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Original Article

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Genetically Predicted Causal Effects of Gut Microbiota and Gut Metabolites on Digestive Tract Cancer: A Two-Sample Mendelian Randomization Analysis

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Abstract

Background: Evidence from numerous observational studies and clinical trials has linked gut microbiota and metabolites to digestive tract cancer. However, the causal effect between these factors remains uncertain.

Methods: Data for this study were obtained from the MiBioGen, TwinsUK Registry, and FinnGen (version R8). Two-sample Mendelian randomization analysis with inverse variance weighting method was primarily used, and the results were validated by heterogeneity analysis, pleiotropy test, and sensitivity analysis.

Results: At $P < 5 \times 10^{-8}$, our analysis identified four gut microbiotas as risk factors for digestive tract cancer and six as risk factors for colorectal cancer. Conversely, one gut microbiota exhibited protection against bile duct cancer, and two showed protective effects against stomach cancer. At $P < 1 \times 10^{-5}$, our investigation revealed five, six, three, eight, eight, and eight gut microbiotas as risk factors for esophageal, stomach, bile duct, liver, pancreatic, and colorectal cancers, respectively. In contrast, four, two, eight, two, two, and five gut microbiotas exhibited protective effects against these cancers. Additionally, *GABA*, a metabolite of gut microbiota, displayed a significant protective effect against colorectal cancer.

Conclusion: In conclusion, specific gut microbiota and metabolites play roles as risk factors or protective factors for digestive tract cancer, and a causal relationship between them has been established, offering novel insights into gut microbiota-mediated cancer development.

Keywords: Gut microbiota; Gut metabolites; Digestive tract cancer; Causal effect; Mendelian randomization

Introduction

The gut microbiota emerges as a pivotal determinant of host well-being, exerting its influence from the earliest stages of life [1, 2]. Among the diverse microbial communities inhabiting the human body, the gut microbiome has garnered considerable attention and extensive exploration [3]. Mounting evidence implicates intricate associations between cancer development and somatic mutations, epigenetic modifications in neoplastic cells, as well as interplay among host genetic variations, immune responses, environmental exposures, and the microbiome [4]. Leveraging cutting-edge molecular tools, researchers are progressively unraveling the intricate dynamics between the host and diverse microbial entities, wherein the gut microbiota has been recognized as a potentially influential risk or protective factor across a spectrum of diseases, including cancer [5, 6]. Despite the weighty epidemiological evidence, discerning the precise contribution of microbes to human cancer pathogenesis remains a formidable challenge, warranting meticulous inquiry into the underlying genetic and molecular mechanisms that underpin their putative causal relationship.

Over the past decade, genome-wide association studies (GWAS) have revolutionized the landscape of complex disease genetics by examining millions of genetic variants to uncover associations between genotypes and phenotypes [7]. By investigating the relationship between common single-nucleotide polymorphisms (SNPs) and diseases or other phenotypic traits on a genome-wide scale, GWAS have opened up novel avenues for comprehending the underlying mechanisms of complex diseases. Additionally, Mendelian randomization (MR) analyses employ genetic variants, typically SNPs, as instrumental variables (IVs) to infer potential causal relationships between exposures and outcomes [8-10]. Since SNPs are randomly allocated at the point of conception and remain unaffected by confounding variables, the impact of such confound-

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ing factors can be minimized. In addition, because the genotype of an individual's exposure is determined at the time of conception, this cannot be changed, whereas the SNPs for the outcome are de-matched by exposure, meaning that the exposure precedes the outcome. Therefore, there will be no reverse causality where causality is associated with genotype, which is another advantage of MR. To summarize, MR analysis offers more robust evidence for inferring causality than traditional observational studies [10, 11].

Given the absence of studies investigating the causal link between gut microbiota, gut metabolites, and digestive tract cancer, we undertook a population-based study on a European cohort to examine this relationship. The objective of our study was to ascertain the existence of a causal relationship between gut microbiota, gut metabolites, and digestive tract cancer. The findings from this study will contribute to a deeper understanding of the involvement of gut microbiota and metabolites in the pathogenesis of digestive tract cancer, leading to the development of more targeted cancer surveillance protocols. These protocols aim to enable early detection of cancer or pre-cancerous lesions, thereby reducing the burden on healthcare resources.

Materials and Methods

A two-sample MR design was employed to investigate the causal relationship between gut microbiota, gut metabolites, and the risk of developing digestive tract cancer. In this MR study, we considered gut microbiota as the exposure factor and digestive tract cancer as the outcome. To fulfill the requirements of the MR approach, independent genetic variants were utilized as IVs, and they needed to satisfy three crucial assumptions [11]: 1) strong association of IVs with the exposure; 2) absence of pleiotropic associations between IVs and any known confounding factors; and 3) absence of prognostic associations, except potentially with the exposure. To ensure the integrity of the analysis, genetic data pertaining to gut microbiota, gut metabolites, and digestive tract cancer were extracted from separate GWAS datasets, thereby eliminating sample overlap. A comprehensive overview of this MR study can be found in Figure 1.

Exposure data

Summary data on gut microbiota were collected from two sources: The MiBioGen (international consortium MiBioGen) and TwinsUK Registry (The UK Adult Twin Registry), which included five levels (phylum, class, order, family, and genus), 212 taxa, 18,340 participants and one level (species), four taxa, 1,126 twin pairs, respectively. Additionally, data on gut metabolites were derived from the Framingham Heart Study (FHS), encompassing eight types of metabolites (*betaine, β-hydroxybutyric acid (BHB), carnitine, choline, γ-aminobutyric acid (GABA), propionic acid, serotonin, trimethylamine N-oxide (TMAO)*), with a total of 2,076 participants included in the analysis. The specifics are shown in Table 1. No weak IVs were identified among the exposure factors, and all F-statistics exceeded 10, indicating minimal bias due to weak IVs (Supplementary Tables S1, S2, and S3, www.wjon.org). The MiBioGen dataset, a large multiethnic GWAS collaboration, consisted of 18,340 participants from 16 cohorts across various countries, including the United States, Canada, Israel, Korea, Germany, Denmark, the Netherlands, Belgium, Sweden, Finland, and the United Kingdom. This dataset included 24S ribosomal RNA gene sequencing and genotype data, enabling the exploration of the association between human autosomal gene variants and the gut microbiome [12]. The Twins UK Registry, established in 1993 at King's College London, is the largest adult twin program in the UK. The registry comprises individuals ranging in age from 16 to 98 years and aims to investigate the genetic and environmental factors underlying complex traits and diseases [13]. FHS is the world's largest population-based family study, characterized by a substantial sample size and a long observation period spanning three generations. The dataset is rich in cardiometabolic phenotype information and is based on family structure. It provides a unique opportunity to examine the impact of genetic, environmental, and clinical factors on the plasma metabolome [14, 15].

IV selection

We conducted a comprehensive analysis of bacterial taxa at six levels (phylum, class, order, family, genus, and species), considering each unique taxon as a characteristic. To ensure the reliability of our conclusion regarding the causal relationship between gut microbiota, gut metabolites, and the risk of developing digestive tract cancer, we implemented a rigorous quality control process to select the most suitable IVs. First, we employed two thresholds to identify SNPs significantly associated with gut microbiota and gut metabolites as IVs. Using a genome-wide significance threshold of $P < 5 \times 10^{-8}$, only a limited number of IVs were available. To achieve a more comprehensive exploration of the potential causal relationship, we employed a lower genome-wide significance threshold of $P < 1 \times 10^{-5}$, resulting in the inclusion of additional IVs. Second, we applied a minor allele frequency (MAF) threshold of 0.01 for the variant of interest. Third, to mitigate bias, we selected exposure factors that were SNPs with no significant linkage disequilibrium (LD) and a closely pairwise correlation ($R^2 < 0.01$), while considering a clumping distance of 10,000 kb. Fourth, we excluded palindromic SNPs (e.g., those with A/T or G/C alleles) to avoid potential issues related to strand orientation or allele coding. Additionally, we carefully compared the alleles with the human genome reference sequence (build 37) and removed ambiguous or duplicated SNPs. These stringent criteria were applied to ensure the validity and robustness of our analyses and to minimize potential biases in our results.

Outcome data

We utilized aggregated data from the FinnGen (R8) database that encompasses cancer-related GWAS studies. The pooled data consisted of information on digestive tract cancer, esophagus cancer, stomach cancer, bile duct cancer, liver cancer,

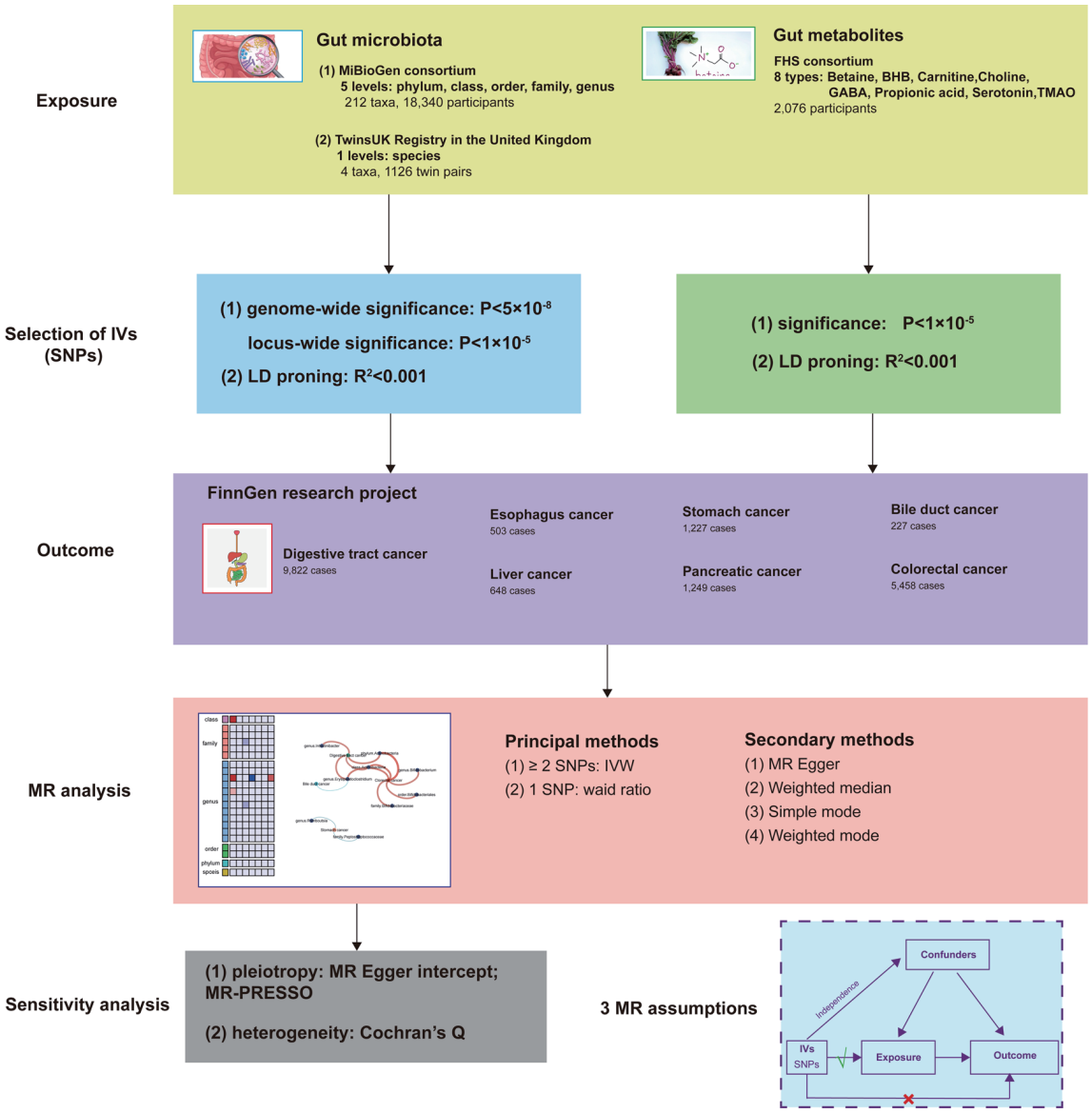


Figure 1. Study design of the two-sample MR for the effect of the genetically predicted gut microbiome and gut metabolites on digestive tract cancer. MiBioGen: international consortium MiBioGen; TwinsUK Registry: the UK adult twin registry; FHS: Framingham Heart Study; LD: linkage disequilibrium; SNP: single-nucleotide polymorphism; MR: Mendelian randomization; IVs: instrumental variables; IVW: inverse-variance weighted.

pancreatic cancer, and colorectal cancer, with respective European population sizes of 9,822, 503, 1,227, 227, 648, 1,249, and 5,458 individuals (Table 1). The FinnGen database was established through a collaborative effort between academia and industry, with the aim of exploring genotype-phenotype correlations within the Finnish population and gaining insights into the impact of the genome on health [16].

MR analysis and sensitivity analysis

To explore potential causal relationships, we conducted a two-sample unidirectional MR study to investigate the association between gut microbiota, gut metabolites, and various digestive tract cancer, including esophagus cancer, stomach cancer, bile duct cancer, liver cancer, pancreatic cancer, and colorectal cancer. The primary analysis method used was the inverse-variance weighted (IVW) test [17], employing the IVW method when the number of SNPs was ≥ 2, and the weighted ratio method for analysis when only one SNP was available. Additional complementary methods, including weighted mode [18], MR-Egger intercept [19], weighted median [20], and simple mode [21], were also employed. The IVW method has been reported to exhibit slightly greater strength compared to other methods under specific conditions [20].

To ensure the robustness of the results, we conducted a

Table 1. Description of Gut Microbiota, Metabolites, and Digestive Tract Cancer

Traits	Consortium	Sample size	Populations	Year	Journal
Gut					
Gut microbiota	MiBioGen	18,340 individuals	European	2021	Nature Genetics
	TwinsUK Registry	1,126 individuals	European	2016	Cell Host & Microbe
Gut metabolites	FHS	2,076 individuals	European	2013	Cell Metabolism
Cancer					
Digestive tract cancer	FinnGen (R8)	9,822 cases	European	2022	Nature
Esophagus cancer	FinnGen (R8)	503 cases	European	2022	Nature
Stomach cancer	FinnGen (R8)	1,227 cases	European	2022	Nature
Bile duct cancer	FinnGen (R8)	227 cases	European	2022	Nature
Liver cancer	FinnGen (R8)	648 cases	European	2022	Nature
Pancreatic cancer	FinnGen (R8)	1,249 cases	European	2022	Nature
Colorectal cancer	FinnGen (R8)	5,458 cases	European	2022	Nature

sensitivity analysis. First, we employed MR-PRESSO [22] and MR-Egger intercept to assess potential horizontal pleiotropy. The MR-PRESSO test helped identify and exclude SNPs that might introduce bias, with a P-value > 0.05 indicating the absence of horizontal pleiotropy. The remaining SNPs were used for MR analysis. Deviation of the MR-Egger intercept from the origin suggested potential horizontal pleiotropy effects of the IVs, with a P-value < 0.05 indicating such effects, while a P-value ≥ 0.05 suggested no evidence of horizontal pleiotropy among the selected IVs. Second, we employed the weighted median as an additional sensitivity analysis to evaluate the robustness of the MR estimates. Third, we calculated the F-statistic to assess weak instrumental bias, considering a F-statistic < 10 as indicative of weak IVs that may introduce bias and should be excluded [23]. Furthermore, Cochran's Q statistic was used to assess heterogeneity in the IVW model, with a Q value greater than the number of instruments minus 1, suggesting the presence of heterogeneity and invalid instruments. A P-value < 0.05 indicated the possible existence of heterogeneity [24].

All statistical analyses were performed using R (version 4.0.2) and the TwoSampleMR package (version 0.5.6), which are open-source software tools.

Ethics approval and consent to participate

Ethical approval was not required for this study because our analysis used publicly available GWAS summary data, and these original GWAS had previously been approved by the appropriate ethical and institutional review boards.

Results

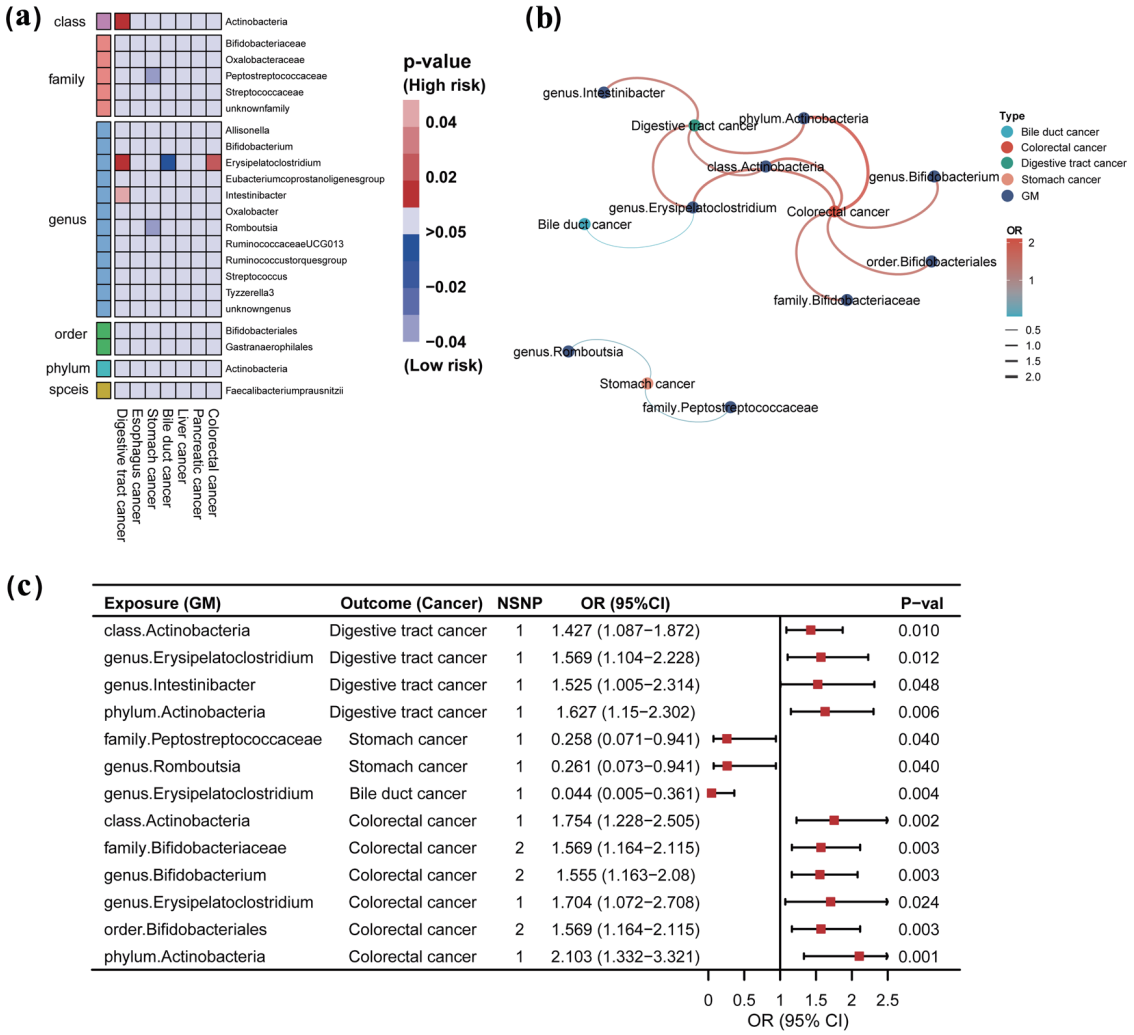
SNP selection

After a quality control step, we identified 215 and 22 gut microbiota exposure phenotypes at the genus, family, order, class, phylum, and species levels of significance at $P < 1 \times 10^{-5}$ and $P < 5 \times 10^{-8}$, respectively, with a significance level of 131, 35, 20, 16, 9, 4 and 18, 5, 2, 1, 1, 2, respectively (Fig. 2a and Supplementary Figure S1A, www.wjon.org). At a significance level of $P < 1 \times 10^{-5}$, eight gut metabolites were finally identified as exposure phenotypes, namely *betaine, BHB, carnitine, choline, GABA, propionic acid, serotonin,* and *TMAO* (Fig. 3). The final number of IVs selected for each gut microbiota and gut microbiota metabolite is detailed in Supplementary Table S4 (www.wjon.org).

Causal relationship between gut microbiota and digestive tract cancer

Genome-wide statistical significance threshold $P < 5 \times 10^{-8}$

At a significance level of $P < 5 \times 10^{-8}$, our analysis revealed a causal relationship between specific gut microbiota and digestive tract cancer, including stomach, bile duct, and colorectal cancers (Fig. 2a, b). The IVW analyses indicated that certain microbial taxa, such as genus *Erysipelatoclostridium* (odds ratio (OR) = 1.569), class *Actinobacteria* (OR = 1.427), phylum *Actinobacteria* (OR = 1.627), and genus *Intestinibacter* (OR = 1.525), were associated with an increased risk of digestive tract cancer when considered as exposure factors. Similarly, for colorectal cancer, the presence of genus *Erysipelatoclostridium* (OR = 1.704), class *Actinobacteria* (OR= 1.754), family *Bifidobacteriaceae* (OR = 1.569), genus *Bifidobacterium* (OR = 1.555), order *Bifidobacteriales* (OR = 1.569), and phylum *Actinobacteria* (OR = 2.103) were associated with an increased risk. Interestingly, we also identified some protective factors, such as genus *Erysipelatoclostridium* (OR = 0.044) associated with a reduced risk of bile duct cancer, and family *Peptostreptococcaceae* (OR = 0.258) and genus *Romboutsia* (OR = 0.261) associated with a reduced risk of stomach cancer when used as exposure factors. For further details, refer to Figure 2c, Supplementary Tables S5 and S6



(c)

Exposure (GM)	Outcome (Cancer)	NSNP	OR (95%CI)	P−val
class.Actinobacteria	Digestive tract cancer	1	1.427 (1.087−1.872)	0.010
genus.Erysipelatoclostridium	Digestive tract cancer	1	1.569 (1.104−2.228)	0.012
genus.Intestinibacter	Digestive tract cancer	1	1.525 (1.005−2.314)	0.048
phylum.Actinobacteria	Digestive tract cancer	1	1.627 (1.15−2.302)	0.006
family.Peptostreptococcaceae	Stomach cancer	1	0.258 (0.071−0.941)	0.040
genus.Romboutsia	Stomach cancer	1	0.261 (0.073−0.941)	0.040
genus.Erysipelatoclostridium	Bile duct cancer	1	0.044 (0.005−0.361)	0.004
class.Actinobacteria	Colorectal cancer	1	1.754 (1.228−2.505)	0.002
family.Bifidobacteriaceae	Colorectal cancer	2	1.569 (1.164−2.115)	0.003
genus.Bifidobacterium	Colorectal cancer	2	1.555 (1.163−2.08)	0.003
genus.Erysipelatoclostridium	Colorectal cancer	1	1.704 (1.072−2.708)	0.024
order.Bifidobacteriales	Colorectal cancer	2	1.569 (1.164−2.115)	0.003
phylum.Actinobacteria	Colorectal cancer	1	2.103 (1.332−3.321)	0.001

Figure 2. Causal effects of gut microbiome and digestive tract cancer based on the IVW method (SNPs with $P < 5 \times 10^{-8}$). (a) Heatmap of the P-value. Red color in the heatmap indicates a positive correlation between gut microbiome and digestive tract cancer and blue color indicates a negative correlation. The color depth represents the size of the P-value, with darker colors indicating more significant P-values. (b) Network interactions based on statistically significant ORs. Color and line thickness in the network diagram indicate the OR of gut microbiota and digestive tract cancer. Darker red color and thicker lines indicate higher OR values. (c) Forest plot with statistically significant ORs. OR > 1 indicates the positive correlation between a particular gut microbiota and a particular digestive tract cancer. OR < 1 indicates the negative correlation between the two. IVW: inverse-variance weighted; GM: gut microbiome; SNP: single-nucleotide polymorphism; OR: odds ratio; 95% CI: 95% confidence interval; NSNP: number of SNP.

(www.wjon.org).

These results were statistically significant ($P < 0.05$), and the MR-Egger regression and Cochran's Q test provided no evidence of bias or heterogeneity (Supplementary Table S7, www.wjon.org).

Genome-wide statistical significance threshold $P < 1 \times 10^{-5}$

At a significance level of $P < 1 \times 10^{-5}$, we observed a broader range of causal associations between gut microbiota and digestive tract cancer, including esophageal, stomach, bile duct, liver, pancreatic, and colorectal cancers. The IVW analyses revealed the following findings (Supplementary Tables S8 and S9, www.wjon.org).

For esophageal cancer, certain microbial taxa such as family *ClostridialesvadinBB60group* (OR = 2.164), genus *unknowngenus.id.1000000073* (OR = 2.164), class *Alphaproteobacteria* (OR = 2.360), genus *Coprococcus2* (OR = 2.396), and genus *Sellimonas* (OR = 1.438) were identified as risk factors, while family *Lachnospiraceae* (OR = 0.501), genus *Butyricimonas* (OR = 0.544), genus *CandidatusSoleaferrea* (OR = 0.532), and phylum *Verrucomicrobia* (OR = 0.5260) were protective factors (Supplementary Figure S1B and D, www.wjon.org).

For stomach cancer, genus *Erysipelatoclostridium* (OR =

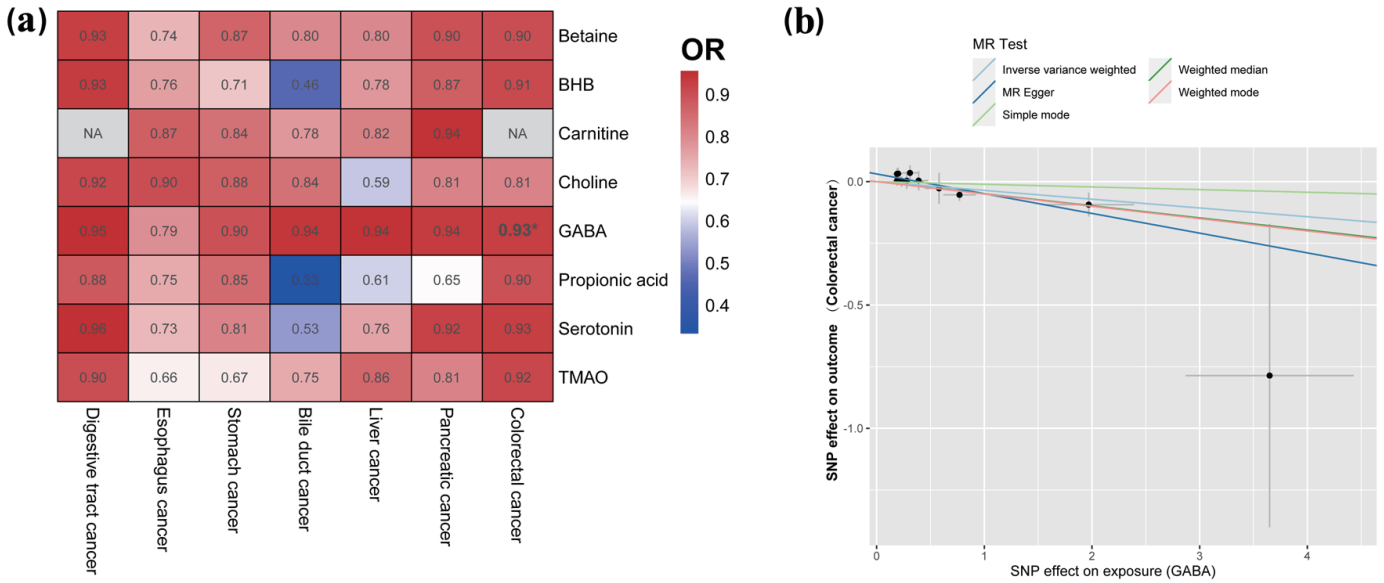


Figure 3. Causal effects of gut metabolite and digestive tract cancer based on the IVW method (SNPs with $P < 1 \times 10^{-5}$). (a) Heatmap of the ORs. Color in the heatmap indicates the OR of gut metabolite and digestive tract cancer. Darker red color indicates higher OR values. The asterisk indicates statistically significant OR. (b) Correlation between *GABA* and colorectal cancer. IVW: inverse-variance weighted; SNP: single-nucleotide polymorphism; OR: odds ratio; MR: Mendelian randomization.

1.540), genus *Olsenella* (OR = 1.257), genus *Roseburia* (OR = 1.589), genus *RuminococcaceaeUCG014* (OR = 1.524), genus *Streptococcus* (OR = 1.641), and genus *unknowngenus.id.959* (OR = 1.344) were associated with an increased risk, while genus *LachnospiraceaeFCS020group* (OR = 0.658) and genus *RuminococcaceaeUCG004* (OR = 0.611) were protective factors (Supplementary Figure S1B and E, www.wjon.org).

For bile duct cancer, genus *Alloprevotella* (OR = 2.824), genus *Tyzzerella3* (OR = 2.688), and order *Lactobacillales* (OR = 3.179) were identified as risk factors, while class *Negativicutes* (OR = 0.310), family *unknownfamily.id.1000001214* (OR = 0.476), genus *Anaerotruncus* (OR = 0.3402), genus *Howardella* (OR = 0.574), genus *unknowngenus.id.1000001215* (OR = 0.476), order *Gastranaerophilales* (OR = 0.476), order *Selenomonadales* (OR = 0.310), and species *Akkermansiamuciniphila* (OR = 0.743) were protective factors (Supplementary Figure S1B and F, www.wjon.org).

For liver cancer, genus *Barnesiella* (OR = 2.177), family *Enterobacteriaceae* (OR = 2.515), family *unknownfamily.id.1000005471* (OR = 1.810), genus *Oscillospira* (OR = 2.121), genus *unknowngenus.id.1000005472* (OR = 1.810), genus *unknowngenus.id.2001* (OR = 1.742), order *Enterobacteriales* (OR = 2.515), and order *MollicutesRF9* (OR = 1.810) were identified as risk factors, while genus *Turicibacter* (OR = 0.577) and phylum *Firmicutes* (OR = 0.570) were protective factors. Furthermore, among the risk factors identified for liver cancer, including family *Enterobacteriaceae* (OR = 1.288), family *unknownfamily.id.1000005471* (OR = 1.159), genus *unknowngenus.id.1000005472* (OR = 1.159), order *Enterobacteriales* (OR = 1.288), and order *MollicutesRF9* (OR = 1.159), we observed that they were also associated with an increased risk of developing digestive tract cancer (Supplementary Figure S1B and G, www.wjon.org).

For pancreatic cancer, genus *Flavonifractor* (OR = 2.124), genus *unknowngenus.id.2041* (OR = 1.555), class *Erysipelotrichia* (OR = 1.616), family *Erysipelotrichaceae* (OR = 1.616), genus *Streptococcus* (OR = 1.653), genus *Terrisporobacter* (OR = 1.671), genus *Victivallis* (OR = 1.258), and order *Erysipelotrichales* (OR = 1.616) were associated with an increased risk, while class *Lentisphaeria* (OR = 0.697) and order *Victivallales* (OR = 0.697) were associated with a decreased risk. Within the identified risk factors, we observed that genus *Terrisporobacter* (OR = 1.326) and genus *Victivallis* (OR = 1.103) were specifically associated with an increased risk of developing digestive tract cancer (Supplementary Figure S1B and H, www.wjon.org).

For colorectal cancer, family *Porphyromonadaceae* (OR = 1.699), class *Coriobacteriia* (OR = 1.319), family *Coriobacteriaceae* (OR = 1.319), family *Enterobacteriaceae* (OR = 1.494), family *Lactobacillaceae* (OR = 1.346), genus *Sellimonas* (OR = 1.142), order *Coriobacteriales* (OR = 1.319), and order *Enterobacteriales* (OR = 1.494) were associated with an increased risk, while class *Bacteroidia* (OR = 0.766), genus *Eubacteriumfissicatenagroup* (OR = 0.854), order *Bacteroidales* (OR = 0.766), phylum *Bacteroidetes* (OR = 0.680), and phylum *Cyanobacteria* (OR = 0.820) were protective factors. Among the identified factors, we observed that family *Coriobacteriaceae* (OR = 1.209), family *Enterobacteriaceae* (OR = 1.288), family *Lactobacillaceae* (OR = 1.213), family *Porphyromonadaceae* (OR = 1.389), genus *Sellimonas* (OR = 1.103), and order *Coriobacteriales* (OR = 1.209) were all associated with an increased risk of digestive tract cancer. On the other hand, phylum *Bacteroidetes* (OR = 0.790) was associated with a reduced risk of developing digestive tract cancer

(Supplementary Figure S1B and I, www.wjon.org).

All these results showed statistical significance ($P < 0.05$). The MR-Egger regression and Cochran's Q test indicated no evidence of bias or heterogeneous associations (Supplementary Table S10, www.wjon.org).

Causal relationship between gut metabolites and digestive tract cancer

In our analysis of the causal relationship between gut metabolites and digestive tract cancer, we conducted an IVW analysis on eight specific metabolites (at a significance level of $P < 1 \times 10^{-5}$): betaine, BHB, carnitine, choline, *GABA*, propionic acid, serotonin, and TMAO. Among these metabolites, our findings revealed that *GABA*, a metabolite produced by the gut microbiota, exhibited a protective effect against colorectal cancer (OR = 0.965) (Fig. 3, Supplementary Tables S11 and S12, www.wjon.org). The P-values obtained from the MR-Egger regression and Cochran's Q test were both greater than 0.05, indicating no evidence of bias or heterogeneous associations (Supplementary Table S13, www.wjon.org).

Sensitivity analysis

To ensure the robustness of our MR causal effect estimates, we performed several sensitivity analyses. These included the utilization of MR-Egger, weighted mode, simple mode, and weighted median estimator (WME) methods. Across all analyses, no evidence of a horizontal pleiotropic effect was detected, as indicated by P-values greater than 0.05. Furthermore, no outliers were identified in the MR-PRESSO analyses, and the Cochran's Q test revealed no significant heterogeneity ($P > 0.05$) (Supplementary Table S14, www.wjon.org).

Discussion

Our study utilized genetic variants from the largest intestinal microflora GWAS, focusing on those that exhibited strong associations with comprehensive genetic data. Through our investigation, we identified several gut microbiota and metabolites that potentially serve as risk or protective factors for digestive tract cancer, establishing a causal relationship between them. These findings hold significant implications for public health interventions aimed at reducing the risk of cancer. Notably, our study represents the first of its kind in exploring the causal relationship between gut microbiota, gut metabolites, and digestive tract cancer using MR analysis, as no prior studies have been published on this subject.

An increasing body of research has revealed a potential causal link between the gut microbiota we have selected and various digestive tract cancer, including esophageal, pancreatic, stomach, bile duct, liver, and colorectal cancers [25-30]. Additionally, several other studies have demonstrated associations between metabolites produced by the gut microbiota and the risk of developing digestive tract cancer [31-34].

In a specific study, *15S rRNA* gene sequencing was utilized to compare the fecal microbiota composition of 16 patients diagnosed with esophageal squamous cell carcinoma (ESCC) and 16 healthy individuals serving as control subjects. The findings of this study indicated that the gut microbiota of ESCC patients exhibited potential enrichment in pro-inflammatory and/or oncogenic bacteria (e.g., *Butyricimonas*, *Veillonella*, and *Streptococcus*), while simultaneously showing depletion of butyrate-producing and/or potentially anti-inflammatory bacteria (e.g., *Butyricicoccus*, *Lachnospiraceae NK4A136 group*, and *Eubacterium eligens group*). Moreover, investigations have identified logarithmic ratios between *Streptococcus* and *Butyricicoccus*, as well as *Streptococcus* and *Lachnospiraceae NK4A136 group*, as potential diagnostic biomarkers for ESCC [35]. However, it is important to note that our study's results suggest a potentially different role for *Butyricimonas* and *Lachnospiraceae* as protective factors against esophageal cancer. Further studies are required to validate these findings and provide more conclusive evidence.

Studies have consistently observed an elevated presence of *Streptococcus* in the intestines of patients diagnosed with stomach cancer [36-40]. Furthermore, investigations examining postoperative specimens of stomach cancer have reported an increased abundance of *Streptococcus* in stomach cancer tissues, suggesting its potential involvement as a causative agent in stomach cancer [41-43]. In alignment with these findings, our study also identified an increased risk of stomach cancer when *Streptococcus* was considered as an exposure factor. Additionally, another study noted that patients with gastric intraepithelial neoplasia exhibited an enrichment of specific intestinal commensals in their gastric microbiota, including *Romboutsia*, *Fusicatenibacter*, *Prevotellaceae-Ga6A1-group*, and *Intestinimonas*, which demonstrated a protective effect [44]. Consistent with these observations, our study revealed a reduced risk of stomach cancer when genus *Romboutsia* was utilized as an exposure factor.

Our study supports the finding that *Lactobacillales* is a risk factor for bile duct cancer. Similarly, a study conducted by Jia et al [45] investigated the fecal microorganisms of patients with intrahepatic bile duct cancer and observed increased levels of *Lactobacillus*, *Actinomyces*, *Peptostreptococcaceae*, and *Alloscardovia* compared to the healthy population. This discovery holds significant implications for the diagnosis and prediction of intrahepatic bile duct cancer.

The role of gut microbiota in the development and progression of liver cancer as a coordinator of the gut-liver axis has been emphasized [46]. Jiang et al [47] conducted a study investigating the gut microbiota of hepatocellular carcinoma (HCC) patients and identified higher abundances of *Barnesiella*, *Firmicutes*, and *Streptococcus* in their intestines. Similarly, Yang et al [48] observed an association between HCC and gut microbiota, with a higher abundance of *Streptococcus* in the gut of HCC patients. Consistent with these studies, our findings also indicate that *Streptococcus* and *Barnesiella* are risk factors for liver cancer. However, in contrast to previous findings, we found that *Firmicutes* are protective factors for liver cancer.

In our study, we identified *Flavonifractor* and *Streptococcus* as risk factors for pancreatic cancer. These findings are

consistent with previous research. A large-scale metabolome-wide association study (MWAS) demonstrated an association between *Flavonifractor sp90199495* and the metabolite *X-21849*, which was found to be related to the risk of pancreatic ductal adenocarcinoma [49]. Additionally, another study observed significant differences in gut microbial composition between pancreatic cancer patients and healthy individuals, with higher levels of *Streptococcus* being associated with an increased risk of pancreatic cancer development and liver metastasis. These findings suggest that *Streptococcus* may serve as a potential biomarker for the early diagnosis of pancreatic cancer and liver metastasis originating from pancreatic cancer [50]. Furthermore, Ogrendik's study revealed a positive correlation between salivary microbiota and the risk of pancreatic cancer [51]. The research specifically identified *Porphyromonas gingivalis* as a significant risk factor for pancreatic cancer.

The gut microbiota exerts a crucial influence on the initiation and progression of colorectal cancer [52]. Certain researchers delve into the microbial mechanisms connected to the pathogenesis and advancement of colorectal cancer [53]. A meta-analytic study demonstrated a link between colorectal cancer and microbiota dysbiosis, with increased abundance of *Porphyromonadaceae* and *Coriobacteriaceae* in the intestines of colorectal cancer patients [54]. Another study conducted by Huo et al [55] investigated the gut mucosal microbiota associated with recurrence and survival in colorectal cancer patients. They observed that *Bacteroidales*, *Coriobacteriaceae*, and *Porphyromonadaceae* were associated with poorer survival and higher recurrence rates. Interestingly, they found that the effects of *Bacteroidales* differed based on their abundance at different tumor sites. High abundance of *Bacteroidales* at the extratumoral site was associated with better overall survival (OS) and disease-free survival (DFS), while high abundance at the tumor site was associated with poorer OS and DFS. They suggested that *Bacteroidales* at the extratumoral site might have a protective role in recruiting beneficial T cells and improving the prognosis of colorectal cancer patients, while *Bacteroidales* at the tumor site could act as pathogens leading to colorectal cancer recurrence. Consistent with these findings, our study also identified *Coriobacteriaceae* and *Porphyromonadaceae* as risk factors for colorectal cancer, while *Bacteroidales* were identified as protective factors. Furthermore, a study conducted by Fortoul et al [56] has identified *Hemophilus influenzae* as a potential protective factor against colorectal cancer. This observation is intriguingly associated with the up-regulation of NLRP3 inflammasome in response to *Hemophilus influenzae* infection. The presence of NLRP3 inflammasome may contribute to the maintenance of gut microbiota homeostasis and decrease the risk of colorectal cancer. This intriguing finding suggests a promising avenue for further research.

The gut microbiota performs complex metabolic activities, generating various metabolites that can have both harmful and beneficial effects [57, 58]. These metabolites play a significant role in the interactions between colorectal cancer cells and the gut microbiota. Multiple studies have demonstrated the inhibitory effect of *GABA*, a metabolite produced by gut microbiota, on the proliferation of colon cancer cells [59]. Furthermore, *GABA*-producing *Lactobacillus* plantarum has been shown to induce apoptosis in drug-resistant colorectal cancer cells and inhibit metastasis [60]. In line with these findings, our study also identified *GABA* as a metabolite associated with a reduced risk of colorectal cancer. However, an animal study revealed that knockdown of the *EphB6* gene in mice promoted tumor growth of colorectal cancer cells in a xenograft model by regulating *GABA* release [61]. Further research is needed to fully understand the role of *GABA* in colorectal cancer.

In addition to some of the known associations between gut microbiota and digestive tract cancer, we have identified additional causal associations between gut microbiota and these tumors, but no studies have yet reported these associations. This is a novel finding that needs to be explored in further studies. Our study has important implications for pre-cancer screening and intervention. Although much progress has been made in identifying genetic variation in human diseases, most genetic risks remain unexplained. How gut microbiota affects the development and progression of digestive tract cancer still needs to be revealed by further biological studies. Furthermore, while our study primarily sought to explore the causal relationship between gut microbiota and digestive tract cancer, a robust association between gut microbiota and cancers not in the digestive tract is also evident. For instance, Cardeiro et al's retrospective study identified a statistically significant correlation between enterococcal infection and a decreased occurrence of breast cancer [62]. This research direction holds great promise as well.

Human behavior and the environments in which they live are complex and are influenced by interactions between genes and the environment [63-66]. To eliminate confounding factors in epidemiologic studies, we used MR methods. The SNPs in our study were closely associated with the gut microbiota and were compared with multiple cancer databases. The results of sensitivity analyses showed statistical robustness, and no pleiotropy or heterogeneity was found. However, our study has some limitations. First, the key assumptions of MR have some limitations, and although we tried our best to exclude confounding factors, we cannot fully guarantee the absence of other confounding factors or potential pleiotropic effects. Second, because GWAS pooled data were used, the results may have been affected by different quality control and selection criteria. Third, the analytical principles of MR can only infer potential causal relationships and cannot identify specific biological pathways. Fourth, our study is mainly based on European populations and has limited generalizability to other ethnicities. Finally, due to the lack of data on applicable SNPs, we were unable to explore whether digestive tract cancer leads to alterations in gut microbiota through bidirectional MR studies. Therefore, the possibility of reverse causality needs to be considered with caution in our conclusions and we hope that data on appropriate SNPs will be available in the future for further study interpretation.

Conclusion

Our study conducted a comprehensive evaluation of the association between gut microbiota, gut metabolites, and digestive

tract cancer. Our findings suggest that certain gut microbiota and metabolites, when considered as exposure factors, can act as either risk factors or protective factors for digestive tract cancer. These results provide valuable new insights into the mechanisms by which the gut microbiota and gut metabolites influence the development of cancer.

Supplementary Material

Table S1. IVs associated with 22 gut microbiota and seven digestive tract cancer ($P < 5 \times 10^{-8}$).

Table S2. IVs associated with 215 gut microbiota and seven digestive tract cancer ($P < 1 \times 10^{-5}$).

Table S3. IVs associated with eight gut metabolites and seven digestive tract cancer ($P < 1 \times 10^{-5}$).

Table S4. SNP number of different gut microbiota and metabolites.

Table S5. Causal effect of MR analysis between 22 gut microbiota and seven digestive tract cancer ($P < 5 \times 10^{-8}$).

Table S6. Causal effects between 22 gut microbiota and seven digestive tract cancer based on IVW method ($P < 5 \times 10^{-8}$).

Table S7. Sensitivity results of MR analysis between 22 gut microbiota and seven digestive tract cancer ($P < 5 \times 10^{-8}$).

Table S8. Causal effect of MR analysis between 215 gut microbiota and seven digestive tract cancer ($P < 1 \times 10^{-5}$).

Table S9. Causal effects between 215 gut microbiota and seven digestive tract cancer based on IVW method ($P < 1 \times 10^{-5}$).

Table S10. Sensitivity results of MR analysis between 215 gut microbiota and seven digestive tract cancer ($P < 1 \times 10^{-5}$).

Table S11. Causal effect of MR analysis between eight gut metabolites and seven digestive tract cancer ($P < 1 \times 10^{-5}$).

Table S12. Causal effects between eight gut metabolites and seven digestive tract cancer based on IVW method ($P < 1 \times 10^{-5}$).

Table S13. Sensitivity results of MR analysis between eight gut metabolites and seven digestive tract cancer ($P < 1 \times 10^{-5}$).

Table S14. MR-PRESSO analysis results of MR analysis between gut microbiota, metabolites and digestive tract cancer.

Fig. 15. Causal effects of gut microbiome and digestive tract cancer based on the IVW (inverse-variance weighted) method (SNPs with $P < 1 \times 10^{-5}$). (A) Heatmap of the P-value. Red color in the heatmap indicates a positive correlation between gut microbiome and digestive tract cancer and blue color indicates a negative correlation. The color depth represents the size of the P-value, with darker colors indicating more significant P-values. (B) Network interactions based on statistically significant odd ratios (ORs). Color and line thickness in the network diagram indicate the OR of gut microbiota and digestive tract cancer. Darker red color and thicker lines indicate higher OR values. (C-I) Forest plot with statistically significant ORs in seven different types of digestive tract cancer. OR > 1 indicates the positive correlation between a particular gut microbiota and a particular digestive tract cancer. OR < 1 indicates the negative correlation between the two. SNP: single nucleotide polymorphism; OR: odds ratio; 95% CI: 95% confidence interval; NSNP: number of SNP.

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Conflict of Interest

The authors declare that they have no competing interests.

Informed Consent

Not applicable.

Author Contributions

Jin Sheng Huang and Xu Jia Li were involved in the conception and design; Jin Sheng Huang, Xu Jia Li, Meng Ge Gao, Ling Li Huang, Xu Xian Chen, and Yu Ming Rong were involved in analysis and interpretation of the data; Jin Sheng Huang and Ling Li Huang were involved in the drafting of the paper or revising it critically for intellectual content. All authors agree to be accountable for all aspects of the work.

Data Availability

The summary statistics of exposures were available on the MiBioGen and TwinsUK Registry; the summary statistics of outcome were available on the FinnGen research (https://www.finngen.fi/fi).

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