Comparatively, CYP26 also comprises three subtypes, namely CYP26A1, CYP26B1 and CYP26C1, among which CYP26A1 has the strongest catalytic activity and can degrade RA into inactive hydroxylated and oxidized derivatives (86). An *in vivo* study (42) on trans-retinoic acid and colon cancer found decreased ALDH1A1 and ALDH1A3 protein expression, while ALDH1A2 protein expression remained unchanged in colon cancer progression with alterations in the gut microbiome. In addition, CYP26A1 colon transcription levels increase 3-8 times during the progression of the disease. In addition, the decrease of ALDH1A1 and increase of CYP26A1 were also corrected to a certain extent after the recovery of gut microbiota with antibiotics. Another study (87) found that feeding mice with Bifidobacterium infantis 35624 increased ALDH content in dendritic cells of the intestinal tract, resulting in a further rise in RA content.

The above studies found that gut microbiota was strongly correlated with retinoic acid (RA) metabolic enzymes. Further investigations have shown that this effect is mediated through lipopolysaccharides (LPS), a microbial product and Gram-negative bacteria's outer membrane component (88). LPS interacts with toll-like receptor 4 (TLR4), its natural immune receptor, leading to the activation of signaling pathways. TLR4 signaling cascades activate the PI3K/Akt and NF-κB signaling pathways, resulting in subsequent biological effects (89). In an *in vivo* study, CYP26A1 and CYP26B1 mRNA expression was significantly suppressed in the liver of rats treated with LPS of P. aeruginosa in the presence of RA. Furthermore, Song et al. demonstrated the induction of dysbiosis in a mouse intestinal model through LPS injection, followed by subsequent administration of LPS into chicken embryos which resulted in an upregulation of retinal dehydrogenase 2 (RALDH2) mRNA expression (90). Additionally, quantitative PCR analysis revealed decreased expression levels of cytochrome P450 enzymes, namely Cyp26a1 and Cyp26c1, which are involved in RA metabolism. Furthermore, the study examined antioxidant enzymes and found that LPS treatment up-regulated mRNA expression of antioxidant enzymes such as glutathione peroxidase (GPX1), catalase (CAT) and NAD(P)H quinone dehydrogenase 1 (NQO1). Based on these, the researchers proposed that LPS induces oxidative stress by activating TLR, thereby influencing the levels of RA metabolic enzymes. Another *in vitro* experiment (91) showed similar results and attributed the results to LPS activation of the NF-κB pathway.

In summary, the gut microbiota can increase RA content by promoting RA synthesis enzymes and inhibiting RA degrading enzymes. This action is likely the result of the interaction between bacterial LPS and TLR signaling. It is worth noting that LPS can be further converted into fat micelles in the gastrointestinal tract, promoting the absorption of β-carotene and retinol.

3.3 Gut microbiota promotes the conversion of β-carotene to retinol in the intestine

After consuming fruits or vegetables rich in β-carotene, the compounds undergo various physical and chemical metabolic processes in the digestive tract, such as chewing and fermentation. Some of them can be processed into chylomicrons, which enter the bloodstream and are eventually transported to target cells. Within the target cells, β-carotene is enzymatically broken down into retinol. Other β-carotenes must be converted into retinol within the intestine before being combined with RBP4 for absorption. In short, plant-derived β-carotene needs to be converted into retinol to exert its biological functions, and this conversion process is primarily mediated by BCO, which is a highly potent enzyme found in various tissues of mammals, including jejunal epithelial cells, intestinal mucosa, liver, kidney, lung and brain. There are three paralogs of BCO: 15,15'-β-carotene oxygenase (BCO1), 9',10'-β-carotene oxygenase (BCO2), and RPE65 (92). In the context of liver and intestinal tissues, BCO catalyzes the cleavage of β-carotene, splitting it in the middle to produce two retinal molecules. These retinal molecules are further oxidized to form RA (93).

As early as 1998, Grolier et al. (94) studied the biotransformation of carotenoids into retinoids in rat intestines and investigated the relationship between their bioavailability and the abundance of intestinal flora. Their results suggested that gut microbiota might influence absorption of carotenoids and retinoids, as well as their bioactivities. However, there have been no studies confirming the direct regulation of BCO by the gut microbiome. So far, only a metagenomics study (95) identified a gene in the human gut genomic library that shares homology with BCO. In a subsequent metagenomic study (96) of the human gut, certain Gram-positive and Gram-negative bacteria were found to possess brp/blh genes encoding bacteriorhodopsin-related protein-like homolog protein (Blh) and bacterioopsin-related protein (Brp), which is not homologous to BCO but has similar activity to BCO. Further experiments (97) were conducted to construct strains with brp/blh gene deletions, and the retinoid levels in the medium were measured. The results demonstrated that β-carotene levels were 3.8 times higher and retinol levels were 3.7 times lower than the wild type, thus validating the genomic prediction results. In recent years, brp/blh genes have been reported in proteobacteria, including Sphingopyxis alaskensis, Novosphingobium aromaticivorans and mycobacteria such as Mycobacterium tuberculosis (98). These genes encode enzymes that convert β-carotene into retinal, which could explain the role of the gut microbiota in facilitating vitamin A metabolism. Therefore, the gut microbiota may enhance the efficiency of vitamin A absorption from food by converting β-carotene to retinal in the gut or liver and encoding enzymes that exhibit similar effects to BCO, which may thus enhance the effect of RA downstream.

3.4 Regulating the expression and activity of RAR/RXR

Once transported into the nucleus by CRABP, RA exerts its signaling function by binding to various nuclear receptors and regulating downstream gene transcription. These nuclear receptors include RARs α, β, and γ, RXRs α, β and γ, and PPAR β/δ. In an investigational study (99), the mRNA expression of RAR was compared between normal and malignant bladder tissue specimens from human patients. The findings revealed a significant reduction in the level of RAR mRNA, particularly RARβ2 mRNA, among individuals with bladder cancer. RARs serve as substrates for various serine/threonine kinases, including PKA, PKC, and CDK7, which can phosphorylate them. The level of phosphorylation significantly impacts the activity of RARs. A study (100) revealed that Akt interacts with RARα and phosphorylates its DNA binding domain at Ser (90) residue, leading to a significant inhibition of its activity. Thus, the number and activity of RA receptors are also crucial for proper RA signaling.

Notably, the gut microbiota has been found to be closely associated with the content and activity of these nuclear receptors. Yuan et al. established an animal model of gut microbiota dysbiosis through administration of antibiotic mixtures to mice and observed a significant increase in serum IGF-1 levels due to dysbiosis-induced elevation of SCFAs. Consequently, this led to the activation of the IGF-1/Akt pathway and subsequent regulation of RAR phosphorylation. suggesting that SCFAs inhibit the RA response by enhancing RAR phosphorylation through the IGF-1/Akt pathway in cases of disrupted intestinal microecology (101). Moreover, additional studies (90, 91), by exposing chicken embryos to dysbacteriosis-derived LPS, revealed that intestinal microbiota can influence RA receptor activity through SCFAs and affect RA receptor expression through LPS induction. qPCR data showed that the mRNA levels of RAR (α, β, γ) and RXR (α, β, γ) in the cells were significantly changed after exposure to LPS. Another *in vitro* study (102) showed that the mRNA expressions of RARα and RARγ in hepatic stellate cells (HSCs) were significantly decreased following LPS treatment with an autophagy regulator. The expression of RARα and RARγ was restored after pretreatment with autophagy inhibitors, confirming that LPS may reduce RA receptor levels by activating autophagy.

4 Potential effect of gut microbiota on bladder cancer, also related to vitamin A

In summary, the intestinal microbiota plays a significant role in the absorption, synthesis, degradation, and regulation of retinoic acid (RA) and its receptors, highlighting the critical role for gut microbiota in RA signaling. However, the impact of the gut microbiota extends beyond RA metabolism. Emerging research has revealed that the gut microbiota can influence bladder cancer through various mechanisms. It is important to note that retinoic acid can also intervene in the anti-tumor effects mediated by the intestinal microbiota. This further reveals the close relationship among gut microbiota, retinoic acid, and bladder cancer, highlighting the critical role of the gut microbiota in this context.

4.1 Effects of gut microbiota on tumor

Intestinal microbiota refers to the trillions of microorganisms, including phages, viruses, bacteria, protists, worms and fungi, that colonize the intestinal tract. According to statistics, about 3.8 × 1013 bacteria colonize the intestinal tract, mainly comprising bacteroidetes and actinomyces (103, 104). Advances in 16SRNA gene sequencing and bioinformatics analysis have deepened our understanding of the intestinal microbiota, revealing its crucial role in human physiology and health (105). In addition to facilitating the digestive process and helping the body absorb nutrients from food (106), the gut microbiome also influences host metabolism (107), produces antibacterial substances that mediate the integrity of the intestinal barrier to protect the host from pathogens (108), and regulates host immunity to aid in the removal of harmful substances from the gut (109). In a number of studies (110, 111), it has been shown that gut microbiota diversity plays a critical role in human health. Under physiological or pathological conditions, an imbalance or disruption in the balance of the intestinal microbiome, known as gut microbiota disorder, can occur. Besides impairing the intestinal microbiome's functions, this disorder may also contribute to cancer's development and progression. For instance, an imbalance in the gut flora can cause inflammation that leads to colon cancer (112), and recent research on esophageal cancer similarly reported an imbalance in the flora (113).

It has been shown that a dysbiotic intestinal microbiota contributes to the onset and progression of cancer in several studies. The impact of intestinal microbiota on tumors can be categorized into several aspects. Firstly, it is thought that the gut microbiome contributes to tumor progression by inducing chronic inflammation and immunological responses. Through antigen presentation and activation of pattern recognition receptors, such as toll-like receptors, NOD-like receptors, and G-protein-coupled receptors, the intestinal microbiota can activate immunoinflammatory signaling pathways and influence inflammatory immune response (114). This imbalance of intestinal flora can regulate changes in inflammatory factors, thereby promoting tumor progression. For instance, certain intestinal bacteria may activate the NF-κB or STAT3 pathway to induce the production of cytokines such as IL-10 and IL-17, which are believed to promote tumor cell proliferation and metastasis (115). Secondly, gut microbiota produces specific metabolites, including short-chain fatty acids, tryptophan metabolites and secondary bile acids, which can either promote or inhibit tumor occurrence and development (116, 117). In colorectal cancer, intestinal secondary bile acids have been found to activate carcinogenic pathways such as TGR5/STAT3, WNT/beta-catenin, and NF-kB signaling, thus promoting tumorigenesis (116, 118, 119). Conversely, some short-chain fatty acids, particularly acetate, propionate, and butyrate, have been shown to inhibit the development of colorectal cancer. A recent meta-genomic and metabonomic analysis revealed decreased levels of butyrate-producing bacteria in colorectal cancer patients, suggesting the potential role of butyrate levels in colorectal cancer development

(120). Furthermore, the gut microbiome can promote tumor development by causing DNA damage, promoting cell growth and apoptosis, and modulating the immune response. Notably, E. coli is a prominent example, as it can directly cause genomic instability and DNA damage (121) (Figure 3).

Furthermore, the impact of gut microbiota on tumors is also manifested in its influence on the tumor immune microenvironment. The tumor microenvironment (TME) serves as an internal milieu for the survival and proliferation of cancer cells, comprising various immune cells such as T lymphocytes, B lymphocytes, natural killer cells, and tumor-associated macrophages. The human immune system functions to conduct immune surveillance by identifying and eliminating abnormal cells; henceforth, tumors must evade or suppress this immunosurveillance to sustain their progression. TME is highly conducive to microbial invasion, colonization, and proliferation. A study (122) has demonstrated that intestinal microorganisms can migrate to the TME and induce immunosuppression. Similarly, Zhang et al. have confirmed that gut flora can prompt hepatocytes to recruit myeloid-derived suppressor cells (MDSCs) and produce tumor-promoting and anti-inflammatory chemicals such as TGF-β and IL-10, thereby establishing an immunosuppressive microenvironment that ultimately contributes to the development of cholangiocarcinoma (123). Furthermore, metabolites derived from gut microbiota also impede anti-cancer immunity. For instance, tryptophan metabolite produced by Lactobacillus, can activate aromatic hydrocarbon receptors in tumor-associated macrophages, thus inhibiting the infiltration of cytokines and immune cells in pancreatic cancer (124).

4.2 Effects of gut microbiota on bladder cancer, needs further exploration

It is worth noting that one study (125) found that immune cells, intestinal microbiota, metabolites, and cytokines can leave the intestinal tract through the blood circulation and induce corresponding pathological changes, indicating that the role of intestinal microbiota in promoting tumors may extend beyond the gastrointestinal tract to other areas, including bladder cancer. However, current studies have mainly focused on the relationship between bladder cancer and the urinary microbiome, leading to a relatively limited number of studies on the intestinal microbiome.

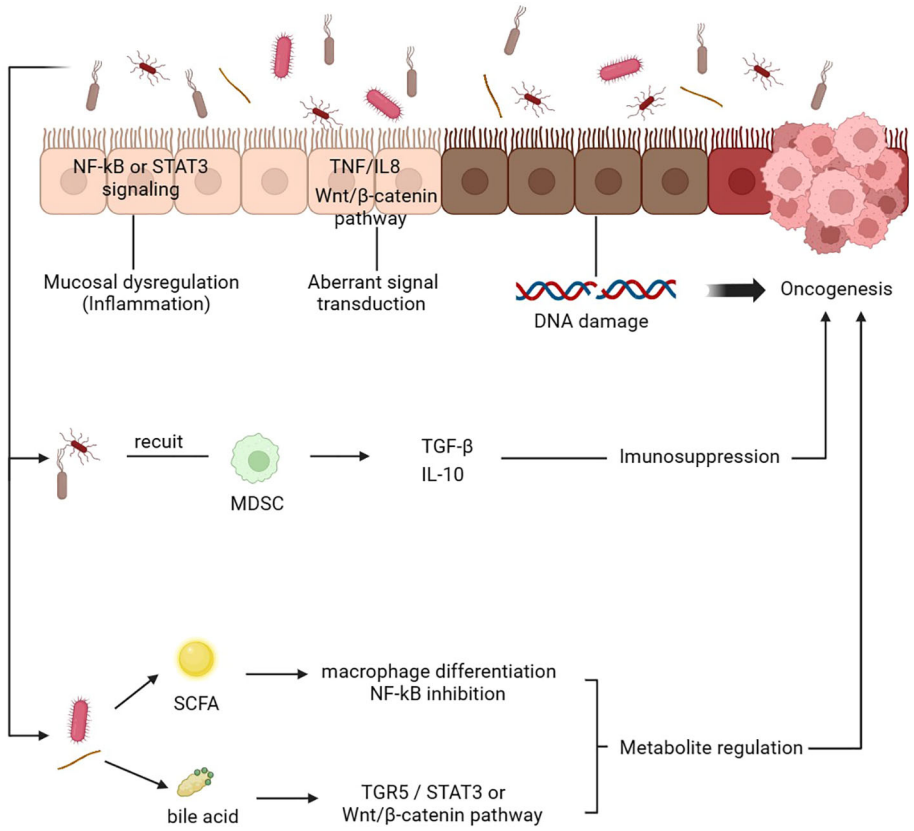

FIGURE 3
Mechanism of gut microbiota mediating tumor development. 1. The gut microbiota induces chronic inflammation by activating the NF-kB or STAT3 pathways and various tumorigenic-related pathways. 2. Abnormal signaling pathways TNF/IL-8 and Wnt/β-catenin promote the metastasis and invasion of tumor cells. 3. The gut microbiota induces DNA damage and cell proliferation. 4. Gut microbiota recruit MDSCs release active mediators, thus mediating immunosuppression and promoting tumorigenesis. 5. The gut microbiota can change the content of various metabolites. For example, SCFA can induce the differentiation of macrophages and inhibit the NF-kB pathway, while bile acids can activate multiple pathways to affect the tumor microenvironment and the occurrence and development of tumors. DC, dendritic cell; SCFA, short-chain fatty acid; MDSC, myeloid-derived suppressor cells.

Although it has been proposed that there might be a correlation between the intestinal microbiome and urinary microbiome, no direct comparisons have been made between the changes in the urinary and intestinal microbiomes in the same patient to confirm this conjecture (126). The most direct evidence linking the gut microbiota and bladder cancer comes from a study comparing the gut microbiota composition of bladder cancer patients with that of a normal population, which revealed alterations in the gut microbiota composition and significant differences in metabolite concentrations, such as butyric acid (29). In addition, the influence of dietary intervention on the intestinal microbial composition of mice on bladder cancer has been assessed in several *in vivo* experiments (28, 127, 128). The results demonstrate that normalizing the intestinal microbial composition through dietary intervention repairs the intestinal physiological barrier, reduces inflammation and immune response, inhibits bladder cancer progression, and enhances sensitivity to radiotherapy and chemotherapy. Lactic acid bacteria have been shown to be beneficial in avoiding the return of superficial bladder cancer in two clinical trials that compared their effectiveness to that of other biologics in preventing tumor recurrence following transurethral excision of bladder tumors (30, 129). These studies have offered preliminary evidence of the tight association between bladder cancer and gut microbiota, but further study is required to clarify the precise processes behind this association.

4.3 Auxiliary effects of vitamin A on tumor toxicity of gut microbiota

Interestingly, it has been observed that not only does the stable intestinal microbiota have a positive effect on the RA signaling pathway, but the level of vitamin A also has an important impact on the homeostasis of the intestinal microbiota, suggesting a mutually reinforcing positive feedback relationship. Micronutrient food sources, such as vitamins A, appear unlikely to have a significant impact on the gut microbiome, but studies have suggested that certain micronutrient signals, such as vitamin A, may first be amplified by inducing secretory mediators in intestinal epithelial cells and other stromal cells, leading to a stronger signal and influence on luminal microbes (130). This notion is supported by studies showing that vitamin A-deficient mice have impaired intestinal structural integrity and reduced Paneth cell numbers but increased secretion of goblet cells and mucins and that these secreted mucins, antimicrobial peptides and proteins have specific effects on the microbiome of these animals (131). Since mucin formation by goblet cells and low levels of antimicrobial peptides are two examples of how vitamin A deficiency affects the phenotypic and function of intestinal epithelial cells, it follows that these changes can impact the number and makeup of symbiotic bacteria in the gut. Studies have shown that vitamin A alleviates inflammation, enhances intestinal epithelial barrier function, and influences gut bacterial diversity *in vivo* (132, 133). Vitamin A deficiency leads to a specific reduction in the gut microbiome, ecological imbalance, impaired immune system function, and increased susceptibility to gastrointestinal infections or injuries (134). Mice treated with vitamin A or RA have shown higher gut microbiota diversity and altered bacterial composition (33, 135). Similar results have also been reported in clinical studies, which revealed significant differences in the gut microbiota composition among vitamin A intake groups (136). A study (34) on the stage-dependent effect of all-trans retinoic acid on lupus found that after two weeks of all-trans retinoic acid treatment, the abundance of intestinal lactobacillus decreased while clostridium increased, indicating that treatment with all-trans retinoic acid significantly altered the abundance of bacteria in the gut.

5 Conclusion

There is clear evidence indicating that the gut microbiota can enhance the absorption of RA by facilitating the transformation of vitamin A and influencing bile acid metabolism. It can also modulate the levels of RA by affecting key enzymes involved in RA synthesis and degradation. In addition, as summarized in the third part of the article, studies have also highlighted the potential impact of intestinal flora on bladder cancer through the production of specific metabolites or modulation of urethral microbiota, and RA has demonstrated a certain efficacy in modulating tumor-associated gut microbiota. Overall, the intestinal microbiota can contribute to the anti-tumor effects of the RA signaling pathway at multiple levels. However, direct evidence linking intestinal microbiota to enhanced inhibitory effects of RA in bladder cancer is currently lacking. Furthermore, the toxic effects of intestinal microbiota on bladder cancer have been demonstrated, and RA has been shown to play a significant role in the anti-tumor effects of the gut microbiota. The interaction between RA and the gut microbiome enhances the anti-tumor effects of each other. Consequently, any alterations in retinoic acid or the gut microbiota can disrupt this positive cycle, leading to an adverse feedback loop. Therefore, solely supplementing exogenous vitamin A may not provide optimal preventive effects for bladder cancer patients, as their gut microbiome may undergo alterations during the development and progression of the disease. Currently, synthetic retinoic acid drugs are being utilized in clinical practice to overcome the limitations of short half-life and poor patient tolerance. Encouragingly, these drugs have exhibited satisfactory therapeutic effects while maintaining good patient tolerability (137, 138). Additionally, promising feedback has been obtained from studies investigating intestinal probiotics (30–32). Although no reports exist regarding their combined application for bladder cancer treatment, it is reasonable to anticipate that further elucidation of the interplay between these three factors will pave the way for novel strategies in bladder cancer prevention and treatment.

Author contributions

BQ conceived the manuscript, and PL performed the literature search and drafted the manuscript. JZ and TC edited tables and figures. WL and QC collected the data. JRZ and LZ reviewed and polished the manuscript. All authors have approved the final version submitted and agree to its submission to this journal.

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Conflict of interest

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

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Association of nicotine dependence and gut microbiota: a bidirectional two-sample Mendelian randomization study

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Background: Nicotine dependence is a key factor influencing the diversity of gut microbiota, and targeting gut microbiota may become a new approach for the prevention and treatment of nicotine dependence. However, the causal relationship between the two is still unclear. This study aims to investigate the causal relationship between nicotine dependence and gut microbiota.

Methods: A two-sample bidirectional Mendelian randomization (MR) study was conducted using the largest existing gut microbiota and nicotine dependence genome-wide association studies (GWAS). Causal relationships between genetically predicted nicotine dependence and gut microbiota abundance were examined using inverse variance weighted, MR-Egger, weighted median, simple mode, weighted mode, and MR-PRESSO approaches. Cochrane's Q test, MR-Egger intercept test, and leave-one-out analysis were performed as sensitivity analyses to assess the robustness of the results. Multivariable Mendelian randomization analysis was also conducted to eliminate the interference of smoking-related phenotypes. Reverse Mendelian randomization analysis was then performed to determine the causal relationship between genetically predicted gut microbiota abundance and nicotine dependence.

Results: Genetically predicted nicotine dependence had a causal effect on *Christensenellaceae* (β: -0.52, 95% CI: -0.934–0.106, P = 0.014). The *Eubacterium xylanophilum* group (OR: 1.106, 95% CI: 1.004-1.218), *Lachnoclostridium* (OR: 1.118, 95% CI: 1.001-1.249) and *Holdemania* (OR: 1.08, 95% CI: 1.001-1.167) were risk factors for nicotine dependence. *Peptostreptococcaceae* (OR: 0.905, 95% CI: 0.837-0.977), *Desulfovibrio* (OR: 0.014, 95% CI: 0.819-0.977), *Dorea* (OR: 0.841, 95% CI. 0.731-0.968), *Faecalibacterium* (OR: 0.831, 95% CI: 0.735-0.939) and *Sutterella* (OR: 0.838, 95% CI: 0.739-0.951) were protective factor for nicotine dependence. The sensitivity analysis showed consistent results.

Conclusion: The Mendelian randomization study confirmed the causal link between genetically predicted risk of nicotine dependence and genetically predicted abundance of gut microbiota. Gut microbiota may serve as a biomarker and offer insights for addressing nicotine dependence.

KEYWORDS

gut microbiota, Mendelian randomization, nicotine dependence, causality, genetics

Introduction

The use of tobacco products is responsible for the deaths of nearly 8 million people worldwide each year (1) and is a significant contributor to lung cancer and cardiovascular disease (2). Although many people are aware of the detrimental effects associated with smoking, the presence of nicotine, a highly addictive substance in tobacco products makes it difficult for individuals to quit (3, 4). Nicotine is known to reinforce smoking and tobacco use behaviors that establish and sustain nicotine dependence. The majority of smokers require some form of assistance to quit, as only approximately 4% of smokers are able to quit on their own successfully (1). Nicotine dependence often presents with physical manifestations, including an increase in tolerance, withdrawal symptoms, and reduced ability to control behavior.

Nicotine is quickly absorbed via the oral mucosa and respiratory tract (5), thereby increasing the risk of related cardiovascular, respiratory, and gastrointestinal diseases (6). Smoking also increases the likelihood of developing gastrointestinal diseases, notably inflammatory bowel disease (7), irritable bowel syndrome (8), peptic ulcer disease (9), and gastrointestinal cancer (10). In addition, smoking heightens the risk of gastrointestinal infections (11), including Helicobacter pylori (12).

Gut microbiota comprises a diverse array of microorganisms that colonize the mammalian gut, including bacteria, fungi, archaea, viruses, and parasites (13). The two-way communication between gut microbes and their hosts may influence many immunity- and metabolism-related biological systems, thereby impacting host health (14). Enhancing host immunity is an important function of the gut microbiota (15). The gut microbiota competes for limited nutrients and regulates host immunity to suppress the colonization of exogenous pathogenic microorganisms (16–18). Additionally, the effects of these immune reactions can extend to almost all parts of the human body (15). When there is an imbalance in the gut microbiota and impaired intestinal barrier function, an increase in harmful pathogenic microorganisms may further induce the occurrence and development of diseases, such as hypertension (19), autoimmune hepatitis (20), cancer (21), and others.

Smoking can modify the microbiome in several regions (22), including the periodontal, intestinal, and respiratory tracts, and augments the mechanisms whereby changes in mucosal immune responses, fluctuations in intestinal cytokine levels, alteration in intestinal permeability, and epigenetic modification alter gene expression (23, 24). Prebiotics are undigestible food elements that can selectively promote the growth and function of the colonic microbiota, ultimately improving host health (25). Supplementation of probiotics and the reconstruction of a healthy microbiota in the gut are now considered effective strategies for treating diseases caused by gut microbiota dysbiosis (26–28). Therefore, using appropriate prebiotics to target specific microbial communities may be an effective approach for preventing and treating nicotine dependence. However, the causal relationship and mechanisms between gut microbiota and nicotine dependence are still unclear, which poses obstacles to the prevention and treatment of nicotine dependence. Thus, it is imperative to study the causal link between the gut microbiota and nicotine dependence.

The Mendelian randomization (MR) method is an epidemiological technique (29) that employs genetic variation as an instrumental variable to explore the putative causal effects of exposure on the onset of disease. Building upon the recent large-scale genome-wide association studies (GWAS) on the gut microbiota (30–32) and disease, we employed the Mendelian randomization approach to investigate the causal link between the gut microbiota and the risk of nicotine dependence in this study. This study aims to explore the impact of genetic prediction of nicotine dependence on the gut microbiota, and elucidate the role of the gut microbiota in the pathogenesis of nicotine dependence through genetic prediction. Furthermore, it aims to uncover the potential of genetic prediction of the gut microbiota to aid in the development of novel preventive strategies.

Methods

Study design

The aim of this study was to evaluate the causal relationship between genetically predicted nicotine dependence risk and genetically predicted abundance of gut microbiota using a Mendelian randomization method. The Mendelian randomization design consisted of three components. Firstly, the selection of genetic variants as instrumental variables for nicotine dependence. Secondly, the acquisition of a summary dataset for genetic instruments derived from a genome-wide association study

of nicotine dependence, and finally, obtaining a summary dataset for single nucleotide polymorphism results. These results were used to investigate the impact of GWAS genetic instruments on gut microbiota. Figure 1 outlines the design of the Mendelian randomization study, while Figure 2 presents an overview of the investigation along with a flow chart. The research design of this study follows the reporting guidelines of STROBE-MR (33), and the supplementary files include the checklist based on STROBE-MR and the checklist based on Critical Appraisal Checklist for evaluating Mendelian randomization studies (34). The checklist is elaborated in detail in the supplementary materials.

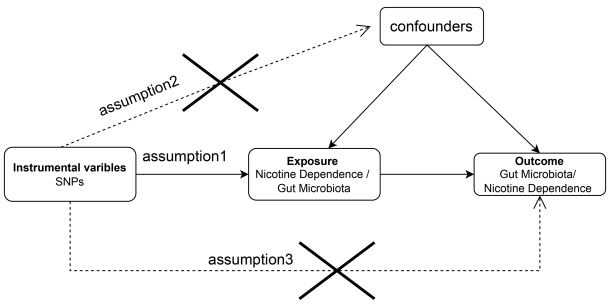

FIGURE 1
Bidirectional two-sample Mendelian randomization between nicotine dependence and gut microbiota abundance outcomes. Directed acyclic graph (DAG) of the causal relationship between nicotine dependence and gut microbiota abundance.

GWAS summary data for nicotine dependence

This study used the genome-wide association meta-analysis data from Hancock DB et al. to investigate nicotine dependence risk (35). The authors conducted a genome-wide meta-analysis on 38,602 former smokers of European and African American descent with mild (N = 17,796; 46.1%), moderate (N = 13,527; 35%), or severe (N = 7,279; 18.9%) nicotine dependence. Genotyping was performed on various genome-wide platforms, and after quality control, 1000G genomic interpolation was used to analyze the genotype data. Linear regression was carried out on the data and adjusted for age, sex, pedigree principal components, and cohort-specific covariates. The Genome-wide association study (GWAS) results were combined using METAL with fixed-effects inverse variance weighting meta-analysis, across all studies with FTND data to maximize statistical power. More than 99% of the former smokers were over 18 years old, and in case of the presence of relatives, family structure was adjusted. Additional information can be found in Table S1.

GWAS summary data for gut microbiota

Data on the composition of human gut microbiota were obtained from the MiBioGen consortium through a large-scale multi-ethnic GWAS study (36). This study involved 18,340 participants from 24 cohorts in countries such as the United

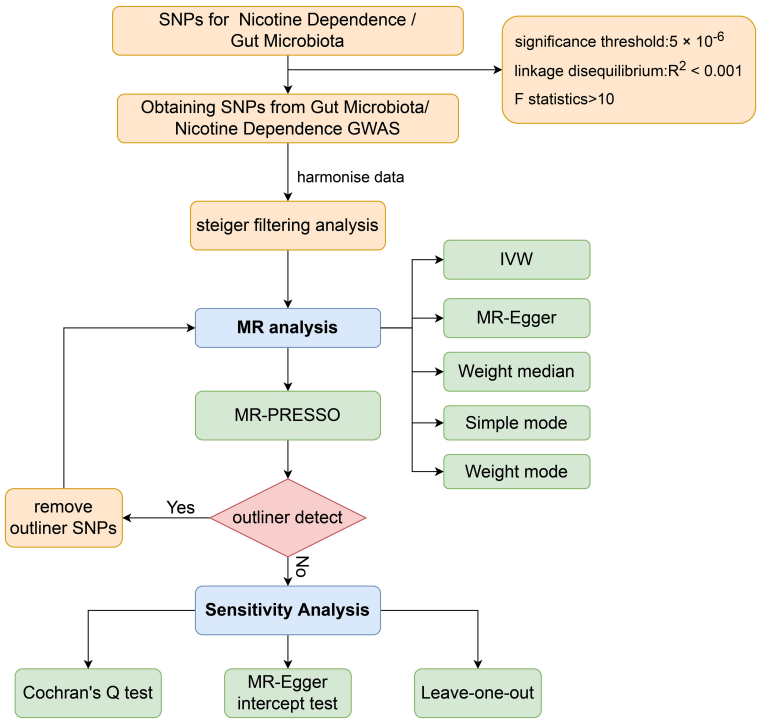

FIGURE 2
Workflow of Mendelian randomization study revealing causality between gut microbiota abundance and risk of nicotine dependence. SNP, single-nucleotide polymorphism; IVW, inverse variance weighted; MR, Mendelian randomization; MR-PRESSO, MR Pleiotropy Residual Sum and Outlier.

States, Canada, Israel, Korea, Germany, Denmark, the Netherlands, Belgium, Sweden, Finland, and the UK. The participants' 16S ribosomal RNA gene sequences and genotyped data were analyzed to investigate the relationship between human autosomal genetic variation and gut microbial communities. The study included 211 taxa comprising 35 families, 20 orders, 16 phyla, 9 orders, and 131 genera.

GWAS summary data for smoking-related phenotypes

The GWAS data for smoking-related phenotypes were obtained from a meta-analysis of GWAS summary association data from 1,232,091 individuals predominantly of European ancestry (37). The smoking phenotypes included age of smoking initiation, smoking initiation, cigarettes per day, and smoking cessation. The authors applied extensive genetic quality control and filtering to the summary statistics provided by each cohort. Imputed variants with an imputation quality below 0.3 (estimated squared correlation between imputed and true dosage) were subsequently removed. Then, the allele labels and allele frequencies of each study were compared with those of the imputation reference panel, and discrepancies were either removed or harmonized. Finally, a meta-analysis was conducted using the software package rareGWAMA based on a fixed-effect model.

Selection of instrumental variables

This study aimed to explore the causal relationship between nicotine dependence and gut microbiota through the Mendelian Randomization analysis of instrumental variables. First, single nucleotide polymorphisms (SNPs) with a genome-wide significance threshold (5×10^{-8}) were selected as instrumental variables (IVs) relating to nicotine dependence. After linkage disequilibrium analysis (R2 < 0.001, clumping distance = 10,000 kb), only one SNP was retained. To ensure a satisfactory number of IVs, a significance threshold of 5×10^{-6} for SNP versus nicotine-dependent phenotypes and a minor allele frequency (MAF) threshold of 0.01 were set. Additional linkage disequilibrium analysis (R2 < 0.001, clumping distance = 10,000 kb) was conducted on the European 1000 Genomes Project data to screen out instrumental variables that could cause biased results.

To assess the potential causal influence of gut microbiota on nicotine dependence, we analyzed genome-wide association data of gut microbes at five taxa levels: order, class, family, genus, and phylum, defining each taxon as a trait. We implemented quality control steps to select the most suitable instrument and ensure the reliability and accuracy of conclusions regarding the causal relationship between the gut microbiome and nicotine risk. Firstly, we selected snps with significance below the genome-wide statistical threshold (5×10^{-8}), but this provided few eligible IVs. Therefore, we lowered the threshold to $P<5\times10^{-6}$, which is more comprehensive. We then used a MAF threshold of 0.01 for variants of interest and performed an LD analysis (R2 < 0.001, with a clumping distance of 10,000 kb) to evaluate LD among the included snps.

We evaluated the strength of instrumental variables by computing the F-statistic as F = R2 × (N - 2)/(1 - R2), where R2 represents the proportion of variation in the exposure factor clarified by each instrumental variable while N represents the sample size for the GWAS that relates to the exposure (38). In turn, R2 is calculated as $(2 \times EAF \times (1 - EAF) \times beta^2)/[(2 \times EAF \times (1 - EAF) \times beta^2) + (2 \times EAF \times (1 - EAF) \times N \times SE(beta)^2)]$, where EAF is the effect allele frequency, beta is the estimated genetic effect of the exposure factor, N is the GWAS sample size for the SNP-exposure correlation, and SE (beta) refers to the genetic effect's standard error (39). Instrumental variables having a F-statistic <10 for weak instruments may suggest a possible bias and need to be removed. Meanwhile, those having a F-statistic >10 are included for further analysis.

Steiger filtering analysis (40) was further used to determine the directional effect of individual instrumental variable SNPs on the outcome. A "TRUE" result predicts the expected direction of association. SNPs that are shown as "False" in the Steiger filtering analysis will be excluded and not included in the subsequent Mendelian randomization analysis.

Statistics analysis

Statistical analyses were performed using R software version 4.2.2, utilizing the R packages "TwoSampleMR" (v.0.5.6) (40), "MRPRESSO" (v.1.0) (41), and "MendelianRandomization" (42) (v.0.7.0) in order to carry out a Mendelian randomization (MR) analysis on the causal relationship between nicotine dependence and gut microbiota. Statistical significance was determined at $p<0.05$ to establish causality.

A multiple test significance threshold was set at 0.05/n (where n represents the number of independent bacterial taxa at the corresponding taxonomic level) due to the numerous comparisons that took place at each character level, such as phylum, class, order, family, and genus. Significance values that fell between the multiple test significance threshold and 0.05 were considered potentially significant.

Two-sample Mendelian randomization

The primary analysis used inverse variance-weighted (IVW) to explore the potential causal relationship between gut microbiota abundance and nicotine dependence. The IVW method is widely applied in Mendelian randomization studies and provides reliable causal estimates in the absence of horizontal pleiotropy. IVW method, namely the meta-analysis of the variant-specific Wald ratios of each variant (i.e., the beta coefficient of the exposure SNP divided by the beta coefficient of the outcome SNP) (43), is used to provide a combined estimate of the causal estimates for each SNP in each potential direction of effect. The IVW method assumes independence of genetic variation and serves as an effective tool for instrumental variable analysis. However, it may ignore the

mediating effects of other risk factors or potential pleiotropy, and bias may occur when there is horizontal pleiotropy between instrumental SNPs (44). In addition to the exposure, it may also affect the outcome of interest through causal pathways, resulting in a violation of the instrumental variable assumption of Mendelian randomization. Therefore, we additionally applied the methods of weighted median, MR-Egger, simple mode, and weighted mode. Based on the assumption of Instrument strength independent of direct effect (InSIDE), the MR-Egger regression method conducts weighted linear regression to generate consistent causal effect estimates independent of IV effectiveness (44). However, the MR-Egger regression method has relatively poor accuracy and is susceptible to the influence of peripheral genetic variation (45). The weighted median method can achieve unbiased estimation of the effect, which does not rely on the InSIDE assumption and thus holds significant advantages over the MR-Egger regression method (46). Specifically, it is an excellent alternative method that allows stable estimation of the causal effect when the weight of the causal effect calculated by effective instrumental variables exceeds 50%, while providing lower Type I error. Finally, the weighted mode method was employed to assess the overall causal effect of a large number of genetic instruments. In many cases, this method yields lower Type I error, less bias, and lower computational complexity compared to the primary methods (47).

Sensitivity analysis

We conducted several sensitivity analyses consisting of tests such as Cochran's Q statistic, funnel plots, leave-one-out analysis, and the MR-Egger intercept test. Cochran's Q test revealed heterogeneity in the instrumental variables in case the p-value was lower than 0.05. The "leave-one-out" method was applied to validate the causal relationship between nicotine dependency and gut microbiota abundance. The fluctuations observed in results before and after SNP removal demonstrate the stability of the causal association between the exposure variable and the outcome. In the MR-Egger intercept test, a non-zero intercept reflects the presence of directional pleiotropy and represents the mean pleiotropic effect of genetic variation (44).

For detecting and correcting pleiotropic outliers, we employed the mendelian randomized pleiotropic residuals and outliers (MR-PRESSO) method (48). The method tested for overall heterogeneity through regressing SNP-outcome associations on SNP-exposure associations. The observed distance of each SNP from the regression was then matched with the expected distance under the original hypothesis of no pleiotropy. Upon detecting outliers in the MR-PRESSO analysis, we removed them and repeated the Mendelian randomization analysis mentioned above.

Multivariable Mendelian randomization

In order to evaluate the moderating effect of smoking-related phenotypes on the causal relationship between nicotine dependence and gut microbiota, SNPs related to smoking-related phenotypes and nicotine dependence were extracted and selected as instrumental variables (IVs) for Multivariable Mendelian randomization (Figure 3). The GWAS p-value threshold between SNPs and phenotypes was set at 5×10^{-6}. A block window of 10,000 kb and r2 = 0.001 were chosen to remove linkage disequilibrium. Cross-instrumental variables were harmonized with the outcome to obtain adjusted assessments for causal effects. A multivariable random-effect IVW model and MR-egger model were constructed in Multivariable Mendelian randomization. Statistical significance was determined at $p<0.05$ to establish causal relationships.

Reverse Mendelian randomization analysis

To investigate whether gut microbiota abundance is associated with the risk of nicotine dependence, we conducted a reverse

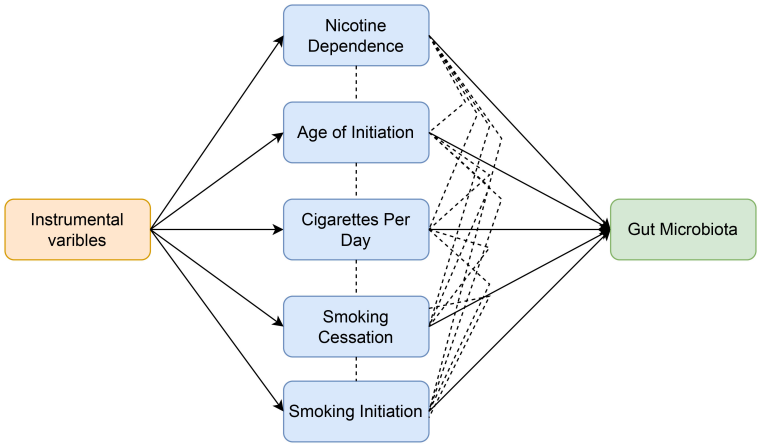

FIGURE 3
Workflow of multivariable Mendelian randomization study revealing causal effect of nicotine dependence on gut microbiota abundance by evaluating the moderating effect of smoking-related phenotypes.

Mendelian randomization (MR) analysis using SNPs related to gut microbiota abundance as instrumental variables (where gut microbiota is the exposure and nicotine dependence risk is the outcome).

Standard protocol approval, registration and patient consent

The GWAS data used in this study were all from publicly available databases. The summary statistics of nicotine dependence, smoking-related phenotypes and gut microbiota abundance do not contain any personal information, and each GWAS has received ethical approval from the relevant ethical review board.

Results

Causal effect of nicotine dependence on gut microbiota

17 SNPs met the instrumental variable screening criteria for nicotine dependence, and all had an F-statistic >10 indicating no weak instrumental bias (Table S2). The F-statistic for the instrumental variable lies between 20.91 and 62.95. In addition, Steiger filtering analysis helped to exclude SNPs with reverse causal directions (three from genus Victivallis and one from genus Prevotella9) (Table S3). As the summary data for SNP results were not extracted for the genera *Erysipelotrichaceae UCG003*, *Lachnospira* and *Blautia*, a mendelian randomization analysis was carried out for the outcomes, including a combined total of 208 gut microbiota classifications to investigate the relationship between nicotine dependence and gut microbiota (Table S4), namely 16 classes, 128 genera, 35 families, 20 orders, and 9 phyla.

The significance thresholds for multiple comparisons at different taxon levels were set at: phylum ($p = 5.560\times10^{-3}$), class ($p = 3.125\times10^{-3}$), order ($p = 2.500\times10^{-3}$), family ($p = 1.429\times10^{-3}$), and genus ($p = 3.906\times10^{-4}$), with adjusted P-values based on Bonferroni correction.

Following univariable Mendelian randomization analysis, a potential causal effect of nicotine dependence on the abundance of five genera, two families, one phylum, and one class was found (Figure 4). According to the results of mendelian randomization analysis based on the IVW method, nicotine dependence caused a causal effect on the abundance of *Actinobacteria*, *Christensenellaceae* (beta: 0.494, 95% CI: 0.113-0.874, P = 0.011) and *Lachnospiraceae UCG001* (beta. 0.254, 95% CI: 0.005-0.503, P =0.045) increased in abundance, where the causal effect of nicotine dependence on Actinobacteria was consistent at both the phylum (beta: 0.215, 95% CI: 0.028-0.402, P = 0.024) and class (beta: 0.198, 95% CI: 0.002-0.394, 0.048) levels. Nicotine dependence was simultaneously induced in *Lactobacillaceae* (beta: -0.426, 95% CI: -0.809-0.043, P = 0.029), *Allisonella* (beta: -0.670, 95% CI: -1.130-0.210, P = 0.004), *Gordonibacter* (beta: -0.480, 95% CI: -0.906-0.053, P =0.027), *Lactobacillus* (beta: -0.416, 95% CI: -0.800-0.032, P =0.034), *Rikenellaceae RC9* gut group (beta: -0.570, 95% CI: -1.070-0.071, P =0.025). The causal effects of nicotine dependence on *Lactobacillaceae* were consistent at the family and genus levels. For the phylum *Actinobacteria*, class *Actinobacteria* and genus *Gordonibacter*, MR-Egger method yielded results in the opposite direction to the IVW method, whereas weighted median, simple mode, and weighted mode methods produced analysis results consistent with the IVW method. However, for family *Christensenellaceae*, family *Lactobacillaceae*, genus *Lachnospiraceae UCG001*, genus *Lactobacillus*, genus *Allisonella*, and genus Rikenellaceae RC9, MRegger, weighted median, simple mode, and weighted mode methods provided effect directions consistent with the IVW method. The scatter plot and forest plot were shown in Figures S1 and S2.

After using multivariable Mendelian randomization to adjust for smoking-related phenotypes (age of smoking initiation, smoking initiation, cigarettes per day, and smoking cessation), nicotine dependence was found to have a causal impact only on *Christensenellaceae* (β: -0.52, 95% CI: -0.934–0.106, P = 0.014). (Table S5 and Figure 5) The detailed information about the instrumental variables used for each covariate in the multivariate Mendelian randomization analysis is recorded in Table S6. Furthermore, conditional F statistics of the instrumental variables for each covariate in the multivariable Mendelian randomization were all greater than 10, indicating no weak instrumental bias (Table S7).

Causal effects of gut microbiota on nicotine dependence

In the first step, 1425 SNPs, which were associated with gut microbiota in phylum, class, order, family, and genus, were identified, excluding *Christensenellaceae* since they did not have suitable instrumental variables. The F statistic of each SNP exceeded 10, ranging from 17.17 to 88.41, indicating no weak instrumental bias (Table S8). Furthermore, Steiger filtering analysis did not identify any SNPs with opposite causal directions (Table S9). We extracted 115 genera, 29 families, 16 orders, and 5 phyla for our instrumental variables. The range of number of IVs from each classification ranged from 3-13.

The significance thresholds for multiple comparisons were set based on the Bonferroni correction: phylum ($p = 5.560 \times 10^{-3}$), class ($p = 3.125 \times 10^{-3}$), order ($p = 2.778 \times 10^{-3}$), family ($p = 1.562 \times 10^{-3}$), and genus ($p = 4.310 \times 10^{-4}$).

By using MR analysis (Table S10), we combined the SNP effects from the gut microbiota and found that one family and seven genera have a potential causal influence on nicotine dependence (Figure 6). According to the IVW approach, the *Eubacterium xylanophilum* group (OR: 1.106, 95% CI: 1.004-1.218, P = 0.041), *Lachnoclostridium* (OR: 1.118, 95% CI: 1.001-1.249, P = 0.048) and *Holdemania* (OR: 1.08, 95% CI: 1.001-1.167, P =0.048) were risk factors for nicotine dependence. Of these, *Lachnoclostridium* had the smallest value of OR. *Peptostreptococcaceae* (OR: 0.905, 95% CI: 0.837-0.977, P =0.011), *Desulfovibrio* (OR: 0.014, 95% CI: 0.819-0.977, P =0.895), *Dorea* (OR: 0.841, 95% CI. 0.731-0.968, P =0.016), *Faecalibacterium* (OR: 0.831, 95% CI: 0.735-0.939, P =0.003) and

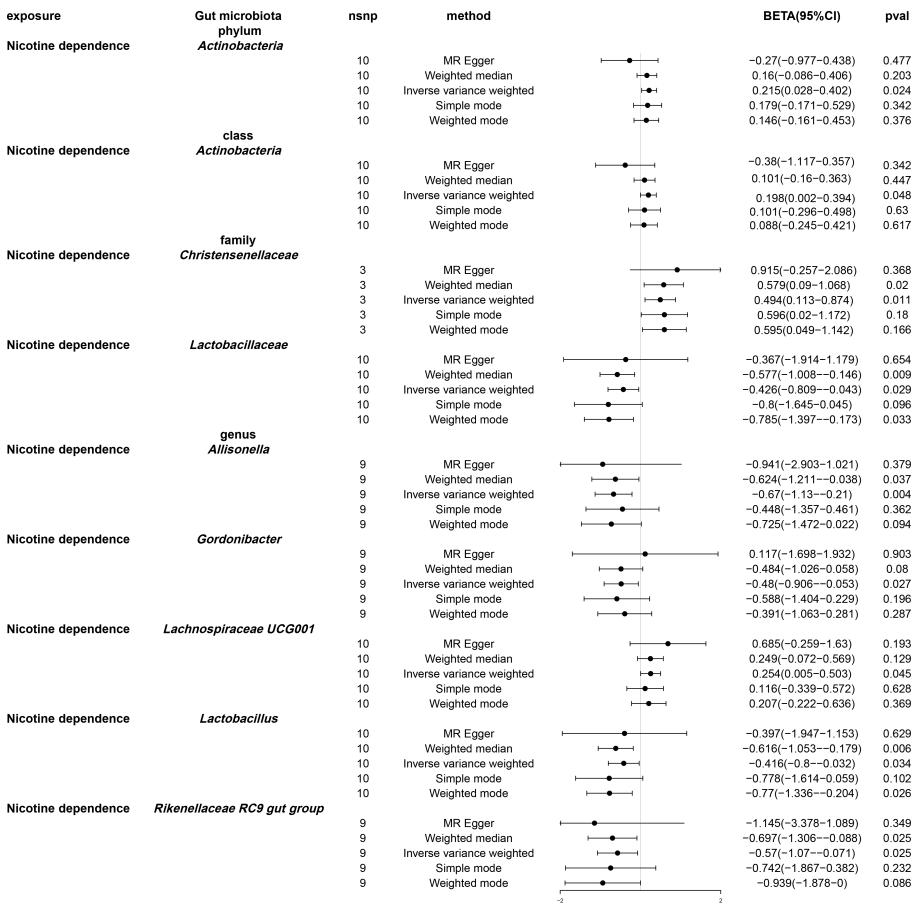

FIGURE 4
Forest plot of Mendelian randomization of two samples as a result of gut microbiota abundance. Causal effects of five Mendelian randomization methods with nicotine dependence as exposure and gut microbiota abundance as outcome. Effect estimates are expressed as effect size (BETA) with 95% confidence intervals (CI). SNP, single nucleotide polymorphism.

Sutterella (OR: 0.838, 95% CI: 0.739-0.951, P =0.006) were protective factor for nicotine dependence, with *Faecalibacterium* having the smallest value of OR. According to the results of other Mendelian analysis methods, for genus *Sutterella* and genus *Dorea*, MR-Egger method yielded results in the opposite direction to the IVW method, while weighted median, simple mode, and weighted mode methods produced analysis results consistent with the IVW method. For the family *Peptostreptococcaceae*, genus *Eubacterium xylanophilum group*, genus *Lachnoclostridium*, genus *Holdemania*, genus *Lachnoclostridium*, and genus *Desulfovibrio*, MR-Egger, weighted median, simple mode, and weighted mode provided effect directions consistent with the IVW method. The scatter plot and forest plot were shown in Figures S3 and S4.

Sensitivity analysis

No evidence was found for horizontal pleiotropy when using the MR-Egger regression intercept method on the gut microbiota and nicotine-dependent instrumental variables (Tables S11–S13). We screened and removed any outliers in the MR-PRESSO analysis and found no horizontal pleiotropy for the gut microbiota or nicotine-dependent instrumental variables (Tables S14 and S15). Furthermore, the majority of Cochrane Q statistics did not show significant heterogeneity ($p > 0.05$) as shown in the Supplementary Material (Tables S16–S18). In cases where heterogeneity was found to be significant, we used a random-effects model with an IVW model. The results of leave-one-out sensitivity and funnel plot were shown in Figures S5–S8.

Discussion

Our study employed a bidirectional Mendelian randomization approach to assess the causality between nicotine dependence and gut microbial abundance. To our knowledge, this is the first mendelian randomization study to examine the causal relationship between nicotine dependence and gut microbial abundance.

Smoking is a complex behavior that encompasses several stages, including initiation, regular smoking, nicotine dependence, cessation, and relapse. While some individuals may maintain low levels of smoking without experiencing dependence (49), others may become heavily dependent, which escalates the challenges

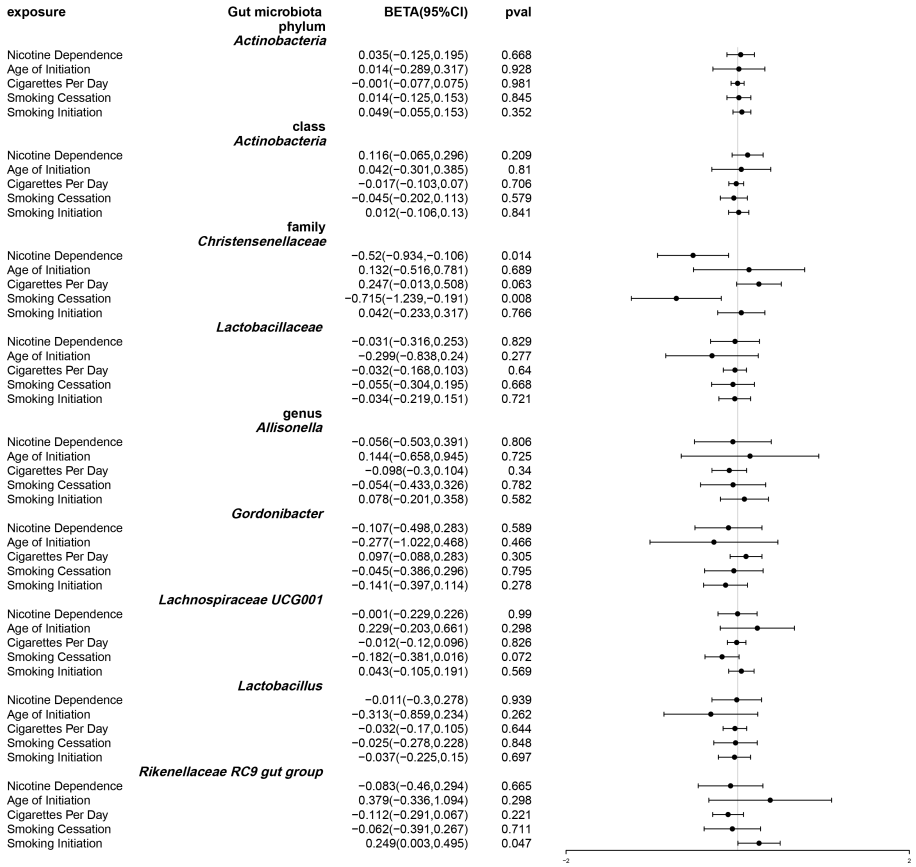

FIGURE 5
Forest plot of multivariable Mendelian randomization. Causal effects of IVW methods with nicotine dependence and four smoking related phenotypes as exposure while gut microbiota abundance as outcome. Effect estimates are expressed as effect size (BETA) with 95% confidence intervals (CI).

associated with stopping smoking and augments the risk of relapse (50, 51). Furthermore, while smoking behavior broadly encompasses various stages, nicotine dependence has a strong genetic component with high heritability rates (up to 75%) coupled with environmental factors (52). Nicotine dependence is a significant predictor of the severity of tobacco withdrawal (53), response to treatments (54), and smoking-related health outcomes (55, 56), which impede smoking cessation success.

Previous studies have found that smokers are often accompanied by alterations in gut microbiota composition. Proposed mechanisms to explain the impact of smoking on the gut microbiota include enhanced oxidative stress (57), alterations in intestinal tight junctions and gut mucin composition (58), and changes in acid-base homeostasis (59). Smoking leads to changes in the composition of the gut microbiota, showing an increased abundance of certain bacteria such as *Prevotella*, *Veloperae*, *Anaplasma*, *Acidophilus*, and *Helicobacter oxysporum*, and a decrease in the abundance of other bacteria such as *Thiotrichales* and *Helicobacter Lachesis* (60). The use of nicotine products leads to known health consequences, but may also be a major cause of intestinal ecological disorders and increased intestinal permeability (14). Smoking cessation partially reversed these microbial alterations, resulting in increased microbial diversity and abundance in certain phyla. In healthy individuals who quit smoking, significant changes were observed in the fecal microbiota, including an increase in the relative abundance of thick-walled bacteria and actinomycetes and a decrease in anaplasma and amoebae (61). Quitting smoking resulted in significant changes in the fecal microbiota of healthy individuals. Although studies have investigated the effects of tobacco use on gut microbiota, previous studies are limited by small sample sizes and inaccurate assessment of tobacco use. Patient inclusion is often based solely on self-reported smoking history, with unstable criteria for assessing smoking frequency, which renders studies susceptible to confounding factors that may not be excluded.

While a prior mendelian randomization study has investigated the relationship between smoking associated phenotypes and gut microbiota (62), no study has examined the relationship between nicotine dependence and gut microbiota. Given the aforementioned limitations of previous studies on smoking and gut microbiota, further research is needed to address the relationship between nicotine dependence and gut microbiota. Therefore, in this study, we used genome-wide association data from patients with nicotine dependence based on FTND scores to investigate the causal relationship between nicotine dependence and gut microbiota using mendelian randomization.

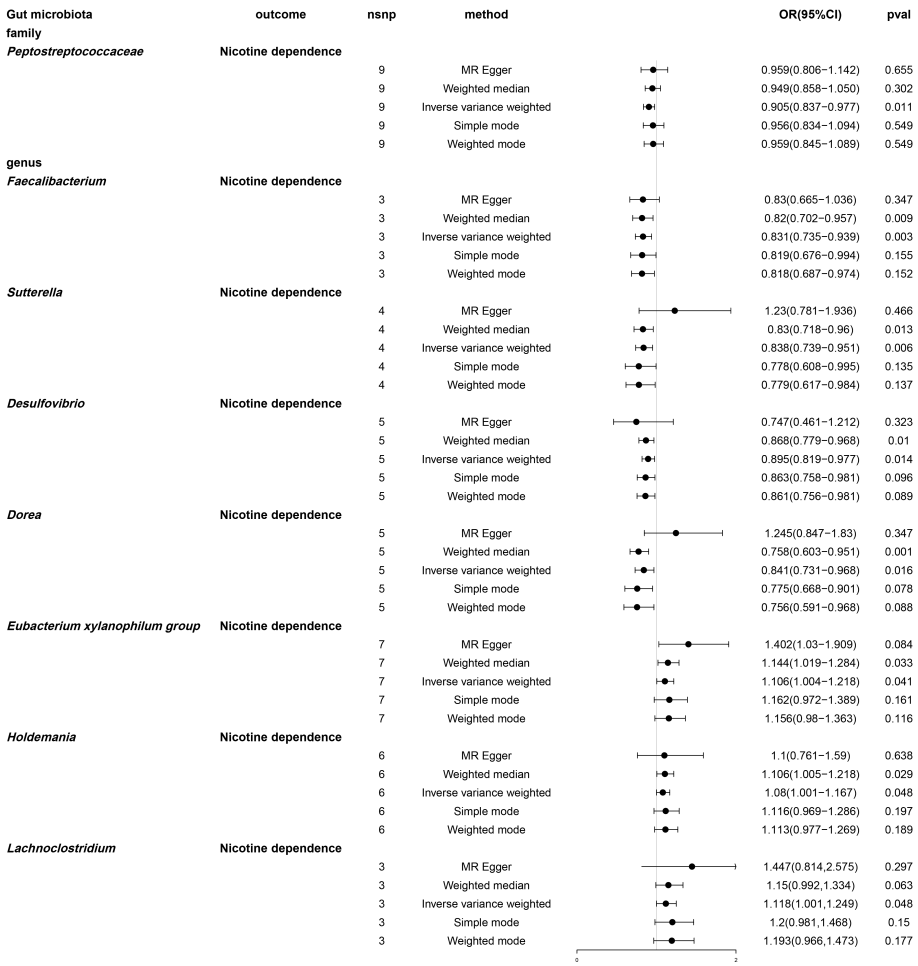

FIGURE 6
Forest plot of two-sample Mendelian randomization with nicotine dependence as an outcome. Causal effects of five Mendelian randomization methods with gut microbiota abundance as exposure and nicotine dependence as outcome. Effect estimates are expressed as odds ratios (OR) with 95% confidence intervals (CI). SNP, single nucleotide polymorphism.

Studies have shown that smoking leads to a higher abundance of *Streptococcus* spp. and *Desulfovibri* in the digestive tract (63), as well as a higher abundance of *Streptococcus* spp. in the upper small intestinal mucosa of smokers (64). Additionally, smokers show a significantly lower abundance of *Faecalibacterium* in the gut (65). In this study, we found that genetically dependent nicotine dependence can lead to a decrease in the gut abundance of *Christensenellaceae*. Furthermore, even after smoking cessation, the effect of decreased *Christensenellaceae* abundance persists. *Christensenellaceae*, belonging to the *Firmicutes* phylum, is widely present in the human gut and mucosa (66–69), and it plays a crucial role in host health. Previous studies have found a negative correlation between the relative abundance of *Christensenellaceae* and host body mass index in different populations and multiple research studies (70). It is also closely associated with glucose metabolism (71, 72) and inflammatory bowel disease (73). *Christensenellaceae* may serve as probiotics to improve health status (74), but further research is needed to elucidate the underlying mechanisms. Previous studies have shown that smoking reduces the abundance of Firmicutes in the gut (60). Our study suggests a causal effect between genetically predicted nicotine dependence and *Christensenellaceae*. Further investigation is needed to explore the potential of *Christensenellaceae* in improving symptoms of nicotine dependence in patients and preventing diseases associated with nicotine dependence.

From the perspective of the effect of gut microbiota on nicotine dependence, a recent study has shown that colonization of *Bacteroides xylanisolvens*, a human gut bacteria, can effectively degrade intestinal nicotine, providing a new target for the treatment of patients with non-alcoholic fatty liver disease (75). This suggests that gut microbiota abundance may influence nicotine metabolism and further impact the disease progression of nicotine dependence. However, there is insufficient evidence from previous studies to assess whether gut microbiota abundance has a preventive or promotive effect on nicotine dependence. Our study found that *Peptostreptococcaceae*, *Desulfovibrio*, *Dorea*, *Faecalibacterium*, and *Sutterella* decrease the risk of nicotine dependence, while the *Eubacterium xylanophilum* group and *Holdemania* increase the risk of nicotine dependence. These findings have not been widely reported previously, indicating a potential contribution of this study to the existing literature.

There are several advantages in our study. Specifically, we employed a biodirectional mendelian randomization analysis to establish the causal association between gut microbiota and nicotine dependence. This approach allowed us to control for confounding factors and minimize the risk of reverse causation. The gut microbiota and nicotine dependence genome-wide association data were retrieved from the largest available GWAS meta-analysis to ensure the statistical robustness of the instrumental variables used in the Mendelian randomization analysis. To minimize the potential impact of weak IV bias, we employed a suitable threshold for the genomic correlation of instrumental variables (p = 5e-06). This threshold was chosen based on the availability of a sufficient number of SNPs with adequate statistical power for most gut flora, effectively avoiding confounding. In contrast, previous studies used a p-value cut-off of 1e-05 (36, 76) or 1e-06 (62) resulting in only a few gut flora receiving 3 or more SNPs for the Mendelian randomization analysis. Consequently, the power of the previous studies might have been insufficient, introducing false negatives. In addition, the previous study ended up including only 41 gut microbiota (62), which may have circumvented the inclusion of a larger number of flora and led to false positives when performing the FDR test for p-value (FDR = p*n/rank). The phenotypes utilized in the prior Mendelian randomization analyses of smoking initiation, lifetime smoking, and daily cigarette consumption, were not clinically practical for age-specific interventions. In contrast, our study of the FTND scale for diagnosing nicotine dependence as a phenotype may offer clinical guidance to those who smoke, but do not meet the diagnostic criteria for nicotine dependence. Employing the FTND scale may help prevent the development of nicotine dependence.

Although our study has several strengths, we acknowledge some limitations. Notably, the p-values in our findings are not robust to the Bonferroni method adjusted for significance. However, it is important to note that our study is hypothesis-driven, based on strong physiological evidence that supports the epidemiologically established link between gut flora and nicotine dependence. To strengthen the results further, future studies may need to include a larger sample size of nicotine-dependent patients. Additionally, the use of multiple comparisons to adjust p-values may increase the risk of false negatives due to the high number of microbial taxa and the multilevel structure (correlation between abundance and microbial strains) and the correlation between nicotine dependence. Therefore, caution should be exercised when interpreting negative results or potentially significant p-values. As with the previous point, future GWAS studies could benefit from increasing the sample size of patients with gut flora and nicotine dependence to reduce the likelihood of such biases. Third, as the majority of participants in the Nicotine Dependent GWAS were of European ancestry, the external validity of our findings to other ethnic groups may be constrained. Given that smoking is more prevalent among men, a disproportionately high number of male patients were included in the nicotine-dependent phenotype. Moreover, gender differences may exist in the composition of the gut microbiota. In our study, out of the nicotine dependence GWAS data employed, 53.2% of the participants were male, and the relatively even sex ratio could alleviate the potential gender bias to some extent. Nevertheless, the summary data from genome-wide association analyses limited our capacity to conduct further subgroup analyses to explore gender-specific discrepancies.

Conclusion

In conclusion, our investigation confirms the causal link between genetically predicted nicotine dependence and gut microbiota, underscoring the interactive impacts of nicotine dependence on gut microbes that might act as novel biomarkers and yield revelations for addressing and avoiding nicotine dependence.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: The GWAS meta-analysis results on nicotine dependence were provided by the dbGaP study of PubMed (study_id=phs001532) and can be downloaded from http://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs001532.v1.p1. The summary data on gut microbiota is from MiBioGen consortium, which can be obtained from the IEU GWAS database (https://gwas.mrcieu.ac.uk/) (GWAS ID: ebi-a-GCST90016908– ebi-a-GCST90017118).The summary data on smoking related phenotypes is from GSCAN consortium, which can be obtained from the University Digital Conservancy Home (https://conservancy.umn.edu/handle/11299/201564).

Ethics statement

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

Author contributions

All authors have been involved in the preparation of the manuscript and approved its submission. Conceptualization: LL, ZC and JZ. Formal analysis: YC, MZ, KJ and JL. Funding acquisition: LL, ZC and JZ. Methodology: JL, SW and LL. Writing original draft: YC, MZ and KJ. Writing-review and editing: YC, MZ, KJ, JL, SW, LL, ZC and JZ. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Genetic association and bidirectional Mendelian randomization for causality between gut microbiota and six lung diseases

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Purposes: Increasing evidence suggests that intestinal microbiota correlates with the pathological processes of many lung diseases. This study aimed to investigate the causality of gut microbiota and lung diseases.

Methods: Genetic information on intestinal flora and lung diseases [asthma, chronic bronchitis, chronic obstructive pulmonary disease (COPD), interstitial lung disease (ILD), lower respiratory tract infection (LRTI), pulmonary arterial hypertension (PAH)] and lung function was obtained from UK Biobank, FinnGen, and additional studies. A Mendelian randomization (MR) analysis was conducted to explore the causal association between gut microbiota and lung diseases.

Results: The genetic liability to lung diseases may be associated with the abundance of certain microbiota taxa. Specifically, the genus *Prevotella* ($p = 0.041$) was related to a higher risk of asthma; the family Defluviitaleaceae ($p = 0.002$) and its child taxon were identified as a risk factor for chronic bronchitis; the abundance of the genus *Prevotella* ($p = 0.020$) was related to a higher risk of ILD; the family Coriobacteriaceae ($p = 0.011$) was identified to have a positive effect on the risk of LRTI; the genus *Lactobacillus* ($p = 0.0297$) has been identified to be associated with an increased risk of PAH, whereas the genus *Holdemanella* ($p = 0.0154$) presented a causal decrease in COPD risk; the order Selenomonadales was identified to have a positive effect on the risk of FEV1($p = 0.011$). The reverse TSMR analysis also provided genetic evidence of reverse causality from lung diseases to the gut microbiota.

Conclusion: This data-driven MR analysis revealed that gut microbiota was causally associated with lung diseases, providing genetic evidence for further mechanistic and clinical studies to understand the crosstalk between gut microbiota and lung diseases.

KEYWORDS

Mendelian randomization, gut microbiota, lung diseases, lung function, chronic lung diseases

Introduction

Respiratory diseases are the leading causes of disability and death worldwide (1) because the lung is a complex and vulnerable organ that is exposed to smoking, environmental degradation, and occupational hazards (2). According to the systematic analysis for the Global Burden Disease Study 2019, lower respiratory infection is the 3rd cause of death. Chronic obstructive pulmonary disease (COPD) is the 6th cause of death (3), and more than 500 million people have chronic respiratory diseases across the world (1). Regardless of the pathophysiological process of infectious or chronic respiratory diseases, the overwhelming immune responses and improper reparative and regenerative processes account for lung structural and functional disorders (4). Gut microbiota is the community of microorganisms living in the digestive tracts, playing a vital role in training host immunity, modulating endocrine function and metabolic rewiring, and producing various biological compounds that affect the host (5). However, the composition of the human gut microbiome is determined and dynamically altered by genetic or exogenous factors, such as diseases, diets, and aging (6). It has been demonstrated that different respiratory diseases can be affected by changes in the intestinal microenvironment and vice versa (7). Emerging studies have indicated that gut microbial species and their derived functional metabolites regulate lung homeostasis, and the dysbiosis of the gut-lung axis contributes to the development and progression of respiratory diseases (8), suggesting that gut microbiota may be a potential causal factor of respiratory diseases. Moreover, gut microbiome-derived small-chain fatty acids (SCFAs) are capable of activating bone marrow hematopoiesis (9). Chiu et al. have shown that the mean proportions of *Acinetobacter* and *Stenotrophomonas* are significantly elevated in COPD patients. Similarly, Wang et al. have found that gut microbiota-derived succinate aggravates acute lung injury after ischemia/reperfusion in mice (10).

However, due to the lack of evidence from randomized controlled studies, it remains unclear whether there is a causality between gut microbiota and lung diseases and lung function. Previous family-based or population-based studies have suggested that many respiratory diseases are associated with genetic variation, and genome-wide association studies (GWAS) have shown that many genetic variants are related to pulmonary traits (11, 12). An MR analysis is capable of employing genetic variants as proxies of exposure to yield the causal estimate of the environmental exposure on the intended outcomes (13) using GWAS, which provides a high degree of evidence and a low susceptibility to confounding factors. Importantly, MR overcomes the constraints of conventional observational studies, such as potential bias from confounding and reverse causation, and produces reliable results (14).

Herein, this study aimed to investigate the causal relationship between gut microbiota and lung diseases and lung function. Using a reverse MR approach, we also explore whether SNPs associated with lung diseases and lung function are causally related to gut microbiota.

Methods

Study settings

A bidirectional two-sample MR (TSMR) analysis was designed to assess the causal relationship between gut microbiota and the risk of lung diseases and lung function. The forward MR analysis was performed to explore the causal effect of each taxon on lung diseases and lung function, while the reverse MR was performed to investigate whether the genetic liability for lung diseases and lung function influenced the abundance of the gut microbiota. The study flowchart is presented in Figure 1.

Data sources

Exposure data sources

Summary statistics of the intestinal flora (211 bacterial taxa) were obtained from a genome-wide meta-analysis by the MiBioGen consortium, comprising 18,340 participants from 24 European cohorts with 122,110 loci of variation (15). After removing 15 taxa without specific species names, 196 bacterial traits (119 genera, 32 families, 20 orders, 16 classes, and 9 phyla) were screened.

Outcome data sources

The traits involved in this study were downloaded in the IEU Open GWAS project (updated to 2023.05.31, $N = 42{,}346$) or FinnGen (https://www.finngen.fi/fi). The genetic variants included in this study were, all or partially, identified from the UK Biobank (16) or FinnGen Research.

Selection of instrument variants

After removing 15 taxa without specific species names, 196 bacterial traits (119 genera, 32 families, 20 orders, 16 classes, and 9 phyla) were screened. Due to the limited number of SNPs available, a locus-wide significance threshold (1×10^{-5}) was adopted to find more potential SNPs related to the outcome, and the minor allele frequency (MAF) threshold with the IVs of interest was 0.01. To ensure the independence of the selected SNPs, the linkage disequilibrium (LD) test was conducted using LD $r^2 < 0.1$ within a clumping distance of 500 kb. However, if SNPs could not be found in the outcome datasets, proxies at the threshold of LD $r^2 > 0.8$ were used if applicable. To avoid weak instrument bias, the F-statistic of each SNP was calculated, and the SNP with $F < 10$ was removed (17). Finally, the process of harmonizing was performed to eliminate the SNPs with incompatible or palindromic (e.g., A/T or G/C alleles) with intermediate allele frequencies (e.g., A/C paired with A/G), and the number of SNPs included in the analysis was more than three.

MR analysis and sensitivity analysis

TSMR was performed to analyze the causality between gut microbiota and lung diseases and lung function. The inverse-variance weighted (IVW) method was adopted as the main method to preliminarily assess the potential causal effects of each bacterial taxon on chronic lung diseases and lung function in the absence of horizontal pleiotropic effects. If the result of the IVW method was statistically significant ($p < 0.05$), a potential causal association between the bacterial taxa and disease was considered. Simultaneously, Cochrane's Q test was used to assess the heterogeneity between IVs, and if heterogeneity was observed ($p < 0.05$), the random-effects IVW model was used to provide a more conservative estimate; otherwise, the fixed-effect IVW

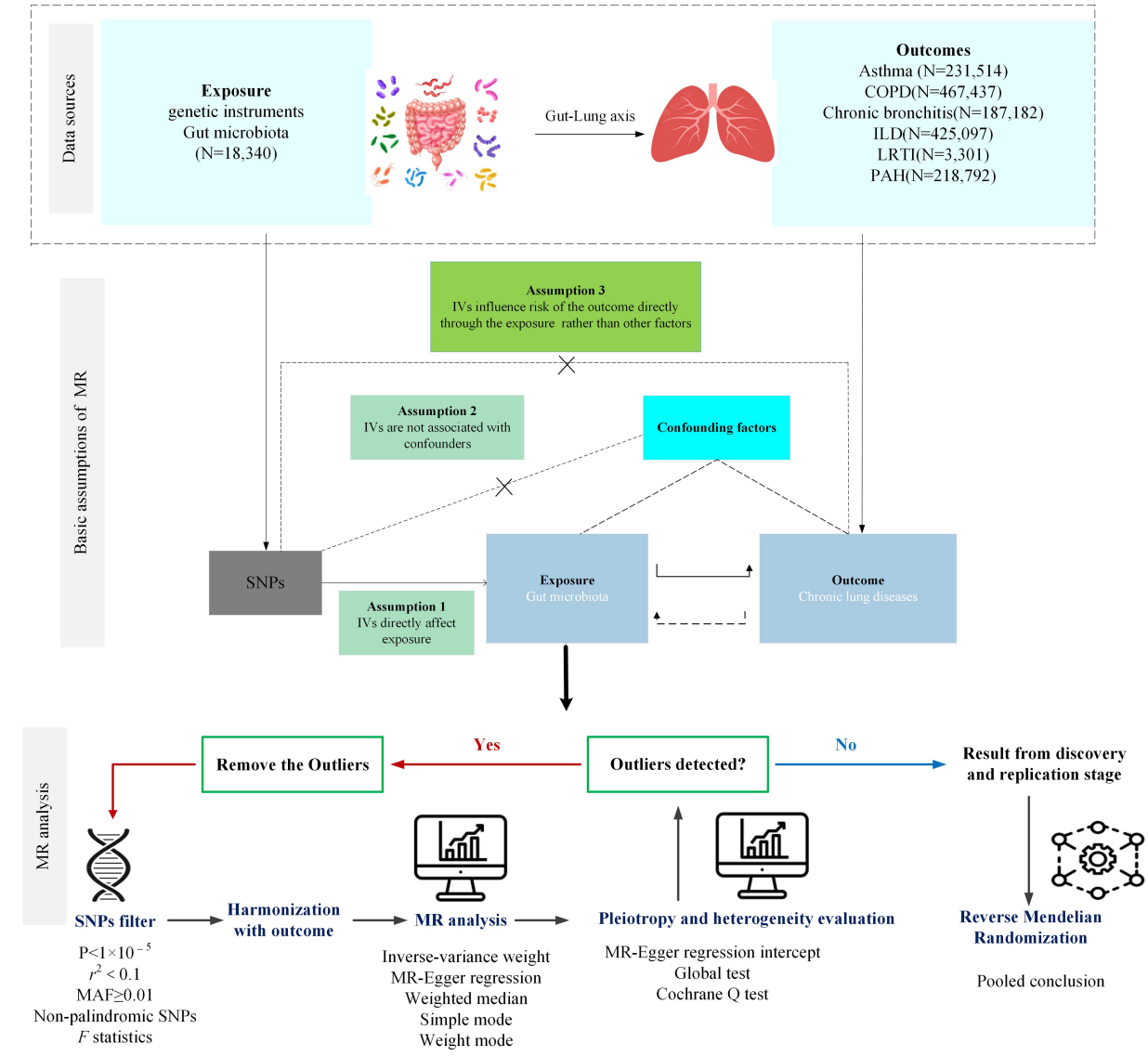

FIGURE 1
Flowchart of MR analysis and assumptions.

model would be applied. Weight median (WM) method, MR-Egger regression, simple mode, and weight mode are the other four MR methods to explore the causality and provide wider confidence intervals (18), of which the WM method could provide a consistent estimate if at least half of the weight comes from valid IVs (19); MR-Egger regression assumes that more than 50% of IVs are influenced by horizontal pleiotropy (20). Similarly, simple mode and weight mode are complementary methods to investigate the causality of the exposure and outcomes.

To test the sensitivity of the results of the above MR analysis, the MR-Egger intercept test and MR-PRESSO global test were applied to test the horizontal pleiotropy among the selected IVs. Leave-one-out analysis was conducted to detect and remove any potential outliers that affect the observed causal correlation. In terms of the significant MR estimates, the Mendelian median pleiotropy residual sum and outlier (MR-PRESSO) test were used to assess the heterogeneity. In detail, the MR-PRESSO global test was used to test whether there exists a horizontal pleiotropy, and the MR-PRESSO outlier test was calculated to remove outliers to adjust horizontal pleiotropy. The value of distributions in the MR-PRESSO analysis was set to 1,000 (21).

Results

Causal effects of gut microbiota and lung diseases

According to the process of selection described above, SNPs of each respiratory disease were screened. The details of the SNPs involved in the TSMR analysis for asthma, COPD, chronic bronchitis, ILD, LRTI, PAH, and lung function are shown in Supplementary Tables S1–S7. Potential causal relationships between gut microbiota and lung diseases were found using TSMR methods before Benjamin and Hochberg correction (Figures 2, 3). MR results

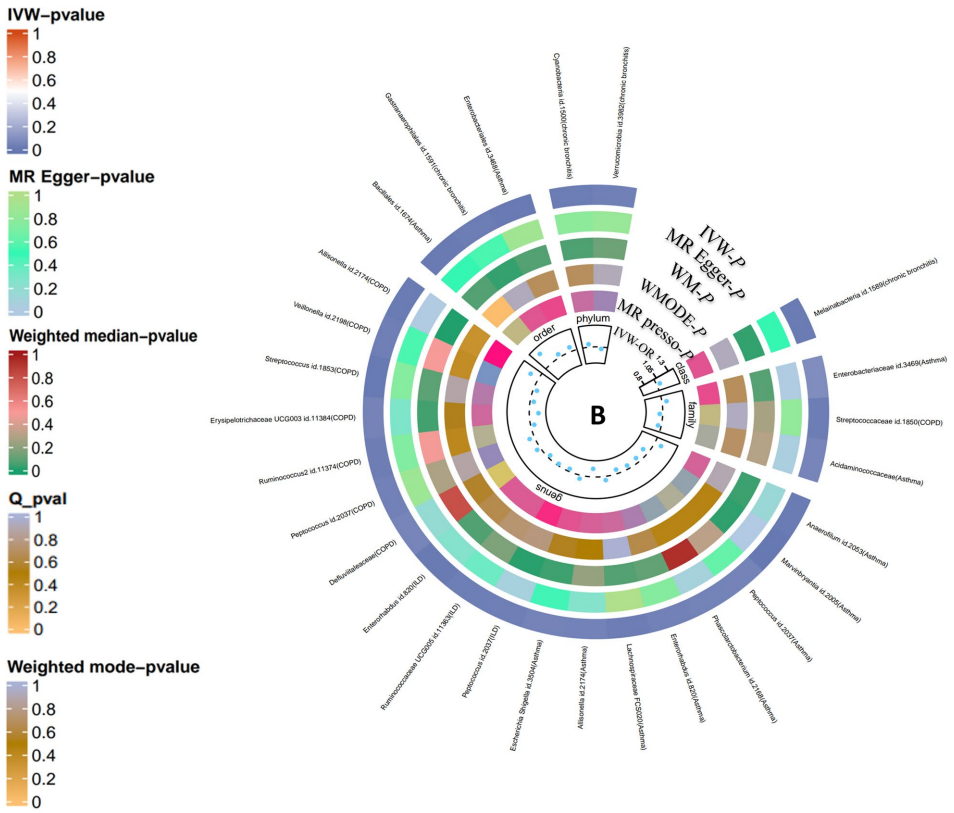

FIGURE 2
Causal analysis of gut microbiota on lung diseases (locus-wide significance, $p < 1*10^{-5}$). From the inner to outer circles, they represent the estimates of: MR-PRESSO, weight mode, weight median, MR-Egger, inverse-variance weighted methods, respectively. And the shades of color reflect the magnitude of p-value.

and sensitivity analysis of the significant relationship between gut microbiota and six lung diseases are shown in Tables 1, 2.

Asthma

The summary GWAS statistics for asthma contain 9,851,867 loci of variants from 21,392 cases and 210,122 controls, according to a definition of asthma. Thirteen causal associations between intestinal taxa and asthma were identified by the IVW method in the sets of IVs ($p < 5 \times 10^{-6}$). The false discovery rate (FDR) method was used to determine the multiple testing significance at each feature level, and after a rigorous Benjamin and Hochberg correction, a marginal significant causal association between gut microbiota and asthma was identified. In particular, only the genus *Prevotella* [IVW: OR 1.14, 95% confidence interval (CI): 1.062–1.227, $p = 0.041$] was related to a higher risk of asthma. To avoid excessive bias effects, Cochrane's Q test was performed to analyze the sensitivity of MR results, and no evidence of heterogeneity was found ($p = 0.688$). Moreover, no horizontal pleiotropy was identified by the MR-Egger intercept test ($p = 0.216$) and the MR-PRESSO global test ($p = 0.657$). The leave-one-out test did not find any horizontal pleiotropy in the genus *Prevotella*, and no outlier was found in the genus *Prevotella* by the MR-PRESSO outlier test. In the reverse MR analyses, family Acidaminococcaceae [IVW: OR 0.942, 95% confidence interval (CI): 0.888–0.999, $p = 0.049$], family Enterobacteriaceae (IVW: OR 1.083, 95% CI: 1.024–1.146, $p = 0.005$), order Bacillales (IVW: OR 1.271, 95% CI: 1.065–1.517, $p = 0.008$), order Enterobacteriales (IVW: OR 1.083, 95% CI: 1.024–1.146, $p = 0.006$), genus *Allisonella* (IVW: OR 0.849, 95% CI: 0.729–0.899, $p = 0.036$), genus *Escherichia–Shigella* (IVW: OR 1.065, 95% CI: 1.003–1.131, $p = 0.041$), genus *Phascolarctobacterium* (IVW: OR 0.936, 95% CI: 0.879–0.997, $p = 0.039$), genus *Anaerofilum* (IVW: OR 1.128, 95% CI: 1.027–1.238, $p = 0.012$), genus *Enterorhabdus* (IVW: OR 0.922, 95% CI: 0.855–0.994, $p = 0.034$), genus *Lachnospiraceae* (IVW: OR 1.069, 95% CI: 1.012–1.131, $p = 0.018$), genus *Marvinbryantia* (IVW: OR 0.89, 95% CI: 0.839–0.946, $p = 1.5e\text{-}03$), and genus *Peptococcus* (IVW: OR 0.914, 95% CI: 0.838–0.997, $p = 0.044$) have been identified to be significantly linked with an elevated or reduced risk of asthma.

COPD

A total of 467,437 individuals (49,647 cases and 417,790 healthy controls) were involved in the causal analysis of gut microflora and COPD (22), including the GWAS data from the international COPD Genetics Consortium (ICGC) and FinnGen with additional studies from the UK Biobank. After the MR analysis, 16 bacterial taxa were identified to be associated with COPD, but only the genus

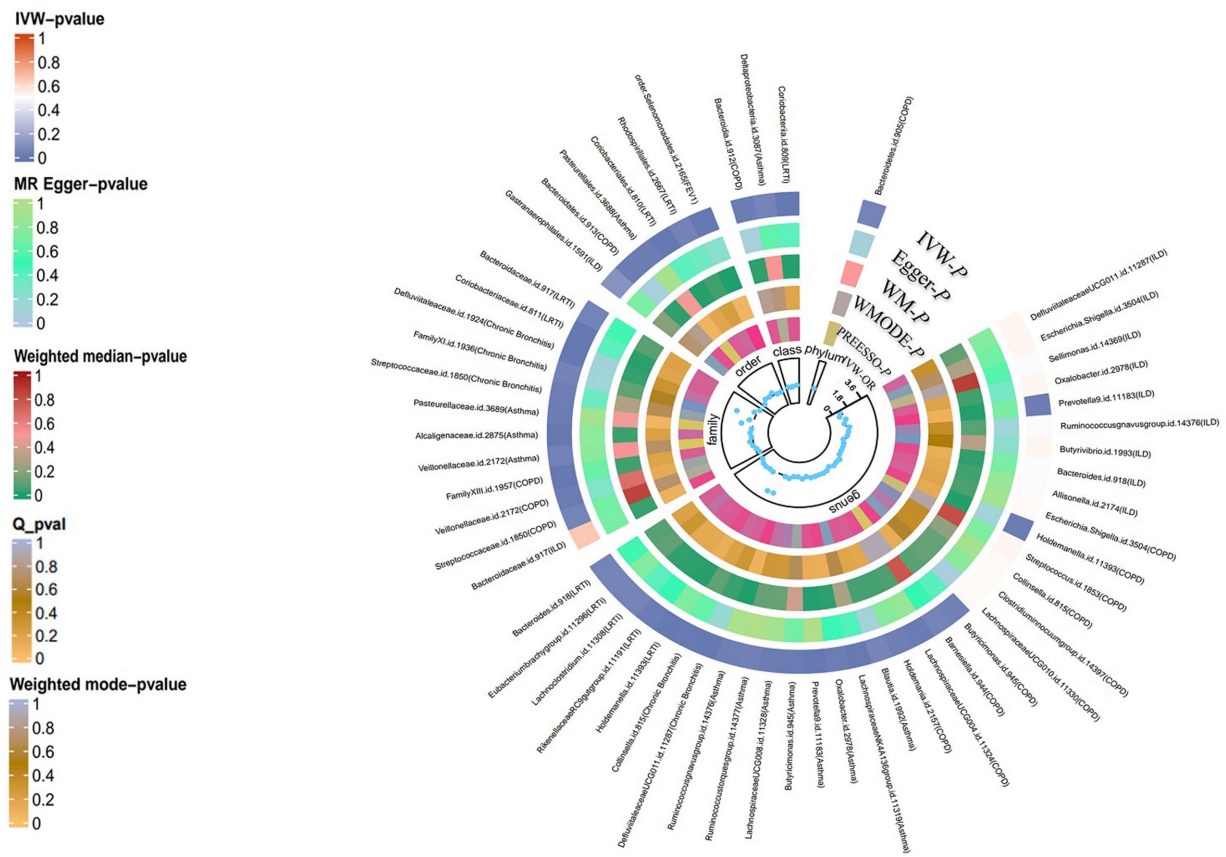

FIGURE 3
Summary-level MR analysis of lung diseases on gut microbiota (locus-wide significance, $p < 1*10^{-5}$). From the inner to outer circles, they represent the estimates of: MR-PRESSO, weight mode, weight median, MR-Egger, inverse-variance weighted methods, respectively. And the shades of color reflect the magnitude of *p*-value.

TABLE 1 MR results of causality of gut microbiota on lung diseases ($p < 1 \times 10^{-5}$).

Exposure	Outcome	Bacterial taxa	N.SNP	IVW		MR-Egger intercept			Cochrane's Q		
				OR	*p*	Intercept	SE	*p*	*Q*	*Q_df*	*Q_p*
Gut microbiota	Asthma	Genus *Prevotella*	15	1.141	4.06E-02	0.014	0.011	0.216	10.981	14	0.688
	COPD	Genus *Holdemanella*	11	0.850	1.54E-02	−0.036	0.028	0.214	6.568	*10*	0.766
	Chronic bronchitis	Genus *Defluviitaleaceae*	9	3.264	2.21E-02	−0.079	0.125	0.548	8.883	*8*	0.352
	Chronic bronchitis	Family *Defluviitaleaceae*	11	3.087	2.17E-03	−0.067	0.099	0.515	9.607	10	0.476
	ILD	Genus *Prevotella*	15	1.347	2.05E-02	0.043	0.027	0.136	21.541	14	0.089
	LRTI	Family *Coriobacteriaceae*	13	1.289	1.06E-02	0.001	0.020	0.977	7.998	12	0.785
	PAH	Genus *Lactococcus*	9	5.593	2.97E-02	−0.102	0.187	0.603	6.001	8	0.647
	FEV1	Order *Selenomonadales*	12	0.931	6.90E-03	0.002	0.005	0.665	18.050	11	0.080

p means *p* value. *Q_df* means degrees of freedom.

Holdemanella (OR: 0.850, 95% CI: 0.782–0.924, $p = 0.0154$) presented a tendency to causally decrease the risk of COPD after the correction. The results of other MR analyses were consistent with their respective IVW results. No heterogeneity was observed by Cochrane's Q test, and the MR-Egger intercept test and MR-PRESSO test also suggested that no horizontal pleiotropy existed. Importantly,

TABLE 2 MR results of causality of lung diseases on gut microbiota ($p < 1 \times 10^{-5}$).

Exposure	Outcome	Bacterial taxa	N.SNP	IVW		MR-Egger intercept			Cochrane's Q		
				OR	*p*	Intercept	SE	*p*	*Q*	*Q_df*	*Q_p*
Asthma	Gut microbiota	family *Acidaminococcaceae*	26	0.942	4.90E-02	0.020	0.012	0.118	20.812	25	0.703
Asthma		family *Enterobacteriaceae*	27	1.083	5.60E-03	0.007	0.012	0.527	22.227	26	0.676
Asthma		order *Bacillales*	19	1.271	7.94E-03	0.001	0.037	0.982	34.485	18	0.011
Asthma		order *Enterobacteriales*	27	1.083	5.59E-03	0.007	0.012	0.527	22.227	26	0.676
Asthma		genus *Allisonella*	14	0.850	3.64E-02	−0.053	0.029	0.097	12.434	13	0.492
Asthma		genus *Escherichia Shigella*	25	1.065	4.06E-02	−0.002	0.012	0.852	23.652	24	0.482
Asthma		genus *Phascolarctobacterium*	26	0.936	3.91E-02	0.017	0.013	0.198	25.246	25	0.449
Asthma		genus *Anaerofilum*	24	1.128	1.16E-02	−0.016	0.019	0.405	14.798	23	0.902
Asthma		genus *Enterorhabdus*	26	0.922	3.41E-02	−0.015	0.015	0.355	22.069	25	0.632
Asthma		genus *Lachnospiraceae*	26	1.070	1.79E-02	0.007	0.012	0.521	11.690	25	0.989
Asthma		genus *Marvinbryantia*	26	0.890	1.54E-04	0.032	0.012	0.015	25.193	25	0.452
Asthma		genus *Peptococcus*	26	0.914	4.36E-03	−0.001	0.018	0.963	25.284	25	0.447
COPD		family *Streptococcaceae*	58	0.946	1.85E-02	−0.006	0.006	0.300	50.268	57	0.724
COPD		genus *Defluviitaleaceae*	58	1.068	4.84E-02	0.017	0.008	0.039	48.007	57	0.796
COPD		genus *Peptococcus*	58	1.093	3.35E-02	0.009	0.010	0.378	61.679	57	0.312
COPD		genus *Ruminococcus*	58	0.934	2.15E-02	0.003	0.010	0.794	68.038	57	0.150
COPD		genus *Erysipelotrichaceae*	6	0.833	3.97E-02	0.036	0.037	0.396	4.054	5	0.542
		genus *Streptococcus*	58	0.934	3.80E-03	−0.004	0.006	0.523	44.913	57	0.877
COPD		genus *Veillonella*	58	0.931	2.58E-02	−0.011	0.008	0.169	59.643	57	0.380
COPD		genus *Allisonella*	46	1.169	1.03E-02	−0.028	0.013	0.040	48.349	45	0.339
Chronic bronchitis		order *Gastranaerophilales*	6	1.061	1.53E-02	−0.007	0.046	0.893	1.313	5	0.934
Chronic bronchitis		phylum *Cyanobacteria*	6	1.055	1.63E-02	0.035	0.042	0.455	3.121	5	0.681
Chronic bronchitis		class *Melainabacteria*	6	1.062	1.39E-02	−0.009	0.046	0.853	1.229	5	0.942
Chronic bronchitis		phylum *Verrucomicrobia*	6	0.965	3.14E-02	−0.008	0.031	0.809	1.388	5	0.926
ILD		genus *Enterorhabdus*	11	0.948	8.33E-03	−0.007	0.009	0.471	7.875	10	0.641
ILD		genus *Peptococcus*	11	0.949	2.45E-02	0.007	0.010	0.490	8.689	10	0.562
ILD		genus *Ruminococcaceae*	11	0.969	2.33E-02	−0.004	0.006	0.498	6.556	10	0.767

the leave-one-out analysis did not detect any outliers in the genus *Holdemanella*. In the reverse analysis, family Streptococcaceae (IVW: OR: 0.946, 95% CI: 0.904–0.924, $p = 0.019$), genus *Defluviitaleaceae* (IVW: OR: 1.068, 95% CI: 1.001–1.141, $p = 0.048$), genus *Peptococcus* (IVW: OR: 1.068, 95% CI: 1.001–1.141, p = 0.048), genus *Ruminococcus* (IVW: OR: 0.934, 95% CI: 0.881–0.989, $p = 0.022$), genus *Erysipelotrichaceae* (IVW: OR: 0.833 95% CI: 0.699–0.991, $p = 0.039$), genus *Streptococcus* (IVW: OR: 0.934, 95% CI: 0.891–0.978, $p = 0.004$), genus *Veillonella* (IVW: OR: 0.931, 95% CI: 0.874–0.991, $p = 0.026$), and genus *Allisonella* (IVW: OR: 1.169, 95% CI: 1.037–1.317, $p = 0.01$) have been found to have a causal relationship with COPD.

Chronic bronchitis

Next, we focused on dissecting the relationship between chronic bronchitis and intestinal flora, and GWAS data were downloaded from FinnGen (www. https://r9.finngen.fi/). In the sets of IVs ($p < 5 \times 10^{-6}$), five causal associations from bacterial taxa to chronic bronchitis were identified by the IVW method. After the Benjamin and Hochberg correction, only two bacterial taxa remained stable. Specifically, family Defluviitaleaceae (OR: 3.086, 95% CI: 1.773–5.374, $p = 0.002$) and its child taxon and genus *Defluviitaleaceae* (OR: 3.264, 95% CI: 1.755–6.071, $p = 0.022$) are identified to have a suggestive positive causal effect on the risk of chronic bronchitis. In the sensitivity analysis, no evidence of heterogeneity in the family Defluviitaleaceae ($p = 0.475$) and genus *Defluviitaleaceae* ($p = 0.298$) was observed by Cochrane's Q test. The results of the MR-Egger intercept test and MR-PRESSO test suggested that no horizontal pleiotropy was found in the family Defluviitaleaceae and its child taxon. The leave-one-out analysis did not detect any outliers in the family Defluviitaleaceae and its child taxon. In the reverse analysis, increasing abundance of the order Gastranaerophilales (IVW: OR: 1.169, 95% CI: 1.037–1.317, $p = 0.01$), phylum Cyanobacteria (IVW: OR: 1.055, 95% CI: 1.01–1.101, $p = 0.0163$), and class Melainabacteria (IVW: OR: 1.062, 95% CI: 1.012–1.114, $p = 0.014$) contributed to the development of chronic bronchitis. In contrast, the abundance of the phylum Verrucomicrobia (IVW: OR: 0.965, 95% CI: 0.934–0.997, $p = 0.0314$) showed a reduced risk with chronic bronchitis.

ILD

Concerning ILD, GWAS data were downloaded from FinnGen (www. https://r9.finngen.fi/). Twelve causal relationships between bacterial taxa and ILD were observed by the IVW method. After the correction and the cross-validation, only one bacterial taxon remained stable. Specifically, a higher abundance of the genus *Prevotella* (OR 1.347, 95% CI: 1.153–1. 573, $p = 0.020$) was related to a higher risk of ILD. Cochrane's Q test was used to test the sensitivity of the MR results, and no heterogeneity was identified ($p = 0.087$). Moreover, the MR-Egger intercept test ($p = 0.136$) and MR-PRESSO global test ($p = 0.111$) suggest that no horizontal pleiotropy exists. Importantly, no outliers were identified by the leave-one-out analysis. In the reverse analysis, the abundance of the genus *Enterorhabdus* (IVW: OR: 0.948, 95% CI: 0.912–0.997, $p = 0.987$), genus *Peptococcus* (IVW: OR: 0.949, 95% CI: 0.907–0.993, $p = 0.024$), and genus *Ruminococcaceae* (IVW: OR: 0.969, 95% CI: 0.943–0.996, $p = 0.023$) presented to have a decreased risk with ILD.

LRTI

As for LRTI, 14,135 cases with 472,349 controls were identified in UK Biobank (23). Ten causal associations from intestinal taxa to LRTI were identified by the IVW method in the sets of IVs ($p < 5 \times 10^{-6}$). After the Benjamin and Hochberg correction, the family Coriobacteriaceae was identified to have suggestive positive causal effects on the risk of LRTI (IVW OR 1.289, 95% CI: 1.122–1.481, $p = 0.011$). The consistent direction and magnitude of the estimates from other MR analyses further confirmed the causal inferences. Similarly, Cochrane's Q test indicated that no heterogeneity was found. Moreover, the MR-Egger intercept test ($p = 0.136$) and the MR-PRESSO global test ($p = 0.111$) were used to avoid horizontal pleiotropy. Finally, leave-one-out analysis further supports that the causalities are not driven by any single SNP. In the context of the causal effects of LRTI on gut microbiota, no bacterial taxon was identified to have a causal association with LRTI.

PAH

PAH is a progressive and incurable vascular disorder characterized by abnormally high blood pressure in the pulmonary artery, contributing to right heart failure with high mortality (24). The genus *Lactobacillus* (OR 5.594, 95% CI: 2.643–14.058, $p = 0.0297$) has been identified to be associated with an increased risk of PAH progression in the set of IVs ($p < 5 \times 10^{-6}$). The sensitivity of the MR results was assessed by Cochrane's Q test, and no heterogeneity was identified ($p = 0.647$). Moreover, the MR-Egger intercept test ($p = 0.603$) and the MR-PRESSO global test ($p = 0.243$) suggest that no horizontal pleiotropy exists. Importantly, no outliers were identified by the leave-one-out analysis. In the reverse analysis, no bacterial taxa were suggested to be associated with PAH.

Lung function

Lung function tests are physiological and non-invasive tests to measure the respiratory function of patients in different situations. In the sets of IVs ($p < 5 \times 10^{-6}$), the aggregate estimate from all SNPs supported a causal impact of gut microbiota on lung function, especially for FEV1. After the Benjamin and Hochberg correction, the order Selenomonadales was identified to have suggestive negative causal effects on the FEV1(IVW OR 0.931, 95% CI: 0.896–0.968, $p = 0.011$). In the reverse analysis, the IVW analysis did not detect any significant causal associations.

Ethics statement

The GWAS datasets used in this study were all publicly available. No additional ethical approval was required.

Discussion

By the use of large-scale GWAS statistics from the UK Biobank and FinnGen, the potential causal relationship between genetically proxied intestinal flora and chronic lung diseases was explored, and five bacterial traits associated with asthma, chronic bronchitis, ILD, LRTI, PAH, and lung function were identified with the framework of TSMR.

Our study suggested that genetic liability to asthma is related to the increased abundance of the genus *Prevotella* among the *Bacteroidetes,* which is a gram-negative bacterium. *Prevotella* is recognized as a member of the oral, vaginal, and gut microbiota and predominates in aspiration pneumonia and pulmonary empyema. In accordance with previous studies, an increased abundance of *Prevotella* at mucosal sites is associated with chronic inflammatory diseases, such as rheumatic diseases and neurodegenerative disorders (25). Santiago et al. have demonstrated that *Prevotella* is one of the most abundant genera among patients with exacerbation-prone severe asthma using deep sequencing of the amplified 16S rRNA gene (26). Moreover, an increasing body of evidence highlights the role of *Prevotella* in

modulating the host immune system by impacting the immune compartment within the intestinal tract (27). Specifically, *Prevotella* triggers the release of interleukin-1β (IL-1β), IL-6, and IL-23 from dendritic cells (DC), which, in turn, facilitate the production of IL-17 by T-helper 17 (Th17) cells, and these Th17 cells activate neutrophils and also influence the generation of regulatory T cells (Treg) (25). Considering that IL-17 exhibits pro-inflammatory properties and has been implicated in autoimmunity, its expression could signify a pro-inflammatory function (28). Simultaneously, Treg cells are essential in preventing inflammatory diseases and maintaining immune homeostasis (29). These cytokines govern crucial processes in inflammation and immune response, leading to airway inflammation and bronchoconstriction. Imbalances in the immune system associated with these processes may contribute to the development and exacerbation of asthma. Lopes et al. have shown that the abundance of *Prevotella* in the subgingival biofilm is associated with the presence of severe asthma using quantitative real-time PCR (30). Conversely, Hilty et al. have found that *Prevotella* spp. is more frequent in healthy controls than adult or child asthmatics in the bronchoalveolar lavage fluid (BALF) using 16sRNA sequencing (31), suggesting that the microenvironment of asthma may not be suitable for the colonization, but only 11 patients with asthma are enrolled in this study. However, to date, the role of *Prevotella* in the gut microbiota on asthma remains unknown in the preclinical or clinical studies; we observed the detrimental effects of the bacteria on asthma using the summary-level data, but further experimental and observational studies are needed to dissect the molecular mechanisms of *Prevotella* on asthma. In a similar vein, reverse MR analyses have identified associations between the family Enterobacteriaceae, the order Bacillales, the order Enterobacteriales, and the genus *Escherichia-Shigella* with asthma. A substantial body of research has shown that these four bacterial groups can potentially induce inflammation and infection by producing endotoxins and pathogenic factors (32, 33). For individuals with asthma, an increase in the abundance of these bacteria may heighten their susceptibility to allergic reactions and inflammation. Conversely, the family Acidaminococcaceae, known for its ability to ferment amino acids, has shown potential therapeutic values in asthma (34). The genera *Allisonella* and *Phascolarctobacterium* produce butyric acid and propionic acid, respectively, and an animal study demonstrated that the levels of both were significantly downregulated in asthmatic mice (35). The genus *Marvinbryantia* is capable of fermenting a wide range of carbohydrates, and the protective effects observed can be attributed to the by-products of carbohydrate fermentation (36), which may contribute to the maintenance of intestinal homeostasis and overall immune health. However, less information is available on the genera *Enterorhabdus*, *Anaerofilum*, *Peptococcus*, and *Lachnospiraceae*, and further research is needed to understand their association with asthma.

COPD is a multidimensional chronic lung disease with progressive obstructive bronchiolitis and airflow obstruction (37). In this study, we showed that the genus *Holdemanella* had suggestive negative causal effects on the risk of COPD. Lai et al. have found that *Parabacteroides goldsteinii* and *P. goldsteinii* are able to ameliorate the severity of COPD in a murine cigarette smoking (CS)-induced model (38). Chiu et al. have suggested that the abundance of *Firmicutes* increased in the declining lung function group (39). Chronic bronchitis is included in the umbrella term COPD, defined as productive cough of more than 3 months occurring within 2 years (40). Zheng et al. have suggested that an increase in the total aerobic, *Clostridium perfringens*, *Enterobacter,* and *Enterococcus* significantly increased on the 20th day in a specific pathogen-free Sprague–Dawley rat model with chronic bronchitis (41). Most previous research studies focused on the role of gut microflora on COPD, and few studies have been conducted to investigate the causality of chronic bronchitis on gut microbiota. We found that genetic liability to chronic bronchitis was related to the abundance of the family Defluviitaleaceae, and the genus *Defluviitaleaceae* had a positive correlation with chronic bronchitis; the family *Defluviitaleaceae* belongs to the order *Clostridiales* which is associated with worse recurrence-free survival (RFS) in patients with non-small cell lung cancer (42). The genus *Defluviitaleaceae* belongs to the family *Lachnospiraceae*, with a sequence similarity of the 16S rRNA gene of approximately 87%. In this study, we also have performed the MR analysis to explore the causality between COPD and gut microbiota and found that the genus *Holdemanella* presented a tendency to causally decrease the risk of COPD after the correction. However, Bowerman et al. have suggested that some bacterial taxa, including *Streptococcus sp000187445*, *Streptococcus vestibularis,* and several members of the family *Lachnospiraceae* correlated with reduced lung function and COPD (22). Similarly, Jang et al. have shown that the increased *Defluviitaleaceae* was found in the gut microbiota of emphysema compared with the healthy controls using pyrosequencing (43).

ILDs are a heterogeneous spectrum of disorders that principally influence the pulmonary interstitium, resulting in dyspnoea, cough, and respiratory failure (44). Chioma et al. have demonstrated that gut microbiota regulates lung fibrosis severity followed by acute lung injury (45). Using GWAS data, we found that the abundance of the genus *Prevotella* has a positive correlation with ILDs. Huang et al. have suggested that the activation of immune response signaling pathways is strongly related to the reduced abundance of *Prevotella* among individuals with fibroblasts responsive to CpG-ODN stimulation (46). Scher and Lou have identified that *Prevotella* in the lungs is associated with the initiation and development of ILD in patients with autoimmune diseases, such as dermatomyositis and rheumatoid arthritis (RA) (47, 48). To date, no relevant data have reported on the gut microbiome of ILD or IPF in humans, but Gong et al. have found that the abundance of *Alloprevotella, Dubosiella, Helicobacter, Olsenella, Parasutterella, Rikenella*, and *Rikenellaceae RC9 gut group* in the gut of the bleomycin (BLM) or silica-induced mice present significant difference compared with the healthy controls by 16S RNA sequencing (49).

The influence of *Prevotella* on ILD encompasses not only the immunological factors, as previously discussed, but also microbial interactions. The gut-lung axis embodies the idea that changes in gut commensal microorganisms can exert distant effects on immune function in the lung (50) while simultaneously involving gastrointestinal functionality and intricate bidirectional communication with the respiratory system (51). Under certain conditions, such as systemic circulation or inhalation of gastroesophageal reflux, *Prevotella* may translocate to the lung tissue via the enteropulmonary axis. The presence of enteric bacteria in the lungs can initiate a local immune response, leading to inflammation and tissue damage, which may subsequently contribute to the development of ILD46. *Prevotella* generates various metabolites and signaling molecules, such as SCFA47. These molecules traverse the gut-lung axis, affect local immune responses, and could potentially induce inflammation and fibrosis in the context of ILD.

LRTI is an umbrella terminology, including acute bronchitis, pneumonia, acute exacerbation of COPD (AECOPD), and acute

exacerbation of bronchiectasis (52), which is the most common cause of death in low-income countries (53). In this study, we found that the abundance of the family Coriobacteriaceae and class Coriobacteriia affects the occurrence of LRTI. It is known that interstitial flora contributes to LRTI pathogenesis and severity through its immunomodulatory properties (54, 55). Goossens et al. have shown that the abundance of the family Coriobacteriaceae tended to be elevated in the gut after intraperitoneal LPS challenge, which is recognized to be associated with the increased expression of matrix metalloproteinases (MMP9) (56). However, Sencio et al. have observed an obvious reduction of Actinobacteria (Bifidobacteriaceae and Coriobacteriaceae families) in the cecal samples from influenza A virus-infected patients (57), and the alteration of SCFAs by the interstitial flora influences the killing activity of alveolar macrophages (57).

PAH is a malignant and devastating pulmonary vascular disorder characterized by precapillary pulmonary hypertension. In this study, we found that the genus *Lactobacillus* had a causal role in PAH. Mounting evidence suggests that the gut-dwelling *Lactobacillus* and its components play a key role in modulating the immune system through stimulating immunological signaling between the gastrointestinal tract and distant organs (58). Consistently, Ma et al. have also demonstrated that the increased abundance of *Lactobacillus* is associated with PAH compared with healthy volunteers and congenital left to right shunt heart diseases (59).

Lung function is used to measure lung volume, capacity, and flow rates, reflecting the functional status of the lungs and the disease severity. We found that the order Selenomonadales pertaining to *Veillonella* presents a reduced risk with FEV1, which is useful to categorize the severity of obstructive lung diseases such as COPD. In contrast, Diao et al. have shown that the abundance of the order Selenomonadales was significantly increased in the throat microbial flora in COPD (60). Moreover, Filho et al. have demonstrated the absence of the order Selenomonadales in the adult lungs, which were independent predictors of mortality in COPD. However, to date, no available studies have been conducted to investigate the role of the order Selenomonadales in the gut on obstructive lung diseases.

It is well-established that microorganisms not only can be found in the gut but also in the respiratory tract (8). In the upper respiratory tract, there are variations in the microbial composition based on the location. For instance, the nasal cavity and nasopharynx are primarily populated by *Moraxella*, *Staphylococcus*, *Corynebacterium*, *Haemophilus*, and *Streptococcus* species, while the oropharynx contains a high abundance of *Prevotella*, *Veillonella*, *Streptococcus*, *Leptotrichia*, *Rothia*, *Neisseria*, and *Haemophilus* species (61). On the other hand, the lower respiratory tract, which includes the trachea and lungs, maintains a relatively low microbial biomass, which is crucial for lower airway mucosal immunology, as it allows for swift microbial clearance through various physiological mechanisms. Extensive research is currently underway to understand how the gut microbiota impacts immune responses and inflammation in the lungs, and conversely, how the lungs influence the abundance of gut microbiota. Various mechanisms, such as the participation of specific subsets of regulatory T cells (62, 63), Toll-like receptors (TLRs), inflammatory cytokines, mediators, and numerous other factors, have been suggested as potential explanations for these interactions (64). However, the precise biological mechanisms remain largely unknown.

The strength of this study is that we employed bidirectional and comprehensive MR that exploits genetic variants to estimate the causal effects of gut microbiota on chronic lung diseases, and MR is capable of minimizing bias due to confounding and reverse causality, thus improving the causal inference (65). Moreover, we performed the analysis with large sample sizes which promotes the power to detect mild-to-moderate associations, and individuals included in this study are all European ancestry to reduce the population selection bias. Nevertheless, this study had several limitations. First, the number of IVs involved in GWAS statistics of gut microbiota is small, and no additional data are available at the species level, which contributes to biased estimates and lack of universality. Second, the methods of sequencing analyses of the gut microbiota and chronic lung diseases may differ, leading to distinct results. Third, the phenotypes of the six lung diseases were not analyzed in this study. Finally, due to the summary-level GWAS data, the demographic data of the studies are absent; further subgroup analysis of the confounding factors such as age and gender on the bacterial taxa and lung diseases remains unknown.

Conclusion

Our bidirectional TSMR study reveals the causal relationship between gut microbiota and chronic lung diseases, providing new insights into the biological mechanisms of gut microbiota-modulated development of chronic lung diseases. To facilitate the dissection of the role of gut microbiota on lung diseases, an integrative approach that uses multiple omics is urgently needed to understand gut-lung signaling.

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

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

Author contributions

YS: Writing – original draft, Writing – review & editing. YZ: Writing – original draft, Writing – review & editing. JX: Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1279239/full#supplementary-material

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Exploring the interplay between posttraumatic stress disorder, gut microbiota, and inflammatory biomarkers: a comprehensive meta-analysis

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Introduction: Posttraumatic stress disorder (PTSD) is the most common mental health disorder to develop following exposure to trauma. Studies have reported conflicting results regarding changes in immune biomarkers and alterations in the abundance of bacterial taxa and microbial diversity in patients with PTSD.

Aim: The purpose of this meta-analysis is to summarize existing studies examining gut microbiota characteristics and changes in immune biomarkers in patients with PTSD.

Methods: Relevant studies were systematically searched in PubMed, Scopus, and Embase, published in English between January 1, 1960, and December 1, 2023. The outcomes included changes in abundance and diversity in gut microbiota (gut microbiota part) and changes in immune biomarkers (immune part).

Results: The meta-analysis included a total of 15 studies, with 9 focusing on changes in inflammatory biomarkers and 6 focusing on changes in gut microbiota composition in patients with PTSD. No differences were observed between groups for all inflammatory biomarkers ($P \geq 0.05$). Two of the six studies found that people with PTSD had less alpha diversity. However, the overall Standardized Mean Difference (SMD) for the Shannon Diversity Index was not significant (SMD 0.27, 95% CI -0.62–0.609, p = 0.110). Regarding changes in abundance, in two of the studies, a significant decrease in Lachnospiraceae bacteria was observed.

Conclusion: This meta-analysis provides a comprehensive overview of gut microbiota characteristics in PTSD, suggesting potential associations with immune dysregulation. Future research should address study limitations, explore causal relationships, and consider additional factors influencing immune function in individuals with PTSD.

Systematic review registration: https://www.crd.york.ac.uk, identifier CRD42023476590.

KEYWORDS

gut microbiome, stress, post-traumatic stress disorder, inflammation, IL-6, *Lachnospiraceae*

1 Introduction

Posttraumatic stress disorder (PTSD) is a debilitating mental health disorder that can develop following exposure to trauma (1). It is characterized by distinct symptoms, including re-experiencing traumatic events, avoiding reminders of the trauma, heightened arousal, and negative changes in cognition and mood (2). PTSD is the most common mental health disorder that arises after trauma and has significant impacts on individuals' well-being and daily functioning (3).

Currently, the world is witnessing new and escalating military conflicts, with the Russian-Ukrainian conflict being one of the largest and most significant. Since February 2022, this conflict has resulted in numerous casualties among both civilian and military populations, as well as massive displacement of people (4–7). The psychological toll of such experiences, including stress and the risk of developing PTSD, is a pressing concern that needs addressing (8, 9).

Recent research has shed light on the intricate relationship between the gut microbiota and mental health (10). The gut microbiota, comprised of trillions of microorganisms residing in the digestive system, has been found to play a crucial role in influencing brain function through the 'microbiota-gut-brain axis (11, 12). Disruptions in the composition and functioning of the gut microbiota have been associated with various psychiatric disorders (13).

Intestinal bacteria are also capable of modulating the immune response, both individually and as consortia, as well as through their metabolites (14–21). These metabolites can exert direct effects on immune cells and can also interact with the gut-brain axis, thereby further influencing brain function (22). Additionally, studies have shown that specific strains of gut bacteria can produce neurotransmitters and other molecules that can directly impact brain activity and behavior (23). Understanding the characteristics of the gut microbiota in individuals with PTSD can provide valuable insights into the underlying mechanisms and potentially open up new avenues for therapeutic interventions.

The purpose of this meta-analysis is to review and summarize existing studies that have examined the gut microbiota characteristics in patients with PTSD. Additionally, the review will compare the levels of inflammatory biomarkers in patients to understand the potential relationship between the gut microbiota, PTSD, and immune biomarkers.

Understanding the characteristics of the gut microbiota in individuals with PTSD can have several implications. Firstly, it can help identify biomarkers or specific microbial signatures associated with the disorder, which can aid in the diagnosis and early detection of PTSD. Secondly, it can inform the development of targeted therapeutic interventions that focus on modulating the gut microbiota to improve mental health outcomes in patients with PTSD. Thirdly, it can contribute to a deeper understanding of the underlying mechanisms of PTSD and potentially uncover novel pathways for intervention. By elucidating the complex interplay between the gut microbiota, PTSD, and immune biomarkers, this research has the potential to revolutionize our approach to the prevention and treatment of PTSD.

2 Methods

2.1 Inclusion and exclusion criteria

The eligibility criteria were established to include studies that investigated the gut microbiota composition in individuals diagnosed with PTSD. The inclusion and exclusion criteria were carefully defined to ensure the selection of relevant studies.

2.2 Search strategy

The search strategy followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (24). We conducted a comprehensive search across multiple databases, including PubMed, Scopus, and Embase, to identify relevant studies published in English between January 1, 1960, and December 1, 2023. The following keywords were used: ("Inflammation" OR "Immune Activation" OR "Interleukin" OR "Cytokine" OR "Interferon" OR "Lymphocyte" OR "Macrophage" OR "Tumor Necrosis Factor-alpha" OR "C-Reactive Protein" OR "IL-1" OR

"IL-2" OR "IL-4" OR "IL-6" OR "IL-8" OR "IL-10" OR "Interferon" OR "IFN" OR "TNF") AND "Posttraumatic Stress Disorder" OR "PTSD". For searching articles related to gut microbiota, the following keywords were used: "Gut Microbiota" OR "Intestinal Microbiota" OR "Microbial Composition" OR "Microbiome" OR "Bacterial Diversity" OR "Microbial Dysbiosis" OR "Bacterial Metabolites" OR "Microbial Modulation" OR "Microorganism Influence") AND ("Posttraumatic Stress Disorder" OR "PTSD" OR "Trauma-Induced Psychopathology" OR "Psychiatric Sequelae of Trauma" OR "Stress-Related Disorders."

2.3 Data extraction and data analysis

Two independent reviewers screened the titles and abstracts of all identified records to assess their eligibility for inclusion in the meta-analysis. The reviewers resolved any disagreements through discussion and consensus. The same reviewers retrieved and reviewed full-text articles of potentially eligible studies to determine final inclusion. Two reviewers performed data extraction independently and resolved any discrepancies through consensus. If other statistics were reported instead of mean and SD, we requested the data from the corresponding author via email. If this approach failed, we used the estimation method to calculate SD according to the Cochrane Handbook for Systematic Reviews of Interventions (25). We used Comprehensive Meta-Analysis V3 for all analyses (24).

If the number of studies that included a specific marker was equal to or exceeded three, we performed meta-analyses on individual immune markers. These main analyses were based on random-effects models. If heterogeneity is significant, a random-effects model is chosen for meta-analysis as it assumes that the underlying true effects vary from one trial to another (26). We used standardized mean difference (SMD) and 95% confidence intervals (CI) to assess the effect size. The significance level was defined as $P< 0.05$. An effect size of 0.2 or less was considered a low effect, 0.5 or more was a large effect.

We obtained publication details, participant demographic and clinical characteristics, and methodological information from systematic reviews and original studies. The main outcomes we focused on were community-level measures of gut microbiota composition, specifically alpha and beta diversity. We also examined taxonomic findings at the phylum, family, and genus levels, specifically looking at relative abundance. Alpha diversity offers a concise overview of the microbial community within individual samples and allows for comparisons between groups to assess the impact of a specific factor (in this instance, psychiatric diagnosis) on the abundance (number of species) and uniformity (representation of each species) within the sample. Beta diversity quantifies the degree of dissimilarity between samples, evaluating the similarity of communities in relation to the other analyzed samples.

2.4 Heterogeneity

We assessed heterogeneity using Q-tests and quantified the proportion of total variability due to heterogeneity with the I^2 statistic. An I^2value <50% was considered low heterogeneity, $I^2 \geq 50\%$ but <75% considered medium heterogeneity, and I2 ≥ 75% considered high heterogeneity. The presence of significant heterogeneity suggests that the characteristics of studies are divergent.

2.5 Quality assessment

Two researchers used the Newcastle-Ottawa Quality Assessment Scale to assess the quality of the literature, and a third reviewer helped resolve differences when necessary (24). The NOS included three criteria: selectivity, comparability, and exposure. Each study could receive up to nine stars. A study with a score of ≥7 was considered of good quality, with a score of 5–6 of average quality, and a score of 0–4 was of poor quality. To visualize the risk of bias, we employed the Robvis tool (27).

3 Results

A comprehensive search was conducted on a total of 5824 articles, out of which 854 were eliminated from consideration due to duplicate findings. Additionally, a significant number of individuals were excluded based on the evaluation of their title or abstract (n = 4841). After analyzing the remaining 39 articles in their entirety, 26 were excluded due to various reasons (refer to Figure 1). Our meta-analysis included a total of 15 studies, with 9 focusing on changes in inflammatory biomarkers and 6 focusing on changes in gut microbiota composition in patients with PTSD. Table 1 describes the characteristics of the included studies, outlining essential details such as sex, sample size, inflammatory markers included in the meta-analysis, and diagnostic criteria for PTSD. In total, among the 9 articles examining inflammatory biomarkers in individuals with PTSD, 401 had a confirmed diagnosis of PTSD, and an additional 642 served as comparison controls. The total number of participants in the gut microbiota sub-part was 489. Risk of bias was assessed using the Newcastle-Ottawa Quality Assessment Scale, as illustrated in Figures 2 and 3.

No differences were observed between groups for all inflammatory biomarkers ($P\geq0.05$), as depicted in Figures 4 and 5. TNF-α (SMD 0.86, 95% CI -0.02 – 1.74, p = 0.057) and IL-6 (SMD 0.72, 95% CI -0.07 – 1.52, p = 0.075) had slightly higher p-values. Additionally, for all inflammatory markers, study heterogeneity was reported to be high ($I^2 > 75\%$), except for the study on interleukin-1β, where heterogeneity was 68% (medium).

Additionally, it was crucial to determine whether there were correlations between inflammation biomarkers and the course's severity. Out of the nine studies, only three conducted a correlational study between inflammatory markers and PTSD severity. Notably, one study found that PTSD patients had significantly higher pro-inflammatory scores compared to combat-exposed subjects without PTSD. However, the pro-inflammatory score was not significantly correlated with depressive symptom severity, CAPS total score, or the number of early-life traumas (31). In another study, TNF-α positively

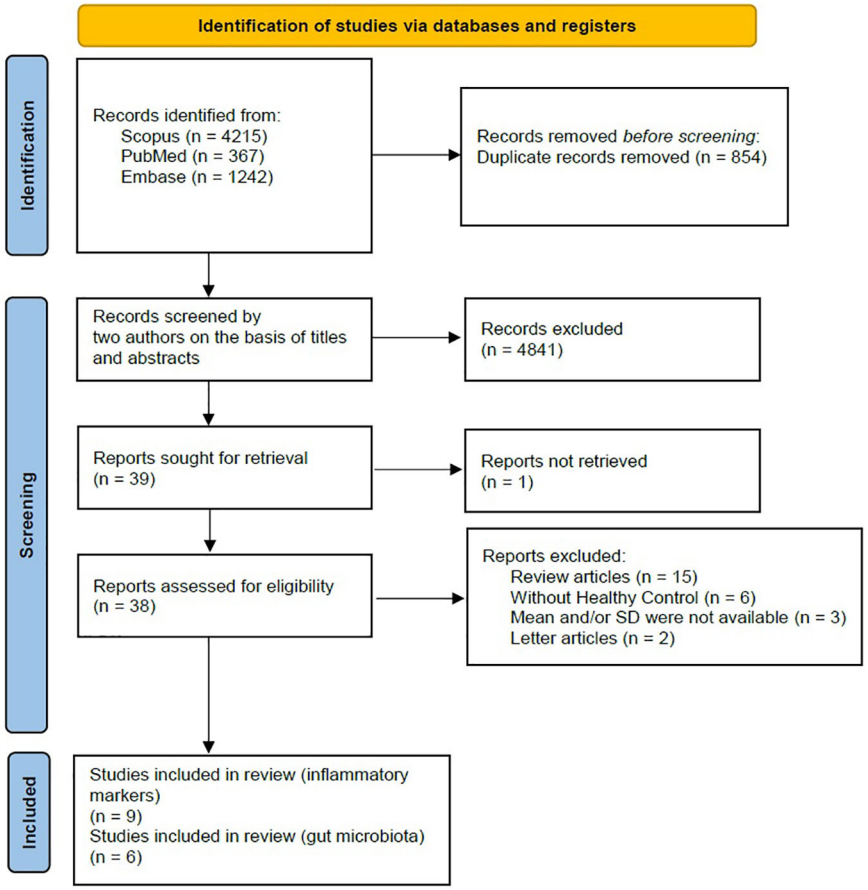

FIGURE 1
PRISMA 2020 flow diagram.

correlated with the total (frequency and intensity) PTSD symptom cluster of re-experiencing, avoidance, and hyperarousal, as well as with the PTSD total symptom score. Controlling for time since trauma attenuated these associations. IL-1β positively correlated with symptoms of anxiety and depression. IL-4 negatively correlated with total hyperarousal symptoms, systolic blood pressure (30). In the study conducted by Eswarappa et al., the biomarkers white blood cell count (OR = 1.27, 95% CI: 1.10–1.47, p = 0.001), C-reactive protein (OR = 1.20, 95% CI: 1.04–1.39, p = 0.02), and erythrocyte sedimentation rate (ESR) (OR = 1.17, 95% CI: 1.00–1.36, p = 0.05) were identified as significant predictors of poorer courses of PTSD (33). Since there were few data and studies, we did not calculate correlations of effects indexes.

The second part of the systematic review involved an analysis of the characteristics of the intestinal microbiota in patients with PTSD (Figure 6). Two out of the six studies showed a decrease in alpha diversity in PTSD patients (SMD for Shannon Diversity Index 0.27, 95% CI -0.62 – 0.609, p = 0.110), while the other two studies found no significant difference between the PTSD and control groups. In two of the studies, a significant decrease in *Lachnospiraceae* bacteria was observed. In one of these studies, bacteria of this taxa were positively correlated with PTSD symptoms score, while in the other study, *Lachnospiraceae* were associated with higher cognitive functioning. The remaining results did not show any consistent patterns and were unique to each study.

4 Discussion

The findings of this meta-analysis provide valuable insights into the characteristics of the gut microbiota in patients with PTSD. The results suggest that there may be alterations in the diversity and composition of the gut microbiota in individuals with PTSD, as well as potential associations with specific bacterial taxa.

Lachnospiraceae are the main SCFA producers of dietary fiber that have anti-inflammatory and modulating effects on the intestinal mucosa, maintaining gut health (10). As we all know, the colon plays a big part in providing energy and trophic factors, as well as controlling T regulatory (Treg) cell colonies (43–45). More and more evidence suggests that SCFAs also have important physiological effects on many organs, including the brain (46–48). Gut microbiota dysbiosis has been linked to behavioral and neurological disorders like autism spectrum disorder (ASD), Alzheimer's disease (AD), and Parkinson's disease (PD) in both

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TABLE 1 Characteristics of studies included in the meta-analysis.

1. Inflammatory markers					
Study (year)	Inflammatory markers included in meta-analysis	Number participants	Gender (male %) (PTSD/HC)	Sample type	PTSD Diagnosis
Dalgard et al. (2017) (28)	IL-1β, IL-2, IL-6,IL-8, IL-10, TNF-α	PTSD (n=16); HC (n=11)	31.2/45.4	Plasma	DSM-IV
Hoge et al. (2009) (29)	IL-1β*, IL-2,* IL-4*, IL-6*, IL-8*, IL-10*, TNF-α*	PTSD (n=28); HC (n=48)	50/50	Plasma	DSM-IV
Kanel et al. (2007) (30)	IL-1β, IL-4, IL-6, IL-10, CRP	PTSD (n=14); HC (n=14)	64/64	Plasma	DSM-IV, CAPS
Lindqvist et al. (2014) (31)	IL-1β, IL-6, IL-10, CRP, TNF-α*	PTSD (n=51); HC (n=51)	100/100	Serum	DSM-IV, CAPS
Guo et al. (2012) (32)	IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α	PTSD (n=50); HC (n=50)	44/50	Serum	DSM-IV
Eswarappa et al. (2018) (33)	IL-6, CRP*	Chronic PTSD (n=170); HC (n=396)	91.2/97	Plasma	DSM IV
Jergović et al. (2014) (34)	CRP	PTSD (n=21); HC (n=23)	100/100	Serum	ICD-10
Gill et al. (2012) (35)	IL-6*, CRP*	PTSD (n=26); HC (n=24)	0/0	Plasma	DSM IV
Muhtz et al. (2011) (36)	CRP	PTSD (n=25); HC (n=25)	36/36	Plasma	PDS/BDI
2. Gut microbiota					
Study (year)	Number participants	Sample type	Gender (male %) (PTSD/HC)	Method of analysis	PTSD Diagnosis
Hemmings et al. (2017) (37)	PTSD (n=18); Trauma-exposed control (n=12)	Stool samples	22.2/41.7	16S rRNA sequencing	CAPS-5
Bajaj et al. (2019) (38)	PTSD (n = 29); Control (n=64);	Stool samples	100/100	16S rRNA sequencing	DSM-V
Malan- Muller et al. (2022) (39)	PTSD (n = 79); Trauma-exposed control (n = 58);	Stool samples	20.26/18.97	16S rRNA sequencing	CAPS-5
Yoo et al. (2023) (40)	Firefighters (n = 15); Control (n = 15);	Stool samples	100/100	16S rRNA sequencing	PCL-C
Zeamer et al. (2023) (41)	Microbiome Sub-study (n = 51)	Stool samples	49	16S rRNA sequencing	DSM-V
Feldman et al. (2022) (42)	Mother-child dyads from Sderot, Israel (n = 148)	Stool samples	47.6	16S rRNA sequencing	DC:0-3R

humans and animals, which supports this idea (49–51). Furthermore, microbiota manipulation and SCFA administration have been proposed as treatment targets for such diseases (52).

While this meta-analysis provides valuable insights into the characteristics of the gut microbiota in patients with PTSD, several limitations should be acknowledged. First, the included studies varied in their sample sizes, diagnostic criteria for PTSD, taking medications (antipsychotics), and taking food. This heterogeneity may have influenced the results and should be considered when interpreting the findings. Second, the cross-sectional nature of the included studies limits our ability to establish causality or determine the temporal relationship between the gut microbiota and PTSD.

One potential mechanism underlying the observed associations between the gut microbiota and PTSD is through the modulation of immune function and inflammation. Disruptions in the gut microbiota composition and functioning have been associated with altered immune responses and increased inflammation, which have been implicated in the pathophysiology of psychiatric

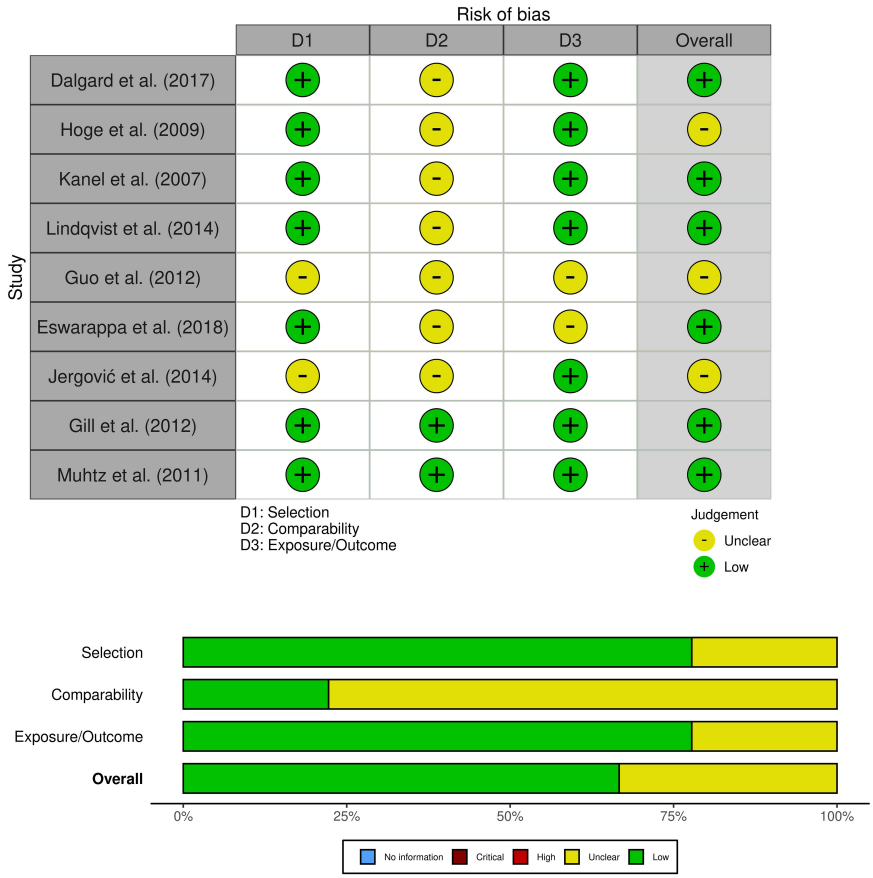

FIGURE 2
Risk of bias (studies of inflammation biomarkers).

disorders, including PTSD (53, 54). Changes in the gut microbiota may lead to dysregulation of the immune system, contributing to the development and maintenance of PTSD symptoms (55). Environmental factors like stress and diet can disturb the gut microbiome, triggering the intestinal epithelium to release pro-inflammatory cytokines, potentially causing intestinal permeability, increased antigen movement, and inflammation (56). Increasing evidence suggests that imbalanced communication within the gut-brain axis plays a role in the development of stress and mood disorders, with observed changes in the gut microbiome in individuals with PTSD (57, 58). Gut microbiome alterations may also mediate the association between early life adversity and symptoms of anxiety in adulthood (59). Along with research showing a strong link between PTSD and inflammatory gastrointestinal conditions like IBD, the data point to a possible role for gut microbiota imbalance in the inflammatory environment linked to PTSD (60).

However, no significant differences were observed in the levels of inflammatory biomarkers between the two groups. There have been two meta-analyses conducted so far. In one meta-analysis by Yang et al. (2020), interleukin-1β, IL-2, IL-6, interferon-γ, TNF-α, C-reactive protein, and white blood cells were higher in PTSD (61). In a meta-analysis, Passos et al. (2015) found that interleukin 6, interleukin 1β, and interferon γ levels were higher in the PTSD group than in healthy controls (62).

PTSD affects the immune system because it overworks the sympathetic nervous system and alters the function of the hypothalamus-pituitary-adrenal (HPA) axis (63). Activating the sympathetic nervous system causes catecholaminergic neurotransmitters, like norepinephrine, to be released, which in turn causes pro-inflammatory cytokines to be released. Catecholamine activates immune responses via the adrenergic-β receptor (64). Previous studies indicated that catecholamine-induced Th1 responses modulated immune cell distribution through the β-adrenergic receptor (65). Interestingly, previous studies found that the concentrations of norepinephrine and the expression of the adrenergic-β2 receptor increased in PTSD patients (66, 67). When the HPA axis was activated, it stopped pro-inflammatory activity by releasing glucocorticoids and stopping the NFκB pathway (68). Previous studies indicated that dysregulation of the HPA axis promoted pro-inflammatory cytokine secretion in PTSD (69). For example, Yehuda et al. found the levels of salivary cortisol were decreased in PTSD, and Klengel et al. found glucocorticoid receptor resistance in PTSD (70, 71). In summary, the results indicated that PTSD patients were in a pro-inflammatory state.

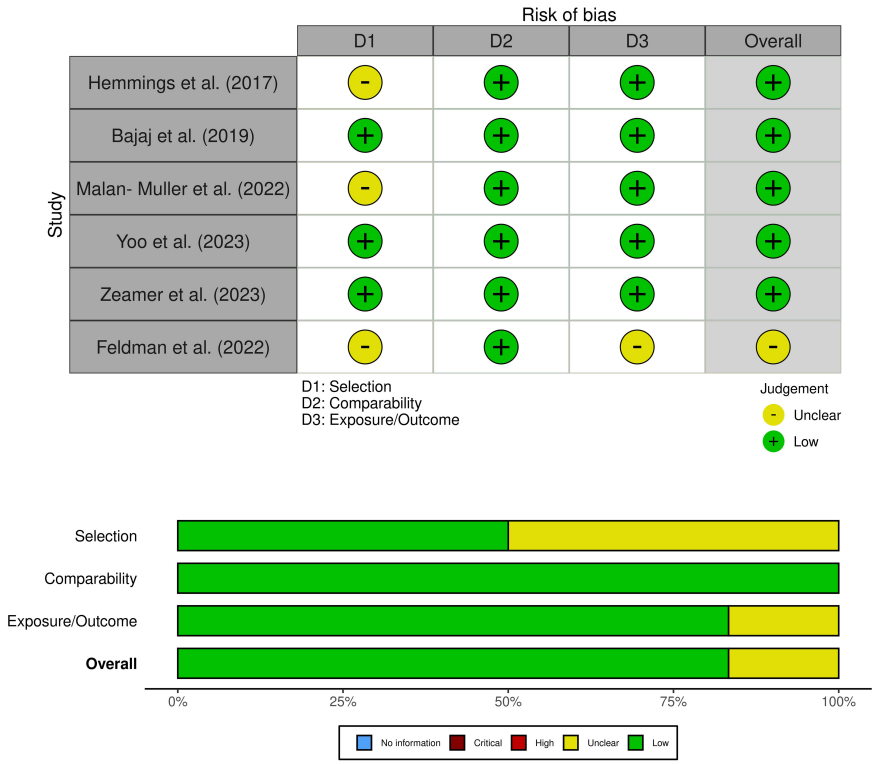

FIGURE 3
Risk of bias (studies of gut microbiota composition).

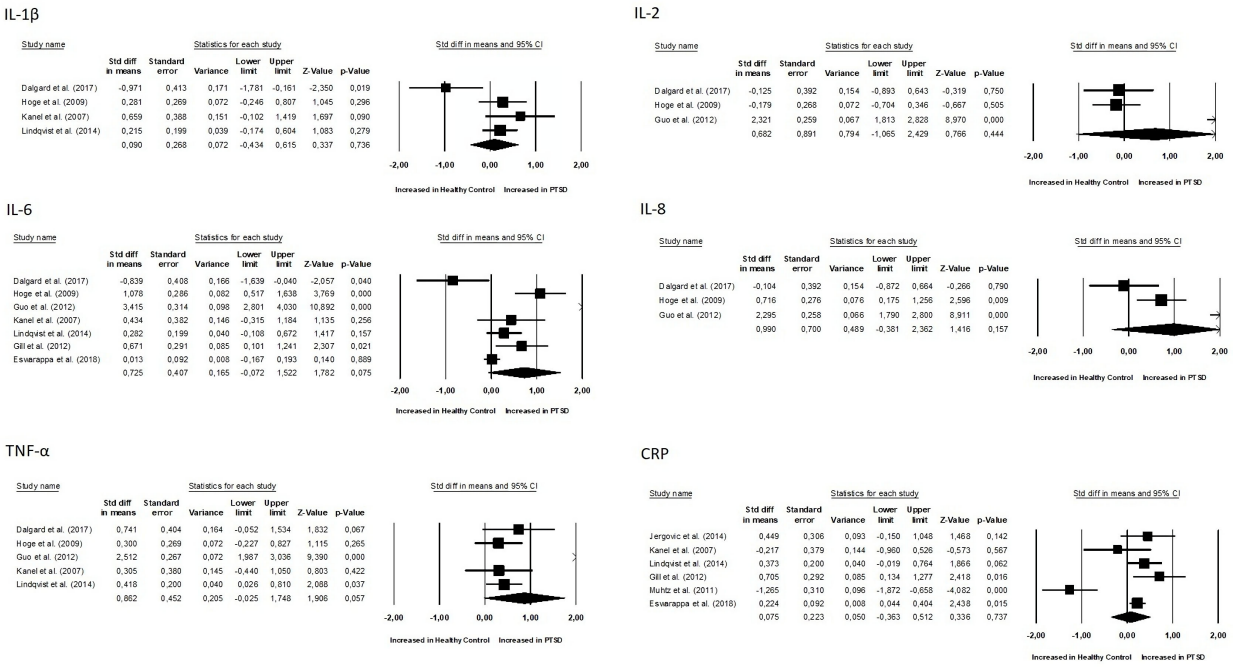

FIGURE 4
Meta-analyses of pro-inflammatory biomarkers (IL-1β, IL-2, IL-6, IL-8, TNF-α, CRP).

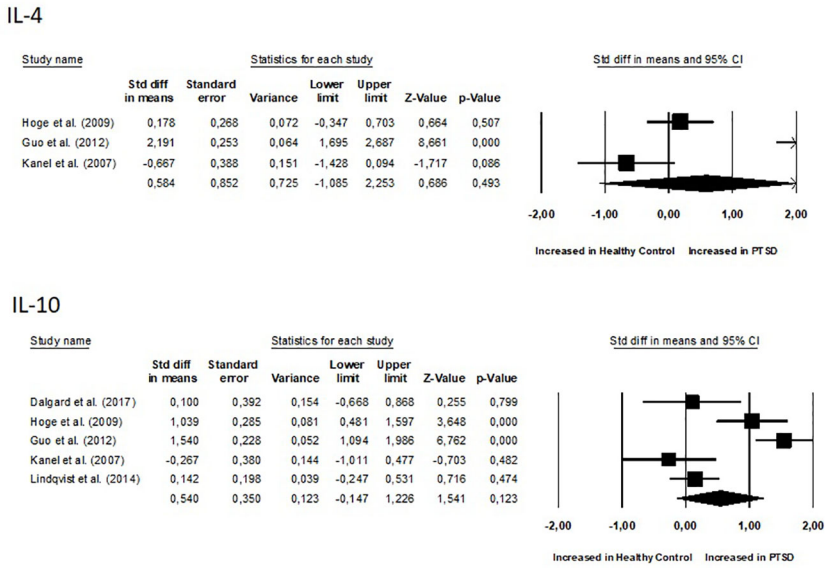

FIGURE 5
Meta-analyses of anti-inflammatory biomarkers (IL-4 and IL-10).

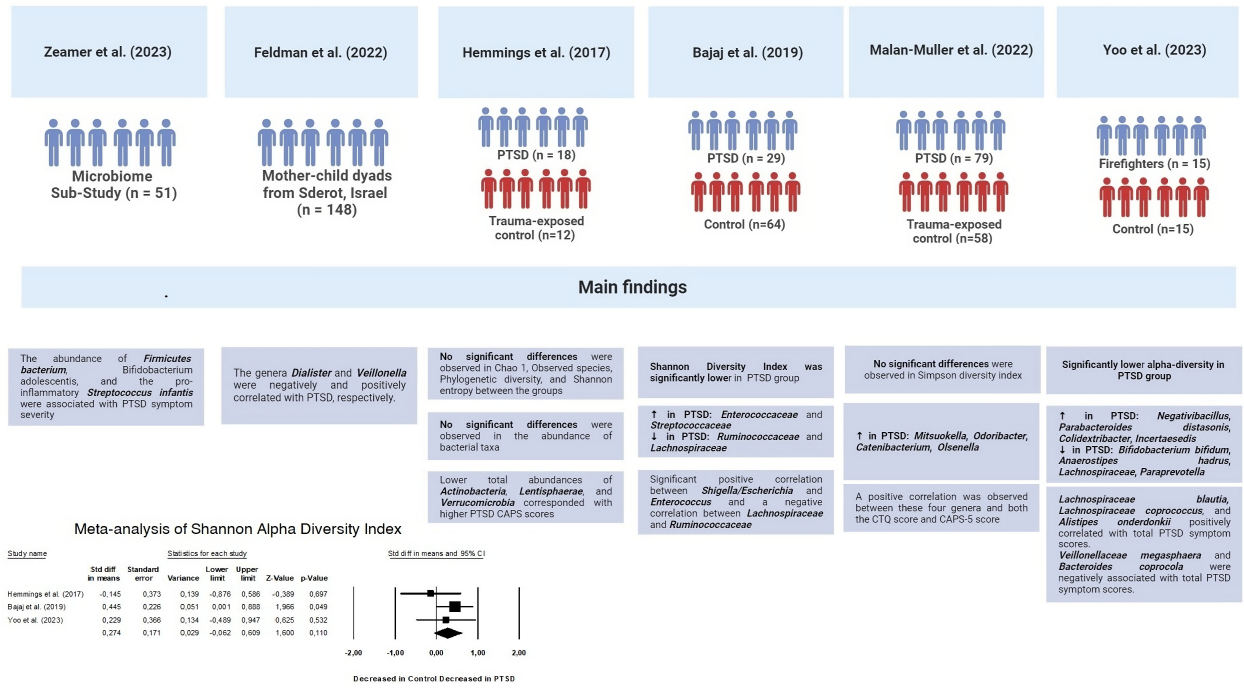

FIGURE 6
Key findings of gut microbiota composition studies.

These findings suggest that dysregulation of the immune system may play a role in the development and maintenance of PTSD symptoms, although further research is needed to fully understand the relationship between inflammatory biomarkers and PTSD. Additionally, it is important to consider other factors that may contribute to immune dysregulation in individuals with PTSD, such as lifetime trauma burden, biological sex, genetic background, metabolic conditions. Experiencing trauma and stress throughout

one's life may contribute to inflammation even before the occurrence of a traumatic incident leading to PTSD (72). A cross-diagnostic meta-analysis of trauma exposure showed that people who had a lot of traumatic events in their lives, like being abused as a child, being in a natural disaster, or being in a violent situation, had higher levels of CRP, IL-1β, IL-6, and TNF-α in their blood. To demonstrate the connection between inflammation and the occurrence of traumatic events, researchers have used animal models such as repeated social defeat stress (RSDS). They found that IL-17A released by meningeal T cells in the brain controlled anxious behavior in mice by connecting to IL-17Ra on neurons (62).

Metabolic conditions coexisting with PTSD can potentially intensify the inflammatory environment associated with the disorder (73). People with PTSD are at a heightened risk of developing type 2 diabetes mellitus, metabolic syndrome (MetS), and its various components, such as obesity, insulin resistance, and dyslipidemia (74, 75). This higher rate of comorbidity can be explained by the unhealthy lifestyles that are linked to PTSD, such as irregular sleep patterns, poor nutrition, drug and tobacco use, and lack of physical activity. These lifestyles make inflammation worse (76, 77). The noradrenergic system is turned on in both MetS and PTSD, which starts an innate immune response (73). Similar to PTSD, MetS, and obesity are marked by elevated levels of proinflammatory markers, including CRP, IL-6, and TNF-α (78).

The metabolic findings suggest inflammation, inefficient energy production, and potential mitochondrial dysfunction in individuals with PTSD (77). Mitochondrial dysfunction could result in heightened production of reactive oxygen species (ROS) in peripheral organs and immune cells, contributing to peripheral inflammation. The proposed connection between inflammation, oxidative stress, and metabolism is further emphasized by Kusminski and Scherer, who suggest that mitochondrial dysfunction plays a linking role in these interconnected processes.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

PP: Conceptualization, Visualization, Writing – original draft. VO: Writing – review & editing. IK: Writing – original draft. IB: Writing – review & editing. KL: Writing – review & editing. OK: Supervision, Visualization, Writing – original draft.

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Conflict of interest

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

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Oral administration of Manuka honey induces IFNγ-dependent resistance to tumor growth that correlates with beneficial modulation of gut microbiota composition

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Background: To investigate the potential of Manuka honey (MH) as an immunomodulatory agent in colorectal cancer (CRC) and dissect the underlying molecular and cellular mechanisms.

Methods: MH was administered orally over a 4 week-period. The effect of MH treatment on microbiota composition was studied using 16S rRNA sequencing of fecal pellets collected before and after treatment. Pretreated mice were implanted with CRC cells and followed for tumor growth. Tumors and lymphoid organs were analyzed by flow cytometry (FACS), immunohistochemistry and qRT-PCR. Efficacy of MH was also assessed in a therapeutic setting, with oral treatment initiated after tumor implantation. We utilized IFNγ-deficient mice to determine the importance of interferon signaling in MH-induced immunomodulation.

Results: Pretreatment with MH enhanced anti-tumor responses leading to suppression of tumor growth. Evidence for enhanced tumor immunogenicity included upregulated MHC class-II on intratumoral macrophages, enhanced MHC class-I expression on tumor cells and increased infiltration of effector T cells into the tumor microenvironment. Importantly, oral MH was also effective in retarding tumor growth when given therapeutically. Transcriptomic analysis of tumor tissue highlighted changes in the expression of various chemokines and inflammatory cytokines that drive the observed changes in tumor immunogenicity. The immunomodulatory capacity of MH was abrogated in IFNγ-deficient mice. Finally, bacterial 16S rRNA sequencing demonstrated that oral MH treatment induced unique changes in gut microbiota that may well underlie the IFN-dependent enhancement in tumor immunogenicity.

Conclusion: Our findings highlight the immunostimulatory properties of MH and demonstrate its potential utilization in cancer prevention and treatment.

KEYWORDS

Manuka honey, immunomodulation, type I/II IFN, tumor immunogenicity, colorectal cancer

Introduction

Cancer represents a crucial global health concern, accounting for 10 million deaths annually (1). Cancer growth results from a multistep process during which cells acquire multiple mutations, eventually leading to continuous cellular growth and division. Although several factors can contribute to cancer development, a compromised immune system is widely recognized as a dominant contributor to the onset and progression of cancer (2, 3).

The role of the immune system in cancer is illustrated by its ability to eradicate emerging transformed cells once they arise, a concept known as "cancer immunosurveillance" (4). However, tumor cells are capable of acquiring characteristics and strategies by which they can evade the immune system and consequently progress in their growth (5). In light of the vital role of the immune system in cancer development and progression, there is a rising interest in employing cancer immune preventive agents to amplify immune responses and reduce cancer susceptibility in healthy individuals.

There is a growing body of evidence suggesting that different types of honey have anti-cancer properties (6). Previously, our lab and others demonstrated the potential of Manuka honey (MH) to impede the growth of various types of human and murine cancer cell lines (7–9) and revealed the underlying molecular mechanisms of its anti-tumor action (8, 10). MH has also been described to possess immunomodulatory properties (11). While some studies highlighted the potential of MH as an anti-inflammatory agent (12, 13), others demonstrated that MH also exhibits pro-inflammatory properties (14–19).

In our previous work, we demonstrated that MH can trigger the activation of macrophages by inducing the expression of pro-inflammatory cytokines, such as TNF-α and IL-1β, and the chemokines CXCL2 and CCL2 which are potent chemoattractants of myeloid cells (15). Additionally, when administered intraperitoneally, MH elicited a peritoneal immune response characterized by a significant increase in the recruitment of neutrophils and an enhancement in the functional maturation of peritoneal macrophages (15). In the present study, we investigated the effect of oral administration of MH, given as part of a preventive or therapeutic regimen, on the host immune system and its potential to modulate anti-tumor immune responses in implantable murine colorectal cancer (CRC) models. Several reports demonstrated that alterations in the composition of gut microbiota and their translocation to secondary lymphoid organs can stimulate immune responses against tumors by influencing various cell types such as $CD8^+$ and $CD4^+$ T cells as well as tumor-associated myeloid cells (20, 21). Therefore, we also assessed the potential changes in microbiota composition following MH treatment in this study. Our findings provide compelling evidence that supports a role for MH as an immunomodulatory anti-tumor agent, highlighting its potential use in cancer prevention and treatment.

Materials and methods

Cell line and reagents

The murine CT26 colon carcinoma cell line was a kind gift from Dr. Siegfried Weiss (Helmholtz Centre for Infection Research, Braunschweig, Germany). The colon adenocarcinoma MC38 cell line was provided by Prof. Jo Van Ginderachter (Vrije University Brussel, Belgium). Cells were maintained as previously described (7, 22). Manuka honey (UMF® 20+ from ApiHealth, Auckland, New Zealand) was used in the current study and diluted in distilled water under aseptic conditions. MH is composed mainly (~76%) of a mixture of sugars (fructose, glucose, maltose, sucrose and galactose) together with a significant component of bioactive compounds, including phenolics and flavonoids (6). As a control for MH, a sugar solution, designated sugar control (SC), containing equivalent concentrations of the three major sugars in honey (38.2% fructose, 31.3% glucose, and 1.3% sucrose) was used (Sigma, St. Louis, MO, USA) (8).

Experimental animals

BALB/c and C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). IFNγ-deficient ($IFN\gamma^{-/-}$) mice were purchased from the Jackson laboratories (USA) and have been described (23). All animals were bred in the animal facility of the College of Medicine and Health Sciences, United Arab Emirates University. For the current study, male mice at the age of 8-10 weeks were used. Female mice were not used to avoid potential physiological variability associated with the estrous cycle. Mice received rodent chow and water ad libitum and were maintained 5-6 mice per cage in a standard 12 h light/12 h dark cycle at a

temperature of 21°C with 40–57% humidity. All studies involving animals were carried out in accordance with, and after approval of the Animal Research Ethics Committee of the United Arab Emirates University (Protocols #A12-13 and ERA-2019-5853).

Oral treatment and tumor studies

BALB/c mice of comparable age and weight were randomly divided into two groups. Mice were gavaged daily with 0.2 mL of a water solution containing 70% SC or 70% MH (w/v). After 4 weeks of treatment, mice were euthanized, and their mesenteric lymph nodes (MLNs), inguinal lymph nodes (ILNs), and spleens were excised for further analysis.

For the tumor model studies, BALB/c or C57BL/6 mice were treated with SC or MH for 4 weeks, then subcutaneously inoculated with CT26 (2×10^5/mouse) or MC38 (1×10^5/mouse) cells, respectively, in the right flank. Tumor dimensions (width and length) were measured using a digital caliper twice a week, and tumor volume was calculated using the formula: tumor volume = $(L\times W^2)/2$, as detailed elsewhere (24, 25). Mice were euthanized 21 days post-implantation, and their tumors were excised for further analysis. In other experiments, oral MH was administered as a therapeutic regimen by first implanting tumor cells and then starting daily MH administration from day 3 post implantation for a total period of up to 3 or 4 weeks, as indicated. For these studies, we utilized wild-type C57BL/6 and IFN$\gamma^{-/-}$ mice.

Processing of lymphoid organs and tumors

Single cell suspensions were prepared from the spleens, MLNs, and ILNs by mechanical dissociation as previously described (26). Tumors were processed using a previously described method, with modification (27). Briefly, dissected tumor tissues were subjected to mechanical and enzymatic digestion in gentleMACS C-tubes (Miltenyi Biotec, Germany) using a tumor dissociation kit (Miltenyi Biotec) and the GentleMACS dissociator (Miltenyi Biotec), according to manufacturer's instructions. Tumor-infiltrating leukocytes (TILs) were subsequently purified from tumor cell suspensions using magnetic CD45$^+$ microbeads and the autoMACS cell separator, according to the manufacturer's protocol (Miltenyi Biotec).

Flow cytometric analysis

Analysis of MLN, ILN, spleen, and tumor cells was carried out using multi-color flow cytometry, following our standard protocol (22, 27). The following antibodies (all purchased from Biolegend, San Diego, CA, USA) were used in the current study: anti-CD45-APC (Cat# 103112), anti-CD19-PE (Cat# 115508), anti-CD19-PE-Texas Red (Cat# 115554), anti-CD3-BV785 (Cat# 100232), anti-CD4-FITC (Cat# 100509), anti-CD8-APC-Cy7 (Cat# 100714), anti-CD8-APC (Cat# 100712), anti-CD11b-Alexa Flour-488 (Cat# 101217), anti- CD11c-PE (Cat# 117308), anti- Ly6G-BV605 (Cat# 127639), Ly-6A/E (Sca-1)-PE-Texas Red (Cat# 108138), anti-MHC II (I-A/I-E)-BV785 (Cat# 107645), anti-MHC I H-2K^d -BV421 (Cat# 116623). Non-viable cells from tumors were excluded using 7-AAD viability dye (Biolegend) and non-viable cells from spleens, MLNs, and ILNs were excluded using Zombie Aqua dye (Biolegend). Data were collected on 10,000-50,000 cells (depending on the organ) using a FACSCelesta flow cytometer (BD Biosciences, Mountain View, CA, USA) and analyzed using FlowJo software (BD Biosciences).

Immunohistochemical analysis

Immunohistochemical staining was performed on tumor tissue sections as per established protocols in our laboratory (22, 28). Sections were incubated overnight with specific monoclonal antibodies to CD8 (ab209775; Abcam, UK), CD4 (ab183685; Abcam), or granzyme-B (44153S; Cell Signaling Technology, Danvers, MA, USA), after which they were incubated with HRP-conjugated goat polyclonal secondary antibody for 45 min at room temperature. Sections were then developed using DAB chromogen substrate (Dako, Carpinteria, CA, USA), counterstained with hematoxylin, and examined using an Olympus BX51 microscope (Olympus Corporation, Japan) at 40× magnification. The positive cells were counted in 10-20 randomly selected high-power fields (HPF), and the average count was calculated.

Quantitative real-time PCR

qRT-PCR was carried out essentially as previously detailed (22, 27). We used premade TaqMan primers and probes (Applied Biosystems, Foster City, CA, USA) for the following genes, CXCL1 (Mm04207460_m1), CXCL2 (Mm00436450_m1), CXCL10 (Mm99999072_m1), IFN-γ (Mm01168134_m1), and granzyme B (Mm00442834_m1). mRNA levels of target genes were normalized according to the comparative ΔCq method to respective mRNA levels of the housekeeping gene HPRT (Mm01545399_m1). The expression of the target gene is reported as the level of expression relative to HPRT and presented as fold change relative to control mice.

Fecal sample collection and DNA extraction

DNA was extracted from stool samples using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Valencia, CA, USA), following standard protocol. DNA concentration was measured using the NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA).

Bacterial 16S rRNA gene amplicon sequencing

16S Metagenomic Sequencing kit (Illumina, San Diego, CA, USA) was used for library preparation. V3–V4 hypervariable

regions of the bacterial 16S rRNA gene were amplified using the primers (5′-CCTACGGGNGGCWGCAG-3′ and 5′ GACTACHVGGGTATCTAATCC-3′) provided by the manufacturer and following the recommended protocol as described before (29). Library concentration was assessed by Qubit Fluorometric Quantitation (Invitrogen, Waltham, MA, USA). Short-read paired-end amplicon sequencing was performed using Illumina® MiSeq Instrument for 600 cycles.

Bioinformatic analysis

Processing of sequencing reads (adaptor trimming and filtering of low-quality reads) followed by taxonomic classification were done using Quantitative Insights Into Microbial Ecology version 2 (QIIME2) software suite (30). After the identification of Operational Taxonomic Units (OTUs), downstream analyses were carried out in RStudio (v 4.1.2). Diversity was measured using BiodiversityR (v 2.15-2) and plotted by ggplot2 (v. 4.1.3). Alpha diversity measures (Observed OUT, CHAO1, Shannon's Diversity, and Simpson's Diversity indices) were compared between the groups using Mann-Whitney U test. For beta diversity, principal coordinate analysis based on Jaccard and Bray Curtis dissimilarity metrics was used to assess differences between the groups using non-parametric multivariate analysis of variance (PERMANOVA). Linear discriminant analysis (LDA) effect size (LEfSe) was used to detect biomarkers from microbial profiles (31) using the Microbiome Analyst 2.0 platform (McGill, Canada), which was also used to generate the graph of relative abundance and the heatmap for groups comparison (32). Venn diagrams were generated to compare the taxa exhibiting significant differences based on the LDA analysis for the identification of shared and unique OTUs (33).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 9.0 (San Diego, CA, USA). Statistical significance between control and treated groups was determined using 2-way ANOVA or the unpaired, two-tailed Student's t-test, as indicated. In all analyses, $p < 0.05$ was considered statistically significant * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

Results

Oral administration of MH induces functional alterations in host immune responses

We have previously demonstrated the ability of i.p. administration of MH to effect changes in the immune system via inducing the recruitment of neutrophils into the peritoneal cavity and the maturation of peritoneal macrophages (15). In our efforts to apply a more physiological route of administration that would be safe and more applicable to humans, we investigated the effect of repeated oral administrations of MH on the immune system of BALB/c mice. Based on our previous experience, a solution of 50-70% MH (w/v) is suitable for *in vivo* use in mice (7). Naïve BALB/c mice were orally gavaged with water (control group), sugar control (SC) solution, or MH for 4 weeks. To address if repeated oral doses of MH are associated with any adverse events, body weights were determined in treated animals over the 4-week period. Baseline body weights were recorded before starting the treatment (Means ± SEM = 22.97 ± 0.397, 23.63 ± 0.783, 23.54 ± 0.818 g for the water, SC and MH groups, respectively) and at weekly intervals after treatment. The percentage change in body weight from baseline was then calculated. The results indicated that, in comparison with water-treated and SC-treated groups, treatment with MH over 4 weeks did not affect normal weight gain, with all 3 experimental groups showing comparable levels of body weight gain over the treatment period (Supplementary Figure 1A). Accordingly, we selected SC as the control for all subsequent experiments. The potential effect of oral MH on blood glucose levels was also investigated by determining non-fasting glucose levels in blood samples of randomly-selected mice collected on a weekly basis in SC or MH-treated groups. The average random glucose blood level in untreated, age-matched, control mice is 172.0 ± 14.9 mg/dL (mean ± SD). At the end of the treatment period, we observed that MH administration did not alter the blood glucose levels, with both SC and MH groups showing comparable glucose levels that lie within the normal range (<200 mg/dL) (Supplementary Figure 1B). Thus, no apparent negative effects were evident following oral administration of MH.

The capacity of MH to effect changes in the immune system was next investigated. Different peripheral lymphoid tissues including gut-draining MLNs, ILNs, and spleens were collected, and their weights and absolute cell counts were recorded. Our results indicated that MH administration did not alter the weights or the total cell counts of the collected tissues in comparison to SC-treated mice (Supplementary Figures 1C–H).

Next, multi-color flow cytometry was utilized to analyze the cellular changes in the collected tissues following MH administration. The gating strategy employed to identify the major immune subpopulations is shown in (Supplementary Figure 2). FACS data indicated that MH administration did not lead to alterations in the cellular landscape of MLNs, ILNs, or spleens between SC or MH-treated mice (Supplementary Figure 3).

In the context of our previous findings demonstrating a functional maturation of macrophages that was observed following i.p. administration of MH, we next sought to investigate if similar alterations were induced following oral MH administration. Upregulation of MHC class II proteins on myeloid cells is a key event that is induced in response to activation through type I/II interferon signaling pathways (34, 35). Therefore, we investigated whether oral administration of MH can induce any alterations within the cellular landscape of the peripheral lymphoid tissues, including MLN, ILN, and spleen. Given that the majority of cells in these tissues comprise T and B lymphocytes, we focused on analyzing changes in the expression of proteins known to be induced by type I/II IFNs. One of the well-known IFN-inducible genes is *Ly6a*, which encodes for Sca-1 protein on T lymphocytes (36–38). The results of the flow cytometric analysis showed that oral administration of MH led to

a significant increase in Sca-1 expression on both $CD8^+$ and $CD4^+$ T cells (Figure 1). The percentage of Sca-1-positive $CD8^+$ T cells in MLNs, ILNs, and spleens increased by 21.6%, 24.4%, and 24.7%, respectively in MH-treated mice in comparison to SC-treated mice (Figures 1A, B, D, E, G, H). Similarly, the percentage of Sca-1-positive $CD4^+$ T cells increased by 36.5%, 39.8%, and 62.9% in the same three organs, respectively (Figures 1A, C, D, F, G, I). These results show that oral MH administration induced IFN-dependent responses in T cells, both at the level of the gastrointestinal tract as well as in systemic lymphoid organs.

Oral administration of MH retards the growth of implanted tumors

The demonstration of the capacity of oral MH administration to activate T lymphocytes supports its potential role as an immunomodulatory agent. We reasoned that oral MH could potentially be used to boost immune responses preventatively in different disease settings. To test this hypothesis, we investigated the capacity of MH to modulate anti-tumor immune responses using two different syngeneic murine CRC models, namely CT26 tumors in BALB/c mice and MC38 tumors in C57BL/6 mice. The treatment protocol followed in this study is illustrated in Figure 2A. Our findings revealed that pre-treating mice with MH resulted in a significant growth retardation of both CT26 (Figure 2B) and MC38 (Figure 2C) tumors. In the CT26 model, tumors grew continuously and rapidly in mice given vehicle (H_2O) or SC solution, reaching a mean volume ± SEM of 897 ± 169 mm^3 and 916 ± 114 mm^3, respectively on day 21 post-implantation (Figure 2B). On the other hand, mice treated with MH exhibited a significant reduction in tumor volume, with a mean of 511 ± 90 mm^3 on day 21 post-implantation. The suppression in tumor growth was observed as early as 9 days post-implantation, and by day 21, it reached 44% compared to SC-treated mice (p = 0.006) (Figure 2B). Tumor growth in individual mice of each of the three experimental

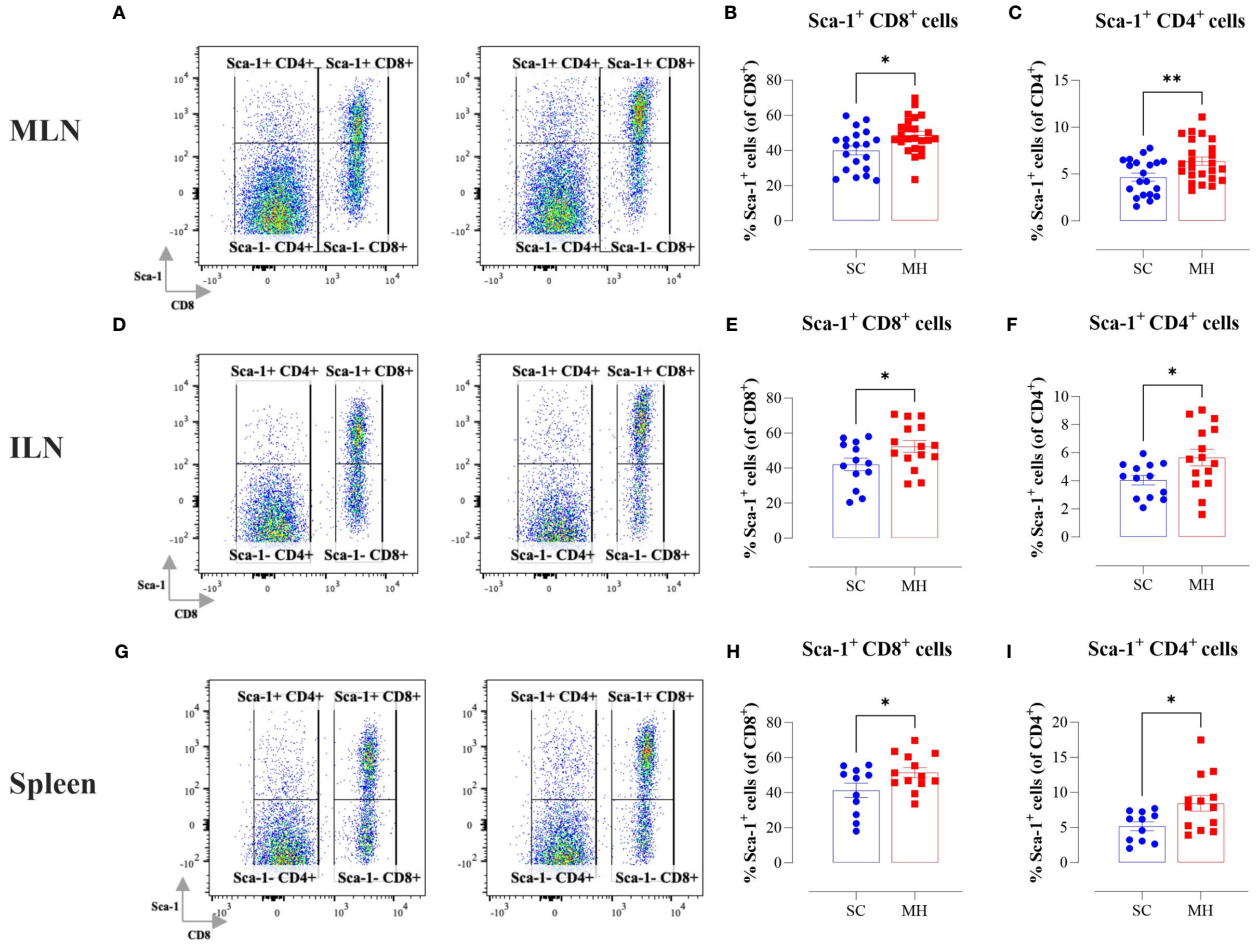

FIGURE 1
Lymphocyte activation in lymphoid tissues following MH treatment. BALB/c mice were orally gavaged with either SC or MH for 4 weeks. Following treatment, lymphoid organs were processed for flow cytometry analysis. Cells from MLNs **(A–C)**, ILNs **(D–F)**, and spleens **(G–I)** were analyzed to quantify the percentage of Sca-1^+ $CD8^+$ T cells **(B, E, H)**, and Sca-1^+ $CD4^+$ T cells **(C, F, I)**. Representative dot plots are shown in **(A, D, G)**. The values for individual mice and mean ± SEM are shown. The data is pooled from 5 **(A–C)**, 4 **(D–F)**, and 3 independent experiments **(G–I)**. p values were calculated using the unpaired Student's t-test, * ($p \leq 0.05$), and ** ($p \leq 0.01$).

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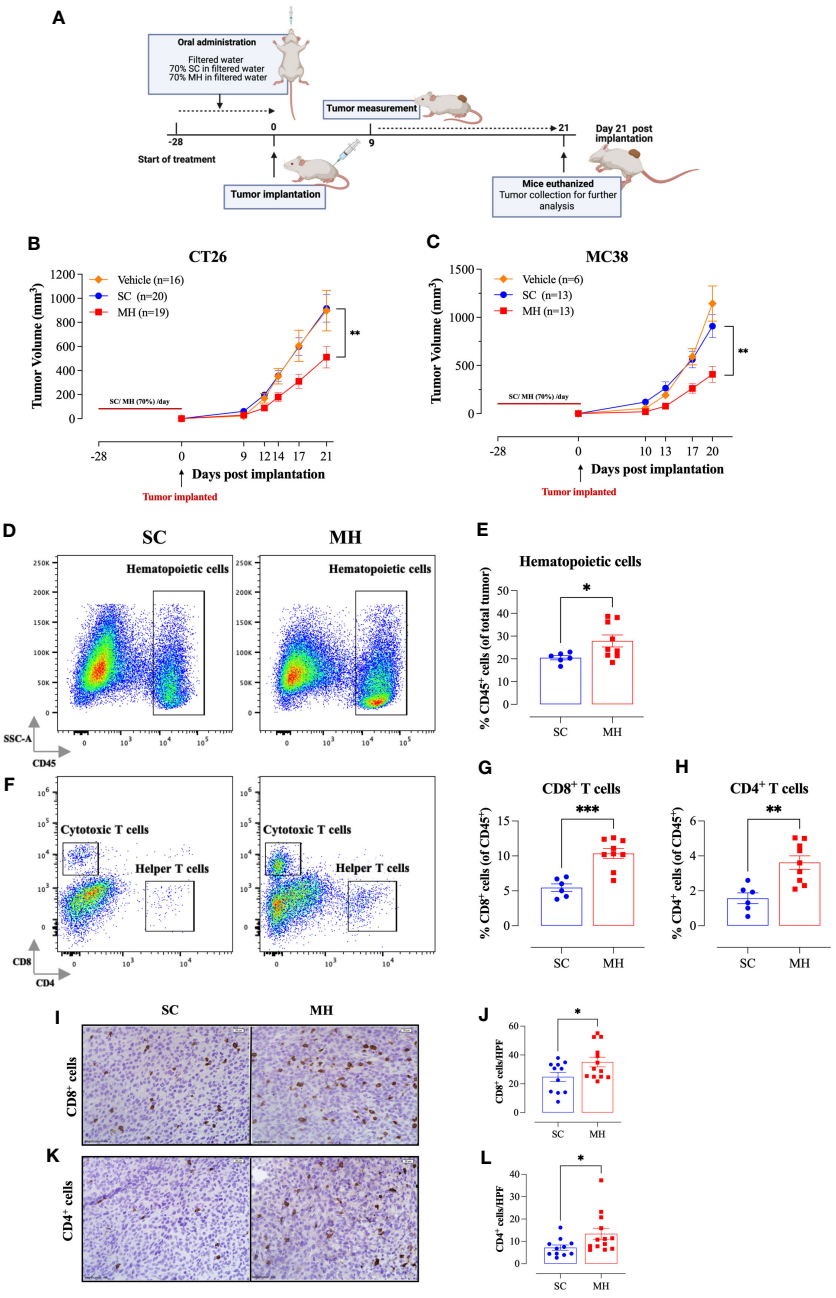

FIGURE 2
Retardation of tumor growth in MH pre-treated mice correlates with enhanced tumor infiltration by immune cells. **(A)** A schematic diagram describing the preventative model treatment protocol. Mice were orally gavaged daily with filtered water, 70% SC or 70% MH for 4 consecutive weeks. Following the treatment period, CT26 or MC38 CRC cells were implanted and tumor growth was followed for the subsequent 3 weeks. Mice were euthanized on day 21 post-implantation, and tumors were excised and processed for further analysis. Tumor growth curves of CT26 **(B)** or MC38 tumor **(C)** in water-treated, SC-treated, and MH-treated mice are shown. Each data point represents the mean ± SEM of 16-20 mice, pooled from 3 individual experiments. Asterisks denote statistically significant differences between the SC-treated and MH-treated groups. *p* values were calculated using 2-way ANOVA. Resected tumors were analyzed for the extent of intratumoral immune cells by flow cytometry **(D–H)** and immunohistochemistry **(I–L)**. Representative dot plots and quantification of percentage of $CD45^+$ immune cells **(D, E)**, $CD8^+$ cytotoxic T cells **(F, G)**, and $CD4^+$ helper T cells **(F, H)**. The values for individual mice and mean ± SEM are shown (SC: n=6, MH: n=9), pooled from 2 independent experiments. **(I–L)** Tissue sections were analyzed by immunohistochemistry to quantify the number of $CD8^+$ and $CD4^+$ cells. Representative images at 40× magnification (scale bar 20 µm), and the quantitative estimation of the number of $CD8^+$ cells **(I, J)** and $CD4^+$ cells **(K, L)** per HPF (high-power field) are presented for each group. The values for individual mice and mean ± SEM are shown (SC: n=11, MH: n=13), pooled from 3 independent experiments. Asterisks denote statistically significant differences between the MH-treated and SC-treated groups. *p* values were calculated using the unpaired Student's t-test, * ($p \leq 0.05$), ** ($p \leq 0.01$), and *** ($p \leq 0.001$).

groups is shown in Supplementary Figures 4A, C, E. Very similar findings were observed using the MC38 tumor model (Figure 2C). Pre-treatment with MH resulted in 55% suppression ($p = 0.002$) in the growth of MC38 tumors by day 20 post implantation (Figure 2C). These results highlight a potential immune-boosting anti-tumor role for MH when given preventively.

MH treatment induces alterations within the tumor microenvironment

To investigate the underlying mechanism for the observed MH-mediated retardation of tumor growth, we analyzed the tumor microenvironment (TME) for alterations in the cellular landscape by flow cytometry. Tumors were excised at the end of the observation period, subjected to mechanical and enzymatic digestion, and processed to a single cell suspension. The cells were then stained with different panels of mAbs to quantify the cellular constituents within the tumor tissue. The gating strategies employed to identify the cellular subpopulations are illustrated in (Supplementary Figure 5).

Tumor-infiltrating immune cells were identified by being positive for the pan-hematopoietic CD45 cell surface marker. There was a significant 36% increase in $CD45^+$ immune cells in the tumors of MH-treated mice compared to the control group (28% *vs.* 20%, respectively) (Figures 2D, E). FACS analysis revealed alterations in both the phenotypic and functional characteristics of the lymphoid and myeloid subpopulations in the TME. Regarding the $CD3^+$ T cell population, we observed a ~2-fold increase in the percentages of both $CD8^+$ and $CD4^+$ T cells (Figures 2F–H) following MH treatment. The increase in the infiltration of T cells was also demonstrated morphologically by immunohistochemistry, where the number of both $CD8^+$ and $CD4^+$ T cells was substantially increased in tumor tissue sections of MH-treated mice (Figures 2I–L).

Further analysis using myeloid cell-specific antibodies showed that the majority (70-80%) of the gated $CD45^+$ population in the TME were $CD11b^+$ myeloid cells (Figure 3A). Interestingly, there was a significant decrease (~18%) in the percentage of intratumoral myeloid cells in the MH-treated group compared to the SC-treated group (Figures 3A, D). This was largely accounted for by a 50% reduction in the percentage of $Ly6G^+$ granulocytes (Figures 3B, E), most likely representing myeloid-derived suppressor cells (39). In contrast, the percentage of $Ly6C^{hi}$ cells increased significantly (~1.7-fold) in MH-treated mice (Figures 3B, F). These cells have been described to be pro-inflammatory in function and are recruited to tumor tissue in response to CCL2/CCR2 signaling (40). They further differentiate into MHC class II (MHC-II) positive or negative tumor-associated macrophages (TAM) dependent on macrophage colony-stimulating factor-mediated signals (41). In terms of the other myeloid subpopulations, there was no major change in the percentages of $Ly6C^{lo/Neg}$ cells (Figures 3B, G) and dendritic cells ($CD11c^+$ cells) (Figures 3C, H) following MH treatment.

To gain insight into the functionality of intratumoral myeloid cells, we analyzed the level of expression of MHC-II proteins on $CD11b^+$ $Ly6G^-$ subpopulation. There was a significant increase in the percentage of myeloid cells expressing MHC-II proteins (2.4-fold) in MH-pretreated mice (Figures 3I, J). Furthermore, the overall level of expression of MHC-II on myeloid cells in MH-treated group tended to be slightly elevated compared to SC-treated mice, but this difference did not reach statistical significance ($p = 0.083$) (Figures 3I, K, L). These data are suggestive of an enhancement in the antigen-presentation capacity of myeloid cells within the TME of MH-treated mice.

Given the evidence of the involvement of type I/II IFN pathways in the observed functional changes in cellular function, we next analyzed whether similar alterations could be observed on the tumor cells. It is well known that the expression of MHC class I (MHC-I) proteins is regulated by type I/II IFN signaling pathways (42). Therefore, we analyzed the expression of MHC-I proteins on $CD45^-$ tumor cells grown in mice after pretreatment with MH in comparison with tumor cells grown in control mice given SC solution. The results of this analysis showed that tumor cells grown in control mice showed bimodal levels of MHC-I expression, with 2 clearly discernible subpopulations being observed. The majority of these tumor cells (~62%) expressed low levels of MHC-I proteins, while the remaining population (~38%) showed high levels of MHC-I (Figures 4A–C). In sharp contrast, approximately 70% of tumor cells grown in mice pre-treated with MH exhibited high levels of MHC-I proteins (Figures 4A, C). Furthermore, a 7-fold increase in the MFI level of MHC-I proteins on tumor cells was observed following MH administration (Figures 4D, E). These results suggest that MH treatment indirectly enhanced the immunogenicity of tumor cells, rendering them more susceptible to killing by anti-tumor $CD8^+$ T effector cells. Taken together, our findings indicate that the ability of MH to effect changes in tumor growth is linked to a series of immunomodulatory alterations within the TME.

Altered expression of cytotoxic effector molecules and immunoregulatory mediators following MH treatment

To elucidate the mechanism by which MH modulates the cellular components of the TME and exerts the observed anti-tumor response, RNA was extracted from purified, intratumoral, $CD45^+$ cells, or whole tumor tissue, of SC-treated or MH-treated mice. The RNA was then used to determine the gene expression levels of key inflammatory chemokines and cytotoxic effector molecules by qRT-PCR. At the level of tumor-infiltrating leukocytes, MH treatment led to a small (1.6-fold) but insignificant increase in the expression level of the chemokine CXCL10 (Figure 5A). At the whole tumor level, there was also a 2.3-fold increase in the expression of CXCL10 in MH-treated mice (Figure 5F; $p = 0.0131$). CXCL10 is secreted in response to IFN-γ and preferentially regulates the recruitment of inflammatory T lymphocytes (43). The expression levels of CXCL2 and CXCL1 chemokines, which are potent chemoattractants that control the recruitment of polymorphonuclear leukocytes in inflammation and tissue injury (44), were also examined. qRT-PCR results indicated a

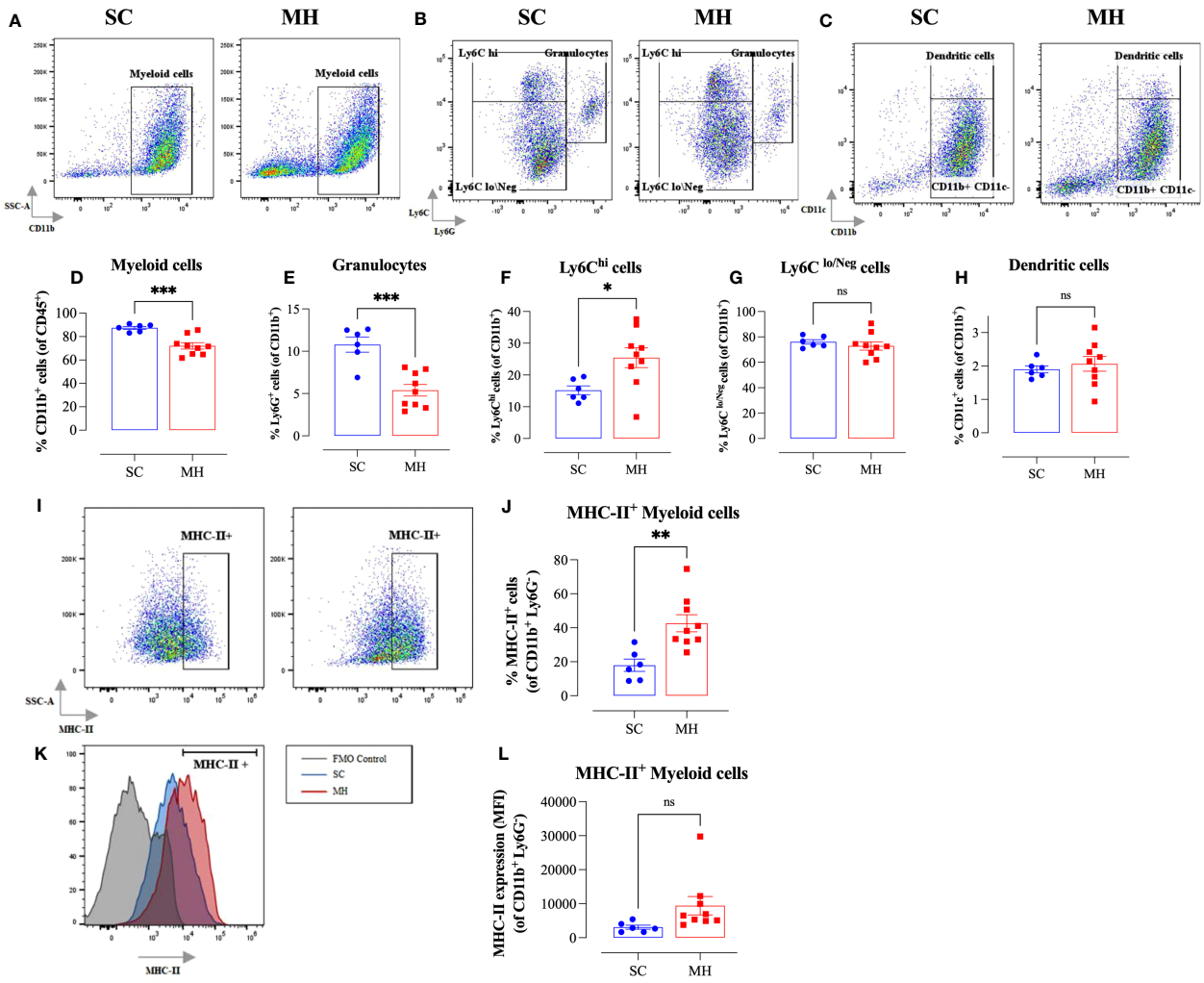

FIGURE 3
MH treatment alters intratumoral myeloid subpopulations and enhances expression of MHC class II on macrophages. BALB/c mice were orally gavaged with either 70% SC or 70% MH for 4 consecutive weeks, then implanted with CT26 tumor cells. Mice were euthanized on day 21 post-implantation, their tumors were resected, and the percentages of intratumoral myeloid cells were determined by flow cytometry. Representative dot plots and the quantification of the percentages of $CD11b^+$ myeloid cells **(A, D)**, $Ly6G^+$ granulocytes **(B, E)**, $Ly6C^{hi}$ cells **(B, F)**, $Ly6C^{lo/Neg}$ cells **(B, G)**, and $CD11c^+$ dendritic cells **(C, H)**. The values for individual mice and mean ± SEM are shown (SC: n=6, MH: n=9), pooled from 2 independent experiments. Analysis of MHC class II protein expression **(I–L)**. Representative flow plots **(I)** and the quantification **(J)** of the percentage of $MHC\text{-}II^+$ cells (gated on $CD11b^+$ $Ly6G^-$ cells) in SC-treated and MH-treated mice. **(K)** Representative overlay histograms showing MHC-II expression on $CD11b^+$ $Ly6G^-$ myeloid cells of SC-treated and MH-treated mice. The grey histogram indicates staining with FMO control. **(L)** Median fluorescence intensity of $MHC\text{-}II^+$ $CD11b^+$ $Ly6G^-$ in SC-treated and MH-treated groups. The values for individual mice and mean ± SEM are shown (SC: n=6, MH: n=9), pooled from 2 independent experiments. Asterisks denote statistically significant differences between the MH-treated and SC-treated groups. *p* values were calculated using the unpaired Student's t-test, * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), and ns (no statistical significance, $p > 0.05$).

statistically significant 1.6-fold decline in the expression level of CXCL2 in MH-treated mice (Figure 5B). A trend toward a decrease in the expression levels of CXCL1 was also observed, but this did not reach statistical significance (Figure 5C). These findings may underlie the observed reduction in the proportion of intratumoral $Ly6G^+$ granulocytes following MH treatment.

MH treatment also resulted in a significant increase in the expression levels of IFN-γ (~1.7-fold) and granzyme B (~2.8-fold), as detectable at the level of TILs (Figures 5D, E). Both of these mediators are secreted by effector immune cells to induce tumor cell apoptosis (45, 46). A significant increase (2.6-fold) in the expression of IFN-γ ($p = 0.0148$) and granzyme B ($p = 0.0261$) was also observed at the level of the whole tumor tissue (Figures 5G, H). Consistent with these findings, immunohistochemical staining of tumor tissues indicated an increase in the number of granzyme B-positive cells following MH treatment (Figures 5I, J), reflecting the presence of activated, anti-tumor, cytotoxic lymphocytes, most likely T cells and/or NK cells.

Therapeutic efficacy of oral MH against implanted CRC tumors

Having demonstrated the capacity of oral MH as a preventive treatment against cancer growth, we assessed its potency in a therapeutic model. Mice were implanted with MC38 CRC cells

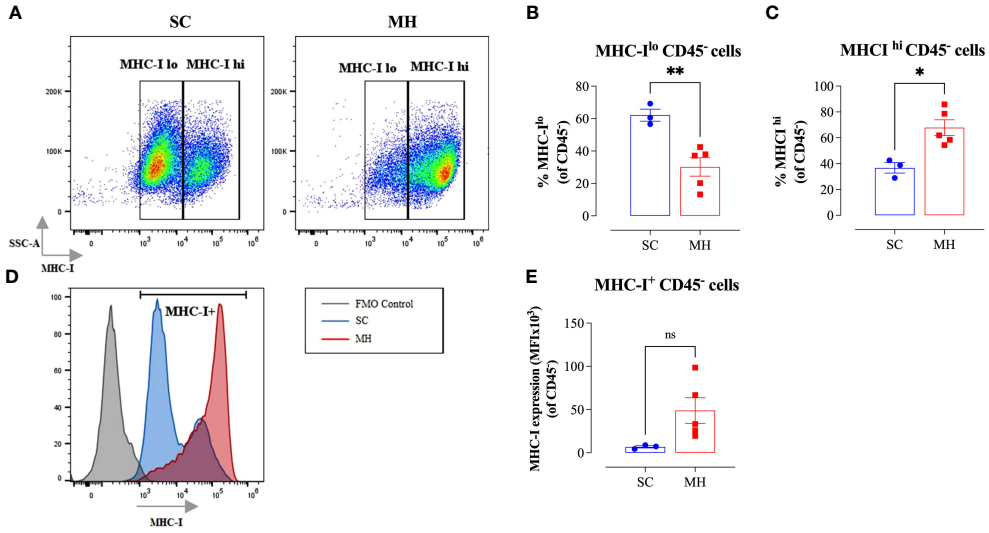

FIGURE 4
Enhancement in tumor immunogenicity as a consequence of MH administration. **(A–C)** Representative flow plots **(A)** and quantification of the percentage of MHC-I^{lo} **(B)** and MHC-I^{hi} **(C)** (gated on CD45$^-$ cells) in SC-treated and MH-treated mice. **(D)** Representative overlay histograms showing MHC-I expression on CD45$^-$ cells of SC-treated and MH-treated mice. The grey histogram indicates staining with FMO control **(E)** Median fluorescence intensity of MHC-I$^+$ CD45$^-$ cells in SC-treated and MH-treated groups. The values for individual mice and mean ± SEM are shown (SC: n=3, MH: n=5), obtained from 1 experiment. Asterisks denote statistically significant differences between MH-treated and SC-treated groups. *p* values were calculated using the unpaired Student's t-test, * ($p \leq 0.05$), ** ($p \leq 0.01$), and ns (no statistical significance, $p > 0.05$).

and oral MH was subsequently administered on a daily basis starting on day 3 post implantation. The data affirm that daily MH administration leads to a significant inhibition of tumor growth in normal, immunocompetent, mice (Figure 6A). The effect of MH on tumor growth was first apparent at about 10 days after the initiation of treatment. In sharp contrast, MH-induced curtailment of tumor growth was completely abrogated in IFNγ-deficient mice (Figure 6B), demonstrating mechanistically that MH most likely exerts its immunomodulatory effect via the activation of the IFNγ pathway.

Oral MH induces changes in gut microbiota

We hypothesized that oral administration of MH could induce changes in gut microbiota that would underlie the enhanced anti-tumor immune responses observed in these mice. To address this possibility, we determined the composition of gut microbiota in fecal samples collected from mice either before treatment or after 4 weeks of treatment with MH or SC solution. Microbiota were profiled at the genus level to detect the alterations in abundance and diversity caused by each treatment. The stacked area plot (Figure 7) shows the relative abundances of genera ranked based on their prevalence in the samples (listed below in the graph) collected from mice before and after treatment with SC or MH. Variations were obvious among the samples. The microbiota fingerprint in the control group was maintained between week 0 and week 4. As for the MH group, microbiota profiles looked more homogenous after treatment and with more similarity compared to the variability seen in week 0. To identify the genera with significant differences before and after each treatment, linear discriminant analysis (LDA) was done. As shown in Figures 8A, B, treatment with either SC or MH caused significant changes in microbiota profiles, with depletion of some genera (red color in the graphs) and enrichment of others (blue color in the graphs). These findings confirm that the microbiota were changed after 4 weeks of either treatment. It is noteworthy that treatment with SC caused depletion of *Lactobacillus* which is generally considered a beneficial bacteria. In sharp contrast, MH treatment caused depletion of pathogenic bacteria namely, *Staphylococcus, Enterococcus*, and *Bacteroides.*

Next, we investigated if microbiota alteration induced by treatment was similar in MH versus SC groups. Significantly changed microbiota identified by LDA analysis in MH and SC groups (from Figures 8A, B) were compared, and Venn diagram (Figure 8C) was used to identify unique and shared genera. Most of the genera altered in response to treatment with MH and SC were unique for each group. Only two genera were shared between the two groups, namely *Methylarcula* (more in MH in week 4, and more in SC in week 0, i.e. enriched after treatment with MH and depleted after treatment with SC), and *Geovibrio* (less in week 4 in both groups, i.e. reduced due to treatment with both SC and MH). Moreover, we compared the genera detected in MH and SC groups after 4 weeks of treatment to explore microbiota differences between these groups post-treatment. The heatmap also shows the genera with significant difference between week 0 and week 4 after each treatment (shown in Figures 8A, B) and reveals the distinct microbiota fingerprints per group. As shown in Figure 8D, significant differences were found in the genera after 4 weeks of treatment with SC or MH (marked with asterisk in the heatmap). For example, MH induced the depletion of several genera, including *Bacteroides*, *Staphylococcus* and *Enterococcus*, which have been

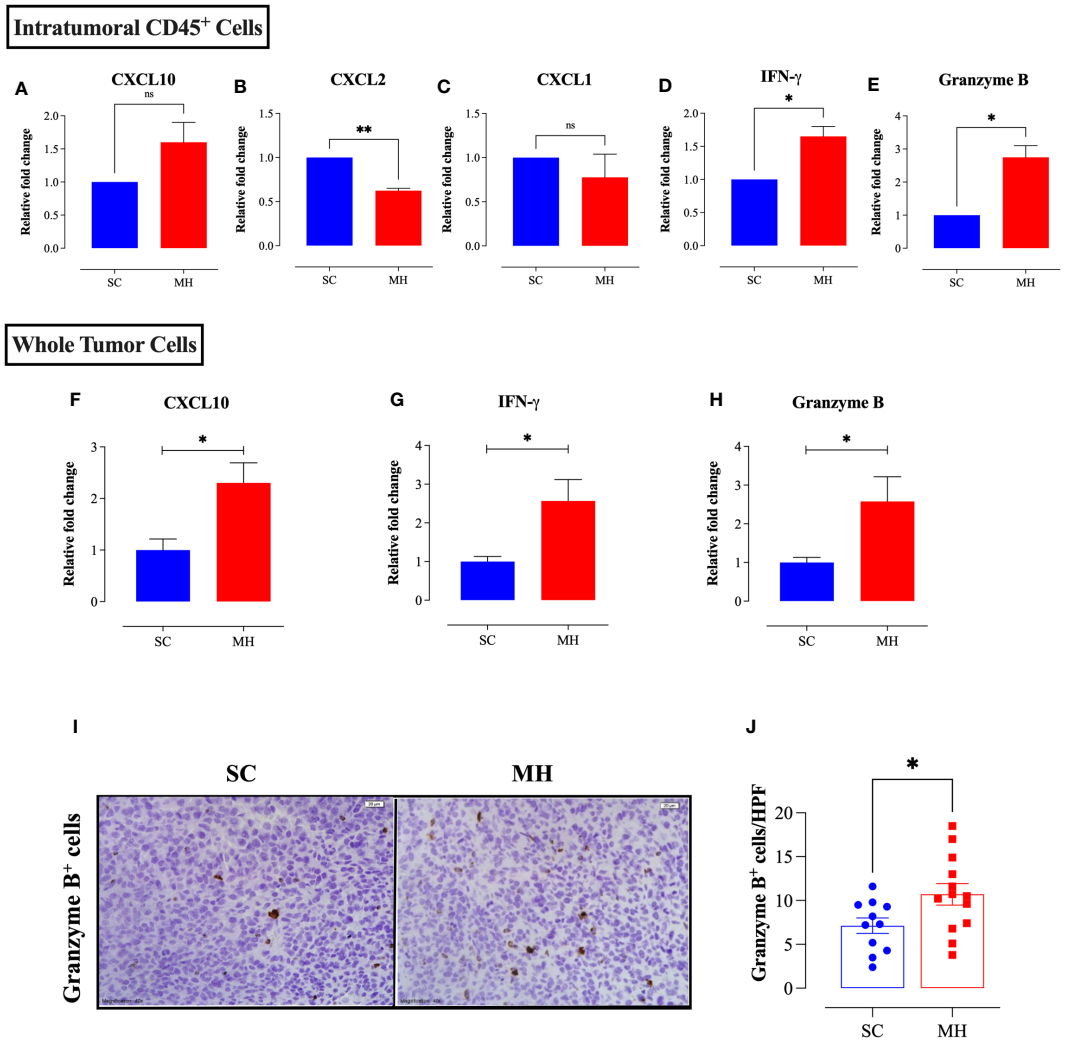

FIGURE 5
MH treatment alters the expression of chemokines and anti-tumor effector molecules within the tumor microenvironment. CT26 tumor tissues were excised from SC-treated and MH-treated mice on day 21 post-implantation. $CD45^+$ cells were purified from a pool of tumor cells obtained from 4 tumors per group. RNA was extracted from the purified $CD45^+$ cells and used to assess the mRNA levels of CXCL10 **(A)**, CXCL2 **(B)**, CXCL1 **(C)**, IFN-γ **(D)**, and granzyme B **(E)**. The data are expressed as means ± SEM of 2 replicates per group and are representative of 2 independent experiments. **(F–H)** RNA was extracted from whole tumor tissue and assessed for the relative expression of CXCL10 **(F)**, IFNγ **(G)** and granzyme B **(H)** genes. **(I, J)** Tissue sections were analyzed by immunohistochemistry to quantify the number of granzyme B^+ cells. Representative images at 40× magnification (scale bar 20 µm) are presented for each group **(I)**. Quantitative estimation of the number of granzyme B^+ cells/HPF (high-power field) is shown in panel **(J)**. The values for individual mice and mean ± SEM are shown (SC: n=11, MH: n=13), pooled from 3 independent experiments. Asterisks denote statistically significant differences between the MH-treated and SC-treated groups. *p* values were calculated using the unpaired Student's t-test, * ($p \leq 0.05$), ** ($p \leq 0.01$), and ns (no statistical significance, $p > 0.05$).

described to have pro-tumorigenic activities (47–51). In contrast, two of the microbiota genera enriched after MH treatment were *Ruminococcus* and *Clostridium* cluster IV, both of which encompassing members that have been recognized for their anti-tumorigenic potential (52, 53). The LDA analysis of the significantly different genera is shown in (Supplementary Figure 6). The relative abundance of each significantly different genus among the groups is shown in (Supplementary Figure 7).

Additionally, we have explored the effect of MH and SC treatment on microbiota diversity. The difference was not statistically significant in alpha and beta diversity (Figures 9A–E), but the only exception was seen in Shannon's index, which is a widely used alpha diversity metric. The latter index is the negative sum of proportional microbiota abundance multiplied by the log of its proportional abundance (54). It is generally useful in predicting diversity by reflecting both richness (the number of microbiota) and evenness (the uniformity of distribution of microbiota) within a community (55). Shannon's index was significantly higher after treatment with MH for 4 weeks compared to the baseline in week 0 (Figure 9A), suggesting an increase in the richness and evenness of microbiota after treatment with MH. This effect was not evident after treatment with SC. Nevertheless, pairwise comparison between SC and MH in week 0 and week 4 did not reveal any significant difference between these groups. Altogether, our findings demonstrate that MH treatment led to distinct changes in microbiota composition that are significantly different from the

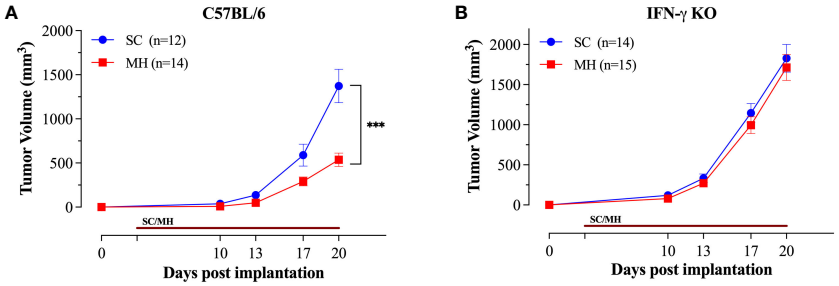

FIGURE 6
Therapeutic treatment with oral MH retards tumor growth in an IFNγ-dependent manner. Normal C57BL/6 **(A)** or IFNγ-deficient **(B)** mice were implanted with tumor cells and were orally gavaged daily starting on day 3 post implantation with 70% SC or 70% MH for 3 consecutive weeks. Each data point represents the mean ± SEM of the indicated mice within each experimental group, pooled from 2 independent experiments. Asterisks denote statistically significant differences between the SC-treated and MH-treated groups. *p* values were calculated using 2-way ANOVA, *** ($p \leq 0.001$).

effect of SC, with identification of key microbiota that were increased or decreased following treatment.

Discussion

Previous reports from our laboratory highlighted the role of MH as an anti-cancer and immunomodulatory agent (6–8, 10, 11, 15). In the current study, we present compelling evidence demonstrating the capacity of orally-administered MH to boost anti-tumor immune defense against an implanted colon adenocarcinoma tumor. To the best of our knowledge, this is the first report to demonstrate mechanistically how preventative, or therapeutic, administration of MH can lead to alterations in the cellular landscape within the TME that promote a more effective anti-tumor immunity.

The present study highlights several novel findings regarding the potential use of MH as a preventative and therapeutic agent against cancer. First, *in vivo* experiments using the oral administration route demonstrated immunological alterations consistent with the induction of IFN signaling pathway. Second, the significance of MH-induced immunological alterations was highlighted in two separate murine CRC models, where pre-treating mice with a daily oral dose of MH over 4 weeks resulted in a retardation of tumor growth. Third, MH-mediated tumor inhibition correlated with a series of cellular changes within the TME. Fourth, these intratumoral cellular alterations were accompanied by changes in the expression levels of various immunomodulatory chemokines and inflammatory cytokines. Fifth, MH-induced improvement in anti-tumor immune responses was also evident when used in a therapeutic regimen and was completely dependent on IFNγ. Lastly, MH treatment modulated gut microbiota composition, enriching for a unique pattern of several bacterial genera and inducing a depletion of pathogenic bacteria. Notwithstanding these findings, a major limitation of the present study is the use of ectopic tumor models. The utilization of a genetic, spontaneous, CRC model would increase the relevance of our findings. While the proportions of immune cells in the peripheral lymphoid tissues remained unaltered after MH treatment, there was a noticeable increase in their activation status, as evidenced by the upregulation of Sca-1 expression on lymphoid cells in the MLNs, ILNs, and spleens. Sca-1 is an interferon-inducible protein (36–38), that is upregulated as a result of inflammatory responses (56). Thus, the data indicate that oral MH treatment most likely triggers an inflammatory response that ultimately leads to an enrichment of type I/II interferons in both the gut and periphery. Induction of the IFN response may well be triggered when pathogen-associated molecular patterns (PAMPs) interact with membrane-bound pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs). TLRs recruit the MyD88 adaptor protein upon binding with their respective ligands, which leads to downstream activation of NF-kB, interferon regulatory factor 3 (IRF3), and interferon regulatory factor 7 (IRF7) transcription factors, which are responsible for inducing type I interferons (57, 58). Our previous findings showed that the immunostimulatory effect of MH following its intraperitoneal administration was significantly reduced in the absence of the MyD88 protein, indicating that TLRs may be involved in MH-triggered response (15). Since TLRs are involved in inducing type I IFN, it is plausible that oral administration of MH stimulates, directly or indirectly, type I IFN through a TLR-dependent pathway. However, further investigation is required to verify this hypothesis. Utilizing mice with known TLR defects would be useful in confirming this notion and elucidating the underlying mechanism in finer detail.

Alternatively, the enhancement in anti-tumor immune responses by MH could be related to the demonstrated changes in gut microbiota. Microbial dysbiosis is known to stimulate the host immune system, particularly T cell immune responses (59, 60). A previous study showed that an increase in Sca-1 expression on lymphoid cells in MLNs and peripheral tissues is linked to microbial dysbiosis in B cell-defective mice. The dysbiosis in the gut mucosal environment leads to type I IFN enrichment in $CD8^+$ T cells, resulting in increased anti-tumor immunity (61). Honey has been shown to acquire protective prebiotic effects due to the presence of oligosaccharides and polyphenols as major constituents (62). MH was shown to improve the growth of probiotic bacteria while inhibiting the growth of pathogens (63). Animal studies have shown that oral administration of 2.2g/kg (44 mg/mouse) of MH to mice for 4 weeks leads to alterations in the concentrations of short-chain fatty acids (64). A small clinical study, involving 20 healthy individuals aged 42-64 years, was conducted to establish the safety of daily MH consumption, particularly in regard to allergic

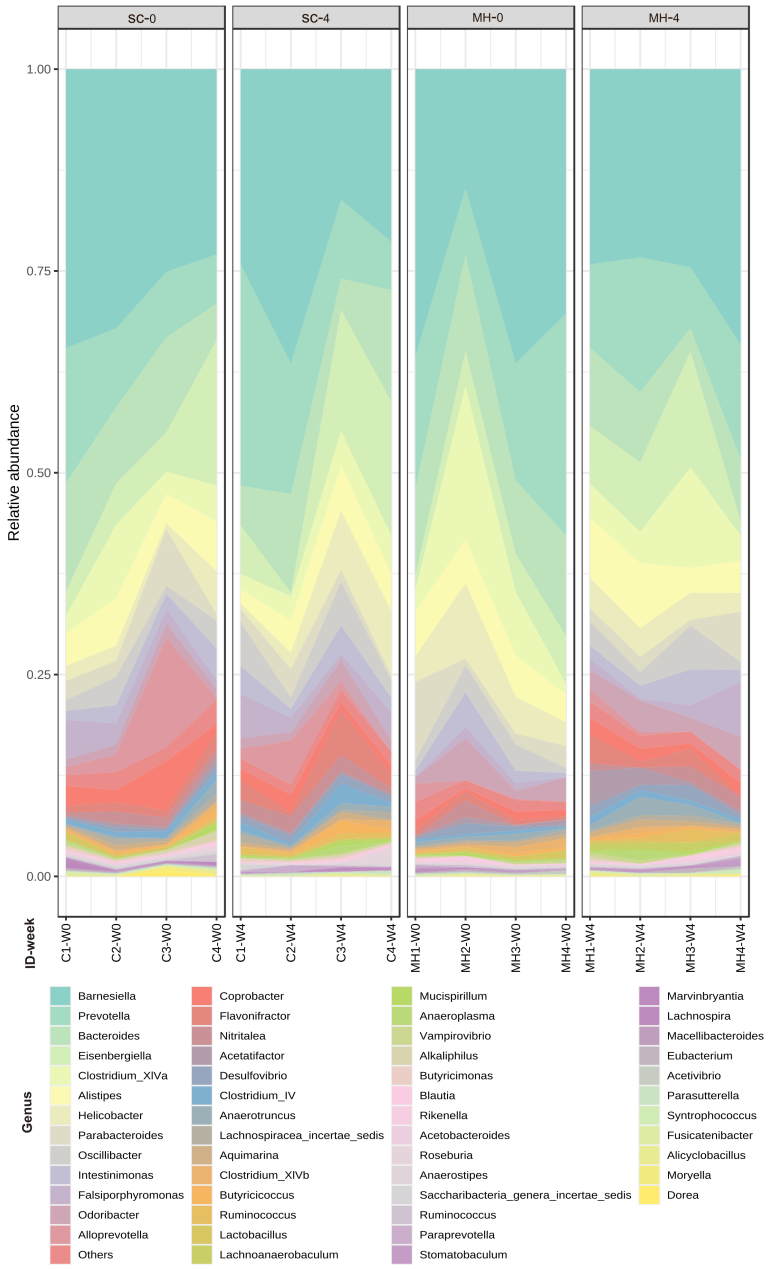

FIGURE 7
Relative abundance of genera detected at the baseline (week 0 before treatment) and in week 4 after treatment with SC or MH. Data shown represent the relative abundance of the genera listed, in each mouse investigated in this study before and after treatment.

responses or changes in gut microbiota (65). The consumption of 20g of MH (UMF 20+) daily for 4 weeks did not result in any significant changes in gut microbiota (65). The authors postulated that the prebiotic effects of MH may have been masked by various factors such as the interaction with other dietary components, the storage conditions of honey, and the relatively low dosage of MH used. This suggests that factors beyond simply the actual dose may have influenced the impact of MH. In the current study, we demonstrate the capacity of orally-administered MH to induce significant changes in gut microbiota composition. It is worth noting that the dose used in our study is a comparatively higher dose than previously used (approximately 140 mg/mouse, equivalent to a human dose of 39.8 g). Our findings suggest that daily consumption of MH could boost immunity against development of cancer in at risk population. While this is perhaps a rather simplistic view, given the complexity of the process of cancer development and progression and the multitude of different cancer types, it is nevertheless an important message from the point of view of MH potentially being an important immunomodulatory agent.

We investigated the impact of oral MH treatment on implanted tumors. By focusing on using a preventative treatment regimen, we

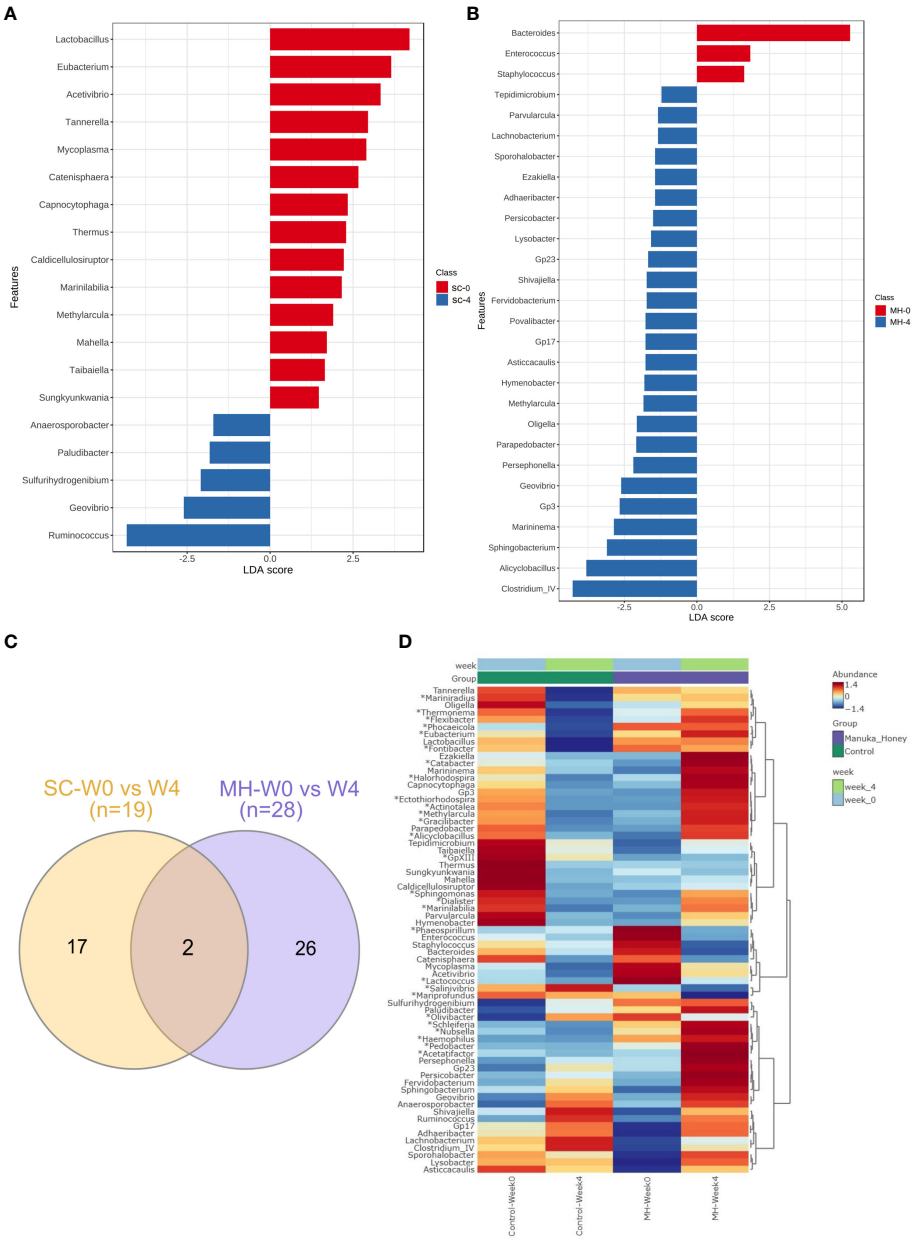

FIGURE 8
Microbiota variations after 4-week treatment. LDA analysis comparing genera pre and post-treatment with CS **(A)** and MH **(B)** showed depleted (red) and enriched (blue) in week 4 post-treatment. Venn diagram **(C)** of shared and unique genera that were significantly different pre- and post-treatment for each of the SC and MH groups. The heatmap **(D)** shows microbiota fingerprints in each group before and after treatment, with significantly altered genera. Asterisks (*) represent genera with significant differences between MH and CS after 4 weeks of treatment, while the rest are those with significant difference in week 0 compared to week 4 in either treatment group.

could delineate the immunomodulatory effect of MH from its anti-tumor effect. Our findings demonstrated a 44% reduction in tumor growth compared to the control group. To gain insights into the underlying mechanism of this effect, we analyzed the immune system components of CT26 tumors by flow cytometry. Our analysis revealed that the observed inhibition of tumor growth was associated with a significant enhancement in $CD45^+$ hematopoietic cell infiltration into the tumor tissue. Further investigation revealed that treatment with MH increased the proportion of intratumoral cytotoxic and helper T cells. T cells have been shown to play a crucial role in inducing anti-tumor immune responses (66). Cytotoxic effector $CD8^+$ T cells can directly recognize and kill cancer cells by releasing cytotoxic molecules, such as granzymes and perforin, as well as pro-inflammatory cytokines like IFN-γ and TNF-α (67). Similar to $CD8^+$ T cells, $CD4^+$ T cells secrete pro-inflammatory cytokines with direct anti-tumor effects (68). Additionally, $CD4^+$ T cells play a crucial role in activating and expanding $CD8^+$ T cells through the secretion of IL-2, which promotes their proliferation and activation. Moreover, $CD4^+$ T cells license dendritic cells (DCs) to activate $CD8^+$ cells by either

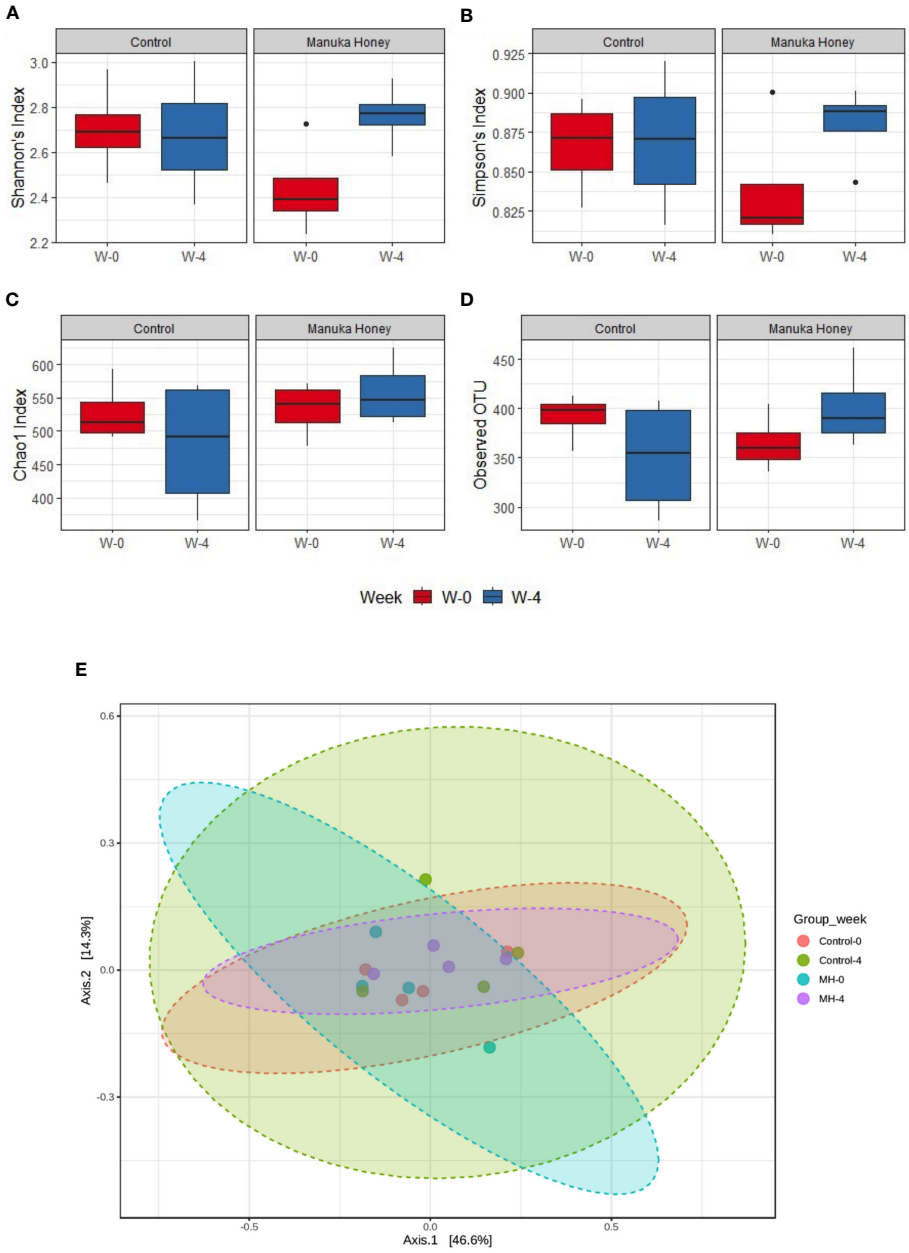

FIGURE 9
Microbiome diversity. **(A–D)** Show alpha diversity comparison between samples grouped based on treatment type and duration, using Shannon's index **(A)**; Simpson's index **(B)**; Chao1 index **(C)**, and observed species **(D)**. Box plots show Q1-median-Q3 with data range. Black dots are outlier values. Principal coordinates (PCo) analysis plots of beta diversity measured by Bray Curtis index **(E)** for control group and MH group in week 0 vs week 4. All groups are color-coded, and each dot represents an experimental mouse in each group. Although the difference was not statistically significant, (PERMANOVA: F-value: 0.92753; R-squared: 0.13389; p-value: 0.461), week 4 (post-treatment) was distinct from week 0 (pre-treatment) in the MH group.

cross-presenting tumor antigens to $CD8^+$ T cells or inducing the production of cytokines and costimulatory molecules (69–71). Our findings revealed that MH treatment not only increased the infiltration of intratumoral T cells but also enhanced their cytotoxic potential, as shown by the elevated levels of IFN-γ and granzyme-B in purified $CD45^+$ immune cells from tumor tissue.

In addition to alterations in TILs, we have observed changes in the intratumoral myeloid populations in response to MH treatment. Specifically, there was a reduction in the proportion of $CD11b^+$ myeloid cells, accompanied by a significant decrease in the proportion of $Ly6G^+$ granulocytes. In addition, MH treatment may enhance the antigen-presenting capacity of intratumoral myeloid cells, as suggested by their increased expression of MHC class II proteins. It is important to acknowledge the limitations inherent in our analysis of myeloid cells in the TME. These cells represent a very heterogeneous and complex subpopulations with different functions (72). The use of only CD11c marker to identify dendritic cells is limited given the fact that these are quite

heterogeneous in nature. Furthermore, another limitation in our analysis of the intratumoral myeloid cells is the absence of additional cell markers to distinguish M1 and M2 macrophages. Myeloid cells, including TAMs, DCs, tumor-associated neutrophils (TANs), and myeloid-derived suppressor cells (MDSCs), are the most abundant immune cells in the TME, and their heterogeneity allows them to exert both pro-tumor and anti-tumor effects during tumor development and progression (73). The role of MDSCs in suppressing anti-tumor immunity and supporting the proliferation of tumors has been well-documented (39). In our study, MH-mediated tumor inhibition was associated with a significant reduction in the percentages of intratumoral $Ly6G^+$ myeloid cells, which resemble granulocytic-MDSCs that are known to contribute to tumor growth promotion and immune response suppression. Various studies have reported the presence of granulocytic-MDSCs within the tumors and organs of CT26-bearing mice (39, 74, 75). CT26 tumors produce proinflammatory mediators and factors that contribute to the development and expansion of granulocytic-MDSCs in both primary tumors and distant organs (76, 77). This alteration in myeloid populations could explain the tumor-inhibiting effects of MH treatment. An increase in both the proportion and functional ability of cytotoxic T cells following MH treatment suggests that the suppressive effect of granulocytic-MDSCs on T cells is reduced. However, to verify this, it is crucial to directly evaluate the immunosuppressive capacity of intratumoral $Ly6G^+$ $CD11b^+$ cells by performing cellular function assays. Our results also indicated an increase in the expression of MHC-II proteins on the intratumoral myeloid cells, implying that type I and/or type II interferons could be responsible for this induction (34, 35). These findings indicate that these cells are potentially more able to act as antigen-presenting cells to $CD4^+$ helper T cells, hence augmenting anti-tumor T cell responses (66).

One major mechanism through which tumors avoid the immune response is by downregulating MHC class I, thereby decreasing their recognition and elimination by cytotoxic $CD8^+$ T cells (78). A promising approach to enhancing the efficacy of anti-tumor therapies involves restoring the expression of MHC class I through type I/II IFN stimulation (79). In the current study, MH treatment enhanced the expression of MHC-I on the $CD45^-$ tumor cells, indicating the involvement of type I and/or type II interferons in this induction. The increase in MHC-I expression is consistent with the observed increase in TILs and IFNγ expression in MH-treated mice. The increased CXCL10 expression, which is also triggered by IFN-γ, may regulate the recruitment of inflammatory T lymphocytes (43). Our findings also demonstrated that MH treatment reduces CXCL2 expression, which plays a crucial role in recruiting intratumoral granulocytic MDSCs and promoting their pro-tumor immunosuppressive function (44, 80). These findings suggest that oral MH treatment enhances the immunogenicity of CT26 tumor cells, making them more susceptible to cytotoxic T cell-mediated killing.

In line with our findings, previous studies demonstrated the potential of natural products like polyphenols to restructure the immunosuppressive microenvironment of tumors and hinder tumor growth (81, 82). These natural products have been shown to downregulate the percentages of immunosuppressive cells, such as MDSCs, Tregs, and M2-MACs, while promoting the proportions and function of anti-tumor effector T cells like $CD8^+$ T cells, $CD4^+$ T cells, and NK cells (81–83). Given that MH comprises a variety of polyphenols, it is perhaps not unreasonable to suggest that these bioactive substances contribute to the elicitation of the observed anti-tumor immune responses following MH treatment. Alternatively, oral administration of MH could alter anti-tumor immunity through changing gut microbiota-derived metabolites. Nutritional regulation of these metabolites and their influence on the immune system has been recently reviewed (84). Although much remains to be elucidated, there is evidence for individual metabolites acting to either improve responses to cancer therapy, such as indole-3-acetic acid (85), or mitigate against high-fat-diet-mediated progression of intestinal tumors, such as butyrate (86). Moreover, butyrate was shown to inhibit gastric tumors by reducing the expression of immunosuppressive factors, such as PD-L1 and IL-10 (87). In the context of colorectal cancer, a recent study demonstrated that oral administration of *Lactobacillus plantarum* CBT could effectively inhibit the growth of colorectal cancer in noth orthotopic as well as ectopic preclinical mouse models (88). Given the evidence that MH could effect changes in gut microbiota content, it would be extremely beneficial to characterize the relative changes in the metabolite abundance with a view of uncovering their influence on cancer growth and response to therapy.

Current studies have shown that certain members of the intestinal microbiota can facilitate colorectal carcinogenesis by generating carcinogenic microbial metabolites and secreting oncogenic virulence factors (89). It is worth noting that some of the bacterial genera that were significantly different after 4-week treatment with MH were previously reported to have an impact on tumorigenesis. For the genus *Enterococcus*, which was depleted after MH treatment, previous studies have reported an association between some species of *Enterococcus* (e.g. *E. faecalis*) and gastrointestinal tumorigenesis related to its interaction with immune cells. Studies in IL-10 deficient mice found that these bacteria can cause macrophage polarization to M1 phenotype, resulting in inflammation and DNA damage of intestinal epithelial cells (90, 91). Furthermore, *Enterococcus* can secret tumor-stimulating metabolites with proliferative and angiogenic effects on CRC (51). As for *Bacteroides*, which was also depleted after MH treatment, some species such as *B. fragilis* can contribute to oncogenic transformation in the colon by producing enterotoxins which can induce c-Myc expression and cellular proliferation in intestinal epithelial cells (92). Recent experimental evidence confirmed that *Bacteroides*-driven colitis can promote colon tumorigenesis. Colonization with enterotoxigenic *B. fragilis* can induce mucosal IL-17 production with subsequent events leading to tumor formation, a process that was ameliorated by IL-17 neutralization (48). Specifically, *Bacteroides* toxin (fragilysin) triggers an IL-17 immune response that activates NF-κB signaling in colonic epithelial cells, leading to pro-tumoral myeloid cells infiltration in the colon (93). Finally, for the genus *Staphylococcus*, which was also depleted after MH treatment, surveillance studies in patients with CRC revealed that some species, such as *S. lugdunensis*, were associated with colon carcinoma (49). Moreover, bidirectional functional effects of *Staphylococcus* species on carcinogenesis have been proposed (50), mostly driven by the many immunoregulatory factors produced by *Staphylococcus* member species. Interestingly, some staphylococcal nucleases have been recognized as oncoproteins (94).

As for the microbiota enriched after MH treatment, many of them are associated with health-promoting effects, and some are recognized as anti-tumorigenic bacteria. For instance, *Ruminococcus* was enriched after MH treatment. Bacteria from this genus are secondary bile acid producers (95). These metabolites are able to suppress colon carcinogenesis through modulation of signaling pathways in colon cancer cells (52, 96). Moreover, bile acids produced by these bacteria have strong antimicrobial properties and can modulate the gut microbiome by selectively eliminating pathogens, supporting the growth of other health-promoting bacteria (97). *Clostridium* cluster IV which encompasses several butyrate producers, was significantly enriched after MH treatment. *Clostridium* cluster IV includes four members, namely *C. leptum, C. sporosphaeroides, C. cellulosi and Faecalibacterium prausnitzii* (98). All these bacteria have potent probiotic characteristics which are essential for intestinal homeostasis, thus providing protection against cancers. For instance, *F. prausnitzii* has proven anti-tumorigenic and anti-proliferative effect by inhibiting the formation of abnormal colonic crypt foci in animal models of CRC. Furthermore, the application of *F. prausnitzii* reduced the level of lipid peroxidation in colonic tissues, which is also protective against CRC (53).

There is mounting evidence indicating that the gut microbiota play a crucial role in cancer development and response to anti-cancer therapies (99, 100). Analysis of the gut microbiota of CRC patients has shown that certain bacteria, such as *Streptococcus gallolyticus*, *Fusobacterium nucleatum*, *Escherichia coli*, *Bacteroides fragilis*, and *Enterococcus faecalis*, are more prevalent in CRC patients compared to the normal population, while the levels of other genera like *Roseburia*, *Clostridium*, *Faecalibacterium* and *Bifidobacterium* decrease in CRC patients (101). Experimental evidence from preclinical as well as clinical studies demonstrated that gut microbiota plays a critical role in influencing the response to anti-cancer therapies. For instance, in a murine melanoma model, the presence of commensal *Bifidobacterium* was linked to differences in response to immune checkpoint inhibitors (ICI), and fecal microbiota transplantation improved the anti-tumor effectiveness of PD-L1 blockade (102). Clinical studies have further revealed that the composition and diversity of the gut microbiota can predict a favorable response to ICI immunotherapy, with specific bacterial strains such as *Ruminococcus*, *Akkermansia muciniphila*, and *Bifidobacterium* being present in the gut microbiome of ICI-responsive patients (103, 104). Therefore, manipulating the gut microbiome may have broad potential in cancer prevention and treatment.

Overall, our study indicates that oral administration of MH has the potential to activate the immune system and enhance anti-cancer immune responses in a preclinical model of CRC. Although the full mechanistic details remain unknown, our findings suggest that pretreatment with MH can activate lymphoid cells in mucosal and peripheral tissues, thereby facilitating a preactivated ready-to-respond state, that is most likely contributing to the superior anti-tumor immunity. Most importantly, MH treatment appears to promote anti-tumor immunity even when given therapeutically post tumor implantation. MH appears to enrich for type I/II IFN signature by either altering the gut microbiota, or via a hitherto unknown mechanism, leading to the upregulation of Sca-1 on $CD4^+$ and $CD8^+$ cells in the gut, and their subsequent migration from the gut to the periphery. These T cells possess superior effector potential, ultimately promoting anti-tumor immune responses. The increased efficacy is linked to a series of immunological alterations within the TME, resulting in the suppression of tumor growth. The proposed mechanism of action for oral MH treatment is summarized in Figure 10. Validation of the proposed mechanism

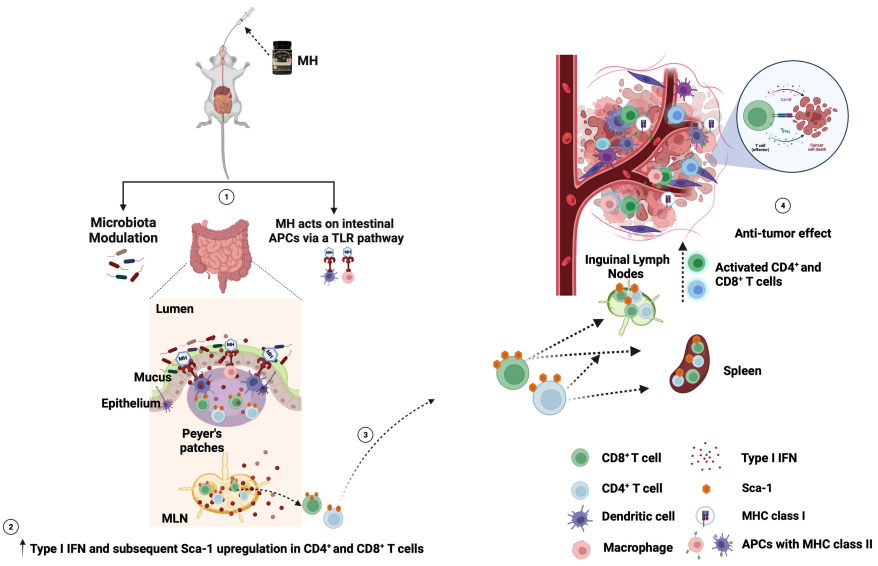

FIGURE 10
Schematic diagram of the proposed mechanism of oral MH on anti-tumor immune response. (1) MH treatment either modulates the gut microbiota or induces the TLR pathway. This leads to (2) the enrichment of type I IFN and the subsequent upregulation of Sca-1 on the $CD4^+$ and $CD8^+$ T cells in the gut environment. (3) The Sca-1^+ $CD4^+$ and $CD8^+$ T cells migrate from the gut to the periphery (spleen and ILNs). (4) these preactivated T cells then migrate to the TME and induce anti-tumor immune responses. Figure adapted from (61) and created with BioRender.com.

is required. For example, the use of mice pre-treated with antibiotics to deplete their microbiota would provide crucial evidence for a role for the microbiota in the observed MH-induced enhancement in anti-tumor immune responses. Furthermore, the potential of combining MH treatment with another modality, such as chemotherapy or immunotherapy, would be immensely rewarding for the ultimate goal of improving the efficacy of anti-cancer therapy.

Data availability statement

The data supporting the findings of this study are available within the article and its supplementary material. The 16S rRNA sequencing data are deposited in the Sequence Read Archive (SRA) in the National Center for Biotechnology Information (NCBI) database under Accession No. PRJNA1063249. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by Institutional Animal Research Ethics Committee of the United Arab Emirates University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

BA: Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. RM: Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. II: Formal analysis, Investigation, Methodology, Writing – review & editing. YM: Formal analysis, Investigation, Methodology, Writing – review & editing. AsA: Formal analysis, Investigation, Methodology, Writing – review & editing. GB: Formal analysis, Investigation, Methodology, Writing – review & editing. FA: Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. AbA: Formal analysis, Investigation, Writing – review & editing. MF: Formal analysis, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1354297/full#supplementary-material

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Effect of the gut microbiome, plasma metabolome, peripheral cells, and inflammatory cytokines on obesity: a bidirectional two-sample Mendelian randomization study and mediation analysis

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Background: Obesity is a metabolic and chronic inflammatory disease involving genetic and environmental factors. This study aimed to investigate the causal relationship among gut microbiota abundance, plasma metabolomics, peripheral cell (blood and immune cell) counts, inflammatory cytokines, and obesity.

Methods: Summary statistics of 191 gut microbiota traits (N = 18,340), 1,400 plasma metabolite traits (N = 8,299), 128 peripheral cell counts (blood cells, N = 408,112; immune cells, N = 3,757), 41 inflammatory cytokine traits (N = 8,293), and 6 obesity traits were obtained from publicly available genome-wide association studies. Two-sample Mendelian randomization (MR) analysis was applied to infer the causal links using inverse variance-weighted, maximum likelihood, MR-Egger, weighted median, weighted mode, and Wald ratio methods. Several sensitivity analyses were also utilized to ensure reliable MR results. Finally, we used mediation analysis to identify the pathway from gut microbiota to obesity mediated by plasma metabolites, peripheral cells, and inflammatory cytokines.

Results: MR revealed a causal effect of 44 gut microbiota taxa, 281 plasma metabolites, 27 peripheral cells, and 8 inflammatory cytokines on obesity. Among them, five shared causal gut microbiota taxa belonged to the phylum *Actinobacteria*, order *Bifidobacteriales*, family *Bifidobacteriaceae*, genus *Lachnospiraceae* UCG008, and species *Eubacterium nodatum* group. Furthermore, we screened 42 shared causal metabolites, 7 shared causal peripheral cells, and 1 shared causal inflammatory cytokine. Based on known causal metabolites, we observed that the metabolic pathways of D-arginine, D-ornithine, linoleic acid, and glycerophospholipid metabolism were closely related to obesity. Finally, mediation analysis revealed 20 mediation relationships, including the causal pathway from gut microbiota to obesity, mediated by 17

metabolites, 2 peripheral cells, and 1 inflammatory cytokine. Sensitivity analysis represented no heterogeneity or pleiotropy in this study.

Conclusion: Our findings support a causal relationship among gut microbiota, plasma metabolites, peripheral cells, inflammatory cytokines, and obesity. These biomarkers provide new insights into the mechanisms underlying obesity and contribute to its prevention, diagnosis, and treatment.

KEYWORDS

obesity, gut microbiota, plasma metabolites, peripheral cells, inflammatory cytokines, Mendelian randomization, mediation analysis

1 Introduction

Obesity, a complex metabolic disease, arises from an imbalance between energy intake and expenditure, leading to excess energy storage in adipose tissues. Its etiology is multifaceted, involving both genetic and environmental factors. Presently, approximately one-third of the global population is overweight (defined as a body mass index [BMI] between 25 and 29 kg/m^2), with 10% classified as obese (BMI ≥ 30 kg/m^2) (1). This global epidemic poses significant risks to physical and mental health, being a primary contributor to various diseases, including cardiovascular issues, allergic conditions, hypertension, type 2 diabetes (T2D), cancer, and mood-related disorders (2–4). Thus, obesity is a serious public health concern.

In recent years, increasing evidence has shown that an imbalance in the gut microbiota may play a major role in obesity (5). The gut microbiota is a microbial community living in the human intestine that plays an important role in human metabolic regulation and immunomodulation via interactions with the host (6, 7). The diversity and richness of the gut microbiota in obese patients are reduced, and the composition of the gut microbiota changes to varying degrees (8, 9). For example, an increased *Firmicutes* to *Bacteroidetes* ratio may play a role in the development of obesity (8, 10). A case-control study found that *Enterobacteriaceae* levels were increased, whereas *Desulfovibrio* and *Akkermansia muciniphila* levels were decreased in overweight and obese children (11).

Metabolomics can reveal correlations between metabolites or metabolic pathways and physiological and pathological changes, thus providing new information for research on disease mechanisms (12). Multiple studies have shown that metabolites and metabolic pathways are closely associated with obesity and that obese patients have metabolic disorders (13, 14). For example, a study using targeted serum metabolomics identified metabolites significantly associated with obesity. In that study, serum concentrations of glycine, glutamine, and glycero-phosphatidylcholine (Pcaa) 42:0 were positively correlated, whereas those of PCaa 32:0, PCaa 32:1, and PCaa 40:5 were negatively correlated with obesity (14). In addition, plasma metabolites, such as branched-chain amino acids and glutamate, may mediate the relationship between the gut microbiota and obesity (15).

Obesity is a chronic inflammatory disease closely related to the immune system and inflammatory responses (16). Adipose tissue macrophages are key contributors to obesity-related inflammation, accounting for less than 10% of the immune cells in lean individuals and up to 50% in obese individuals (17). Additionally, a higher white blood cell count may be associated with an increased risk of obesity. After weight loss, total white blood cells, major components, neutrophils, and lymphocytes significantly decrease (18, 19). There is also an increasing number of reports on the relationship between inflammatory cytokines and the risk of obesity. Previous studies have shown that the increase in the levels of pro-inflammatory cytokines interleukin (IL)-1, IL-6, and tumor necrosis factor alpha is closely related to the occurrence and development of obesity (20, 21). In addition, research has shown decreased serum levels of IL-27 in obese individuals. IL-27 can act directly on adipocytes and lead to adipocyte differentiation and thermogenesis, thus reducing weight and improving metabolic diseases, such as T2D (22).

While previous studies have identified associations between the gut microbiome, metabolome, immune inflammation, and obesity, the precise causal relationships and their respective mediation proportions remain unclear. Mendelian randomization (MR) analysis is an effective method that uses genetic variation as an instrumental variable (IV) to evaluate the potential causal relationship between exposures and outcomes (23). This minimizes the impact of confounding factors on causal estimation, as genetic variations are randomly assigned at conception. Mediation analysis is used to evaluate the effects of an exposure on an outcome through a mediator (24). Therefore, we conducted MR analyses based on publicly available genome-wide association study (GWAS) summary data to evaluate the causal relationship among the gut microbiota, plasma metabolites, peripheral cells, inflammatory cytokines, and obesity, and to identify pathways from the gut microbiota to obesity mediated by plasma metabolites, peripheral cells, and inflammatory cytokines.

2 Methods

2.1 Study design

The study flowchart is illustrated in Figure 1. First, we obtained published GWAS summary data that included traits such as gut microbiota, plasma metabolites, peripheral cells, inflammatory cytokines, and obesity (Supplementary Table S1). Second, two-sample MR analyses were used to evaluate the causal relationship among gut microbiota, plasma metabolites, peripheral cells, inflammatory cytokines, and obesity. Finally, two-step and multivariable MR (MVMR) analyses were used to identify the mediation effect of plasma metabolites, peripheral cells, and inflammatory cytokines on the relationship between gut microbiota and obesity. Our MR study was conducted in accordance with the STROBE-MR guidelines (Supplementary Table S2) (25).

2.2 Data sources

The summary statistics of gut microbiota were retrieved from the largest multi-ethnic GWAS meta-analysis exploring the host

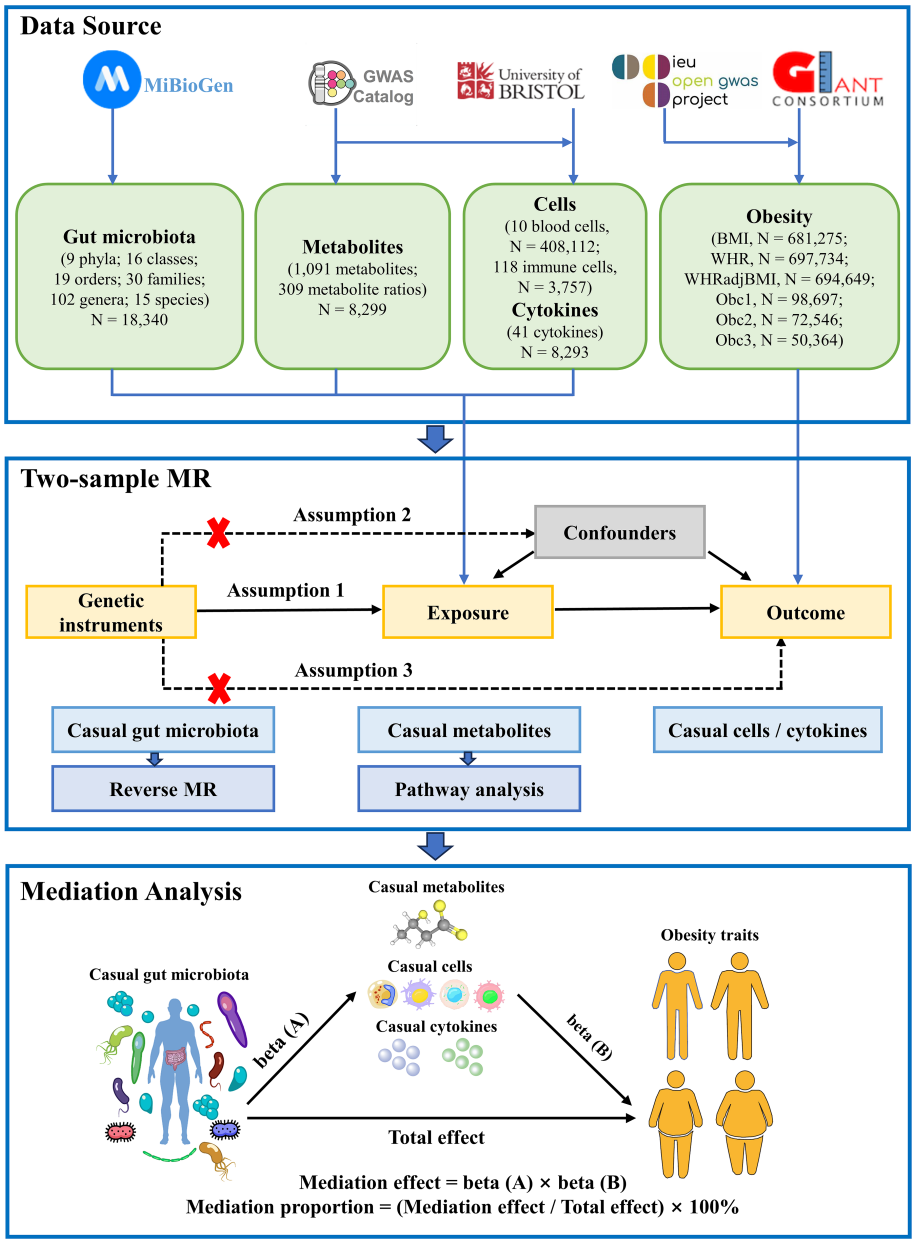

FIGURE 1
Flow chart of the study. Mendelian randomization study rationale: assumption 1, genetic instruments are associated with exposure; assumption 2, genetic instruments are not associated with confounders; assumption 3, genetic instruments are not associated with outcome, and genetic instruments act on outcome only through exposure. BMI, body mass index; WHR, waist-to-hip ratio; WHRadjBMI, WHR adjusted for BMI; Obc1, Obesity class 1; Obc2, Obesity class 2; obc3, Obesity class 3; MR, Mendelian randomization.

genetic impact on gut microbiota, which was based on the MiBioGen consortium (https://mibiogen.gcc.rug.nl/), including 18,340 individuals from 24 cohorts (26). The gut microbiota was identified using 16S rRNA sequencing, and the patients were genotyped using a genome-wide single nucleotide polymorphism (SNP) microarray to determine the genetic locus affecting the relative abundance of the gut microbiota. The GWAS summary data of 191 gut microbiome components (including 9 phyla, 16 classes, 19 orders, 30 families, 102 genera, and 15 species) were included in this study for subsequent MR analyses.

Summary statistics of plasma metabolomics were acquired on the GWAS Catalog (https://www.ebi.ac.uk/gwas/) under the study accession numbers GCST90199621–GCST90201020, which included 1,091 plasma metabolites and 309 metabolite ratios from 8,299 European individuals (27). In that study, there were 850 known metabolites among 1,091 plasma metabolites, which could be divided into 8 broad metabolic groups: lipid (395), amino acid (210), xenobiotics (130), nucleotides (33), cofactors and vitamins (31), carbohydrates (22), peptides (21), and energy (8); the remaining metabolites were partially characterized molecules (21) and unknown (220).

Summary statistics for blood cell traits included 408,112 European participants (28); summary statistics for peripheral immune cells included that of 3,757 European individuals analyzed using flow cytometry (29). The GWAS data were downloaded from the GWAS Catalog, and we selected 10 blood cell count traits and 118 immune cell absolute count traits for subsequent analyses (accession numbers for each trait can be found in Supplementary Table S1). GWAS data for 41 inflammatory cytokines were collected from the University of Bristol (https://data.bris.ac.uk/data/dataset), including three Finnish cohort studies (N = 8,293): the Cardiovascular Risk in Young Finns Study, FINRISK1997, and FINRISK2002 (30, 31).

GWAS summary data for obesity-related traits were collected from large-scale GWAS or the corresponding meta-analyses. Obesity-related traits included BMI, waist-to-hip ratio (WHR), WHR adjusted for BMI (WHRadjBMI), and Obesity classes 1, 2, and 3. GWAS summary data on BMI (32), WHR (33), and WHRadjBMI (33) were obtained from the meta-analysis of UK Biobank and Genetic Investigation of Anthropometric Traits (GIANT) consortium (https://portals.broadinstitute.org/collaboration/giant/index.php/GIANT_consortium_data_files), which contained approximately 700,000 European individuals. Three obesity clinical classification datasets were downloaded at the IEU OpenGWAS database (https://gwas.mrcieu.ac.uk/) and obtained from a genome-wide meta-analysis (34), which contained 263,407 European individuals: Obesity class 1 (BMI ≥ 30 kg/m^2) contained 32,858 patients and 65,839 controls; Obesity class 2 (BMI ≥ 35 kg/m^2) included 9,889 patients and 62,657 controls; and Obesity class 3 (BMI ≥ 40 kg/m^2) included 2,896 patients and 47,468 controls. Control was defined as an individual with a BMI < 25 kg/m^2.

2.3 Instrumental variable selection

To estimate causal effects using genetic variation, three basic assumptions of IVs must be satisfied: 1) IVs are related to exposure factors; 2) IVs are not associated with confounding factors; and 3) IVs are not related to outcome variables and only act on outcome variables through exposure factors. Specifically, the IVs included in this study were screened to meet the following conditions: 1) The SNP obtained at the locus-wide significance threshold of $P < 1 \times 10^{-5}$ is used when there are too few whole-genome significance loci in the original GWAS results (35), or the genome-wide significance threshold of $P < 5 \times 10^{-8}$ is used as a potential tool variable related to each exposure trait. 2) SNPs related to outcome variables were excluded ($P < 0.05$). 3) The clumping process was performed to avoid the impact of linkage disequilibrium ($r^2 < 0.01$, window size = 500 kb; or $r^2 < 0.001$, window size = 10,000 kb). 4) The MR pleiotropy residual sum and outlier (MR-PRESSO) test was applied to detect horizontal pleiotropy, and the pleiotropy effect was eliminated by removing the outliers (36). In summary, the SNPs were sorted in ascending order according to the *P*-values of the MR-PRESSO outlier test, and the remaining were eliminated one by one until there was no pleiotropy (MR-PRESSO global test *P*-value > 0.05). 5) The strength of the selected SNPs was evaluated using F-statistic, where SNPs with F-statistic < 10 were excluded to avoid weak instrument bias in the MR analysis (37). The F statistic formula is $F = [R^2 \times (n - k - 1)] / [k \times (1 - R^2)]$, where R^2 is the portion of the exposure variance explained by the IVs, n is the sample size, and k represents the number of IVs (37). 6) IVs with a stronger association with the outcome than exposure were removed by Steiger filtering.

2.4 Statistical analysis

2.4.1 Two-sample Mendelian randomization

The MR method was used to evaluate the causal relationship among the gut microbiota, plasma metabolites, peripheral cells, inflammatory cytokines, and obesity. The Wald ratio was used to infer the causality for exposure, which included only one IV. For exposure comprising multiple IVs, inverse variance-weighted (IVW), maximum likelihood, MR-Egger, weighted median, and weighted mode methods were used to infer causality. IVW usually provides the highest statistical power (38); therefore, it is preferred, whereas other methods are used as supplements. IVW uses a meta-analysis to combine the Wald ratio estimates of each IV, where the intercept is limited to zero (38). In the absence of horizontal pleiotropy, IVW can provide an unbiased causal estimate (39). When there was heterogeneity, the random-effect IVW test provided more conservative and robust estimates; otherwise, a fixed-effect model was used. Similar to IVW, the maximum likelihood method assumes a linear relationship between exposure and outcome (40). MR-Egger verifies the existence of multiple horizontal effects; when pleiotropy exists, it can provide an effective causal estimation (41). Even when up to 50% of the IVs are ineffective, the weighted median can provide effective causal estimates (42). The weighted mode approach is still valid if most IVs with similar causal estimates are valid instruments, even if other IVs do not meet the requirements of the MR analysis (43).

Sensitivity analysis was performed to assess the robustness of causality. MR-Egger regression and MR-PRESSO were used to assess the horizontal pleiotropy. The non-zero intercept of the

MR-Egger regression suggested directional pleiotropy (41). Cochran's Q test was used to assess the heterogeneity among the IVs. Additionally, leave-one-out sensitivity analysis was used to assess whether a single SNP drove the causal estimation. MR Steiger analysis was used to assess the direction of the potential causal association between exposure and outcomes. Only causal microbial characteristics, plasma metabolites, peripheral cells, and inflammatory cytokines with no heterogeneity or pleiotropy were included in the subsequent analysis when the IVW MR method results reached a significance threshold of $P < 0.05$.

Furthermore, considering the potential chance to increase the overall type I error during multiple comparisons, we implemented the false discovery rate (FDR) correction using the Benjamini–Hochberg procedure (44) on the primary IVW results. A significance threshold of FDR < 0.1 indicates a significant association, whereas $P_{IVW} < 0.05$ but FDR > 0.1 implies a suggestive association.

All MR analyses were performed in R (version 4.3.1) software, using the "TwoSampleMR" (version 0.5.7) (https://github.com/MRCIEU/TwoSampleMR) (39) and "MR-PRESSO" (version 1.0) (https://github.com/rondolab/MR-PRESSO) (36) packages.

2.4.2 Reverse Mendelian randomization analysis

To explore whether obesity had a causal effect on the identified gut microbiota ($P_{IVW} < 0.05$), a reverse MR analysis was performed. In this scenario, obesity-related SNPs were regarded as IVs, obesity as the exposure, and gut microbiota taxa as the outcomes. The reverse MR analysis procedure was similar to that used for the MR analysis.

2.4.3 Metabolic pathway analysis

For identified known plasma metabolites ($P_{IVW} < 0.05$), we used MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) (45) to conduct metabolic pathway analysis to identify potential metabolic pathways that may be related to the biological processes of obesity. This study used two libraries: the Small Molecule Pathway Database (SMPDB) (46) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (47).

2.4.4 Mediation analysis

Mediation analysis aims to evaluate the pathway from exposure to outcome through a mediator, which helps explore the potential mechanisms by which exposure affects outcome (24). The mediation analysis in this study focused on obesity-related gut microbiota, plasma metabolites, peripheral cells, and inflammatory cytokines. First, the causal relationship between gut microbiota and plasma metabolites, peripheral cells, and inflammatory cytokines was evaluated using two-sample MR methods to obtain beta (A). Second, MVMR was used to screen plasma metabolites, peripheral cells, and inflammatory cytokines that still had a causal relationship with obesity after correction for gut microbiota to obtain beta (B) and ensure that the mediating effects on outcomes are independent of exposure (24). The mediation effect was calculated using a two-step MR: mediation effect = beta (A) × beta (B). The total effect of the gut microbiota on obesity was obtained in the previous two-sample MR, and direct effect = (total effect – mediation effect). The mediation proportion used the following formula: mediation proportion = (mediation effect / total effect) × 100%. The 95% confidence intervals (CI) for the mediation effects and proportions mediated were estimated using the delta method (24). Based on the results, we categorized the identified mediators into different levels of evidence. When only a triangular relationship existed, representing that exposure was causally associated with outcome, mediator was causally associated with outcome, and exposure was causally associated with mediator. The identified metabolites, peripheral cells, or cytokines were considered to have potential mediation effects in the pathway from gut microbiota to obesity. If the identified metabolites, peripheral cells, or cytokines did not only exist in a triangular relationship but also had mediation effects significantly different from 0, they were considered as mediators with strong evidence.

3 Results

3.1 Causal effects of gut microbiota on obesity

Using two-sample MR, we identified 50 suggestive associations between gut microbiota and obesity ($P_{IVW} < 0.05$, FDR > 0.1; corresponding to 44 unique gut microbiota taxa). The causal microbial counts of the obesity traits BMI, WHR, WHRadjBMI, Obesity classes 1, 2, and 3 were 13, 12, 9, 9, 4, and 3, respectively (Figure 2; Supplementary Table S3). Five bacterial features were associated with more than one obesity trait, which may be a common molecular mechanism in the GWAS datasets of different obesity phenotypes. The phylum *Actinobacteria* (BMI; Obesity class 3), order *Bifidobacteriales* (BMI; WHR), and family *Bifidobacteriaceae* (BMI; WHR) had a negative causal relationship with obesity. In contrast the genus *Lachnospiraceae* UCG008 (WHR; WHRadjBMI) and species *Eubacterium nodatum* (BMI; WHR; WHRadjBMI) had a positive causal relationship with obesity (Supplementary Figure S1). *Lachnospiraceae* is closely related to obesity, and we found that the genus *Lachnospiraceae* FCS020 may increase the risk of obesity (BMI), whereas the family *Lachnospiraceae* and genus *Lachnospiraceae* NK4A136 may reduce the risk (WHRadjBMI). Moreover, the family belonged to the order subcategory; therefore, SNP sets included in families and their relevant orders might heavily overlap. These include SNPs of the family *Bifidobacteriales* and order *Bifidobacteriaceae* when applying MR analysis between the gut microbiota and obesity. Sensitivity analysis further verified the robustness of the MR results (Supplementary Table S4). The Q statistics showed no evidence of heterogeneity. Furthermore, the results of MR-Egger regression and MR-PRESSO analyses suggested no evidence of horizontal pleiotropy. Based on the MR-Steiger test, we did not find any reverse causality.

For causal associations between gut microbiota and obesity identified above, we conducted reverse MR and found a negative causal relationship between WHR and the genus *Ruminococcaceae*

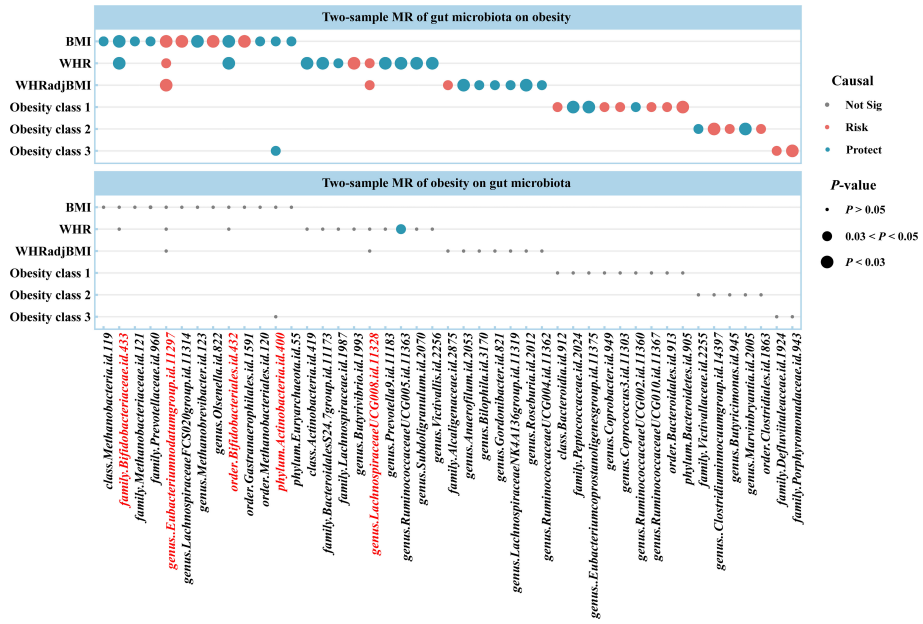

FIGURE 2
Causal estimates of bidirectional MR between gut microbiota and obesity. *Top*: Estimates from the IVW analysis of gut microbiota on obesity. *Bottom*: Estimates from the IVW analysis of obesity on gut microbiota. The bacterial features underlined in red were related to more than one obesity traits. BMI, body mass index; WHR, waist-to-hip ratio; WHRadjBMI, WHR adjusted for BMI; MR, Mendelian randomization; Not Sig, not significant.

UCG005 (odds ratio [OR] = 0.877, 95% CI [0.776–0.992], P = 0.037) (Figure 2; Supplementary Tables S5, S6).

3.2 Causal effects of plasma metabolites on obesity

Based on the IVW method, the results suggested 327 causal relationships between plasma metabolomics and obesity (P_{IVW} < 0.05, corresponding to 281 unique plasma metabolites, 229 unique plasma metabolite levels, and 52 unique metabolic ratios). Among BMI, WHR, WHRadjBMI, Obesity classes 1, 2, and 3, there were 84 (73 metabolites and 11 ratios), 82 (67 metabolites and 15 ratios), 54 (44 metabolites and 10 ratios), 41 (34 metabolites and 7 ratios), 29 (20 metabolites and 9 ratios), and 37 (27 metabolites and 10 ratios) associations detected, respectively (Figure 3; Supplementary Table S7). Additionally, we observed 32 shared causal metabolites and 10 shared causal ratios for different obesity traits. Among them, plasma metabolites included lipids (13), amino acids (7), xenobiotics (2), nucleotide (1), cofactor and vitamins (1), carbohydrates (1), peptide (21), energy (1), partially characterized molecules (1), and unknown (4) (Figure 3). For example, 2-oxoarginine* had a positive causal relationship with WHR (OR = 1.0145, 95% CI [1.0035–1.0256], P = 0.0093), WHRadjBMI (OR = 1.0113, 95% CI [1.0001–1.0.225], P = 0.0471), and Obesity class 1 (OR = 1.1374, 95% CI [1.0190–1.2696], P = 0.0217). However, following FDR correction, only 1-(1-enyl-palmitoyl)-2-oleoyl-GPC (P-16:0/18:1)* maintained a significant negative causal relationship with BMI (OR = 0.9744, 95% CI [0.9619–0.9870], P = 0.0001, FDR = 0.0874) (Supplementary Table S7). These results were validated no heterogeneity and horizontal pleiotropy using sensitivity analyses (Supplementary Table S8).

The metabolic pathway analysis identified 17 significant metabolic pathways (12 unique pathways) (Supplementary Figure S2). We discovered metabolic pathways shared between different obesity phenotypes: "D-Arginine and D-ornithine metabolism" for WHR (P = 0.0332), WHRadjBMI (P = 0.0154), and Obesity class 1 (P = 0.0205); "Linoleic acid metabolism" for BMI (P = 0.0382), Obesity classes 1 (P = 0.0256), and 3 (P = 0.0192); "Glycerophospholipid metabolism" for BMI (P = 0.0299) and WHR (P = 0.0348) (Supplementary Table S9).

3.3 Causal effects of blood cells, peripheral immune cells, and inflammatory cytokines on obesity

The IVW method revealed 35 associations between peripheral cells and obesity (P_{IVW} < 0.05, corresponding to 27 unique peripheral cells: 7 unique blood cells and 20 unique immune cells), including BMI, WHR, WHRadjBMI, Obesity classes 1, 2, and 3, with 6, 9, 9, 5, 3, and 3 associations, respectively (Figure 4; Supplementary Table S10). We identified seven shared causal cells, of which six cell traits had consistent causal effects among multiple obesity traits. The risk of obesity may be increased by two blood cell traits, high light scatter reticulocyte count (WHR, WHRadjBMI) and platelet count (WHR, WHRadjBMI), and three immune cell traits, $CD14^+$ $CD16^-$ monocyte absolute count (BMI, WHR), $CD28^-$ $CD8^+$ T cell absolute count (Obesity classes 1 and 3), and monocytic myeloid-derived suppressor cells absolute count (Obesity classes 1 and 2). One immune cell trait, effector memory $CD4^+$ T cell absolute count (WHR, WHRadjBMI), may reduce the risk of obesity. Next, we performed FDR correction on the results of the

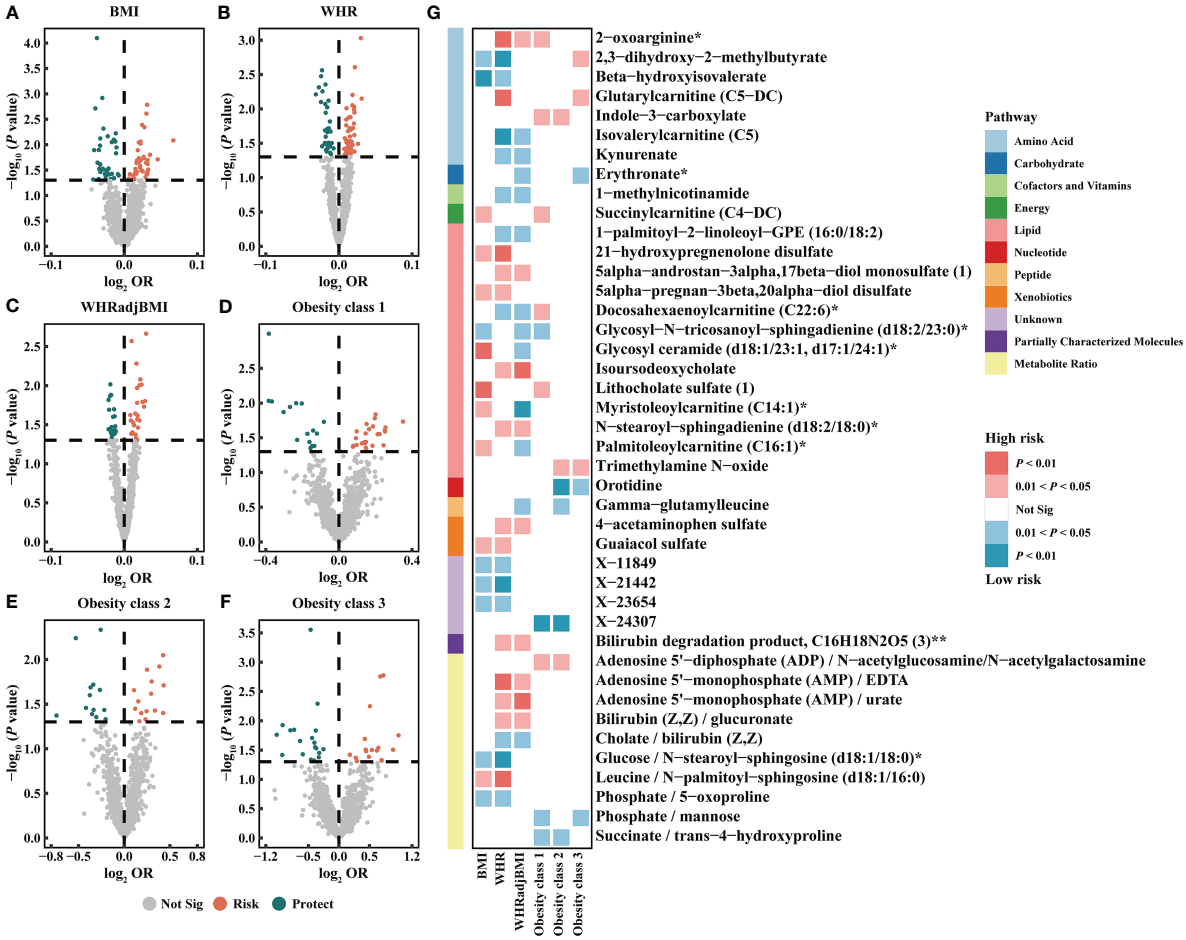

FIGURE 3
Causal estimates of plasma metabolites on obesity. **(A–F)** Volcano plots of the IVW MR for the associations between plasma metabolites and each obesity trait. **(G)** Heatmap of the 42 shared plasma metabolites that showed a causal association with more than one obesity trait at nominal significance (P_{IVW} < 0.05). BMI, body mass index; WHR, waist-to-hip ratio; WHRadjBMI, WHR adjusted for BMI; OR, odds ratio; Not Sig, not significant.

IVW method for blood cell and immune cell traits separately. The results indicate that after correction, five blood cell traits still exhibit a positive causal relationship with WHRadjBMI: high light scatter reticulocyte count (OR = 1.0113, 95% CI [1.0018–01.0209], P = 0.0194, FDR = 0.0484), neutrophil count (OR = 1.0159, 95% CI [1.0035–1.0285], P = 0.0119, FDR = 0.0396), platelet count (OR = 1.0109, 95% CI [1.0025–1.0194], P = 0.0112, FDR = 0.0396), reticulocyte count (OR = 1.0137, 95% CI [1.0038–1.0236], P = 0.0066, FDR = 0.0396), and white blood cell count (OR = 1.0116, 95% CI [1.0002–1.0232], P = 0.0469, FDR = 0.0938) (Supplementary Table S10). Additionally, the findings indicate that the immune cell trait IgD^+ $CD24^-$ B cell absolute count continues to demonstrate a negative causal relationship with BMI (OR = 0.9774, 95% CI [0.9651–0.9899], P = 0.0004, FDR = 0.0477) (Supplementary Table S10). The MR results remained stable in the sensitivity analyses, suggesting the absence of significant heterogeneity and horizontal pleiotropy (Supplementary Table S11).

Moreover, the causal relationship between cytokines and obesity was evaluated using MR, and the results supported the existence of nine suggestive associations between cytokines and obesity (P_{IVW} < 0.05, FDR > 0.1; corresponding to eight unique cytokines), including four, four, two, and one association of BMI, WHR, Obesity classes 1, and 2, respectively (Figure 4; Supplementary Table S12). We found that growth-regulated alpha protein (GROA) (BMI: OR = 0.9929, 95% CI [0.9868–0.9990], P = 0.0236; Obesity class 1: OR = 0.9506, 95% CI [0.9067–0.9965], P = 0.0354) was the only shared causal cytokine that could reduce the risk of obesity. The sensitivity analyses further indicated the absence of heterogeneity and horizontal pleiotropy in these MR analyses (Supplementary Table S13).

3.4 Mediation analysis results

To explore the potential mechanisms of obesity occurrence and development, we conducted a mediation analysis to identify the causal pathway from gut microbiota to obesity mediated by plasma metabolites, peripheral cells, and inflammatory cytokines (please refer to the Mediation analysis section of Methods for details). This analysis focused on previously identified gut microbiota, metabolites, cells, and cytokines associated with obesity in the two-sample MR (Supplementary Tables S3, S7, S10, S12).

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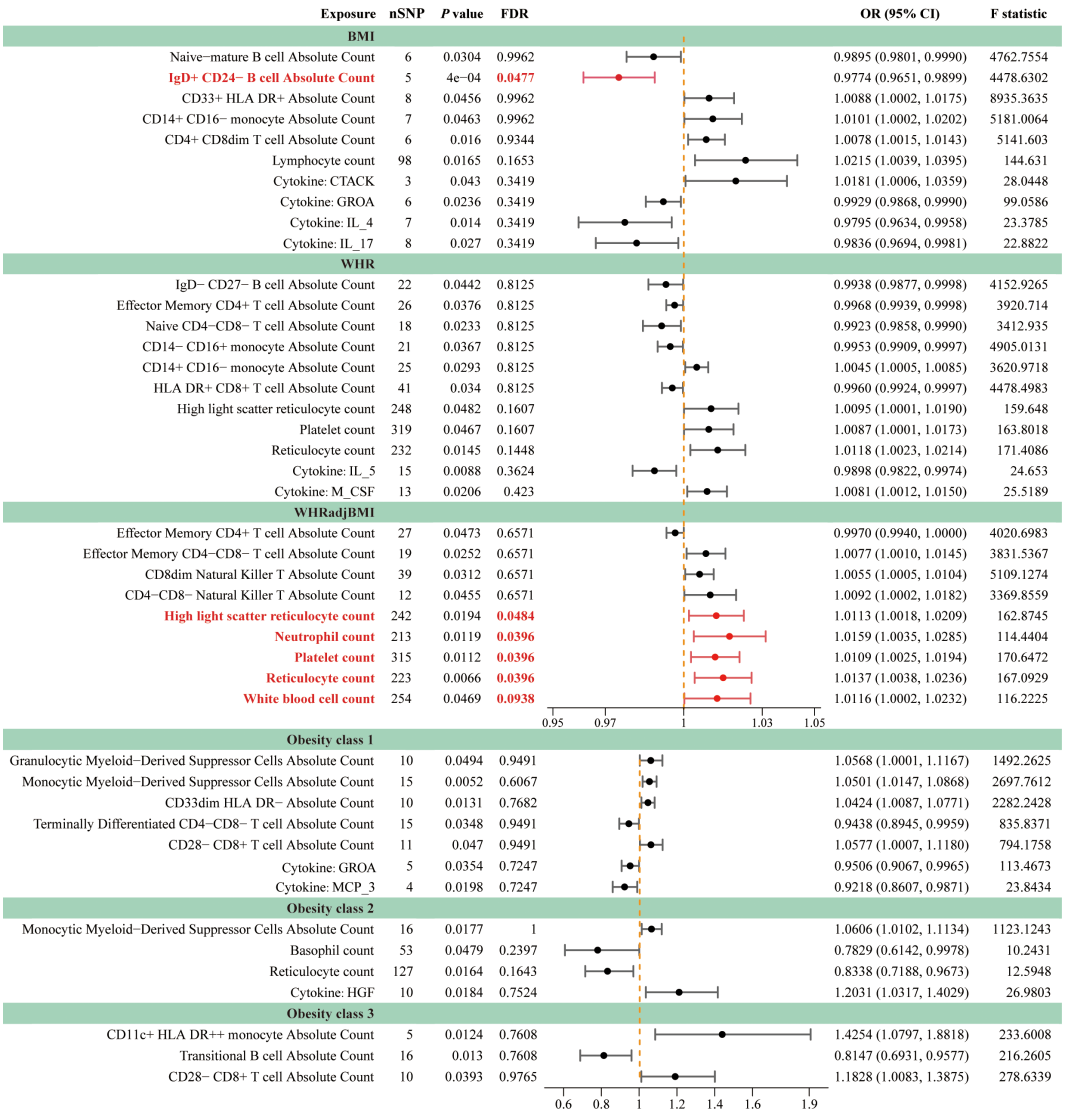

FIGURE 4
Forest plots for causal effects of peripheral cells and inflammatory cytokines on obesity. The horizontal bars correspond to the estimated OR with 95% CI using the IVW method for peripheral cells and inflammatory cytokines on obesity. Causal relationships that remain statistically significant after FDR correction were emphasized using red font and lines. FDR, false discovery rate; BMI, body mass index; WHR, waist-to-hip ratio; WHRadjBMI, WHR adjusted for BMI; OR, odds ratio; CI, confidence interval.

Firstly, the causal relationship among causal gut microbiota, metabolites, cells, and cytokines was evaluated via two-sample MR. We identified 95 associations of gut microbiota to metabolites (BMI, 41; WHR, 31; WHRadjBMI, 9; Obesity class 1, 8; Obesity class 2, 5; Obesity class 3, 1), 10 associations to cells (WHR, 2; WHRadjBMI, 4; Obesity class 1, 1; Obesity class 2, 1; Obesity class 3, 2), and 2 associations to cytokines (WHR, 1; Obesity class 1, 1) (Supplementary Table S14). Furthermore, MVMR analysis was used to screen for metabolites, cells, and cytokines that exhibit a causal relationship with obesity after correcting for the gut microbiota. The results showed that after microbial adjustment, there were 34 metabolite–obesity associations (BMI, 14; WHR, 14; WHRadjBMI, 5; Obesity class 2, 1), 7 cell–obesity associations (WHR, 2; WHRadjBMI, 4; Obesity class 2, 1), and 2 cytokine–obesity associations (WHR, 1; Obesity class 1, 1) (Supplementary Figure S3; Supplementary Table S15). These MR results were validated through the sensitivity analysis, further suggesting the absence of heterogeneity and horizontal pleiotropy (Supplementary Tables S16, S17).

In summary, we identified 20 mediating relationships (1 with strong evidence, 19 with potential evidence), including 17, 2, and 1 gut microbiota–obesity causal pathways mediated by metabolites, cells, and cytokines, respectively (Table 1). The mediation analysis reveals that only 1-(1-enyl-palmitoyl)-2-oleoyl-GPC (p-16:0/18:1) levels exhibit significant negative mediation effects (beta = −0.0043, 95% CI [−0.0085, −0.0001], P = 0.0462) on phylum *Actinobacteria* and BMI with 13.55% (95% CI: 0.23%, 26.87%) proportion. The pathway from phylum *Actinobacteria* to BMI was also potentially mediated by 1-(1-enyl palmitoyl)-2-palmitoyl-GPC (P-16:0/16:1) levels with 4.97% proportion. Additionally, three additional microbial features exhibited more than one mediator. The mediation ratios from class

TABLE 1 Mediation effect of gut microbiota on obesity via plasma metabolites, peripheral cells and inflammatory cytokines.

Exposure	Mediator	Outcome	Total effect	Direct effect	Mediation effect (95% CI)	*P*-value	Mediation Proportion (95% CI) #
p_Actinobacteria	1-(1-enyl-palmitoyl)-2-oleoyl-GPC (P-16:0/18:1)*	BMI	-0.0318	-0.0275	**-0.0043 (-0.0085, -0.0001)**	**0.0462**	**13.55% (0.23%, 26.87%)**
p_Actinobacteria	1-(1-enyl-palmitoyl)-2-palmitoleoyl-GPC (P-16:0/16:1)*	BMI	-0.0318	-0.0302	-0.0016 (-0.0036, 0.0004)	0.1171	4.97%
c_Methanobacteria	3-hydroxybutyrate	BMI	-0.0149	-0.0133	-0.0016 (-0.0039, 0.0007)	0.1696	10.74%
o_Methanobacteriales	3-hydroxybutyrate	BMI	-0.0149	-0.0133	-0.0016 (-0.0039, 0.0007)	0.1696	10.74%
f_Methanobacteriaceae	3-hydroxybutyrate	BMI	-0.0149	-0.0133	-0.0016 (-0.0039, 0.0007)	0.1696	10.74%
c_Actinobacteria	methionine sulfone	WHR	-0.0189	-0.0166	-0.0023 (-0.005, 0.0004)	0.0896	12.29%
c_Actinobacteria	X-16935	WHR	-0.0189	-0.0161	-0.0028 (-0.006, 0.0003)	0.0784	15.06%
o_Bifidobacteriales	myo-inositol	WHR	-0.0196	-0.018	-0.0016 (-0.0039, 0.0007)	0.1783	8.14%
f_Bifidobacteriaceae	myo-inositol	WHR	-0.0196	-0.018	-0.0016 (-0.0039, 0.0007)	0.1783	8.14%
f_Lachnospiraceae	deoxycarnitine	WHR	-0.0215	-0.0198	-0.0017 (-0.0038, 0.0004)	0.1175	7.85%
g_Lachnospiraceae UCG008	1-palmitoyl-2-oleoyl-GPE (16:0/18:1)	WHR	0.016	0.0148	0.0012 (-0.0005, 0.0028)	0.1592	7.21%
g_Lachnospiraceae UCG008	1,2-dilinoleoyl-GPE (18:2/18:2)*	WHR	0.016	0.0147	0.0013 (-0.0006, 0.0032)	0.1765	8.24%
g_Subdoligranulum	docosapentaenoate (n3 DPA; 22:5n3)	WHR	-0.0292	-0.0259	-0.0032 (-0.0072, 0.0008)	0.1121	11.11%
g_Victivallis	X-13431	WHR	-0.0169	-0.0162	-0.0008 (-0.0016, 0.0001)	0.0761	4.51%
f_Alcaligenaceae	gamma-glutamylvaline	WHRadjBMI	0.0192	0.0168	0.0025 (-0.0008, 0.0058)	0.1443	12.89%
f_Alcaligenaceae	high light scatter reticulocyte count	WHRadjBMI	0.0192	0.0188	0.0004 (-4.52E-05, 0.0009)	0.0773	2.15%
g_Lachnospiraceae NK4A136	pimeloylcarnitine/3-methyladipoylcarnitine (C7-DC)	WHRadjBMI	-0.0165	-0.0147	-0.0018 (-0.0041, 0.0005)	0.1289	10.88%
s_Eubacterium nodatum	glycosyl-N-palmitoyl-sphingosine (d18:1/16:0)	WHRadjBMI	0.011	0.0096	0.0013 (-0.0004, 0.0030)	0.1261	12.14%
g_Anaerofilum	white blood cell count	WHRadjBMI	-0.0147	-0.0144	-0.0003 (-0.0006, 1.30E-05)	0.0613	1.87%
g_Ruminococcaceae UCG010	GROA	Obesity class 1	0.2152	0.1966	0.0186 (-0.0054, 0.0425)	0.1280	8.64%

When the 95% CI of the mediation effect spans 0, the 95% CI for mediation proportion is not calculated, as the direction of the upper or lower limit of the mediation effect is opposite to the total effect. Bold formatting indicates that the *P*-value is less than 0.05. BMI, body mass index; WHR, waist-to-hip ratio; WHRadjBMI, WHR adjusted for BMI; CI, confidence interval.

Actinobacteria to WHR through methionine sulfone and X-16935 (an unknown metabolite) levels were 12.29% and 15.06%, respectively. The mediating ratios of genus *Lachnospiraceae* UCG008 to WHR through 1-palmitoyl-2-oleoyl-GPE (16:0/18:1) and 1,2-dilinoleoyl-GPE (18:2/18:2) levels were 7.21% and 8.24%, respectively. The mediating ratios of family *Alcaligenaceae* to WHRadjBMI through gamma-glutamylvaline levels and high light scatter reticulocyte count were 12.89% and 2.15%, respectively. Two mediators mediated more than one relationship: metabolite 3-hydroxybutyrate levels mediated class *Metanobacteria*, order *Metanobacteriales*, and family *Metanobacteriaceae* to BMI, all with a mediation ratio of 10.74%. The ratios of myo-inositol levels mediated order *Bifidobacteriaceae* and family *Bifidobacteriaceae* to WHR (both 8.14%). White cells are a very important type of immune cell in human blood. The white blood cell counts mediated genus *Anaerofilum* to WHRadjBMI, with 1.87% proportion. GROA is a typical inflammatory chemokine with a mediating ratio of 8.64% between the genus *Ruminococcaceae* UCG010 and Obesity class 1. These results showed the consistent direction of the total, indirect, and direct effects, and that the leave-one-out analysis supported the reliable causal relationship in the two-sample MR study of exposure to outcome, exposure to mediator, and mediator to outcome (Table 1; Supplementary Figure S4; Supplementary Table S18).

4 Discussion

This study comprehensively evaluated the causal relationship among gut microbiota, plasma metabolome, blood cells, peripheral

immune cells, inflammatory cytokines, and obesity using MR analysis. We found potential causal associations between 44 bacterial features, 281 plasma metabolites (229 metabolites and 52 ratios), 27 peripheral cells (7 blood and 20 immune cells), and 8 inflammatory cytokines and obesity. Pathway analysis of known plasma metabolites indicated that D-arginine, D-ornithine, linoleic acid, and glycerophospholipid metabolism play important roles in the occurrence and development of obesity. In addition, the mediation analysis results supported the mediating effects of plasma metabolites, peripheral cells, and inflammatory cytokines on the gut microbiota in obesity pathogenesis.

Our findings suggest that higher bacterial abundance within phylum *Actinobacteria*, order *Bifidobacteriales* (the subcategory of phylum *Actinobacteria*), and family *Bifidobacteriaceae* (the subcategory of order *Bifidobacteriales*), may confer protection against obesity (48, 49). The abundance of *Bifidobacterium* (a subcategory of the family *Bifidobacteriaceae*) decreased significantly in individuals with increased visceral adipose tissue, BMI, blood triglycerides, and fatty liver (49). Obese patients can reduce their total blood sugar after short-term *Bifidobacterium*-based probiotic treatment and adjust the gut microbiota structure by increasing beneficial and decreasing pathogenic or opportunistic bacteria (50). In addition, many studies have shown that some strains of *Bifidobacterium* can function as probiotics to protect against obesity (51), such as *Bifidobacterium animalis subsp. Lactis* GCL2505, *Bifidobacterium breve* strain B-3, *Bifidobacterium breve* BR03, and *Bifidobacterium breve* B632 strains (52–54). *Bifidobacterium* can absorb sugars and produce short-chain fatty acids, especially acetate, which modulates host energy metabolism (e.g., inhibits fat accumulation in adipose tissue, increases insulin sensitivity, and enhances fatty acid / glucose metabolism) via the short-chain fatty acid receptor, G protein-coupled receptor 43, which is a common mechanism of probiotic activity (52).

The family *Lachnospiraceae* (phylum *Firmicutes*, class *Clostridia*) is one of the most important families of the intestinal microbiota in healthy adults, including 58 genera and several unclassified strains with complex functions and controversial roles in diseases (55, 56). Most human and mouse studies have revealed that an increased abundance of *Lachnospiraceae* is associated with metabolic diseases (57); however, certain controversies remain. For instance, some reports indicate positive and negative correlations between *Lachnospiraceae* ND3007, *Lachnospiraceae* NK4A136, and obesity, respectively (58). *Lachnospiraceae bacterium* 3_1_57FAA_cT1 is a potentially beneficial microorganism that is inversely proportional to homeostatic model assessment of insulin resistance (HOMA-IR) and fasting insulin levels and may mediate the impact of obesity on insulin resistance (59). The beneficial effects of *Lachnospiraceae* NK4A136 and *Lachnospiraceae bacterium* 3_1_57FAA_cT1 can be explained by the production of butyrate in the intestine (56, 58, 59). The genus *Lachnospiraceae* UCG008 emerged as a shared-risk bacterium across multiple obesity-related traits, consistent with its association with an elevated risk of various diseases, such as hemorrhagic stroke and periodontitis (60–62). Our findings suggest that genera *Lachnospirace* UG008 and *Lachnospirace* FCS020 may increase the risk of obesity, while the family *Lachnospiraceae* and genus *Lachnospiraceae* NK4A136 may reduce this risk.

Contrary to a previous study highlighting statistical associations between *Enterobacteriaceae*, *Desulfovibrio*, and *Akkermansia* with obesity (11), our MR results did not support these findings. This disparity in interpretation may be partly attributed to residual confounding and reverse causation observed in observational studies, rather than a validated causal correlation. It's worth noting that the gut microbiota encompasses not only the bacteriome but also the mycobiome and virome, both of which contribute to obesity pathogenesis (63, 64). While our present study solely focuses on the gut bacteriome, future research will explore the relationship between fungi, viruses, and obesity. Nonetheless, controversies persist regarding the relationship between gut microbiota and obesity (65).

We found that D-arginine, D-ornithine, linoleic acid, and glycophospholipid metabolism were key pathways associated with obesity. Previous studies have shown that these metabolic pathways are mainly affected by gestational diabetes (66) and that D-arginine and D-ornithine metabolism are the main pathways associated with perinatal obesity (67). Linoleic acid is an omega-6 polyunsaturated fatty acid commonly found in the diet and is crucial for human health. Moderate intake of linoleic acid has a positive effect on maintaining cell membrane health and nervous system function. However, a high intake of linoleic acid may contribute to the obesity epidemic as the rich content of omega-6 fatty acids in modern diets is generally imbalanced by the intake of omega-3 fatty acids. Moreover, linoleic acid is converted into arachidonic acid in the body, which plays a role in inducing inflammation and fat synthesis (68, 69). Glycerophospholipid metabolism involves the synthesis, degradation, and remodeling of glycerophospholipids. Glycerophospholipids are the most abundant phospholipids in mammalian cell membranes and can be divided into subcategories, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine. Animal studies have shown that abnormal levels and proportions of PC and PE can lead to dyslipidemia (70), obesity (71), and insulin resistance (72). Human studies have also shown that PC and PE are associated with T2D (73) and the risk of metabolic syndrome (74).

Myo-inositol is a sugar alcohol containing six carbon atoms that helps improve insulin sensitivity, and its deficiency may be related to the pathogenesis of metabolic diseases, such as metabolic syndrome, polycystic ovary syndrome, and diabetes (75). Myo-inositol has potential therapeutic effects on metabolic diseases (76). Our study supports a negative causal relationship between myo-inositol and obesity. Additionally, the mediation ratio of the myo-inositol-mediated order *Bifidobacteriales* and family *Bifidobacteriaceae* to the obesity trait WHR was 8.14%. 3-Hydroxybutyrate is a normal metabolic product of fatty acid oxidation and can be used as an energy source without sufficient blood sugar. It is also an important regulatory molecule that can affect gene expression, lipid metabolism,

neuronal function, and the overall metabolic rate (77). The levels of 3-hydroxybutyrate are high in obese patients and decrease after weight loss surgery (78, 79). Our study supports a positive causal relationship between 3-hydroxybutyrate and obesity. Mediation analysis showed that the mediating proportion of the 3-hydroxybutyrate mediating class *Methanobacteria*, order *Methanobacteriales*, and family *Methanobacteriaceae* to the obesity trait BMI was 10.74%.

Obesity is a chronic inflammatory disease, and an increase in the white blood cell count has been widely associated with these diseases. Observational research has shown that the white blood cell count is positively correlated with the incidence of diabetes, hypertension, obesity, dyslipidemia, and metabolic syndrome (19). White blood cell count is a marker of inflammation and an indicator of whether obesity increases the risk of T2D. A high white blood cell count is associated with reduced insulin sensitivity (80, 81). This study indicated a positive causal relationship between white blood cell counts and obesity, and white blood cell counts mediated 1.87% of the effect of genus *Anaerofilum* on obesity trait WHRadjBMI. The pro-inflammatory cytokine GROA, also known as C-X-C motif chemokine ligand 1 (CXCL1), is a CXC chemotactic factor that helps in the recruitment and migration of various immune cells and plays an important role in regulating immune and inflammatory responses (82). Previous studies have shown that an increase in serum CXCL1 is associated with obesity, hyperglycemia, and pancreatic dysfunction (83). However, using MR, we found that CXCL1 may reduce the risk of obesity. In addition, a mediating ratio of 8.64% was observed for the CXCL1, which mediated genus *Ruminococcaceae* UCG010 to the obesity trait Obesity class 1.

This is the first time that a comprehensive MR framework has been used to analyze the causal relationship among gut microbiota, plasma metabolites, blood cells, peripheral immune cells, inflammatory cytokines, and obesity. Furthermore, a pathway from the gut microbiota to obesity was constructed through a two-step MR and mediation analysis via plasma metabolites, blood cells, peripheral immune cells, and inflammatory factors. This study used a series of sensitivity analyses to maximize the robustness of the MR results. However, this study has certain limitations. First, the lack of demographic information, such as age and sex, in the initial study hindered further subgroup analyses. Second, the majority of people studied by the GWAS were of European ancestry; therefore, the generalizability of the research results to other populations is limited. In addition, although the MR method is effective in evaluating the causal relationship between exposure factors and outcomes, this result needs to be further validated based on more experimental and clinical studies.

5 Conclusion

In summary, our MR study identified 44 gut microbiota taxa, 281 plasma metabolites, 27 peripheral cells, and 8 inflammatory cytokines that were causally linked to obesity; among them, 5 shared bacterial features, 42 shared metabolites, 7 shared cells, and 1 shared cytokine. Pathway analysis revealed 12 obesity-related metabolic pathways, with particular emphasis on D-arginine, D-ornithine, linoleic acid, and glycerophospholipid metabolism which were closely related to obesity. Moreover, we found 20 mediating relationships, including the causal pathways mediated by 17 metabolites, 2 peripheral cells, and 1 inflammatory cytokine from gut microbiota to obesity.

This MR analysis supports the causal effects of the gut microbiota, plasma metabolites, peripheral cells, and inflammatory cytokines on obesity. In addition, mediation analysis revealed that plasma metabolites, peripheral cells, and inflammatory cytokines mediate the pathway from the gut microbiota to obesity. The identified gut microbiota, plasma metabolites, and cellular and inflammatory factors may serve as biomarkers for the diagnosis and treatment of obesity and contribute to the study of obesity mechanisms.

Data availability statement

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

Ethics statement

All analyses were based on publicly available summary statistics, which do not require ethical approval and consent.

Author contributions

YL: Investigation, Methodology, Software, Writing – original draft, Formal analysis. XW: Methodology, Software, Validation, Visualization, Writing – original draft, Formal analysis. ZZ: Data curation, Resources, Writing – original draft. LS: Conceptualization, Funding acquisition, Methodology, Supervision, Writing – original draft, Project administration. LC: Conceptualization, Methodology, Writing – review and editing. XZ: Conceptualization, Funding acquisition, Writing – review and editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1348347/full#supplementary-material

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Dangers of the chronic stress response in the context of the microbiota-gut-immune-brain axis and mental health: a narrative review

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More than 20% of American adults live with a mental disorder, many of whom are treatment resistant or continue to experience symptoms. Other approaches are needed to improve mental health care, including prevention. The role of the microbiome has emerged as a central tenet in mental and physical health and their interconnectedness (well-being). Under normal conditions, a healthy microbiome promotes homeostasis within the host by maintaining intestinal and brain barrier integrity, thereby facilitating host well-being. Owing to the multidirectional crosstalk between the microbiome and neuro-endocrine-immune systems, dysbiosis within the microbiome is a main driver of immune-mediated systemic and neural inflammation that can promote disease progression and is detrimental to well-being broadly and mental health in particular. In predisposed individuals, immune dysregulation can shift to autoimmunity, especially in the presence of physical or psychological triggers. The chronic stress response involves the immune system, which is intimately involved with the gut microbiome, particularly in the process of immune education. This interconnection forms the microbiota-gut-immune-brain axis and promotes mental health or disorders. In this brief review, we aim to highlight the relationships between stress, mental health, and the gut microbiome, along with the ways in which dysbiosis and a dysregulated immune system can shift to an autoimmune response with concomitant neuropsychological consequences in the context of the microbiota-gut-immune-brain axis. Finally, we aim to review evidenced-based prevention strategies and potential therapeutic targets.

KEYWORDS

physiological stress, microbiome, mental health, psychopathology, autoimmunity

1 Introduction

Over 1 in 5 youth and adults live with mental illness in the United States alone, and 1 in 25 live with a serious mental illness (e.g., schizophrenia, bipolar disorder, major depressive disorder (MDD), which further increases risk for a plethora of physical diseases (1). Pharmacotherapy interventions are rapidly evolving but often carry significant side effects, such as weight gain, metabolic dysfunction, extrapyramidal symptoms, and tardive dyskinesia, a drug-induced movement disorder (2). What's more, many psychiatric medications as well as polypharmacy possess antimicrobial properties, which alter the gut and neural milieu (3). As such, there is a dire need for effective prevention and treatment strategies, not only for the primary illness, but also for the wide array of common comorbidities.

Since Hans Selye's (1946) (4) pioneering work on stress and the general adaptation syndrome, stress has become a well-established key risk factor for psychiatric disorders (5). Psychosocial stressors and diathesis stress models are the core of many etiological theories of mental illness (6). More specifically, the pathoetiology of psychiatric disorders continues to unravel complex multidirectional relationships involving neuroendocrine, immune, and inflammatory mechanisms in the gut, microbiome, and brain. Much research to date has elucidated the complex matrices of the microbiota-gut-immune-brain axis. In this narrative review, we aim to highlight the important role of the chronic stress response and its relationship to the microbiota-gut-immune-brain axis in the pathophysiology of psychiatric disorders, focusing primarily on human studies. To do so, we will address these complex mechanisms by discussing the multidirectional communications between the chronic stress response and the microbiota-gut-brain-axis, immune tolerance, and mental health (see Figure 1). Specific psychopathologies and their relationship with microbiome and immune dysregulation will be discussed, along with associated physical, mental, and nutritional triggers. Finally, we will briefly discuss potential prevention strategies and therapeutic targets.

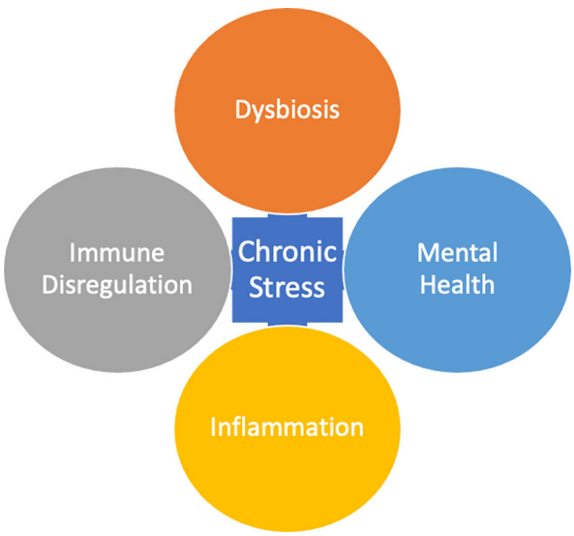

FIGURE 1
Chronic Stress is a compounded response that occurs in positive feedback loop with Dysbiosis, Immune Dysregulation, Inflammation, and Mental Health.

2 The crossroads of chronic stress, immune activation, and the microbiome

2.1 Microbiota-gut-immune-brain axis

We coexist in an incredible symbiotic relationship with our commensal organisms, which contribute to the prevention or triggering of disease. The gut-brain axis (GBA) is a complex communication system that involves multiple interactions between gut functions and the emotional and cognitive centers of the brain. These interactions are mediated by various mechanisms, including neuro-immuno-endocrine mediators. The GBA includes the central nervous system (CNS), autonomic nervous system (ANS), enteric nervous system (ENS), and hypothalamic-pituitary-adrenal (HPA) axis (7). The HPA axis is considered the core stress axis that coordinates the adaptive responses of the organism to stressors of any kind. The gut microbiota plays an important role in the GBA, interacting not only locally with intestinal cells and ENS, but also directly with the CNS through neuroendocrine and metabolic pathways. Dysbiosis, an imbalance in the gut microbiota and therefore the gut microbiome, has been linked to various mental disorders, including anxiety, depression, and autism (8, 9). Dysbiosis also occurs in functional gastrointestinal disorders (FGID) that are strongly associated with mood disorders and have been linked to a disruption of the GBA. The ANS is a neural relay composed of a complex network with neurons located in both the CNS and peripheral nervous system. These are responsible for body functions that occur without conscious effort such as digestion, heart rhythm, and breathing. Combined with activity from the ENS and modulated by the CNS, the ANS promotes physiological homeostasis (7). To facilitate homeostasis, the ANS interfaces with endocrine, motor, autonomic, and behavioral areas, all of which comprise the larger network of the bidirectional GBA (7). The ANS works in conjunction with the other systems that comprise the GBA to enact CNS-driven changes to the gut (7). Communication between the gut and the CNS is mediated by the ANS. Because the vagus nerve (the tenth cranial nerve) directly innervates the gut, ENS, and ANS, it provides the most direct neurological response. This rapid neurological response may take the form of pain and stress responses (7). It follows then that ANS activity can induce ENS responses. Potential triggers to the ENS are often responsible for changes in gut motility, leading to differing delivery and uptake of microbiota-accessible carbohydrates (MACs) needed for the proliferation and maintenance of diverse gut microbiome composition. As part of the ANS circuit directly interfacing with the gut, the vagus nerve also serves as the fastest information exchange route between the gut and the CNS (7). The gut is

enmeshed in hepatic and celiac branches of the vagus nerve (7). Density of these branches decreases caudally from the proximal duodenum, ileum, and the ileocecal junction; though, they continue to extend to the colon (7). The vagus nerve forms intraganglionic laminar endings, intramuscular arrays, terminal axonal endings in the mucosa, and neuropods, as a subset of enteroendocrine cells that comprise synapses with neurons of the vagus nerve (7).

The potential interplay between signaling molecules in the body and the microbial communities of the gut microbiome is another key feature of the bidirectional relationship between microbiota and behavior. The gut microbiota play a significant role in regulating the GBA and local and systemic immunity. The microbiome metabolites from the gut, particularly short-chain fatty acids (SCFA), have immunomodulatory properties and can interact with nerve cells by stimulating the ANS and the sympathetic nervous system (SNS) via G-protein-coupled receptors (10). SCFAs can also regulate the release of gut peptides from enteroendocrine cells, affecting gut-brain hormonal communication (10). The gut microbiota are capable of producing a variety of other neuroactive and immunomodulatory compounds, including dopamine, histamine, and acetylcholine (10, 11). Moreover, the gut microbiome is an important regulator of bile acid pool size and composition, which, in turn, affects blood-brain barrier (BBB) integrity and HPA function. The microbiota that constitutes the gut microbiome interact with bile salts in the gut to achieve this modulation of bile acid (7). Recently, certain taxa were shown to express bile salt hydrolase (BSH), which can deconjugate taurine and glycine from bile acids (7). The presence of microbiota expressing BSH in the gut microbiome has been linked to an increase in diversity of bile acids in the host gut (7). Additionally, deconjugated bile salts are shown to be less efficiently reabsorbed by the small intestine (7). This in turn has implications on host metabolism and weight gain (7). The gut microbiota may also contribute to the regulation of brain function by influencing tryptophan metabolism. Once absorbed from the gut, tryptophan can cross the BBB and participate in serotonin synthesis; however, the availability of tryptophan is heavily influenced by the gut microbiota (10, 11). Resident gut microbiota can utilize tryptophan for growth or, in some cases, production of indole or serotonin (10, 11). The gut microbiota could influence serotonergic neurotransmission by limiting the availability of tryptophan for serotonin production in the CNS (7, 11).

The gut microbiota also influences the development of the HPA axis and the stress response, which will be covered in detail subsequently. In short, activation of the HPA axis leads to the release of corticosterone releasing factor (CRF) from the hypothalamus, which stimulates the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary, inducing the synthesis and release of glucocorticoids from the adrenal cortex (12). Germ-free mouse models have shown that the microbiota play a key role in the development of the HPA axis and its stress response (12). Germ-free animals exhibit exaggerated HPA axis activity with elevated ACTH and corticosterone in response to stress, which is normalized ('rescued') after fecal microbiota transplant from control mice (12). The gut microbiota and stress response are also linked in humans, with probiotic supplements shown to improve stress and emotional responses (12).

2.2 The chronic stress response and mental health

Chronic stress exposure has severe lasting biological consequences (13). The field of psychoneuroimmunology, which elucidates the relationship between immune function, brain health, and psychosocial factors (14) has highlighted the impact of chronic stress exposure throughout the lifespan, from early life and prenatal stress to adulthood (15, 16). Threats come in many forms, including external (noise, physical abuse) and internal (illness, sleep deprivation, emotions) and are processed by the brain exteroceptively (from outside) and interoceptively (from within) to trigger the stress response (17). The acute response to real or perceived stress is an adaptive mechanism designed to protect the host and maintain homeostasis through neural, endocrine, and immune processes, primarily via the SNS and hypothalamic-pituitary-adrenal (HPA) axis (18). The (CNS), ANS (sympathetic and parasympathetic divisions) and HPA-axis work in concert to activate and deactivate the stress response as needed. However, chronic HPA-axis activation with associated cortisol dysregulation is maladaptive and associated with a myriad of negative health outcomes, including increased susceptibility to infection, metabolic syndrome obesity, cancer, cardiovascular disease, mental health disorders (18), and neurological disorders such as Alzheimer's disease (19). In fact, the profound impact of psychological stress on inflammatory processes is an often-overlooked management strategy in numerous disease states (20).

In brief, the HPA-axis, SNS, and CNS are activated in response to stress with an ultimate release of catecholamines (i.e., epinephrine and norepinephrine), glucocorticoids (i.e., cortisol) and subsequent negative feedback to the CNS to prevent sustained activation and prolonged glucocorticoid exposure (19). Chronic exposure to stress alters neurotransmitter and hormone levels and increases immune activation centrally and peripherally, as evidenced by elevated proinflammatory cytokines (e.g., IL-6), increased monocytes and neutrophils, and activated microglia (21). Microglia, the resident immune cells in the brain, upregulate several immune markers (e.g., Iba1, CD11b, CD86, TLR4, CD14, and CD68), proinflammatory cytokines (e.g., IL-1β, CCL2), and reactive oxygen species (ROS), leading to phagocytosis of neuronal elements in response to stress (21). Furthermore, microglia highly express glucocorticoid receptors, pointing to their role in the stress response (22), and produce toll-like receptor 4 (TLR-4), for which the ligand is lipopolysaccharides (LPS), underscoring their involvement in immune and inflammatory processes as well as intestinal hyperpermeability (23). Microglia are key regulators of stress and neuroinflammation (22). With chronic activation, microglia promote sustained neuroinflammation, synaptic dysfunction, and altered brain network connectivity seen in various neuropsychiatric disorders (24, 25). Additionally, early life stressors may prime microglia, such that responses to stress are potentiated with accompanying neuroinflammation and susceptibility to mental illness (16). Early life stress during developmental periods, whether pre- or post-natal, is a predominant risk factor for an altered stress response and negative physical and neuropsychiatric outcomes, accounting for an estimated 45% of mental illness in children and up to 30% in adults (26).

Owing at least in part to altered immune responses and neuroinflammation, chronic stress exposure is one of the strongest risk factors for developing various psychiatric disorders such as burnout, depression, posttraumatic stress disorder, bipolar disorder, schizophrenia, anxiety disorders, substance abuse disorders, and addiction (5, 13, 21). Mounting evidence suggests that chronic stress and trauma can result in epigenetic changes that contribute to the development of psychopathology (13). Likewise, mental health has a profound effect on inflammatory processes throughout the body including the brain, indicating the importance of including mental health in the management of physical disorders and vice versa (20). These systemic and neuroinflammatory processes also contribute to dysbiosis via the microbiota-gut-immune-brain axis and are often associated with elevated IL-1β, IL-6, LPS, and decreased brain-derived neurotrophic factor (BDNF), a neurotrophin required for neuronal development and survival as well as synaptic plasticity and cognition (27). Dysbiosis also decreases concentrations of tight junction proteins (e.g., occludins, zonulin) in the intestine and BBB (23). As such, the intestine and BBB become hyperpermeable with compromised integrity–the "leaky gut, leaky brain" that can affect physical, cognitive, and mental health (whole-person well-being) (see Figure 2).

2.3 The chronic stress response and the microbiome

Microbiome research has emerged as a promising new frontier in disease and health. We are continuing to learn how chronic stress not only "gets under the skin," but also "into the belly" and can further emerge *from* the belly (28). Evidence supports the critical role of gut microbes and their metabolites in central neurochemistry, mood, and behavior, particularly stress-related processes (6, 29), including but not limited to neurotransmitters, neuropeptides, SCFAs, bile acids, endocrine hormones, and immunomodulators (3). Notably, the ecosystem of the ENS shares many structural and chemical components, such as neurotransmitters, neurons, and glia. As previously mentioned, chronic stress leads to HPA-axis activation and ultimately, to dysbiosis, and the inflammation resulting from dysbiosis also leads to HPA-axis activation (27). *Via* the ENS by way of the microbiota-gut-immune-brain axis, psychological stress leads to chronically elevated glucocorticoid and results in monocyte- and TNF-mediated inflammation by inducing inflammatory enteric glia to promote monocyte recruitment via CSF-1, and consequential transcriptional immaturity in enteric neurons, acetylcholine deficiency, and TGF-b2-induced gut dysmotility (30).

The gut microbiome is a key player in both the top-down and bottom-up processes involving the relationship between neurobiology and the intestinal ecosystem, and clinically reflected in comorbidities of gastrointestinal (GI) symptoms with anxiety, depression, spectrum disorders, and neurodegenerative disorders (27). Stress therefore shifts the microbiome ecosystem into a state of dysbiosis that leads to intestinal and BBB hyperpermeability, endotoxemia, and neuroinflammation (31). This bidirectional dysbiosis-inflammatory-brain/mood connection can be observed in many disorders and is exemplified in inflammatory bowel disease (e.g., Crohn's disease, ulcerative colitis), which are not only accompanied by psychological symptoms, but also exacerbated by psychological stress (30); this produces a vicious cycle of physical and mental symptoms seen in many disease states. Furthermore, as early life stressors may prime microglia to increase susceptibility to psychopathology, as discussed earlier, early life trauma may also

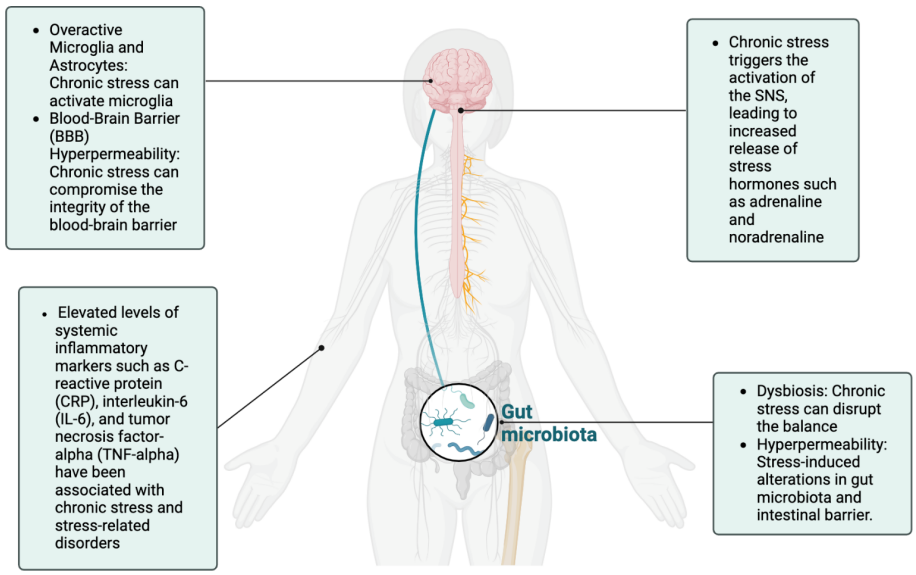

FIGURE 2
The chronic stress response, characterized by sympathetic overdrive and HPA-axis dysregulation, can have profound effects on inflammation across multiple systems, including the gut, systemic circulation, and the brain. These interconnected pathways underscore the bidirectional relationship between stress and inflammation, which plays a pivotal role in the pathophysiology of stress-related disorders.

prime the gut microbiome toward a state of dysbiosis and facilitate inflammatory cascades, thereby increasing the risk of post-traumatic stress disorder (32).

In addition to the immune and inflammatory contributions of the gut microbiome and stress, the gut microbiota also produce neurotransmitters including as gamma-aminobutyric acid (GABA), norepinephrine, epinephrine, dopamine, acetylcholine, melatonin, and histamine, which likely exert their effects via the ENS and vagal afferents (33–37). An estimated90% of serotonin alone is produced in the gut, primarily from enterochromaffin cells (36). Many psychopathologies and neurological disorders are associated with disruptions in neurotransmitter levels, which can also act as hormones affecting numerous receptor sites (38). While still under exploration, it has been shown that bacterial species from the gut microbiota dramatically alter chemicals that mediate neurotransmitters and neural transmission (38). Serotonin, which affects both mood and GI function, is the most studied neurotransmitter in the field of psychiatry (39) and is directly related to microbiota composition (38). Serotonin dysregulation is associated with anxiety and depression, and studies have shown that supplementation with probiotics from the genus *Bifidobacterium* and *Lactobacillus* may improve the symptoms of depression (39). Understanding the mechanisms behind microbiome perturbations that lead to dysregulation of neurotransmitters is a potential target for prevention and management of several psychopathologies, some of which will be discussed in following sections.

2.4 Immune dysregulation leads to autoimmunity

Immune dysregulation is a complex phenomenon that is key to the pathogenesis of autoimmunity. The immune system is designed to defend against pathogens, largely through antigen recognition, while at the same time maintaining tolerance to self by limiting self-reactive lymphocytes (reactive to self-antigens). This is maintained in two parts. First, the complex gene rearrangements during lymphocyte development, which lead to a diverse pool of antigen receptors, allow for robust immune responses to even yet unseen pathogens (40). Second, these lymphocytes are tested for self-tolerance and those that fail are eliminated or at least held in check either during development in the primary lymphoid tissue (central tolerance) or after leaving the primary lymphoid tissue (peripheral tolerance) under normal circumstances (40).

Autoimmunity occurs due to escape from the normal self-tolerance screening and/or elimination process (central and peripheral tolerance). This results in an aberrant immune reaction to self, which is termed autoimmunity. It is well known that genetics are an important predisposing factor for autoimmunity; this affects the ability of the immune system to perform the processes of central and/or peripheral tolerance (40). The environment is also a contributor to the ability of the immune system to maintain tolerance including infection, stress, and environmental exposure to certain chemicals. Additionally, this loss of tolerance can propagate through epitope spreading, in which the immune response expands to include additional self-antigens, and molecular mimicry, when foreign antigens share structural similarities with self-antigens resulting in confusion between foreign and self-antigens (40).

It is impossible to discuss immune tolerance without highlighting regulatory T cells (Tregs), a subset of T lymphocytes that promote tolerance and resolution of immune responses (return to homeostasis). Tregs can extrinsically mediate autoimmunity, as they are able to suppress self-reactive lymphocytes. This is regulatory tolerance, a type of self-tolerance. Further, Tregs can stimulate peripheral tolerance when antigens are delivered orally (oral tolerance), providing a potential mechanism for therapies against issues as diverse as food allergies and autoimmunity (40). A balance between the classically activated M1 (Th1 responses) and alternatively activated M2 (Th2 responses) macrophages is also important in the role of immune tolerance. M2 macrophages produce IL-10 and TGF-beta to promote wound healing (40) and can also play a role in immunosuppression. A complex system of checks-and-balances is required to have a robust immune response without autoimmunity. Therefore, for autoimmunity to be established, multiple mechanisms must fail; this is further exacerbated by an inflammation positive feedback loop (40).

3 Stress, psychopathology, and the microbiome

Dysbiosis and psychopathology appear to be related in many ways. The literature suggests a dual relationship, functioning in a manner through which changes in gut microbiota affect behavior, while conversely, changes in behavior result in alterations in gut microbiome composition (41, 42). Of note, medications used to treat mental disorders are often associated with GI side effects, suggesting further effects on the gut microbiome (43). Likewise, evidence suggests that certain probiotic strains, fecal microbiota transplantation (FMT), prebiotics, postbiotics (i.e., SCFA), and dietary modifications can alleviate some symptoms associated with mental illness (44), although evidence is limited and research ongoing. Many psychiatric disorders have been shown to have significant differences in microbiome composition (see Table 1), including depression, bipolar disorder, schizophrenia, autism spectrum disorder, attention deficit hyperactivity disorder (ADHD), and pediatric autoimmune psychiatric disorder associated with streptococcal infections (PANDAS) (44, 45). It is important to note, however, that much of the available literature involves animal models and considerable differences in assessment methods. At present, mechanistic contributions, at least in animals, seem to be clearer than appropriate intervention strategies in human studies.

3.1 Depression & anxiety

The incidence of depression and anxiety has significantly increased over the past several decades (46). Depression is the

TABLE 1 Examples of microbial alterations in pyschopathology.

Depression and Anxiety	Lower levels of *Faecalibacterium*, *Dialister*, *Coprococcus* spp.. *Flavonifractor* (formerly *Eubacterium*) was increased in participants with depression
Obsessive Compulsive Disorder	Lower diversity and abundance of butyrate-producing genera, specifically *Pseudomonas*, Caulobacteraceae (family), *Streptococcus*, *Novosphingobium*, and *Enhydrobacter*
Bipolar Disorder	Lower abundance of the phylum Bacillota (formerly Firmicutes) specifically *Faecalibacterium*, the family Lachnospiraceae, and the genuses *Akkermansia* and *Sutterella*
Schizophrenia	Noted presence of the phylum Pseudomonadota and increased lactic acid bacteria
Alcohol Use Disorder	Lower abundance of *Akkermansia muciniphilia*, *Faecalibacterium prausnitzii*, *Bacterioides*, and at the phylum level more Pseudomonadota (formerly Proteobacteria), and more members of the Enterobacteriaceae family

leading cause of medical disability worldwide (47) and major depressive disorder (MDD), the more severe form, is the second leading cause of disability in the United States; both are associated with pathological shifts in gut microbiota (47). The relationship between the gut microbiome and depression and anxiety was established more than a decade ago but continues to be refined (48).

Recent work suggests that the microbiota-gut-brain axis functions in a bidirectional manner in the regulation of depressive-like behaviors (7, 11). Data from mouse models demonstrate that changes in behavior caused by stress, knockout of caspase-1, or pharmacological treatments result in changes in the gut microbiome (41). In germ-free mice, the absence of gut microbiota results in decreased immobility time in the forced swimming test compared to conventionally-raised, healthy, control mice (41). From clinical sampling, gut microbiome composition significantly differed between MDD patients and healthy controls (7, 42). FMT from MDD patients to germ-free mice resulted in depression-like behaviors compared with FMT from healthy controls (12, 41). Generally speaking, MDD is associated with reduced numbers of *Bifidobacterium* and *Lactobacillus* versus healthy controls (12).

Ghrelin is a gut hormone that regulates energy homeostasis, eating and sleeping behavior, cognition, reward mechanisms, and mood (7, 49). Ghrelin interacts with acylated ghrelin receptors expressed by the hypothalamus, dentate gyrus, and other regions of the brain. This hormone has been shown to regulate mood and has close links to depression (49). Both acute and chronic stress results in elevated ghrelin, whereas prolonged stress also leads to chronic increased dysregulation of the HPA axis, serotonin signaling, and increased depressive behaviors–perhaps due to prolonged overexpression of ghrelin. Ghrelin has been associated with MDD, and elevated ghrelin has been found to act as a measure of treatment response (7, 42). Ghrelin is primarily produced in the gut, and germ-free mice have lower circulating ghrelin (7, 41, 49). Prebiotic treatment, which increases SCFA production, has been found to alter ghrelin, the production of which is regulated by SCFA signaling (7). Gastric infusion of the SCFA acetate increases plasma ghrelin, indicating potential bidirectional communication from the gut to the brain in the control of ghrelin secretion from cholinergic cells.

Due to its role in mediating mood and its connection to the gut microbiota, ghrelin could serve as an optimal biomarker to identify treatment response to prebiotic and probiotic treatment in the MDD population (7, 42).

Patients with irritable bowel syndrome (IBS) and inflammatory bowel disorders (IBD) are at higher risk of depression (50). The relationship between dysbiosis and MDD has been hypothesized to involve the microbiota-inflammasome-brain connection, whereby dysbiosis caused by stress and GI conditions exacerbates MDD via upregulation of pro-inflammatory pathways including the caspase-1-dependent, pro-inflammatory NLRP3 inflammasome present in several immune cell types (e.g., microglia, monocytes, granulocytes, T cells, B cells) (51). Furthermore, C-reactive protein (CRP), which tends to be chronically elevated in depression and anxiety, has been shown to interact with the gut microbiome and affect the risks of anxiety and depression (46). Messaoudi et al.found that taking a probiotic formulation of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 for 30 days decreased anxiety and depression scores while concomitantly lowering cortisol (52). A more recent study by Valles-Colomer et al.surveyed a large microbiome cohort from the Flemish Gut Flora Project (n=1,054) to examine the relationship between microbiome alterations and quality of life in depression (53). They found that butyrate-producing bacteria (e.g., *Faecalibacterium* and *Coprococcus*) were associated with higher quality of life measures: *Flavonifractor* was increased in depression; *Dialister*, *Coprococcus* spp. were reduced in depression (53). Further,the dopamine metabolite 3,4-dihydroxyphenylacetic acid was positively correlated with quality of life, suggesting the potential role of microbial neurotransmitter production in depression (53).

Furthermore, changes in serotonergic signaling in germ-free mice may contribute to the altered anxiety-related phenotype observed (12, 41). Studies in germ-free animals have shown that microbial colonization of the gut is central to the development and maturation of both ENS and CNS (41). The absence of the gut microbiome is associated with altered expression and turnover of neurotransmitters in both nervous systems and neuromuscular abnormalities (12, 41). These anomalies are restored after microbial colonization in a bacterial species-specific manner; in other words, the germ-free animals are rescued by gut microbiome colonization. This link reinforces the bidirectional mechanism of the GBA and the role that the microbiome plays in influencing the development of behavior in mice, with germ-free mice exhibiting an anxiolytic phenotype in the elevated plus maze (EPM), an ethologically and pharmacologically validated tool for the assessment of rodent anxiety-like behavior (12, 41, 54). The molecular data provide initial insights into the neurobiological pathways underlying this behavioral phenotype. In germ-free mice the subunit of N-methyl-D-aspartate (NMDA) receptors, NR2B, was severely downregulated. This downregulation was particularly acute in the central amygdala and is thought to contribute to the anxiolytic-like phenotype that was observed in the EPM (41). NMDA receptors are heteromeric complexes and are made up of both NR1 and NR2 subunits. The NR2B subtype is the

critical receptor in amygdala synaptic plasticity and development and in learning and memory. Additionally, NMDA receptor antagonists are known to block anxiety-like behavior in both mice and rats (12, 41, 54). Antagonists specific to NR2B block the acquisition of amygdala-dependent fear-learning, further illustrating the role that this NMDA receptor subtype plays in the expression of anxiety, fear, and CNS plasticity. Up-regulation of BDNF mRNA in the dentate region of the hippocampus in the germ-free mice is consistent with literature identifying a role for this molecule in anxiety-like behaviors (41). Recent work has demonstrated that impaired BDNF signaling in the dentate gyrus of adult mice results in a marked increase in anxiety-like behavior (41). Hence, it is reasonable to suggest that increased BDNF may be related to the observed reduction in anxiety-like behaviors in germ-free mice.

3.2 Obsessive compulsive disorder

Obsessive compulsive disorder (OCD) is a chronic debilitating mental illness with an unclear etiology involving immune, neurotransmitter, endocrine, and microbiome dysregulations (45). Immune status affects the evolution of OCD and is marked by an alteration in the type of immune response, especially Th1 versus Th2 (55). In concert with HPA-axis dysregulation, cytokines such as IL-6 and TNF-α tend to be increased in OCD (55). However, a recent study suggests that CRP may be more clinically relevant than IL-6 and TNF-α. Turna et al. compared the microbiomes and inflammatory markers of 21 non-medicated OCD patients to 22 age- and sex-matched controls and found elevated CRP compared to controls, but not IL-6 or TNF-α (45). Further, CRP was associated with severity of symptomatology and the OCD group presented lower diversity and abundance of butyrate-producing genera in their gut microbiomes (45). Larger studies are needed for clarification.

A more recent study by Kang et al., compared the microbiome of OCD patients to health controls using circulating bacterial extracellular vesicles in serum and found that at the genus level *Pseudomonas, Caulobacteraceae(f), Streptococcus, Novosphingobium*, and *Enhydrobacter* were significantly reduced in the OCD group, and microbial composition of the genera *Corynebacterium* and *Pelomas* were significantly different in the early-onset versus late-onset types (56).

3.3 Bipolar disorder

Bipolar disorder is a serious mental disorder that has been associated with systemic immune alterations and chronic inflammation, mediated by the gut microbiota (57). Some evidence suggests that microbiome alterations are associated with bipolar disorder, particularly a decrease in Bacillota, formerly Firmicutes, especially *Faecalibacterium* (3). Surprisingly, pharmacological treatment of bipolar disorder may further exacerbate dysbiosis. In a study involving atypical antipsychotics in females with bipolar disorder, the treatment group demonstrated decreases in the family Lachnospiraceae and in the genuses *Akkermansia* and *Sutterella* compared to treatment-naïve controls (58). What's more, patients with bipolar mania are almost twice as likely to have been recently treated with systemic antibiotics, and studies examining probiotic use suggest that it could reduce the rate of re-hospitalization following a manic episode (3). More research is warranted and needed.

3.4 Schizophrenia

Schizophrenia is a lifelong, serious mental disorder and, like many other pathologies, is associated with immune dysfunction, chronic inflammation, and dysbiosis (57). In the limited studies of patients with schizophrenia, the gut microbiome has shown increases in the phylum Pseudomonadota, formerly Proteobacteria, (predominantly the genus *Succinivibrio*), increased abundance of lactic acid bacteria and *Lactobacillus* phage in the oral microbiome, and a relationship between dysbiosis and first episode psychosis severity (44). Further alterations have been observed but a clear pattern has not yet emerged. While speculative, dysbiosis may be related to psychosis as a result of microglial activation through SCFA production alteration (44).

3.5 Alcohol use disorder

Alcohol use disorder (AUD) is one of the most significant preventable contributors to morbidity and mortality (59, 60). Alcohol directly alters the permeability of the intestinal barrier, causes dysbiosis, and is associated with peripheral and central inflammation (60). A systematic review of 17 studies of AUD and the gut microbiome by Litwinowicz et al. found that individuals with AUD demonstrated lower abundance of *Akkermansia muciniphilia* and *Faecalibacterium prausnitzii*, less of the genus Bacterioides, at the phylum level more Pseudomonadota (formerly Proteobacteria), and more members of the Enterobacteriaceae family (61). A recent study by Litwinowicz & Gamian compared the microbiomes of participants with AUD, alcoholic liver disease (ALD), and healthy controls (59).

They found a significantly lower abundance of butyrate-producing families (especially the species *Ruminococcaceae*, *Lachnospiraceae*, and *Oscillospiraceae*) in AUD compared to controls, which was more severe in ALD, and, at the phylum level, an increase in endotoxin-producing Pseudomonadota (formerly Proteobacteria) in AUD, again worse so in ALD compared to controls. Fungal microbiome studies have suggested significantly increased abundance of the genera *Candida*, *Debaryomyces, Pichia, Kluyveromyces*, and *Issatchenkia* and of the species *Candida albicans* and *Candida zeylanoides* compared to controls, some of which decreased with alcohol abstinence (62).

Therefore, there is the potential for gut microbiome targeted therapeutics to support management of AUD and ALD with substantial additional research necessary.

4 Immune dysregulation and the microbiome

4.1 Autoimmunity and the microbiome

Immune dysregulation can result from inappropriate immune reaction to commensal microbiota, the residents of human microbiomes. When this reaction causes disease, it is technically termed xenoimmunity, as the microbiota are not built by the host but rather acquired from foreign sources (e.g. maternal microbiome, dietary intake, the environment, etc.) (40). However, the presentation of such disease is virtually identical to autoimmunity. Further, many are beginning to consider the various microbiomes as vital components of their host, meaning they cannot be separated from the host. Thus, immune responses to commensal microbiota that result in disease are typically included in the autoimmunity category, and we will continue to refer to these as autoimmunity here. The classic example of autoimmune disease stimulated by a microbiome is Crohn's disease, a type of IBD in which T lymphocytes react to commensals in the gut microbiome (40). Crohn's disease is characterized by waves of severe inflammation, diarrhea, pain, and fatigue and often results in weight loss, malnutrition, and granulomatous lesions in the intestinal mucosa and submucosa. There is a strong genetic component to Crohn's disease including NOD2, a gene that regulates lymphocytes and antigen recognition in the intestine through autophagy (recycling of cellular components or whole cells) (63).

Further, the microbiota plays an important role in immune education, the process by which an immune system learns to recognize appropriate targets, mount a robust immune response, and return to homeostasis accordingly (40). Without proper immune education during the early life window of opportunity, aberrant immune responses are more likely including allergy and autoimmunity (64–66). Therefore, microbiota may be both a trigger for self-reactive lymphocytes and for aberrant immune responses required to support autoimmunity.

All of this contributes to function or dysfunction of the microbiota-gut-immune-brain axis, which is exaggerated by an inflammation positive feedback loop. Multidirectional links have been made between the gut microbiota, the HPA axis, and mental health issues; however, this work has been mostly in animal models, which have translated very poorly to humans, especially in this field, to-date (66, 67). Therefore, caution is warranted when interpreting the literature until more translational research is conducted showing strong relationships in humans. At that point, animal models will be very useful for mechanistic studies.

4.2 Physical/mental triggers of autoimmune reactions and dysbiosis overlap

Several physical and psychological triggers have been associated with the dynamic interplay between immune dysregulation, autoimmunity, inflammation, and dysbiosis – the effects of which are widespread and far-reaching. In addition to psychological stressors and trauma discussed previously, physical triggers have also been associated with this detrimental cycle. Examples of environmental triggers include diet, alcohol, medication, infections, pollution, physical activity, and calorie intake (68). In genetically predisposed individuals, environmental triggers can result in loss of self-tolerance, and dysbiosis may be one important pathway by which the immune system erroneously favors proinflammatory pathways that instigate autoimmune states (69). Recently, novel research questions are exploring the role of microbiome-induced autoimmunity as a novel pathoetiological factor, primarily involving intestinal hyperpermeability, dysbiosis, toll-like receptor (TLR) ligands, and B cell dysfunction, as well as potential therapeutic implications (70). Altered microbial composition and this inflammatory-dysbiosis-autoimmune process has been identified in a number of autoimmune diseases, such as IBD, multiple sclerosis, lupus, rheumatoid arthritis, and type I diabetes (69, 71–74). This lends further credence to the common neuropsychiatric comorbidities in autoimmune and other inflammatory states (71). A predominance of Th1/Th17 lymphocytes, plasma cells, and antigen presenting cells (APCs) can instigate the process by presenting luminal microbiota-derived antigens and toxins to T and B lymphocytes, which then become inappropriately activated in autoimmune states (69). Probiotic and FMT studies have shown some promise but need further refinement to establish clear therapeutic recommendations. Nonetheless, treatment with probiotics, prebiotics, and other microbiome-altering therapies may facilitate eubiosis and symptom management in autoimmune diseases (74) by utilizing gut microbes to promote immunomodulatory responses that balance autoimmune-related inflammation (73).

4.3 Nutrition contributions to dysbiosis and inflammation

Certain dietary components have been identified as pro-inflammatory, contributing to an increase in inflammatory markers. These include saturated fats, trans fats, refined sugars, and excessive intake of omega-6 fatty acids, commonly found in ultra-processed and fast foods, over omega-3 fatty acids, especially the non-essential omega-6 fatty acids (68).

Saturated fats, commonly found in red meat and ultra-processed foods, have been associated with increased production of pro-inflammatory molecules. In contrast, monounsaturated fats found in olive oil, seeds, nuts, and legumes and omega-3 fatty acids present in fatty fish, seafood, seeds, nuts, and legumes exert anti-inflammatory effects (68). Refined carbohydrates, such as those found in sugary beverages and white bread, can contribute to inflammation while whole grains, rich in fiber and antioxidants, have been linked to lower inflammatory markers (44, 68). Anti-inflammatory foods, such as vegetables, fruits, whole grains, and omega-3 fatty acids, possess properties that help modulate the immune response and reduce inflammation (68). In fact, omega-3 fatty acids are necessary to turn off the inflammatory process via

specialized pro-resolving mediators (SPMs) (75, 76). Antioxidants found in vegetables and fruit play a crucial role in mitigating inflammation by neutralizing free radicals; these compounds include vitamins C and E, beta-carotene, and various polyphenols.

Research by David et al. demonstrated that a short-term shift to a low-fiber, high-fat diet could rapidly induce significant changes in the composition of the gut microbiome. This shift was marked by a decrease in beneficial bacteria, such as *Bifidobacteria*, and an increase in potentially harmful bacteria, including members of the phylum Bacillota, formerly Firmicutes (75). These alterations are indicative of dysbiosis and inflammation. Another study by Esposito et al. demonstrated that a Mediterranean diet, rich in fruits, vegetables, whole grains, and omega-3 fatty acids, significantly reduced inflammatory markers in individuals with MDD (76). Conversely, diets high in refined sugars, saturated fats, and ultra-processed foods have been associated with increased inflammation. Additional studies from other researchers studying inflammatory diseases offer support for the important role of nutrition in dysbiosis and inflammation. Attur et al. and de Oliveira et al. studied the role of intestinal dysbiosis and nutrition in the context of inflammatory diseases such as rheumatoid arthritis (RA) (77, 78). These studies demonstrated the intricate connections between the gut microbiome, dietary patterns, and systemic inflammation, emphasizing the potential link between diet-induced microbial changes and the pathogenesis of RA. Gill et al. demonstrated how various diets impact the gut microbiota and lead to inflammation, which plays a crucial role in GIand inflammatory diseases (79).

Potrykus et al. demonstrated microbial contributions to chronic inflammation and proposed potential modifications of the gut microbiome as therapeutic interventions (80, 81). Their work suggested that targeted microbiome specific strategies to modulate dysbiosis-related inflammatory responses can be explored as a potential treatment strategy for inflammatory diseases and other disorders linked to the inflammation. Taken together, the evidence suggests that nutrition can contribute to dysbiosis and inflammation, which can in turn impact the GBA and the development of mental health disorders. Ouabbou et al. describe the microbiome as "a potential missing link" when studying the impact of inflammation in mental disorders (80). This suggests that the intricate relationship between inflammation and the gut microbiome plays a crucial role in mental health conditions like depression and anxiety (82).

Vitamin D, classically known for its role in bone health, has been established as an immunoregulatory hormone and is now being linked to the gut microbiome. Seasonal Affective Disorder (SAD), a subtype of depression with a seasonal pattern, is characterized by recurrent depressive episodes during specific seasons, linked to reduced sunlight exposure. Seasonal changes in sunlight impact vitamin D synthesis, and vitamin D deficiency has been associated with increased inflammation (83). Vitamin D deficiency is associated with dysbiosis (84), suggesting a role for vitamin D in microbiome dysbiosis. Further, as an immunoregulatory hormone, vitamin D impacts the immune response in general and likely specifically to commensal microbiota. There has been limited research on this specific topic, but there is strong mechanistic plausibility.

5 Prevention strategies and therapeutic targets

A major advantage of microbiota-targeted therapy lies in the dynamic nature of the microbiome that can be readily altered by several interventional strategies, such as diet, exercise, and stress management (3). Dietary interventions have long been known to affect inflammation and continue to serve as a main lifestyle intervention for the prevention and treatment of various diseases. Nutritional psychiatry is one field of research that recognizes the impact of nutrition on the brain, mood, behavior, and mental health and respective methods to improve mood and treat mental illness (85).

The Western diet, for example, is associated with increased depression risk while the Mediterranean diet reduces the risk (12). Dietary origins of mood changes and inflammation have ignited novel approaches to the treatment of depression, for example, with polyunsaturated fats (PUFA) (86). Serotonin production and release in the gut, for example, is largely the product of dietary choices such as complex carbohydrates and tryptophan containing foods (87). The anti-inflammatory effects mediated by microbial metabolites of dietary fiber and polyphenols confer multidirectional benefits to brain and mental health and hold the potential to serve as nonpharmacological approaches to mental illness to improve treatment outcomes (87). Population studies and clinical trials support positive effects of diet in mood disorders such as depression and anxiety, even in severe presentations (85). While specific micro- and macronutrients are important considerations, consuming a wide variety of nutritious foods has been demonstrated to provide the most beneficial effects on physical and mental health, rather than a focus on single nutrients that are not reflective of real-life eating habits (87).

The Mediterranean diet is one example of a dietary lifestyle that has demonstrated efficacy in decreasing the risk of depression in numerous studies, including randomized controlled trials (88–90), including in older adults (91). One of the more recent randomized controlled trials (HELFIMED) examined the Mediterranean diet supplemented with fish oil in 152 adults suffering from depression and found the intervention group had greater reduction in depression and improved mental health (88). They also found increased vegetable diversity, nuts, and legumes, along with increased omega-3 fatty acids, decreased omega-6 fatty acids were all correlated with improved mental health (88).

The Mediterranean-DASH diet Intervention for Neurodegenerative Delay (MIND), which emphasizes green leafy vegetables, berries, and low intake of red meat, is commonly recommended to prevent and slow cognitive decline, has also been inversely associated with odds of depression and psychological distress (92). A recent prospective cohort study in older adults found that high adherence to the MIND diet was associated with lower rates of depression over time (93).

Considering the influence of the microbiome on the brain, mood, and behavior, as well as altered eating habits and weight gain that often occur with stress-related mental disorders (e.g. MDD, PTSD), diet and nutrition are therapeutic strategies worth consideration, especially as adjunct therapy (86).

Taking into account the considerable role of the chronic stress response in dysbiosis and mental illness, stress management techniques are a vital component of whole-person well-being. Herein lies another way in which the microbiota-gut-immune-brain axis may be regulated, although this research area is still in its nascency. Cognitive behavioral therapy (CBT) is a well-established intervention for various psychological disorders and is a first-line treatment for anxiety-related disorders (e.g., generalized anxiety disorder, social anxiety disorder, phobias, OCD, PTSD) (94). An interesting recent study by Jacobs et al. examined the effect of CBT in 84 IBS patients on IBS symptoms and the microbiome (93). Prior to CBT, participants had increased fecal serotonin, increased the order Clostridiales and decreased *Bacterioides*, whereas post CBT participants demonstrated improved functional connectivity-related brain changes that correlated with *Bacteriodes* expansion (95). Mindfulness and meditation are mind-body techniques that are utilized for stress management and a broad range of disorders, and therefore also merit mention in this regard. Mindfulness is posited to facilitate a healthy gut microbiome and gut-barrier function by its ability to reduce inflammation and modulate the stress response (96). More specific microbiome-mind-body intervention studies are beginning to emerge. A recent study by Wang et al. examined the efficacy of mindfulness-based cognitive therapy (MBCT) in high trait anxiety individuals and its impact on gut microbiota in 21 young adults with high trait anxiety compared to 29 healthy controls (97). In the high trait anxiety group they found markedly decreased bacterial diversity with distinct clusters (significant overgrowth of *Streptococcus*, *Blautia*, and *Romboutsia;* decreased *Faecalibacterium, Coprococcus*,and *Lachnoclostridium*) compared to healthy controls. They also found that the MBCT intervention decreased anxiety and depression, improved mindfulness and resilience, and shifted microbial populations to more similarly diverse profiles as the healthy controls (97). The experience of stress is also affected by one's daily environmental conditions and lifestyle habits, such as physical activity, screen time, and time outdoors. The literature suggests that intentional exposure to outdoor environments, in the form of outdoor walks and exercise, gardening, and nature viewing may reduce the experience of stress and improve well-being (98). This is a critical consideration for those who have restricted access to outdoor environments, such as those living in facilities for mental illness or cognitive decline.

Exercise confers innumerable health benefits, the mechanisms by which far exceed the scope of this paper. The physiological processes of stress and inflammation reduction are firmly established and continue to unravel additional connections, such as microbiota involvement. Research is beginning to support a mutual benefit of antioxidant overexpression and exercise on the microbiome (99). Exercise also serves to modulate several metabolic processes and neurotransmitters related to metabolic, psychological, and gut health (100). Exercise therefore occupies an important role in mitigating the stress response and health of the microbiota-gut-immune-brain axis.

6 Conclusion

This brief narrative review highlights several compelling research areas that support the complex matrix of the chronic stress response, immune dysregulation, mental illness, and the microbiota-gut-immune-brain axis. Many of these topics merit their own in-depth review, but the mechanistic insight of a systems biology approach to mental and autoimmune disorders may inform clinically relevant approaches to prevention and management strategies. Chronic stress is a key constituent in a multitude of negative physiological and psychological consequences, and although managing the stress response is far from a novel idea, the ways in which we intervene to treat mental health disorders and avoid physical consequences are sorely needed. Incorporating the role of the microbiome into this dynamic interplay is one avenue with which the clinical landscape can shift from reactive to proactive.

Author contributions

AW: Conceptualization, Project administration, Writing – original draft, Writing – review & editing, Supervision. YN: Conceptualization, Project administration, Writing – original draft, Writing – review & editing. AB: Writing – original draft, Writing – review & editing. LAF: Conceptualization, Project administration, Writing – original draft, Writing – review & editing.

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The microbiota-gut-brain-immune interface in the pathogenesis of neuroinflammatory diseases: a narrative review of the emerging literature

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Importance: Research is beginning to elucidate the sophisticated mechanisms underlying the microbiota-gut-brain-immune interface, moving from primarily animal models to human studies. Findings support the dynamic relationships between the gut microbiota as an ecosystem (microbiome) within an ecosystem (host) and its intersection with the host immune and nervous systems. Adding this to the effects on epigenetic regulation of gene expression further complicates and strengthens the response. At the heart is inflammation, which manifests in a variety of pathologies including neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Multiple Sclerosis (MS).

Observations: Generally, the research to date is limited and has focused on bacteria, likely due to the simplicity and cost-effectiveness of 16s rRNA sequencing, despite its lower resolution and inability to determine functional ability/alterations. However, this omits all other microbiota including fungi, viruses, and phages, which are emerging as key members of the human microbiome. Much of the research has been done in pre-clinical models and/or in small human studies in more developed parts of the world. The relationships observed are promising but cannot be considered reliable or generalizable at this time. Specifically, causal relationships cannot be determined currently. More research has been done in Alzheimer's disease, followed by Parkinson's disease, and then little in MS. The data for MS is encouraging despite this.

Conclusions and relevance: While the research is still nascent, the microbiota-gut-brain-immune interface may be a missing link, which has hampered our progress on understanding, let alone preventing, managing, or putting into remission neurodegenerative diseases. Relationships must first be established in humans, as animal models have been shown to poorly translate to complex

human physiology and environments, especially when investigating the human gut microbiome and its relationships where animal models are often overly simplistic. Only then can robust research be conducted in humans and using mechanistic model systems.

KEYWORDS

human gastrointestinal microbiome, gut-brain axis, neuroimmunomodulation, enteric nervous system, neurogenic inflammation, neurodegenerative diseases, neuropathology, neuroinflammatory disease

1 Introduction

While bacteria are the most commonly studied member, the gut microbiome consists of trillions of microbes including fungi, archaea, viruses, phages, and bacteria, which develop early in life and are influenced by genetic and environmental factors, including those that affect brain health (1). We cannot define what a 'healthy' gut microbiome is at present; however, low diversity is a common marker of an 'unhealthy' gut microbiome, which is often termed 'dysbiosis.' Dysbiosis is associated with many disease states, especially those becoming increasingly common in Western societies, likely due to limited exposure to diverse microbiota and inflammatory environmental exposures such as diet (2–4). This includes neuroinflammatory and neurodegenerative diseases, as dysbiosis contributes to gut and brain hyperpermeability, commonly termed 'leaky gut' and 'leaky brain,' by way of reduced tight junction proteins such as occludins (5). Microbial metabolites produced during dysbiosis are able to induce barrier dysfunction in preclinical models, leading to the passage of abnormal substances across barriers (6). The barrier function of the gut and the brain are an important part of innate immunity, without which the immune system cannot function properly, resulting in chronic inflammation locally and, perhaps eventually, systemically. This is a hallmark of dysregulation of the microbiota-gut-brain-immune interface. Moreover, dysbiosis can occur in any tissue containing a microbiome, including the oral and nasal cavities, lungs, skin, bladder, and vagina (7). While less studied than the gut microbiome, new evidence suggests the resident microbiota in these tissues can also contribute to immunoregulation and therefore a broad spectrum of disease states (7–9). Clues to the importance of microbiomes outside of the gut suggest some involvement in neuropsychiatric and neurodegenerative diseases, such as the presence of oral bacteria in the postmortem brains of persons with Alzheimer's disease (7). Notably, the extent to which these localized microbiota contribute to disease is in an early stage of exploration; this paper will therefore focus on the more widely-studied gut microbiome. Accordingly, this narrative review will assess the state of the science in the emerging literature behind the microbiota-gut-brain-immune interface and the pathogenesis of neuroinflammatory diseases.

2 The microbiota-gut-brain-immune interface and neuroinflammation

Multidisciplinary research is emerging around the microbiota-gut-brain-immune interface, moving from animal models to human studies. This research is finding that the gut microbiota mediate the relationship between the enteric nervous system (ENS), autonomic nervous system (ANS), central nervous system (CNS) largely through regulation of the immune response and inflammation. The idea that brain function is tied to the gut microbiome and involves epigenetic and immunoregulatory changes is becoming common place in the clinic as well as in research. Further, nervous system epigenetic changes mediated by the gut microbiota show great promise to elucidate the pathogenesis of and novel therapeutics for neurological disorders (10). What is more, intimate and sophisticated relationships between diet, the gut microbiome, and cognition are emerging. Indeed, transdisciplinary perspectives intersecting neuroscience, psychology, and philosophy are further exploring the role of the gut microbiome in perception and cognition, and posit the idea that the microbiome possesses its own proto-cognition independent of, yet interrelated to the rest of the body (11). Interestingly, the hormones ghrelin and leptin (involved in hunger and satiety, respectively) have also been tied to cognition (12). Our microbiome, therefore, may not only affect the quality of our cognition but also how we perceive our internal and external worlds.

While external factors are important contributors to well-being and neuroinflammatory disease by way of epigenetic changes, internal factors (e.g. psyche, lifestyle, age, chronic inflammation, microbiomes) are at least equally important and interact with each other to potentiate a signal, perhaps synergistically. Changes to the epigenetics of the nervous system are typically acquired since neurons do not divide (13); the microbiota and their metabolites influence neurons (14–16). Microbiota-gut-brain-immune interface dysregulation has been associated with neuropathologies commonly linked with inflammation including mild cognitive impairment (MCI), Alzheimer's disease, Parkinson's disease, and multiple sclerosis and gastrointestinal (GI) symptoms are common in these disorders or even predate their onset (5, 17–23). For

instance, GI symptoms predate the onset of Parkinson's disease; this and a growing body of research support the theory that Parkinson's starts in the gut and dysregulates the microbiota-gut-brain-immune interface, resulting in CNS and movement-related symptoms (21). GI symptoms may include nausea, constipation, dysphagia, abnormal salivation and defecatory dysfunction. Further, there is likely a bidirectional relationship, e.g. neuropsychiatric comorbidities are prevalent in inflammatory bowel disease (24). Thus, the microbiota-gut-brain-immune interface is a vital mediator of neuroinflammation likely to affect many facets of brain health including neurodevelopment, cognition, and behavior (20, 23). The most vulnerable aspects of the microbiota-gut-brain-immune interface to these effects involve multi-way physiological communication along the microbiota-gut-brain axis. Direct communication in the microbiota-gut-brain axis occurs predominantly via the vagus nerve while indirect signaling is diverse and complex including the ENS, ANS, CNS, immune system (e.g. glial activation), neuroendocrine system, tryptophan metabolism, and microbial metabolites (e.g. short-chain fatty acids, SCFAs) (18). Additionally, the gut microbiota are crucial to nutrient harvesting and produce some nutrients themselves that are co-factors for epigenetic pathways (25).

The spleen serves a crucial role in facilitating communication within the microbiota-gut-brain-immune interface by acting as a reservoir for various immune factors. Although the precise involvement of the spleen in the gut-brain axis is not completely understood, research indicates a correlation between antibiotic treatment and splenic function. Studies on mice subjected to antibiotic treatment have demonstrated a significant decrease in spleen weight, NK cells, macrophages, and neutrophils compared to control groups. Conversely, there is an observable increase in the percentage of CD8+ T cells within the spleen (26). Moreover, a proposed gut-spleen axis has been identified in patients with asplenia and common variable immune deficiency, wherein the reduction of IgM memory B cells induced by splenectomy may affect secretory IgA production in the gut. Numerous diseases, ranging from traumatic brain injury to conditions like Crohn's disease, inflammatory bowel disease, septic shock, Alzheimer's disease, Parkinson's disease, schizophrenia, and depression, have been linked to the gut–brain–spleen axis. It is suggested that the vagus nerve reflex and systemic circulation serve as potential regulatory routes for these diseases (27).

In the following sections, we discuss the major elements of the microbiome-gut-brain-immune interface. An overview of this relationship can be seen in Figure 1.

2.1 Microbiome – gut – brain communication

2.1.1 The vagus nerve

Also known as the tenth cranial nerve or 'the great wanderer,' the vagus nerve spans the vast majority of the human body and is the key nerve for interoception (internal sensing), and communication between the body and brain to maintain homeostasis or react accordingly (28–30). This communication is bidirectional and involves neuronal, neuroimmune, and neuroendocrine signaling and reaches beyond the parenchyma to include muscle, mucosa, ENS neurons, and the gut microbiome (the gut microbiota and their metabolites, typically measured with metagenomics and metabolomics) (5, 18, 19, 30–32).

2.1.2 Additional vagal connections

The vagus nerve innervates the gut mucosa including the gut associated lymphoid tissue (GALT). Included in the GALT, the lamina propria are part of the immune system and play a crucial role in immune education, which is why numerous immune cells reside here (20). The proximity of the lamina propria to the vagus nerve, (nascent) immune cells, and the gut microbiome make it central to the microbiota-gut-brain-immune interface. Here, the gut

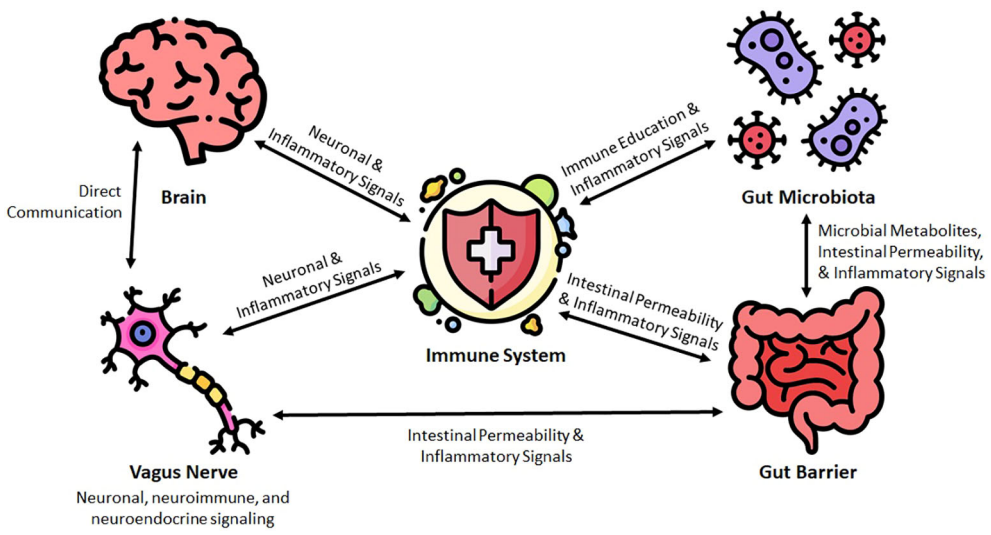

FIGURE 1
Conceptual framework for the microbiota-gut-brain-immune interface in the pathogenesis of neuroinflammatory diseases.

microbiota can influence vagal signaling and/or the immune system and therefore affect brain health (30). For instance, bacterial taxa (i.e. *Campylobacter jejuni, Lacticaseibacillus rhamnosus JB-1* a.k.a. *Lactobacillus rhamnosus/reuteri JB-1, Limosilactobacillus reuteri* a.k.a. *Lactobacillus reuteri*) have been shown to affect brain, cognition, and behavior via vagal signaling, resulting in positive and negative outcomes (28, 33). Another demonstration of the interplay between the vagus nerve, the brain and the gut microbiota is found in preclinical data obtained from rodent models. Performing subdiaphragmatic vagotomy on rodents treated with cuprizone blocked demylination in the brain and restored the gut microbiota dysbiosis induced by cuprizone (34). This supports the concept that the vagus nerve plays a critical role in the microbiota-gut-brain axis.

Further, the vagus nerve regulates peripheral inflammation and intestinal permeability via the ENS cholinergic anti-inflammatory pathways, and, therefore, plays a key role in the prevention or pathogenesis of so-called 'leaky gut' (31). In times of stress or disease, vagal signaling is inhibited (low vagal tone), which hampers the microbiota-gut-brain-immune interface and can results in negative outcomes in the microbiome, gut, brain, and immune system (31). Extra-vagal signaling in the microbiota-gut-brain-immune interface involves microbial metabolites like SCFAs, secondary bile acids, and tryptophan metabolites including the neurotransmitter serotonin a.k.a. 5-hydroxytryptamine (5-HT) and the gut hormones cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), and peptide YY (PYY), which are propagated via enteroendocrine cells (EECs) (31, 35). However, stress and disease also affect the gut microbiota and their metabolites; therefore, altering extra-vagal signaling as well.

2.1.3 Secondary bile acids

Bile acids are synthesized from cholesterol. Primary bile acids like chenodeoxycholic acid (CDCA) and cholic acid (CA) are produced first (36). After production in the liver, primary bile acids are transported by way of the small intestine to the colon where they are metabolized by the gut microbiota into the secondary bile acids such as lithocholic acid (LCA) and deoxycholic acid (DCA) (36). These secondary bile acids are then transported across the gut barrier where they may travel to the liver or circulate systemically, likely also crossing the blood-brain barrier (36). While the gut microbiota determines the production of secondary bile acids, secondary bile acids also seem to alter the composition of the gut microbiome, indicating a bidirectional relationship (35, 37). In the brain, no less than 20 bile acids have been found, where they likely alter neurological function and promote disease (36, 37). In a healthy, normal state, bile acids appear to be neuroprotective in the brain; however, in a dysbiotic and/or diseased state, this metabolism and regulation is perturbed, degrading or eliminating the neuroprotective effect (36, 37). Bile acids interact with receptors like the farnesoid X receptor (FXR) and G-protein-coupled bile acid receptor 1 (GPBAR1), which are present in many different immune cells, thereby influencing neuroinflammation (38). Secondary bile acids can also exert an indirect influence on neurological function; for example, LCA and DCA can modulate serotonin production by interacting with the enterochromaffin cells (ECCs) of the gut, thus impacting gut-brain axis signaling (39). The dysregulation of secondary bile acids has been correlated with neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis, and Multiple Sclerosis (40, 41) Secondary bile acids are one of many elements of the microbiota-gut-brain-immune interface that appears to play a major role in the pathogenesis of neurodegenerative disease.

2.1.4 Short-chain fatty acids

Many of the bacterial members of the gut microbiome rely on microbiota-accessible carbohydrates (MACs)—fiber and resistant starch—for fuel, producing metabolites in the process. Of these metabolites the most well-known and, perhaps, beneficial class is the SCFAs, which are an energy source for colonocytes and, therefore, support gut barrier function, microbiome balance, and reduce neuroinflammation, likely to within 'optimal' ranges (23, 42). Further, SCFAs promote tolerance and homeostasis via regulatory T cells (Tregs) among many other effects on the immune system and are, thus, considered anti-inflammatory (43–45). Communication between the gut microbiota-gut-brain axis is also mediated by SCFAs via receptors on ECCs, a type of EEC that 1) is involved in serotonin production and 2) directly interacts with the vagus nerve (20, 35).

As with most elements of biochemistry and nutrition, there is an optimal range of SCFAs, both above and below which negative health outcomes are seen. In dysbiotic and/or diseased states, SCFAs are produced in too much (e.g. irritable bowel syndrome) or too little (e.g. low MACs diet) quantities. Some of the SCFAs are transported across the gut barrier and into circulation, where they appear to cross the blood-brain barrier, affecting CNS function (46). SCFAs such as butyrate have been shown to promote gene expression via epigenetic regulation (i.e. enhanced chromatin accessibility), which may aid in memory consolidation (46). However, not all SCFAs are created equal. For example, butyrate is particularly neuroprotective via Treg induction while acetate may exacerbate neurodegeneration (23).

2.1.5 Neurotransmitters

Transmitting electrochemical signals between neurons and to effector sites, neurotransmitters can act as hormones, promoting function and health in peripheral tissues including the brain (47, 48). Many neurotransmitters are also produced by gut microbiota including γ-aminobutyric acid (GABA), norepinephrine, epinephrine, dopamine, and acetylcholine (20, 49, 50). While these microbial neurotransmitters clearly play a role in the gut (see Tables 1, 2), in proximal regions, and likely in circulation, it is unclear if all or some of these interact with the CNS in sufficient concentrations to have a meaningful effect; however, they can exert an effect via the ENS including the vagus nerve (51, 52). Further, SCFAs may add to this effect on vagal signaling, as SCFAs play a key role in neurotransmitter metabolism; for example, SCFAs modulate the production of tryptophan by ECCs. Tryptophan is a required pre-cursor to serotonin (20).

Serotonin has been extensively studied for its role in gastrointestinal and brain health and in gut-brain communication (the gut-brain axis) (51). The majority of serotonin (~90%) is stored in ECCs in the gut, produced from tryptophan (47, 53, 54). Tryptophan to serotonin metabolism involves the kynurenine pathway and, therefore, leads to production of quinolic acid (neurotoxic) and kynurenic acid (neuroprotective)—the balance of which is regulated by the gut microbiota and may contribute to neuroinflammation and ultimately neurodegeneration (55). While neurotransmitter-producing gut microbiota are still in the early stages of elucidation, there are a few bacterial taxa of note (see Tables 1, 2).

Throughout the body, the microbiota-gut-brain axis and subsequently the microbiota-gut-brain-immune interface has a profound impact via direct and indirect pathways and is influenced by host genetics, lifestyle, environmental exposures, etc. As many of these are modifiable risk factors, this is an important line of research to support mental and physical health (well-being) and may prove to be crucial for prevention, management, and treatment of neuroinflammatory and neurodegenerative disorders, for which we currently have very few tools at our disposal.

TABLE 1 Bacterial effects on neurotransmitters, by neurotransmitter.

Neurotransmitter	Observation	Bacterial Taxa*
Acetylcholine	Support/Produce	*Lactobacillus*
Acetylcholine	Support/Produce	*Bifidobacterium*
Acetylcholine	Support/Produce	*Enterococcus*
Acetylcholine	Support/Produce	*Streptococcus*
GABA	Support/Produce	*Lactobacillus*
GABA	Support/Produce	*Bifidobacterium*
GABA	Support/Produce	*Enterococcus*
GABA	Support/Produce	*Streptococcus*
GABA/glutamate	Metabolize intermediate	*Lactobacillus* spp.
GABA/glutamate	Metabolize intermediate	*Bifidobacterium* spp.
Serotonin	Produce	*Lactobacillus*
Serotonin	Produce	*Bifidobacterium*
Serotonin	Produce	*Streptococcus*
Serotonin	Downregulate	*Enterococcus*

*This is likely not the case for all species/strains but has been observed within this domain.

TABLE 2 Bacterial effects on neurotransmitters, by taxa.

Bacterial Taxa*	Observation	Neurotransmitter
Bifidobacterium	Support/Produce	Acetylcholine
Bifidobacterium	Support/Produce	GABA
Bifidobacterium	Produce	Serotonin
Bifidobacterium spp.	Metabolize intermediate	GABA/glutamate
Enterococcus	Support/Produce	Acetylcholine
Enterococcus	Support/Produce	GABA
Enterococcus	Downregulate	Serotonin
Lactobacillus	Support/Produce	Acetylcholine
Lactobacillus	Support/Produce	GABA
Lactobacillus	Produce	Serotonin
Lactobacillus spp.	Metabolize intermediate	GABA/glutamate
Streptococcus	Support/Produce	Acetylcholine
Streptococcus	Support/Produce	GABA
Streptococcus	Produce	Serotonin

*This is likely not the case for all species/strains but has been observed within this domain.

3 Neuroinflammation

Physical and/or psychological stress can also cause inflammation, including chronic neuroinflammation, through dysregulation of the hypothalamic-pituitary-adrenal axis (HPA axis). Inflammation can originate anywhere in the body, typically via inflammatory cascades that include cytokines, chemokines, reactive oxygen species (ROS), and trafficking of immune cells (e.g. T and B cells). Neuroinflammation also involves specialized members of the immune system called glia (microglia and astrocytes) resident in the CNS. A robust, acute immune response is a necessary response to injury or invasion; it is equally important for this inflammation to resolve in a timely manner, avoiding chronic inflammation. This is true of neuroinflammation as well. While acute neuroinflammation is protective, chronic neuroinflammation increases risk of neurodegenerative disorders, the most common of which are Alzheimer's disease, Parkinson's disease, prion disease, amyotrophic lateral sclerosis, motor neuron disease, Huntington's disease, spinal muscular atrophy, and spinocerebellar ataxia (56). Further, local or systemic inflammation can sensitize the immune system, leading to exacerbation of inflammation, including neuroinflammation.

Gut microbiota influence brain function by way of maintenance of homeostasis in innate and adaptive immunity, limiting acute and chronic inflammation in the gut and CNS and, therefore, risk of neurodegenerative disorders even independent of other pathogenesis features like amyloid plaques (23, 55, 57). Neuroinflammation is a key element in the pathogenesis, prevention, and treatment of neurodegenerative disorders; once at a critical point, the epigenetic profile changes dramatically (13). Epigenetic changes due to neuroinflammation may include substantial changes in DNA methylation, histone methylation and acetylation, and non-coding RNA expression (13). Further, the relationship between neuroinflammation and epigenetic changes has long suspected to be bidirectional, as neuroinflammation is also strongly influenced by epigenetic mechanisms. For instance, DNA methylation may be a regulator of activated microglia that drive AD pathology, and presence of neuroinflammatory conditions (e.g. psychiatric disorders) demonstrate altered patterns of DNA methylation (58). The origin of neuroinflammation is often outside of the CNS, commonly certain bacterial taxa in the gut microbiome. For example, *Heliobacter pylori*, which is present in about half of human gut microbiomes, leads to DNA

methyltransferase inhibition, destabilizing the genome in a manner typical of certain disease states (46).

It is important to note the possibility that dysbiosis resulting in altered protein express may also contribute to neuropathology, independent of the inflammatory response. In a mouse model, fecal microbiota transplant (FMT) from aged donors to young adult mice resulted in impaired spatial memory in conjunction with altered protein expression associated with hippocampal synaptic plasticity and neurotransmission with concomitant reduction in SCFA-producing bacteria; yet, gut permeability and cytokines were not affected (59). However, the authors note that cytokines were assessed at the end of the intervention and were unaware if cytokines fluctuated in the early-stage post-FMT.

The role of vascular endothelial growth factor (VEGF) may also serve an underrecognized role in the multifactorial pathophysiology related to alteration of the gut microbiome, protein expression, and inflammation. Of note, in AD mouse models in which alterations in RNA/protein expression and microglia occur with elevated amyloid-beta-peptides in the ENS of the small intestine, some evidence suggests that VEGF mediates neuroprotective and neurodegenerative effects in both the CNS and PNS (60).

3.1 Glia: astrocytes and microglia

These immune cells reside in the nervous system; in the CNS glia are involved in the production, potentiation, and resolution of neuroinflammation. Also known as glial cells or neuroglia, glia support neuronal functions, e.g. synapse formation, neuronal plasticity, neurotransmission, injury response, and protection from neurodegenerative disorders. Glia release cytokines and chemokines that are potential mediators of neurotoxicity (Table 3). The most plentiful glia are astrocytes, which are "master regulators of synapse formation, ion homeostasis, and neurovascular coupling" (13).

In response to changes in their environment, in addition to producing cytokines and chemokines, microglia express antigenic markers, regulate neurotransmitters, and undergo morphological changes (61). Once activated, microglia can cause neuronal damage by producing reactive oxygen species and nitric oxide—both neurotoxic—and cross-reacting with astrocytes to magnify the effect, resulting in loss of neurotrophic functions. Microglia have been proposed to promote neuroinflammation and neurotoxicity; however, recent research suggests that their impact can be context-dependent, contingent upon their polarization phenotype, activation status, and cellular context (62). These effects are modulated through neuron-microglia communication facilitated by various neurotransmitter receptors expressed on microglia. Notably, receptors for neurotransmitters such as glutamate, GABA, norepinephrine, cannabinoid, and acetylcholine play significant roles in mediating these interactions. Microglia may modulate neurotransmitter release, thus, coordinating either positive or negative feedback loops tailored to the needs of the organism. Moreover, these interactions may extend to indirect effects on neighboring microglia, further expressing the role of neuron-microglia communication.

TABLE 3 Immune response via glia.

	Relevant Roles
Cytokines (secreted by immune cells)	
Interleukins (IL)	
•IL-1β	Crucial for host defense against pathogens but can also worsen damage in chronic disease and acute tissue injury. It aids in combating microbes and facilitating tissue repair mechanisms.
•IL-6	Regulates innate immunity and initiates inflammation. Known to contribute to pain, hypersensitivity, neuropathy, and cancer by interacting with immune cells, glia cells, and neurons along the pain pathway.
•IL-8	A chemoattractant cytokine. Produced by various tissue and blood cells. Uniquely targets neutrophils (minimal effects on other blood cells) specifically in inflamed areas.
•IL-33	Produced by synapse-associated astrocytes; essential for normal synapse numbers and circuit function in the thalamus and spinal cord. Primarily signals via microglia to enhance synaptic engulfment under normal conditions. In mice, hippocampal IL-33 triggers inflammation, resulting in cognitive impairment.
Tumor necrosis factor-alpha (TNF-α)	3 interconnected vicious cycles: 1) Microglia release TNF-α, which stimulates release of TNF-α and glutamate, activating microglial receptors, leading to more TNF-α release. 2) TNF-α prompts astrocytes to release glutamate, which accumulates due to inefficient uptake, raising extracellular glutamate. 3) TNF-α disrupts the balance of synaptic activity, causing excessive calcium entry and neuronal death; dying neurons sustain microglial activity, further increasing TNF-α release.
Chemokines (cytokines that attract immune cells)	
CCL2 a.k.a. monocyte chemoattractant protein-1 (MCP-1)	Pro-inflammatory mediators that attracts or enhances the expression of other inflammatory factors/cells. Up-regulated in many central nervous system (CNS) disorders with blood brain barrier (BBB) breakdown.
CCL5 a.k.a. Regulated upon Activation, Normal T cell Expressed and presumably Secreted (RANTES)	

3.2 Chronic neuroinflammation

The relationship between the microbiota-gut-brain-immune interface, microbial metabolites, and glia is a potent regulator of GI and actionable target in neuroinflammatory and neurodegenerative disorders (12). In chronic neuroinflammation, pro-inflammatory cytokines are habitually upregulated and glia are overactive, resulting in damage to neurons, synapse function, cortical tissue, and functional connectivity—commonly observed in neurological disorders (57, 63). The multidirectional relationship of the microbiota-gut-brain-immune interface governs this chronic neuroinflammation, which may start at any point, including moving from the gut into the CNS, which often results in systemic inflammation. Systemic inflammation is a core feature of

chronic neuroinflammation. Neuroinflammatory diseases arecharacterized by elevated C-reactive protein (CRP), TNF-α, and IL-1β (23). The overactivation of glia that drives pathogenesis is affected by the gut microbiome and implicated in neuroinflammation and subsequent neurological disorders (e.g. Alzheimer's and Parkinson's), as well as gut microbiome composition (dysbiosis) and intestinal inflammation and permeability ('leaky gut') (18, 23, 64). Neurodegenerative disorders are likely multifactorial in cause, and a key element of this is the gut microbiota due to their production of neuroactive metabolites (5). In specific, gut microbiota that produce the endotoxin lipopolysaccharides (LPS) may contribute to amyloid deposition and neuroinflammation in Alzheimer's (65). LPS interacts with the microbiota-gut-brain-immune interface via Toll-like receptor (TLR) 4 and the NF-κB pathway, stimulating an inflammatory cascade, triggering leaky gut, and leading to neuroinflammation (18, 23, 64–66).

Interleukins (ILs) are a category of cytokines: some of which have pro-inflammatory/immune reaction stimulating effects while others have anti-inflammatory/homestasis stimulating effects. Therefore, the change in the concentrations of these cytokines (the cytokine milleau) can have great effects on neuroinflammation. ILs are a promising target for treating neuroinflammation and subsequent neurodegeneration (67). Many of the Preventative and Therapeutic Interventions discussed below alter the cytokine milleau, meaning this is at least in part their method of action.

TLRs are pattern recognition receptors (PRRs) that recognize molecules and patterns of molecular structure, pathogen-associated molecular patterns (PAMPs), from bacteria that are extracellular or have been engulfed into vesicular pathways via phagocytosis. They signal through cytokines such as ILs. TLRs could be used as targets to quench neuroinflammation, according to research from preclinical to clinical trials (68). Futher, small phytocompounds such as curcumin have been shown to target TLRs (68). The dose and other details for such an intervention have yet to be elucidated and show the possiblity of a hormetic response, meaning higher doses are actually detrimental.

No matter the cause(s), dysbiosis fosters a damaging, inflammatory environment via the microbiota-gut-brain-immune interface via LPS and other stimulators of the inflammatory cascade, i.e. pro-inflammatory cytokines and chemokines, T-helper cells, and monocytes (5). Further, dysbiosis contributes to aberrant HPA axis activation that can result in cortisol dysregulation, which exacerbates leaky gut (5). Therefore, dysregulation of the microbiota-gut-brain-immune interface can create a circular inflammatory feedback loop between dysbiosis, leaky gut, and chronic systemic and CNS inflammation.

4 Microbial alterations in neuropathology

The emerging patterns of gut microbiome changes specific to neurological disorders may aid in the development of treatment options for these recalcitrant disorders (described in subsequent sections). This data is largely correlational and focused solely on the composition of the gut microbiome, meaning it is not yet ready for the clinic. Further, some of this compositional work has been done at fairly high order such as phyla/phylum, which is extremely non-specific. While other work is done at the genus level, this is still fairly non-specific with a great deal of diversity of function within a single genus. Keeping this in mind, one must take the research on the composition of the microbiome, especially that at the phyla or genus level, with more than a grain of salt. Functional data on the gut microbiome are beginning to emerge and will grow substantially in the years to come as the cost of advanced technologies such as shotgun metagenomics continue to decline and are adopted more widely. This coupled with more robust study designs including longitudinal studies may lead to groundbreaking therapies and even means of prevention.

Currently, it appears that there are common mechanisms among neurodegenerative disorders like Alzheimer's disease, Parkinson's disease, multiple sclerosis, and stroke/traumatic brain injury. These include a dysbiotic gut microbiome, insufficient SCFA production, elevated LPS, and leaky gut that stimulate pro-inflammatory immune, neuroendocrine, and neuroinflammatory pathways. It is likely that the differences between these disorders is due to variation in the microbial alterations of the affected gut microbiomes. This may or may not also be related to baseline microbiome composition and function prior to the onset of neuroinflammation, which are largely determined by environmental exposures including diet and lifestyle in addition to seeding of the gut microbiome during crucial developmental phases as well as host genetics (e.g. propensity for chronic inflammation) (25, 69). These relationships are summarized in Supplementary Table S1.

4.1 Alzheimer's disease

The hallmarks of Alzheimer's disease are amyloid-beta and hyperphosphorylated tau protein accumulation with neuronal degeneration, which is thought to develop decades prior to symptoms. The microbiota-gut-brain-immune interface has been implicated in its pathogenesis with human studies finding dysbiosis in the gut microbiomes of those with Alzheimer's (69–71). For example, the gut microbiomes of those with Alzheimer's compared to healthy controls has decreased Bacillota (formerly Firmicutes) and *Bifidobacterium* and increased Bacteroidota (formerly Bacteroidetes), and Escherichia and Shigella (two inflammatory genus) as well as lower abundance of the species *Eubacterium rectale* (*E. rectale* is believed to be anti-inflammatory), all of which correlates with increased pro-inflammatory cytokines in Alzheimer's (72, 73). However, these compositional changes have not been confirmed in cohorts in other countries, such as China, indicating a role for the environment and/or the need for higher resolution data, likely at the species or strain level (74, 75). There is a need for further research, especially that better control for potential confounders, to be able to use this as a screening tool for research or clinically.

4.2 Parkinson's disease

In Parkinson's disease, misfolded α-synuclein accumulates in the neuronal cell body with motor impairments at least in part due to progressive dopaminergic neuron damage, resulting in decreased dopamine (5, 76). Of note, digestive symptoms are common in Parkinson's and typically develop prior to hallmarks of the disease. This points to a key role of the microbiota-gut-brain-immune interface and lends some support for a causative role via temporality *à la* the Bradford Hill criteria (5, 21, 77). While it is not yet clear that α-synuclein causes neuronal loss (it may be an intermediate step or a symptom), the vagus nerve may transmit pathology to the brainstem, resulting in deposition of α-synuclein (78, 79). In fact, correlations have been established between α-synuclein and composition of the gut microbiome, which have been supported by a recent meta-analysis (76). Specifically, compared to healthy controls, the gut microbiomes of those with Parkinson's have: depletion of *Prevotellaceace* that are involved in SCFA production, which leads to leaky gut and endotoxin exposure; depletion of important SCFA producers belonging to the Lachnospiraceae family and of which key players include *Blautia*, *Roseburia*, and *L-Ruminococcus*; increased *Enterobacteriaceae*, which can raise LPS and eventually lead to neuroinflammation; and enrichment of *Lactobacillus*, *Akkermansia*, and *Bifidobacterium* (5, 76). However, it is unclear if these changes are the cause or consequence of disease at present.

4.3 Multiple sclerosis

Gut dysbiosis appears to promote the pathogenesis of Multiple Sclerosis (MS) via the microbiota-gut-brain-immune interface: leaky gut, leading to immune activation, leading to systemic inflammation, leading to disruption of the blood brain barrier, leading to neuroinflammation, leading to neurodegeneration (52, 80). The impetus for this cascade and the leaky gut that drives it is currently unknow but dysbiosis has been found in MS patients and is mechanistically plausible (52, 80–83). While it is possible that this may actually be a consequence of the disease process rather than its cause, the research to date does not clearly support this concept. Further, the types of MS seem to have their own distinct versions of dysbiosis (52, 80–83). When compared to healthy controls, the gut microbiomes of those with MS have fewer SCFA-producing bacteria, *Butyricimonas*, *Faecalibacterium*, *Clostridium* cluster IV and XIVa, *Faecalibacterium prausnitzii*, and Blautia species and more *Akkermansia muciniphila*, *Ruthenibacterium lactatiformans*, *Hungatella hathewayi*, *Eisenbergiella tayi*, and *Clostridium perfringens* (82–84).

4.4 Overlap in neurodegenerative disorders

In a 2022 systematic review involving 52 studies and 5,496 participants with Alzheimer's disease, Parkinson's disease, MS, amyotrophic lateral sclerosis, and stroke, the strongest overlap was seen between Parkinson's disease and MS with 8 shared genera (85). Interestingly, Parkinson's also shared 6 genera with stroke. While there was overlap between Alzheimer's and Parkinson's the sample size is small, making this an unreliable association at present. Among these CNS disorders, *Akkermansia*, *Faecalibacterium*, and *Prevotella* were most commonly indicated. Again, this work is done at high order (low resolution) and is still mostly correlational and, therefore, may not be causative. However, these overlapping trends may better inform researchers and clinicians about preventative and interventional measures that could be more broadly applicable to neuroinflammatory disorders. Thus, this may represent a research priority for funding agencies. Key relationships for all of these disorders are highlighted in Supplementary Table S1 *Observed Functional Relationships with the Gut Microbiota and Neurodegenerative Disorders*; of note, only bacterial taxa have been characterized sufficiently at present.

4.5 Preventative and therapeutic interventions

Much of the excitement around the research on the microbiota-gut-brain-immune interface, especially around altering the gut microbiome, is the possibility of preventative and therapeutic interventions. This is especially poignant in neurodegenerative disorders, where there is little to offer in terms of such interventions. Most of the research has been in animal models with limited human studies, however. Translating these findings into the clinic requires further investigation in general and in how best to personalize such interventions to maximize their impact for an individual.

4.6 Nutrition for neuroinflammation

Nutrition is an important, modifiable risk factor that has a major role in the microbiota-gut-brain-immune interface, affecting each aspect of the interface. Gene-diet interactions have been linked to the microbial theory of inflammation, neuroinflammation, and neurodegenerative disorders like dementia (86, 87). Diet induced changes to the gut microbiome are key in the microbiota-gut-brain-immune interface. Gut microbiome changes are associated with shifts in the production of SCFAs and secondary bile acids, which in turn can impact inflammation and the release of neurotransmitters like serotonin (35). Therefore, resolving inflammation, dysbiosis, and leaky gut are likely to prevent and/or manage neurodegenerative disorders. The quantity of MACs present in the diet is linked to the production of SCFAs (88). A diet with adequate calories, rich in MACs promotes health in the microbiota-gut-brain-immune interface, while a high calorie, low MACs diet is associated with cognitive decline (25, 89, 90). SCFAs clearly play a role in this, especially in light of their role as fuel for colonocytes, preventing leaky gut and the inflammatory cascade (25, 89, 90). Polyphenols may also play a key role and have been shown continually and repeatably to be health-promoting elements of a healthy diet (25, 91, 92). Thus, diets low in MACs, high in fat/protein (i.e. Western-style diets) are

associated with gut dysbiosis and inflammation (25, 93). While some research on ketogenic diets, which are often low in MACs, has shown promise in reducing neuroinflammation and improving cognitive function in animal models of Alzheimer's and Parkinson's diseases, microbiome research poorly translates from animal models, which are overly simplistic. Therefore, the results in humans are often greatly attenuated or even lost due to a much more complex physiology, meaning they are no longer meaningful let alone clinically meaningful. This necessitates research in humans, which is currently lacking for ketogenic diets.

The nutrients and nutraceuticals often recommended for brain health mostly support the gut microbiome and the barrier function of the gut and blood brain barrier (94). However, any individual food is more than the sum of its parts—the concept of the 'food matrix' (95–98). Therefore, focusing on nutrients alone is insufficient to promote a healthy diet. Instead, an emphasis on the inclusion of whole, minimally processed foods and limiting ultra-processed foods is necessary and likely better able to support the microbiota-gut-brain-immune interface and prevent the inflammatory cascade and consequently neurodegeneration. Dietary patterns that embody this include the Mediterranean diet and the Mediterranean-DASH Diet for Neurodegenerative Delay (MIND) diet.

The Mediterranean diet is a style of dietary pattern that emphasizes vegetables, fruit, olive oil, and low-moderate alcohol intake (i.e. red wine) and is considered to be health promoting. It is also rich in polyphenols and omega-3 fatty acids, the latter of which is required for the resolution of the inflammatory response via resolvins and are associated with reduced neuroinflammation (99, 100). This dietary pattern has been linked to lower risk of neurodegenerative disorders and cognitive impairment and better global cognition and episodic memory (101). Many of the disorders that are seen to be decreased in those on this type of diet (coronary artery disease, hypertension, diabetes, metabolic syndrome, dyslipidemia) are also risk factors for cognitive impairment and involve the microbiota-gut-brain-immune interface (99).

The MIND diet is a version of the Mediterranean diet with an emphasis on neuroprotection and cardioprotection through anti-inflammatory foods that has been shown to slow cognitive decline with aging (99, 101, 102). It consists of high intake of whole plant foods emphasizing berries and green leafy vegetables, nuts, beans, fish, poultry, and olive oil while limiting animal foods, processed foods, and foods high in saturated fat. The focus on the food matrix likely plays a role in the MIND diet's effect on the microbiota-gut-brain-immune interface, potentially making it a more comprehensive tool.

Adherence to both the Mediterranean and MIND diets are associated with decreases in all-cause dementia independent of genetic risk and numerous studies support reduction in the risk of Alzheimer's disease specifically (19, 86, 103). Both the Mediterranean and MIND diets are associated with reduced pathology in Alzheimer's (104). Evidence to date demonstrates support for the Mediterranean and MIND diets in the prevention of a multitude of disease states, and the MIND diet appears to impart the greatest neuroprotection. However, there are limitations to this research: small cohorts, lacking a gold-standard to measure dietary adherence, potential for reverse causality because of short durations/follow-up (86, 105). In an attempt to account for this, a recent population-based study by de Crom et al. found an association between both diets and reduced dementia risk; however, there is still potential for confounding from lifestyle (105).

4.6.1 Lifestyle modifications

Many modifiable risk factors fall under the category of lifestyle. Stress management, restorative sleep, and other lifestyle factors have been shown to affect epigenetic regulation, the microbiota-gut-brain-immune interface, and neurodegenerative disease risk (106–112). As discussed above, chronic stress can trigger gut dysbiosis and the inflammatory cascade, so it follows that stress management has been linked to improvements in the gut microbiome composition and in stress-related epigenetic regulation (93, 113, 114). Mind-body therapies, e.g. yoga and meditation, are multifaceted interventions with numerous health benefits including stress management. Such mind-body interventions are promising to promote a diverse, non-dysbiotic/eubiotic gut microbiome and to reduce chronic inflammation (115–118). Similarly, restorative sleep is negatively associated with gut dysbiosis and cognitive decline (119, 120).

4.6.2 Physical activity

Effecting both the gut microbiome and epigenetics, physical activity is a potentially powerful modifier of neurodegenerative disorder risk. Physical activity (natural movement and exercise) alters gut composition and function by promoting beneficial gut bacteria and SCFA production; it also supports resolution of inflammation and return to immune homeostasis (121–125). All of these effects are likely to reduce the risk of neurodegenerative disorders. Additionally, physical activity is generally neuroprotective: increasing cerebral blood flow and circulation-related benefits (i.e. oxygenation and nutrient delivery), the production of neurotrophic factors including brain-derived neurotrophic factor (BDNF), and neurotransmitter (e.g. dopamine and serotonin) release that improves mood, cognition, and well-being (126–128). BDNF promotes neuronal growth, survival, and synaptic plasticity, supporting learning and memory (127). This orchestrated interplay of neurotrophic, anti-inflammatory, and metabolic processes work hand in hand with the gut microbiome in conferring neuroprotection associated with physical activity. Epigenetic changes from physical activity also contribute to its neuroprotective role (106, 112, 128–130). Given that yoga is both exercise and a mind-body therapy, both processes are likely contributing to the beneficial effects of yoga on the microbiota-gut-brain-immune interface and therefore reduced risk of neurodegenerative disease.

4.6.3 Probiotics, prebiotics, and fermented foods

Probiotics are, by definition, live microorganisms that confer health benefits when administered in adequate amounts (131). In animal models of neurodegenerative disorders, supplementation with probiotics shows great promise for improving neuroinflammation, cognitive function, gut microbiome composition and function,

epigenetic profiles, inflammation, and gut barrier function (132–139). A better understanding of the dysbiosis in neurodegenerative diseases as well as how certain taxa (e.g. keystone species) affect the microbiota-gut-brain-immune interface is necessary to rationally design probiotics that may be preventative or therapeutic for neurodegenerative disorders. At present, the majority of probiotic strains on the market are taken from yogurt, as they are easily granted generally recognized as safe (GRAS) status by the Food and Drug Administration (FDA). This greatly limits the possibility of their efficacy as therapeutics in an ecosystem that is much more diverse than yogurt.

Prebiotics are MACs that stimulate the growth and activity of microbes already present in the gut microbiome, thereby also altering the composition and function of the gut microbiome (140). Prebiotics have been shown to increase beneficial gut bacteria, reduce neuroinflammation, and support cognitive function (134, 137, 139). Given that prebiotics are likely to have a broader effect than the limited types of probiotics currently available, this may be a more promising avenue. However, a healthy diet emphasizing whole foods and limiting ultra-processed foods (e.g. the Mediterranean or MIND diets) can supply sufficient MACs to fuel the gut microbiota. Hence, the use of prebiotics over the emphasis of a healthy diet, which brings many other health-promoting elements, is currently under debate. In those unwilling or unable to adopt a diet rich in MACs, it is possible that prebiotics may support the gut microbiota sufficiently to avoid neurodegenerative disorders or it may be insufficient or missing other key elements from the diet and the food matrix.

The microbiota-gut-brain axis involves bidirectional communication through multiple pathways. These pathways, both direct and indirect, can facilitate epigenetic reprogramming within the microbiota-gut-brain axis, mediated by histone tail modification, DNA methylation, and non-coding RNA. Alterations in the composition of the microbiota can induce epigenetic changes that ultimately influence behavior; for example, *Helicobacter pylori* in the gut increases CpG-methylation in the promoter region of 06-methylguanine DNA methyltransferase, consequently reducing DNA methyltransferase activity in the gastric mucosa (141). Furthermore, studies have discovered a correlation between the gut microbiome and gene expression within the CNS, particularly in regions controlling the development of mood and neurological disorders; for instance, dietary supplementation of mice with *Lactobacillus rhamnosus* has been found to modulate the expression and transcription of GABA subunits across various brain regions (141). Interventions involving the supplementation of pre- or probiotics may ameliorate neurobehavioral abnormalities through epigenome alteration, often resulting in phenotypic attenuation.

Fermented foods were traditionally used to extend the storage of perishable food substrates; however, recent studies have highlighted their roles in the introduction of beneficial microbes and molecules to the gut microbiome. The connecting pathways of the microbiota-gut-brain axis have been used to understand the effects of fermented foods on the permeability of the intestinal and blood-brain barrier and their role in the treatment of neuroinflammation and mental health disorders. Various studies have demonstrated a decrease in circulating cytokines, especially IL-6, IL-10, IL-12, and TNF-α, among patients on fermented food diets (142). Consumption of fermented foods has also been shown to reduce corticosterone when exposed to stress; a proposed mechanism of cortisol modulation is via attenuating the response to peripheral immune challenges through a reduction in circulating cytokines and other inflammatory mediators (142). The administration of fermented products has also been shown to improve anxiety and depressive features and improved memory-associated tasks (142).

4.6.4 Other supplements

4.6.4.1 Gut barrier: glutamine and zinc carnosine

Supplements that support gut barrier function include glutamine (143–145) and zinc carnosine a.k.a. polaprezinc, which is important for wound and mucosa (e.g. the gut) healing and likely also beneficial for neuroprotection and reducing neurodegenerative disorder risk (146–149).

4.6.4.2 inflammation

4.6.4.2.1 Omega-3 fatty acids

As mentioned previously, omega-3 fatty acids help to resolve inflammation (anti-inflammatory); thus, supplemental omega-3 may be beneficial for the microbiota-gut-brain-immune interface. In fact, a reduced risk of Alzheimer's disease and cognitive decline is associated with intake of omega-3, especially docosahexaenoic acid (DHA) (150). However, this effect has not been consistent perhaps due to issues with dose, formulation, rancidity, study design, etc. (151, 152). In Parkinson's disease, several studies have demonstrated a reduction in dopaminergic neuron degeneration and neuroinflammation with greater intake of omega-3 (151).

4.6.4.2.2 Curcumin

A polyphenol found in turmeric, curcumin's anti-inflammatory properties have been studied as a potential therapeutic in Alzheimer's disease with the exploration of several modified formulations include nanotization to improve its bioavailability and pharmacokinetic properties, the major limitation to its therapeutic benefits (153–155). However, given the food matrix/entourage effect, one wonders if the extract is as potent as the whole food (turmeric) and/or if there are synergistic effect in food combinations. For instance, it is well known that black pepper improves the bioavailability and action of turmeric (156–158).

4.6.4.2.3 Resveratrol

Another polyphenol, resveratrol and its sources (i.e. red grapes and wine) have been linked to improved cognitive function and neuroinflammation and are being studies for Alzheimer's disease and Parkinson's disease (159–163). However, resveratrol research is still in its early stages and has hit some barriers, including concerns for the need for high doses. Again, the food matrix/entourage effect may be an important component to explore, potentially limiting the need for high dose therapy.

4.6.5 Fecal microbiota transplantation

Fecal Microbiota Transplantation (FMT) is the transfer of fecal matter from a healthy donor into the gut of a recipient after the administration of antibiotics to clear the way (164). While

historically thought of as radical and reserved for the life-and-death struggle of recurrent *Clostridioides difficile*, FMTs are being developed by industry, including two that have been FDA-approved recently (164–167). These standardized FMTs open up the possibility of broader use including for neurodegenerative disorders (168–172). To understand the long-term safety and efficacy of FMT in neurodegenerative disorders and for neuroinflammation, more research is needed.

5 Generalizability of microbiome research findings

In an emerging field like this, some demographic and ethnic groups are underrepresented, limiting the generalizability of its findings (173). Why is this so important for the microbiota-gut-brain-immune interface and the pathogenesis of neurodegenerative disorders? In a 2015 study, gut microbiome compositions were shown to vary significantly by geographic location, meaning the composition of those in the US, are likely different than those in other countries and the findings from US research may not be translatable in other countries (174). This has since been confirmed by many other studies and researchers. The gut microbiome is similar to a finger print in that there is a huge amount of interindividual variability, meaning averages are often not representative as well. Therefore, small sample sizes, especially from groups with limited diversity, are only able to describe the population in the sample—they are not generalizable (175). Despite this, high-income countries (HICs) lead microbiome research output due to well-established research infrastructure and funding availability. Contrastingly, lower-middle-income countries (LMICs) are underrepresented in microbiome research (175). Access is limited to advanced sequencing technologies, funding, and expertise, all of which encumber research efforts in LMICs (173, 176). Further, the emphasis on infectious disease and more immediate concerns to public health likely redirect consideration and resources from more long-term research projects such as the microbiome (176).

In order to truly understand the gut microbiome and the microbiota-gut-brain-immune interface, we must study diverse populations. This will ensure the equitable advancement of personalized medicine and healthcare generally. Diverse populations with distinct lifestyles and dietary patterns reside in LMICs. Microbiome studies in these regions are likely to elucidate population-specific variations in susceptibility to disease, response to therapies, and interaction with the environment. Likely barriers in some of the most underrepresented groups are highlighted subsequently.

5.1 Sub-Saharan Africa

Perhaps the most underrepresented region in gut microbiome research is Sub-Saharan Africa. With its immense genetic, cultural, and environmental diversity, increasing research in this region will greatly advance our understanding of unique microbial interactions and the related implications for human health. Research in this area is limited largely due to barriers such as insufficient research infrastructure, funding scarcity, access to advanced technologies, and ethical issues such as informed consent and sharing samples (176, 177). In addition to addressing these barriers to gut microbiome research, promoting collaborative research in this field within Africa will lead to priceless insights into the diverse human population in this continent as well as a more complete comprehension of the human microbiome globally.

5.2 Latin America

Gut microbiome research remains comparatively limited in Latin America as a region. In contrast, some countries have made advances in this field; however, even these are still lagging behind high-income countries. Major barriers include funding scarcity, insufficient research infrastructure, and inadequate expertise pipelines. Collaboration between Latin American and international researchers can help bridge this gap and foster knowledge exchange to enhance gut microbiome research regionally and globally (178).

5.3 Southeast Asia

Due to the large population in Southeast Asia, this region represents one of the most significant for global health research, generally, and gut microbiome research, specifically. Despite this, this region continues to lag behind in gut microbiome research. Barriers include a dearth of well-established research institutions (insufficient research infrastructure) and funding scarcity. As in Latin America, collaboration may fill some of this gap; however, regional capacity building will also be required (179). Both are necessary to advance gut microbiome research in this region and lead to a wholistic understanding of the gut microbiome globally.

5.4 Middle East

The final key, underrepresented region in gut microbiome region is the Middle East with relatively limited gut microbiome research (180, 181). Barriers in this region include political instability, funding scarcity, and ethical issues including cultural norms and sharing data. Again, international collaboration can overcome some of this with the ultimate need for local capacity building.

5.5 Considerations for improving the generalizability of microbiome research

Capacity building is a key step in addressing the underrepresentation of LMICs in gut microbiome research. To build capacity locally, investments will need to be made to provide training and support to local researchers, equipping them with the latest technology, and developing collaborations between

HIC and LMIC institutions. An example of this being success is reported by Maduka et al. in 2017, where African researchers were empowered through bioinformatics training and development of the necessary infrastructure (182).

Another element of support is international partnerships and exchange programs, which can cultivate knowledge exchange and resource sharing. It is common to see large-scale collaborations in HICs (e.g. the Human Microbiome Project), which have facilitated data and resource sharing to advance gut microbiome research with comprehensive datasets and groundbreaking discoveries (141, 183). Some of these HIC projects have promoted international collaboration (i.e. Earth Microbiome Project, American Gut Project, MetaHIT Consortium) and could serve as models for bringing LMICs in as well (184–186).

Additionally, community engagement is indispensable to ensuring appropriate, diverse representation in gut microbiome research. To conduct research in these populations, culturally appropriate approaches must be used, which require respecting cultural practices and beliefs (187). Further, such approaches (e.g. culturally sensitive recruitment strategies and community-based participatory research design) promote diversity, equity, and inclusion in the research and the study population, enhancing the applicability of the findings (188). To assure fair and equitable development of this emerging field, such ethical considerations must be prioritized including as they relate to sharing of data and resources as well as the informed consent process, particularly when vulnerable populations (i.e. those in LICs) are involved.

The generalizability of gut microbiome research can also be improved by the inclusion standardization, longitudinal study designs, and multi-omics analysis. Standardizing methodologies ensures the comparability and reproducibility of gut microbiome research findings (189, 190). To establish guidelines for data generation, processing, and analysis and facilite harmonization across studies, the International Human Microbiome Standards project was established (173, 186); this data will lay the foundation for personalized and innovative methods of prevention, treatment, and management of disease. Longitudinal studies are necessary to understand how the gut microbiome evolves over time and how this relates to health outcomes and/or the pathogenesis of disease. Further, such long-term studies are likely to elucidate unknown relationships between alterations of the gut microbiome and disease development that cannot be studies through observational or cross-sectional studies. Finally, integrating multi-omics data (e.g. genomics, epigenomics, metagenomics, metabolomics) will provide a wholistic understanding of the interactions among the microbiota-gut-brain-immune interface, revealing novel biomarkers and therapeutic targets for numerous disease states. The combination of these will advance personalized healthcare globally.

Author contributions

AW: Conceptualization, Project administration, Writing – original draft, Writing – review & editing. YN: Conceptualization, Project administration, Writing – original draft, Writing – review & editing. NZ: Writing – original draft, Writing – review & editing. AM: Writing – review & editing. LF: Conceptualization, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1365673/full#supplementary-material

SUPPLEMENTARY TABLE 1
Observed functional relationship with the gut microbiota.

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Causal association of juvenile idiopathic arthritis or JIA-associated uveitis and gut microbiota: a bidirectional two-sample Mendelian randomisation study

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Background: The gut microbiota significantly influences the onset and progression of juvenile idiopathic arthritis (JIA) and associated uveitis (JIAU); however, the causality remains unclear. This study aims to establish a causal link between gut microbiota and JIA or JIAU.

Methods: Using publicly available genome-wide association studies (GAWS) summary data, we conducted a two-sample Mendelian randomisation (MR) analysis employing various methods, namely inverse variance weighted (IVW), simple mode, weighted mode, weighted median and MR-Egger regression methods, to assess the causal association between JIA or JIAU and gut microbiota. Sensitivity analyses, including Cochrane's Q test, MR-Egger intercept test, leave-one-out analysis and MR-PRESSO, were performed to evaluate the robustness of the MR results. Subsequently, reverse MR analysis was conducted to determine causality between gene-predicted gut microbiota abundance and JIA or JIAU.

Results: The MR analysis revealed a causal association between gut microbiota abundance variations and JIA or JIAU risk. Specifically, the increased abundance of genus *Ruminococcaceae UCG013* (OR: 0.055, 95%CI: 0.006–0.103, p = 0.026) and genus *Ruminococcaceae UCG003* (β: 0.06, 95%CI: 0.003–0.117, p = 0.041) correlated with an increased risk of JIA, while genus *Lachnospiraceae UCG001* (OR: 0.833, 95%CI: 0.699~0.993, p = 0.042) was associated with a reduced risk of JIA, among others. Sensitivity analysis confirmed MR analysis robustness.

Conclusions: This study provides substantial evidence supporting a causal association between genetically predicted gut microbiota and JIA or JIAU. It highlights the significant role of intestinal flora in JIA or JIAU development, suggesting their potential as novel biomarkers for diagnosis and prevention. These findings offer valuable insights to mitigate the impact of JIA or JIAU.

KEYWORDS

juvenile idiopathic arthritis, uveitis, gut microbiota, causality, bidirectional, Mendelian randomisation analysis

Introduction

Juvenile idiopathic arthritis (JIA) is a heterogeneous condition characterised by arthritis of unknown origin manifesting before the age of 16 (1), often resulting in functional limitations and, in severe cases, disability (2). Globally, approximately three million children are affected by JIA (3, 4), making it the most prevalent chronic inflammatory rheumatic disease in childhood. Severe cases may exhibit persistent systemic symptoms, joint inflammation, severe drug side effects and macrophage activation syndrome (MAS) occurrence, which can pose life-threatening consequences, thereby placing a considerable burden on children's health and socioeconomic systems (5). Juvenile idiopathic arthritis-associated uveitis (JIAU) is commonly acknowledged as a prevalent and severe extra-articular manifestation of JIA (6), characterised by chronic bilateral recurrent anterior uveitis, a leading cause of disability and visual impairment. The incidence of JIAU varies from 5.0% to 19.1% among JIA populations across different geographical regions (7). Although multiple factors, such as genetics, environment and immunity, are hypothesised to contribute to the pathogenesis and underlying mechanisms of JIA and JIAU (6, 8), their precise aetiology and pathogenesis remain unclear, necessitating further investigation to enhance diagnosis, treatment and reduce associated disease burden. The human intestinal microbiota, comprising approximately 100 trillion bacteria of 1000–1150 species, forms a symbiotic relationship with the host, playing critical roles in human metabolism, nutrient absorption, immune responses and other aspects (9). Additionally, the intestinal microbiota is intricately linked to the onset and progression of various human diseases. In the context of predictive, preventive and personalised medicine, systemic inflammation serves as an essential communication bridge between the human host and the gut microbiota (10). Recent evidence suggests a potential role of gut microbiota in immune-mediated diseases such as rheumatoid arthritis (RA), wherein dysbiosis of the gut microbiota disrupts intestinal barrier function, leading to increased permeability and immune imbalance. Consequently, this dysregulation allows immune cells to migrate to extraintestinal sites, including joints, thereby triggering localised inflammation (11, 12).

Recent case-control studies spanning three continents provide compelling evidence of the influence of gut microbiota dysbiosis on the onset and progression of JIA (13–16). A prospective study focussing on the gut microbiota in patients with JIA in Italy and the Netherlands reports the presence of gut microbial dysbiosis in this population. Moreover, significant differences in microbial diversity and composition exist between patients with JIA and healthy individuals (17). Another study conducted in China yielded similar findings, indicating that patients with JIA display a significantly lower abundance of *Anaerostipes*, *Dorea*, *Lachnospira* and *Roseburia* compared to the control group. The study also identified 12 genera that could potentially serve as biomarkers and predictive factors for JIA (18). Nevertheless, the causal relationship between JIA and the gut microbiota remains uncertain, necessitating further investigation. Furthermore, observational studies are prone to the impact of confounding variables and reverse causality, potentially biasing results (19).

To address these limitations, we employ Mendelian randomisation (MR) to investigate the genetic-level association between JIA or JIAU and gut microbiota. MR utilises single nucleotide polymorphisms (SNPs) as instrumental variables (IVs) to estimate potential causal links between exposure variables and health outcomes (20). Leveraging extensive genome-wide association studies (GWAS) data, we employ a bidirectional two-sample MR approach to explore the causal relationship between the gut microbiota and these diseases, offering novel perspectives into potential therapeutic strategies targeting the microbiota (21–23).

Methods

Description of the research design

This study employs a bidirectional two-sample MR approach to evaluate the causal associations between the genetic prediction of JIA or JIAU and the genetic prediction of gut microbiota. Three key assumptions guide the selection of SNPs as IVs: 1) relevance, requiring a substantial correlation between the SNP and the exposure variable; 2) exclusion restriction, indicating that the SNP influences the outcome solely through the exposure variable and not by any alternative causal pathway; and 3) independence, implying that the

SNP is independent of the outcome variable and potential confounding factors. In forward MR analysis, gut microbiota serves as the exposure variable, while JIA or JIAU is considered the outcome variable. Conversely, in reverse MR analysis, JIA or JIAU is regarded as the exposure variable, while gut microbiota is treated as the outcome variable. This approach aims to investigate potential causal links between JIA or JIAU and gut microbiota in both directions. Figure 1 illustrates the schematic representation of the MR causality research design, elucidating the underlying principles of MR studies. Figure 2 presents a flowchart outlining the step-by-step process involved in conducting such a study. This study adheres to the reporting guidelines outlined in STROBE-MR, supplemented with materials that include a checklist according to STROBE-MR and a checklist based on the Critical Appraisal Checklist for examining MR research (24, 25). The Supplementary Materials provide a detailed explanation of the checklist items.

Data source

JIA and JIAU

GWAS data for JIA are based on a recent meta-analysis comprising 6,056 patients with JIA and 25,086 European ancestry controls (26). The detailed research process is described in previous studies by Lopez-Isac E and McIntosh LA (26, 27). The diagnostic criteria for JIA follow international standards published by the International League of Associations for Rheumatology (ILAR), with the specificity and common susceptibility loci for the various JIA ILAR subtypes systematically examined using the JIA GWAS data. The GWAS data for JIAU were sourced from Haasnoot et al.'s study (28), encompassing 192 patients with JIAU of European ancestry and 330 JIA control patients without uveitis.

Gut microbiota

GWAS data related to gut microbiota were obtained from the Mi BioGen consortium, representing the most extensive dataset available (https://mibiogen.gcc.rug.nl/). The GWAS dataset includes 16S rRNA gene information from the faecal microbiomes of 18,340 individuals across 24 cohorts, alongside whole-genome genotyping data. Specifically, the study investigated the species composition of gut microbiota utilising the 16S rRNA gene regions V1-V2, V3-V4 and V4, representing three distinct variable regions. The dataset comprises 211 intestinal flora, categorised by kingdom, phylum, order, family, genus and species. These species exhibit relative abundance or genetic variations and can be further categorised into 9 phyla, 16 classes, 20 orders, 35 families and 131 genera. After excluding 12 unknown genera, we incorporated a total of 119 genera as classification units for the bidirectional MR study. Supplementary Table 1 presents the features of the data sources utilised in this investigation.

The selection of IVs

To ensure the validity of the causal relationship between gut microbiota composition and JIA or JIAU risk, rigorous quality control measures were applied in selecting appropriate genetic IVs.

Given the limited number of genetic variants associated with gut microbiota, we established a significance threshold of $p< 1.0\times10^{-5}$ based on the majority of MR studies on gut microbiota to ensure adequate candidate instrument numbers for forward MR analysis (29–31). Additionally, to ensure independence between IVs and avoid bias from linkage disequilibrium (LD) between SNPs, we applied LD clustering with a cutoff of $r^2< 0.01$ and a clustering distance of 500 kb. Finally, to minimise instrument bias, IVs with an F statistic less than 10 were excluded, where $F = (N - 2) \times \frac{R^2}{1-R^2}$, with N representing the sample size and R^2 the squared correlation coefficient (32).

For reverse MR, the significance threshold was set at $p< 5.0\times10^{-6}$ to screen SNPs as IVs for JIA and $p< 1.0\times10^{-5}$ for JIAU, with LD set at $r^2< 0.001$ and a clumping distance of 10,000 kb.

To determine the direction of influence of specific IVs on the outcome, Steiger filtering analysis was employed (33). IVs with a 'TRUE' result indicating the expected association direction were included in subsequent analysis, with those classified as 'False' were excluded.

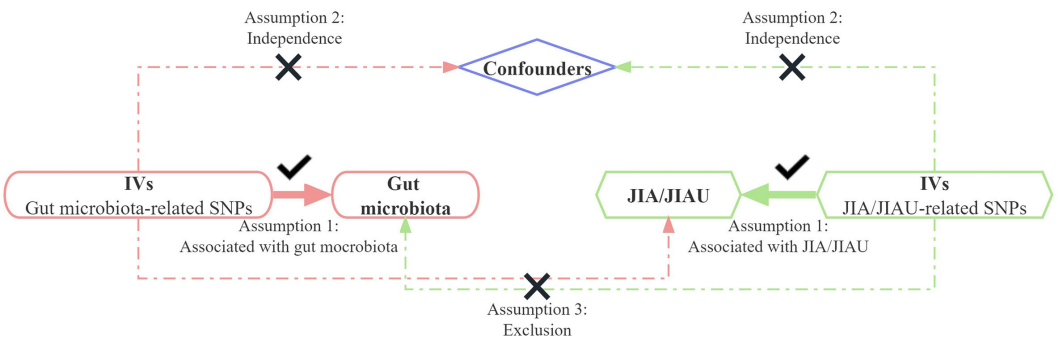

FIGURE 1
Bidirectional two-sample Mendelian randomisation between JIA/JIAU and gut microbiota abundance outcomes. Directed acyclic graph (DAG) of the causal association between JIA/JIAU and gut microbiota abundance. IVs, instrumental variables; SNPs, single nucleotide polymorphisms; JIA, juvenile idiopathic arthritis; JIAU, JIA-associated uveitis.

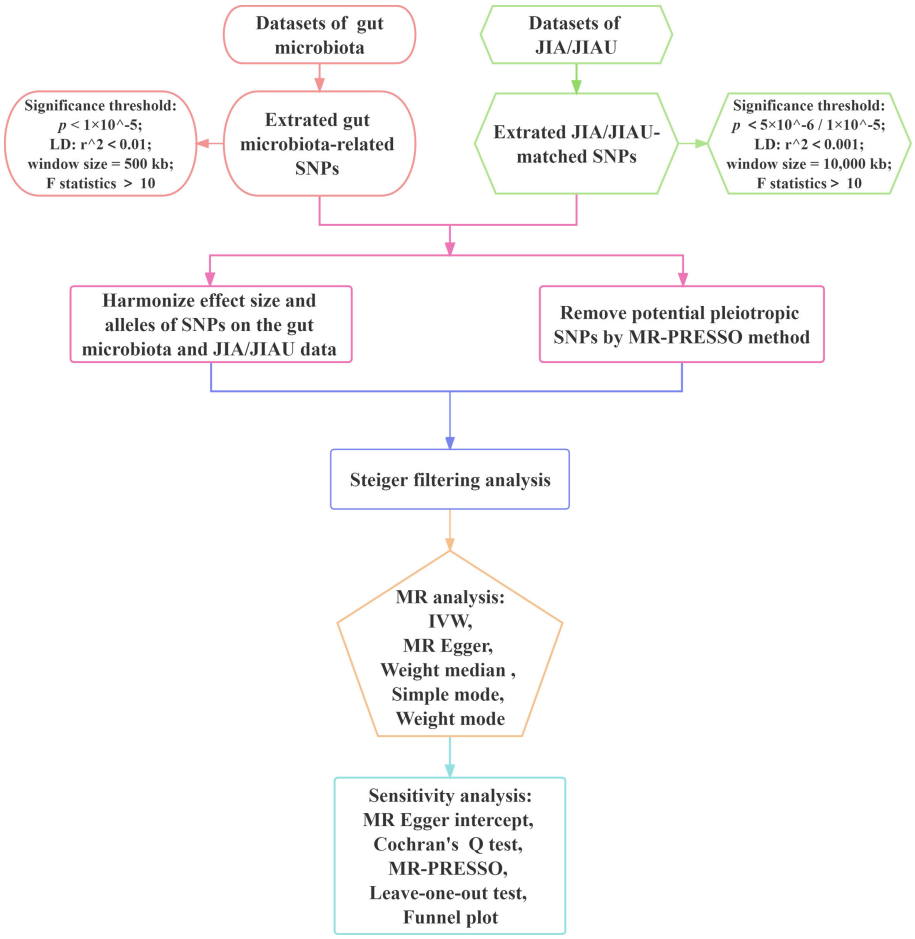

FIGURE 2
Workflow of Mendelian randomisation study revealing causality between gut microbiota abundance and JIA/JIAU risk. JIA, juvenile idiopathic arthritis; JIAU, JIA-associated uveitis; SNPs, single nucleotide polymorphisms; LD, linkage disequilibrium; IVW, inverse variance weighted; MR, Mendelian randomisation; MR-PRESSO, MR pleiotropy residual sum and outlier.

Statistics analysis

MR analysis was conducted to explore the causality between JIA/JIAU and gut microbiota using R software version 4.2.2 and three specific R packages: 'TwoSampleMR' (v.0.5.6) (33), 'MRPRESSO' (v.1.0) (34) and 'MendelianRandomization' (35). A significance threshold of $p< 0.05$ was considered for causation. To account for multiple comparisons at every taxonomic level (phylum, class, order, family and genus), a significance threshold of 0.05/n was set, where n represents the number of distinct bacterial species at that particular taxonomic level.

Five statistical methodologies were employed: inverse variance weighted (IVW), simple mode, weight mode, weighted median and MR-Egger regression. IVW, considered the primary method, was used for outcome determination, supplemented by the other four methods. Different methods possess different underlying assumptions about horizontal pleiotropy. IVW employs the inverse of the squared standard error (SE) of the outcome as weights and does not include an intercept component in regression. Additionally, random effects were utilised for IVW modelling, with overall estimates derived from weighted linear regression of the Wald estimate for each SNP (36). The other four methods complement IVW by providing more robust estimates in broader scenarios, albeit with lower effect values (wider CI) (34). Moreover, the MR-Egger method offers a consistent causal effect test when SNPs directly linked with the outcome or exhibiting horizontal pleiotropy are excluded (37). Weighted median methodology can produce reliable estimates even when up to 50% of findings are from erroneous SNPs (38). Meanwhile, the simple mode provides an unweighted mode of estimating causal effects' empirical density function (39). Supplementary Table 2 lists the advantages, disadvantages, efficacy and applications of these five methods.

Sensitivity analysis

Following MR analysis, sensitivity analysis was conducted to assess the robustness of the results. Firstly, the intercept of the MR-Egger regression was examined to detect the presence of horizontal pleiotropy, with $p > 0.05$ suggesting a weaker potential for pleiotropy in the causal analysis (34). Additionally, MR-PRESSO

was employed to identify and address possible outliers by screening for heterogeneity and outliers, followed by a re-analysis of the MR. To assess heterogeneity in the IVW method, Cochran's Q test (40) and funnel plots were used. Furthermore, to evaluate the impact of individual SNPs on the primary causal relationship, a leave-one-out analysis was conducted by sequentially excluding individual SNPs.

Approval, registration and consent

All GWAS data utilised in our analysis were obtained from publicly available datasets with ethical permission granted by the relevant ethical review boards. These datasets do not contain personal information, ensuring compliance with ethical standards.

Results

Forward MR

In the initial steps, a total of 3036 SNPs relevant to gut microbiota at the phylum, class, order, family and genus levels were identified, each with an F statistic > 10, indicating minimal instrumental bias (Supplementary Table 2).

Additionally, Steiger filtering analysis revealed no SNPs with a reverse causal direction for gut microbiota on JIA (Supplementary Table 3), while 849 SNPs were eliminated for JIAU (Supplementary Table 5). For our analysis, a total of 131 genera, 35 families, 16 classes, 20 orders and 9 phyla were identified as IVs, with 5 to 26 IVs obtained from each classification.

To address multiple comparisons, significance thresholds for gut microbiota on JIA and JIAU were determined using the Bonferroni correction: For JIA, thresholds were set at phyla ($p = 5.56 \times 10^{-3}$), class ($p = 3.13 \times 10^{-3}$), order ($p = 2.50 \times 10^{-3}$), family ($p = 1.35 \times 10^{-3}$) and genus ($p = 3.82\times 10^{-4}$). Similarly, for JIAU, thresholds were set at phyla ($p = 5.56 \times 10^{-3}$), class ($p = 3.13 \times 10^{-3}$), order ($p = 2.94 \times 10^{-3}$), family ($p = 1.43 \times 10^{-3}$) and genus ($p = 3.82\times 10^{-4}$).

Utilising the IVW method, an association between JIA risk and gut microbiota at one phylum and four genera was identified (Figure 3). MR analysis revealed that family *FamilyXI* (OR: 1.148, 95%CI: 1.008~1.307, $p = 0.037$) increased the risk of JIA, while genera *Rikenellaceae RC9* gut group (OR: 0.843, 95%CI: 0.74~0.96, $p = 0.01$), *Lachnospiraceae UCG001* (OR: 0.833, 95%CI: 0.699~0.993, $p = 0.042$), *Intestinimonas* (OR: 0.840, 95%CI: 0.709~0.994, $p = 0.0426$) and *Clostridium innocuum group* (OR: 0.862, 95%CI: 0.73~1.0, $p = 0.0495$) decreased the risk of JIA. Notably, consistent effect directions were observed for family *FamilyXI*, genus *Lachnospiraceae UCG001*, genus *Intestinimonas*, and genus *Clostridium innocuum group* across multiple MR methods. However, for the genus *Rikenellaceae RC9* gut group, the results from the MR-Egger method differed from the IVW method but remained consistent with the weighted median, simple mode, and weighted mode methods. A circular heat map visualised the outcomes of MR analysis conducted on two sample groups,

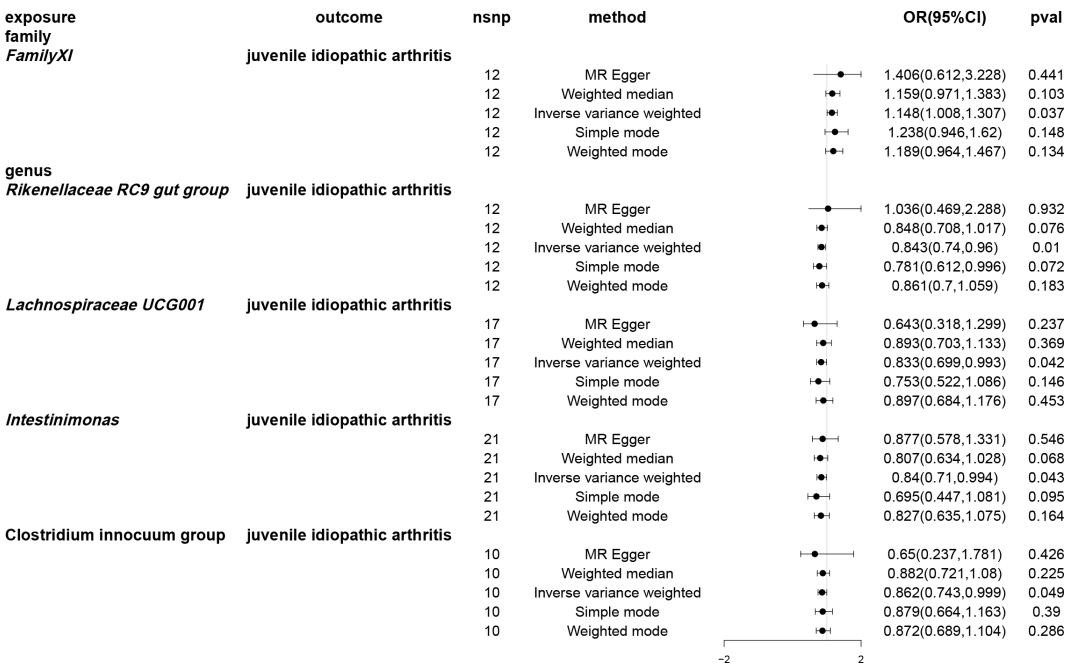

FIGURE 3
Forest plots of Mendelian randomisation for two samples indicated the causal effects of five different Mendelian randomisation methods with gut microbiota abundance as exposure and juvenile idiopathic arthritis (JIA) as the outcome. The effect estimates are presented as the effect size (OR) and 95% confidence interval (CI). snp, single nucleotide polymorphism.

illustrating the causal effects estimated by the five different MR methods, with gut microbiota abundance as the exposure variable and JIA as the outcome variable (Figure 4). Supplementary Figures 1, 2 display the scatter plot and forest plot, respectively.

A causal relationship was found between one genus of gut microbiota and the risk of JIAU using the IVW method (Figure 5). MR analysis showed that genus *Prevotella7* reduced the likelihood of JIAU (OR: 0.398, 95% CI: 0.191–0.83, $p = 0.014$). Additionally, the MR-Egger, weighted median, simple mode and weighted mode methods revealed consistent effect directions. A circular heat map provided insights into the relationship between gut microbiota abundance (exposure variable) and JIAU (outcome variable) (Figure 6), with scatter plots (Supplementary Figures 5) and forest plots (Supplementary Figure 6) further illustrating these relationships.

Reverse MR

For JIA, 13 SNPs and for JIAU, 6 SNPs met the IV screening criteria, each with an *F*-statistics > 10, indicating minimal instrumental bias (Supplementary Tables 13, 19). Additionally, Steiger filtering analysis revealed no SNPs with opposing causal orientations for either condition (Supplementary Tables 14, 20).

The SNP findings summarised data from a total of 208 gut microbiota classifications, including 16 classes, 126 genera, 33 families, 20 orders and 9 phyla, used to study the connection between JIA and gut microbiota (Supplementary Table 15). Significance thresholds for multiple comparisons at distinct taxonomic classifications were set as follows: phylum ($p = 5.56\times10^{-3}$), class ($p = 3.13\times10^{-3}$), order ($p = 2.50\times10^{-3}$), family ($p = 1.52\times10^{-3}$) and genus ($p = 3.97\times10^{-4}$), and were adjusted using the Bonferroni correction method. Similarly, for JIAU and gut microbiota, 208 different gut microbiota classifications were included in the data summary for the SNP results (Supplementary Table 21). These classifications encompassed 16 classes, 128 genera, 34 families, 20 orders and 9 phyla. Significance thresholds for multiple comparisons at different taxonomic classifications were set as follows: phylum ($p = 5.56\times10^{-3}$), class ($p = 3.13\times10^{-3}$), order ($p = 2.50\times10^{-3}$), family ($p = 1.47\times10^{-3}$) and genus ($p = 3.91\times10^{-4}$), and were adjusted using the Bonferroni correction method.

Using the IVW method, causal associations between JIA and gut microbiota were identified at one phylum, one order, one family and eight genera (Figure 7). MR analysis revealed that JIA increased the abundance of class *betaproteobacteria* (β: 0.061, 95%CI: 0.014–0.109, $p = 0.012$), order *Burkholderiales* (β: 0.061, 95%CI: 0.013–0.109, $p =$

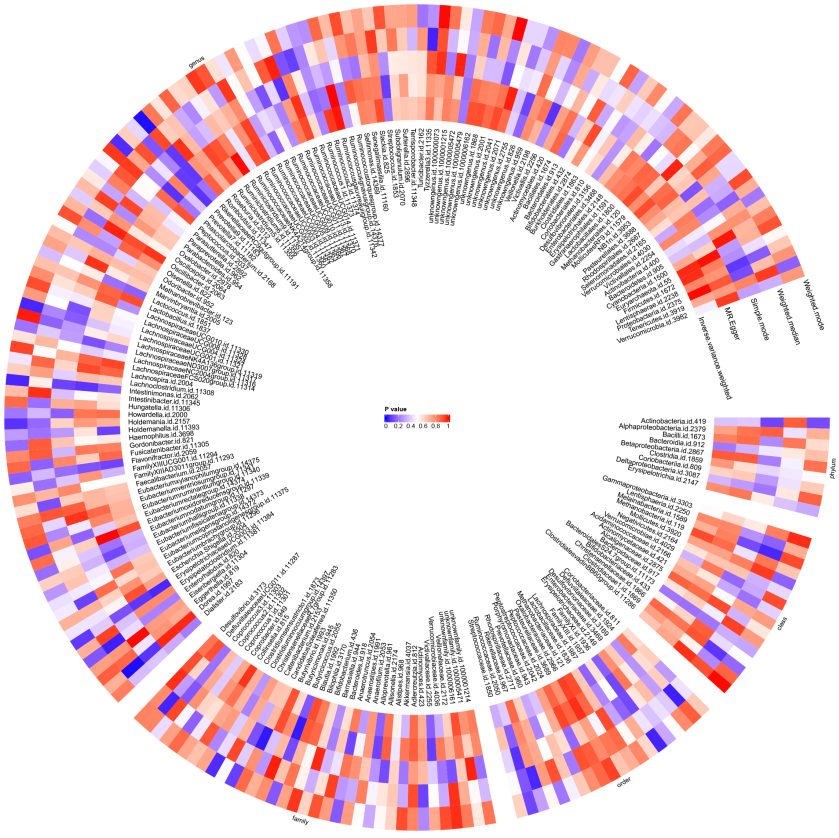

FIGURE 4
Circular heat map was generated to visualise the results of Mendelian randomisation analysis conducted on two sample groups, illustrating the causal effects estimated by five different Mendelian randomisation methods, with gut microbiota abundance as the exposure variable and juvenile idiopathic arthritis (JIA) as the outcome variable.

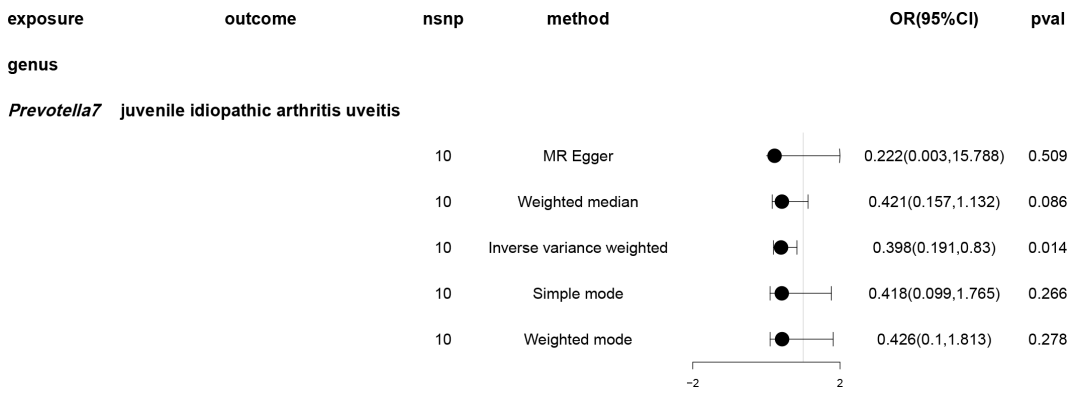

FIGURE 5
Forest plots of Mendelian randomisation for two samples demonstrated the causal effects of five different Mendelian randomisation methods with gut microbiota abundance as exposure and juvenile idiopathic arthritis associated uveitis (JIAU) as the outcome. The effect estimates are presented as the effect size (OR) and 95% confidence interval (CI). snp, single nucleotide polymorphism.

0.013), family *Alcaligenaceae* (β: 0.056, 95%CI: 0.008–0.104, p = 0.023), genus *Ruminococcaceae UCG013* (β: 0.055, 95%CI: 0.006–0.103, p = 0.026), genus *Roseburia* (β: 0.051, 95%CI: 0.003–0.099, p = 0.036), genus *Ruminococcaceae UCG003* (β: 0.06, 95%CI: 0.003–0.117, p = 0.041) and genus *Anaerofilum* (β: 0.097, 95%CI: 0.003–0.192, p = 0.044), while decreasing the abundance of genus *Olsenella* (β: -0.137, 95%CI: -0.24- -0.033, p = 0.01), genus *Coprococcus2* (β: -0.065, 95%CI: -0.123- -0.006, p = 0.03), genus *Romboutsia* (β: -0.057, 95%CI: -0.11, -0.005, p = 0.033) and genus *Eisenbergiella* (β: -0.145, 95%CI: -0.285- -0.006, p = 0.041). For class *beta proteobacteria*, order *Burkholderiales*, family *Alcaligenaceae*, genus *Ruminococcaceae UCG013*, genus *Roseburia*, genus *Ruminococcaceae UCG003*, genus *Olsenella*, genus *Romboutsia* and genus *Eisenbergiella*, the MR-Egger, weighted median, mode-based estimator and weighted mode methods revealed directional effects that were consistent with the IVW method. However, for genus *Anaerofilum* and genus *Coprococcus2*, the results from the MR-Egger method differed from those of the IVW method, while the results from the weighted median, mode-based estimator and weighted mode methods aligned with the IVW method. A circular heat map visually represented the outcomes of MR analysis performed on the two sample groups, depicting the causal effects estimated by the five different MR methods and providing insights into the relationship between JIA (exposure variable) and gut microbiota abundance (outcome variable) (Figure 8). Scatter plots (Supplementary Figure 9) and forest plots Supplementary Figure 10) further illustrated these relationships.

Employing the IVW approach, a causal association was found between JIAU and the gut microbiota at one phylum, one class, one order, two families and three genera (Figure 9). MR analysis demonstrated that JIAU increased the abundance of genus *Phascolarctobacterium* (β: 0.023, 95%CI: 0.001–0.044, p = 0.04) but decreased the abundance of phylum *Lentisphaerae* (β: -0.041, 95%CI: -0.074–0.007, p = 0.017), class *Lentisphaeria* (β: -0.043, 95%CI: -0.076- -0.009, p=0.012), order *Victivallales* (β: -0.043, 95%CI: -0.076- -0.009, p=0.012), family *Peptostreptococcaceae* (β: -0.019, 95%CI: -0.036- -0.001, p = 0.041), family *Victivallaceae* (β: -0.045, 95%CI: -0.082- -0.008, p = 0.016), genus *Slackia* (β: -0.04, 95%CI: -0.075- -0.005, p = 0.026) and genus *Alloprevotella* (β: -0.086, 95%CI: -0.168- -0.005, p = 0.038). For phylum *Lentisphaerae*, class *Lentisphaeria*, order *Victivallales*, family *Victivallaceae*, genus *Slackia* and genus *Phascolarctobacterium*, MR-Egger, weighted median, simple mode and weighted mode methods revealed effect directions consistent with the IVW results. However, for the family *Peptostreptococcaceae*, the results from the MR-Egger method differed from that of the IVW method; however, the weighted median, simple mode and weighted mode methods revealed results consistent with the IVW findings. A circular heat map was generated to visually represent the outcomes of MR analysis performed on the two sample groups, depicting the causal effects estimated by the five different MR methods, thereby providing insights into the relationship between the exposure (JIAU) and outcome (gut microbiota abundance) (Figure 10). Supplementary Figures 13, 14 present the scatter and forest plots, respectively. Supplementary Table 25 lists the top five microbial genera and species and their characterisation with potent effects associated with JIA or JIAU as derived from forward MR and reverse MR.

Sensitivity analysis

When the variables of the gut microbiota or JIA or JIAU samples were analysed using the MR-Egger regression intercept method, no evidence of horizontal pleiotropy was detected (Supplementary Tables 8, 11, 17, 23). We conducted outlier screening and removal in the MR-PRESSO analysis, and the global p-value test of MR-PRESSO did not indicate horizontal pleiotropy for gut microbiota or JIA or JIAU (Supplementary Tables 9, 12, 18). Furthermore, the majority of Cochrane's Q test

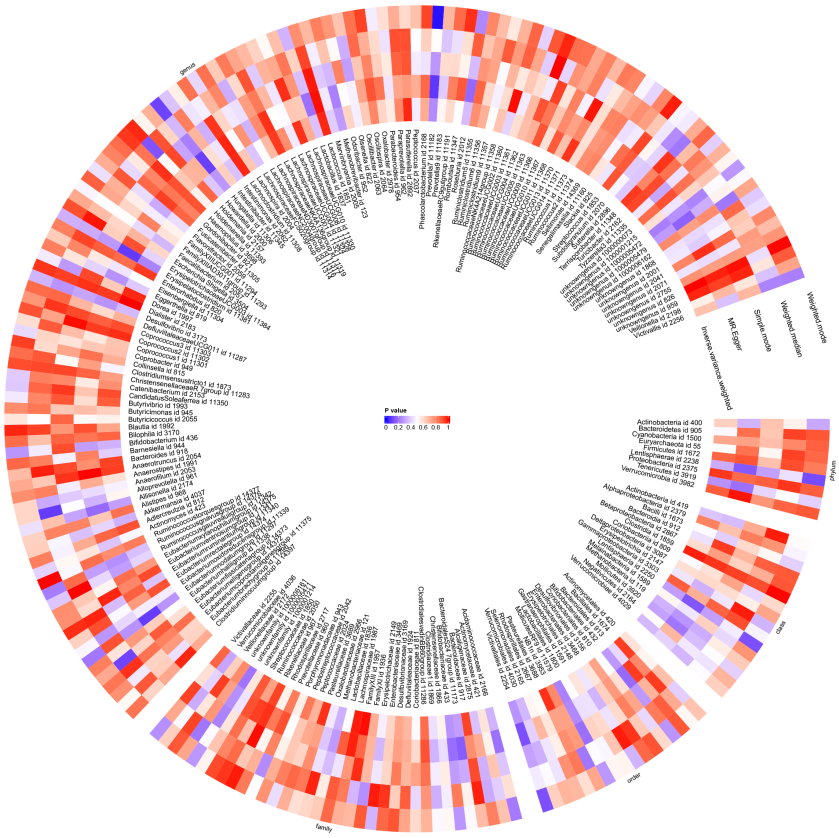

FIGURE 6
With gut microbiota abundance as the exposure variable and juvenile idiopathic arthritis associated uveitis (JIAU) as the outcome variable, a circular heat map was generated to visualise the findings of the Mendelian randomisation analysis performed on two sample groups. The heat map illustrated the causal effects estimated by five different Mendelian randomisation methods.

results did not reveal significant heterogeneity ($p > 0.05$) (Supplementary Table 7, Supplementary Tables 10, 16, 22). In the presence of significant heterogeneity, we employed random effects models and the IVW model as the main analytical results. Sensitivity analyses using the leave-one-out method and funnel plots are depicted in Supplementary Figures 3, 4, 7, 8, 11, 12, 15, 16.

Discussion

JIA is a common autoimmune rheumatic disease that poses a significant threat to the overall health and well-being of children, triggering joint deformities, functional impairments, stunted growth and osteoporosis, among other serious complications. Meanwhile, JIAU, a prevalent extra-articular complication of JIA, further complicates the clinical picture with chronic, non-granulomatous and non-infectious uveitis, often affecting the anterior segment of the eye. Approximately 10% to 14% of patients with JIA exhibit signs of uveitis before arthritis onset (41). Current treatment modalities for JIA and JIAU primarily focus on symptomatic relief, lacking definitive curative options (8). Previous studies have reported that siblings of patients with JIA have a higher risk of developing JIA than the general population, with the onset age, disease type and disease course of twin siblings exhibiting a similar trajectory (42). These findings indicate a genetic factor underlying JIA pathogenesis (43, 44). Moreover, subsequent studies have reported a genetic susceptibility locus for JIA, further confirming this speculation. However, as research advances, scientists have discovered that these genetic susceptibility factors can only explain 18% of the causes of JIA. Currently, the progression of JIA is speculated to be the outcome of the interaction between genetic susceptibility genes, environmental stimuli and immune dysregulation (45–47).

Emerging evidence underscores the microbiota's involvement in immune-related disorders like RA and inflammatory bowel disease (IBD) (11). The intestinal microbiota predominately contributes to JIA pathogenesis by altering intestinal mucosal permeability and regulating the host immune system (48). Notably, altered microbial compositions, such as increased *phyla Bacteroidetes* and decreased *Firmicutes*, have been observed in paediatric patients with JIA compared to healthy individuals (15). This study suggests an increase in the abundance of class *betaproteobacteria* in patients with JIA, consistent with previous research findings (15).

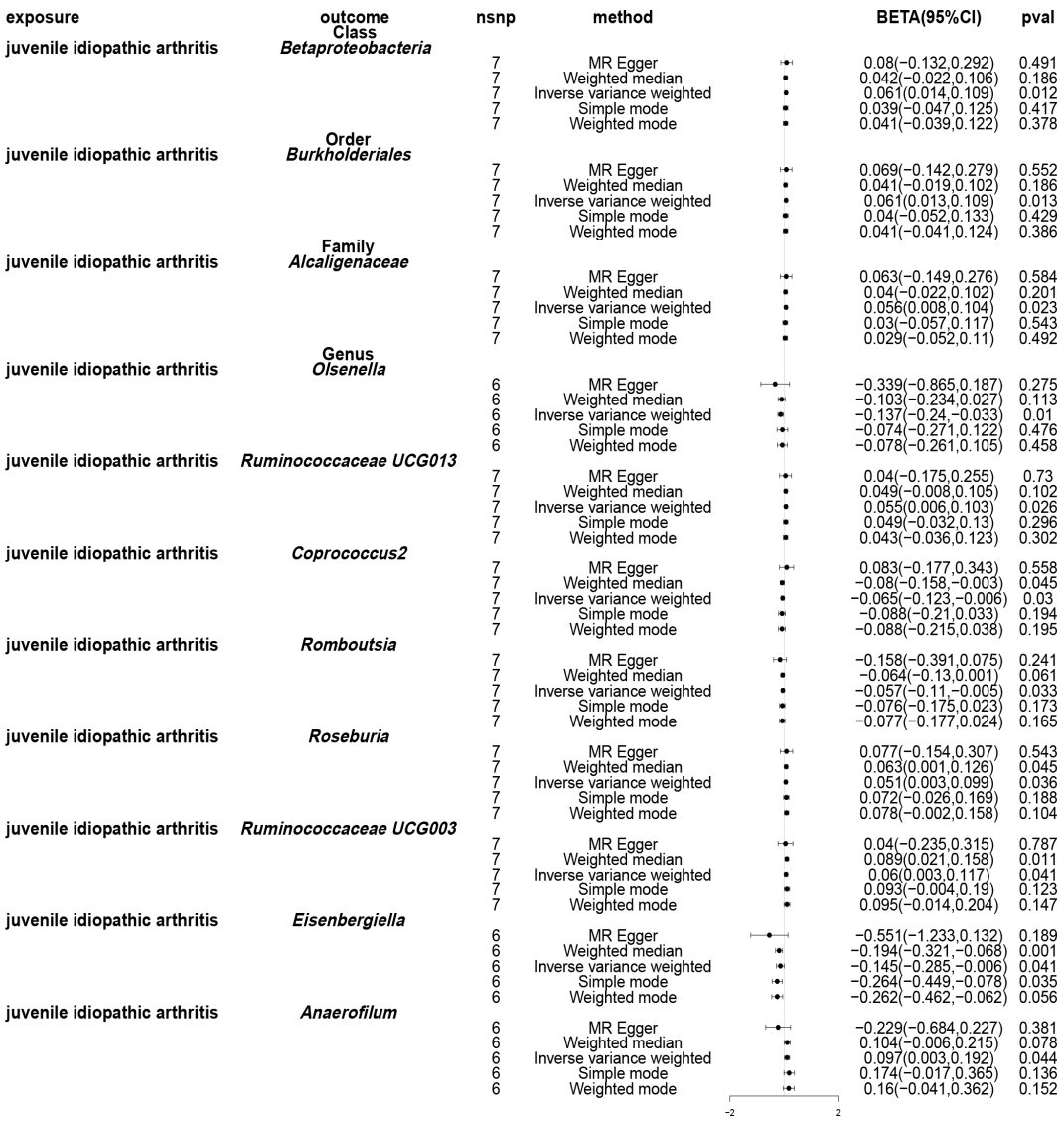

FIGURE 7
Forest plots of Mendelian randomisation for two samples, depicted the causal effects of five different Mendelian randomisation methods with juvenile idiopathic arthritis (JIA) as exposure and gut microbiota as the outcome. The effect estimates are presented as the effect size (BETA) and 95% confidence interval (CI). snp, single nucleotide polymorphism.

In animal experiments, an extraperitoneal injection of intestinal bacterial cell wall components into mice has been demonstrated to induce arthritis in a conventional environment, but not in a sterile environment (49), suggesting a close relationship between gut microbiota alterations and arthritis development. Early investigations by Malin et al. (50) highlighted elevated bacterial urease activity in the stool samples of children with JIA compared to those of healthy children, suggesting an anaerobic dysbiosis of the intestinal flora. Interestingly, oral *Lactobacillus* administration to patients with JIA reduced urease activity. Results from a multicentre study conducted by van Dijkhuizen et al. (78 Italian children and 21 Dutch children) (17) revealed that Italian children with JIA exhibited an elevated abundance of *Faecalibacterium prausnitzii*, *Erysipelotrichaceae*, *Enterococcus*, *Parabactteroides* and *Ruminococccaceae*, but reduced levels of *Allobaculum*, *Gemellaceae*, *Propionibacterium acnes* and *Turicibacter* compared to the healthy control group. Similarly, our study also suggests an increase in the abundance of genera *Ruminococcaceae UCG013* and *Ruminococcaceae UCG003* in patients with JIA. Additionally, a significant decrease in the level of intestinal flora has been reported in samples from active and inactive states compared to healthy children (17). Kindgren et al.'s study on population queues (51) revealed a higher content of *Acidaminococcales*, *Prevotella 9* and *Veillonella parvula* in JIA cases, while *Coprococcus, Subdoligranulum, Phascolarctobacterium, Dialister spp, Bifidobacterium breve, Fusicatenibacter saccharivorans, Roseburia*

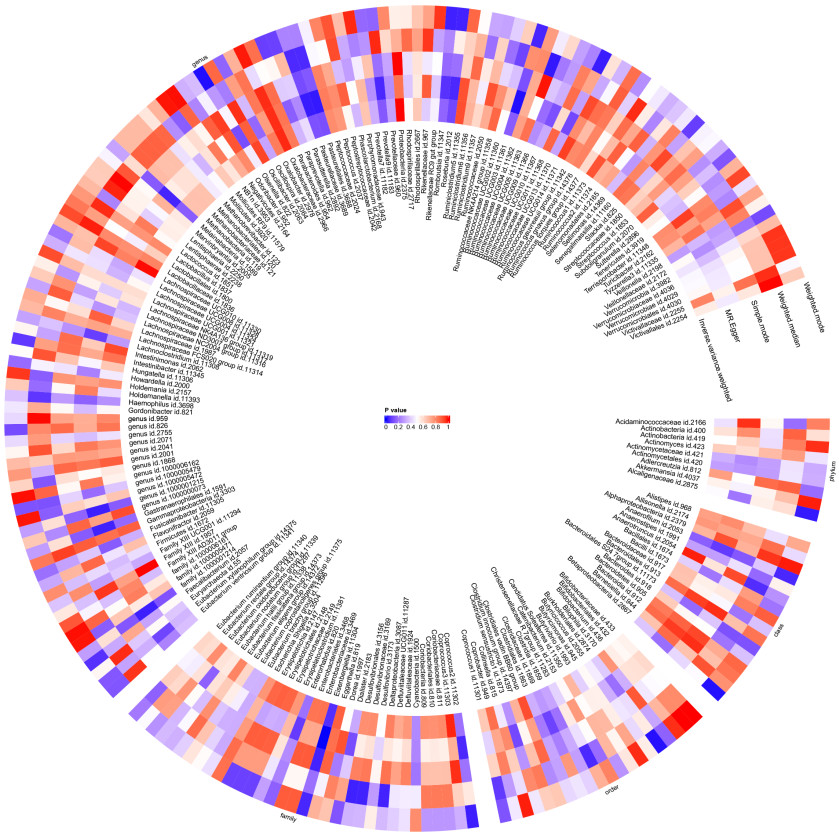

FIGURE 8
Circular heat map was generated to visually represent the outcomes of Mendelian randomisation analysis performed on two sample groups, depicting the causal effects estimated by five different Mendelian randomisation methods, with juvenile idiopathic arthritis (JIA) as the exposure variable and gut microbiota abundance as the outcome variable.

intestinalis and *Akkermansia muciniphila* exhibited reduced abundance. Notably, studies have linked the presence of *Parabacteroides distasonis* to an increased risk of subsequent JIA occurrence, alongside shorter breastfeeding duration and increased antibiotic exposure, particularly in genetically susceptible populations. Therefore, it can be concluded that environmental stimuli have a stronger effect on genetically predisposed infants and that microbial dysbiosis during infancy may initiate or accelerate the development of JIA. However, t our MR study findings indicate an increase in the abundance of genus *Phascolarctobacterium* in JIAU, contrasting previous results. This inconsistency may stem from JIAU's nature as a complication of JIA, potentially exerting different effects on the gut microbiota. However, these findings support the notion that environmental stimuli exert a significant influence. In another cross-sectional investigation, Qian et al. reported that the JIA group exhibited a significant reduction in the relative abundance of four genera (*Anaerostipes*, *didiister*, *Lachnospira* and *Roseburia*) compared to the control group (18). These four genera are known to produce short-chain fatty acids (SCFAs), whose decrease is associated with severe clinical complications. Additionally, the study identified the genus *Lachnospiraceae UCG001* as potentially reducing the risk of JIA. However, contrary to previous findings (18), an increase in the level of genus *Roseburia* in JIA was observed in our study.

Previous research consistently highlights differences in the gut microbiota composition between children with JIA and their healthy counterparts. However, the causal relationship between gut microbiota and the disease remains unclear. It is uncertain whether microbiota imbalance precedes JIA or arises as a consequence of long-term inflammation, abnormal metabolism or behavioural changes associated with JIA symptoms. Given the limitations of previous studies, including susceptibility to confounding factors and reverse causation effects, further investigation is warranted to elucidate the link between JIA or JIAU and the gut microbiota. In our study, leveraging GWAS data and MR analysis, we investigated the causality between JIA or JIAU and gut microbiota. In addition to our primary findings, we observed an elevated risk of *FamilyXI*, while genera *Rikenellaceae RC9* gut group, *Intestinimonas* and *Clostridium* innocuum group were linked to decreased JIA risk. Conversely, a reduced abundance of genus *Prevotella7* was associated with a decreased likelihood of JIAU. Furthermore, the abundance of order *Burkholderiales*, family *Alcaligenaceae* and genus *Anaerofilum* increased, while the abundance of genera *Olsenella*, *Coprococcus2*, *Romboutsia* and

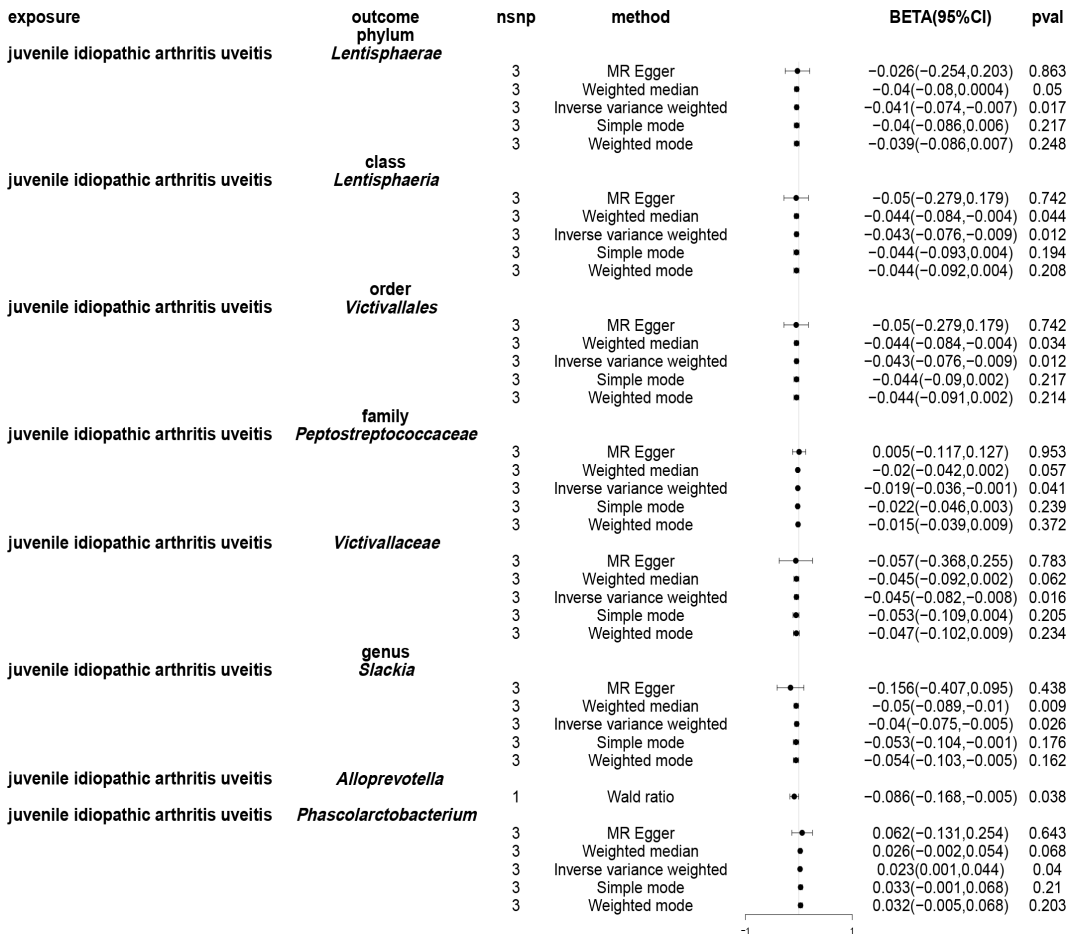

FIGURE 9
Forest plots of Mendelian randomisation for two samples illustrated the causal effects of five different Mendelian randomisation methods with juvenile idiopathic arthritis associated uveitis (JIAU) as exposure and gut microbiota as the outcome. The effect estimates are presented as the effect size (BETA) and 95% confidence interval (CI). snp, single nucleotide polymorphism.

Eisenbergiella decreased in JIA cases. JIAU decreased the abundance of phylum *Lentisphaerae*, class *Lentisphaeria*, order *Victivallales*, family *Peptostreptococcaceae*, family *Victivallaceae* and genus *Slackia*. These novel findings contribute to existing knowledge by uncovering previously unexplored associations.

Given the pivotal role of gut microbiota in arthritis, targeted probiotics are emerging as a novel therapeutic avenue for rheumatic diseases. Since the mature and stable state of the microbiota once formed is difficult to change, and childhood is a critical period to acquire basic functions (such as immune tolerance to commensal microbiota), these findings present a unique opportunity for early intervention and potentially modifying the disease progression by targeting the microbiota (12). Clinical trials investigating prebiotics and probiotics are on the rise, with an expanding body of evidence reporting favourable tolerance and potential benefits for restoring infant microbiota to health (52, 53). However, a randomised controlled trial of probiotics by Shukl et al. demonstrated good tolerance in patients with Enthesitis-related arthritis (ERA) but did not show any favourable clinical or immunological effects compared to non-steroidal anti-inflammatory medication therapy (54). Therefore, to assess the efficacy and safety of probiotics for JIA, further clinical data are warranted to ensure better recommendations for clinical practice.

Our research offers several advantages. To the best of our knowledge, this is the first investigation to explore the causal relationship between JIA/JIAU and gut microbiota abundance using MR analyses. Relative to other research methods, MR analyses have several advantages in the study of the causal relationship between gut microbiota and JIA or JIAU. First, in terms of evidence-based medicine levels of evidence, MR has the third highest evidence-based rating after systematic reviews of randomised controlled trials (RCTs) and RCTs when RCTs are feasible, and the highest evidence-based rating when RCTs are not feasible, ranking first (55). Whereas, due to practical and ethical reasons, random assignment of specific gut flora cannot be done, RCT studies are not feasible when conducting causal studies of gut flora with JIA and JIAU. Observational study data are relatively more readily available and closer to real-world situations, but problems such as smaller sample sizes, confounding factors, and causal inversions often limit inferences about cause and effect (56), so appropriate causal modelling is needed to infer causal associations between exposure factors and disease outcomes. Animal studies have an even lower evidence-based rating. Therefore, for the study of the causal

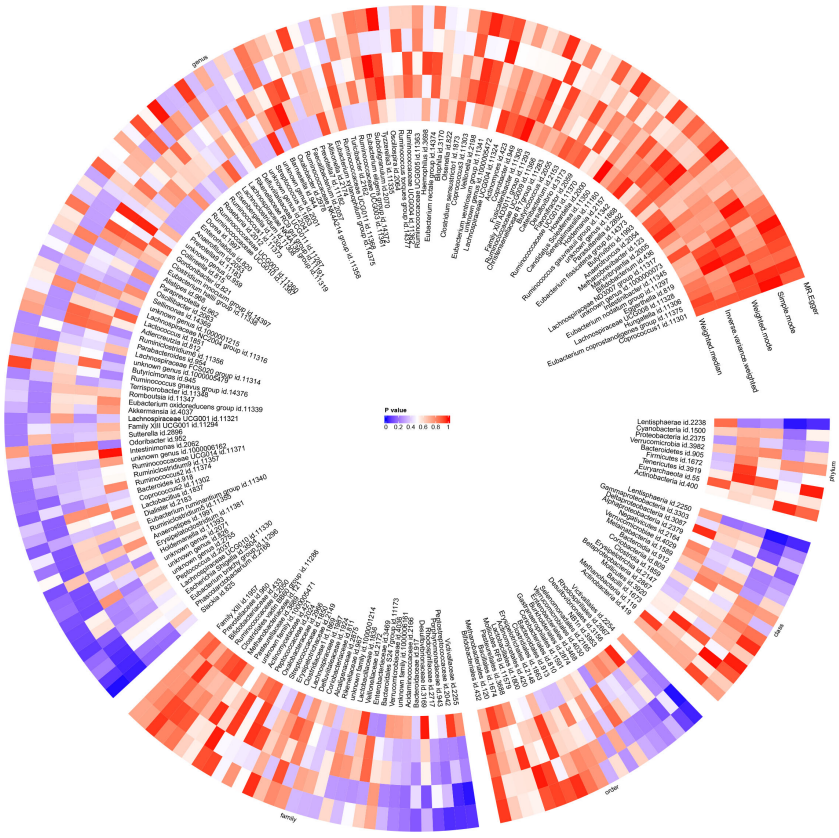

FIGURE 10
The results of Mendelian randomisation study on two sample groups were visualised via a circular heat map, which showed the causal effects estimated by five different Mendelian randomisation methods, with juvenile idiopathic arthritis associated uveitis (JIAU) as the variable of exposure and gut microbiota abundance as the variable of outcome.

relationship of gut microbiota with JIA or JIAU, MR studies provide an effective way to solve the above problems. By utilising MR, which relies on the random allocation of allelic genes at conception, we established a temporal relationship ('cause before effect') between genetic variations and disease development, which is free from the influence of postnatal environmental factors and social behaviours. This approach minimised the risk of reverse causal linkages and allowed for more effective control of confounding variables. Moreover, to ensure the robustness of the auxiliary variables used in the MR analysis, we sourced GWAS data for gut microbiota, JIA and JIAU from the largest available database. Furthermore, to minimise the possible effects of weak IV bias, we adopted appropriate thresholds for the genomic instruments based on a threshold of $p< 1.0\times10^{-5}$ in this bidirectional study. This criterion was selected based on the availability of an adequate number of SNPs with suitable statistical power for the majority of gut microbiota to effectively prevent confounding.

While our study boasts several strengths, it also has certain limitations. Firstly, although our analysis yields statistically significant *p*-values, they may not be as robust as those corrected using the Bonferroni method for significance. Nevertheless, our research is hypothesis-driven and supported by substantial biological data and past studies that establish the epidemiological link between gut microbiota and JIA or JIAU. However, future research may need to include samples from a larger population of patients with JIA and JIAU to further strengthen these results. Secondly, adjusting *p*-values for multiple comparisons may increase the risk of false positives and potentially weaken the number and multi-level structure of microbial communities (abundance and correlation between microbial strains) as well as the correlation between JIA or JIAU. Therefore, cautious interpretation is warranted when considering unfavourable outcomes or potentially significant *p*-values. Furthermore, the two-sample MR method is a theoretical causal analysis method, and the conclusions of our study may contradict epidemiological studies suggesting the impact of environmental risk factors on JIA or JIAU. Additionally, as the specific mechanisms of the gut microbiome in the onset of JIA or JIAU remain unclear, further research, including clinical studies and other ex vivo and *in vivo* studies such as animal experiments, is needed to verify the results of this study. New research techniques and methods such as metagenomics and metabolomics technologies, where available, are needed to further validate the results of the present study and to understand the relationship between the observed intestinal flora and JIA, as well as the mechanisms behind the relationship of JIAU. Lastly, as our study primarily includes participants of European descent in the JIA group, generalising our results to other ethnic populations should be done with caution. When GWAS summary data on JIA, JIAU and gut microbiota from other races become available in the future, we also hope to analyse and study them to improve the applicability and generalisability of these findings.

Conclusion

This study presents compelling evidence supporting a causal association between genetically predicted gut microbiota and JIA or JIAU development. This study underscores the significant interactive influence of intestinal flora on these conditions, suggesting their potential as novel biomarkers for diagnosis and prevention. These findings offer valuable insights that can aid in addressing and mitigating the impact of JIA or JIAU.

Data availability statement

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

Author contributions

J-BH: Conceptualization, Writing – original draft, Writing – review & editing, Formal analysis, Methodology, Validation, Visualization. Y-XC: Formal analysis, Writing – original draft, Writing – review & editing, Methodology, Validation, Visualization. Z-YS: Formal analysis, Validation, Writing – original draft, Writing – review & editing. X-YC: Methodology, Validation, Writing – review & editing. Y-NL: Conceptualization, Validation, Writing – review & editing. J-HY: Conceptualization, Funding acquisition, Writing – review & editing, Validation.

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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.2024.1356414/full#supplementary-material

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Impact of cooperative or competitive dynamics between the yeast *Saccharomyces cerevisiae* and lactobacilli on the immune response of the host

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Fungi and bacteria can be found coexisting in a wide variety of environments. The combination of their physical and molecular interactions can result in a broad range of outcomes for each partner, from competition to cooperative relationships. Most of these interactions can also be found in the human gastrointestinal tract. The gut microbiota is essential for humans, helping the assimilation of food components as well as the prevention of pathogen invasions through host immune system modulation and the production of beneficial metabolites such as short-chain fatty acids (SCFAs). Several factors, including changes in diet habits due to the progressive Westernization of the lifestyle, are linked to the onset of dysbiosis statuses that impair the correct balance of the gut environment. It is therefore crucial to explore the interactions between commensal and diet-derived microorganisms and their influence on host health. Investigating these interactions through co-cultures between human- and fermented food-derived lactobacilli and yeasts led us to understand how the strains' growth yield and their metabolic products rely on the nature and concentration of the species involved, producing either cooperative or competitive dynamics. Moreover, single cultures of yeasts and lactobacilli proved to be ideal candidates for developing immune-enhancing products, given their ability to induce trained immunity in blood-derived human monocytes *in vitro*. Conversely, co-cultures as well as mixtures of yeasts and lactobacilli have been shown to induce an anti-inflammatory response on the same immune cells in terms of cytokine profiles and activation surface markers, opening new possibilities in the design of probiotic and dietary therapies.

KEYWORDS

yeasts, lactobacilli, fermented food, host immune system modulation, *Saccharomyces cerevisiae*, microbial ecology, short-chain fatty acids, trained immunity

1 Introduction

Bacterial-fungal communities exist in virtually all habitats (1, 2), engaging in a variety of interactions within and between species, from symbiosis to competition and predation (3). The human body is an ecological habitat of special importance since numerous populations of bacteria and fungi, that compose the human microbiota together with archaea and viruses, regulate many aspects of human health (4–6). Fungi and bacteria interact in different modes throughout the human body environment: they can directly bind through physical interaction, release, and uptake chemical molecules, proliferate in mixed biofilms, and compete (7). For example, numerous studies on *C. albicans* describe mutualistic interactions with streptococci and competition with lactobacilli (8). On the other hand, *S. cerevisiae* is shown to exert inhibitory effects against *E. coli* (9) and enhance bacterial exopolysaccharide (EPS) production in *Lacticaseibacillus rhamnosus* (10). The interactions between yeasts and lactobacilli as members of the microbiota are not limited to humans, as confirmed by the evidence that *S. cerevisiae* strains reduce potentially pathogenic bacterial genera in the guts of wasps (11).

Humans have benefited from a life-long coexistence with bacterial-fungal communities for millennia, to produce food, antibiotics, and secondary metabolites for pharmacological and biotechnological purposes (12). Fermented foods, such as yogurt, kefir, and kombucha, known to host rich microbial communities, are traditionally part of diets around the world (13). The symbiotic relationship that exists within these microbial communities are not completely understood. An important study by Ponomarova and colleagues (14) demonstrated the role of specific *S. cerevisiae*-produced amino acids in promoting the growth of *Lactiplantibacillus plantarum*, *Lactobacillus plantarum* and *Lactococcus lactis in vitro* and in kefir.

Among the multiple factors that are known to shape both the bacterial and fungal components of the microbiota (15, 16), numerous studies demonstrate diet as a key factor (17–20). Rates of chronic inflammation statuses and non-communicable chronic diseases (NCCDs) are on the rise due to changes in diets, e.g. the so-called "Westernization", a lifestyle condition also characterized by an unbalanced diet in terms of fat-fiber ratio (21, 22), which causes a strong reduction in microbiota diversity (23). On the contrary, fermented foods are the dietary basis for many populations with traditional lifestyles, enriching their microbiota with probiotic microbes with anti-inflammatory properties (24). Several studies support these findings by comparing microbiotas of traditional and industrialized lifestyles (25–28). Experimental diet interventions show significant changes in microbiota composition and immune status (29–31), confirming that the composition and functions of the human gut microbiota strongly influence the overall health status of an individual.

Dysbiosis statuses may impair the resistance to microbial colonization as well as host immune responses and cause the insurgence of several diseases under those with predisposed conditions. In the last decade, scientific research has increasingly explored the links between the microbiota and human health. Evidence has emerged that the composition of the entire community of microbial inhabitants, and not just one or two dominant species, influences a balanced immune response (24, 32). Aberrant immune responses to the gut microbiota, caused by dysbiosis, favor the onset of chronic inflammation and lead to inflammatory bowel diseases (IBD), i.e. ulcerative colitis and Crohn's disease (33–36). Several studies connect dysbiosis with colorectal cancer (37); metabolic diseases such as obesity, type 2 diabetes, and food intolerances (38); neurological disorders through the microbiota-gut-brain (MGB) axis (39, 40); autoimmune and allergic diseases (41–44). In exchange for a favorable colonizing environment, a balanced gut microbiota carries out several beneficial functions for the host. It plays a fundamental role in the synthesis of vitamins and nutrients, as well as in the inhibition of pathogen invasion, by competing for intestinal ecological niches (45) and through the production of metabolites, such as short-chain fatty acids (SCFAs), which reflects in a reduction of virulence gene expression and growth rates of pathogens (46). Under healthy conditions, SCFAs are absorbed by intestinal epithelial cells, leading to the expression of antimicrobial peptides and the maintenance of epithelial integrity (47, 48), and exerting positive effects on the immune system cells in terms of inflammation and gut homeostasis (49).

Human Intestinal Epithelial Cells (IECs) and immune cells recognize microbe-associated molecular patterns (MAMPs) through pattern-recognition receptors (PRRs) and then discriminate between those harmless and pathogenic (50, 51). Effective immune response requires the direct action of innate immunity cells and the production of cytokines for adaptive immunity activation (52). Modulation of the host immune system exerted by the gut microbiota also relies on a so-called trained immunity, defined as a long-term functional modification of innate immune cells that leads to a greater response in case of a second unrelated immune challenge (53). A recent review of trained immunity during mucosal diseases highlights the potential for clinical treatment and emphasizes the importance of microbiota composition in modulating immunity (54).

Present research largely focuses on host-microbiota dynamics and their consequences for health, but the interactions between members of the microbiota are still poorly investigated, especially between beneficial microbes (7). Health status relies on all the microbiota interactions, including those between diet-derived microorganisms (55–57). Here, we performed an explorative investigation of the relationships within co-cultures of *S. cerevisiae* and *Lactobacillus* spp. isolated from different sources, including fermented milk (similar to kefir) previously collected by our group from the Yaghnob Valley in Tajikistan (58), a commercial probiotic, and the stool of a Crohn's disease patient. Our results indicated that co-culture growth yield, trained immunity potential in humans *in vitro*, and SCFA production strongly depend on the natural sources of microorganisms.

2 Materials and methods

2.1 Selection of microbial strains

The bacterial strain of *Lactiplantibacillus plantarum* (B1) was isolated with the manufacturer's consent from a commercial probiotic product that contained mixes of other lactobacilli. The *Lactobacillus delbrueckii* bacterial strain (TJA9) and *Saccharomyces*

cerevisiae yeast strain (CL4) were isolated from a fermented goat milk beverage produced in the Yaghnob Valley in Tajikistan (58). The yeast strain *Saccharomyces cerevisiae* (YH1) was isolated from human fecal samples of a pediatric patient with Crohn's disease (59, 60).

2.2 Culture assays

Given the high nutritional requirements of lactobacilli, which include adequate amino acids, vitamins, carbohydrates, and nucleotides (61, 62), the co-cultures were carried out in in De Man Rogosa Sharpe selective liquid medium (MRS) (Oxoid) + 0.05% cysteine HCl (63). Pre-cultures of lactobacilli were incubated overnight at 37°C in anaerobic conditions in MRS medium + 0.05% cystein HCl, whereas pre-cultures of yeasts were incubated overnight at 30°C in aerobic conditions in Yeast Peptone Dextrose (YPD) medium. Each strain pre-culture was diluted in fresh medium at a concentration of 2 x 10^6 cells/ml for yeast and 2 x 10^6 cells/ml or 2 x 10^7 cells/ml for bacteria, with a yeast:bacteria ratio of 1:1 or 1:10. The inocula were incubated for 24 hours at 37°C in a shake at 200 r.p.m. The choice of temperature was due to the overall aim of the work, i.e. investigating how yeast and lactobacilli grow together in human-related conditions like the gut environment. To ensure that our yeast species could survive and reproduce at such temperature, we measured their growth after 24 hours at 30°C and 37°C. Although, as expected, 30° resulted in a more optimal condition, yeast cells could also grow at 37°C. We also assessed 37°C as optimal for the growth of the co-cultures. A representative result of the growth assays is available in Supplementary Material (Supplementary Figure S2). Several experiments were set up with the goals of studying the growth yield of different yeast strains of *Saccharomyces cerevisiae* and Lactobacillus in co-cultures and investigating the possible interactions that may occur between them. Microbial growth in experimental co-cultures were compared to those in single cultures. The growth of the strains was measured by Bürker chamber counts at different time points, and each experiment was set up in triplicate. The viability of the strains (both in mono-cultures and in co-cultures) was demonstrated by conducting colony counts on agar plates in three different experiments (three technical replicates for each experiment). The statistical test applied to assess differences in viability is the Wilcoxon-Mann-Whitney test (Supplementary Table S1).

2.3 SCFA's analyses

For each GC-MS analysis, single and co-cultures were kept for 24 hours at 37°C in CDM_35, a chemically defined medium already validated for the co-cultures of lactobacilli and yeasts (14). Throughout the experiment, 2 ml of culture supernatants were collected at separate time points every hour for 8 hours in a row and again at completion. The cell growth was monitored by counting at the Bürker chamber. All conditions were set up in triplicate, and then the samples were stored at -80°C.

2.3.1 Chemicals

Methanol and tert-butyl methyl ether (Chromasolv grade), sodium bicarbonate, sodium chloride and hydrochloric acid (Reagent grade), [2H5]Propionic, [2H7]iso-Butyric and [2H9]iso-Valeric (used as internal standards or ISTDs), acetic, propionic, butyric, iso-butyric, valeric, iso-valeric, 2-Methylbutyric, hexanoic, heptanoic, octanoic, and nonanoic acids (analytical standards grade) were purchased by Sigma-Aldrich (Milan, Italy). MilliQ water 18 MΩ cm was obtained from Millipore's Simplicity system (Milan - Italy).

2.3.2 GC-MS method

The qualitative and quantitative evaluation of fatty acids (FAs) was performed using the Agilent gas chromatography-mass spectrometry (GC-MS) system composed of a 5971 single quadrupole mass spectrometer, a 5890 gas-chromatograph, and a 7673 autosampler, through our previously described GC-MS method (64). The FAs were extracted as follows: an aliquot of 1.5 ml of medium culture sample was added to 10 µl of ISTD mixture, 0.5 ml of tert-butyl methyl ether (MTBE), and 100 µl of 6 M HCl, 0.5 M NaCl solution in 2 ml centrifuge tube. Afterward, each tube was stirred in a vortex for 3 minutes, centrifuged at 10,000 rpm, and finally the solvent layer was transferred into an autosampler vial and analyzed. The FAs in the samples were analyzed as free acid form using an Agilent J&W DB-FFAP column 30 m in length, 0.25 mm internal diameter, and 0.25 m of film thickness by using the oven temperatures' program, as follows: initial temperature of 50°C for 1 min, then it was increased to 150°C at 30°C/min, finally grow up to 250°C at 20°C/min was held for 6.67 min. A 1 µl aliquot of extracted sample was injected in splitless mode (splitless time 1 min) at 250°C, while the transfer line temperature was 280°C. The used carrier gas was helium and its flow rate was maintained at 1 mL/min for the whole run time. The MS acquisition was carried out in single ion monitoring (SIM) by applying a proper dwell time (20 ms for each ion monitored) to guarantee an acquisition frequency of 4 cycle/s. The quantitative determination of FAs in each sample was carried out by the ratio between the area abundance of the analytes and the area abundance of the respective labeled internal standard (isotopic dilution method). The value of this ratio was named Peak Area Ratio (PAR) and it was used as the abundance of each analyte in the quantitative evaluation. The ionic FAs' signals and the reference internal standards used for the quantitation of each FAs were reported in Supplementary Table S2.

2.3.3 Data analysis

Before analyzing the FAs concentrations, negative control (fresh medium) values were subtracted from each sample. Samples were analyzed with GraphPad Prism version 9.5.0 for Windows. The performed statistical analysis were Linear regression and a Repeated Measures two-way ANOVA with the Geisser-Greenhouse correction. Time points were compared with Tukey's multiple comparisons test, with individual variances computed for each comparison. Figures and statistics for CL4-B1 and YH1-B1 setups are available in Supplementary Material (Supplementary Tables S3-S14, Supplementary Figures S3, S4).

2.4 Immunological assays

All work with human study participants was approved by the Ethical Committees of the Azienda Ospedaliera Universitaria

(AOU) Careggi (Ref. n. 87/10) and AOU Meyer Children's Hospital (Ref. n. 103/2021), Florence, Italy. The research was carried out according to the principles set out in the Declaration of Helsinki 1964 and all subsequent revisions. Buffy coats were collected from fifteen anonymous healthy donors at the Transfusion Unit at Careggi University Hospital in Florence, Italy. The utilization of donor material, not destined to diagnostic standard procedures and registered with a traceable numeric code, was authorized by the Careggi Transfusion Unit.

2.4.1 Immunomagnetic separation of monocytes

Differential centrifugation with Lympholyte®-H Cell Separation Media (EuroClone) at 2000 rpm for 20 minutes at room temperature separated peripheral blood mononuclear cells (PBMCs) from the remaining blood cell components. The ring of mononuclear cells at the plasma/lymphocyte interface was collected and washed twice with DPBS (Dulbecco's Phosphate Buffered Saline, EuroClone) before centrifuging for 5 minutes at 2000 and 1200 rpm. The cells were resuspended in a sterile saline solution containing DPBS, 1% FBS (fetal bovine serum), and 2 mM EDTA (Buffer MACS). The cells were resuspended and centrifuged at 1200 rpm for 5 minutes. 100 µl of CD14 MicroBeads Human (Miltenyi Biotec) were added to the remaining pellet and incubated at 4°C for 15 minutes. Following the addition of 10 mL of MACS Buffer, CD14+ monocytes were isolated by positive magnetic separation using LS columns and the MidiMACSTM Separator immunomagnetic separator, according to the manufacturer's protocol (Miltenyi Biotec). A volume of 5 ml MACS Buffer was added to the column to recover CD14+ cells. After elution, the cell suspension was centrifuged at 1200 rpm for 5 minutes, and the cell pellet was washed and resuspended in complete RPMI medium supplemented with 10% FBS (100X), 1% sodium pyruvate, 1X nonessential amino acids (100X), 1X Penicillin-Streptomycin (500X), and 20mM L-glutamine. Using the 0.22 m Vacuum Filter System, the entire RPMI culture medium was sterilized by vacuum filtration (EuroClone).

2.4.2 Preparation of stimuli

In vitro experiments of trained immunity induction were carried out using the method described by Rizzetto and colleagues (59). Monocytes (10^6 cells/ml) from fifteen healthy donors were first exposed to low concentrations (10^4 cells/ml) of *S. cerevisiae*, Lactobacillus, or the combination *S. cerevisiae* + Lactobacillus in a 96- well flat bottom plate for 24 hours in a CO_2 incubator at 37°C before being washed to remove all stimuli and incubated for 5 days preserving the same conditions. The cells were subsequently re-stimulated with pure Lipopolysaccharide (LPS, 10 ng/ml) and incubated with the second stimulus for 24 hours.

2.4.3 Quantification of cytokines

The concentration of cytokines IL-6 and TNF-α was determined by ELISA immunoassay, employing the ELISA MAX™ Deluxe Set Human IL-6 and ELISA MAX™ Deluxe Set Human TNF-α (BioLegend), according to the protocol provided by the manufacturer. The inflammatory cytokine panel was measured simultaneously using the MILLIPLEX system (Merck Millipore) to determine whether co-cultures or mixtures induced more production of anti-inflammatory cytokines, such as IL-10, than pro-inflammatory cytokines, such as IL-6 and TNF.

2.4.4 Phenotypic and functional characterization of monocytes

Monocyte surface markers were studied by cytofluorometry using the CyFlow Space 6-color (Sysmex Partec). The anti-human monoclonal antibodies used (Invitrogen) specifically recognized the following antigens (the fluorochrome tags are given in brackets): CD11b (FITC), CD14 (PE), CD80 (FITC), CD86 (APC), HLA-DR (PERCP). Data were acquired with the Sysmex Partec software FloMax.

2.4.5 Data analysis

For the statistical analysis of immunological data, GraphPad Prism 9.5.0 software and the programming environment R 4.3.1 (65) were used. The monocyte markers analysis figure was graphically generated by the ggplot2 package (66). Results were expressed as means ± SEM and the performed statistical tests were one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was for p values < 0.05.

3 Results

3.1 Microbial growth yield in co-culture

To study whether the interactions between yeast and Lactobacillus modify their growth, 24-hour co-cultures have been made between a bacterial and a yeast organism within the four selected strains. Although *S. cerevisiae* and lactobacilli are known to proliferate when grown together, we tested the viability of the strains by co-culturing YH1 and B1. We chose to test these two strains since they come from different biological matrices, while CL4 and TJA9 have been isolated from the same matrix, so we expected them to thrive in co-cultures. As expected, there were no statistically significant differences in the viability of both bacteria and yeasts between mono-cultures and co-cultures (Supplementary Figure S1).

3.1.1 *Lactiplantibacillus plantarum's* growth yield in co-culture depends on cellular concentration

As depicted in Figure 1, in co-cultures of *S. cerevisiae* YH1 (human-derived) and *L. plantarum* B1 (commercial probiotic), the bacteria improved their growth yield after 8 hours of co-culture with the yeast, when the cell concentration ratio was 1:10 (Figure 1A). No significant differences were observed in the growth yield of the bacteria when the concentration ratio was 1:1 (Figure 1B). No change in the growth yield of YH1 in co-culture with B1 was observed (data not shown).

3.1.2 *Lactobacillus delbrueckii's* growth yield is not enhanced in co-culture with *S. cerevisiae*

The increase in the bacterial growth yield shown in Figure 1 led us to wonder whether other lactobacilli experienced the same effect. Figure 2 showed that the L. delbrueckii TJA9 strain did not benefit

from the co-culture with the yeast. On the contrary, its growth yield significantly decreased in co-culture (Figure 2A). The same result was observed using the CL4 yeast strain in co-culture, even if it was isolated from the same biological matrix as TJA9 (Figure 2B).

3.1.3 *S. cerevisiae* growth yield increases in co-culture with *L. delbrueckii*

Given the growth yield reduction of TJA9 with both yeast strains, we checked if it was related to an increase in yeast growth yield under the same conditions. In Figure 3 we showed that both strains of *S. cerevisiae* showed better growth in co-culture with TJA9 than in the single culture, even if this effect seems to reduce after 8 hours (Figure 3).

3.2 Mass spectrometry analysis of short-chain fatty acids

To evaluate if the co-culture between yeast and lactobacillus increases SCFAs production compared to the single cultures, gas-chromatography mass-spectrometry (GC-MS) analyses were performed. Yeast and lactobacilli were grown in a chemically defined medium, which allowed precise quantification of SCFAs produced by the microorganisms.

On average, SCFAs production followed the same pattern between all the co-cultures studied, showing that the values for yeast single cultures were significantly higher than those for bacterial cultures and co-cultures. Three exceptions were found: 2-MethylButyric acid and Valeric acid for the CL4-B1 setup, and Butyric acid for the YH1-B1 setup (Figure 4). Among these, the values of 2-MethylButyric acid for the CL4-B1 setup and Butyric acid for the YH1-B1 setup were significantly higher in the co-cultures than in the yeast cultures in at least one time point (Supplementary Tables S3-S14, Supplementary Figures S3, S4).

3.3 Immune assays

Multiple immune assays were performed to assess the inflammatory and immune-training potential of yeasts and lactobacilli both in single and co-cultures. The tests consisted of an *in vitro* first stimulation of human monocytes with the microorganisms, and subsequent stimulation with LPS after five days, followed by cytokine production levels assessment and immunophenotype analysis (the flow cytometry gating strategy is displayed in Supplementary Figure S5). To investigate whether the effect was due to the mere presence of the two organisms or the co-culture dynamics, a condition made of a mixture of yeast and lactobacilli was added as control. Before other immune assays, the first test was the assessment of monocytes' viability at day 6 after 24 hours of treatment with yeast or bacteria single cultures, co-cultures, and mixtures. The assessment was performed through a specific kit that discriminates alive cells from apoptotic and necrotic ones. The results (Supplementary Figure S6) showed that in almost all conditions the monocytes' viability was above 60% of the total cells. The decrease in viability can be attributed to a physiological death rate of human blood cells cultivated *in vitro* for 6 days.

3.3.1 Yeasts and lactobacilli alone elicit trained immunity responses

Firstly, we assessed the ability of yeasts and lactobacilli, both in single and co-cultures, to stimulate the production of the pro-inflammatory cytokines TNF-α (Figure 5) and IL-6 (Figure 6).

As depicted in Figure 5, both yeast strains have the ability to enhance TNF-α production in monocytes after a second stimulus with LPS, compared with the control (LPS stimulus alone without the previous interaction with the microbe).

Neither the co-cultures nor the mixtures increased the TNF-α production compared to the control. Interestingly, the co-cultures resulted in induced the fewest production of TNF-α, showing a result comparable to the control in the case of CL4-TJA9 (both fermented milk).

Similarly, the IL-6 production by yeasts in single cultures showed an increase compared to the control (Figure 7). In the same way as with TNF-α, co-cultures did not stimulate a higher production of pro-inflammatory cytokines compared to the control. Here, each of the co-cultures resulted in statistically significant lower production of IL-6 compared to that produced by the single yeast culture, with results comparable to the control.

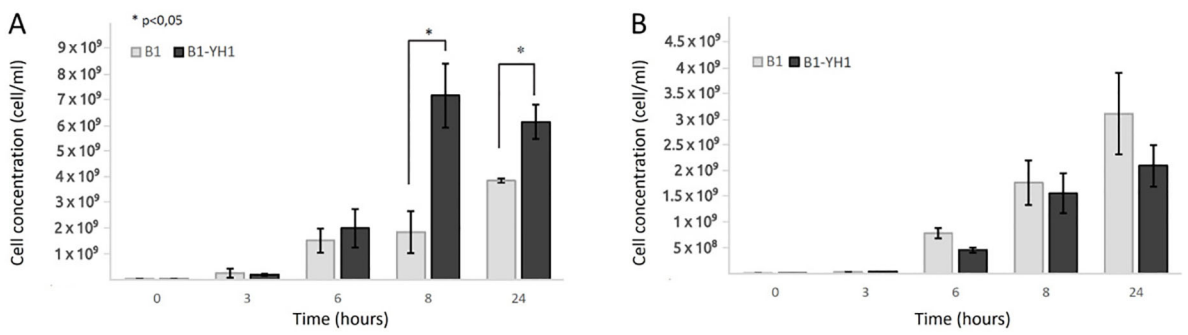

FIGURE 1
Counts over time of *L. plantarum* B1 in single culture and co-culture with *S. cerevisiae* YH1 in MRS medium at 37°C for 24 hours in triplicates, with a yeast-bacteria concentration ratio of 1:10 **(A)** or 1:1 **(B)**. Cellular concentrations were determined by cell counting at the Bürker chamber. Statistical significance was assessed by T-test, * for $p < 0.05$.

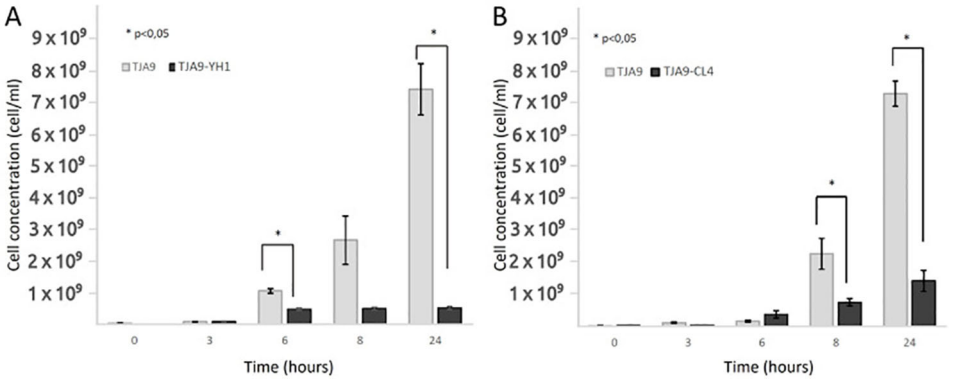

FIGURE 2
Counts over time of *L. delbrueckii* TJA9 in single culture and co-culture with *S. cerevisiae* strains YH1 **(A)** and CL4 **(B)** in MRS medium at 37°C for 24 hours in triplicates. Cellular concentrations were determined by cell counting in the Bürker chamber. Statistical significance was assessed by T-test, * for $p < 0.05$.

3.3.2 Co-cultures and mixtures enhance anti-inflammatory responses

Since co-cultures did not induce the production of the pro-inflammatory cytokines IL-6 and TNF-α, and in some cases a decreased production was observed compared to the control, we wanted to assess whether co-cultures and mixtures were able to induce the production of anti-inflammatory cytokines such as IL-10.

The most noticeable results, which refer to the production of the anti-inflammatory cytokine IL-10 and the pro-inflammatory TNF-α, are shown in Figure 7, while the complete panels are available in Supplementary Material (Supplementary Figure S6).

Here we confirmed that single cultures yeast strains (Figure 7A) induce the production of the pro-inflammatory cytokine TNF-α, whereas both the mixture of YH1 (Crohn's disease) with B1 (commercial probiotic) and the co-culture of CL4 with TJA9 (both isolated from the fermented beverage) strongly increase the production of the anti-inflammatory cytokine IL-10 compared to the control (Figure 7B).

3.3.3 Monocytes treated with co-cultures and mixtures show an anti-inflammatory immunophenotype

To understand if the cytokine profiles were linked to a change in the monocytes' expressions of surface activation markers, an immunophenotype assay was performed in the same treatment conditions.

Results of immunophenotyping (Figure 8) showed that monocytes treated with single cultures of both yeasts and lactobacilli present a surface marker profile resembling those of the control group, i.e. monocytes treated with LPS only. On the contrary, monocytes treated with both co-cultures and mixtures present decreased expression of markers CD14 and CD86.

4 Discussion

Our study explored the relationships within co-cultures of S. cerevisiae (strains YH1 and CL4) and lactobacilli (*Lactiplantibacillus*

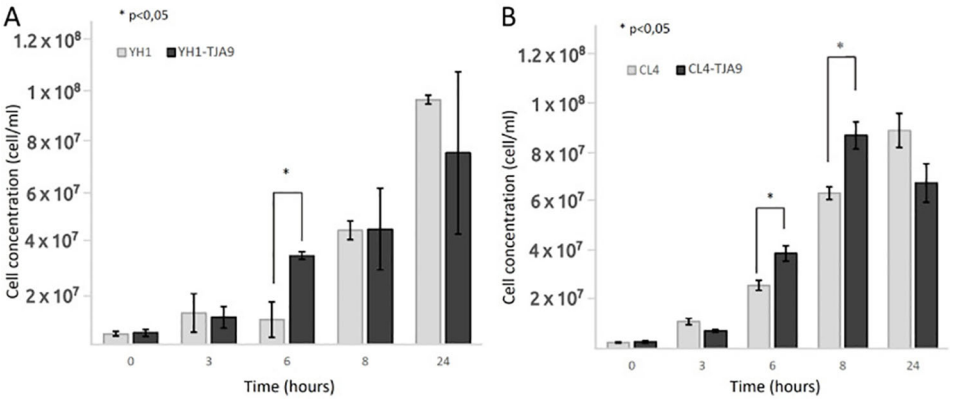

FIGURE 3
Counts over time of *S. cerevisiae* strains YH1 **(A)** and CL4 **(B)** in single culture and co-culture with *L. delbrueckii* TJA9 in MRS medium at 37°C for 24 hours in triplicates, with a yeast-bacteria concentration ratio of 1:10. Cellular concentrations were determined by cell counting at the Bürker chamber. Statistical significance was assessed by T-test, * for $p < 0.05$.

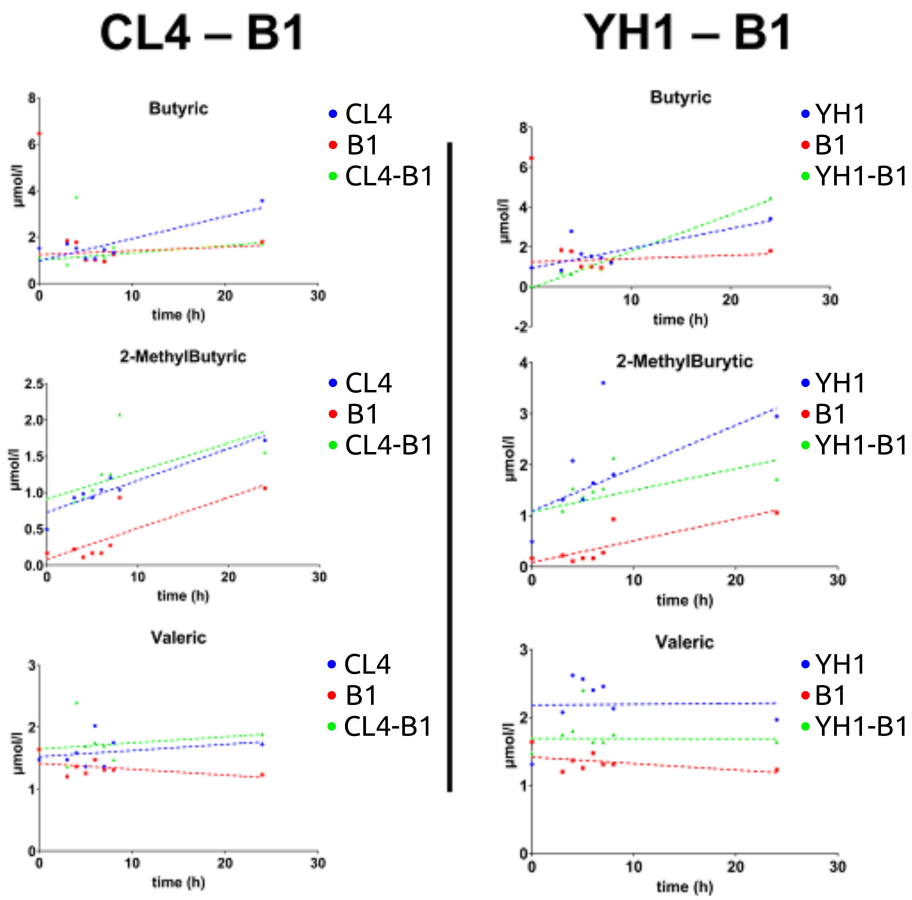

FIGURE 4
SCFAs (Butyric acid, 2-MethylButyric acid, and Valeric acid) production in chemically defined medium represented as time-scaled points and relative linear regression models (dotted lines). The SCFAs production is plotted as concentration (on the y-axis) in 8 different time points (on the x-axis) for the three studied conditions. Blue stands for yeast single cultures, red stands for bacterial single cultures, and green stands for co-cultures. Each value is the mean of three different biological replicates. Statistical significance for each experiment is shown in Supplementary material (Supplementary Tables S3, S6, S7, S10, S12, S13).

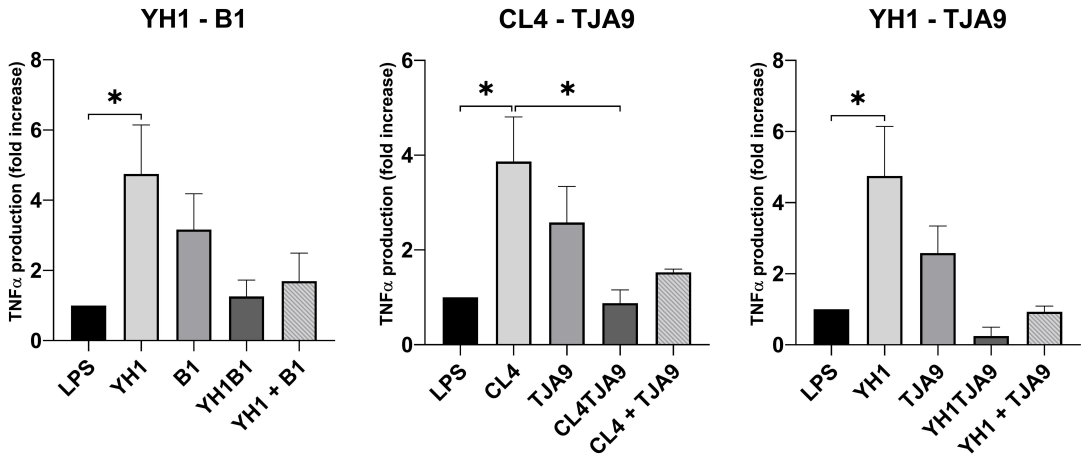

FIGURE 5
TNF-α production by healthy human monocytes after stimulation with diverse single, co-cultures and mixtures of the selected yeast and lactobacillus strains and subsequent stimulation with LPS after 5 days, compared to stimulation with LPS only (LPS columns). Graphs show means and standard errors for 15 independent experiments (N = 15). Statistical significance was assessed by one-way ANOVA; * for $p < 0.05$.

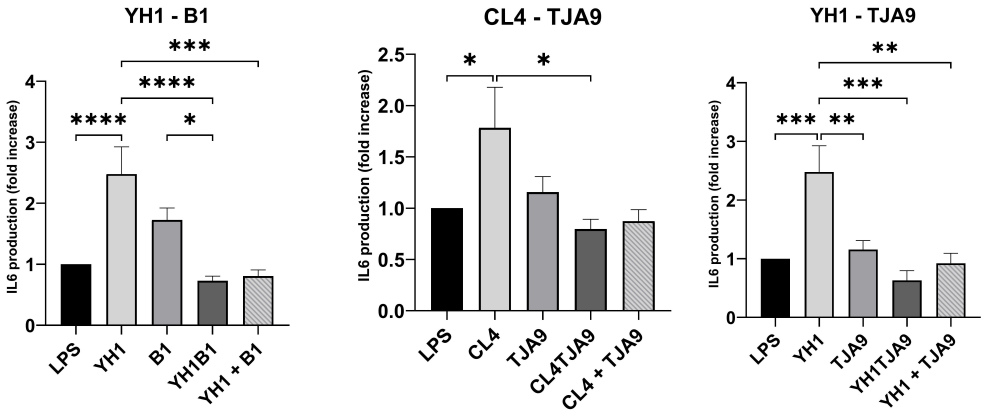

FIGURE 6
IL-6 production by healthy human monocytes after stimulation with diverse single, co-cultures and mixtures of the selected yeast and lactobacillus strains and subsequent stimulation with LPS after 5 days, compared to stimulation with LPS only (LPS columns). Cytokine production is expressed as fold increase compared to control. Graphs show means and standard errors for 15 independent experiments (N = 15). Statistical significance was assessed by one-way ANOVA; * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, **** for $p < 0.0001$.

plantarum, strain B1, and *Lactobacillus delbrueckii*, strain TJA9). Firstly, we identified an increase in *L. plantarum* B1 growth yield when co-cultured with *S. cerevisiae*, but only with a yeast-bacterial cells ratio of 1:10 (which is the ratio commonly found in diverse ecological niches between lactobacilli and yeasts), whereas no changes in bacterial growth yield were observed in the 1:1 ratio. Several studies show the interactions between microbes depend on cellular density, whose increase is due to the increasing production of molecules related to the quorum sensing mechanism (67, 68). These results led us to hypothesize that the growth yield increase of *L. plantarum* only

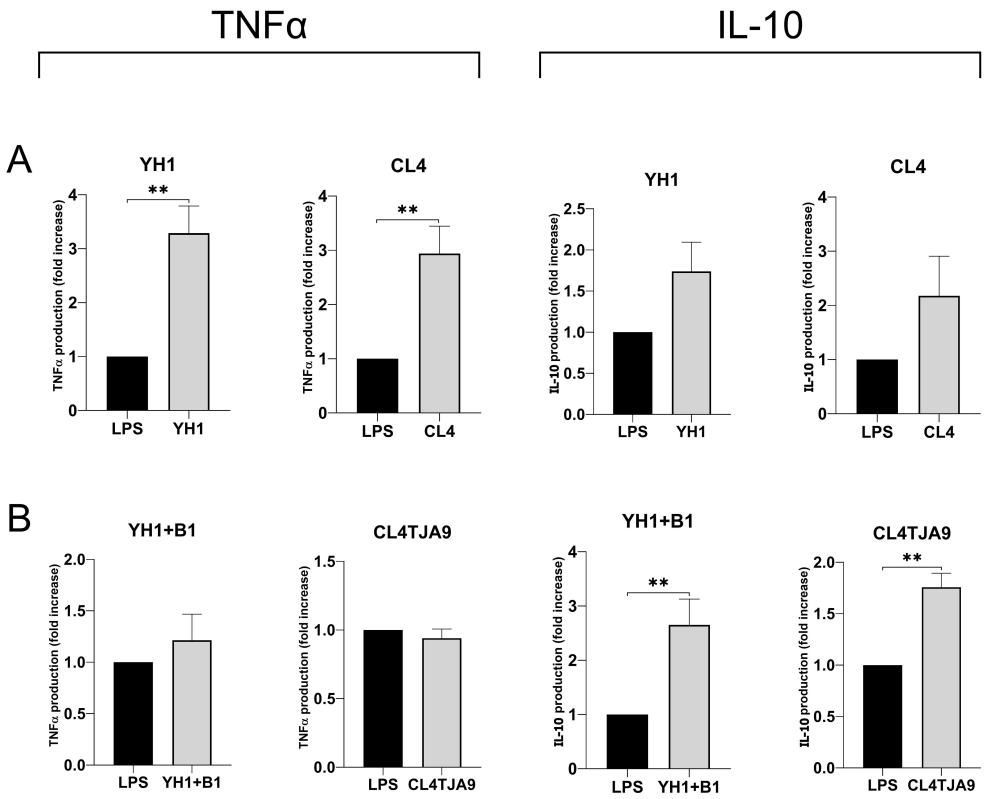

FIGURE 7
TNF-α and IL-10 productions by human monocytes at Day 6 after 24-hour incubation with single culture **(A)**, co-cultures, and mixtures **(B)** of selected yeast and bacterial strains and a subsequent stimulus with LPS at Day 5. Graphs show means and standard errors for 6 independent experiments (N = 6). Statistical significance was assessed by the Mann-Whitney nonparametric test; ** for $p < 0.01$.

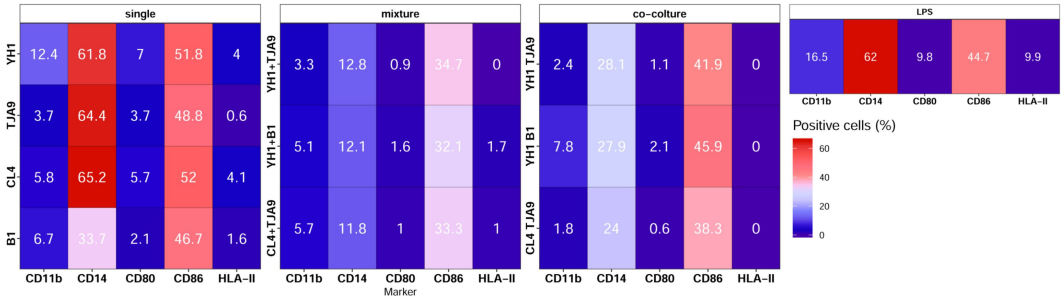

FIGURE 8
Surface markers pattern of human monocytes at Day 6 after 24-hour incubation with single culture, co-cultures, and mixtures of selected yeast and bacterial strains and a subsequent stimulus with LPS at Day 5. Results are expressed as the percentage of cells that showed a specific marker on their surface. Percentages of positive cells are reported on a color scale from the lower values (blue) to the higher ones (red).

in the 1:10 ratio co-culture depends on quorum sensing-associated dynamics. During the observation of microbial growths on the optical microscope, we observed that the 1:10 ratio (and not 1:1) produced yeasts with swollen vacuoles, a marker of cell distress due to hypo-osmotic conditions or glucose deprivation (69). Presumably, yeast cells suffer when largely outnumbered by a bacterial presence. An alternative possible explanation for the growth yield increase of B1 relies on competition mechanisms exerted by some bacterial species (including *L. plantarum*) which have shown the ability to induce the prion [GAR+] in laboratory yeasts, reducing the yeast's ability to ferment glucose (70).

On the contrary, the *L. debrueckii* TJA9 strain's growth yield was reduced in the co-cultures with both yeast strains, suggesting that lactobacilli growth yield in co-cultures depends on the bacterial strain and that the increase of yeast growth yield was not related to the biological matrix of isolation.

We then investigated if yeast metabolites, produced in the co-culture with B1, could favor the growth of TJA9, by cultivating this species in the exhausted media of yeast-B1 co-cultures (data not shown). *L. delbrueckii* did not seem to benefit from the metabolites of the exhausted media, indicating the released metabolites did not modify its growth yield.

One of the goals of this study was to explore the potential of probiotic approaches for Inflammatory Bowel Diseases (IBD), conditions where patients typically show decreased levels of both SCFAs and SCFAs-producing microorganisms (71, 72). To achieve a broader understanding of the possible beneficial effects of yeast-lactobacilli communities, we included an evaluation of the SCFAs production in our investigation. We cultivated all the species, both as single and co-cultures, in a chemically defined medium previously used for the detection of metabolic products of yeast-bacterial co-cultures (14) with the scope of assessing the differences in SCFA's production between strains and conditions through GC-MS. Taking into consideration the B1-CL4 and B1-YH1 co-cultures, results showed that the bacterial single culture produced fewer SCFAs than the other two conditions (yeast single culture and co-cultures), and that, with the exception of Valeric acid, all the SCFAs production increased over time. Another aspect to be noticed is a relatively higher production of 2-MethylButyric acid by B1-CL4 co-cultures with respect to both the single cultures. Butyric acid also showed the same results for the B1-YH1 co-culture. Despite providing insights into the metabolic production dynamics between yeast and lactobacilli communities, the overall SCFAs production resulted in small amounts. This outcome can be convincingly explained by the fact that yeast and lactobacilli are not great producers of SCFAs when compared to other members of the human microbiota, such as bacteria of the Bifidobacteriaceae, Lachnospiraceae, Prevotellaceae and Ruminococcaceae families (73, 74).

Together with SCFAs-producing microorganisms, also commensal fungi have been shown to play a crucial role in IBD pathogenesis and chronicity (35, 36, 75–78). It is worth considering that the first clues on the potential involvement of fungi in IBD came from the observation of an abnormal response to *S. cerevisiae* in Crohn's disease (CD) patients (47). In recent years, Sokol and colleagues observed a reduction of *S. cerevisiae* proportion in CD patients compared with healthy subjects (75). In contrast, Liguori and colleagues observed that *S. cerevisiae* was enriched in CD patients' non-inflamed gut mucosa (77). On the other hand, a recent study reported that *S. cerevisiae* can exacerbate colitis and affect gut barrier permeability (78). All these studies have highlighted the relevance of *S. cerevisiae* in gut inflammation but with controversial outcomes. The wide genetic and phenotypic variability observed for *S. cerevisiae* (48, 60) could explain the inconsistencies in the results of different studies. Therefore, it is likely that both the strain-specificity and the multispecies cross-kingdom interactions taking place in the gut are associated with different patterns of immunomodulation, balancing inflammatory and tolerogenic responses.

Yeasts have been shown to interact with bacteria and other yeasts also through the activation of innate immune responses such as "trained immunity" (51,52, 57). To gain further insight into the immune consequences of our findings, the immunomodulatory potential of lactobacilli and yeasts in single and co-cultures was assessed through multiple immunological assays on human monocytes *in vitro*. Single

cultures of both lactobacilli and yeasts induced a significantly higher production of the pro-inflammatory cytokines TNF-α and IL-6, showing great potential as inducers of trained immunity in human monocytes.

The mixture of the lactobacillus B1 and the yeast YH1, as well as the co-culture of the lactobacillus TJA9 and the yeast CL4 (both isolated from the fermented milk), induced markedly increased production of the anti-inflammatory cytokine IL-10, suggesting a role in dampening the inflammation response. Given the complex composition of the gut microbiota, it is conceivable that the joint activation of receptors for both yeast and bacterial commensal species induces an immune response towards tolerance, resembling a common situation in the gut. In contrast, the exclusive activation of specific receptors for either bacteria or yeasts induces an inflammatory response. These results were consistent with the monocytes' surface markers activation profiles assessed through immunophenotype assays. In fact, monocytes treated with single cultures of bacteria or yeasts presented a profile similar to that of the control group (monocytes treated with LPS only), showing a high activation. When monocytes were treated with mixtures or co-cultures, they showed a reduction in the expression of CD14 and CD86. CD14 is implicated in the recognition of LPS, while CD86 is crucial for the activation of T cells (49, 79, 80). Reduction in the expression of both these receptors could lead to the induction of a tolerance response towards the co-presence of bacteria and yeasts as harmless organisms.

This study took an explorative approach to investigate community dynamics between lactobacilli and yeasts. The results demonstrate that the nature of the interaction, the strains involved, and the concentrations of the cells are crucial factors in determining the outcome in terms of growth yield, metabolic products, and immunomodulatory effects. While single cultures of yeasts and lactobacilli appear to be ideal candidates for developing immune-enhancing products, probiotics containing co-cultures of yeasts and lactobacilli appear as useful tools to induce tolerogenic responses on the same immune cells both in terms of cytokine profiles and activation surface markers.

Since this study was based on *in vitro* interactions between two strains at a time, there are some areas for improvement. Future studies could employ three-dimensional models of reconstituted intestinal tissue and richer microbial communities to obtain results that are more representative of strain interactions in an *in vivo* system. At the same time, our findings, thanks to the use of yeast and lactobacilli strains that can be part of the human gut microbiota and the broad-spectrum analysis of their interactions, open new possibilities in the design of probiotic and dietary therapies.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Ethical Committees of the Azienda Ospedaliera Universitaria (AOU) Careggi (Ref. n. 87/10) and AOU Meyer Children's Hospital (Ref. n. 103/2021), Florence, Italy. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

SN: Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. DR: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. AC: Writing – review & editing, Methodology, Investigation, Conceptualization. RA: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. BC: Writing – review & editing, Methodology, Investigation. MP: Writing – original draft, Methodology, Investigation. GB: Writing – review & editing, Supervision, Methodology. CB: Writing – review & editing, Supervision, Methodology, Conceptualization. DC: Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, 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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1399842/full#supplementary-material

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