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The role of microorganisms in the development and progression of cancer

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Published in
Frontiers in Cellular and Infection Microbiology

ISSN 1664-8714
ISBN 978-2-83252-118-2
DOI 10.3389/978-2-83252-118-2

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The role of microorganisms in the development and progression of cancer

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Citation

Barbugli, P. A., De La Fuente, M. K., Weniger, M., eds. (2023). *The role of microorganisms in the development and progression of cancer*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83252-118-2

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Editorial
PUBLISHED 24 March 2023
DOI 10.3389/fcimb.2023.1153372

OPEN ACCESS

EDITED AND REVIEWED BY
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SPECIALTY SECTION
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 29 January 2023
ACCEPTED 14 March 2023
PUBLISHED 24 March 2023

CITATION
Weniger M (2023) Editorial: The role of microorganisms in the development and progression of cancer.
Front. Cell. Infect. Microbiol. 13:1153372.
doi: 10.3389/fcimb.2023.1153372

Editorial: The role of microorganisms in the development and progression of cancer

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KEYWORDS

cancer, microbiome, lung cancer, colorectal cancer, cervical cancer

Editorial on the Research Topic
The role of microorganisms in the development and progression of cancer

The human body is home to trillions of microorganisms, collectively known as microbiota, which play a crucial role in maintaining health and well-being. Recent research has revealed that the gut microbiome in particular can significantly affect the development and progression of cancer. In this edition, we will present state-of-the art articles on the current understanding of the link between microbiota and cancer, with a specific focus on lung and colorectal cancer.

Lung cancer is the leading cause of cancer death worldwide, and non-small-cell lung cancer (NSCLC) is the most common form of the disease. Recent studies have found that the gut microbiome is altered in lung cancer patients, with specific changes in microbial composition and function.

The two studies included in this edition investigate the important links between lung cancer and the gut microbiome, which is altered significantly in lung cancer patients compared to healthy individuals (Chen et al.). Interestingly, gut microbiota and serum metabolic profiles have been closely related, providing new biomarkers for the diagnosis of early-stage NSCLC (Ni et al.).

Colorectal cancer (CRC) is one of the most common forms of cancer worldwide, and the gut microbiome is known to play a vital role in its etiology. The articles published in this edition analyze specific functions of the microbiome in CRC and their effect on CRC-related miRNA production, as well as the role of several bacteria including Fusobacterium nucleatum, Escherichia coli, Bacteroides fragilis, and Faecalibacterium prausnitzii (Xing et al.). Importantly, Fusobacterium nucleatum (Fn) seems to play a critical role in the development of CRC (Ou et al.). As discussed, one might hypothesize that prevention and treatment based on the relationship between Fn and CRC might be possible. Additionally, not only does the composition of the microbiome seem to play a critical role, but also metabolites produced by the intestinal microbiota influence colorectal cancer. Specifically, sodium butyrate can positively affect the immune system, intestinal barrier, anti-cancer treatment efficiency, and reduce mucositis induced by chemotherapy, making it a

promising option for colorectal cancer patients (Kaźmierczak-Siedlecka et al.). Similarly, fecal metabolites not only seem to play a role in colorectal cancer, but they might also facilitate the diagnosis of gastritis. Interestingly, heptadecanoic acid and pentadecanoic acid in crosstalk with gut microbiota Erysipelotrichaceae_UCG-003 and Haemophilus correlate with chronic atrophic gastritis and could serve as novel biomarkers in the future (Gai et al.).

Furthermore, research on the microbiome has not only gained a foothold in gastrointestinal and pulmonary oncology, but also in studies on breast and cervical cancer. In this regard, a review included in this special issue illustrates the unique microbial composition in breast tissue and tumors, which could help develop novel therapeutic drugs (Song et al.).

Cervical cancer is a disease caused by the abnormal growth of cells in the cervix and is probably the best example of how the microbiome affects tumor biology. The well-established link between the human papilloma virus, cervical cancer, and the now-available vaccine is an excellent example of how microbiome research can lead to changes in real-world tumor therapy and outcomes (https://www.ncbi.nlm.nih.gov/pubmed/30638582). In this edition, an article investigates how HPV screening can detect cervical cancer (Zhang et al.). In addition, the impact of male HPV infection on both male and female HPV-associated cancers must not be overlooked (Zou et al.).

This edition of Frontiers in Cellular and Infection Microbiology serves to underline the importance of the microbiome in cancer and seeks to help increase our knowledge about this pivotal topic.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

Conflict of interest

The author declares 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

frontiers | Frontiers in Cellular and Infection Microbiology

REVIEW
published: 14 April 2022
doi: 10.3389/fcimb.2022.804689

Impacts of MicroRNAs Induced by the Gut Microbiome on Regulating the Development of Colorectal Cancer

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OPEN ACCESS

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Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: 29 October 2021
Accepted: 18 March 2022
Published: 14 April 2022

Citation:
Xing J, Liao Y, Zhang H, Zhang W, Zhang Z, Zhang J, Wang D and Tang D (2022) Impacts of MicroRNAs Induced by the Gut Microbiome on Regulating the Development of Colorectal Cancer. Front. Cell. Infect. Microbiol. 12:804689. doi: 10.3389/fcimb.2022.804689

Although a dysfunctional gut microbiome is strongly linked to colorectal cancer (CRC), our knowledge of the mediators between CRC and the microbiome is limited. MicroRNAs (miRNAs) affect critical cellular processes, such as apoptosis, proliferation, and differentiation, and contribute to the regulation of CRC progression. Increasingly, studies found that miRNAs can significantly mediate bidirectional interactions between the host and the microbiome. Notably, miRNA expression is regulated by the gut microbiome, which subsequently affects the host transcriptome, thereby influencing the development of CRC. This study typically focuses on the specific functions of the microbiome in CRC and their effect on CRC-related miRNA production and reviews the role of several bacteria on miRNA, including *Fusobacterium nucleatum*, *Escherichia coli*, enterotoxigenic *Bacteroides fragilis*, and *Faecalibacterium prausnitzii*. Based on the important roles of miRNAs and the gut microbiome in CRC, strategies for modulating miRNA expression and regulating the gut microbiome composition need to be applied, such as bioactive dietary components and fecal microorganism transplantation.

Keywords: colorectal cancer, microRNAs, gut microbiome, bioactive dietary components, modulation

INTRODUCTION

The intestinal microenvironment averagely hosts more than 100 trillion bacteria, known as the gut microbiome. A healthy microbiome contributes to the maintenance of colonic microenvironment homeostasis, immune system development, and intestinal epithelial function (Yuan et al., 2018). When the composition and function of the microbiome are affected, diseases will occur accordingly, including colorectal cancer (CRC) (Wang et al., 2012). Although a dysfunctional gut microbiome is strongly linked to CRC, our knowledge of the mediators between CRC and the microbiome is limited.

In recent years, microRNAs (miRNAs) have increasingly caught the eye of scientists because of their important roles in the development and treatment of CRC. miRNAs are 20- to 22-nucleotide-long noncoding single-stranded RNAs with a highly stable structure (Bartel, 2004). In mammalian cells, miRNAs act as gene regulatory elements through posttranscriptional modifications *via*

binding to target mRNAs (Fabian and Sonenberg, 2012). miRNAs regulate approximately 90% of the genes encoding proteins and affect critical cellular processes, such as apoptosis, proliferation, and differentiation. Its deregulation has also been implicated in tumor pathobiology, such as angiogenesis, immune surveillance, invasion, and metastasis (Chen et al., 2015; Chen et al., 2016). Studies have found that intestinal profiles of miRNA expression were differently expressed in the colon of colonized mice relative to germ-free mice (Dalmasso et al., 2011). This finding suggested that the microbiome can affect the expression of miRNAs, which in turn target its downstream genes and activate a new pathway, resulting in an influence on intestinal epithelial cells. However, because of the poor knowledge about the interactions between numerous unique miRNAs and the microbiome, it is challenging to study all possible pairwise interactions. Nevertheless, potential connections between unique miRNAs and the microbiome identified in CRC patients can be considered candidates for functional inspection. In this review, we present our understanding of the role of miRNAs in mediating CRC, thereby providing an idea that we can turn to diet regulation to treat and prevent CRC.

CANCER-RELATED miRNAs AND THEIR INTERACTIONS WITH THE MICROBIOME AND HOST IN CRC

Intestinal epithelial cells are the main producer of host-derived miRNAs. miRNAs are synthesized in the nucleus and processed and then function in the cytoplasm (Liu et al., 2016). miRNA genes are transcribed into primary miRNA transcripts (pri-miRNA) through RNA polymerase II or RNA polymerase III and are subsequently cleaved by the microprocessor complex Drosha–DGCR8 (Han et al., 2004; Lee et al., 2004; Borchert et al., 2006). The resulting precursor hairpins, the precursor miRNAs (pre-miRNAs), are exported from the nucleus to the cytoplasm by exportin-5–Ran-GTP (Yi et al., 2003). In the cytoplasm, pre-miRNAs are cleaved into mature length by the Dicer-TRBP complex. Functional strands of mature miRNAs are assembled with argonaute (AGO) proteins and a glycinetryptophan protein of 182 kDa (GW182), and then miRNA-induced silencing complexes (miRISC) mediating target mRNAs silencing are recruited, while passenger strands are degraded (Winter et al., 2009). miRNAs regulate gene expression, especially in mammalian cells, through two different albeit paired mechanisms. miRNAs have a wide range of complementary base pairs with mRNA and will guide the miRISC to the target mRNA and cause mRNA degradation, resulting in the instability or suppression of translation. Second, if the miRNA has partial complementary sequences to the 3′-untranslated region (3′-UTR) of the mRNA, the miRISC will inhibit mRNA translation through the AGO protein (Fabian and Sonenberg, 2012) (**Figure 1**). Many of these target mRNA transcripts play important roles in tumor proliferation, differentiation, and apoptosis (Winter and Diederichs, 2011), and studies have uncovered that each miRNA can target hundreds of mRNAs (Baek et al., 2008). Based on this vast and complex regulatory network, the regulatory function of miRNAs is immensely important in many signaling pathways, such as Wnt and APC, thereby influencing many aspects of tumor pathobiology in CRC (Li et al., 2016; Slattery et al., 2018).

In general, miRNA expression is strictly controlled in normal cells; however, defects in miRNA processing might occur in cancer cells, thereby enhancing tumorigenesis (Kumar et al., 2007). More and more data identified a large number of abnormal miRNA expression patterns in CRC, such as miR-21, miR-29, miR-34a, miR-124a, and miR-155 (Yi et al., 2016). These dysregulated miRNAs could be functionally delivered into the tumor microenvironment (TME) through tumor-derived exosomes) (Raposo and Stoorvogel, 2013). Since this seminal discovery, miRNAs' ability to shape the complex inflammatory TME has emerged as a critical role in cell-to-cell communications. Studies demonstrated that miRNAs in the TME play critical roles in modulating the composition of the gut microbiome by regulating bacterial species, such as *Fusobacterium nucleatum* and *Escherichia coli*, thereby affecting gene regulation and growth effects (Liu et al., 2016). Similar regulation mediated by miRNAs is also found in stromal cells and immune cells (Bullock et al., 2013; Kohlhapp et al., 2015). In cancer cells, miRNAs encapsulated in microvesicles will be selectively transported to stromal cells and immune cells (Fanini and Fabbri, 2017), influencing their development, maturation, and antitumor activities (Fanini and Fabbri, 2017). A growing body of evidence has pointed to a central role of miRNAs in the dialogue between cancer and the immune system, with associated effects on the overall balance between immune-stimulation and immune escape (Kim et al., 2005; Keller et al., 2011; Fanini and Fabbri, 2017). In fact, the dysregulated oncogenic microbiome and immune system will create a more favorable TME for CRC cells. In addition to tumor-derived miRNAs, changes in the expression of miRNAs can also be attributed to an introduction of a foreign organism in the colon (Hu et al., 2015b). In the ileum of mice infected by *Listeria monocytogenes*, miR-378, miR-200c, miR-194, miR-200b, miR-148a, and miR-143 were usually downregulated. Meanwhile, miR-194 was downregulated, and miR-378 was upregulated in germ-free mice, with the rest having no influence (Archambaud et al., 2013). The abnormal expression of miRNAs subsequently activates the signaling pathways and regulates all aspects of tumor pathobiology in CRC (Li et al., 2014). Taken together, the bidirectional interaction between the host and microbiome mediated by miRNAs presents a new layer of complexity in the study of miRNAs.

MICROBIAL REGULATION OF CRC MEDIATED BY miRNAs

F. nucleatum Affects Cell Proliferation and Induces Chemoresistance in CRC Patients by Modulating miRNAs

F. nucleatum, an anaerobic gram-negative bacterium, usually enriched in CRC and is closely related to colorectal carcinogenesis (Fukugaiti et al., 2015). Several recent studies have investigated

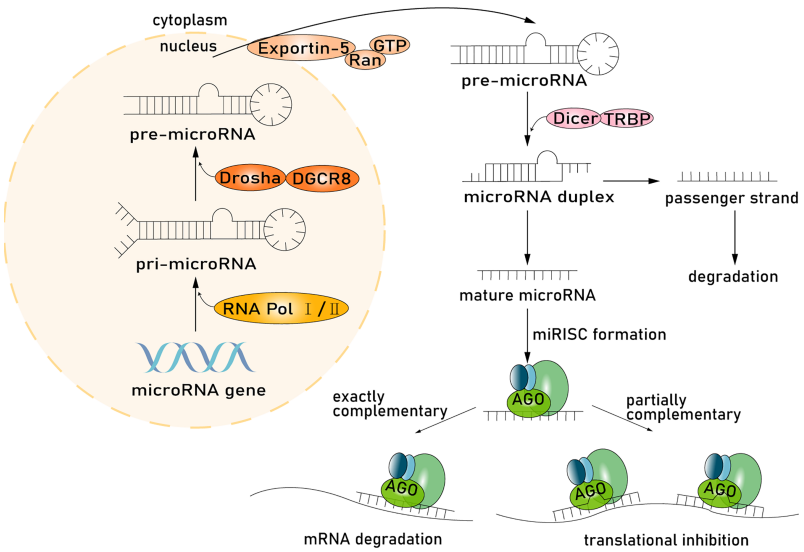

FIGURE 1 | miRNA processing pathway. Host-derived miRNAs are synthesized in the nucleus and processed and then function in the cytoplasm. miRNA genes are transcribed into pri-miRNA through RNA polymerase II or RNA polymerase III subsequently cleaved by the microprocessor complex Drosha-DGCR8. The resulting precursor hairpins, the pre-miRNAs, are exported from the nucleus to the cytoplasm by exportin-5–Ran-GTP. In the cytoplasm, pre-miRNAs are cleaved into mature length by the Dicer in complex with TRBP. Functional strands of mature miRNAs are assembled with AGO proteins and a glycinetryptophan repeat-containing protein of 182 kDa (GW182), and then miRISC mediating target mRNAs silencing are recruited, whereas passenger strands are degraded. miRNAs regulate gene expression through two mechanisms. First, miRNA, with a wide range of complementary base pairs with mRNA, will guide miRISC to degrade mRNA, resulting in the instability or suppression of translation. Second, if miRNA and mRNA have partially complementary sequences, the miRISC will inhibit mRNA translation through the AGO protein.

the role of abnormal miRNA expression resulting from *F. nucleatum* infection in CRC development. Based on the miRNA expression profiles extracted from CRC tissues that were detected positive for *F. nucleatum* infection, Feng et al. (2019) demonstrated that miR-4474 and miR-4717 were significantly increased in early and advanced stage CRC. More importantly, the downregulation of CREB-binding protein (CREBBP) in CRC patients has also been studied and analyzed, which identified CREBBP as a novel target of miR-4474 and miR-4717. CREBBP, a transcriptional cofactor, is able to influence Wnt/β-catenin signaling and promote cell proliferation, likely affecting colonic tumorigenesis (Bordonaro and Lazarova, 2015). Thus, the overexpression of miR-4474 and miR-4717 in *F. nucleatum*–positive CRC tissues can inhibit cell tumor proliferation *via* degrading the mRNA of CREBBP. In addition to miR-4474 and miR-4717, miR-21 was also demonstrated to be dysregulated in *F. nucleatum*–positive CRC tissues (Shi et al., 2016; Yang et al., 2017) (**Figure 2**). It is widely acknowledged that *F. nucleatum* potentiates CRC development through Toll-like receptor 4 (TLR4) signaling, where TLR4 binds to myeloid differentiation factor 88 (MYD88) (Yang et al., 2017; Proenca et al., 2018; Sun et al., 2019) and subsequently activates the key downstream effector nuclear factor κB (NF-κB) (Ogawa et al., 2005; Mukherji et al., 2013). NF-κB is a transcription factor that can bind to the promoter region of miR-21 and upregulate miR-21 expression, thereby resulting in the downstream target gene RASA1 being downregulated (Yang et al., 2017). RASA1 is a member of the RAS GTPase activating proteins (RAS-GAP) family and acts as a suppressor of RAS function (Donovan et al., 2002). The partial functional loss of RASA1 in miR-21 overexpressing cells can activate the GTPase activity of RAS, consequently triggering the RAS-RAF-MEK-ERK (RAS–mitogen-activated protein kinase [MAPK]) signaling pathway (Sun et al., 2013; Sun et al., 2015; Kent et al., 2016). The activation of the MAPK signaling pathway has proven to play an important part in increasing cell proliferation, eventually leading to tumorigenesis (Fang and Richardson, 2005). Taken together, miR-21 upregulation in CRC cells plays an important role in promoting colorectal carcinogenesis by *F. nucleatum via* activating the RAS-MAPK signaling pathway.

Regarding the chemotherapeutics of CRC, the combination of chemotherapeutic agents, including 5-fluorouracil (5-FU), oxaliplatin, and bevacizumab, is widely used to shrink tumor size and reduce tumor growth in advanced CRC patients (Cartwright, 2012). Although most patients with advanced CRC initially respond to combined chemotherapy, treatment outcomes may still be disappointing because of drug resistance leading to tumor recurrence, and the 5-year survival rate of patients is lower than 10% (Dahan et al., 2009). Some studies have shown that the enrichment of *F. nucleatum* is associated with recurrence postchemotherapy and shorter survival duration (Mima et al., 2016; Yu et al., 2017). In the *F. nucleatum*–mediated chemoresistance of CRC cells, miR-4802 and miR-18a*, which similarly depend on the TLR4 and MYD88 signaling pathways, are significantly downregulated (**Figure 2**). The selective loss of

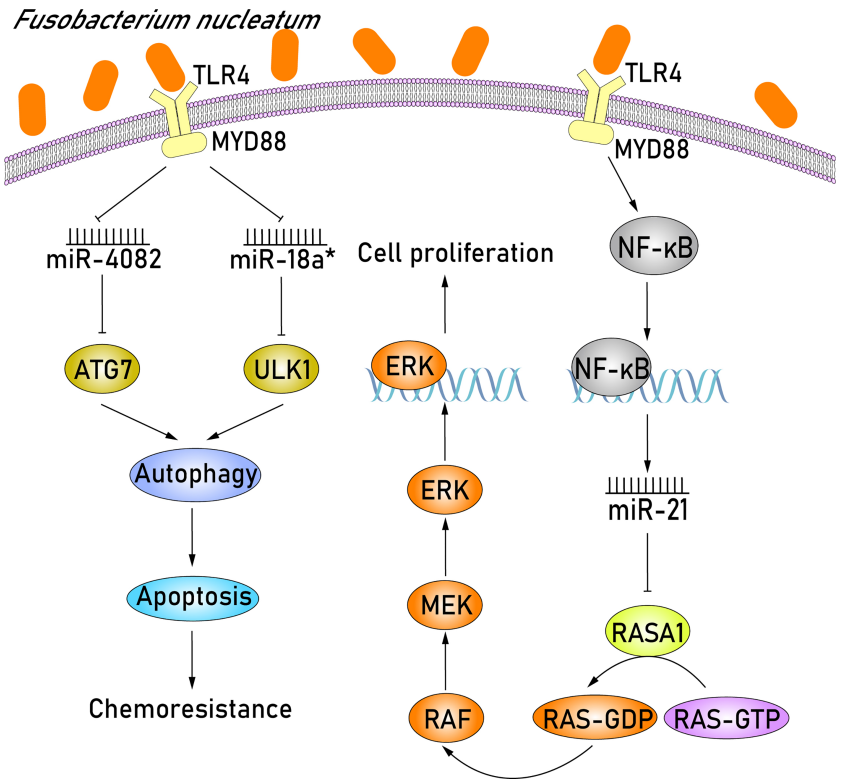

FIGURE 2 | *F. nucleatum* promotes cell proliferation by upregulating miR-21 and induces chemoresistance in CRC patients by downregulating miR-4802 and miR-18a*. *F. nucleatum* initially recognizes TLR4/MYD88 signaling pathway and activates the key downstream effector NF-κB. NF-κB can bind to the promoter region of miR-21 and upregulate miR-21 expression, resulting in the subsequent downregulation of the downstream target gene RASA1. The partial functional loss of RASA1 can activate the GTPase activity of RAS, triggering the RAS-RAF-MEK-ERK (RAS-MAPK) signaling pathway. MAPK signaling pathway plays an important part in increasing cell proliferation, eventually leading to tumorigenesis. Furthermore, in the *F. nucleatum*–mediated chemoresistance of CRC cells, miR-4802 and miR-18a*, which are both dependent on the TLR4 and MYD88 signaling pathways, are significantly downregulated. The selective loss of miR-4802 and miR-18a* induces the upregulation of ATG7 and ULK1, respectively, which are members of autophagy signaling elements. Therefore, when these *F. nucleatum*–infected CRC cells are treated with chemotherapy agents, the autophagy pathway will be activated and consequently promote chemoresistance *via* suppressing cell apoptosis.

miR-4802 and miR-18a,* respectively, induces the upregulation of ATG7 and ULK1, which are members of autophagy signaling elements. Therefore, when these *F. nucleatum*–infected CRC cells are treated with chemotherapy agents, such as 5-FU and oxaliplatin, the autophagy pathway will be activated and promote chemoresistance *via* suppressing cell apoptosis. Altogether, these results indicate that *F. nucleatum* may promote chemoresistance in patients with CRC *via* the selective loss of miR-4802 and miR-18a* expression and subsequent cancer autophagy activation.

E. coli Promotes Cell Proliferation and Inflammation by Modulating miRNAs

E. coli is a facultative anaerobic gram-negative bacteria with pathogen-like features, such as downregulating DNA mismatch repair proteins or producing various toxins exhibiting carcinogenic features (Dalmasso et al., 2014). Certain strains of *E. coli* in group B2, containing the polyketone acid synthetase (*pks*) island, can produce the colibactin toxin, induce proproliferative effect, and are frequently associated with CRC (Buc et al., 2013; Cougnoux et al., 2014; Dalmasso et al., 2014). c-MYC, a transcription factor involved in DNA damage response, is activated in *pks*$^+$ *E. coli*–infected CRC cells, and c-MYC subsequently binds to the miR-20a-5p promoter, resulting in the upregulation of miR-20a-5p. Upregulation of miR-20a-5p can cause the translational silencing of target SENP1 by binding to the SENP1 mRNA 3′-UTR (O'Donnell et al., 2005; Guerra et al., 2010). SENP1 is a key enzyme involved in the regulation of the SUMOylation process and blocks the modification of the small ubiquitin-like modifier 1 (SUMO1)–conjugated p53 patterns. Thus, studies have observed an accumulation of SUMO1-conjugated p53 in *pks*$^+$ *E. coli*–infected CRC cells by downregulating SENP1. Moreover, the SUMOylation of p53 was identified as the key regulator of cellular senescence, characterized by the induction of megalocytosis and cell cycle arrest (Nougayrède et al., 2006; Yates et al., 2008). The senescence of intestinal epithelial cells in *pks*$^+$ *E. coli*–infected CRC cells induces the secretion of growth factors, including hepatocyte growth factor, fibroblast growth factor, and granulocyte-macrophage colony-stimulating factor, which play a crucial role in stimulating tumor growth. These studies reveal a

new paradigm in CRC whereby *pks*$^{+}$ *E. coli*–infected CRC cells activate the c-MYC/miR-20a-5p/SENP1/senescence/growth factors pathway, consequently promoting the proliferation of uninfected cells and, in turn, stimulating tumor growth (**Figure 3**). In addition, among the miRNAs previously reported, the expression of miR-30c and miR-130a was also upregulated significantly in adherent-invasive *E. coli* (AIEC)–infected epithelial cells *via* activating the NF-κB pathway (Fasseu et al., 2010; Nguyen et al., 2014). Overexpression of miR-30c and miR-130a subsequently downregulates the expression of ATG5 and ATG16L1, respectively, by binding to the 3′-UTRs of target mRNAs. ATG5 and ATG16L1 are members of autophagy signaling elements, and their downregulation will result in defective autophagy. Autophagy is a tightly regulated homeostatic process in various physiological states, and therefore, dysregulated autophagy is associated with numerous human pathologies, such as colorectal carcinogenesis (Rubinsztein et al., 2012) (**Figure 3**). AIEC bacteria are able to invade and replicate within epithelial cells and macrophages. Studies have shown that impaired autophagy can enhance the intracellular replication of AIEC and induce the secretion of proinflammatory cytokines (Lapaquette et al., 2010; Lapaquette et al., 2012). Moreover, impaired nucleotide-binding oligomerization domain-containing protein 2 (NOD2) expression also affects bacterial autophagy and can be conducive to AIEC persistence and replication within epithelial cells and macrophages (Lapaquette et al., 2012; Nguyen et al., 2014; Negroni et al., 2016). NOD2 is a member of the nucleotide-binding oligomerization domain (NOD)–like receptors subfamily, which contains a caspase recruitment domain (CARD), and can recruit ATG16L1 to the plasma membrane at the bacterial entry site, thereby activating the autophagic response to invasive bacteria (Travassos et al., 2010; Brooks et al., 2011). Thus, NOD2 and ATG16L1 can activate an autophagy-mediated, anti-bacterial pathway, suggesting a novel method to inhibit AIEC invasion. Altogether, AIEC may increase the proinflammatory response and consequently lead to colorectal carcinogenesis *via* upregulating miR-30c and miR-130a and inducing defective autophagy, while, NOD2 and ATG16L1 may provide an approach to prevent AIEC invasion.

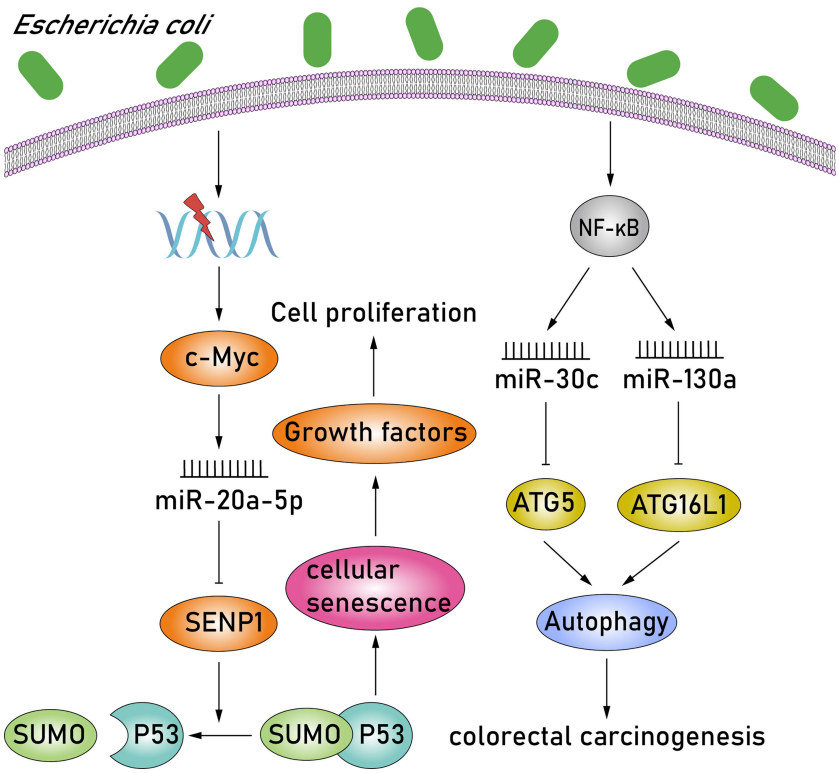

FIGURE 3 | *E. coli* promotes cell proliferation and inflammation by modulating miR-20a-5p, miR-30c, and miR-130a. In *pks*$^{+}$ *E. coli*–infected CRC cells, c-MYC is activated, and it subsequently results in the upregulation of miR-20a-5p. Upregulation of miR-20a-5p can cause the translational silencing of target SENP1. SENP1 is a key enzyme that blocks the modification of the SUMO1-conjugated p53 patterns. Moreover, the SUMOylation of p53 is identified as the key regulator of cellular senescence. The senescence of intestinal epithelial cells in *pks*$^{+}$ *E. coli*–infected CRC cells consequently induces the secretion of growth factors, which play a crucial role in stimulating tumor growth. In addition, expressions of miR-30c and miR-130a were also upregulated significantly in AIEC-infected epithelial cells *via* activating the NF-κB pathway. Overexpression of miR-30c and miR-130a subsequently downregulates the expression of ATG5 and ATG16L1, respectively. ATG5 and ATG16L1 are members of autophagy signaling elements, and their downregulation will result in defective autophagy. Moreover, dysregulated autophagy is associated with numerous human pathologies, such as colorectal carcinogenesis.

Enterotoxigenic *Bacteroides fragilis* Induces Tumor Growth and Promotes Inflammation by Modulating miRNAs

Enterotoxigenic *Bacteroides fragilis* (ETBF), a subtype strain of *B. fragilis*, is one of the most prevalent species in CRC (Vétizou et al., 2015). The pathogenicity of ETBF originates mainly from the bft gene, which encodes for the *B. fragilis* toxin, and this toxin has been widely acknowledged to play a part in colorectal carcinogenesis (DeStefano Shields et al., 2016). In addition to the bacterial toxin, the mechanistic links between miRNAs and ETBF in CRC have also been explored (**Figure 4**). Studies found that *B. fragilis*–associated lncRNA1 (BFAL1) was upregulated in ETBF-infected CRC cells and confirmed that the overexpression of BFAL1 functioned as an activator of ETBF-induced CRC (Bao et al., 2019). BFAL1 is a long noncoding RNA (lncRNA) with limited coding potential; however, it acts as a regulator of numerous biological functions and pathological processes (Quinn and Chang, 2016). At the same time, overexpression of BFAL1 decreased the levels of miR-155-5p and miR-200a-3p and attenuated their suppressive function on target RHEB mRNA expression by competitively binding with target miRNAs. With the help of miRNAs, BFAL1 consequently activates the Ras homolog, which is the MTORC1 binding/mammalian target of the rapamycin (RHEB/mTOR) pathway, thereby mediating ETBF-induced tumor growth (Bao et al., 2019).

In addition, miR-149-3p, which has been proven to inhibit tumorigenesis in other cancers, was also significantly downregulated in both ETBF-infected CRC cells and exosomes derived from ETBF-infected cells (Yao and Wu, 2019). Regulation level of miR-149-3p was attributed to methyltransferase-like 14 (METTL14)–dependent N^6-methyladenosine (m^6A) modification *via* modifying pri-miRNA splicing (Ma et al., 2017; Cao et al., 2021). METTL14 modulates the splicing process of pri-miR-149 by recognizing the microprocessor protein DGCR8 and inducing m^6A modification, which is a predominant internal modification of RNA in higher eukaryotes (Niu et al., 2013; Ma et al., 2017). METTL14 was downregulated in ETBF-infected CRC cells, inducing the level of miR-149-3p, which led to the upregulation of the miR-149-3p target PHD finger protein 5A (PHF5A). PHF5A plays a critical role in mRNA precursor splicing, enhances the stability of the splicing factor 3b (SF3b) complex in CRC cells, and further contributes to decreased exon skipping level. The overexpression of PHF5A subsequently upregulates the target mRNA level of KAT2A in CRC cells, which participates in the regulation of the cell cycle (Cao et al., 2021). KAT2A can regulate gene transactivation *via* H3K9ac and H3K14ac and significantly upregulate target superoxide dismutase 2 (SOD2) mitochondrial in ETBF-infected CRC cells *via* directly binding to the promoter region of SOD2 (Sun et al., 2016; Cao et al., 2021). Several studies have reported that the overexpression of SOD2 is relevant to poor clinical outcome in gastric cancer, and SOD2 may promote intestinal inflammation and tumorigenesis (Janssen et al., 2000; Chen et al., 2013). In addition to miRNA, lncRNA can similarly modify the transcription of SOD2 and promote gastric carcinogenesis, revealing a new angel in elucidating the potential mechanisms behind CRC development. Moreover, exosomes derived from ETBF-infected cells, which encapsulated downregulated miR-149-3p, can also be successfully delivered to $CD4^+$ T cells and significantly reduce the overexpression of interleukin 17A, tumor necrosis factor α, and RORC, thereby resulting in an increased proinflammatory response. Similarly, these exosomes in TME can be delivered to adjacent epithelial cells and promote CRC development *via* downregulating miR-149-3p (Cao et al., 2021). Therefore, ETBF can significantly promote CRC development by regulating miR-149-3p in ETBF-infected CRC cells and by shaping the complex inflammatory TME.

Butyrate Produced by *Faecalibacterium prausnitzii* Suppresses the Proliferation of CRC Cells by Modulating miRNAs

Given the various niche metabolic pathways that the microbiome possesses, there is undoubtedly a metabolic interaction between cancer cells and the microbiome. Some of them, including short-chain fatty acids (SCFAs), vitamins, secondary bile acids, and phytochemicals, play important roles in the composition of the TME and have been found to modulate the expression of miRNAs, affecting the apoptosis, invasion, and proliferation of cancer cells (Louis et al., 2014; Johnson et al., 2016; O'Keefe, 2016). *Faecalibacterium prausnitzii* is a well-known tumor-inducing bacterium in the human gut and is considered to be an important producer of butyrate (Lenoir et al., 2020). Butyrate is the most studied SCFA, and it is synthesized by glycolysis from microbiome-accessible hydrocarbons (Louis and Flint, 2017). There is growing scientific evidence indicating that butyrate can suppress the proliferation of CRC cells and induce apoptosis and differentiation *via* dysregulating the expression of miRNAs. Hu et al. (2015) found that butyrate suppressed oncogenic miR-92a overexpression in human CRC cells and detected a rapid decrease in the levels of c-MYC and pri-miR-17-92a after butyrate treatment. Previous studies have found a highly conserved c-MYC binding site in the intronic C13orf25 promoter of the upstream pri-miR-17-92a coding sequence, suggesting that butyrate treatment reduced miR-92a levels at all processing stages (Ji et al., 2011). miR-92a downregulation subsequently stimulated p57 expression *via* reducing the inhibition of p57 translation. Butyrate-stimulated p57 protein, which is epigenetically silenced in cancer, blocks cell proliferation by promoting apoptosis, inhibiting angiogenesis and cell cycle arrest (Kavanagh and Joseph, 2011). Thus, butyrate, produced by *F. prausnitzii*, decreases oncogenic miR-92a levels by suppressing c-MYC protein levels, thereby activating p57 translation and inhibiting CRC proliferation (**Figure 5**). In contrast, miR-203 expression is upregulated after butyrate treatment and consequently suppresses the proliferation of CRC cells *via* directly inhibiting NEDD9 expression. NEDD9, a significant tumor-promoting factor, is a member of the crk-associated substrate family and was found highly expressed in CRC tissues (Han et al., 2016). Studies have demonstrated that NEDD9 can induce the epithelial–mesenchymal transition (EMT) by activating c-Jun NH-terminal kinase (JNK), thereby promoting tumor invasion and metastasis (Meng et al., 2019). In this pathway, EMT is a reversible process of differentiation that results in the absence of E-calcium adhesion protein (the main ingredient of adhesion) in epithelial cells and JNK, a member of the MAPK family, which has been reported to be closely associated with

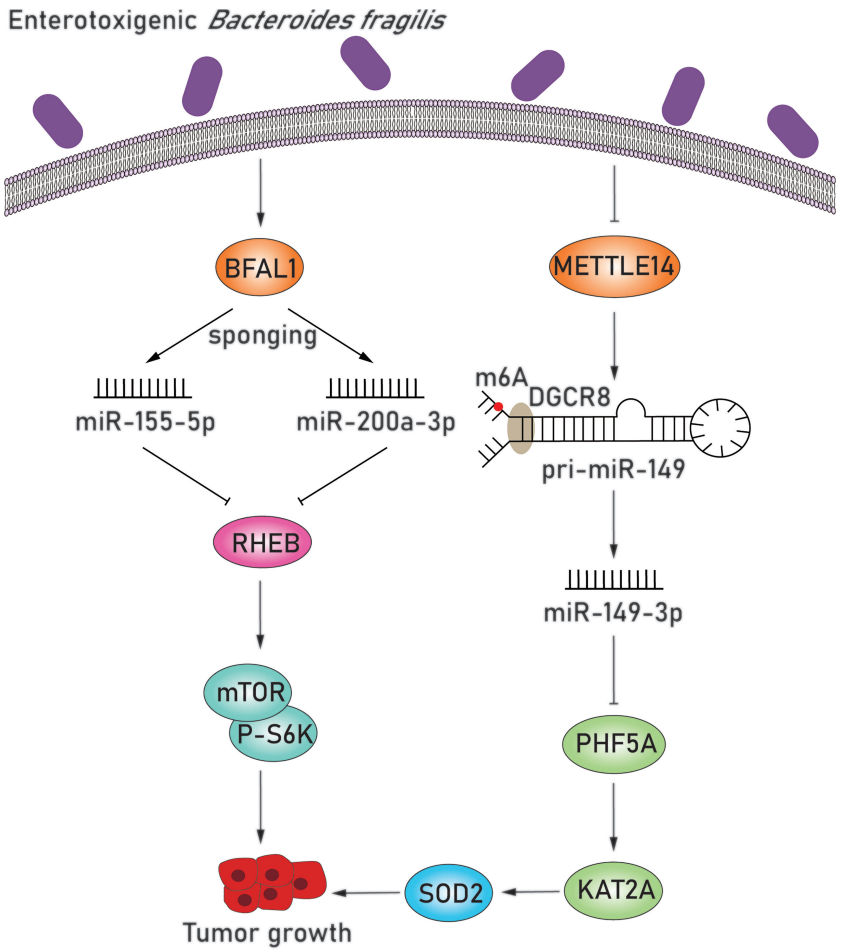

FIGURE 4 | ETBF induces tumor growth by downregulating miR-155-5p, miR-200a-3p, and miR-149-3p. BFAL1 was upregulated in ETBF-infected CRC cells and subsequently decreased the levels of miR-155-5p and miR-200a-3p, which attenuated their suppressive function on target RHEB mRNA expression. The downregulated RHEB consequently activates the RHEB/mTOR pathway, thereby mediating ETBF-induced tumor growth. miR-149-3p is also significantly downregulated in ETBF-infected CRC cells. Regulation of miR-149-3p was attributed to METTL14-dependent m6A modification *via* modifying pri-miRNA splicing. In ETBF-infected CRC cells, METTL14 was downregulated so that the level of miR-149-3p was reduced, which further upregulated target PHF5A. The overexpression of PHF5A upregulated the target mRNA level of KAT2A in CRC cells, which can upregulate SOD2 *via* directly binding to the promoter region of SOD2. More importantly, the overexpression of SOD2 is relevant to poor clinical outcomes in gastric cancer, and SOD2 may promote intestinal inflammation and tumorigenesis.

proliferation, differentiation, apoptosis, and migration (Wagner and Nebreda, 2009; Min et al., 2015). Notably, it has been extensively studied that EMT is characterized by the loss of E-cadherin in epithelial cells, resulting in the downregulation of cell–cell adhesion, suggesting the great impact of E-cadherin inactivation in colorectal carcinogenesis (Meng et al., 2019). Hakai is the first posttranslational regulator described for the E-cadherin stability and has been reported to cause the alteration of cell–cell contacts involved in colorectal carcinogenesis (Castosa et al., 2018). Upregulated miR-203 induced by butyrate can also lower Hakai expression by binding to the 3′-UTR of target mRNA, eventually suppressing the proliferation of CRC cells (Abella et al., 2012) (**Figure 5**). Taken together, butyrate produced by *F. prausnitzii* can suppress the proliferation of CRC cells by upregulating miR-203, which can not only inhibit NEDD9 expression, but also inhibit Hakai expression.

CONCLUSION

Modulation of miRNAs by the microbiome during bacterial pathogen infection and its effects on the host transcriptome have been investigated. Given the role of miRNAs in affecting many critical cellular processes, such as apoptosis, proliferation, and differentiation, we believe that miRNAs play a central, if not critical, role in influencing the development of CRC. In order to fully understand the interactions between unique miRNAs and microbiome, we investigated several possible pairwise interactions in this article and elaborated on the mechanisms involved. The four pathogens *F. nucleatum*, *E. coli*, ETBF, and *F. prausnitzii* are introduced; however, the interactions between other pathogens and CRC-related miRNA still need to be studied. As miRNAs have an important role in CRC, regulating miRNAs for therapeutic interventions is needed. Of note, regulating the composition of the

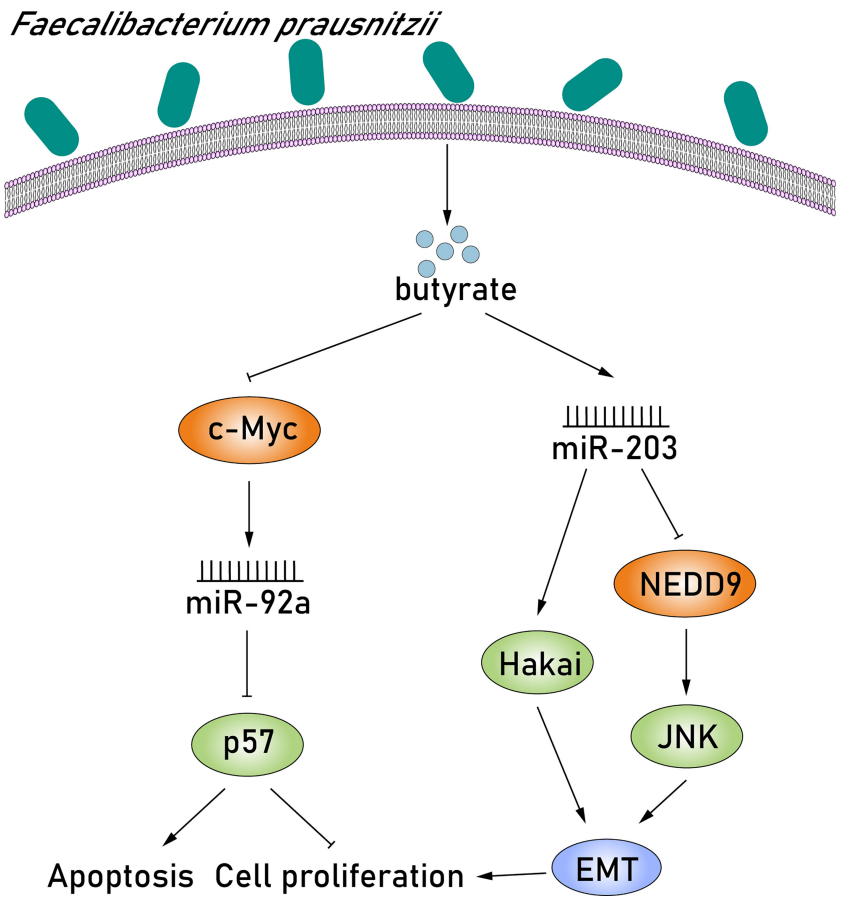

FIGURE 5 | Butyrate produced by *F. prausnitzii* suppresses the proliferation of CRC cells by modulating miR-92a and miR-203. First, butyrate produced by *F. prausnitzii* suppressed oncogenic miR-92a overexpression in human CRC cells and detected a rapid decrease in the levels of c-MYC after butyrate treatment. miR-92a downregulation subsequently stimulated p57 expression, which is epigenetically silenced in cancer, blocks cell proliferation by promoting apoptosis, inhibiting angiogenesis and cell cycle arrest. In contrast, miR-203 expression is upregulated after butyrate treatment and consequently suppresses the proliferation of CRC cells *via* directly inhibiting NEDD9 expression. NEDD9, a significant tumor-promoting factor, can induce the EMT by activating JNK, thereby promoting tumor invasion and metastasis. Upregulated miR-203 induced by butyrate can also lower Hakai expression, eventually suppressing the proliferation of CRC cells.

gut microbiome and reducing the occurrence of CRC, such as fecal microorganism transplantation, need to be applied. In the future, it will be imperative to use a combination of approaches to comprehensively treat CRC to effectively reduce the recurrence of CRC.

AUTHOR CONTRIBUTIONS

JX and YL have contributed equally to this work and share first authorship. The study was conceptualized by JX and YL. HZ and ZZ were responsible for literature search. JX and YL were responsible for writing of the original draft preparation. DT and DW were responsible for writing, review, and editing. Elaboration of the tables and figures was performed by JZ and WZ. JX and YL were responsible for Supervision. All authors have read and agreed to the published version of the manuscript.

FUNDING

This study was supported by Training Project of Key Talents of Youth Medicine in Jiangsu province, China [No. QNRC2016330], The Key Disease Standardization Diagnosis and Treatment Project in Jiangsu Province [NO. BE2015664], The Academic Science and Technology Innovation Fund for College Students [No. ×20180714], The Social Development-Health Care Project of Yangzhou, Jiangsu Province [No. YZ2018087], The Social Development Project of Yangzhou, Jiangsu Province [No. YZ2021075], The Graduate Research and Practice Innovation Plan of Graduate Education Innovation Project in Jiagsu Province [NO. SJCX211644] and High-level talent "six one project" top talent scientific project of jiangsu Province [No. LGY2019034].

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frontiers | Frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 02 May 2022
doi: 10.3389/fcimb.2022.885093

Combined Microbiome and Metabolome Analysis Reveals a Novel Interplay Between Intestinal Flora and Serum Metabolites in Lung Cancer

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Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: 27 February 2022
Accepted: 04 April 2022
Published: 02 May 2022

Citation:
Chen S, Gui R, Zhou X-h, Zhang J-h, Jiang H-y, Liu H-t and Fu Y-f (2022) Combined Microbiome and Metabolome Analysis Reveals a Novel Interplay Between Intestinal Flora and Serum Metabolites in Lung Cancer. Front. Cell. Infect. Microbiol. 12:885093. doi: 10.3389/fcimb.2022.885093

As the leading cause of cancer death, lung cancer seriously endangers human health and quality of life. Although many studies have reported the intestinal microbial composition of lung cancer, little is known about the interplay between intestinal microbiome and metabolites and how they affect the development of lung cancer. Herein, we combined 16S ribosomal RNA (rRNA) gene sequencing and liquid chromatography-mass spectrometry (LC-MS) technology to analyze intestinal microbiota composition and serum metabolism profile in a cohort of 30 lung cancer patients with different stages and 15 healthy individuals. Compared with healthy people, we found that the structure of intestinal microbiota in lung cancer patients had changed significantly (Adonis, $p = 0.021$). In order to determine how intestinal flora affects the occurrence and development of lung cancer, the Spearman rank correlation test was used to find the connection between differential microorganisms and differential metabolites. It was found that as thez disease progressed, L-valine decreased. Correspondingly, the abundance of *Lachnospiraceae_UCG-006*, the genus with the strongest association with L-valine, also decreased in lung cancer groups. Correlation analysis showed that the gut microbiome and serum metabolic profile had a strong synergy, and *Lachnospiraceae_UCG-006* was closely related to L-valine. In summary, this study described the characteristics of intestinal flora and serum metabolic profiles of lung cancer patients with different stages. It revealed that lung cancer may be the result of the mutual regulation of L-valine and *Lachnospiraceae_UCG-006* through the aminoacyl-tRNA biosynthesis pathway, and proposed that L-valine may be a potential marker for the diagnosis of lung cancer.

Keywords: lung cancer, intestinal microorganism, intestinal flora, serum metabolites, biomarker

INTRODUCTION

Lung cancer has the characteristics of high morbidity and high mortality, which seriously endangers human health and quality of life. As the leading cause of cancer death, there would be an estimated 2.2 million new cases and 1.79 million deaths worldwide in 2020 (Sung et al., 2021). Many early-stage lung cancer patients have no symptoms or only have mild symptoms with no specificity, making it difficult to attract attention. However, the diagnosis of lung cancer requires complicated procedures such as pathological biopsy and imaging examinations, and there is a lack of simple bedside detection methods. Therefore, many lung cancer patients are often in the middle or advanced stages when they are diagnosed, so there are always different degrees of cancer metastasis, leading to a poor prognosis (Rudin et al., 2021). The current non-invasive diagnostic method for lung cancer is mainly liquid biopsy. There is a small amount of circulating tumor cells (CTC) shed from the tumor site in the blood of cancer patients, and necrotic cancer cells release a small amount of circulating tumor DNA (ctDNA) into the blood; therefore, it can help to judge the occurrence of cancer by detecting CTC and ctDNA. In addition, non-invasive biomarkers based on protein and microRNA have also been widely studied (I and Cho, 2015; Farooq and Herman, 2020). In recent years, a growing number of studies have discovered the connection between intestinal flora and disease diagnosis, treatment, and prognosis. At present, many studies involve the sequencing of gut microbes in lung cancer patients, and Zheng et al. (2020) have developed an operational taxonomic unit (OTU)-based prediction model for the early diagnosis of lung cancer. However, relying on single omics for prediction does not seem to be sufficient, and so far, the connection and interaction between the gut microbiome and metabolome of lung cancer patients with different stages have not been recorded.

A cohort study found that the diversity of intestinal flora was at a similar level in healthy children and adults, but the composition and function of the microbiome were different (Wen et al., 2019). Herein, we assume that there are differences in the composition or diversity of the intestinal flora of lung cancer patients with different stages. We recruited 30 different-stage lung cancer patients and 15 healthy individuals and performed the corresponding detection and analysis on their stool and serum specimens. The composition of the intestinal flora and serum metabolites was compared by bioinformatics analysis. We are trying to combine microbiology and metabolomics to find out the pathogenesis of lung cancer and potential biomarkers, so as to provide new insights for the diagnosis and treatment of lung cancer in the future.

MATERIALS AND METHODS

Participants and Sample Collection

Thirty newly diagnosed lung cancer patients from Hunan Cancer Hospital and 15 individuals undergoing physical examination from the Health Management Center of the Third Xiangya Hospital of Central South University were included in our study. Stool and serum samples were collected according to the protocol approved by the Ethics Committee of the Third Xiangya Hospital of Central South University, and written informed consent from all participants was obtained. The exclusion criteria were as follows: 1) individuals with primary carcinoma of other organs, 2) individuals suffering from other cancers, and 3) individuals receiving antibiotics or probiotics within the past 3 months. The control group was matched according to age and sex ratio.

Fresh stool samples of each participant were collected and then placed on ice immediately. The temperature was ensured to be below 4°C, and the samples collected were stored at −80°C within 1 h until DNA extraction. Intravenous blood collection was carried out by professional nurses in strict accordance with aseptic standard procedures. Serum was collected by centrifugation and stored at −80°C until being tested.

Microbial DNA Extraction and Sequencing

QIAamp 96 Power Fecal QIAcube HT kit (Qiagen, Hilden, Germany) was used to extract total DNA from stool samples according to the manufacturer's instructions. NanoDrop2000 (Thermo Fisher, USA) and 1% agarose gel electrophoresis were used to detect the concentration and quality of DNA. Barcode-specific primers (primers 5′-TACGGRAGGCAGCAG-3′ and 5′-AGGGTATCTAATCCT-3′) were used to perform PCR amplification on the 16S V3–V4 region.

The PCR products were separated on a 2% agarose gel and further purified using AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA). The purified PCR products were quantified using Qubit dsDNA Assay Kit (Life Technologies, Waltham, MA, USA). Finally, equal amounts of samples were mixed according to the concentration of PCR products, and NovaSeq PE250 paired-end sequencing was performed.

16S rRNA Sequencing Result Data Processing

The Trimmomatic software (Bolger et al., 2014) was used to remove impurity from the original double-ended sequence (FASTQ format), and the FLASH software (Reyon et al., 2012) was used to merge after impurity removal. Then, sequences containing ambiguous bases, single-base homologous regions, and chimeras were removed to achieve accurate impurity removal to ensure the accuracy of the results. After sequencing data that were being preprocessed to generate high-quality sequences, the OTU with sequence similarity ≥97% was defined as a taxon by VSEARCH software (Caporaso et al., 2010). All representative reads were annotated and blasted against the SILVA database (version 132) using RDP classifier v2.11 (confidence threshold of 70%) (Wang et al., 2007). The microbial diversity was estimated using alpha diversity that included the Chao1 index (Chao and Bunge, 2002), Shannon index (Hill et al., 2003), and Simpson index. The UniFrac distance matrix performed by the QIIME software was used for weighted UniFrac principal coordinates analysis (PCoA).

LC/MS Non-Targeted Metabolomics Analysis

Metabolites were extracted after sample pretreatment. The LC-MS system composed of a Dionex U3000 UHPLC ultra-high performance liquid chromatograph (Thermo Fisher Scientific, USA) and a QE PLUS high-resolution mass spectrometer (Thermo Fisher Scientific, USA) was used as the analytical instrument in this experiment. The operating conditions of the instrument were set as follows: chromatographic conditions—chromatographic column, ACQUITY UPLC HSS T3 (100 mm × 2.1 mm, 1.8 μm); column temperature, 45°C mobile phase, 0.1% formic acid–water (A) and acetonitrile (B); flow rate, 0.35 ml/min; injection volume, 2 μl; and mass spectrum condition—ion source, ESI. The sample mass spectrum signal acquisition adopted the positive and negative ion scanning modes, respectively. After obtaining the original data, Progenesis QI v2.3 software was used to perform standardized preprocessing and qualitative and relative quantitative analyses.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (San Diego, CA, USA), QIIME, and R package (V.2.15.3). Student's *t*-test and Fisher's exact test were used to compare sample baseline data. Adonis analysis, Wilcoxon rank-sum test, and Kruskal–Wallis test were used to compare the differences between microbial groups. The metabolomics data were processed and analyzed using the Progenesis QI v2.3 software. Student's *t*-test and fold change analysis were used to compare metabolites between groups. Pearson correlation coefficient was used to measure the degree of linear correlation between two metabolites. The Spearman rank correlation test was used to assess the correlation between microorganisms and metabolites. $p < 0.05$ was considered as statistically significant.

RESULTS

Changes in the Structure of the Intestinal Flora Associated With Lung Cancer

This study included 30 lung cancer patients and 15 healthy individuals. The average age of the early-stage lung cancer (ESLC) group, non-early-stage lung cancer (NESLC) group, and healthy control (HC) group was 61.25 ± 6.09, 57.82 ± 9.1, and 59.6 ± 5.45 years old, respectively. No significant difference was found. Similarly, the gender distribution of the three groups has also been verified (Fisher's exact test, $p = 0.923$). Adenocarcinoma was the main type of lung cancer (6/8 in ESLC, 12/22 in NESLC) (**Table 1**).

In order to explore the intestinal microbial composition of lung cancer patients with different stages, we performed 16S rRNA sequencing on stool samples of 30 lung cancer patients (8 of ESLC, 22 of NSLC) and 15 healthy individuals. The VSEARCH (version 2.4.2) software (Rognes et al., 2016) was used to classify OTU according to 97% of similarity. The sequence with the largest abundance in each OTU was selected as the representative sequence of the OTU, compared, and annotated with the SILVA (v132) database using the RDP classifier (v2.11) as the annotation tool. A total of 6,053 annotated OTUs were obtained. Compared with HC, the total OTUs and unique OTUs of ESLC and NESLC increased, and there were 2,378 annotation OTUs shared by the three groups (**Figure 1A**).

We analyzed the community structure of intestinal microbial (**Supplemental Material 1**). At the phylum level, *Bacteroides*, *Firmicutes*, and *Proteobacteria* were the main components in the three groups. At the family level, besides similar families with higher abundance (*Bacteroidaceae*, *Lachnospiraceae*, *Ruminococcaceae*), the abundance of *Enterobacteriaceae* was higher in ESLC (15.01%), *Enterobacteriaceae* and *Prevotellaceae* in NESLC (10.55%, 9.71%), and *Prevotellaceae* in HC (14.87%). At the genus level, apart from the similar higher abundance genus (*Bacteroides*), *Faecalibacterium* (10.71%), *Prevotella_9* (12.56%), and *Bacteroides* (7.33%) were the most abundant genera identified in HC; *Faecalibacterium* (8.25%), *Klebsiella* (7.76%), and *Escherichia–Shigella* (6.77%) in ESLC; and *Prevotella_9* (7.77%) and *Escherichia–Shigella* (7.23%) in NESLC (**Figures 1B–D**).

The microbial abundance was statistically analyzed (**Supplemental Material 2**). The Chao1 index showed that there was no significant difference in community richness among groups, while the Shannon and Simpson indexes both showed that each group had similar community diversity. When comparing the structure of the microbial community, β diversity showed differences among the groups (**Figure 1E**, Adonis, $p = 0.021$).

Analysis of Differences in Intestinal Microbes

The Kruskal–Wallis algorithm was used to further identify microbes with different abundances, and 3 phyla, 21 families, and 55 genera were identified (**Supplemental Material 3**). At the genus level, *Escherichia–Shigella*, *Anaerotruncus*, *Ruminiclostridium*, *Lactobacillus*, *Pediococcus*, *Sphingobium*, *Prevotellaceae*, *Prevotella_1*, and *Olsenella* were rich in the two lung cancer groups and also *Cryptobacterium* in ESLC and *Lachnospira*, *Roseburia*, *Brevundimonas*, *Lachnospiraceae_UCG-006*, and *Lachnospiraceae_UCG-004* in HC.

Linear discriminant analysis (LDA) effect size (LEfSe) was used to identify key microbial taxa. Eight, five, and two key genera were identified in HC, ESLC, and NESLC, respectively (**Figure 2**). The genus with an average relative abundance of less than 0.01% was excluded. The key species were *Roseburia* (LDA score 4.22, $p = 0.012$), *Lachnospira* (LDA score 4.21, $p = 0.001$), *Anaerostipes* (LDA score 3.83, $p = 0.007$), and *Lachnoclostridium* (LDA score 3.60, $p = 0.042$) in HC; *Lactobacillus* in ESLC (LDA score 3.89, $p = 0.029$); and *Escherichia_Shigella* in NESLC (LDA score 4.53, $p = 0.010$).

Changes of Plasma Metabolite Profile in Lung Cancer Patients and Crucial Metabolites

Metabolites and fermentation products produced by intestinal flora can enter the blood and have a functional impact on the host's physiology. Therefore, in order to further explore the changes in the intestinal microbe–host interaction, we examined

TABLE 1 | Participants' baseline data.

Group		ESLC group (*n* = 8)	NESLC group (*n* = 22)	HC group (*n* = 15)	*p*-value
Age, mean ± SD, years		61.25 ± 6.09	57.82 ± 9.1	59.6 ± 5.45	0.519
Gender, male, *n*		4	12	7	0.923
Type				NA	
	Adenocarcinoma	6	12		
	Squamous cell carcinoma	0	6		
	Small cell carcinoma	0	4		
	Large cell carcinoma	1	0		
	Carcinoma *in situ*	1	0		
Tumor staging				NA	
	0	1			
	I	5			
	II	2			
	III		6		
	IV		16		
Tumor metastasis		1	15	NA	

NA, not applicable.

the metabolic profile in the serum. Based on the abundance of metabolites detected by non-targeted metabolomics, the orthogonal partial least-squares discriminant analysis (OPLS-DA) was performed. According to the scatter plot, samples from different groups were largely separable, indicating different metabolic patterns (**Figures 3A–C**). The permutation test showed that there was no overfitting to the data, and verified the OPLS-DA model (**Figures 3D–F**). Generally, the closer the slopes of the R2Y and Q2Y lines were to zero, the more likely the model was to be overfitted. A total of 5,514 metabolites were identified, consisting of 2,793 of positive ion and 2,721 of negative ion (**Supplemental Material 4**).

Variable importance of projection (VIP) value was obtained through the OPLS-DA model. The biologically significant differential metabolites were mined according to the screening criteria: the VIP value of the first principal component of the OPLS-DA model >1 and the *p*-value of the *t*-test <0.05. The larger the value, the greater the contribution of the variable to the grouping. ESLC vs. HC, NESLC vs. HC, and ESLC vs. NESLC screened out 272, 319, and 68 differential metabolites, respectively (**Supplemental Material 5**). Hierarchical clustering was performed on the expression of the differential metabolites with the top 50 of VIP value to show the relationship among samples and the expression differences of metabolites among samples more intuitively. The result is shown below (**Figure 4**). 9-Hydroxy-7-megastigmen-3-one glucoside, 1-[6-(3)-ladderane-hexanoyl-2-(8-(3)-ladderane-octany])-sn-glycerophosphocholine, and perilloside A were more abundant in the lung cancer groups, while indoleacrylic acid, L-isoleucine, L-valine, PC(O−16:0/2:0), and LysoPC (16:0) decreased.

Correlation analysis can help measure the correlation between differential metabolites. Differential metabolites with the top 50 of VIP value were selected for visual analysis. It was found that perilloside C, which was richer in the lung cancer groups, was negatively correlated with PC(O−16:0/2:0), which was richer in the HC group (**Supplemental Material 6**).

The KEGG ID of the metabolites was used for pathway enrichment analysis. The differential metabolites of ESLC vs. HC were mainly involved in aminoacyl-tRNA biosynthesis; valine, leucine, and isoleucine biosynthesis; ABC transporters; and sphingolipid signaling pathway ($p < 0.01$); those of NESLC vs. HC were involved in caffeine metabolism; valine, leucine, and isoleucine biosynthesis; aminoacyl-tRNA biosynthesis; Fc gamma R-mediated phagocytosis; and choline metabolism in cancer ($p < 0.01$). On the other hand, the differential metabolites of ESLC vs. NESLC were involved in butanoate metabolism, aminoacyl-tRNA biosynthesis, and apoptosis ($p < 0.01$) (**Figure 5**).

We found that the aminoacyl-tRNA biosynthesis pathway seemed to be closely related to the progression of lung cancer, so we focused on the differential metabolites involved in it, L-valine, L-lysine, L-isoleucine, L-histidine, and L-glutamate, to seek new biomarkers (**Supplemental Material 7**). We reviewed the expression levels of these metabolites in different samples (**Figure 6**). The results showed that L-lysine, L-isoleucine, and L-histidine decreased significantly in the lung cancer groups. Compared with ESLC, L-glutamate increased significantly in NESLC. L-valine showed significant differences in the pairwise comparisons, and the trend of change was first down and then up, but always lower than the healthy level.

Conjoint Analysis

Correlation analysis was performed to help us better understand the correlation between the microbiome and plasma metabolome. Based on metabolomics and genomics datasets, network analysis was performed to determine broader associations between the two (**Figure 7** and **Supplemental Material 8**). The CorNetwork diagram showed the relationship between *Anaerostipes*, *Coprococcus_3*, *Lachnospiraceae_UCG-004*, *Lachnospiraceae_UCG-006*, and various metabolites. The Spearman correlation coefficients between the top 100 differential metabolites and the top 100 differential microbes were calculated (**Supplemental Material 9**). The results showed that there was a significant correlation between L-valine and *Lachnospira*, *Anaerostipes*, *Coprococcus_3*, *Fusicatenibacter*, *Lachnospiraceae_UCG-004*, *[Eubacterium]_xylanophilum_group*, *Lachnospiraceae_UCG-006*, and *Burkholderia–Caballeronia–Paraburkholderia*. Among them, *Lachnospiraceae_UCG-006* was the most correlated genus

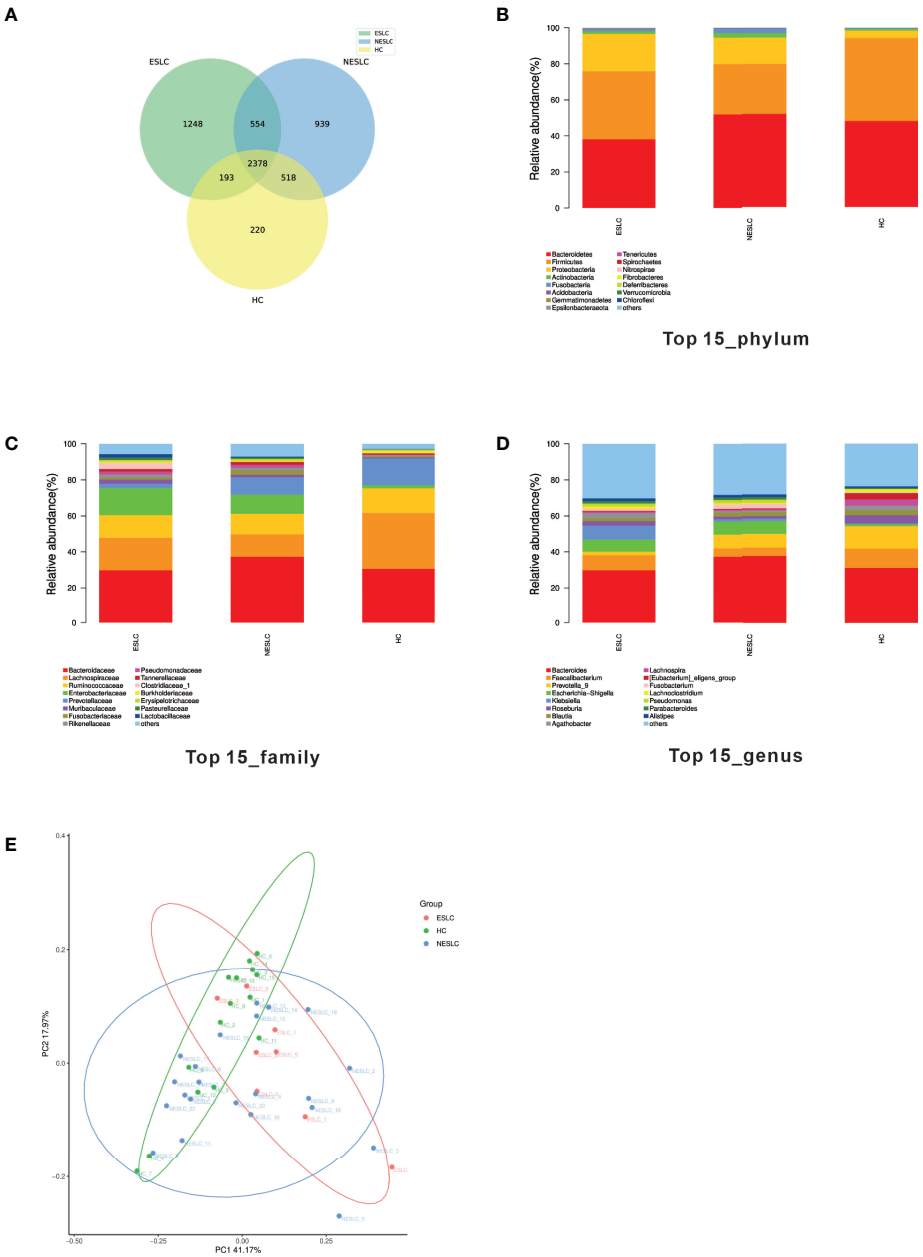

FIGURE 1 | Intestinal flora community structure. **(A)** The Venn diagram showed each group of unique and common OTUs. **(B–D)** The top 15 representative species and their proportions in the three groups at the level of phylum, species, and genus. **(E)** PCoA shows differences between individuals or groups. The abscissa (PC1) and the ordinate (PC2) are the two main coordinates that explain the greatest difference between samples. The points in the graph represent samples, and different colors represent different sample grouping information; similar samples are clustered together.

(correlation = 0.49, $p < 0.001$), which was also a differential genus enriched in HC.

DISCUSSION

Our research has proved the changes of intestinal microbiota and serum metabolic spectrum in lung cancer patients. We combined the two omics to find the possible pathogenesis and potential biomarkers of lung cancer.

The control group consisted of individuals undergoing physical examination from the Health Management Center, while lung cancer patients were newly diagnosed without treatment. Medication use is an important consideration when we screen study subjects, which is mainly because any medication, such as antibiotics and anticancer medication, will

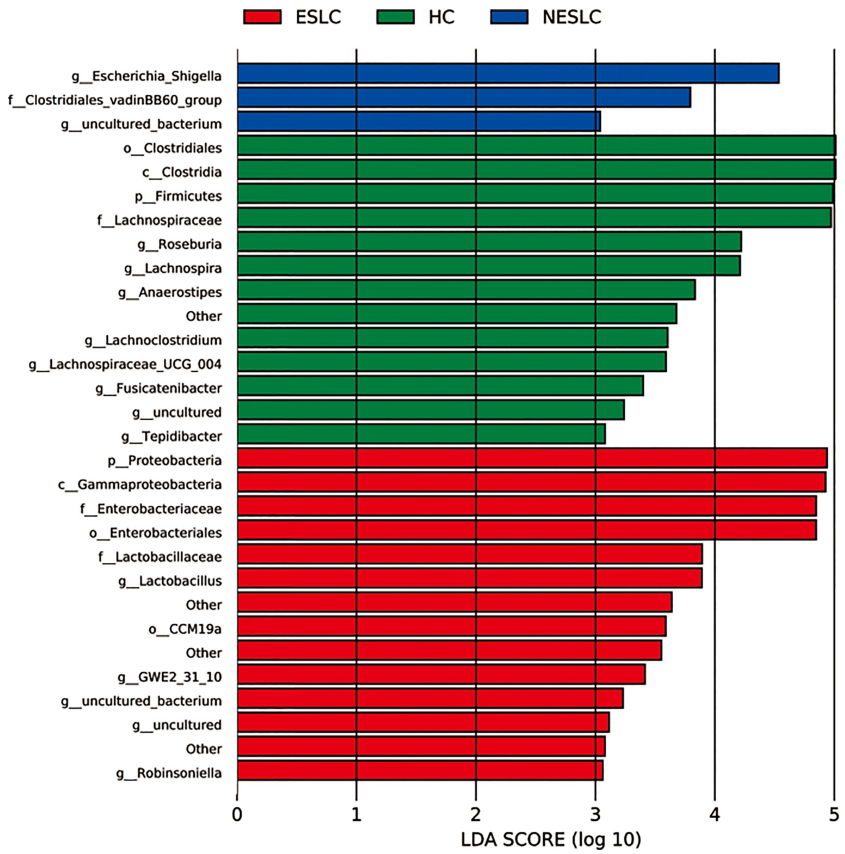

FIGURE 2 | Key genera selection. Differential microbial score chart: the higher the score, the greater the contribution of the microbe to the difference.

cause changes in intestinal flora to varying degrees. We have observed differences in the composition and structure of gut microbes between lung cancer patients with different stages and healthy people. *Faecalibacterium* has a higher abundance in HC, and its relative and absolute abundances in ESLC and NESLC decreased sequentially. *Faecalibacterium* is considered to be a marker of healthy intestines and is a type of butyrate-producing bacteria (Wang et al., 2007). In previous studies, it was also found that its abundance decreased in lung cancer patients (Gui et al., 2020; Zheng et al., 2020). In addition, ESLC has abundant *Klebsiella* and *Escherichia–Shigella*, while NESLC has abundant *Prevotella_9* and *Escherichia–Shigella*. *Klebsiella*, *Escherichia–Shigella*, and *Prevotella_9* all contain many opportunistic pathogens. The latest research has linked the increase in *Prevotella* abundance with local and systemic diseases, including rheumatoid arthritis, hypertension, and metabolic disorders (Horta-Baas et al., 2017; Li et al., 2017; Ding et al., 2020).

Previous studies have shown that the changes of intestinal flora in patients with lung cancer are often related to *Firmicutes*, *Bacteroides*, and *Proteobacteria* (Zhang et al., 2018; Liu et al., 2019; Zhuang et al., 2019; Zheng et al., 2020; Georgiou et al., 2021). When using the Kruskal–Wallis algorithm to further identify microbes with different abundances, it was found that the abundance of *Firmicutes* decreased and *Proteobacteria* increased in the lung cancer group. Compared with previous studies, the change of *Firmicutes* was consistent and *Proteobacteria* was also consistent with most studies, which only decreased in the study of Zhang et al. However, *Bacteroides* has been found to increase in lung cancer, while *Actinomycetes* has been found to decrease (Zhuang et al., 2019; Zheng et al., 2020), which was not found in our study, even in the comparison between HC and NESLC. We try to find the reasons for the differences from the included population and experimental design. The study of Zheng et al. included patients with early-stage lung cancer. Although one of its exclusion criteria was antibiotic use in the last 8 weeks, the use of anticancer drugs was not specified. In the study of Zhuang et al., subjects did not use any drugs in the past 3 months and seemed to have not received chemotherapy, but it was uncertain whether there were therapeutic behaviors other than chemotherapy. Our subjects were all newly diagnosed and treated patients, so we consider that the difference may be due to the selection criteria of subjects or the heterogeneity of patients. It is worth mentioning that although no difference was found at the level of *Bacteroides*

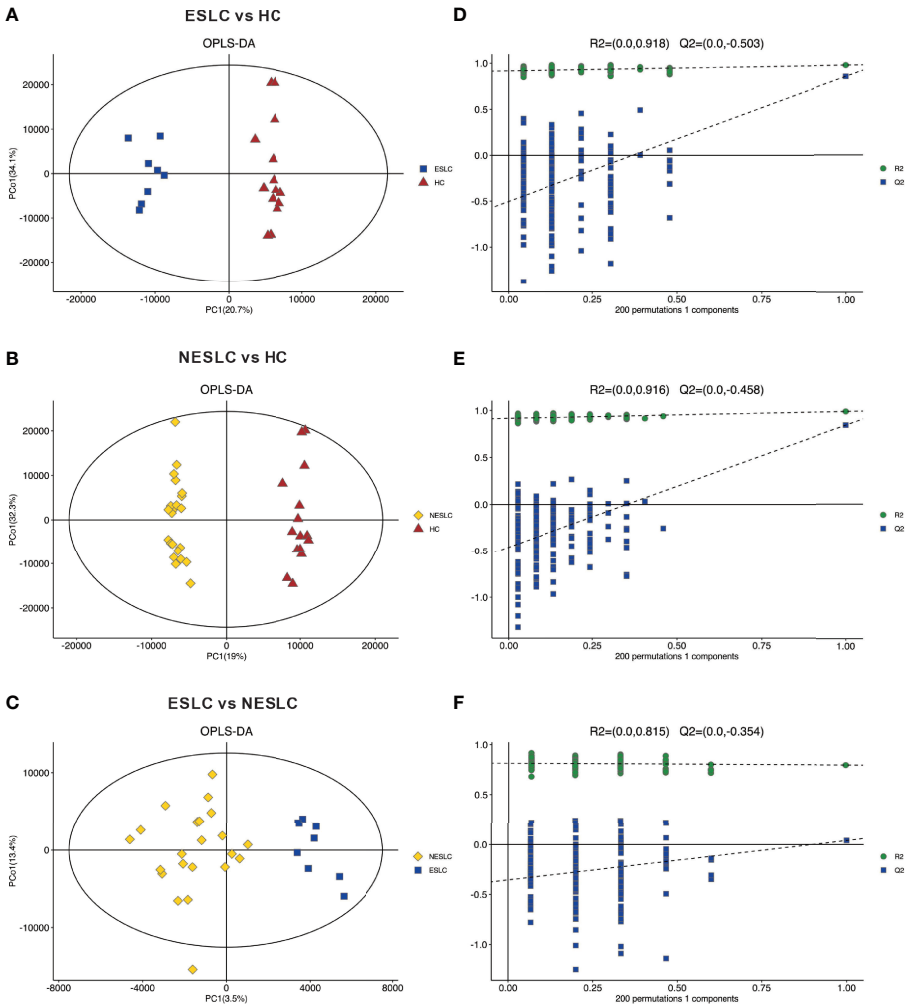

FIGURE 3 | Principal component analysis. **(A–C)** OPLS-DA score chart shows the difference in metabolites between groups. The abscissa represents the variation between groups, and the ordinate represents the variation within groups. **(D–F)** Comparison of the true model parameters in the validation test and those of permutated models.

and *Actinomycota*, differential genera mined such as *Roseburia*, *Lachnospira*, *Olsenella*, and *Cryptobacterium* belonged to *Bacteroides or Actinomycota*.

When looking for key differential microorganisms, it was found that *Anaerostipes*, *Lachnoclostridium*, *Roseburia*, and *Lachnospira* were the key differential genera in HC. These genera are closely related to the production of short-chain fatty acids (Liu et al., 2019; Chen et al., 2021; Yi et al., 2021). Short-chain fatty acids play an important role in human health because they regulate the function and differentiation of almost all intestinal immune cells (Surono et al., 2021). It has been found that short-chain fatty acids maintain intestinal homeostasis by promoting the production of IL-10 in Th1 cells (Sun et al., 2018). Xia et al. (2020) made preparations from plant flower buds to induce SCFA-producing bacteria to produce SCFAs to achieve an anticancer effect. Correspondingly, the key differential genus in ESLC was *Lactobacillus*. *Lactobacillus* itself is a kind of beneficial bacteria, which acts in preventing pathogenic bacteria from invading and colonizing the intestines, enhances the body's immunity, and has anticancer effects (Hendler and Zhang, 2018; Eslami et al., 2019; Han et al., 2021). In colorectal cancer, *Lactobacillus* showed reduced abundance (Loftus et al., 2021). However, in a large cohort study, it was found that the increased abundance of *Lactobacillus* bacteria in the oral cavity was closely related to the risk of lung cancer (Hosgood et al., 2021). The key differential genus in NESLC was *Escherichia_Shigella*. The increased abundance of *Escherichia/Shigella* was considered to be a characteristic of intestinal flora imbalance in Crohn's disease (Pascal et al., 2017). These indicate that compared with HC, patients with early/advanced lung cancer have different degrees of intestinal flora imbalance. Random forest algorithm, a kind of machine learning algorithm, was used to screen important

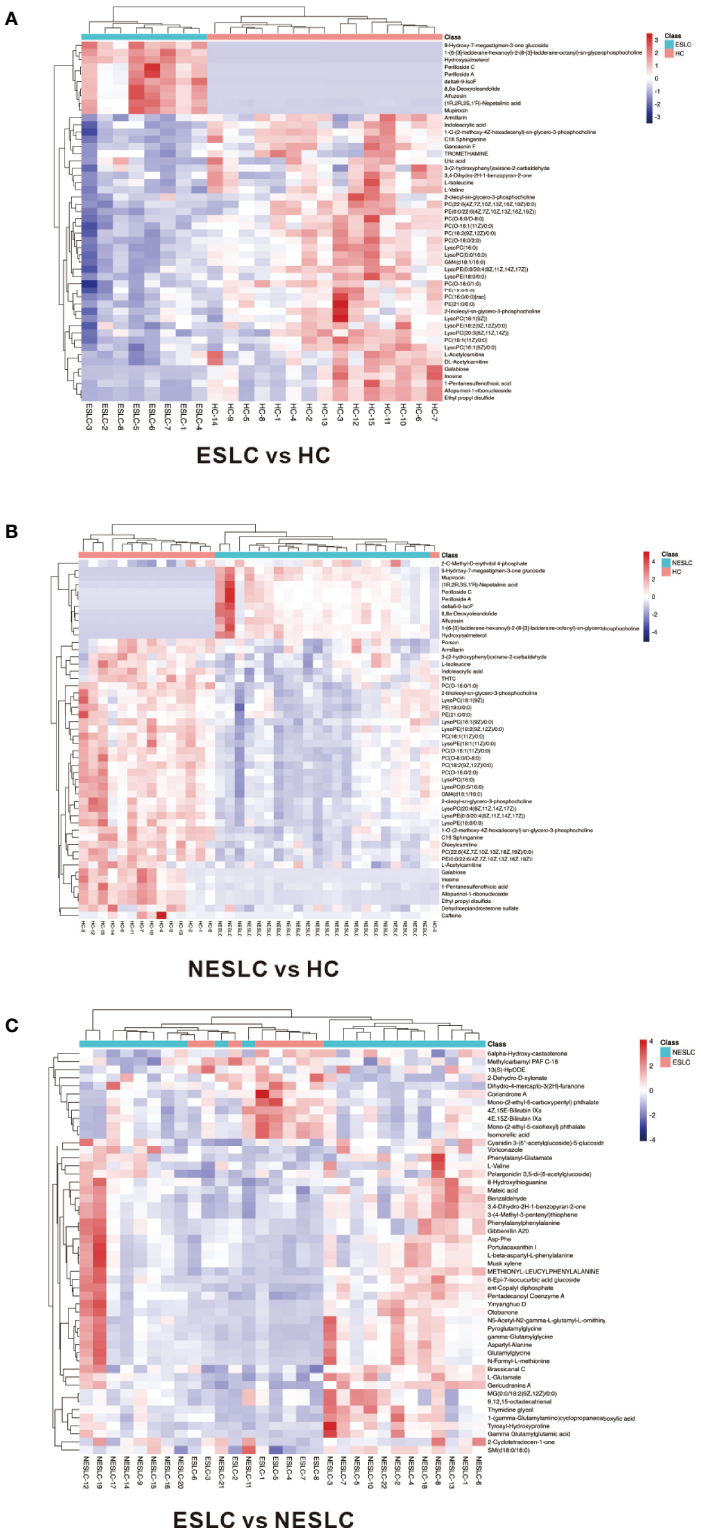

FIGURE 4 | Heatmap of differential metabolites. **(A)** Differential metabolites in ESLC vs. HC. **(B)** Differential metabolites in NESLC vs. HC. **(C)** Differential metabolites in ESLC vs. NESLC. The abscissa represents the sample name, and the ordinate represents the differential metabolite. The color from blue to red indicates that the expression abundance of metabolites is from low to high.

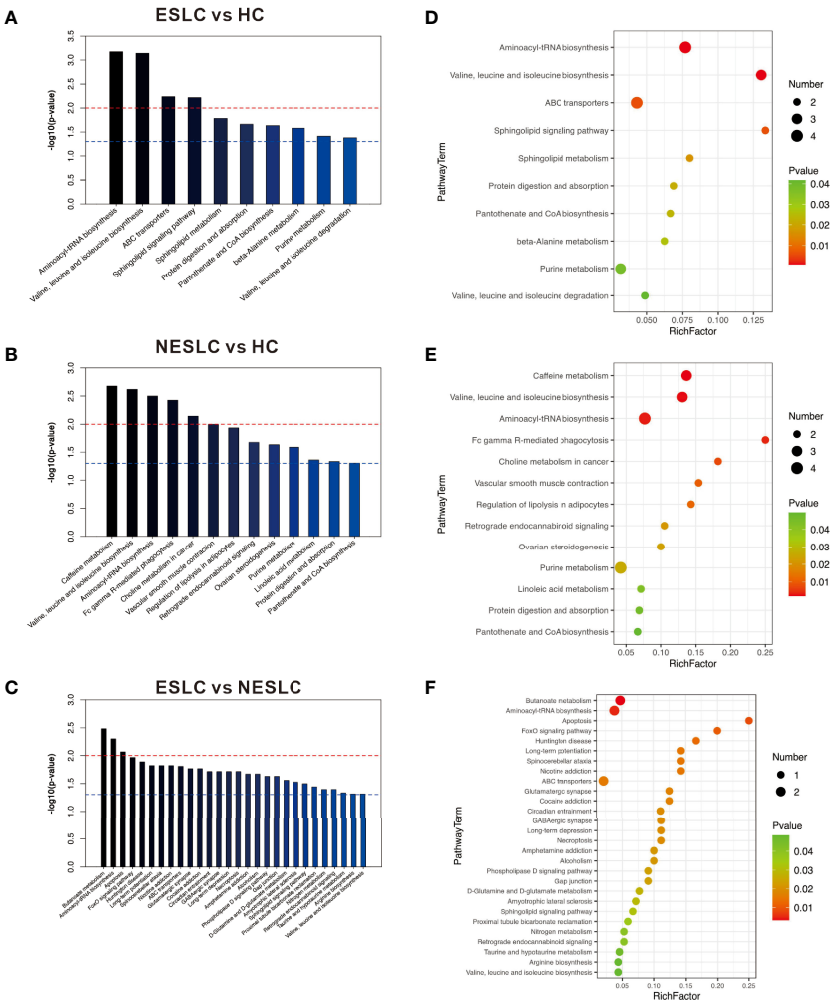

FIGURE 5 | **(A–C)** The *p*-value is the significance of enrichment in metabolic pathways. The red line indicates that the *p*-value is 0.01, and the blue line indicates that the *p*-value is 0.05. When the top of the bar is higher than the blue line, the signal pathway it represents is significant. **(D–F)** The ordinate is the name of the metabolic pathway, and the abscissa is the rich factor (rich factor = the number of significantly different metabolites/the total number of metabolites in the pathway); the greater the rich factor, the greater the degree of enrichment. The color from green to red indicates that the *p*-value decreases sequentially; the larger the point, the more metabolites enriched on the pathway.

microorganisms that distinguish differences between groups. The screened genera were mostly the same as the key genera in each group. However, it should be noted that due to the hierarchical structure of the algorithm, there may be no linear relationship between the filtered features and the output. For example, *Pseudomonas*, although selected as the most important genus, was not a differential genus, and the difference between groups was not statistically significant.

Pathway enrichment analysis was performed using differential metabolites to understand the mechanism of metabolic pathway changes in different samples. Results showed that the aminoacyl-tRNA biosynthesis pathway was enriched in HC vs. ESLC, HC vs. NESLC, and ESLC vs. ESLC, which seemed to be closely related to the progression of lung cancer. In previous studies, the aminoacyl-tRNA biosynthesis pathway was an enrichment pathway for UAP1 expression-related genes in lung adenocarcinoma (Wang et al., 2020). It was closely related to cisplatin resistance in non-small cell lung cancer (Shi et al., 2019). Moreover, it has also been found to be upregulated in gastric cancer, and researchers even proposed a new therapeutic strategy for gastric cancer targeting the aminoacyl-tRNA biosynthesis pathway (Gao et al., 2021). Differential metabolites in this pathway, L-lysine, L-isoleucine, and L-histidine, were significantly reduced in the lung cancer groups, but there was no significant difference between ESLC and NESLC, which meant that these indicators could not be used as reference indicators for staging. L-glutamate was only significantly different between ESLC and NESLC; thus, it was not the best predictor. The serum concentration of L-valine showed differences in the pairwise comparison, so it may be used

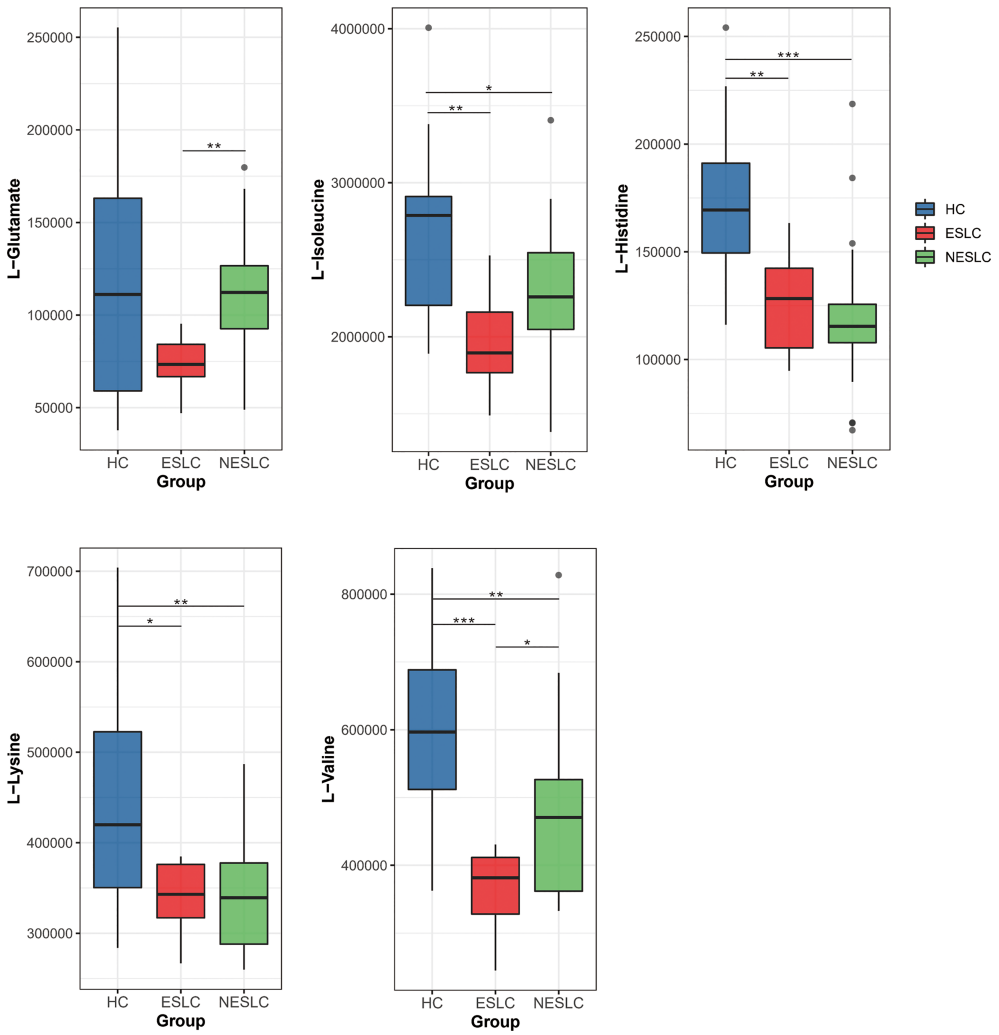

FIGURE 6 | Distribution of different metabolites in each group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

as a potential marker. In the correlation analysis, we found that the most strongly related genus of L-valine was *Lachnospiraceae_UCG-006*, which was the differential genus we screened earlier. The level of valine in early-stage non-small cell lung cancer was lower than that in advanced stage (Puchades-Carrasco et al., 2016). In the study of Ni et al. (2019), valine declined in the lung cancer group as a potential biomarker, although this research was based on two existing data sets. In addition, valine has been reported as a potential biomarker for the differential diagnosis of seronegative rheumatoid arthritis and psoriatic arthritis (Souto-Carneiro et al., 2020). Upregulated concentrations of branched-chain amino acids were detected in stool samples from colorectal cancer patients and in gastric tissue fluid from mice with gastric cancer (Yachida et al., 2019; Gu et al., 2020).

It is worth mentioning that although this study describes the changes in the microbiome and metabolome in lung cancer and the relationship between the two, we cannot explain the causal relationship between them. This requires more and larger cohort studies to explore in the future.

This study has some limitations. Prognosis is an important part of disease research. However, due to the inability to track the prognosis of all patients in the short term, we are temporarily unable to study the prognosis of the disease. On the other hand, the strict entry conditions lead to a small number of participants. We hope to expand the sample size and conduct further studies through multicenter cooperation in our future research.

CONCLUSION

This study describes the characteristics of intestinal flora and serum metabolic profiles of patients with lung cancer in different stages. It reveals that lung cancer may be the result of the mutual

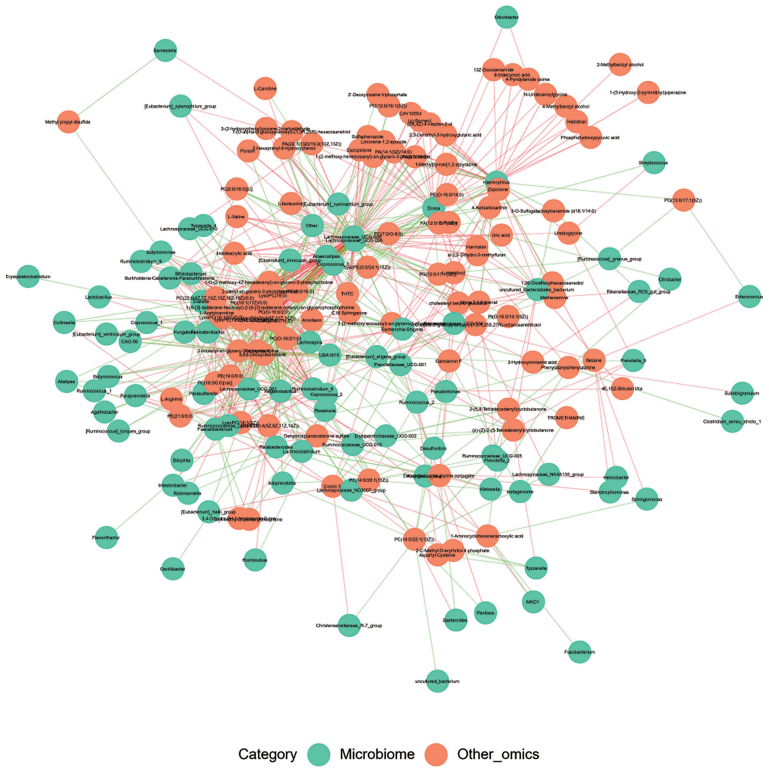

FIGURE 7 | The CorNetwork diagram. The connection indicates correlation. The red connecting line represents a positive correlation between nodes, while the blue line represents a negative correlation.

regulation of L-valine and *Lachnospiraceae_UCG-006* through the aminoacyl-tRNA biosynthesis pathway, and proposes that L-valine may be a potential marker for the diagnosis of lung cancer.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://data.mendeley.com/datasets/8rftx9ybnm/1, genomic sequencing; https://data.mendeley.com/datasets/nj4cz7mmj5/1, metabolomics.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the IRB of The Third Xiangya Hospital of Central South University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Design of the study: SC, RG, and Y-FF. Methodology: SC and X-HZ. Formal analysis: SC and X-HZ. Data curation: SC and X-HZ. Software: SC. Writing—original draft preparation: SC and X-HZ. Writing—review and editing: J-HZ, H-YJ, H-TL, SC, RG, and Y-FF. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation of China (Grant No. 82002452), and the Fundamental Research Funds for the Central Universities of Central South University (Grant No. 2021zzts1090).

ACKNOWLEDGMENTS

We thank Hunan Cancer Hospital for the help provided in specimen collection, Qin-yu Zhao for data analysis, and all the patients involved in the study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.885093/full#supplementary-material

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frontiers | Frontiers in Cellular and Infection Microbiology

REVIEW
published: 16 June 2022
doi: 10.3389/fcimb.2022.849022

The Relationship Between Microbial Community and Breast Cancer

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Breast cancer (BC) is the most common cancer in women and the leading cause of cancer-related deaths in women worldwide. Recent research studies have shown that the intestinal flora is related to the occurrence and progression of BC. Notably, some evidence identifies a unique microbial community in breast tissue, a site previously thought to be sterile. In addition, breast tumors have their own specific microbial community, distinct from normal mammary gland tissue, and all of them may result from intestinal flora. Some microbial community in breast tissue may lead to the occurrence and development of BC. This review focuses on the relationship between the microbial community and breast cancer, which will lay a solid theoretical foundation for further understanding the local microenvironment of BC and developing effective targeted therapeutic drugs.

Keywords: breast cancer, microbial community, local microenvironment, immunity, relationship

OPEN ACCESS

Edited by:
Edith Claudia Kordon, University of Buenos Aires, Argentina

Reviewed by:
Karen Blyth, University of Glasgow, United Kingdom
Albana Gattelli, Molecular Biology and Neurosciences (IFIBYNE), Argentina

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Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *07 January 2022*
Accepted: *24 May 2022*
Published: *16 June 2022*

Citation:
Song X, Wei C and Li X (2022) The Relationship Between Microbial Community and Breast Cancer. Front. Cell. Infect. Microbiol. 12:849022. doi: 10.3389/fcimb.2022.849022

INTRODUCTION

Though accounting only for 2-7% of biomass owing to the miniscule size of microbes, the human microbiome encodes for 100-fold more genes than the human genome indicating an important role in human health (Bhatt et al., 2017). Microbiota and host maintain a dynamic equilibrium referred to as eubiosis that actively influences many physiological processes and is generally beneficial to the host. However, a state of disequilibrium or dysbiosis may evolve contributing to various disease states (Parida et al., 2021). One of the most major achievements in the microbiome field was identified that the role of microbes in the gastrointestinal tract (Turnbaugh et al., 2007; Peterson et al., 2009; Proctor et al., 2019). More recent developments show the existence of microbiota in other body sites, initially considered 'sterile', such as breast (Urbaniak et al., 2014a; O'Connor et al., 2018).

Presently, breast cancer (BC) is the most common malignant tumour and the most important health burden among women worldwide. Recently, statistics have shown that the incidence of BC in various countries around the world is increasing at an accelerated rate, and the affected population is becoming younger (Siegel et al., 2020). With the development of imaging technology, surgery and medical treatments, the diagnosis and treatment of BC have improved. The survival rate of BC patients is increased, and the recurrence rate and mortality rate have decreased correspondingly but

remain high (Desantis et al., 2019). Therefore, exploring the aetiology and pathogenesis of BC is still a top priority. BC is a complex disease that is influenced by many factors, including genetic factors, diet, obesity, endocrine hormone levels and others. Recent research studies have shown that the microbial community is related to the occurrence and progression of BC (Xue et al., 2018).

Some evidence identifies a unique microbial community in breast tissue, a site previously thought to be sterile (O'Connor et al., 2018). In addition, breast tumours have their own specific microbial community, distinct from normal mammary gland tissue (Donnet-Hughes et al., 2010; Chiba et al., 2020). Some microbial community in breast tissue may lead to the occurrence and development of BC. In addition to breast microbiota, some studies have shown that gut microbiota may also influence breast cancer. Furthermore, microbial signatures may differ between different breast cancer patients. For example, an analysis of fecal microbiota shows that postmenopausal women with breast cancer harbor compositionally different gut microbiota than healthy volunteers and exhibit enrichment of several bacterial species (Goedert et al., 2015a; Zhu et al., 2018). In addition, breast cancer is a heterogeneous disease with multiple subtypes and interestingly, microbial signatures may differ between the subtypes (Banerjee et al., 2018).

Furthermore, breast cancer cells are able to repurpose pre-existing metabolic symbiosis, leading to profound alterations in the local microenvironment (Nunes and Serpa, 2020), thus further promoting the development of BC. There are encouraging signs that the breast tumour microbial community is modified by therapy and affects the molecular signaling pathway and the internal environment (Chiba et al., 2020), thus achieving a therapeutic effect. In summary, this review focuses on the relationship between the microbial community and tissue and points out the carcinogenic mechanism of the microbial community in the occurrence and development of BC, as well as the treatment methods, which will lay a solid theoretical foundation for further understanding the carcinogenic mechanism of BC and developing effective targeted therapeutic drugs.

MICROBIAL COMMUNITY IN BREAST TISSUE

Most studies have focused on the intestinal microflora by exploring the relationship between microorganisms and cancer (Tsilimigras et al., 2017), but the increasing understanding of the existence of microorganisms in and adjacent to tumour sites has also brought some new discoveries, which are useful for revealing the carcinogenic mechanism of microflora and the related microenvironment (Chen et al., 2017). Considering the different effects that the microbial community has in distinct organs, recent studies have focused on examining colonizing bacteria in breast tissue. Breast cancer is one of the earliest and most intensively studied diseases using genomic technology (Xuan et al., 2014), but it is only recently that the existence of microorganisms in breast tissue and the potential role of mammary duct microbial community have been explored (Urbaniak et al., 2014b; Chan et al., 2016). In this regard, specific microbial community have been identified in breast milk (Arthur and Jobin, 2013), and several authors postulated that bacteria are capable of using the nipple to gain access to the breast ducts and create a specific microbial community in the breast. This is not surprising considering that skin and oral bacteria have access to the breast ducts through the nipple (Ramsay et al., 2004), we guess that means that breastfeeding could play an important role. But interestingly, recent studies have suggested that their origin is the mother's gastrointestinal tract (Donnet-Hughes et al., 2010). Now, let us look closer at what microbial community are in the breast tissue (**Table 1**).

In Normal Breast Tissue

The breast is composed of epithelial, interstitial and mucosal immune systems, which constitute a complex microenvironment (Going and Moffat, 2004). Since the development of the mucosal immune system is the direct result of microbial exposure, inflammation is partly related to the changes in the microenvironment induced by bacterial infections (Schwabe and Jobin, 2013; Garrett, 2015), so the presence of immune effects in the complex microenvironment of the breast indicates the breast microbial community. At present, there are some predominant microbial community in the normal breast tissue, such as *Proteobacteria* and *Firmicutes* (Urbaniak et al., 2014b), *Sphingomonas yanoikuyae* (Xuan et al., 2014), *Actinobacteria* (Thompson et al., 2017), *Methylobacterium* (Wang et al., 2017), *Ralstonia* (Constantini et al., 2018), *Bacteroidaceae* (Meng et al., 2018), *Prevotella*, *Lactococcus*, *Streptococcus*, *Corynebacterium*, *Staphylococcus* (Urbaniak et al., 2016), unclassified genus of the *Sphingomonadaceae* family in NAF (Chan et al., 2016), and others, which can be seen in the **Table 1**.

In Breast Tumour Tissue

Compared with the normal breast tissue, the microbial spectrum in breast tissues of breast cancer patients is significantly different. Among them, proteobacteria are the most abundant species in normal breast tissue (Mani, 2017). Generally, microbial community enriched in malignant tumour tissues include *Proteobacteria, Firmicutes, Escherichia coli, Methylobacterium radiotolerans, Mycobacterium fortuitum, Mycobacterium phlei, Corynebacterium, Staphylococcus, Actinomyces, Propionibacteriaceae, Propionicimonas, Micrococcaceae, Caulobacteraceae, Rhodobacteraceae, Nocardioidaceae, Methylobacteriaceae, Bacillus, Enterobacteriaceae, Comamondaceae, Bacteroidetes, Alistipes, Brevundimonas diminuta, Arcanobacterium haemolyticum, Peptoniphilus indolicus, Prevotella nigrescens, Propiniobacterium jensenii, Capnocytophaga canimorsus, Fusobacterium, Atopobium, Gluconacetobacter, Hydrogenophaga,*

TABLE 1 | Summary of studies about the microbial community in breast tissue.

Tissue Samples	Test Group	Main Methodology	Microbial Expression	Reference
Breast tissue	43 Canadian women (11 with benign tumors, 27 cancerous tumors and 5 healthy individuals) and 38 Irish women (33 women with BC and 5 healthy individuals)	V6 16S rRNA sequencing (Ion Torrent) Pipeline: UCLUST	↑*Proteobacteria* and *Firmicutes* in breast tissue. ↑*Bacillus* (11.4%) and *Acinetobacter* (10%) in Canadian women. ↑*Enterobacteriaceae* (30.8%) and Staphylococcus (12.7%) in Irish women. ↑*Escherichia coli* in BC tissue.	(Urbaniak et al., 2014b)
Breast tumor tissue and its paired normal adjacent tissue	20 patients ER+ BC	Pyrosequencing V4 16S rDNA Pipeline: QIIME	↑*Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes* and *Verrucomicrobia* (96.6%) in breast tissue. ↑*Methylobacterium radiotolerans* in BC tissue. ↑*Sphingomonas yanoikuyae* in paired normal tissue.	(Xuan et al., 2014)
Breast tissue	668 tumor tissues (HER2+, ER+, TNC) and 72 normal adjacent tissues from The Cancer Genome Atlas (TCGA)	V3-V5 16S rRNA amplified sequencing data	↑*Proteobacteria, Actinobacteria* and *Firmicutes* in breast tissues. ↑*Proteobacteria, Mycobacterium fortuitum* and *Mycobacterium phlei* in BC samples. ↑*Actinobacteria* in normal adjacent tissue.	(Thompson et al., 2017)
Breast tissue	57 women with invasive breast carcinoma and 21 healthy women	V3-V4 16S rRNA sequencing (Illumina) Pipeline: UCLUST	↓*Methylobacterium* and ↑*Corynebacterium*, *Staphylococcus*, *Actinomyces* and *Propionibacteriaceae* in patients with invasive breast carcinoma compared to healthy individuals.	(Wang et al., 2017)
Breast tissue	16 Mediterranean patients with BC (12 samples were collected from core needle biopsies (CNB) and 7 from surgical excision biopsies (SEB); 3 patients were processed with both procedure)	V3 16S-rRNA gene amplicons sequencing (Ion Torrent)	↑*Ralstonia* in breast tissue. No significant differences between healthy adjacent breast tissues and BC tissues.	(Constantini et al., 2018)
Breast tissue	22 Chinese patients with benign tumor and 72 malignant BC patients	V1-V2 16S rRNA sequencing (Illumina HiSeq)	↑*Propionicimonas, Micrococcaceae, Caulobacteraceae, Rhodobacteraceae, Nocardioidaceae and Methylobacteriaceae*, in BC tissues (ethno-specific). ↓*Bacteroidaceae* and ↑ *Agrococcus* are related with malignancy	(Meng et al., 2018)
Breast tissue	58 women: 13 benign, 45 cancerous tumors and 23 healthy women	V6 16S rRNA gene sequencing (Illumina MiSeq) Pipeline: QIIME	↑*Bacillus, Enterobacteriaceae, Staphylococcus, Comamondaceae* and *Bacteroidetes* and ↓ *Prevotella*, *Lactococcus*, *Streptococcus*, *Corynebacterium* and *Staphylococcus* in BC patients compared to healthy controls.	(Urbaniak et al., 2016)
Nipple aspirate fluid (NAF) and aerolar breast skin	25 women with breast ductal cancer and 23 healthy women	V4 16S rRNA gene sequencing (Illumina MiSeq) Pipeline: Mothur	↑*Alistipes* and ↓ unclassified genus of the *Sphingomonadaceae* family in NAF from women with BC compared to healthy controls.	(Chan et al., 2016)
Breast tissue	100 women with triple negative BC (TNBC), 17 matched controls and 20 non-matched controls	PathoChip array	↑*Brevundimonas diminuta, Arcanobacterium haemolyticum*, *Peptoniphilus indolicus, Prevotella nigrescens, Propiniobacterium jensenii* and *Capnocytophaga canimorsus* in TNBC. Among virus, ↑ *Herpesviridae*, *Retroviridae, Parapoxviridae, Polyomaviridae*, *Papillomaviridae* in TNBC.	(Banerjee et al., 2015)
Breast tissue and breast skin	28 women undergoing non-mastectomy breast surgery: 13 benign breast disease and 15 invasive BC (100% ER/PR+ and 29% HER2+)	V3-V5 16S rDNA hypervariable taq sequencing (Illumina MiSeq) Pipeline: IM-TORNADO	↑*Fusobacterium*, *Atopobium*, *Gluconacetobacter*, *Hydrogenophaga* and *Lactobacillus* in BC tissue compared to healthy breast tissue.	(Hieken et al., 2016)
Breast tissue	20 normal breast tissue and 148 BC tissue (50 ER or PR+, 34 HER2+, 24 TP and 40 TN)	Pathochips array	↑*Proteobacteria* and ↑*Actinomyces* in the four BC subtypes studied.	(Banerjee et al., 2018)
Snap-frozen breast tumor tissue	15 women with BC who were treated with neoadjuvant chemotherapy, 18 women with no prior therapy at time of surgery and 9 women who had tumor recurrence	V4 16S rRNA amplicon sequencing (Illumina Miseq) Pipelinee: Mothur (v.1.39.5) Microarray for confirmation	↑*Pseudomonas* spp. in BC tissue after neoadjuvant chemotherapy. ↓*Prevotella* in the tumor tissue from non-treated patients. ↑*Brevundimonas* and *Staphylococc*us in the primary breast tumors in patients developing distant metastases.	(Chiba et al., 2020)

Lactobacillus, and some others (Xuan et al., 2014; Urbaniak et al., 2014b; Banerjee et al., 2015; Chan et al., 2016; Urbaniak et al., 2016; Hieken et al., 2016; Thompson et al., 2017; Wang et al., 2017; Constantini et al., 2018; Meng et al., 2018), which are different from the normal tissue. Notably, *Pseudomonas* spp. in BC tissue increase after neoadjuvant chemotherapy. Compared with tumour tissue from treated patients, Prevotella are decreased in non-treated patients with BC.

Furthermore, focusing on the shift in microbial community composition in breast tissue from patients with disease compared to normal breast tissue, researchers have identified the presence of Bacteroides fragilis in cancerous breasts. Mammary gland and gut colonization with enterotoxigenic Bacteroides fragilis (ETBF), which secretes B. fragilis toxin (BFT), rapidly induces epithelial hyperplasia in the mammary gland. Breast cancer cells exposed to BFT exhibit 'BFT-memory' from the initial exposure. Intriguingly, gut or breast duct colonization with ETBF strongly induces the growth and metastatic progression of tumour cells implanted in mammary ducts in contrast to non-toxigenic Bacteroides fragilis. This work sheds light on the oncogenic impact of the pro-carcinogenic colon bacterium ETBF on breast cancer progression (Parida et al., 2021).

In fact, breast cancer is a heterogeneous disease. Using a whole genome and transcriptome amplification and a pan-pathogen microarray (PathoChip) strategy, Banerjee's research group investigated the diversity of the microbiome in the four major types of breast cancer: endocrine receptor (ER) positive, triple positive, Her2 positive and triple negative breast cancers (Banerjee et al., 2018). The microbial communities for each breast cancer molecular subtype shown in **Table 2**.

In addition, some researchers have compared the microbial community profiles in different histological grades of malignant tumour tissues and found that with the development of tumours, the relative abundance of the Bacteroides family decreases, and the relative abundance of Agrococcus increases (Mani, 2017). The specific correlation between these potential microbial markers and advanced disease may have broad significance in the diagnosis and staging of breast cancer.

INFLUENCE OF GUT MICROBIAL COMMUNITY ON BREAST CANCER

Until now, in addition to the microbial community in breast tissue, evidence from animal experiments also confirmed the relationship between the gut microbial community and breast tissue. The gut microbial community may have an effect on the occurrence and development of BC, and possible mechanisms include estrogen metabolism, diet and obesity, inflammation, immune regulation and bacterial toxin production (Yang JQ et al., 2017).

Estrogen Metabolism

In addition to traditional risk factors such as family history, age, and atypical proliferative breast disease, elevated levels of endogenous or circulating estrogen are directly associated with an increased risk of breast cancer in postmenopausal women (Dallal et al., 2014). Studies have suggested that the gut microbial community may be associated with BC through a response to estrogen metabolism (Goedert et al., 2015b).

The gut microbial community regulates estrogens through secretion of β-glucuronidase. β-glucuronidase de-conjugates estrogen to enable the binding to estrogen receptors (Alizadehmohajer et al., 2020). And then, the estrogen receptor complex could regulate the intestinal function and micro-environment and increase the breast cancer risk (**Figure 1**). In addition, in postmenopausal women, some circulating estrogens in the body are determined by the estrogens involved in the liver-gut circulation, and some gut bacteria are more likely to enter the liver-gut circulation by binding to the estrogens that are excreted in the gut bile; therefore, estrogen and estrogen-like substance concentrations in the body may increase the incidence of BC (Yang JQ et al., 2017).

Notably, dietary estrogens, or phytestrogens, are exogenous estrogens that compete with endogenous estrogen receptor 1 (ESR1). However, the difference is that phytestrogens can reduce the incidence of breast cancer. Enterolactone is a phytestrogen that is the result of the fermentation of lignans by intestinal bacteria. Some experts believe that enterolactone may be used as a drug to inhibit the proliferation of BC cells (Shapira et al., 2013).

Diet and Obesity

Some potential risk factors for BC, such as endogenous and exogenous substance metabolism and obesity status, are related to gut microbial community (Attraplsi et al., 2013). Diet is an important external factor that affects the gut microbial community (Yang JQ et al., 2017), and people who eat different diets over a long period of time have very different microbiomes (Ou et al., 2013). Early great milestone-style work by Doll and Peto suggested that diet is responsible for approximately 35% of cancers

TABLE 2 | Microbial communities in different molecular subtypes of breast cancer.

Molecular Subtypes of BC	Microbial Community			
	Bacteria	Viruses	Parasites	Fungus
ER+	*Arcanobacterium, Bifidobacterium, Cardiobacterium, Citrobacter, Escherichia*		*Brugia, Paragonimus*	*Filobasidilla, Mucor, Trichophyton*
triple positive	*Bordetella, Campylobacter, Chlamydia, Chlamydophila, Legionella, Pasteurella*	*Birnaviridae, Hepeviridae*	*Ancylostoma, Angiostrongylus, Echinococcus, Sarcocystis, Trichomonas, Trichostrongylus*	*Penicillium*
Her2+	*Streptococcus*	*Nodaviridae*	*Balamuthia*	*Epidermophyton, Fonsecaea, Pseudallescheria*
TNBC	*Aerococcus, Arcobacter, Geobacillus, Orientia, Rothia*		*Centrocestus, Contracaecum, Leishmania, Necator, Onchocerca, Toxocara, Trichinella, Trichuris*	*Alternaria, Malassezia, Piedraia, Rhizomucor*

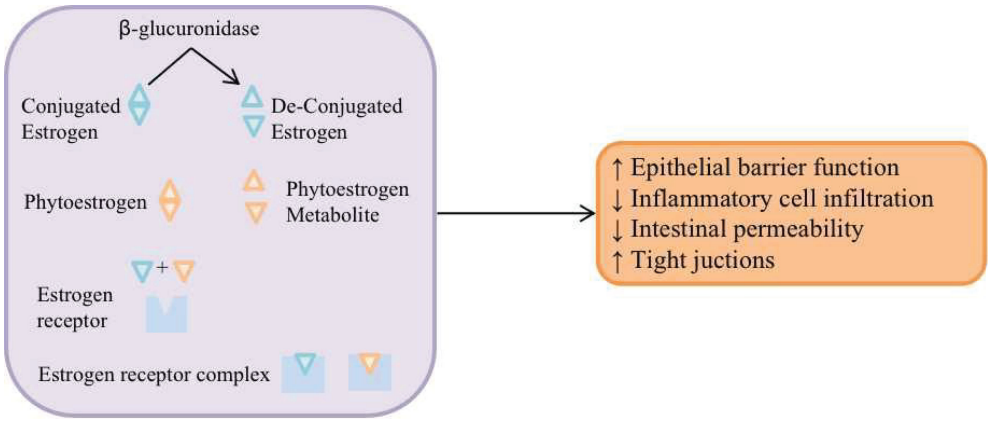

FIGURE 1 | The gut microbial community regulates estrogens through secretion of β-glucuronidase. β-glucuronidase de-conjugates estrogen to enable the binding to estrogen receptors, and then, regulates the intestinal function and micro-environment and increase the breast cancer risk.

(Doll and Peto, 1981). It has been demonstrated that impaired absorption of nutrients can alter the morphology of the gut in non-reproductive mice, leading to a reduction in the number and function of immune cells and a reduction in the production of antimicrobial peptides and immunoglobulins (Round and Mazmanian, 2009), thus indirectly promoting the occurrence of BC. Under a normal diet, intestinal flora can regulate the content of lipopolysaccharide and the production of short-chain fatty acids, directly or indirectly affecting the process of lipid metabolism and affecting the energy balance and body weight of individuals (Velagapudi et al., 2010). The gut microbial community is involved in the occurrence and development of obesity mainly by promoting the production of short-chain fatty acids, inhibiting fasting-induced adipose factor (FIAF), mediating chronic mild inflammatory reactions and inhibiting fatty acid oxidation (Fandriks, 2017). A long-term high-sugar and high-fat diet will change the distribution of gut microbial community, thus leading to the occurrence and development of BC.

Inflammation and the Immune Response

It is known that the expansion of dysbacteriosis in the gut and the extravasation of microbial products can lead to a chronic pro-inflammatory state, which negatively affects the immune system and is unconducive to the elimination of mutant and senescent cells, thus promoting the growth of tumours (Biragyn and Ferrucci, 2018). Changes in intestinal microflora are associated with the development of both intra- and extra-intestinal cancers through the initiation of chronic inflammation and changes in the microenvironment and metabolism (Dapito et al., 2012). Notably, this state may be irreversible (**Figure 2**).

The gut microbial community may be associated with the occurrence and development of BC by influencing T cells, neutrophils and some related inflammatory factors (Yang JQ et al., 2017). Rutkowski et al. confirmed that in breast cancer patients, there is an interaction between symbiotic bacteria, IL-6, and neutrophils (Rutkowski et al., 2015). Such findings have led

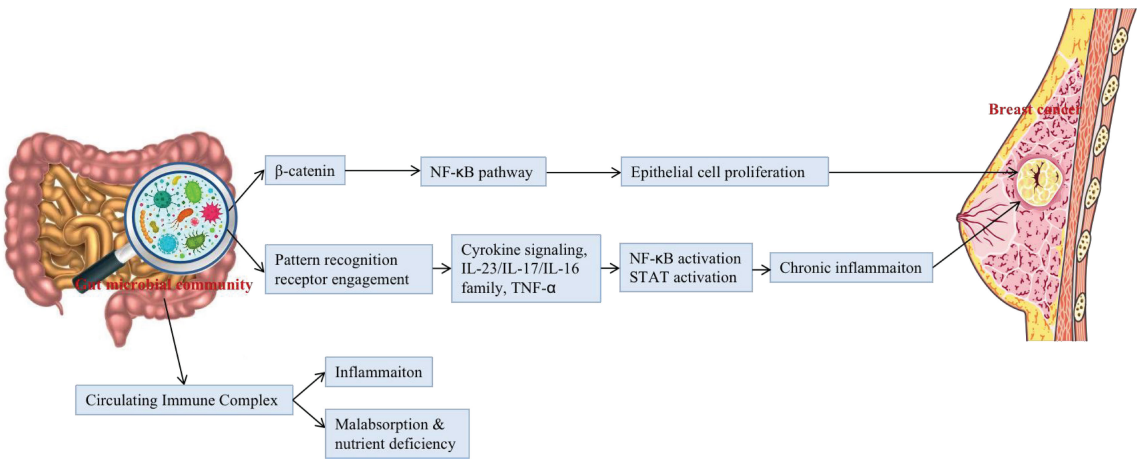

FIGURE 2 | The potential mechanisms that gut microbial community may be associated with the occurrence and development of BC.

researchers to wonder whether downregulation of inflammatory factors and neutrophils could reduce the risk of BC. Studies by Poutahidis have shown that oral administration of Lactobacillus reuteri isolated from human milk to mice can reduce the expression of inflammatory factors (Poutahidis et al., 2013; Poutahidis et al., 2013). Subsequent experiments have shown that the risk of BC could be reduced by downregulating the expression of inflammatory factors in mice.

Bacterial Toxin Production

Studies have shown that some bacterial in humans can release some bacterial toxins, such as the enterotoxin released by *Proteobacteria*, inducing inflammatory bowel disease such as colitis, and damage the intestinal barrier leading to translocation of non-pathogenic bacteria, thus affecting the stability of the immune system and inducing oncogenic-related immune responses that lead to the development of intestinal or extra-intestinal cancers (Rubin et al., 2012), such as BC. Bacterial toxins bind specifically to pattern recognition receptor receptors, such as Toll-like receptors and Nod-like receptors; activate corresponding signalling pathways that cause the expression of chemokines, inflammatory factors, and antimicrobial peptides; promote the proliferation of tumour cells; inhibit the apoptosis of tumour cells; and inhibit the anti-tumour immune response, thus promoting the invasion and metastasis of tumour cells, tumour angiogenesis and other malignant biological behaviours (Kinnebrew and Pamer, 2012). Moreover, some bacterial species may contribute to chronic inflammatory disease by increasing reactive oxygen species production that may eventually mediate genotoxicity. Carcinogenesis can also be modulated by releasing different bacterial toxins that cause DNA damage. As bacteria cross the epithelial barrier, they can directly insert the toxins into the cell of the host. Various bacterial toxins such as Bacillus fragilis, colibactin, and cytolethal cause a carcinogenic cell responses; specifically against DNA damage (Raza et al., 2019).

CLINICAL SIGNIFICANCE

A new role for microflora is as biomarkers. Biomarkers are indicators of the presence or severity of disease. Several studies have reported associations between bacterial markers and clinical or treatment outcomes. Because the intestinal microflora is a rich source of potential biomarkers (Wong and Yu, 2019), the gut microbial community make it possible to predict the responses to disease levels and to treatment. Currently, there is growing evidence that microbial-host interactions may influence or serve as biomarkers for the pathogenesis of BC.

Preventive Effect

The composition of the gut microbiome is not set in stone and instead depends on internal and external factors, such as diet, lifestyle, infection, ageing, antibiotics, activation of the immune response, and IGA produced by B cells. Therefore, the gut microbial community can be regulated to play a preventive role in BC. We can do some beneficial things in our daily life, such as consuming a healthy diet, balancing regular work and rest, and performing the necessary amount of exercise.

Furthermore, several *in vitro* and *in vivo* studies investigated the effect of probiotics on BC; for instance, significant inhibition of cell proliferation, induction of apoptosis, and cell cycle arrest of Enterococcus faecalis and Staphylococcus hominis are proved (Hassan et al., 2016). Lakritz et al. studied two groups of mice: a group manipulated to develop human breast tumors and the other group fed by a Western-style diet (high fat and sugar, low vitamin D3, vitamin C, and fiber) to develop mammary tumors. The two groups were treated with oral intake of probiotic lactic acid microbes. The results showed that the probiotic Lactobacillus reuteri inhibited early-stage carcinogenesis and raised breast cell sensitivity to apoptosis (Lakritz et al., 2014).

Additionally, it was confirmed that oral administration of L. acidophilus represents anticancer activity in mice bearing breast tumors (Yazdi et al., 2010). Another *in vivo* study showed that drinking milk fermented with Lactobacillus helveticus R389 elevated IL- 10 and decreased IL-6 levels both in serum and mammary cells of mice, which lead to breast tumor cell inhibition (Alejandra et al., 2005). Moreover, anticancer effects of probiotics on cancer cell lines are well gathered in the review by Mendoza et al. They showed anti-proliferative activity, apoptosis, cytotoxicity, and cell cycle arrest of probiotics (Mendoza, 2019). Long-term exposure to probiotics such as L. casei Shirota and soy isoflavones in Japanese females demonstrated their chemopreventive effect on cancer development (Toi et al., 2013). Many experts also believe that human symbiotic microbes are more flexible and manoeuvrable than genomes, so we can try to regulate the gut microbial community to prevent tumours. In addition, it may be possible to prevent cancer by using drugs that target bacterial inflammation or genetic toxins (Garrett, 2015).

Therapeutic Effect

Altering the microbial community can affect the growth of cancer and prevent its recurrence. Previous studies have found that injecting Lactobacillus acidophilus into mice with breast tumours alters the production of cytokines and the growth of tumours, possibly altering microbes in the gut, tumour or elsewhere (Maroof et al., 2012). Recent studies in mouse models of colon cancer suggest that oral probiotic supplements containing Lactobacillus helveticus may reduce the production of IL-17-producing T cells by altering the gut microbiome, thus reducing the proliferation of tumour cells (Rong et al., 2019). Taking into account the epidemiological similarities between colon cancer and BC and referring to the hypothesis of gut microbial community and the aetiology of BC, it is important to think deeply whether interfering with the gut microbial community has an effect on BC treatment.

In addition, microbiome regulation may be used as an adjunct to standard cancer therapy (Mani, 2017). Studies have shown that the regulation of microbial community during treatment may help to mitigate the adverse effects of cancer treatment (Montassier et al., 2015). For example, cisplatin, a platinum-based chemotherapy drug, can lead to destruction of the

intestinal epithelial barrier and translocation of intestinal bacteria. Cisplatin was found to destroy the intestinal epithelium and change the gut microbial community in a mouse tumour model, thus leading to a series of adverse reactions. However, interestingly, these reactions could be eliminated by administering medicine with Micrococcus or faecal granules (Perales-Puchalt et al., 2018). In addition, the selective use of specific subgroups of the gut microbial community in chemotherapy can promote anti-tumour immunity. For example, fragile bacteriocin is a key factor in targeting the anti-tumour effect of antibodies against cytotoxic T lymphocyte-associated protein 4 (CTLA-4) (Vetizou et al., 2015). Similarly, the relationship between Bifidobacterium and anti-programmed death ligand receptor (PD-L1)/anti-programmed death receptor (PD-1) therapy has been demonstrated (Sivan et al., 2015; Fessler et al., 2019; Strouse et al., 2019). Dysbiosis, prevalent in non-responders to anti-PD-1 therapy, may cause inflammation and the arrest of T cell differentiation into CD8+ effector cells, and has been associated with a significant reduction in the proportion of Sphingomonas. Oral Bifidobacterium can increase tumor cell control and contributes to interferon (IFN)-γ production by CD8 + tumor-specific T cells, and further increases the activation of intratumoral dendritic cells to improve anti-PD-L1 efficacy. What's more, it is worth mentioning that the intestinal injury and alteration of microflora caused by cisplatin may be a part of its anti-tumour effect (Gui et al., 2015).

Furthermore, microbiome engineering may open new horizons in prevention, diagnosis, and treatment of cancer. As mentioned above, alterations in gut bacterial community may increase the risk of cancer. Therefore, designing antibiotics that target a particular spectrum of the microbiome might help regulate the gastrointestinal microbiome as a possible way to reduce the BC risk (Yang J et al., 2017). It may occur through changes in the activation of signaling pathways as well as the innate and acquired immune responses (Zahra et al., 2020). Engineered probiotics might be useful in targeting these signaling pathways. But, the diversity of bacterial community may make it challenging to developing the antibiotics and identify the cancer.

DISCUSSION AND CONCLUSION

With the discovery and exploration of microbial community, people not only understand the existence of specific microbial community in the breast tissue but also realize the correlation between BC and microbial community in the breast tissue or in the gut. However, notably, regarding the local microflora of breast tissue, not all of them play a certain role in the occurrence and development of BC, and some may have no significance to the development of diseases, such as Ralstonia.

In addition, it is not clear from where the local microflora of the breast actually originates. It is also not clear what the relationship between the microbial community of the breast tissue and the microbial community of the gut is. Currently, there may be three hypotheses as follows: 1) the bacteria enter the ducts of the breast by the nipple and produce a specific microbial community in the breast tissue; 2) bacterial translocation from the gut microbial community; and 3) bacterial invasion originates from the mother's intestinal mucosa. The first assumption is the one that most people accept. For the second hypothesis, the related reason is that the bacteria can spread through the blood and migrate to breast tissue. Indeed, studies have shown that, especially during lactation, cells from gut-associated lymphoid tissue travel to the breast *via* the lymphatics and peripheral blood (Donnet-Hughes et al., 2010). Furthermore, in a mouse model, increased bacterial translocation from the gut during pregnancy and lactation and the presence of bacterially loaded dendritic cells in lactating breast tissue have been shown (Donnet-Hughes et al., 2010). Therefore, some researchers proposed the third hypothesis:bacterial invasion originates from the mother's intestinal mucosa.

New findings are always worth exploring, so when a hypothesis is raised, it also raises some questions that need to be considered: 1) Is BC caused by bacterial translocation or invasion? If so, is it possible to intervene in infants during the breastfeeding phase? 2) Is it possible to change the local microbial community of the breast tissue by altering the gut microbial community? 3) In addition to the carcinogenicity of gut microbial community, can microbial community play a role in inhibiting BC? Which microbial community are involved, and how can we increase their number?

In addition, chemotherapy drugs for BC, such as paclitaxel, have previously been thought to work by acting directly on tumour cells. However, recently, paclitaxel was found to inhibit tumour metabolism by modifying the gut microbial community (Su et al., 2019). Therefore, can paclitaxel also change the local microenvironment of breast tumour tissue? In other words, is its therapeutic effect on tumours due to changes in local microorganisms, which thus allows the dominant bacteria to play a role in killing tumours?

At present, research on the microbial community related to breast tissue is still at the initial stage. The relatively definite finding is that there are some specific microbial community in the breast tissue. However, whether these bacteria are related to the development of BC is not clear. In addition, whether the microbial community in the breast tissue is the cause or a result of BC needs to be considered. Interestingly, some studies by Urbaniak et al. have shown that bacterial communities do not differ between tumour tissue and normal adjacent tissue at either the population or individual level (Fernández et al., 2018). So what is the point of microbial community in breast tissue?

In conclusion, the relationship between microbial community and breast tissue is a new field of mammary gland research. Notably, the microbial community is a double-edged sword that regulates the local and systemic immune responses. On the one hand, it can lead to the occurrence and development of cancer. On the other hand, microbial community is of great significance in preventing the occurrence and development of cancer. Therefore, in-depth study of the microbial community is necessary. Perhaps in the future, microbial community will

provide an unprecedented treatment for BC, especially TNBC, which currently lacks an efficient method to cure.

AUTHOR CONTRIBUTIONS

XS wrote this manuscript. CW and XL revised the manuscript. All authors contributed to the review and approved the submitted version.

FUNDING

The present study was supported by the National Natural Science Foundation of China (grant no. 81473687), the Academic Promotion Program of Shandong First Medical University (grant no. 2019QL017), the Natural Science Foundation of Shandong Province (grant no. ZR2020MH357, ZR2020MH312), Tai'an Science and Technology Innovation Development Project (grant no.2020NS092).

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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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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 29 August 2022
DOI 10.3389/fcimb.2022.935071

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 03 May 2022
ACCEPTED 08 August 2022
PUBLISHED 29 August 2022

CITATION
Zhang J, Yang D, Cui X, Liu G, Cui Z, Wang C and Piao H (2022) Performance of human papillomavirus E6/E7 mRNA assay for primary cervical cancer screening and triage: Population-based screening in China. *Front. Cell. Infect. Microbiol.* 12:935071. doi: 10.3389/fcimb.2022.935071

Performance of human papillomavirus E6/E7 mRNA assay for primary cervical cancer screening and triage: Population-based screening in China

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Objective: Cervical cancer screening is very important in the prevention and treatment of cervical cancer. In China, the cervical screening strategy needs to be improved. To explore a suitable cervical screening strategy in China, we evaluated the performance of the human papillomavirus (HPV) E6/E7 mRNA (Aptima HPV (AHPV)) assay in primary screening and different triage strategies for women undergoing routine cervical screening.

Methods: A total of 10,002 women aged 35 to 65 years of age were recruited in Liaoning Province and Qingdao City, China. Specimens were tested by liquid-based cytology (LBC) and the AHPV assay, and women who tested positive on any test were referred for colposcopy. Genotyping was performed on all high-risk HPV (HR-HPV)-positive samples. Test characteristics were calculated based on histological review.

Results: We identified 109 women with high-grade squamous intraepithelial lesion or worse (HSIL+), including six with cervical cancer. The sensitivity of AHPV was clearly higher than that of LBC (92.7 [95% CI: 87.2, 97.2] *vs*. 67.9 [95% CI: 59.6, 76.1], $p < 0.001$). The specificity of AHPV was 93.0 (95% CI: 92.5, 93.5), which was lower than that of LBC (95.2 [95% CI: 94.8, 95.6], $p < 0.001$). There was no statistical difference between the positive predictive value of AHPV and LBC (13.5 [95% CI: 11.2, 16.2] *vs*. 14.3 [95% CI: 11.4, 17.6], $p = 0.695$). The difference of area under the curve (AUC) values between the AHPV test (0.928 [95% CI: 0.904, 0.953]) and LBC test (0.815 [95% CI: 0.771, 0.860]) in detecting HSIL+ was statistically significant ($p < 0.001$). Finally, among the three triage strategies, both the sensitivity (73.4 [95% CI: 65.1, 81.7]) and AUC (0.851 [95% CI: 0.809, 0.892]) of AHPV genotyping with reflex LBC triage were the greatest.

Conclusion: In summary, the AHPV assay is both specific and sensitive for detecting HSIL+ and may be suitable for use in primary cervical cancer screening in China. AHPV genotyping with reflex LBC triage may be a feasible triage strategy.

KEYWORDS

Cervical screening, HPV E6/E7 mRNA, HR-HPV prevalence, cytology, triage

Introduction

Cervical cancer is one of the main malignant tumors threatening women's health. The incidence and mortality rates of cervical cancer in China have increased year by year in the past 20 years, with a trend for this disease to increasingly affect younger women (Yu and Chen, 2015; Chen et al., 2016; Sung et al., 2021) . Persistent infection with high-risk human papillomavirus (HPV) is the main pathogenic factor for cervical cancer. The prevention of cervical cancer has been widely performed globally. However, cervical cancer vaccination started late in China, and the vaccinated population coverage is low. It is thus important to improve our current screening systems. The performance of traditional Pap smear in developing countries and regions is not satisfactory, with a sensitivity of only 30%–40% (Gay et al., 1985). Liquid-based cytology has improved its performance, but the number of cytopathologists in China remains insufficient, and the diagnostic skills are uneven, which hinders the popularization of this technology for routine screening. The visual inspection with acetic acid and Lugol iodine (VIA/VILI) screening method does not depend on specific equipment and is simple and inexpensive to operate but has low sensitivity (40%–60%) (Denny et al., 2005; Wei et al., 2012). In view of the oncogenic etiology, HPV testing could serve as an accurate means of detecting women at risk of cervical cancer. High-risk HPV (HR-HPV) testing was recommended in Europe in 2008 for primary cervical cancer screening in women older than 25 years, and in April 2014, the United States Food and Drug Administration (FDA) approved the Cobas 4800 HPV-DNA test for primary cervical cancer screening in women older than 25 years (Huh et al., 2015). The latest guidelines published by the WHO in 2021 also recommend HPV for primary screening (World Health Organization, 2021). The results of HPV testing were shown to be relatively accurate and consistent irrespective of the assays used, and HPV primary screening increased the rate of detection of cervical intraepithelial neoplasia lesions of grade 2 or more (CIN2+) by 25% (Zhang et al., 2021). Primary HPV screening has the advantage of high sensitivity but lacks specificity. The four HPV tests currently approved by the FDA include three DNA-based assays and one RNA-based assay. The detection of HPV E6/E7 mRNA could theoretically have higher specificity (Iftner et al., 2015). The E6/E7 oncogenes are well known to play critical roles in the development of cervical cancer. Since E6/E7 overexpression occurs after the integration of HPV into the genome, direct testing of HR-HPV E6/E7 in cervical samples may turn out to be more specific than HR-HPV-DNA testing in detecting high-grade cervical lesions (Ge et al., 2018). Upon comparison with HPV-DNA testing using the non-inferiority score test, the HPV E6/E7 mRNA assay met the cross-sectional clinical and reproducibility criteria of the international guidelines for HPV test requirements for cervical screening in the detection of CIN2+ (Heideman et al., 2013). At present, several studies, including those from Shenzhen, China (Wu et al., 2010), Henan Province, China (Zhang et al., 2020b), Wenzhou, China (Pan et al., 2019), and Tehran, Iran (MOUSAVI et al., 2020), also confirmed the efficacy and feasibility of the HPV E6/E7 mRNA assay. However, these studies were limited by their small sample size and were mostly hospital-based studies. There is thus a need for a large prospective population-based screening study to assess the performance of the HPV E6/E7 mRNA assay in cervical cancer screening in China. Moreover, seldom did previous reports evaluate possible triage strategies in Aptima HPV (AHPV)-positive women (Wang et al., 2019).

Therefore, in this study, we analyzed and assessed the performance of different primary screening schemes and various triage strategies by conducting a large cross-sectional study of population-based cervical cancer screening in Liaoning Province and Qingdao City in China, in order to advance the level of prevention and treatment of cervical cancer in China.

Materials and methods

Study population

The study population was recruited from Liaoning Province (Shenyang City for an urban population and Benxi County and Sujiatun District for rural populations) and Qingdao City (for an urban population) between April 2018 and December 2021. The

criteria for inclusion in the community screening population were as follows: resident population with household registration (living locally for more than 3 years) in the screening area, aged 35–65 years, no severe organ dysfunction or mental illness, volunteering to participate, and being able to complete the questionnaire. Meanwhile, the exclusion criteria were as follows: women with a history of hysterectomy, pelvic radiation therapy, pregnancy, or lactation, and those with other serious medical and surgical conditions under treatment. This study was approved by the ethics committee of Liaoning Cancer Hospital (approval number: 20180106).

Study design

In terms of the study design, upon enrollment, a single cervical specimen was collected from all participants using a Cytobrush and suspended in PreservCyt collection medium (Hologic Inc., Marlborough, MA, USA), in accordance with the manufacturer's instructions. Each specimen was used for liquid-based cytology (LBC) and the AHPV assay (Hologic, San Diego, CA, USA). Participants who had atypical squamous cells of undetermined significance (ASC-US) or a worse cytologic diagnosis and/or were HPV-positive on either assay were referred for colposcopy and biopsy. Colposcopy was performed by specialized colposcopists at a cervical lesion clinic. Colposcopy-guided biopsy was performed if abnormal epithelium was observed. If the colposcopy assessment was inadequate, random biopsies at 3, 6, 9, and 12 o'clock positions in the cervix and endocervical curettage (ECC) were performed. Patients who had an ASC-US or low-grade squamous intraepithelial lesion (LSIL) cytology test result and showed no visible lesion during colposcopy at the first visit were not subjected to biopsy and were considered to have a histological status of "no HSIL". If cervical cancer was suspected during sampling, a cervical biopsy was performed immediately. Women with negative co-screening results were considered to have a histological status of "no HSIL". The biopsy results were categorized into the following three general groups: benign (including no pathological alteration and benign or reactive lesions), low-grade squamous intraepithelial lesions (LSIL, CIN 1, and HPV effect), and high-grade cervical lesions or worse (HSIL+). All CIN 2 lesions were confirmed by immunohistochemical staining for p16 and Ki-67.

Liquid-based cytology

All samples were first analyzed by ThinPrep® LBC (Hologic Inc., USA). LBC results were evaluated according to the 2014 Bethesda System.

Human papillomavirus testing and genotyping

The LBC specimens were tested under blinded conditions with the Aptima® HPV assay (Gen-Probe; Hologic, San Diego, CA), an FDA-certified HPV E6/E7 mRNA assay that detects 14 HR-HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). All HR-HPV-positive samples were further genotyped by the Aptima® HPV 16 18/45 Genotype (GT) assay (AHPV-GT) (Gen-Probe; Hologic, San Diego, CA, USA). The AHPV-GT can detect HPV16 and a subset of HPV18 and HPV45 cases (Wang et al., 2019). Detection and result reporting were performed by professional technicians in accordance with the manufacturer's instructions.

Data analysis

Positivity in primary screening with LBC was defined with ASC-US+. Positivity in primary screening with AHPV was defined as positivity for any HR-HPV infection. Positivity in combined screening with co-testing (LBC+AHPV) was defined as positivity for either ASC-US+ or HR-HPV infection. Three kinds of possible triage strategies are shown in Figure 1. 1) LBC-AHPV: patients with an LBC test result with ASC-US were referred for colposcopy if the AHPV test was positive, and patients with an LBC test result with LSIL or worse were referred directly. 2) AHPV-LBC: AHPV test-positive cases were referred if the LBC test gave a result of ASC-US or worse. 3) AHPV genotyping with reflex LBC triage: AHPV-positive cases were further tested by HPV genotyping and referred if HPV16/18/45-positive, or if positive for other HR-HPV genotypes with an LBC test result of ASC-US or worse. Histological confirmation of HSIL+ served as the clinical observation endpoint. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), Youden's index, and the area under the receiver operating characteristic (ROC) curve (AUC) were determined in line with standard definitions when comparing different diagnostic tests. The 95% confidence intervals (CIs) of proportions were calculated. Sensitivity and specificity were compared using McNemar's test for paired data, and Pearson's chi-square test for comparing predictive values of diagnostic tests was used to compare PPVs and NPVs. AUC was compared by the Delong test. The number of referred colposcopies to detect one case of HSIL+ was calculated as a measure of the screening efficiency of the screening method. Age was presented with median and interquartile range, as age was not normally distributed. Differences between categorical variables were compared by chi-squared (χ^2) tests. Statistical analysis was performed using SPSS 22.0, and $p < 0.05$ was considered statistically significant.

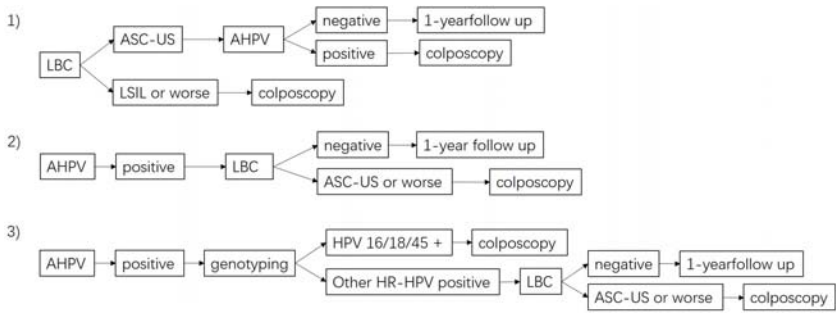

FIGURE 1
Flow diagram of the different triage strategies. 1) LBC-AHPV triage, 2) AHPV-LBC triage, and 3) AHPV genotyping with reflex LBC triage. LBC, liquid-based cytology; AHPV, Aptima human papillomavirus.

Results

Characteristics of the study population

A total of 10,002 eligible women were recruited in this study within 34 communities between April 2018 and December 2021. They provided both a specimen and a completed questionnaire. The median age of the participants was 49 years (interquartile range, 44–55). Of these, 7,978 (79.8%) were from Liaoning Provence, and 2,024 (20.2%) were from Qingdao City; 5,994 (59.9%) were from an urban population and 4,008 (40.1%) from a rural one. Overall, 7,789 (77.9%) had never previously participated in cervical cancer screening. However, HPV results were not available in 11 women due to ineligible specimens. The baseline characteristics of the 9,991 women with both cytology and HPV screening results are shown in Table 1. Among them, 720 (7.2%) women had positive cytology results (ASC-US or worse), and 1,244 (12.5%) women were infected with HPV. Of the 1,571 women who tested positive on any screening test, 989 (63.0%) underwent colposcopy, and 965 (97.6%) had either adequate negative colposcopy findings or an adequate biopsy specimen. Two women were suspected of having cervical cancer at the time of sampling, so a cervical biopsy was also performed at that time (Figure 2). HPV genotyping results were available in 1,213 women. Among them, 184 (15.2%) were HPV16-positive, 65 (5.4%) were HPV18/45-positive, and 980 (80.8%) were positive for other HR-HPV types, including 16 women with multiple infections of these three groups of HPV genotypes.

TABLE 1 The baseline characteristics of the 9,991 women with both LBC and AHPV test results.

		N (%)
Age	35–44	2,603 (26.1)
	45–55	5,225 (52.3)
	56–65	2,163 (21.6)
Age of first sex	≤19 years	236 (24.8)
	>19 years	9,262 (75.2)
No. of sexual partners	≥2	613 (6.4)
	1	8,893 (93.6)
No. of parturitions	>1	1,043 (12.1)
	≤1	7,557 (87.9)
Smoking	Yes	619 (6.2)
	No	9,371 (93.8)
Cytology	Positive	720 (7.2)
	Negative	9,271 (92.8)
AHPV	Positive	1,244 (12.5)
	Negative	8,747 (87.5)

LBC, liquid-based cytology; AHPV, Aptima human papillomavirus.

Detection rate of high-grade squamous intraepithelial lesion or worse

A total of 109 women with HSIL+ (1.1% of the cohort) were identified, among whom six (6.0/100,000 of the cohort) had confirmed cervical cancer. The rates of HSIL+ detection did not differ significantly between the urban and rural cohorts (1.0% *vs.* 1.2%, $\chi^2 = 0.419$, $p = 0.556$).

In addition, the rate of HSIL+ detection was not correlated with the menopausal status of women ($\chi^2 = 0.000$, $p = 0.982$). The rate of HSIL+ detection in HPV-negative women was significantly lower than that of cytologic normality (0.1% *vs.* 0.4%, $\chi^2 = 16.294$, $p < 0.001$). The rate of HSIL+ detection was significantly higher in HPV16-positive women than in HPV18/45-positive women and those positive for other high-risk genotypes ($\chi^2 = 44.685$, $p < 0.001$). HSIL+ was detected significantly more often in women infected with HPV16/18/45 than in women with a cytological status of ASC-US+ (35.5% *vs.* 14.2%, $\chi^2 = 11.769$, $p = 0.001$) (Table 2).

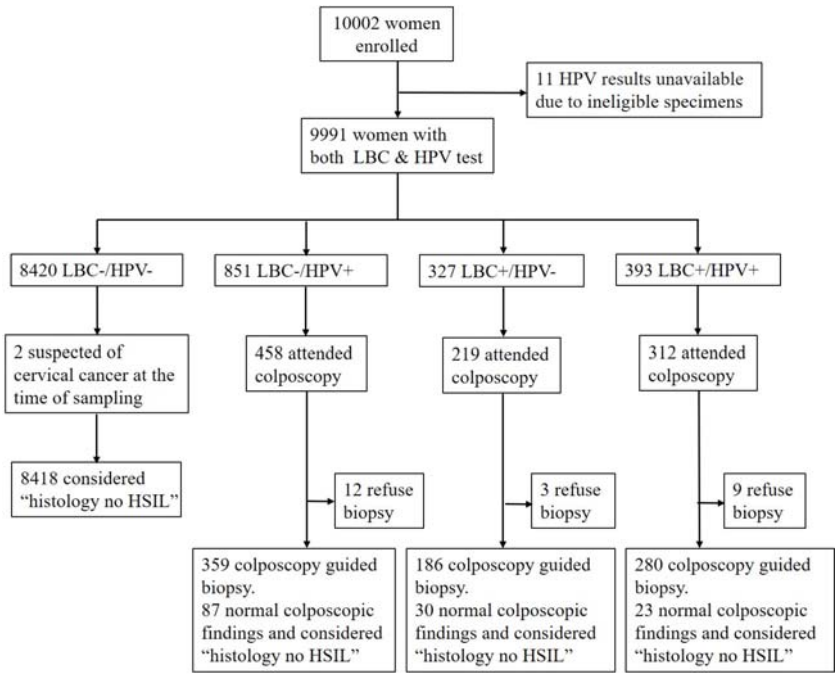

FIGURE 2
Flow diagram of the study population.

Comparison of performance of different primary screening tests, combined screening, and triage strategies

A comparison of the performance of the primary screening tests and combined screening for the detection of HSIL+ is shown in Table 3. The sensitivity of AHPV was 92.7 (95% CI: 87.2, 97.2), which was significantly higher than that of LBC at 67.9 (95% CI: 59.6, 76.1) ($p < 0.001$). This difference was associated with LBC missing 27 (24.8%, 27/109) cases of HSIL+ in the cohort. However, the specificity of AHPV was lower than that of LBC (93.0 [95% CI: 92.5, 93.5] *vs.* 95.2 [95% CI: 94.8, 95.6], $p < 0.001$). The difference between PPVs of AHPV and LBC had no statistical significance (13.5 [95% CI: 11.2, 16.2] *vs.* 14.3 [95% CI: 11.4, 17.6], $p = 0.695$). Compared to the LBC or AHPV primary screening, although co-testing had the highest sensitivity of 98.2 (95% CI: 95.4, 100.0) ($p < 0.001$, $p = 0.03$ respectively), it had the lowest specificity of 90.8 (95% CI: 90.2, 91.3) (both $p < 0.001$). The AUC of co-testing was the greatest at 0.945 (95% CI: 0.932, 0.958), followed by AHPV at 0.928 (95% CI: 0.904, 0.953). The difference in the AUC of co-testing and the AHPV test for HSIL+ was not statistically significant ($p = 0.1407$); however, the AUC of the AHPV test was significantly greater than that of the LBC test (0.815 [95% CI: 0.771, 0.860], $p < 0.001$) (Figure 3).

In Table 3, three triage strategies were analyzed. Based on the primary screening test being either the LBC test or AHPV test, triage using LBC, AHPV, or genotyping would yield higher PPVs, but a smaller proportion of cases would be referred to colposcopy. The sensitivity of AHPV genotyping with reflex LBC triage was the highest at 73.4 (95% CI: 65.1, 81.7). The difference between the sensitivity of AHPV genotyping with reflex LBC triage and AHPV-LBC triage (62.4 [95% CI: 53.2, 71.6]) was statistically significant ($p < 0.001$). The specificity of AHPV genotyping with reflex LBC triage at 96.7 (95% CI: 96.4, 97.1) was lower to that of AHPV-LBC triage at 97.5 (95% CI: 97.1, 97.8) ($p < 0.001$). The AUC of AHPV genotyping with reflex LBC triage strategy was the greatest (0.851 [95% CI: 0.809, 0.892]), which was significantly greater than that of AHPV-LBC triage (0.799 [95% CI: 0.754, 0.845], $p = 0.0006$), and there was a tendency that the AUC of AHPV genotyping with reflex LBC triage is slightly greater than that of LBC-AHPV triage (0.816 [95% CI: 0.771, 0.861], $p = 0.0564$) (Figure 3).

Screening efficiency

With AHPV, 749 women were referred for colposcopy, among whom 101 HSIL+ cases were detected. Therefore, seven to eight women (749/101) need to be referred for each case of HSIL+ detected. Moreover, for each case of HSIL+ detected in LBC, seven women (519/74) need to be referred, so AHPV is nearly equivalent to LBC in terms of screening efficiency (Figure 4).

TABLE 2 Distribution of histological diagnosis results stratified by menopause status, cytology, HPV, and genotyping test results (N [%]).

	Normal	LSIL	HSIL+	Total	χ^2	*p*
Total	8,987 (95.8)	289 (3.1)	109 (1.2)	9,385		
Menopause status						
Premenopause	4,861 (95.9)	150 (3.0)	59 (1.2)	5,070	0.000	0.982
Menopause	4,126 (95.6)	139 (3.2)	50 (1.2)	4,315		
Cytology						
NILM	8,666 (97.7)	165 (1.9)	35 (0.4)	8,866	16.294*	<0.001*
ASCUS	257 (74.9)	65 (19.0)	21 (6.1)	343		
ASC-H	14 (35.9)	10 (25.6)	15 (38.5)	39		
LSIL	37 (36.6)	47 (46.5)	17 (16.8)	101		
HSIL	0 (0.0)	2 (10.0)	18 (90.0)	20		
AGC	13 (81.3)	0 (0.0)	3 (18.8)	16		
HPV test						
Negative	8,575 (99.3)	53 (0.6)	8 (0.1)	8,636		
Positive	412 (55.0)	236 (31.5)	101 (13.5)	749		
HPV16	37 (35.2)	32 (30.5)	36 (34.3)	105	44.685	<0.001
HPV18/45	30 (68.2)	11 (25.0)	3 (6.8)	44		
Other HR-HPV	341 (57.7)	188 (31.8)	62 (10.5)	591		

HPV, human papillomavirus; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; HR-HPV, high-risk HPV.
*Comparison of the detection rate of HSIL+ between cytology-negative women and HPV-negative women. NILM, negative for intraepithelial lesion or malignancy; ASC-US, atypical squamous cell of undetermined significance; ASC-H, atypical squamous cells, HSIL cannot be excluded; AGC, atypical glandular cells.

Discussion

This study evaluated and compared the performance of HPV E6/E7 mRNA (AHPV) assay, liquid-based cytology (LBC), and co-testing as primary screening and three different triage strategies in a routine community screening cohort of 10,002 women in China. We found that HPV E6/E7 mRNA had high sensitivity and good specificity in detecting high-grade cervical precancerous lesions.

Large population-based studies in China previously reported HPV prevalence ranging from 9.9% to 27.5% (Li et al., 2013). In this study, the prevalence of HPV infection was 12.5%, which was at the low end of the reported range of HPV prevalence in China. In terms of the reasons for this low rate, first, Liaoning Province and Qingdao City are not areas with a high HPV prevalence. Second, the population in this study was population-based, while some previous studies were instead hospital-based. Finally, it was found that the rate of positivity in the HPV E6/E7 mRNA assay was lower than that in the HPV-DNA assay (Mousavi et al., 2020).

HPV testing has the advantages of being objective, the results being obtained in a short time and being easy to repeat. HPV testing can detect precancerous cervical lesions earlier than cytology. A negative result of HR-HPV testing was reported to predict a lower risk of future CIN2+ and could enable screening to be performed less regularly (Ogilvie et al., 2012; Ronco et al., 2014; Zhang et al., 2021). In theory, HPV E6/E7 mRNA is produced after the genomic integration of HPV viral genes and might represent a state of active HPV infection, so the detection of HPV E6/E7 mRNA transcripts may provide greater specificity for CIN2+ (Zhang et al., 2017). HPV E6/E7 mRNA assay has been focused on in recent years, and several studies have compared its performance to that of the HPV-DNA assay for cervical cancer screening. For example, a study enrolling 9,451 women aged 30–60 years attending routine cervical cancer screening in Germany compared an RNA-based test (AHPV) and a DNA-based test (HC2), finding no statistically significant difference in sensitivity in detecting CIN2+ ($p = 0.180$) or CIN3+ ($p = 0.0625$) lesions between them. The specificity (<CIN2) and positive predictive value (CIN2+) of the AHPV test were significantly higher than those of the HC2 test ($p < 0.001$) (Iftner et al., 2015). The RNA-based assay detected actively infected cells, whereas DNA-based assays, such as HC2, could not distinguish between intracellular and extracellular viral DNA, leading to the results potentially being affected by contamination with extracellular viral particles. Consistent with this, the slightly lower sensitivity of the RNA-based HPV test in detecting CIN2+ was reported in other earlier studies (Monsonego et al., 2011). Moreover, more recent reports demonstrated equal (Ratnam et al., 2011; Nieves et al., 2013) or higher (Wu et al., 2010; Monsonego et al., 2012; Kuroki et al., 2021) sensitivity of the AHPV test compared with that of the HC2 test. In addition, compared to the Cobas HPV test, AHPV and GT demonstrated significantly higher specificity and PPV (Ge et al., 2018). After long-term follow-up, the future risk of HSIL+ in women with a negative HPV E6/E7 mRNA test result was found to be quite low, as was that of women with negative results in DNA-based assays (Reid et al., 2015; Iftner et al., 2019).

TABLE 3 Comparison of performance of different primary screening tests and different triage strategies.

Primary screening tests	No. of HSIL+	The rate of referred to colposcopy (%)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Youden's index (95% CI)	AUC (95% CI)	*p*
LBC	74	7.2	67.9 (59.6, 76.1)	95.2 (94.8, 95.6)	14.3 (11.4, 17.6)	99.6 (99.4, 99.7)	0.63 (0.54, 0.72)	0.815 (0.771, 0.860)	<0.001*
AHPV	101	12.5	92.7 (87.2, 97.2)	93.0 (92.5, 93.5)	13.5 (11.2, 16.2)	99.9 (99.8, 100.0)	0.86 (0.80, 0.91)	0.928 (0.904, 0.953)	
Co-testing	107	15.7	98.2 (95.4, 100.0)	90.8 (90.2, 91.3)	11.1 (9.7, 10.9)	100.0 (99.9, 100.0)	0.89 (0.86, 0.91)	0.945 (0.932, 0.958)	0.1407#
LBC-AHPV	72	4.4	66.1 (56.9, 75.2)	97.2 (96.8, 97.5)	21.6 (17.7, 26.4)	99.6 (99.4, 99.7)	0.63 (0.54, 0.73)	0.816 (0.771, 0.861)	0.0564**
AHPV-LBC	68	3.9	62.4 (53.2, 71.6)	97.5 (97.1, 97.8)	22.4 (18.0, 27.6)	99.5 (99.4, 99.7)	0.60 (0.50, 0.69)	0.799 (0.754, 0.845)	0.0006##
AHPV genotyping with reflex LBC	80	5.5	73.4 (65.1, 81.7)	96.7 (96.4, 97.1)	20.9 (17.0, 25.4)	99.7 (99.5, 99.8)	0.70 (0.62, 0.79)	0.851 (0.809, 0.892)	

LBC-AHPV, LBC tests with ASCUS were referred if AHPV test was positive, and LBC test with LSIL or worse was referred directly. AHPV-LBC, AHPV test-positive cases were referred if LBC test with ASCUS or worse. AHPV genotyping with reflex LBC, AHPV-positive cases were further tested by HPV genotyping and referred to colposcopy if HPV16, 18/45-positive, or if other HR-HPV genotypes positive with LBC test ASCUS or worse.

HSIL, high-grade squamous intraepithelial lesion; AUC, area under the receiver operating characteristic curve; PPV, positive predictive value; NPV, negative predictive value; LBC, liquid-based cytology; AHPV, Aptima human papillomavirus; LSIL, low-grade squamous intraepithelial lesion.

*Comparison of AUC between LBC and AHPV.

#Comparison of AUC between AHPV and co-testing.

**Comparison of AUC between LBC-AHPV and AHPV genotyping with reflex LBC.

##Comparison of AUC between AHPV-LBC and AHPV genotyping with reflex LBC.

In this study, HPV E6/E7 mRNA testing showed good performance in population-based cervical cancer screening with high sensitivity of 92.7 (95% CI: 87.2, 97.2) and high specificity of 93.0 (95% CI: 92.5, 93.5). Therefore, HPV E6/E7 mRNA testing could be suitable for the primary screening of cervical cancer.

Current practice in China mainly involves annual cytological screening. The detection rate of precancerous cervical lesions and cervical cancer by cytology testing was reported to be only 124.87–491.03/100,000 in China (Song et al., 2015; Song et al., 2021). However, the age-standardized prevalence of CIN2+ lesions was 2.7% among women in rural China and 1.3%

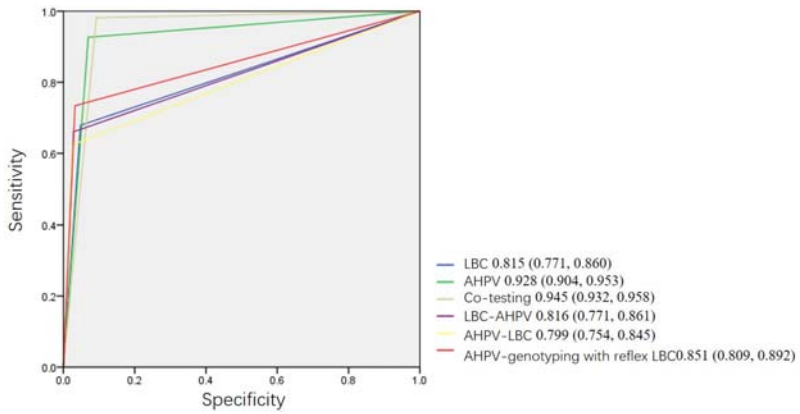

FIGURE 3
Receiver operating characteristic curves (ROC) of different primary screening tests, combined screening, and triage strategies.

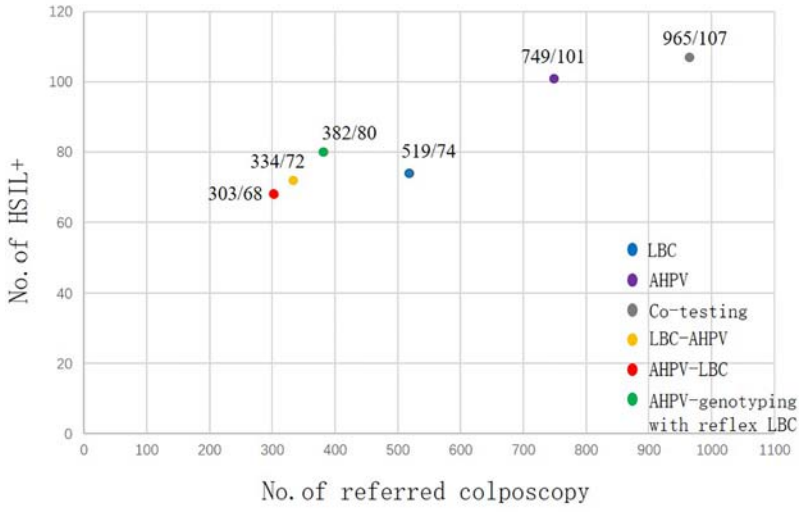

FIGURE 4
Screening efficiency of the different primary screening and three triage strategies for HSIL+. HSIL, high-grade squamous intraepithelial lesion.

among women in urban China, which are high among Asian women. Therefore, the poor sensitivity of cytology demands the development of more accurate screening approaches. Strategies for improving the detection of CIN2+ cases have been assessed for the HPV E6/E7 mRNA assay based on the primary screening test being cytology (Monsonego et al., 2011; Ratnam et al., 2011; Monsonego et al., 2012; Nieves et al., 2013). In this study, the rate of HSIL+ detection in the co-testing screening was 1.1% in the total population, 1.0% in the urban population, and 1.2% in the rural population. The detection rate in the urban population was close to the prevalence of CIN2+ in women in urban China, but the detection rate in the rural population still needs to be improved. In this study, HSIL+ was detected at a rate of 1.0% by RNA-based HPV assay screening alone but at 0.74% by LBC screening alone. The AUC value of AHPV screening alone of 0.928 (95% CI: 0.904, 0.953) was significantly higher than that of LBC screening alone of 0.815 (95% CI: 0.771, 0.860; $p < 0.001$). Although the AUC value of AHPV screening alone was slightly lower than that of co-testing screening, the difference was not statistically significant ($p = 0.1407$). Co-testing screening might only be applicable in economically developed parts of China because of its high cost. Considering the screening efficiency, this study also implied that only slightly more women would need to be referred to detect one HSIL+ case (eight women with the AHPV assay versus seven with cytology alone), but a larger proportion (36.5%) of additional HSIL+ cases would be identified (101 cases for the AHPV assay versus 74 for cytology alone), compared with the use of AHPV with cytology as a primary screening test. Therefore, compared with the cytology test, the HPV E6/E7 mRNA assay may be a better option in primary cervical cancer screening in China.

HPV E6/E7 mRNA assay was an effective triage method in women with a cytological result of ASC-US (Stoler et al., 2013). In this study, compared with the LBC test alone, with the use of AHPV as triage for the cytology test, although the sensitivity decreased slightly (67.9%, 66.1%), the specificity increased (95.2%, 97.2%), the colposcopy referral decreased (7.2%, 4.4%), and the AUC value of the two methods was close (0.815, 0.816). Moreover, with the use of AHPV as triage for the cytology test, 334 women would have been referred, with 72 cases of HSIL+ being detected immediately. This means that the number of referrals may be drastically reduced by more than 35%, while the number of detected HSIL+ cases remained unchanged.

Although the sensitivity of the AHPV alone strategy reached 92.7%, the colposcopy referral was achieved at 12.5%, and its PPV for HSIL+ was 13.5%, consistent with the literature at 6.3%–21.1% in cervical cancer primary screening (Cuzick et al., 2013; Iftner et al., 2015). Primary HPV testing with triage methods was necessary. There were few studies on AHPV-based screening triage strategy. Wang et al. reported the sensitivity of AHPV-positive women triaged with cytology was 59.5% (Wang et al., 2019). In this study, the sensitivity of AHPV-LBC triage was 62.4%. AHPV with cytology-only triage had relatively low sensitivity. On the one hand, the limited sensitivity of cytology might be a detriment to HPV testing sensitivity, especially in places where strict quality assurance cannot be ensured (Almonte et al., 2020). On the other hand, AHPV-LBC triage would miss to detect HSIL+ in HPV-positive women with

normal cytology, especially in HPV16/18-positive women with normal cytology (Wentzensen et al., 2016). HPV genotyping triage provided better risk stratification and required fewer women to attend close testing (Zhao et al., 2021). In this study, the rate of referred to colposcopy for AHPV genotyping with reflex LBC triage was only 5.5%. Among the three triage strategies, AHPV genotyping with reflex LBC triage had the highest AUC value, which highlights the importance of HPV16/18 genotyping. Previous studies reported that the sensitivities of AHPV genotyping with reflex LBC triage were 84.6% (Wang et al., 2019) and 86.6% (Iftner et al., 2015) in the HPV-positive population. However, in this study, the sensitivity of AHPV genotyping with reflex LBC triage was relatively low (73.4%). First, AHPV-GT detects HPV16 and a subset of HPV18 and HPV45 cases. A summarized global meta-analysis indicated that HPV16 was the most frequently detected type; HPV18 ranked second place in CIN3 and invasive cervical cancer (ICC); HPV45 was more common than other non-HPV16/18 types in ICC (Guan et al., 2012). However, in China, HPV31/33/52/58 has a higher risk of HSIL+ than HPV18/45 in HPV-positive and cytology-negative women (Zhang et al., 2020a). Women with non-HPV16 18/45-positive and cytology-negative are followed up, which may be the reason for the low sensitivity of AHPV genotyping with reflex LBC triage. In addition, only 53.8% (458/851) of the AHPV-positive and LBC-negative women who needed to be referred for colposcopy actually had colposcopy. The low performance of colposcopy referral may also reduce the sensitivity of AHPV genotyping with reflex LBC triage. To summarize, AHPV genotyping with reflex LBC triage may be a feasible triage strategy for AHPV-based screening. However, in China, HPV extended genotyping is worth further study.

Excessive colposcopy referral not only wastes medical resources but also brings unnecessary mental burden to women. A good screening method should balance lesion detection and colposcopy referral. The number of colposcopes to be referred for each case of HSIL+ can be used as an indicator to measure the screening efficiency. In this study, the screening efficiency of AHPV (7.4, 749/101) is nearly equivalent to LBC (7.0, 519/74). The screening efficiency of AHPV genotyping with reflex LBC triage and AHPV-LBC triage was higher than that of the AHPV test alone. However, the detection rate of HSIL+ is also lower than that of AHPV primary screening. The requirements for medical resources and organization of each triage strategy are higher than those of AHPV primary screening. Therefore, AHPV screening has achieved a good balance in the detection of lesions and colposcopy referral, which is suitable for cervical cancer screening in middle-income areas.

This study has some limitations that need to be taken into consideration. First, 37% of women who needed to refer for colposcopy were not recalled. In future research, we should find ways to improve the colposcopy referral compliance of HPV-positive but cytology-negative women. Second, longitudinal follow-up should be carried out in women with both negative LBC and AHPV results. Finally, the performance of primary HPV screening with different triage strategies differed among age groups (Bao et al., 2022). Evaluation of the age-specific effectiveness of primary AHPV screening and possible triage strategies is warranted.

In conclusion, the HPV E6/E7 mRNA assay was found to be more sensitive than cytology and to have good specificity. This study suggests that primary screening with HPV E6/E7 mRNA assay is a candidate protocol suitable for cervical cancer screening in China. AHPV genotyping with reflex LBC triage may be a feasible triage strategy. Further longitudinal studies and extending genotyping studies are warranted for triage strategies in primary HPV screening.

Data availability statement

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

Ethics statement

This study was reviewed and approved by the Ethics Committee of Liaoning Cancer Hospital and Institute. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

All the authors contributed to the conception and design of the study. JZ, DY, and XC organized the database. GL performed the statistical analysis. JZ wrote the first draft of the manuscript. JZ, DY, XC, GL, ZC, and CW contributed to the acquisition and analysis of data. All authors contributed to manuscript revision and read and approved the submitted version.

Funding

This study was supported by the National Key Research and Development Program (2016YFC1303001).

Acknowledgments

We would like to express our gratitude to the staff of the Department of Cancer Prevention and Treatment at Liaoning

Cancer Hospital and Institute and community workers in Liaoning Province and Qingdao City.

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Review
PUBLISHED 26 October 2022
DOI 10.3389/fcimb.2022.1023806

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Intestinal Microbiome, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 20 August 2022
ACCEPTED 05 October 2022
PUBLISHED 26 October 2022

CITATION
Kaźmierczak-Siedlecka K, Marano L, Merola E, Roviello F and Połom K (2022) Sodium butyrate in both prevention and supportive treatment of colorectal cancer. *Front. Cell. Infect. Microbiol.* 12:1023806.
doi: 10.3389/fcimb.2022.1023806

Sodium butyrate in both prevention and supportive treatment of colorectal cancer

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Accumulating evidence suggests that selected microbiota-derived metabolites play a significant role in both tumor prevention and supportive treatment of cancer. Short-chain fatty acids (SCFAs), i.e., mainly acetate, proprionate, and butyrate, are one of them. Nowadays, it is known that butyrate is a key microbial metabolite. Therefore, in the current review, we focused on butyrate and sodium butyrate (NaB) in the context of colorectal cancer. Notably, butyrate is characterized by a wide range of beneficial properties/activities. Among others, it influences the function of the immune system, maintains intestinal barrier integrity, positively affects the efficiency of anti-cancer treatment, and may reduce the risk of mucositis induced by chemotherapy. Taking into consideration these facts, we analyzed NaB (which is a salt of butyric acid) and its impact on gut microbiota as well as anti-tumor activity by describing molecular mechanisms. Overall, NaB is available as, for instance, food with special medical purposes (depending on the country's regulation), and its administration seems to be a promising option for colorectal cancer patients.

KEYWORDS

colorectal cancer, short-chain fatty acids, butyrate, sodium butyrate, gut microbiome, metabolome

Introduction

Short-chain fatty acids (SCFAs) are known as significant microbial metabolites (Mohseni et al., 2020; Fang et al., 2021; Zhang et al., 2022). The group of SCFAs mainly includes acetate (C2), proprionate (C3), and butyrate (C4) (Liu et al., 2018; Guan et al., 2021). They are produced in different amounts; i.e., in the colonic lumen, the proportion is as follows: 60% acetate, 25% proprionate, and 15% butyrate (Canani et al., 2011). An appropriate ratio is 3:1:1 for acetate, proprionate, and butyrate, respectively (Nogal et al., 2021). The highest concentration of SCFAs is observed in the proximal colon (7–140 mM) (Sun et al., 2017; Liu et al., 2018).

Butyrate (a key bacterial metabolite) is produced in the colon through bacterial fermentation using dietary fibers and starch as sources (Louis and Flint, 2017). Butyrate-producing microbes are bacteria belonging to the Firmicutes phylum. According to some data, the most important butyrate-producer is *Faecalibacterium prausnitzii* (Kaźmierczak-Siedlecka et al., 2022). Butyrate provides multiple effects in the human body. Among others, it has anti-inflammatory properties (inhibits the pro-inflammatory mediators TNF-α, IL-1β, IL-6, and IL-8 as well as upregulates the anti-inflammatory IL-10) and affects intestinal mucosal immunity (for instance, through regulation of immune cell migration) (Liu et al., 2018). Sodium butyrate (NaB), which is a salt of butyric acid, is characterized by a wide range of beneficial properties/activities. NaB has been investigated in case of several diseases, such as inflammatory bowel diseases (IBDs) (Chen et al., 2018; Facchin et al., 2020), non-alcoholic fatty liver disease (NAFLD) (Zhou et al., 2017; Zhang et al., 2021), and obesity (Fang et al., 2019; Beisner et al., 2021). During the last several years, an increasing interest in the effect of NaB usage in the context of colorectal cancer has been observed.

Currently, colorectal cancer is one of the most commonly diagnosed cancer. Notably, 1.8 million new cases were noted in 2018 and 1.93 million in 2020, globally (Picard et al., 2020; Li et al., 2022). Despite the fact that the treatment of this cancer is quite well established and it regards neoadjuvant therapy as well as surgery, there is a need to search for new therapeutic mini-invasive or non-invasive methods that, among others, support the preparation of patients for surgical treatment, reduce the side effects of anti-cancer therapy, and inhibit the progression of disease. Modification of gut microbiota and metabolome aspects seems to be extremely significant in the case of colorectal cancer. Using of NaB may be a promising strategy for colorectal cancer patients.

The general properties/activities of butyrate

Immune system-related aspects

Butyrate is known as one of the most important metabolites produced through bacterial fermentation in the gut. The biosynthesis of butyrate is done in two metabolic pathways. The first of which regards the phosphorylation of butyryl-CoA to butyryl-phosphate and then the transformation to butyrate with enzyme butyrate kinase. The other metabolic pathway regards the transformation of butyryl-CoA to acetate and then to butyric acid (Liu et al., 2018). It has a wide range of beneficial properties including immunomodulatory functions and anti-tumor activity (Wu et al., 2018; Fu et al., 2019; Siddiqui and Cresci, 2021). It affects immune system by, among others, regulating the expression of pro- and anti-inflammatory mediators. Butyrate inhibits the expression of pro-inflammatory IL-1β and IL-6 while promoting the expression of anti-inflammatory cytokine IL-10 (Hui et al., 2019). Additionally, butyrate promotes the anti-tumor immunity of CD8+ T cells (He et al., 2021). The activity, proliferation, and apoptosis of multiple immune cells may also be directly regulated by butyrate (Danne and Sokol, 2021). Zhou et al. have shown that butyrate is produced by *F. prausnitzii*, and it maintains Th17/Treg balance (Zhou et al., 2018). *Clostridium butyricum*, which has probiotic properties, is also known as a butyrate-producing bacterium (Chen et al., 2020; Stoeva et al., 2021). In the Li et al. study, it was observed that butyrate constrained neutrophil functions as well as ameliorated mucosal inflammation in case of inflammatory bowel disease (Li et al., 2021). Butyrate provides anti-inflammatory effects and increases apoptosis in cancer cells (Jaye et al., 2022). Butyrate and proprionate are more effective in inhibiting HT29 cell growth compared to acetate (Jaye et al., 2022). Moreover, butyrate is a main source of energy for colonocytes (Fu et al., 2019). Additionally, it influences mucosal immunity and maintains gut microbiota stability, providing gut microbial homeostasis (Wu et al., 2018; Fu et al., 2019).

Maintenance of intestinal barrier integrity

Epithelial cells have G-protein-coupled receptors (GPCRs), which are the receptor of butyrate (Hanus et al., 2021). It modulates cellular functions by the above-mentioned GPCRs, which are expressed in multiple cells and tissues (for instance, adipose tissue, monocytes, neutrophils, B and T lymphocytes, and colonic myeloid cells) (Siddiqui and Cresci, 2021). In adipose tissues, butyrate promotes production and secretion of leptin; thus, it may be mentioned as a body weight regulator (Coppola et al., 2021). Butyrate enhances epithelial cell proliferation and the mucus layer, and improves tight junctions (Peng et al., 2009). Notably, tight junctions are intercellular junctions essential for maintaining epithelial barrier integrity (Otani and Furuse, 2020). The claudin family of membrane proteins are a significant part of tight junctions (Otani and Furuse, 2020). The mucus layer is made up of butyrate, mucins, immunoglobulins, and glycoproteins (Zhang et al., 2019). It should be emphasized that butyrate upregulates the expression of the MUC2 gene; thus, it promotes the synthesis of mucins, which protect the epithelial cells from lumen toxins (Zhang et al., 2019). Bacteria such as *Fusobacterium nucleatum*, *Bacteroides fragilis*, and *Escherichia coli* are known colorectal cancer-associated pathogens, and they contribute to the dysfunction of intestinal barrier integrity (Kaźmierczak-Siedlecka et al., 2020; Ahmad Kendong et al., 2021). Butyrate increases the expression of claudin-1 and Zonula Occludens-1; thus, it is important in the context of maintenance of intestinal barrier integrity (Hajjar et al., 2021). In the Feng et al. study (regarding Caco-2 monolayers), it has been revealed that acetate (0.5 mM),

proprionate (0.01 mM), and butyrate (0.01mM) both alone or in combination significantly increase transepithelial electrical resistance (Feng et al., 2018). They protect intestinal barrier function by inhibition of NLRP3 (cytosolic protein) inflammasome (Feng et al., 2018; Sharma and Kanneganti, 2021). Nevertheless, according to Huang et al., butyrate is the main SCFA that alleviates intestinal barrier dysfunction by downregulating the level of claudin-2 (Huang et al., 2021). Moreover, the authors concluded that claudin-2 is the major target of butyrate (Huang et al., 2021). The improvement of the intestinal barrier after butyrate supplementation was also confirmed in the Zhao et al. study regarding 48 rats with severe acute pancreatitis and intra-abdominal hypertension (Zhao et al., 2020). In this study, *C. butyricum* (butyrate-producing probiotic bacteria) and butyrate were given orally. It was noted that rats that consumed *C. butyricum* or butyrate had reduced intestinal injury and decreased the plasma level of inflammatory cytokines, diamine oxidase, and lipopolysaccharide (Zhao et al., 2020).

To summarize this part, butyrate is a key microbial metabolite that extremely affects the intestinal barrier function, and it seems to be needed for colorectal cancer patients in which increased intestinal permeability is noted.

Butyrate and apoptosis of cancer cells

Butyrate can induce the apoptosis of colorectal cancer cells (Bordonaro, 2020). In the Xiao et al. study, the apoptosis of colon cancer cells (HCT116) was detected using flow cytometry (Xiao et al., 2014). These cells were exposed to NaB at a dose of 10 mmol/L per 24 h. Previously, colon cancer cells were also treated with ERK inhibitor or siRNA. It was noted that NaB modulates ERK and sphingosine kinase 2 and consequently induces apoptosis of colon cancer cells (Xiao et al., 2014). The anti-carcinogenic effect of butyrate on colon cancer cells (SW480) was also confirmed in the Elimrani et al. study (Elimrani et al., 2015). Similarly, Roy et al. have shown that butyrate at a dose of 2.5–20 mM (and also carnitine) induces apoptosis of colon cancer cells and inhibits Caco-2 cell proliferation (Roy et al., 2009). Taking together the results of the above-mentioned studies, it can be concluded that butyrate may provide anti-carcinogenic effects.

Impact on anti-cancer treatment

Recently, it was shown that butyrate may improve the efficiency of radiotherapy (Park et al., 2020). Moreover, butyrate may enhance the irinotecan effect; thus, it may enhance the effect of chemotherapy (Encarnação et al., 2018). Similarly, the results of another study revealed that butyrate promotes the effects of 5-fluorouracil on cancerous colonocytes (Geng et al., 2021). It is estimated that approximately 40% of patients who underwent chemotherapy developed mucositis (Ferreira et al., 2012). Butyrate can reduce the side effects of treatment with 5-fluorouracil, which was shown in a mouse model study with mucositis induced by chemotherapy (Ferreira et al., 2012).

The reduction of butyrate-producing bacteria is observed in colorectal cancer patients (Wang et al., 2012). The intracellular concentration of butyrate in colonic cells is regulated by transporters, such as monocarboxylate transporter 1 (MCT1), sodium-coupled monocarboxylate transporter 1 (SMCT1), and breast cancer resistance protein (BCRP) (Gonçalves and Martel, 2016). Notably, the alterations of these transporters' expression may occur in colorectal cancer patients (Gonçalves and Martel, 2016). During the discussion of transporters MCT1 and SMCT1, the term "butyrate paradox" was mentioned. The basis of this paradox is found deeply in epigenetics (Salvi and Cowles, 2021). In case of differentiated intestinal epithelial cells, butyrate is oxidized and utilized as a fuel (energy), and in this time, it is not able to inhibit histone deacetylase (HDAC), whereas cancerous colon cells use preferentially glucose as fuel instead of butyrate (Salvi and Cowles, 2021). Then, butyrate accumulates and acts as an inhibitor of HDAC. There is an observed prevalence of glycolytic metabolism over oxidative phosphorylation in these cells. The modification of histone (H3), which is induced by butyrate, is linked to the activation of genes participating in both cell cycle inhibition and apoptosis (Salvi and Cowles, 2021).

The summary of butyrate properties is presented in Figure 1.

Sodium butyrate

Anti-tumor activity of NaB

NaB is able to provide an anti-tumor effect in the context of colorectal cancer. Notably, there are several mechanisms by which NaB may be involved in this process (Wang et al., 2020). In the Xi et al. study, the effect of NaB on colorectal cancer cells (lines SW480, LOVO, HCT116, and HCT8) was investigated (Xi et al., 2021). The results of this study explore the anti-cancer activity of this component; i.e., NaB is able to induce apoptosis as well as inhibit colorectal cancer cell proliferation (Xi et al., 2021). In another study, the impact of NaB on colorectal cell lines (HCT116 and SW480) was also assessed (Zhou et al., 2019). The authors used next-generation RNA sequencing. It was noted that 7,192 genes were differently expressed in cells treated with NaB in comparison with untreated cells. Recently, it was also observed that NaB is able to inhibit colorectal cancer cells' (lines HCT116 and LOVO) migration through enhancement of miR-200c expression-mediated downregulation of Bmi-1 (Xu et al., 2018). Notably, in another study it was noted that the miR-200c/FUT4 axis prevents the proliferation of colon cancer cells (Cong et al., 2021). This effect is obtained by downregulation of the Wnt/β-catenin pathway

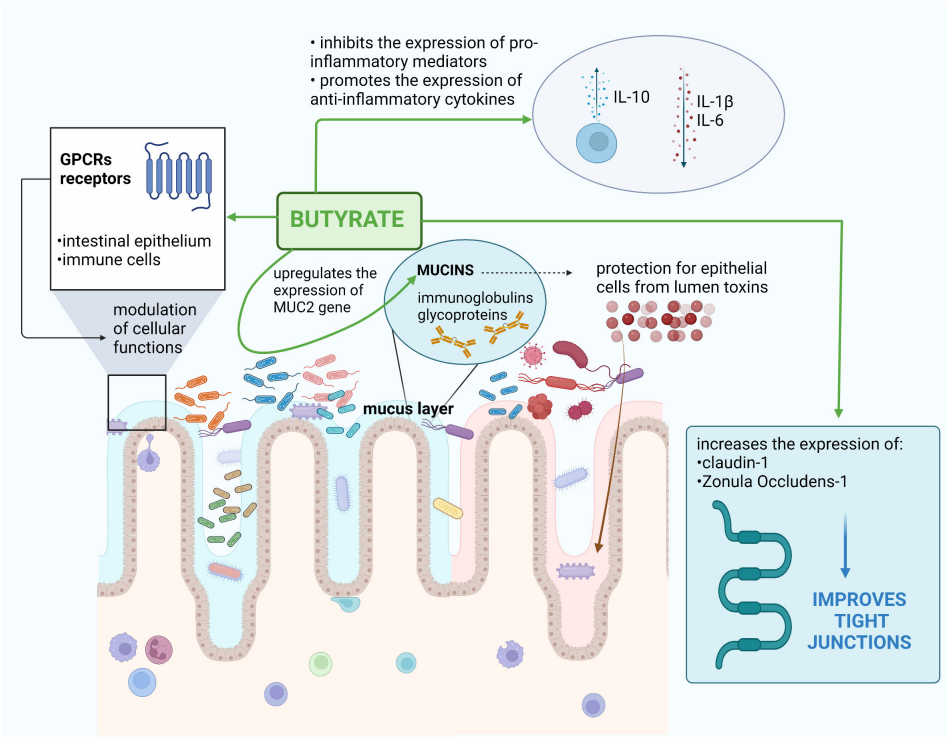

FIGURE 1
Butyrate-associated beneficial effects in oncological aspects. Own elaboration based on literature (Gonçalves and Martel, 2016; Fu et al., 2019; Hui et al., 2019; Zhang et al., 2019; Hajjar et al., 2021; Huang et al., 2021; Siddiqui and Cresci, 2021).

(Cong et al., 2021). Interestingly, Sanaei et al. investigated the effect of NaB on both AsPC-1 (pancreatic cancer) and HCT-116 (colon cancer) cell lines (Sanaei and Kavoosi, 2022). It was observed that NaB increased p16INK4a, p14ARF, and p15INK4b, and reduced class I and II HDACs (Sanaei and Kavoosi, 2022). The authors also reported that colon cancer cell lines were more sensitive to NaB than pancreatic cell lines (Sanaei and Kavoosi, 2022). Notably, p16INK4a is a tumor suppressor protein (Serrano, 1997). It was observed that the loss of p14ARF expression is related to more advanced tumors, which has been confirmed in the Kim et al. study (Kim et al., 2020) (Table 1).

Thioredoxin and thioredoxin reductase are important modulators of tumor development (Zhang et al., 2017). Wang et al. have reported that NaB significantly inhibited the growth of colorectal cancer cells as well as decreased the expression of thioredoxin-1 (Trx-1) protein in these cells (Wang et al., 2020). Notably, these results were not observed in normal colon epithelial cells and that can explain the selective inhibition of cell growth by NaB (Wang et al., 2020). Trx-1 is defined as a

TABLE 1 Summary of the anti-tumor activity of NaB. CRC—colorectal cancer.

Cells lines	NaB's mechanism of action	Reference
CRC cells lines: SW480, LOVO, HCT116, HCT8	≻ induces apoptosis ≻ inhibits CRC cell proliferation	Xi et al., 2021
CRC cells lines: HCT116, SW480	≻ 7,192 genes were differently expressed in cells treated with NaB in comparison with untreated cells	Zhou et al., 2019
CRC cells lines: HCT116, LOVO	≻ inhibits CRC cell migration *via* enhancement of miR-200c expression-mediated downregulation of Bmi-1	Xu et al., 2018
Pancreatic cell line: AsPC-1, colon cell line: HCT-116	≻ increases p16INK4a, p14ARF, and p15INK4b	Sanaei and Kavoosi, 2022
CRC cell lines: HT29, SW480	≻ NaB significantly inhibits the growth of CRC cells and decreases the expression of thioredoxin-1 (Trx-1) protein in these cells	Wang et al., 2020
CRC cell lines: HCT-116, HT-29	≻ induces autophagy in CRC cells by LKB1/AMPK signaling	Luo et al., 2019

small redox-active protein (Shao et al., 2020; Liu et al., 2022). The downregulation of Trx-1 seems to be important in the context of colorectal cancer (Wang et al., 2020). It can be associated with inflammation and oxidative stress (Shao et al., 2020). According to the results obtained in the Shao et al. study, Trx-1 can also be a potential therapeutic target in case of sepsis, due to the fact that it plays a significant role in inflammation and oxidative stress (Shao et al., 2020).

The deficiency of folic acid alters the cytosine methylation in DNA (Lu et al., 2008). Recently, Lu et al. investigated the role of folic acid and NaB in the prevention of colorectal cancer in a mouse model study (Lu et al., 2008). The results of this study have shown that the lower level of p21WAF1 gene expression was found in colorectal cancer samples compared to normal colorectal mucosa. The administration of NaB beneficially increased the level of p21WAF1 mRNA and p21WAF1 protein. Thus, it can prevent tumorigenesis in a mouse model of colorectal cancer induced by 1,2-dimethylhydrazine (Lu et al., 2008).

AMPK regulates glucose and cholesterol metabolism (Shackelford and Shaw, 2009). Liver kinase B1 (LKB1) is a tumor suppressor gene on human chromosome 19p13. LKB1 controls both cell metabolism and oxidative stress, and together with AMPK, it controls cell growth (Shackelford and Shaw, 2009; Ciccarese et al., 2019). NaB induces autophagy in colorectal cancer cells by LKB1/AMPK signaling, which has been demonstrated in the Luo et al. study (Luo et al., 2019). The authors concluded that NaB may be a novel target for colorectal cancer patients (Luo et al., 2019).

NaB and its impact on gut microbiota

The supplementation with NaB can beneficially affect gut microbiota. In the Ma et al. study, the effect of NaB on modulation of gut microbiota in mice with colorectal cancer liver metastasis was investigated (Ma et al., 2020). The composition of gut microbiota was assessed using 16S rRNA gene sequencing. It was observed that NaB beneficially altered gut microbiota. Moreover, it modulated immune system by decreasing Treg cells and increasing NK cells as well as T helper cells (Ma et al., 2020). In another animal model (C57BL/6J mice) study, it was revealed that exercise and NaB supplementation reversed metabolic dysfunctions, which have been induced by high-fat diet ($p < 0.05$), and inhibited the amount of microbes producing lipopolysaccharide ($p = 0.001$) (Yu et al., 2019).

The impact of diet on the concentration of SCFAs

The "Western" diet, which regards the consumption of high amounts of saturated fatty acids, simple sugars, and highly processed food, negatively alters the gut microbiome, causing its imbalance and promoting inflammatory environment (Bibbò et al., 2016; Christ et al., 2019). This type of diet is associated with an increased amount of opportunistic bacteria, lipopolysaccharide, and trimethyloamine-N-oxide as well as the decrease of SCFA production (Beam et al., 2021). As a consequence, it contributes to maintain chronic inflammation and development of nutrition-related diseases. Recently, it was shown that the dysbiotic microbial changes that are caused by a "Western" type of diet can be reversed by supplementation with butyrate (van den Berg et al., 2021). These results were obtained in an animal model study (C57BL/6 mice) (van den Berg et al., 2021).

The amount of bacteria that produce butyrate may be increased by the administration of omega-3 fatty acids [eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] (Gheorghe et al., 2022). In the Zhuang et al. study, it was noted that both EPA and DHA contribute to the increase of butyrate-producing bacteria while decreasing the bacteria that produce lipopolysaccharide, such as *Bilophila* and *Escherichia/Shigella* (Zhuang et al., 2020). Overall, omega-3 fatty acids have an impact on the composition of gut microbiota (Costantini et al., 2017). Moreover, they improve intestinal barrier integrity and reduce the amount of bacteria-producing trimethylamine (TMA) (Rousseau, 2021). The Mediterranean diet, which regards the consumption of food with a high content of dietary fiber, fish, olives, and olive oil (thus also omega-3 fatty acids), is associated with a high level of bacteria that produce SCFAs, and as a consequence, it contributes to the production of a high level of SCFAs and provides gut homeostasis (Merra et al., 2020; Calabrese et al., 2021; Gibiino et al., 2021; Nogal et al., 2021).

Directions for the future and recommendations for clinical practice in the context of microbiota

First of all, specialists should take into consideration the analysis of gut microbiota-related aspects in case of colorectal cancer patients. They should put more attention to fungi and viruses instead of only bacteria, because they are also a significant part of gut microbiota participating in carcinogenesis. According to some data, the components of gut microbiota can be used as novel biomarkers for the early detection of tumors, or they can be a promising marker for anti-cancer treatment including immunotherapy (Temraz et al., 2019; Tsiaoussis and Souglakos, 2021). Moreover, specialists should search for not only a local but also a distal association. For instance, there is a link between the imbalanced changes of oral microbiota and colorectal carcinogenesis and the progression of colorectal cancer. The gut microbial community and key phenotypes are also a significant field that should be further discussed. Notably, microbes can modulate host phenotypes (Han et al., 2021). Consequently, it affects many pathways and even changes the response to

immunotherapy (Han et al., 2021). In this context, the production of metabolites by gut microbiota seems to be crucial. Therefore, not only the composition of gut microbiota but also microbiota-derived metabolites should be analyzed in colorectal cancer patients. The specialists should consider the supplementation of NaB in colorectal cancer but in combination with components that stimulate the production of butyrate, such as not only dietary fiber but also omega-3 fatty acids. Notably, these data are still deeply undiscovered. Some of the products/supplements (not only probiotics) can also change gut microbiota and even the microenvironment of the tumor. Interestingly, oral immunonutrition can alter the microenvironment of the tumor, which has been shown in the D'Ignazio et al. study (D'Ignazio et al., 2020).

Another challenge for the future of gut microbiota is shallow shotgun sequencing. Currently, in most of the studies, gut microbiome is analyzed with 16S rRNA gene sequencing, whereas the shallow shotgun method allows one to obtain a more functional point of view.

Conclusions

SCFAs are an integral part of gut microbiome functioning/homeostasis. Butyrate is a key C4 SCFA with a wide range of beneficial properties. NaB, which is a salt of butyric acid, may open a new promising option and can be a potential strategy for colorectal cancer patients. It provides anti-tumor effects *via* several molecular mechanisms, and they have been described in this paper. Nevertheless, studies that were published assess the effect of NaB on colorectal cancer cell lines or regard animal model studies. There is a large shortage of studies that investigate the results of supplementation with NaB in patients with colorectal cancer. It can be linked to the popularity of this type of products as well as the formal regulation of NaB, because it may differ depending on country. For instance, in Poland, NaB is available as food with special medical purposes. Overall, NaB should be considered as a supportive product in the complex interdisciplinary anti-cancer treatment.

Author contributions

All authors read and accepted the current form of the article. All authors contributed to the article and approved the submitted version.

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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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Mini Review
PUBLISHED 24 November 2022
DOI 10.3389/fcimb.2022.1077651

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 23 October 2022
ACCEPTED 07 November 2022
PUBLISHED 24 November 2022

CITATION
Zou K, Huang Y and Li Z (2022) Prevention and treatment of human papillomavirus in men benefits both men and women. *Front. Cell. Infect. Microbiol.* 12:1077651. doi: 10.3389/fcimb.2022.1077651

Prevention and treatment of human papillomavirus in men benefits both men and women

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Men should not be overlooked in research on human papillomavirus (HPV) and its associated genital diseases. This is because men infected with HPV are not only at higher risk of genital cancers, but also increase their partners' risk of HPV infection and reinfection through sexual contact. Herein, we summarized the state of knowledge regarding the prevention and treatment of HPV infection in men as well as the possible effects of the prevention and treatment of HPV in men on their female partners. Condom use, smoking cessation, male circumcision, and HPV vaccination for men each play an important role in preventing HPV infection within heterosexual couples. Additionally, men could choose to test for certain types of HPV, such as the oncogenic HPV16 or HPV18 strains, as part of a routine screening program when their partner is positive for HPV. Although there is no recognized treatment for HPV infection as of yet, immunotherapy drugs, such as toll-like receptor agonists, therapeutic HPV vaccines, and immune checkpoint inhibitors, have shown promising results in clinical trials and in actual clinical practice. HPV infection in men also increases the risk of cervical cancer in their female partners. Because of the high partner concordance for HPV demonstrated in prior research, the prevention and treatment of HPV in men should be explored more comprehensively in future research.

KEYWORDS

couple, heterosexual, human papillomavirus, infection, sexually transmitted

Introduction

Human papillomavirus (HPV) is the most common reproductive tract virus. HPV belongs to the Papillomaviridae family. Every year, HPV causes 630,000 cancer cases in men and women (i.e., 4.5 percent of all cancer cases), thereby posing a serious threat to public health on a global scale. In 2012, HPV-related malignancies accounted for 8.6% of

Abbreviations: HPV, human papillomavirus; hr-HPV, high-risk human papillomavirus; lr-HPV, low-risk human papillomavirus; MC, male circumcision; ICI, immune checkpoints inhibitor.

all cancer cases in women and 0.8% of all cancer cases in men (Serrano et al., 2018). The Martel study, based on the data from 2012, was updated to report that cervical cancer accounted for 83% of HPV-attributable cancers, while other HPV-attributable cancers were head and neck cancer, anus cancer (half for each sex), penile cancer, vaginal cancer, and vulvar cancer (de Martel et al., 2017). Moreover, HPV is one of the most common causes of sexually transmitted diseases in sexually active women and men, causing the proliferation of scaly epithelium on the mucous membranes of human skin (Burd, 2003). At present, more than 200 HPV subtypes have been identified, of which more than 85 types have been identified in the human body (Burd, 2003). Approximately, 40 species can be transmitted to the anogenital organs and surrounding skin through sexual activity (Munoz et al., 2003).

HPV is divided into broad high-risk (hr-HPV) and low-risk types (lr-HPV) according to carcinogenicity. Infection with hr-HPV can cause head and neck cancer and oropharyngeal cancer in addition to anogenital cancer. In contrast, benign skin lesions, such as genital warts, are generally attributed to lr-HPV (Arbyn et al., 2012). Although most HPV infections and their related precancerous lesions can resolve spontaneously, HPV infection and its sequelae is still an important cause of cancers of the cervix, vulva, vagina, penis, prostate, and anus (Giuliano et al., 2015). HPV infections can also influence sperm status, potentially leading to infertility (Foresta et al., 2010). The prevention and treatment of HPV-related diseases remains a major focus in the field of medicine.

Recent studies suggest that men can take a series of measures to reduce the incidence of certain HPV types, for example, men should be vaccinated against HPV (this is especially true for adolescents) (Petrosky et al., 2015; Schmeler and Sturgis, 2016) and circumcision should be performed for males (Smith et al., 2021). However, current prevention and treatment protocols in regard to HPV infections focus only on female-specific HPV-related diseases, especially cervical cancer (which ranks fourth among female-specific diseases worldwide). There is presently sparse literature and few clinical compasses that recommend prevention, screening, and treatment for HPV infections in men.

At present, the joint prevention and treatment of HPV in men and women (which is especially critical for sexual partners) is not at the core of the screening and treatment protocols that are currently in place. However, cross-infection between couples results in persistent HPV infections more easily than in other situations, thereby elevating the risk of developing high-grade cervical lesions and eventually cervical cancer (de Lima Rocha et al., 2012). Hence, the aim of this paper is to provide an overview of the current understanding of the prevention and treatment of HPV for men and new ideas for heterosexual couples' joint health.

Prevention of HPV

Condom use

Sexual contact is the primary transmission route for HPV. Males act as both virus carriers and vectors and this is an important component of the epidemiological chain for HPV (Castellsague et al., 2003). Further, women are more likely to transmit HPV to their male partners than men are to transmit HPV to their female partners (Malagon et al., 2021).

The fact that cross-infection occurs between members of a couple should not be ignored, as this is one of the reasons for the poor control of HPV evident in the literature and in clinical practice. Condoms are effective in physically isolating HPV infection, and men who do not use condoms have higher rates of HPV infection (Vardas et al., 2011). According to finding by Nielson et al. (2007), the number of condoms used in the previous three months is linked to a lower prevalence of HPV. One cross-sectional analysis covering three countries suggested that consistent condom use is an important factor in the low detection rate of any HPV type, any oncogenic type, and multiple types (Repp et al., 2012). Another cross-sectional study of 393 men showed that regular condom use during sexual intercourse is correlated with a reduction in oncogenic and overall HPV risk, which is similar to the results of the former study (Baldwin et al., 2004). Therefore, the use of condoms during sexual intercourse is essential to preventing HPV transmission and infection.

Smoking cessation

Smoking is a known independent risk factor for HPV infection. Schabath and colleagues successively demonstrated that current smoking overall as well as current smoking with a history of greater than five pack-years of smoking was associated with a higher incidence of HPV infection (especially oncogenic infection) and a lower probability of infection clearance in men (Schabath et al., 2012; Schabath et al., 2014). Researchers have also found that smoking 10 or more cigarettes per day was associated with HPV infection in men (Nielson et al., 2007). For women, the prevalence of HPV was found to be 40.8% for smokers versus 25.2% for non-smokers; the corresponding values for men were 68.2% versus 63.2% (Kaderli et al., 2014). This suggests that men with a history of smoking are more likely to be infected with HPV than women with a history of smoking. Therefore, timely cessation of smoking in men may play an especially critical role in HPV prevention.

Male circumcision

Numerous studies have demonstrated that male circumcision (MC) is effective in reducing the incidence of

multiple HPV infection strains in men (Castellsague et al., 2002; Svare et al., 2002; Gray et al., 2010; Smith et al., 2021), thereby also decreasing the incidence of HPV-related diseases. In Baldwin and colleagues' cross-sectional study, it was suggested that circumcision reduces the risk of overall HPV in addition to oncogenic and non-oncogenic HPV, respectively (Baldwin et al., 2004). Men who have been circumcised may be less likely to allow viral invasion through epithelial abrasions, subsequent viral shedding, and viral persistence. Thus, circumcision is also the most effective factor in reducing the clearance of oncogenic and any HPV infection (Lu et al., 2009). Similar to HPV vaccines, not only can MC help men avoid acquiring certain genital diseases, but also is beneficial to women's health. Men who undergo MC reduce the risk of HPV infection to their female sexual partners (Morris et al., 2019). Moreover, the presence of foreskin in a woman's sexual partner is considered a risk factor for cervical cancer (Agarwal et al., 1993). Evidence is emerging that MC can meaningfully reduce the prevalence of cervical cancer in female partners within heterosexual couples (Castellsague et al., 2002; Svare et al., 2002; Morris et al., 2019). We draw the conclusion that MC should be included as a primary preventive measure for cervical cancer, penile cancer, and other HPV-related cancers within updated medical guidelines.

HPV vaccines

To date, 107 countries have introduced HPV vaccination programs, among which developed countries (led by Australia) have high vaccine implementation coverage; in contrast, in middle and low-income developing countries, the scale of HPV vaccine introduction has not yet been satisfactory (Bruni et al., 2021). It is thought that the HPV vaccine plays an indispensable role in preventing cervical cancer in women. However, HPV vaccination is not just an issue for women. Men are also exposed to the possibility of developing various diseases as sequalae of HPV infection. Moreover, high HPV vaccine coverage in men strongly benefits women by reducing the risk of cervical cancer (Lehtinen et al., 2018). Therefore, the HPV vaccine is undoubtedly equally needed for men and women.

A growing number of studies on gender-neutral HPV vaccination have emerged, and investigators have suggested that HPV16 eradication in the general population is predicted when 75% coverage of early adolescents (both boys and girls) is achieved (Lehtinen et al., 2019; Vanska et al., 2020). We additionally note that the herd effect refers to the indirect protective effect of vaccination on the unvaccinated population by reducing infection transmission within the susceptible population. A community-randomized trial previously reported that gender-neutral vaccination of early adolescents produced a striking population effect, substantially increasing the protective impact of vaccination on women's health (Lehtinen et al., 2018).

Nevertheless, barriers still exist to achieving prevalent HPV vaccination in men, including but not limited to a lack of knowledge regarding HPV, prejudices against the vaccine, various sociodemographic and religious factors, fear of side effects, and concerns about cost (Grandahl and Neveus, 2021). Consequently, it is necessary to enrich knowledge regarding HPV-related diseases in the general population so that more people are willing to get vaccinated. Simultaneously, the existing healthcare system should be modified with respect to making HPV vaccines more accessible.

There are three types of HPV vaccines: a bivalent HPV vaccine, four-valent HPV vaccine, and nine-valent HPV vaccine. The bivalent vaccine protects against HPV16 and HPV18; the four-valent vaccine protects against HPV6, HPV11, HPV16, and HPV18; and the nine-valent vaccine protects against HPV6, HPV11, HPV16, HPV18, HPV31, HPV33, HPV45, HPV52, and HPV58. These three types of vaccines are all effective against HPV16 and HPV18, including HPV-related cancers, as it can be prevented effectively through the use of vaccination campaigns, as the majority of these cancers are caused by HPV16.

According to the recommendation of the Advisory Committee on Immunization Practices (Petrosky et al., 2015; Oshman and Davis, 2020), females aged 11 or 12 years should be routinely vaccinated with bivalent, four-valent, or nine-valent HPV vaccines, while males of the same age should be vaccinated with four-valent or bivalent HPV vaccine. The Advisory Committee on Immunization Practices also recommended vaccination for females and males aged 13–26 years who have not received the HPV vaccine or who have not completed the required three doses. We note that each type of vaccine is administered in a three-dose schedule. According to current recommendations, the second dose should be administered 1–2 months after the first dose and the third dose should be administered 6 months after the first dose.

Detection of HPV

As most HPV infections clear spontaneously without intervention, a positive result does not indicate the need for immediate treatment of the patient or his or her sexual partners. Nevertheless, asymptomatic HPV infection in men is thought to be an important cause of ongoing transmission to female partners, and HPV infection in men increases the risk of cervical cancer in women (Barrasso et al., 1987). HPV infection also poses a risk for genital warts, penile cancer, and anal cancer in men. Therefore, HPV screening is necessary for men. Currently, however, only standardized HPV screening for women is emphasized, and there are no routine HPV screening programs in place for men. To our knowledge, data on the most reliable sampling site, the standardized sampling method, and the quality of sampling for men are not currently available. Some

researchers suggest that, in men, samples collected from the external genital region yield a higher detection rate for oncogenic HPV than samples collected in the anal region, and the penile shaft is recommended as the optimal anatomical site for HPV detection (Nielson et al., 2007; Giuliano et al., 2007). Moreover, testing for HPV DNA appears to be the best strategy for detecting HPV infection in males, as revealed by Nicolau and colleagues. Brush material obtained from the distal urethra as well as from the external surface of the penis tends to be the most effective approach to diagnosing HPV infection in men (Nicolau et al., 2005). Targeted screening for certain types of HPV may also be used as a testing tool for HPV detection; for example, E6 seropositivity for HPV16 has been used as a prognostic and surveillance tool for oral cancer (Holzinger et al., 2017).

HPV testing for men should remain a focus within future research and clinical endeavors. We recommend several models herein: 1) men could choose to test for HPV when their female partner is positive for HPV (especially for those whose partners are positive for hr-HPV); 2) HPV screening programs for men should be developed as part of an easy and routine program; and 3) certain types of HPV infection, such as HPV16 and HPV18, should be highlighted because these infection strains are linked to the majority of malignancies of the penis, anus, and head and neck as compared with other HPV strains.

Treatment of HPV-related lesions

Current treatment is focused on addressing individual HPV-related lesions, such as cervical cancer, vulvovaginal cancer, and penile cancer. Surgery, radiotherapy, chemotherapy, and targeted therapy are widely used in clinical practice. There is presently no standardized treatment for HPV infection only.

However, numerous studies regarding immunotherapy for HPV infection, a treatment modality that aims to achieve therapeutic goals by restoring local immune cell function, have been emerging as of now. The specific mainstream immunotherapy approaches currently under evaluation are described later in this report.

Toll-like receptor agonists

It is well known that toll-like receptors activate innate immunity by activating downstream signaling pathways through the recognition of pathogen associated molecular patterns, thereby stimulating the production of proinflammatory cytokines and type I interferons (Mifsud et al., 2014; Owen et al., 2020). In summary, toll-like receptor agonists stimulate the body's innate immune system and enhance innate immune function to clear pathogens and protect the body from infection (Mifsud et al., 2014). Imiquimod, a typical toll-like receptor agonist, has been increasingly used in the treatment of HPV-associated intraepithelial neoplasia and squamous cell carcinoma *in situ* of the penis as an alternative treatment option to surgery, with fewer adverse events and tumor recurrence (Schroeder and Sengelmann, 2002; Tristram et al., 2014).

Therapeutic HPV vaccines

Among the HPV proteins, E6 and E7 (proteins involved in tumorigenesis and progression) are considered ideal targets for cervical cancer immunotherapy (Pal and Kundu, 2019). Therapeutic HPV vaccines targeting E6 and E7 proteins have therefore been proposed; these vaccines are capable of enhancing the T cell immune response (Garbuglia et al., 2020). Live-vector-based, peptide-based, protein-based, dendritic cell-based, and genetic vaccines each have great advantages as well as demonstrated effectiveness in the treatment of HPV-related diseases (Chandra et al., 2021). Although no vaccine has presently been approved for clinical use, the therapeutic HPV vaccine has a promising future as an effective treatment strategy.

Immune checkpoint inhibitors

Immune checkpoints and their ligands have been found to be constantly upregulated in the tumor microenvironment of diverse malignancies, representing a major obstacle in the initiation of the body's effective innate anti-tumor immune response (Toor et al., 2020). Immune checkpoints inhibitors (ICIs) have become a popular research target in the field of tumor immunotherapy and are currently the main therapeutic strategy employed in immunotherapy. ICIs induce the blockade of programmed cell death protein 1, programmed death-ligand 1, and cytotoxic T-lymphocyte-associated protein 4. Ipilimumab (targeting cytotoxic T-lymphocyte-associated protein 4), and nivolumab and pembrolizumab (both targeting programmed cell death protein 1) are presently licensed for marketing. Moreover, programmed cell death protein 1 inhibitors have entered clinical trials with respect to the treatment of advanced cervical cancer following HPV infection (Chung et al., 2019; Naumann et al., 2019). Still, the clinical efficacy of any particular ICI alone is limited; according to current findings, ICIs should instead be combined with other therapeutic modalities to improve treatment outcomes. In summary, more convincing clinical trials proving the efficacy of this treatment modality are needed to address the current difficulties in the application of immunotherapy in HPV-related diseases.

Necessity of HPV treatment for men

Reciprocal transmission of HPV is prevalent between couples. In a previous study conducted by Burchell and colleagues that evaluated couples in which both partners were positive for any type of HPV, 87% were found to be concordant for at least one type of HPV strain (Burchell et al., 2010). Compelling evidence from a well-designed cross-sectional study conducted in 2014 showed that 68% of the evaluated couples in which both partners were positive for HPV had at least one genotype (i.e., strain) in common; moreover, if male partners were positive for at least one HPV genotype, this had a substantial impact on their female partners' positivity status (de Lima Rocha et al., 2012). Moreover, Bleeker et al. demonstrated a high concordance of partner HPV types, and presented findings that this concordance may be associated with an increased viral load (Bleeker et al., 2005).

Among heterosexual partners, female patients diagnosed with cervical intraepithelial neoplasia have been shown to increase the risk of HPV infection in their sexual partners (Martin-Ezquerra et al., 2012). Although the majority of infections are cleared through enhanced immune function, there is still a risk of progression to severe disease among those infected with HPV.

Discussion

Men are an important component of the cycle of the transmission of sexually transmitted diseases, and that HPV-positive men are also responsible for their female partners' reinfection status (Skoulakis et al., 2019). As carriers and vectors of hr-HPVs, male partners may also cause a significant impact on the development of cervical cancer in their female partners (Skegg et al., 1982; Barrasso et al., 1987; Bosch et al., 1996). This reminds us that HPV-specific treatment and preventive medicine efforts should not only be targeted to women, but also toward men with HPV infections.

Hence, in addition to various screening and preventive efforts, the co-treatment of HPV for male and female partners is extremely crucial to slow down HPV transmission. Moreover, partner co-therapy for HPV infection should gradually be included within clinical treatment practices and formal medical guidelines informing clinical decision-making.

Conclusion

HPV infection in men and the effects of HPV infection on their partners are increasingly being emphasized in the medical literature, in ongoing epidemiologic and clinical research efforts, and in clinical practice. Since sexual transmission is the main route of HPV infection, condoms can help prevent HPV transmission by physically isolating contact with the mucous membranes of the skin. Smoking has also been recognized as an independent factor contributing to HPV infection, and quitting smoking is one of the known measures for preventing HPV. Moreover, studies have successively proven that MC and male vaccination exert a crucial effect on preventing HPV in female sexual partners.

We also note that, on the one hand, HPV infection in men may be a potential risk factor for cervical cancer in women. On the other hand, the high concordance of HPV types in partners suggests that cross-infection is a barrier to HPV control. Therefore, in summary, the treatment of HPV infection in men should undoubtedly be addressed equally as that for women.

Early intervention in men can protect against the transmission of HPV infection, while also reducing the incidence of cervical cancer in female partners and facilitating the treatment of female patients with HPV. However, there is still much to learn about the prevention and treatment of HPV and its related malignant diseases. We strongly recommend that this disparity be investigated in male patients as well in female patients, and that this should become a key focus and hotspot for future research.

Author contributions

KZ contributed to the manuscript drafting and final approval. YH contributed to manuscript revising and critical discussion. ZL provided practical suggestions and critically revised the manuscript. All authors have read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by the Medical Science and Technology Project of Sichuan Provincial Health Commission (No. 21PJ050).

Acknowledgments

We acknowledge the staff of the Key Laboratory of Birth Defects and Related Diseases of Women and Children (Sichuan University), Ministry of Education, Chengdu, People's Republic of China, for the methods instruction of the study.

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Review
PUBLISHED 29 November 2022
DOI 10.3389/fcimb.2022.1020583

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Intestinal Microbiome, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 16 August 2022
ACCEPTED 09 November 2022
PUBLISHED 29 November 2022

CITATION
Ou S, Wang H, Tao Y, Luo K, Ye J, Ran S, Guan Z, Wang Y, Hu H and Huang R (2022) Fusobacterium nucleatum and colorectal cancer: From phenomenon to mechanism. *Front. Cell. Infect. Microbiol.* 12:1020583. doi: 10.3389/fcimb.2022.1020583

Fusobacterium nucleatum and colorectal cancer: From phenomenon to mechanism

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Colorectal cancer(CRC) is the third most frequent malignant tumor. The gut microbiome acts as a vital component of CRC etiology. Fusobacterium nucleatum(Fn) is a key member of colorectal cancer-associated bacteria. But we lack a systematic and in-depth understanding on its role in CRC evolution. In this article, We reviewed the abundance changes and distribution of Fn in CRC occurrence and development, potential effect of Fn in the initiation of CRC, the source of intratumoral Fn and the cause of its tropism to CRC. In addition, We described the mechanism by which Fn promotes the malignant biological behavior of CRC, affects CRC response to therapy, and shapes the tumor immune microenvironment in great detail. Based on the relationship between Fn and CRC, we proposed strategies for CRC prevention and treatment, and discussed the feasibility and limitations of specific cases, to gain insights into further basic and clinical research in the future.

KEYWORDS

Fusobacterium nucleatum, colorectal cancer, metastasis, metabolic reprogramming, stemness, immune microenvironment, therapy response, prevention

1 Introduction

Colorectal cancer (CRC) is a major public health issue. It ranked third in incidence and second in mortality among all cancers in 2020 (Sung et al., 2021). Although both hereditary and environmental risk factors contribute to the development of CRC, genetic variables account for only 12 to 35% of CRC risk factors (Dekker et al., 2019). This indicates that CRC is influenced heavily by environmental risk factors, of which the gut microbiota is a key component (Avril and DePaolo, 2021). Infection with pathogens such as pathogenic *E. coli, Salmonella enterica, toxigenic Bacteroides fragilis, Fusobacterium nucleatum (Fn), Peptostreptococcus anaerobius* and *Helicobacter pylori* has been reported

to be associated with the risk of CRC (Tsoi et al., 2017; Dai et al., 2019; Hernández-Luna et al., 2019). Fn is a gram-negative, anaerobic bacillus that doesn't generate spores (Han, 2006), and classified into four subspecies, including *nucleatum, polymorphum, vincentii(fusiforme)*, and *animalis* (Nie et al., 2015). It exists not only in the human oral cavity but also in the digestive and genitourinary tracts, related with periodontitis (Signat et al., 2011), inflammatory bowel disease (Ohkusa et al., 2003; Minami et al., 2009; Strauss et al., 2011), pancreatic abscess (Brook and Frazier, 1996), hepatic abscess (Athavale et al., 2002; Yoneda et al., 2011), brain abscess (Han et al., 2003; Kai et al., 2008), osteomyelitis (Gregory et al., 2015), pericarditis (Truant et al., 1983), appendicitis (Swidsinski et al., 2011), chorioamnionitis (Altshuler and Hyde, 1988). Although all subspecies of Fn can be detected in CRCs, their intratumoral colonization is heterogeneous. Among them, animalis subspecies is the most prevalent subspecies in CRCs (Ye et al., 2017; Borozan et al., 2022). In addition, in one tumor, colonization by one or two subspecies is commonly observed, whereas colonization by three or four subspecies is detected rarely (Bi et al., 2021). The abundance of Fn is generally elevated in feces, cancer tissues from CRC patients. As a bacterium that colonizes inside CRC, Fn has a great impact on the growth and evolution of CRC. Numerous researches have focused on the role of Fn in CRC in the past decade. Although some breakthroughs have been made in the mechanism of interaction between Fn and CRC, We still lack In-depth and comprehensive understanding of Fn's role in CRC. In this review, We drew a comprehensive landscape of Fn in the occurrence and development of CRC from the macro and micro perspective, and proposed CRC prevention and treatment strategies based on current understanding of the role of Fn in CRC.

2 Temporal and spatial distribution of Fn in CRC

The malignant transformation process of normal intestinal epithelium-precursor lesion-CRC involves two distinct carcinogenesis pathways: the traditional adenoma-carcinoma pathway(70–90%) characterized by Adenomatous polyposis coli (APC) mutation, chromosomal instability, and lack of CpG island hypermethylation; and the alternative/serrated neoplasia pathway(10–20%) characterized by BRAF mutation, chromosomal stability, and high CpG island hypermethylation (Dekker et al., 2019). These pathways reflect a series of genetic and epigenetic events that occur in a logical order. Serrated adenomas/polyps, as precursor lesions of the serrated pathway, are subdivided into sessile serrated adenoma or polyp, traditional serrated adenoma and hyperplastic polyp (JE et al., 2015). Several studies showed that Fn was overabundant in adenomas tissue or stools of adenoma patients compared to healthy people or non-adenoma patients (Kostic et al., 2013; McCoy et al., 2013; Flanagan et al., 2014). Therefore, Fn is already enriched in the lesions (precancerous lesions) before intestinal epithelium cell completing malignant transformation in a subset of patients. Another study showed that hyperplastic polyp and sessile serrated adenomas had a higher prevalence of invasive Fn compared with traditional adenomas(65.7%, 78.8% vs. 28.9% respectively) in proximal colon (Yu et al., 2016). However, the research by Rezasoltani et al. came to a contrary conclusion. In comparison to fecal samples from the normal, hyperplastic polyp, and sessile serrated adenoma groups, traditional adenoma cases, including tubular adenoma and villous/tubuvillous polyp, had a higher amount of Fn. Interestingly, their research showed that there was a positive correlation between the polyp size, dysplasia grade, and the amount of Fn, which were consistent with the finding of Flanagan (Flanagan et al., 2014). In comprehensive consideration, Fn have no preference for carcinogenesis pathways. And the study by Park et al. supports this view because the relative abundance of Fn doesn't differ between tubular adenoma and sessile serrated adenoma/polyp groups (Park et al., 2016).

Increasing evidence has shown that Fn enrichment is prevalent in CRC. When compared to healthy controls, Fn has a greater overall abundance or presence rate in the fecal microbiota of CRC patients (Kostic et al., 2013; Mira-Pascual et al., 2015; Amitay et al., 2017). Gut microbiota can be classified into two groups: fecal-luminal microbiota and mucosa-associated microbiota (Fung et al., 2014). Mucosa-associated microbiota interacting with intestinal epithelium directly and intimately, is considered more closely related to CRC (Yoon et al., 2017). A study has reported that the amount of Fn in stool is not proportional to the Fn of tissue from the same individual (Flanagan et al., 2014). Therefore, the evidence from the mucosa or tissues is more convincing than stool. The Fn abundance in the tumor tissues of CRC patients is relatively higher than that in mucosal tissues of non-tumor subjects (Xu and Jiang, 2017). Likewise, Fn abundance is substantially higher in the tumor than in neighboring normal mucosal tissue from the same individual (Castellarin et al., 2012; Kostic et al., 2012; Flanagan et al., 2014; Li et al., 2016; Gao et al., 2017; Yamaoka et al., 2018). Interestingly, in the surrounding tissue of CRC, the abundance of Fn is gradually decreased with the distance from the tumor (Cuellar-Gómez et al., 2021; Kono et al., 2021). Fn is also positively associated with the progress of CRC. The study demonstrated that tissues and fecal samples from the patients with invasive CRC(T1b-T3) had a higher Fn abundance than the patients with early CRC(Tis and T1a)(9.65% vs. 0.95%) (Zorron Cheng Tao Pu et al., 2020). Furthermore, another study found that Fn abundance in fecal samples from patients with stage II or III CRC was higher than stage I (Amitay et al., 2017). There is also a positive relationship between Fn and lymph node metastases. The research from Japan showed that the frequency of lymph node metastases in CRC patients with

high Fn abundance was higher than low Fn abundance in cancer tissue (59.1% vs. 0%) (Li et al., 2016). These researches above indicates that there is a intense association between Fn and CRC, particularly in the development after initiation.

Overall, CRC in right side has a higher detection rate or relative abundance of Fn than left side (proximal colon> distal colon> rectum) (Salvucci et al., 2021; Borozan et al., 2022). More specifically, the proportion of Fn-high CRC gradually increases from rectum to cecum (Mima et al., 2016a). This may due to the nutritional and environmental preferences of Fn in the gut. According to the data from numerous nations, the presence of Fn in CRC is significantly connected with the molecular characteristics of high microsatellite instability (MSI-H), high CpG island methylator phenotype (CIMP-H), BRAF mutation, TP53 wild type, consensus molecular subtypes 1 (CMS1) (Table 1). Among them, the association between Fn and MSI-H is independent of CIMP and BRAF mutation status (Mima et al., 2016b). Interestingly, these are also the molecular characteristics of right-sided CRC (Dekker et al., 2019), and the proportions of CRC with specific molecular features such as MSI-H, CIMP-H, and BRAF and PIK3CA mutations gradually increase along the bowel subsites from rectum to ascending colon (Yamauchi et al., 2012). Whether the presence of Fn leads to these molecular changes, or these molecular features cause the enrichment of Fn in CRC is unknown. Currently, there is no sufficient evidence to support that Fn induces oncogenic mutations in intestinal epithelial cells. Fn gavage, even if the frequency is as high as once a day, was not sufficient to induce colon tumorigenesis in either wild-type mouse model or chronic intestinal inflammation mouse model with IL10 deficiency or T-bet and Rag2 double deficiencies (Kostic et al., 2013; Yu et al., 2015). Fn promotes tumorigenesis only in the presence of oncogenic gene defects, or mutagenic chemical agents AOM or DMH (Table 2). Therefore, we tend to hold the thinking that Fn plays a promoting role after the occurrence of key oncogenic mutations in intestinal epithelial cells, rather than directly inducing oncogenic mutations in cells. But research by Lesiów et al. demonstrated that complexes formed by fragments of Fn FomA adhesin with copper significantly stimulated reactive oxygen species production and strong oxidative stress response in CT26 cells (Lesiów et al., 2019). This may trigger DNA damage and further canceration in colon cells. The study by Stokowa-Sołtys et al. provided further direct evidence that copper(II) complexes with fragments of adhesin YadA from Fn possessed DNA-cleaving activity (Stokowa-Sołtys et al., 2022). But these studies lack comparisons with other bacteria. We need to further evaluate the effects of Fn on inducing DNA damage or mutation *in vitro* and *vivo*.

3 Ability of Fn to colonize normal gut

To determine whether Fn can induce the transition from normal mucosa to adenoma, we need to assess the ability of Fn to colonize the gut. Fn can't stably colonize the gut of specific-pathogen-free (SPF) mice raised conventionally, even with repeated inoculations (Queen et al., 2022). However, Fn is able to colonize the gut of germ-free (GF) mice for a long time after oral administration. Furthermore, the study by Brennan et al. showed that the strain Fn7-1 could colonize the gut of neonatal mice and ASF mice(colonized with specific eight murine-isolated bacterial strains). Those suggest that it's easy for Fn to colonize the gut with simple components of microbiota but difficult to colonize the gut with balanced and complex microbiota. As an alien species, Fn may be repelled by native gut flora. In addition, Fn subspecies differs in the ability of

TABLE 1 Molecular characteristics of CRC related to Fn.

Country	Sample size (patients)	Positively correlated molecular features	Negatively correlated molecular features	References
South Korea	246	MSI-H; CIMP-H	/	(Lee et al., 2018)
Japan	511	MLH1 methylation; CIMP-H; MSI-high	/	(Ito et al., 2015)
New Zealand	34	CMS1	/	(Purcell et al., 2017)
Japan and USA	149	CIMP; hMLH1 methylation; CHD7/8 mutation;	TP53 mutation	(Tahara et al., 2014)
USA	1069	MSI-H; MLH1 hypermethylation; CIMP; BRAF mutation	/	(Mima et al., 2016b)
USA	1994	Hypermutated status; MSI; CIMP; ERBB3; POLE	TP53 mutation	(Borozan et al., 2022)
South Africa	55	MSI-H	/	(Viljoen et al., 2015)
Ireland, UK, and USA	1430	CMS1; BRAF mutation;	/	(Salvucci et al., 2021)

MSI-H High microsatellite instability, CIMP CpG island methylator phenotype, CMS1 Consensus molecular subtypes 1, MLH1 mutL homolog 1, ERBB3 erb-b2 receptor tyrosine kinase 3, POLE DNA polymerase epsilon, catalytic subunit, BRAF B-Raf proto-oncogene, serine/threonine kinase.

10.3389/fcimb.2022.1020583

TABLE 2 Effects of Fn gavage on inflammatory factor and tumorigenesis of colon in various mouse models.

Murine model	Gut microbiota status before Fn inoculation	Breeding environment	Frequency of Fn inoculation	Sample source	Upregulated factors	Non-upregulated factors	Tumorigenesis promotion	References
WT	GF	GF	Once a week	Distal colon	IL17	TNF-a; IL-1β; CXCL1; CXCL2; IFN-γ; IL6; IL10; IL17	/	(Queen et al., 2022)
IL10$^{-/-}$	/	/	Once a day	/	/	/	NO	(Kostic et al., 2013)
T-bet$^{-/-}$ Rag2$^{-/-}$	/	/	Once a day	/	/	/	NO	(Kostic et al., 2013)
Apc$^{Min/+}$	GF	GF	Once a week	Distal colon	/	TNF-a;IL-1β; CXCL1; CXCL2; IFN-γ; IL6; IL10; IL17	NO	(Queen et al., 2022)
Apc$^{Min/+}$	GF	GF	Once a week	Distal colon	/	/	NO	(Tomkovich et al., 2017)
Apc$^{Min/+}$	GF	SPF	Once a week	Distal colon	/	/	NO	(Tomkovich et al., 2017)
Apc$^{Min/+}$IL10$^{-/-}$	GF	SPF	Once a week	Distal colon	/	TNF-a;IL-1β; IFN-γ; IL6; IL22; IL17a	NO	(Tomkovich et al., 2017)
Apc$^{Min/+}$	/	/	Once a day	Colon tumor	IL6; IL8; COX2; TNF-a	IL1b; IL24	YES	(Kostic et al., 2013)
Apc$^{Min/+}$	SPF	SPF	Once a day	/	/	/	YES	(Chen et al., 2020)
Apc$^{Min/+}$	SPF	SPF	Once a day	Serum	MIP3α; IL22; IL21; IL17F	TNF-a; TGFβ1; IFN-γ; IL28; IL23; IL17; IL13; IL12p70; IL6; IL4; IL2; IL1β	YES	(Yang et al., 2017)
Apc$^{Min/+}$	SPF	SPF	Once a day	Colon	IL17F; IL21; IL22; IL23p19; IL31		YES	(Yu et al., 2015)
Apc$^{Min/+}$	SPF	SPF	D0, D14, D18-28*	Colonic epithelium and lamina propria	IL17a	IL6; IL8; TNF; CCL2; COX-2; IL23p19	YES	(Brennan et al., 2021)
ASF WT	a community of eight murine-isolated bacterial strains	GF	Once at the 8th week and an additional two weeks before sacrifice	Colonic lamina propria	IL17a; IL23p19	/	/	(Brennan et al., 2021)
AOM/DSS	SPF	SPF	Twice per DSS-water cycle	Serum	IL1β; IL6	/	YES	(Yu et al., 2020)
AOM	GF	GF	Once	/	/	/	YES	(Ternes et al., 2022)
AOM	SPF	SPF	Twice per DSS-water cycle	Serum	/	IL1β; IL6	NO	(Yu et al., 2020)
AOM/DSS	SPF	SPF	Once every 2 days	Colon tumor	CCL20	/	YES	(Xu et al., 2021)
AOM/DSS	GF	SPF	Once a day	/	/	/	YES	(Kong et al., 2021)
DMH	SPF	SPF	Once a day	Colon	IL17F; IL21; IL22; IL23p19; IL31	MIP3α; IL33	YES	(Yu et al., 2015)

DMH, 1,2-dimethylhydrazine; AOM, azoxymethane; DSS, dextran sodium sulfate; GF, germ-free; SPF, specific-pathogen-free; WT, wild type; IL10$^{-/-}$, IL10 deficiency; T-bet$^{-/-}$Rag2$^{-/-}$, T-bet and Rag2 double deficiencies; Apc$^{Min/+}$, Apc deficiency; Apc$^{Min/+}$IL10$^{-/-}$, Apc$^{Min/+}$ and IL10$^{-/-}$ double deficiencies.

colonizing the gut. This emphasizes that Fn is a heterogeneous species. Under what circumstances can Fn colonize the gut with complex microbiota? The study by Tomkovich et al. demonstrated that Fn could colonize the gut of SPF $Apc^{Min/+}$ mice (Tomkovich et al., 2017). $Apc^{Min/+}$ mice with APC gene deficiency, spontaneously develop intestinal tumors in an aberrant crypt foci-adenoma-adenocarcinoma sequence. Disruption of the mucosal barrier allows Fn easily to invade the mucosa. Moreover, adenoma, as an intermediate of CRC formation progress, highly expresses Gal-GalNAc, which is recognized and bound by Fap2 of Fn (Abed et al., 2016). Consistent with this, Fn is enriched in tumor tissue compared to adjacent normal tissue in Fn-fed $Apc^{Min/+}$ mice (Kostic et al., 2013). In addition, Fn has been reported to be isolated more frequently from mucosal biopsy samples of inflammatory bowel disease patients compared with those from non-inflammatory bowel disease controls (Strauss et al., 2011). Therefore, we infer that Fn can't stably colonize the normal gut harboring balanced complex microbiota, unless the normal mucosal structure is disrupted, the normal flora is disturbed, or inflammation or dysplasia occurs. These changes may provide Fn with competitor inhibition, more attachment niches, and a more suitable nutrient environment in the gut.

4 Oral Fn translocate to CRC *via* two routes

Fn in CRC is thought to originated from the mouth cavity since it is the primary resident member of the human oral microbiota and is rarely seen in the gut (Dewhirst et al., 2010; Faust et al., 2012; Human Microbiome Project Consortium, 2012). This view is further confirmed by Komiya and Abed. The study by Komiya et al. showed that identical strains were detected in more than 40% of CRC patients' tumors and saliva specimens (Komiya et al., 2019). The study by Abed et al. confirmed the fairly close evolutionary relationship between oral and matched tumor isolates by genome sequence analysis (Abed et al., 2020). Oral Fn can translocate to CRC through two routes. One is the oral cavity-circulatory system-CRC, and the other is the oral cavity-alimentary tract-CRC. In humans, transient bacteremia is frequent during periodontal disease, with bacterial burdens exceeding 104 bacteria/ml blood 15 minutes after tooth brushing (Ashare et al., 2009). The studies by Abed et al. demonstrated that blood-borne Fn can colonize the tumor of the CT26 and MC38 mouse orthotopic CRC models (Abed et al., 2016; Abed et al., 2020). Therefore, oral Fn can enter the circulatory system through broken gums and eventually reach the tumor. In addition, Fn can also translocate to the gut where the tumor is located *via* the digestive tract after swallowing, and then invade the tumor through the damaged mucosa. This route has been confirmed by multiple preclinical studies (Kostic et al., 2013; Wu et al., 2018).

5 Affinity molecule mediate Fn's tropism to CRC

Fn prefers localizing colorectal adenocarcinoma. This phenomenon is partly attributed to the high expression of polysaccharide D-galactose-β(1-3)-N-acetyl-D-galactosamine (Gal-GalNAc) in CRC cells (Abed et al., 2016). The level of Gal-GalNAc is higher in colorectal adenocarcinomas cells compared with normal cells in adjacent tissue. The surface protein Fap2 of Fn is a galactose-binding lectin and has a high affinity to Gal-GalNAc. The attachment of Fn to CRC cell was mediated by the recognization-combination of Gal-GalNAc and Fap2. After O-glycanase treatment or using Fn strains with inactivated Fap2 mutants, the attachment is reduced. The adhesion of Fn to CRC cell is validated not only *in vitro* but also *in vivo* (orthotopic rectal CT26 adenocarcinoma mouse model and $Apc^{Min/+}$ mice model) (Abed et al., 2016). Except for adhesion, Fap2 also mediates intracellular invasion of Fn to CRC cells (Casasanta et al., 2020). Another factor that promotes the enrichment of Fn in CRC is the adhesin FadAc. FadA is a virulence factor of Fn. It appears in two types: secreted mature FadA (mFadA) and non-secreted intact pre-FadA. Neither mFadA nor pre-FadA can bind to E-cadherin alone. Only the complex FadAc formed by mFadA and pre-FadA has binding activity. The surface FadAc presented by Fn has a high affinity to E-cadherin on the surface of CRC cells. The interaction of FadAc and E-cadherin is critical for Fn's attachment and invasion (Rubinstein et al., 2013). The ability of Fn to bind and invade cells is severely impaired by either deletion of FadA of Fn or downregulation of E-cadherin on CRC cells. However, E-cadherin is expressed in various types of cells. The affinity of FadAc for E-cadherin can't fully explain Fn's tropism to CRC. Fap2 and FadA are likely to play a synergistic role in the CRC enrichment of Fn. The binding of Fap2 and Gal-GalNAc allows Fn to selectively localize to CRC. Then, the combination of FadA and E-cadherin strengthens the attachment of Fn to CRC cells. Invasion to CRC cells mediated by FadAc allows Fn to hide within CRC cells, thereby avoiding clearance by the immune system.

6 Inflammation associated tumorigenicity of Fn in mouse model

Inflammation-induced genetic and epigenetic alterations are important aspects of CRC initiation, particularly in colitis-associated CRC. Studies on whether Fn promotes tumorigenesis by inducing inflammation are inconsistent (Table 2). Multiple studies have shown that in wild-type murine model, Fn gavage failed to trigger colitis characterized by increased histological colitis score, colon shortening and upregulation of inflammatory

factors (Brennan et al., 2021; Queen et al., 2022). Besides, Fn was inferior to pks+ Escherichia coli and failed to promote colon inflammatory factors expression and colon tumorigenesis in GF/SPF $Apc^{Min/+}$mice and SPF $Apc^{Min/+}IL10^{-/-}$ mice (Tomkovich et al., 2017; Queen et al., 2022). Differently, other two studies showed that Fn successfully promoted colon tumorigenesis and the expression of a cluster of the inflammatory gene, such as IL6, IL8, COX2, TNF-a, MIP3a(also named CCL20), IL22, IL21, IL17F in SPF $Apc^{Min/+}$ mice (Kostic et al., 2013; Yang et al., 2017). It should be noted that the gavage frequency in the latter two studies was higher than the former two studies(once a day vs. twice a week). Since Fn is difficult to stably colonize the normal gut of mice, high-frequency oral administration may increase the colonization and abundance of Fn in the mucosa. Therefore, we infer that Fn-induced colitis and tumorigenesis may be dose-dependent. Furthermore, these studies were conducted in different places, and the mice lived in different environments. The differences of gut microbiota may cause inconsistence of the research results. Fn-induced inflammation may also require a specific consortium of cross-communicating or synergistic bacteria. Oral feeding of Fn was shown to be tumorigenic in the Azoxymethane(AOM)-Dextran sodium sulfate(DSS) murine model(a colitis-associated CRC model). However, the tumorigenic effect of Fn was abolished without pro-colitis agent DSS, indicating that Fn and inflammation induced by other factors exert a synergistic effect in colon tumorigenesis (Yu et al., 2020).

7 Fn accelerates CRC proliferation and metastasis

Fn infection aggravates the malignancy proliferation of CRC (Figure 1). In CRC cells HCT116, LoVo, HT29 and SW480, incubating with Fn activates TLR4/MYD88/NF-κB signaling to promotes miR-21 transcription (Yang et al., 2017). Upregulated miR-21 inhibits the expression of RASA1, subsequently accelerates cancer cell proliferation *via* activating the MAPK cascade. Except for MAPK, abnormal activation of the Wnt/β-catenin signal is the initiation factor of most CRC (Dekker et al., 2019). Fn adhesin FadAc binding to E-cadherin on the CRC cell membrane leads to E-cadherin phosphorylation and internalization, leading to the cytoplasmic accumulation and nuclear translocation of β-catenin (Rubinstein et al., 2013). Increased nuclear translocation of β-catenin activates the transcription of downstream genes, such as CCND1 and MYC, and promots CRC cell proliferation. Besides, Annexin A1 is an important CRC growth stimulator, with increased expression in CRC cells (Rubinstein et al., 2019). The Fn FadAc up-regulates Annexin A1 through E-cadherin, activating β-catenin signaling, finally accelerating the proliferation of CRC cells (Rubinstein et al., 2019). P21-activated kinase 1 (PAK1) is a member of the PAK family of serine/threonine kinases. Fn and its lipopolysaccharides activate PAK1 through TLR4, and activated PAK1 (p-PAK1) phosphorylates β-catenin at Ser675. β-catenin phosphorylation enhances its transcriptional activity,

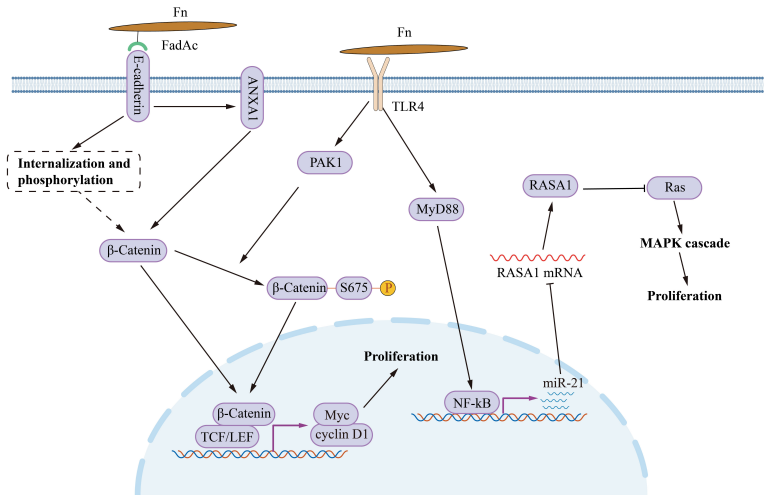

FIGURE 1
The mechanism of Fn promotes CRC cell proliferation. Fn adhesin FadAc binding to E-cadherin on CRC cell membrane leads to E-cadherin phosphorylation and internalization, enhancing the cytoplasmic accumulation and nuclear translocation of β-catenin. FadAc also up-regulates Annexin A1 *via* E-cadherin, activating β-catenin signaling. Fn lipopolysaccharides activate PAK1 through TLR4, and phosphorylates β-catenin at Ser675 by activating PAK1 (p-PAK1). Phosphorylation increases the nuclear accumulate of β-catenin. Nuclear β-catenin activates the transcription of downstream genes, such as CCND1, and MYC, and then promotes CRC cell proliferation. Fn activates TLR4/MYD88/NF-κB signaling to promotes miR-21 transcription. miR-21 activates the MAPK cascade by inhibiting RASA1, subsequently accelerates cancer cell proliferation.

and activates the expression of CCND1 and MYC (Chen et al., 2017).The activation of TLR4/PAK1/β-catenin cascade was also observed in Fn-gavaged $Apc^{Min/+}$ mice (Wu et al., 2018).

Fn promotes metastasis by regulating signaling molecules between CRC cells (Figure 2). Fn's direct adhesion and invasion to CRC cells induces the expression and secretion of cytokines CXCL1 and IL-8, which promote chemotactic migration of non-Fn-exposed CRC cells (Casasanta et al., 2020). Fn outer membrane adhesins Fap2 are critical for the upregulation and secretion of IL-8 and CXCL1. Fn-induced cell migration can be reduced by blocking Fn-host-cell binding and internalization with Fap2 knockout, galactose sugars, l-arginine, or neutralizing antibody. However, exhaustion of the cytokines IL-8 and CXCL1 in the culture medium can only partially reverse CRC migration, indicating that other factors or mechanisms may contribute to CRC cell migration. CCL20 is also involved in Fn-mediated CRC metastasis. Fn upregulates CCL20 by activating NF-κB/miR-1322 signaling, and promotes the migration and metastasis of CRC cells (Xu et al., 2021). Fn infection increases CRC cells exosome release which is rich in miR-1246/27a-3p/92b-3p and CXCL16/IL-8/RhoA (Guo et al., 2020).The exosomes delivers these signal molecules from Fn-infected cells into non-infected cells. Among them, miR-1246/27a-3p/92b-3p suppress GSK3β expression by directly targeting the mRNA 3'-untranslated region, finally activating the Wnt/β-catenin pathway, promoting epithelial-mesenchymal transition and metastasis of CRC cells. CXCL16 in exosomes also plays an important role in promoting the migration of recipient CRC cells through the CXCL16/CXCR6 axis. In summary, Fn-infected CRC cells can transmit information molecules to uninfected CRC cells in two ways, direct secretion and exosomes to promote migration and metastasis.

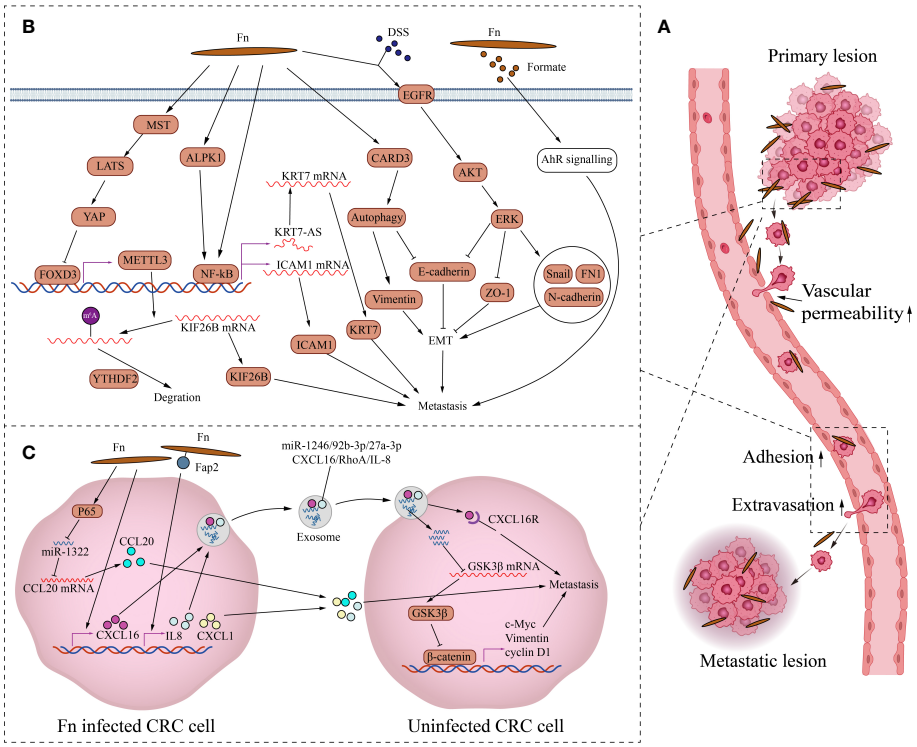

FIGURE 2
The mechanism of Fn promoting CRC metastasis. **(A)** This is the schematic diagram of CRC distant metastasis. Fn exists in primary and metastatic lesions. Fn infection can increase vascular permeability, CRC cells attachment to endothelial cells and extravasation to promote metastasis. **(B)** Fn promotes metastasis by regulating intercellular signals. Fn inhibits transcription factor FOXD3 through activating MST/LATS/YAP axis axis. The downregulation of FOXD3 inhibits the expression of its target gene METTL3, finally increases the level of KIF26B by reducing the YTHDF2-dependent mRNA degradation, to promote metastasis. Fn activates NFKB through ALPK1 or other paths, Activated NFKB promotes CRC metastasis by KRT7-AS/KRT7, and also by increasing ICAM1. Fn induces epithelial mesenchymal transformation of CRC cells to promote metastasis by activating CARD3 mediated autophagy, and also by activating EGFR/AKT/ERK siganaling. Formate, metabolite of Fn promotes the metastatic dissemination by activating the AhR signal. **(C)** Fn promotes metastasis by activating intracellular signals. Fn infection induces the expression and secretion of cytokines CXCL1 and IL-8, which promote metastasis of non-Fn-exposed CRC cells. Fn upregulates CCL20 by activating NF-κB/miR-1322 signaling, and promotes metastasis of CRC. Fn infection increases CRC cells the release of exosome full of miR-1246/27a-3p/92b-3p and CXCL16/IL-8/RhoA to promotes metastasis of other CRC cells.

Fn also promotes CRC metastasis by activating intracellular signaling (Figure 2). As a structural fibrous protein, keratin 7 (KRT7) plays a role in maintaining cell structural integrity and promoting cell motility (Helfand et al., 2004). LncRNA KRT7-AS is a single antisense RNA transcribed from the KRT7 negative strand. Fn infection increases its transcription level of KRT7-AS by activating the NF-κB pathway in CRC cells (Chen et al., 2020). Increased KRT7-AS transcript promotes CRC metastasis by enhancing KRT7 mRNA stability and translation. Besides, Fn infection promotes CRC metastasis by activating the autophagy pathway *via* upregulating the receptor-interacting protein CARD3 (Chen et al., 2020). The upregulation of CARD3 leads to activation of autophagy, characterized by upregulation of LC3-II, Beclin1, downregulation of P62, and increased formation of autophagosomes. The upregulation of CARD3 also leads to elevated epithelial-mesenchymal transition (EMT) and metastasis ability of CRC cells characterized by upregulation of Vimentin and downregulation of E-cadherin. And these effects are weakened by autophagy inhibitor chloroquine. DSS is a well-known colitis-inducing agent. DSS and Fn co-treatment increased the motility and EMT characteristics of CRC cells compared with Fn infection alone (Yu et al., 2020). The potential mechanism is that Fn and DSS stimulate EMT through epidermal growth factor receptor (EGFR) activation. The co-treatment of CRC cells with Fn and DSS increases the phosphorylated levels of EGFR, inducing the activation of downstream effector kinases, including protein kinase B (AKT) and extracellular signal-regulated kinase (ERK), finally leading to the upregulation of EMT transcription factor Snail and mesenchymal markers fibronectin and N-cadherin, the downregulation of epithelial markers E-cadherin and ZO-1. Fn also promotes tumor formation and the expression of EMT markers in the AOM-DSS murine model. In addition, N6-methyladenosine (m6A), an important epitranscriptome modification, affects various fundamental biological processes by regulating mRNA (Patil et al., 2018). By inhibiting the Hippo pathway, Fn treatment activates YAP signaling in CRC cells (Chen et al., 2022). YAP nuclear translocation suppresses the expression of the downstream target gene METTL3 by inhibiting the transcription of FOXD3,which is the transcription factor of METTL3. Downregulation of METTL3, a major m6A methyltransferase, reduces the YTHDF2-dependent KIF26B mRNA degradation and promotes KIF26B expression by reducing the m6A modification of KIF26B mRNA, ultimately promoting CRC metastasis. To spread to other sites, tumor cells need to undergo a process of infiltration, adhesion, and extravasation (Reymond et al., 2013). The metastatic spread is an inefficient process because only a small fraction of cancer cells in the primary tumor have the potential to form metastases. Even if a patient has cancer cells in their blood, distant metastases may not necessarily occur. Adhesion of cancer cells to endothelial cells and transendothelial invasion of cancer cells are critical steps in the metastatic process. Intercellular adhesion molecule 1 (ICAM1) is a transmembrane protein that participates in direct interactions between cells, including cancer cell and vascular endothelial cell (Wai Wong et al., 2012). Fn upregulates ICAM1 to promote CRC cells attachment to endothelial cells and extravasation by activating the alpha-kinase 1(ALPK1)/NF-κB pathway, ultimately facilitating the distant metastasis of CRC (Zhang et al., 2022). In addition, Fn-derived metabolite formate drives CRC cells' metastatic dissemination by triggering aryl hydrocarbon receptor (AhR) signaling (Ternes et al., 2022).

Tumor metastasis is also related to the destruction of the vascular endothelial barrier. Fn adhesion to endothelial cells requires the affinity between Vascular endothelial-cadherin (VE-cadherin) and FadA on Fn. Fn adhesion to endothelial cells causes VE-cadherin to be repositioned away from cell-cell junctions, increasing permeability between endothelial cells and allowing bacteria to pass through loosened junctions (Fardini et al., 2011). Fn infection also reduces CD31 expression in endothelial cells, implying decreased cell-cell contact and increased vascular permeability (Mendes et al., 2016). There is no doubt that the destruction of the vascular endothelial barrier not only contributes to the invasion of Fn but also contributes to the hematogenous metastasis of cancer cells. Overall, increased vascular endothelial permeability, enhanced attachment of cancer cell to vascular endothelium, and enhanced epithelial-mesenchymal transition and motility of tumor cells by Fn jointly promote the metastasis of CRC cells.

8 Fn enhances stemness of CRC cell

Cancer stem cells (CSCs), a subset of tumor cells, are considered to be responsible for tumor initiation, progression, resistance to chemotherapy, and recurrence (Ishizawa et al., 2010; Nassar and Blanpain, 2016). Compared with other tumor cells, CSCs have the following characteristics: unlimited self-renewal ability, stronger spheroidization ability *in vitro* and tumorigenic ability and reconstruction of tumor heterogeneity *in vivo*, high expression of stem cell marker, epithelial-mesenchymal transition, radiotherapy and chemotherapy resistance (Batlle and Clevers, 2017). Both CSCs and non-CSCs are plastic. CSCs can undergo a phenotypic transition in response to appropriate stimuli. In 2019, we proposed the conjecture that Fn may promote CRC drug resistance by increasing the proportion of CSCs in CRC (Luo et al., 2019). Consistent with our hypothesis, studies suggested that Fn infection increased the expression of stem cell marker CD44 and CD133, and the ability of spheroid formation in CRC cells (Yu et al., 2020; Wang et al., 2020).The research by Liu et al. not only confirmed the Fn-induced transformation of the stem cell-like phenotype of CRC cells but also further elucidated the mechanism (Liu et al., 2022). Numb is a cell fate determinant

that promotes CSCs differentiation by inhibiting Notch signaling, thus regarded as a suppressor in various cancers (Colaluca et al., 2008). In colorectal cancer stem cells(CCSCs), Fn reduces lipid accumulation by promoting fatty acid oxidation and provides energy for CCSCs self-renewal and proliferation. On the other hand, Fn increases lipid droplet in non-CCSCs by promoting fatty acid and triglyceride synthesis. Accumulated lipid droplets activate Notch signaling by recruiting Numb and E3 ubiquitin-protein ligase MDM2 to promote the ubiquitinated degradation of Numb. Fn infection inhibits CCSCs differentiation, and allows non-CCSCs to acquire stemness features. In addition to Fn itself, its metabolite formate can also induce stemness in CRC cells. Treatment of mice bearing CRC xenografts with formate increases the CSC markers Aldehyde dehydrogenase(ALDH), CD44, and octamer-binding transcription factor 4(OCT4) expression in tumors (Ternes et al., 2022). The underlying mechanism could be that Fn-derived formate activates the aryl hydrocarbon receptor signaling, because the aryl hydrocarbon receptor is involved in regulating the stemness of cancer cell (Stanford et al., 2016).

9 Fn regulates tumor metabolic reprogramming

Metabolic disorder is one of the hallmarks of cancer (Hanahan and Weinberg, 2011). Normal cells use glucose for energy production mainly through the oxidative phosphorylation pathway in the presence of sufficient oxygen. Unlike normal cells, cancer cells process glucose primarily through glycolysis. It is well known that adenosine triphosphate (ATP) production by oxidative phosphorylation is 18 times more than glycolysis. This is not a wise choice from an energy production efficiency standpoint. Cancer cells not only require a lot of energy but also need to synthesize many substances to generate new cells, because cancer cells are constantly proliferating. Increased glycolysis allows more glycolytic intermediates to be diverted into various biosynthetic pathways, including nucleotides, amino acids, and lipids (Vander Heiden et al., 2009). Fn promotes glycolysis in CRC cells by upregulating a key glycolytic enzyme Enolase 1 (ENO1) (Hong et al., 2020). Fn activates transcription of lncRNA ENO1-IT1 by upregulating transcription factor SP1. Elevated ENO1-IT1 directs the histone acetyltransferase KAT7 to its target gene ENO1, increasing the level of acetylation of histone H3 lysine 27(H3K27Ac) in the promoter region. H3K27Ac modification in the promoter region of ENO1 enhanced the transcriptional activity of ENO1 and consequently accelerates the glycolysis and proliferation of CRC cells. In addition, Fn also promotes glucose uptake and glycolytic activity in CRC cells by up-regulating angiopoietin-like 4(ANGPTL4) (Zheng et al., 2021). Fn infection up-regulated the transcription factor HIF1a level, and also enhanced the H3K27Ac level of the HIF1a-binding site in the ANGPTL4 promoter region by down-regulating histone deacetylases. Promoter region H3K27Ac and upregulation of transcription factors synergistically promote ANGPTL4 transcription and expression. The up-regulation of ANGPTL4 not only promotes glycolysis but also promotes glucose uptake in CRC cells by upregulating the glucose transporter GLUT1. Interestingly, the upregulation of ANGPTL4, which is induced by Fn, promotes Fn intracellular colonization in CRC cells.

Fn regulates lipid metabolism in CRC. Cytochrome P450 2J2 (CYP2J2), a member of the cytochrome P450 superfamily of enzymes, can metabolize linoleic acid to 9,10-epoxyoctadecaenoic acids(9,10-EpOME) and 12,13-epoxyoctadecaenoic acids (12,13-EpOME). EpOMEs have been reported to have cancer-promoting activity in a mouse model of CRC (Wang et al., 2019). CYP2J2 has also been found to be upregulated in a variety of cancers and associated with drug resistance (Karkhanis et al., 2017). Fn infection upregulates CYP2J2 expression in CRC cells *via* TLR4/AKT/Keap1/NRF2 signaling (Kong et al., 2021). NRF2 is a transcription factor of CYP2J2 and promotes its expression. Elevated CYP2J2 increases the production of 12,13-EpOME, which finally accelerates the EMT, metastasis, and development of CRC.

10 Fn interacts with tumor immune microenvironment

The components of tumor microenvironment (TME), such as immune cells, fibroblasts, signaling molecules, extracellular matrix, and blood vessels, constantly interact with the tumor, affecting its growth and evolution. Fn, as the overabundant intratumoral bacteria in CRC, cross-communicate with the tumor microenvironment. We focus on the crosstalk mechanism of Fn and immune cells (Figure 3). In the ApcMin/+ mouse model, Oral feeding of Fn promoted colon tumorigenesis and altered the intratumoral immune microenvironment. Although CD3+CD4+and CD3+CD8+T lymphocytes were not significantly affected, CD11b+ myeloid immune cells in the colon tumor were expanded by Fn. Tumor-associated macrophages(TAMs,especially M2), myeloid-derived suppressor cells(MDSCs), tumor-associated neutrophils, and dendritic cells were enriched in colon tumors of Fn-fed mice (Kostic et al., 2013). Consistently, the presence of Fn was also associated with a higher density of MDSCs in liver metastases of human CRC (Sakamoto et al., 2021). As we know, these components of the tumor immune microenvironment are closely related to tumor progression.

10.1 Fn and monocyte/macrophage polarization

Macrophages, as one of the most abundant infiltrating leukocytes in TME, play an important role in cancer

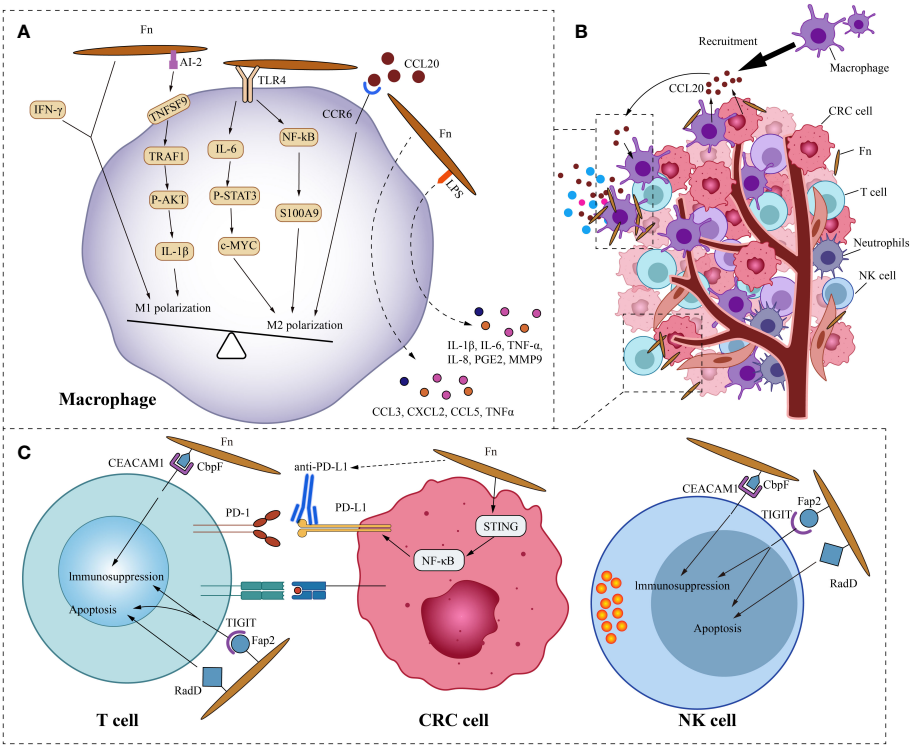

FIGURE 3
Fn interacts with CRC immune microenvironment. **(A)** This is the schematic diagram of communication of Fn and CRC immune microenvironment. **(B)** In general, Fn induces macrophages to M2 polarization. Fn promotes macrophages M2 polarization by activating TLR4/IL6/p-STAT3/c-Myc and NFKB/S100A9 signals. But some components such as AI-2 of Fn and IFN-γ induced by Fn mediate macrophages MI polarization. CRC cells and macrophages derived CCL20 induced by Fn infection also promotes macrophages M2 polarization. Other molecules derived from macrophages induced by Fn may also have important effects in the immune microenvironment, such as IL-6, IL-1β, TNF-a, IL-8, PGE2 and MMP9. **(C)** Fn binds to the inhibitory receptors TIGIT and CEACAM1 on T cells and NK cells through Fap2 and CbpF, respectively, to mediate their immunosuppression and apoptosis. Fn upregulates the expression of PD-L1 in CRC cells by activating the STING/NFKB axis, and enhances the response of CRC to PD-L1 therapy.

progression by stimulating proliferation, metastasis, and angiogenesis while creating an immunosuppressive environment (Cendrowicz et al., 2021). Two retrospective studies from Korea showed that intratumoral Fn was positively correlated with macrophage infiltration and M2 polarization in tumors (Park et al., 2017; Lee et al., 2021). This is consistent with the study by Kostic et al (Kostic et al., 2013). The study by Ye et al. found that Fn and the cytokine C-C motif chemokine ligand 20 (CCL20) were significantly increased in CRC compared with normal mucosa (Ye et al., 2017). This implies a potential link between Fn and CCL20. Their further study found that co-culture with Fn induces CCL20 expression in CRC cells and monocytes. They also observed that Fn-induced monocyte migration was partially inhibited by CCL20 neutralizing antibody, suggesting that CCL20 signal is involved in the regulation of monocyte/macrophage motility. Consistent with the findings of Ye, the research by Xu et al. showed that Fn infection stimulated the expression of CCL20 in CRC cells. Moreover, CRC cell-derived CCL20 promoted macrophage migration and M2 polarization *in vitro*, and also promoted intratumoral infiltration of macrophages *in vivo* (Xu et al., 2021). Macrophages are extremely plastic cells that may modify their function dramatically based on the microenvironmental signals in the TME. Based on their diverse functions, macrophages are divided into two opposite subtypes: classically activated macrophages (M1, a pro-inflammation subtype) and alternatively activated macrophages (M2, an anti-inflammation and tumor-promotion subtype) (Wu et al., 2019). Fn not only induces the recruitment of macrophages into the tumor but also regulates the polarization of macrophages. The ability of Fn to induce M2 polarization of macrophages is stronger than *E. coli*. Fn stimulates S100A9 expression *via* TLR4/NF-kB signaling in CRC cells and macrophages (Hu et al., 2021). S100A9 may activate macrophages and M2 polarization in an autocrine or paracrine manner, ultimately promoting the progress of CRC. Similarly, the study by Chen et al. showed that Fn induced macrophage M2 polarization *via* a TLR4/IL-6/p-STAT3/c-MYC cascade (Chen et al., 2018).

However, Fn doesn't always induce macrophage M2 polarization. Liu and colleagues found that in a mouse model of DSS-induced enteritis, Fn increased the infiltration of M1 macrophages in colon tissue (Liu et al., 2019). Further study of the mechanism found that under the co-stimulation of Fn and IFN-γ, Fn can induce the M1 polarization of mouse bone marrow-derived macrophages, but Fn alone can't stimulate it. Sakamoto and colleagues found that in the liver metastases of human CRC, the presence of Fn was not significantly correlated with M2 macrophages (Sakamoto et al., 2021). Fn induces robust CCL3, CXCL2, CCL5, and TNFα secretion in mouse macrophages *in vitro* (Casasanta et al., 2020). Unlike Fn-induced CRC cell inflammatory factor secretion, the cytokine secretion of these macrophages does not depend on Fap2 of Fn. CCL3 stimulates lymphocyte recruitment, while CXCL2 promotes angiogenesis in CRC. Grenier et al. found that the lipopolysaccharide of Fn significantly increased the secretion of pro-inflammatory cytokines IL-6, IL-1β, TNF-a, IL-8, and PGE2 in macrophages (Grenier and Grignon, 2006). Among them, the increase of IL-8 was the most significant. The lipopolysaccharide derived from Fn also promoted the secretion of MMP9 in macrophages, which may play a role in promoting tumor cell invasion and metastasis. Therefore, we speculate that the influence of Fn on the polarization of macrophages depends on the microenvironment, where the macrophages are located. In addition, not all components of Fn induce macrophage M2 polarization. Autoinducer-2 (AI-2) is a non-species-specific autoinducer that is involved in interspecies communication and is a main signal-type molecule of the quorum-sensing system in bacteria (Thompson et al., 2015). Wu and his colleagues found that AI-2 from Fn promoted macrophage M1 polarization *via* a mechanism involving TNFSF9/TRAF1/p-AKT/IL-1β signaling (Wu et al., 2019).

10.2 Fn and lymphocyte immunosuppression

A cross-sectional study of 598 CRC cases found that the amount of tissue Fn was inversely related to the density of CD3+ T cells in CRC tissue, but not to the density of FOXP3+ or CD8+ T cells (Mima et al., 2015). This is consistent with the research on murine model by Kostic et al (Kostic et al., 2013). Another cross-sectional study based on 933 CRC cases in two US-wide prospective cohort studies, showed an inverse association of Fn with the densities of CD3+ T cells and CD3+ CD4+ CD45RO+ memory helper T cells in CRC (Borowsky et al., 2021). Similarly, Fn was observed to associate with a lower density of CD8+ T cells in human CRC liver metastases (Sakamoto et al., 2021). Several studies have shown that Fn has immunosuppressive activity in lymphocytes. On the one hand, Fn can induce apoptosis of Jurkat T-cells (Jewett et al., 2000). On the other hand, Fn suppresses human T-cell activation by stopping cells in the mid-G1 phase of the cell cycle (Shenker and Datar, 1995). Additionally, Fn can inhibit human T-cell response to mitogens and antigens (Shenker and DiRienzo, 1984). Fap2 and RadD are large proteins localized to the outer membrane of Fn. The lymphocyte death induced by Fn mainly depends on Fap2 and RadD (Kaplan et al., 2010). Incubating lymphocytes with membranes carrying the Fap2 or RadD mutations resulted in partial decreases in cell death of 51% and 27%, respectively, whereas incubating lymphocytes with membranes containing the Fap2 and RadD double mutations led in a 77% reduction in cell death. Fn membranes containing Fap2 and RadD trigger cell death in lymphocytes at levels similar to incubation with whole cells, suggesting that the lethal action of Fn on lymphocytes is not energy-dependent. Natural killer cells (NK cells) are a type of cytotoxic lymphocyte that plays an important role in the body's innate immune system. Tumors, viruses, parasites, and bacteria can be killed by NK cells directly or indirectly (Koch et al., 2013). T cell immunoglobulin and ITIM domain(TIGIT) is an inhibitory receptor present on human NK cells as well as a variety of T cells. The Fap2 protein on Fn interacts directly with TIGIT, inhibiting cytotoxicity of NK cells and T cells, weakening the killing effect on cancer cells (Gur et al., 2015). In addition, Fn also binds and activates the human inhibitory receptor CEA cell adhesion molecule 1(CEACAM1), thereby inhibiting the activity of T and NK cells (Gur et al., 2019). The trimeric autotransporter adhesin CbpF of Fn has been reported to bind specifically to CEACAM1 and mediate the inhibition of T Cell Function (Brewer et al., 2019; Galaski et al., 2021). Consistent with the result above, a study by Kim et al. showed that Fn inhibited the viability of NK cells *in vitro*, and reduced NK cell density in mouse intestinal tissues (Kim et al., 2021).

11 Fn affects CRC response to therapy

The 5-year survival rate in advanced CRC patients is less than 10% (Dahan et al., 2009). Chemotherapy is one of the effective treatments for patients with advanced CRC. Although most CRC patients initially respond to chemotherapy,cancer progression eventually occurs due to the drug resistance. Fn, as the intratumoral bacteria in CRC, is one of the driving factors of CRC resistance to chemotherapy. Autophagy can assist cells to cope with intracellular and external stressors such as hypoxia, nutritional deficiency, or cancer therapy, eventually promoting cancer development (Amaravadi et al., 2019). Fn promotes CRC chemoresistance by influencing autophagy (Yu et al., 2017). Fn targets TLR4/MYD88 signaling, leading to downregulation of miR-4802 and miR-18a. The loss of miR-18a and miR-4802 result in increased ULK1 and ATG7 gene expression, subsequently enhancing the activity of Autophagy, leading to CRC cells' chemoresistance to Oxaliplatin and 5-FU. Baculoviral

IAP repeat containing 3(BIRC3), as an inhibitor of apoptosis protein family member, mediates cell apoptosis resistance by inhibiting caspase cascade (Bertrand et al., 2008). Fn infection upregulates BIRC3 by activating TLR4/NF-κB pathway (Zhang et al., 2019). The NF-κB P65 enhances BIRC3 gene transcription by binding to its upstream promoter region. The upregulation of BIRC3 induced by Fn confers CRC cells chemoresistance to 5-Fu.

Immune checkpoint therapy activates antitumor immune response by blocking interactions between T cell inhibitory receptors and their cognate ligands, resulting in durable tumor regression. In a subset of patients, drugs of blocking immune checkpoints, such as programmed cell death protein 1 (PD-1) and its ligand PD-L1, have shown significant efficacy. However, anti-PD-1/PD-L1 therapy is deemed ineffective for the majority of CRC patients, and only those with high MSI-H and a high mutation burden respond to the treatment (Kreidieh et al., 2020). The presence of Fn in CRC tissue may be a double-edged sword. On the one hand, infection of high dose Fn can induce PD-L1 expression in CRC cells by activating the stimulator of interferon response cGAMP interactor 1 (STING) signaling. It is well known that PD-L1 mediates the immune escape of cancer cells. On the other hand, due to the upregulation of PD-L1, CRC is more sensitive to immune checkpoint blockade therapy. The study based on 41 CRC patients undergoing PD-1 blockade therapy, found that patients with Fn positive CRC had longer progression-free survival than Fn negative CRC. Experiments based on organoid and mouse subcutaneous homograft tumor model showed that Fn infection enhances the antitumor effect of PD-L1 blockade (Gao et al., 2021). This may provide us with a new perspective: whether Fn levels in CRC tissue can be used as an indicator for screening patients who potentially respond to immune checkpoint therapy. In addition, a study based on 1041 CRC patients shows that the presence of Fn was inversely associated with tumor-infiltrating lymphocytes(TIL) in MSI-H tumors, but positively correlated with TIL in non-MSI-H CRC (Hamada et al., 2018). We need further studies to determine whether the effect of Fn on lymphocytes depends on MSI status in CRC. If Fn can increase lymphocyte recruitment in patients with non-MSI-H CRC (account for nearly 85% of all CRC patients) for immune therapy.

12 Strategies for CRC prevention and treatment by anti-Fn

Given the deleterious effects of Fn in tumors of CRC patients, we still lack effective countermeasures. we should focus on how to accurately eliminate Fn and its adverse effects. At present, in this field, researchers have carried out various tentative studies (Figure 4).

12.1 Prevention of Fn infection and invasion

It is well known that we have successfully prevented Mycobacterium tuberculosis infection in humans by vaccination with Bacillus Calmette-Guérin (BCG). Similarly, can we prevent the invasion of Fn into the human body by vaccination? Guo et al. have found that the Fn-AhpC recombinant protein could be specifically recognized by antibodies present in the serum of CRC patients. Systemic prophylactic immunization with AhpC/alum significantly protected 77.3% of mice from infection (Guo et al., 2017). Such results are encouraging. Fn-derived proteins such as AhpC may serve as a potential vaccine candidate against Fn inhabitation or infection in the gut, which is of great significance for the prevention of CRC associated with Fn infection. Whether there is other antigen in Fn that is more suitable to be used as a vaccine still needs more exploration and verification by scientific researchers.

12.2 Inhibition/Elimination of intratumoral Fn

Bullman and colleagues treated mice bearing colon cancer xenografts with the antibiotic metronidazole and found that metronidazole treatment reduced intratumoral Fn burden and overall tumor growth significantly (Bullman et al., 2017). Although antibiotics such as metronidazole have a significant killing effect on Fn, they can also cause intestinal flora disturbance, affecting the colonization and activity of some probiotics. Phages can infect and lyse bacteria, replicate and degrade biofilm substrates, and are promising drugs for the treatment of bacterial infections (Agarwal et al., 2018). Since the attack mechanism of phages is highly specific to bacterial species, phage therapy should be particularly suitable for the accurate removal of tumor-promoting bacteria (Servick, 2016). Zheng et al. isolated a phage strain from human saliva that could specifically destroy Fn. They constructed a phage-guided biotic–abiotic hybrid nanosystem by using irinotecan(a first-line drug against CRC), dextran nanoparticles, and phages. This nanosystem exhibits powerful advantages, not only eliminating intratumoral Fn, reversing Fn-induced chemoresistance and enhancing chemoresponse, but also possessing remarkable tumor-specific targeting ability, thereby reducing the toxic and side effects of chemotherapeutic drugs. In addition, the system enhanced the proliferation of the anticancer Clostridium butyricum. Silver nanoparticles (AgNPs) exhibit excellent antibacterial activity but lack bacterial specificity. Dong et al. electrostatically assembled AgNPs on the surface of a specifically Fn-binding M13 phage (Dong et al., 2020). AgNPs were precisely directed to Fn in the tumor microenvironment due to the ability of phages to target Fn. AgNPs and M13 phage synergistically remove intratumoral Fn

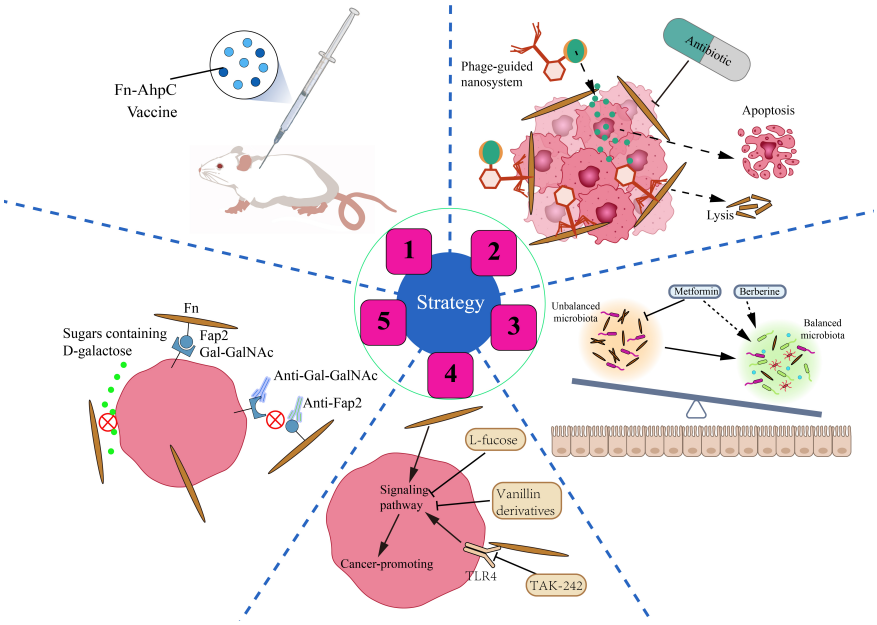

FIGURE 4
Strategies for CRC prevention and treatment by anti-Fn. 1: Prevention of Fn infection. Fn derived vaccine enhances the immunity of mice to Fn infection. 2: Inhibition/Elimination of intratumoral Fn. Antibiotics can significantly reduce the intratumoral Fn load. Phage guided nano system with drug can kill two birds with one stone in CRC. 3: Rebalance of gut microbiota. Some non-antibiotic drugs such as berberine and metformin can not only inhibit Fn but also improve gut flora. 4: Reverse Fn-activated signaling. A small part of compounds and molecular inhibitors such as L-fucose, vanillin derivatives, TAK-242 can selectively inhibit the Fn activated oncogenic signaling pathway. 5: Blockade of Fn's cell adhesion and internalization. Antibodies such as antibodies targeting Gal-GalNAc or Fap2 and sugars such as D-galactose can block the adhesion of Fn to CRC cells, and eliminate the malignant effect of Fn from the source.

and reverse immunosuppressive TME, characterized by increased maturation of dendritic cells, M1 polarization of macrophages, and enhanced anti-tumor effect of T cells. Experiments in mouse allograft tumor models showed that it inhibited tumor growth and enhanced the therapeutic response to FOLFIRI chemotherapy and anti-PD-1 therapy.

12.3 Rebalance of gut microbiota

Fn gavage changes the structure of the intestinal microflora in mice and also promotes tumorigenesis (Yu et al., 2015). Fn may disturb the balance of gut microbiota by increasing opportunistic pathogens and reducing probiotics. Therefore, the disordered gut microbiota and the enrichment of Fn in the intestinal lumen and tumor tissue of patients with CRC may be mutually causal. Berberine, an isoquinoline alkaloid, is the pharmacological component of the Chinese herbal medicine Coptis Rhizoma, which has been used in China for thousands of years to treat intestinal infections, especially bacterial diarrhea (Tang et al., 2009). Berberine not only reverses the microbiota imbalance induced by Fn gavage but also downregulates mucosal immune cytokine secretion and activation of JAK/STAT and MAPK/ERK pathways (Yu et al., 2015). This implies the potential value of berberine in CRC treatment, especially in people with high levels of Fn. A study published in 2018 screened more than 1,000 non-antibiotic drugs for their effects on gut bacteria and found that metformin significantly inhibited Fn at colonic concentrations of about 1.5 mM (Maier et al., 2018). Given this, Huang et al. conducted a study and found that metformin reduced the Fn abundance, alleviated symptoms caused by Fn, and rescued Fn-induced tumorigenicity in $APC^{Min/+}$ mice (Huang et al., 2020). Drugs such as metformin, which both inhibit specific pathogens and regulate intestinal flora disturbances, may maximize the benefits of treatment, especially in those with CRC who have diabetes. Another advantage that cannot be ignored is that these drugs have been used in clinics, and their side effects and adverse reactions have been mastered by people.

12.4 Reverse Fn-activated signaling

The fourth strategy is to target Fn-activated signaling pathways and reverse Fn-induced malignant transformation. L-fucose is a naturally occurring monosaccharide found in food or the body. Duan et al. revealed that L-fucose reversed the tumor-promoting properties of Fn by inhibiting its ability to

activate jak-stat3 signaling and EMT in colon cancer cells *in vitro* (Duan et al., 2021). Despite the lack of support from *in vivo* research data and rigorous validation of the mechanism, this finding is still eye-catching. Zhou et al. found that vanillin derivatives IPM711 and IPM712 reversed Fn-induced proliferation and migration of CRC by inhibiting the activation of the E-Cadherin/β-Catenin pathway (Zhou et al., 2022). IPM712 has been reported to show better anticancer activity than 5-Fu with low toxicity at therapeutic concentrations (Ma et al., 2020). TLR4 is a key receptor that mediates the tumor-promoting effect of Fn. Therefore, TLR4 inhibitor TAK-242 is a potential effective treatment drug for Fn-infected CRC patients (Chen et al., 2017; Chen et al., 2018).

12.5 Blockade of Fn's adhesion and internalization for CRC cell

The promoting effect of Fn on the malignant behavior of CRC cells is mainly based on the high adhesion and invasiveness of Fn to CRC. As described in the previous, the molecular basis of Fn adhesion and invasion is the specific binding of lectin Fap2 and adhesins FadAc to Gal-GalNAc and E-cadherin respectively. We speculate that it is promising to develop antibodies targeting Gal-GalNAc or Fap2 to selectively block Gal-GalNAc/Fap2 binding, because Gal-GalNAc is highly expressed in CRC cells and low or not expressed in non-CRC cells. Furthermore, it has been reported that sugars containing D-galactose can inhibit Fn adhesion and invasion of CRC cells significantly (Abed et al., 2016). However, whether oral administration of sugars containing D-galactose reverses the cancer-promoting effect of Fn remains to be further investigated.

13 Conclusion and future direction

In the past ten years, from the appearance to the cause and mechanism, the understanding of Fn as a "driver" of CRC development has gradually become clear. However, the role and mechanism of Fn in the initial stage of CRC remain unknown for us. CRC includes various pathological subtypes and molecular subtypes. To our knowledge, different CRC cell lines have different responses to Fn infection. The question that the malignant biological behavior of which subtype of CRC is aggravated most obviously by Fn infection deserves further study. It will help us accurately identify which subgroup of patients in risk needs to be intervened if Fn is present in their tumors. In addition, we need to determine the threshold value of bacterial load in tumor, beyond which, biological intervention is required for patients. As the previous studies have confirmed that the promoting effect of low-dose Fn infection on the malignant biological behavior of CRC cells is weak. If we use drugs to interfere with them excessively, the harmful side effects may be far greater than the therapeutic effects.

Previous studies on the effect of Fn in the tumor immune microenvironment mainly involve monocytes, macrophages, T cells and NK cells. How other components of the tumor microenvironment, such as tumor-associated fibroblasts, MDSCs, and dendritic cells, respond to Fn infection remain covered. The effect of Fn on macrophage polarization is still controversial. Whether macrophages are polarized to cancer-promoting or cancer-suppressing direction may depend not only on Fn, but also on the components of microenvironment. The current mechanism studies on Fn and TAM are based on animal cells or animal models or human leukemia cell models, which are far from the real state of TAM in human CRC. In addition, most of the current studies on Fn and the tumor immune microenvironment are binary studies, and we should consider their responses to Fn invasion as a whole. CRC contains multiple subtypes. They have different immune cell infiltration profiles. Previous studies on Fn and tumor immunity ignored the molecular subtypes of CRC. We need to determine whether the effects of Fn on immune cells are consistent among various CRC subtypes. This will provide reference for the determination of clinical immunotherapy strategy.

We have already uncovered part of mechanisms of Fn in CRC exacerbation. Next, we should pay more attention to translating these mechanisms into clinical applications. In terms of disease control strategies, prevention is better than treatment. As a prevention method, immunization with antigen derived-from Fn deserves further exploration. Since Fn activates multiple cancer promoting signal pathways, we advise researchers to try to use small molecule inhibitors to reverse the harmful effects of Fn infection on CRC. Besides, Nano-systems coupled with chemotherapeutics are very promising, as not only enhance the tumor-targeting ability of chemotherapy but also reverse Fn-induced resistance and immunosuppression. In view of the immunosuppressive effect of Fn on lymphocytes, we believe that TIGIT and PD-L1 dual immune checkpoint blockade therapy may have better efficacy in patients with high intratumoral Fn levels. In addition, we should also consider introducing probiotics to antagonize Fn and activate anti-tumor immune responses, such as Lactobacillus rhamnosus and Bifidobacterium Breve (Yoon et al., 2021; Si et al., 2022).

Author contributions

All authors contributed to the article and approved the submitted version. SO wrote the manuscript draft. HW, YT were responsible for the literature search. SO and SR drew the figures, JY and KL were responsible for language polishing. ZG, YW and HH contributed to furtherly editing the manuscript. RH provided direction and instruction and revised the manuscript.

Funding

This work was supported by National Natural Science Foundation of China under Gant [No. 81872034], the Natural Science Foundation of Heilongjiang Province under Gant [No. H2017016], Wu Jieping Medical Foundation under Gant [No. 320.6750.19092-41], Chen Xiao-ping Foundation for The Development of Science and Technology of Hubei Province under Gant [No. CXPJJH12000002-2020025].

Acknowledgments

Thanks to Yifan Mu, School of Architecture, Harbin Institute of Technology, Harbin, China, for her assistance in pictures.

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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Glossary

CRC	Colorectal cancer
Fn	Fusobacterium nucleatum
APC	Adenomatous polyposis coli
MSI	Microsatellite instability
CMS	Consensus molecular subtypes
SPF	Specific-pathogen-free
GF	Germ-free
Gal-GalNAc	D-galactose-b(1-3)-N-acetyl-D-galactosamine
IL6	Interleukin 6
IL8	Interleukin 8;
COX2	Cytochrome c oxidase subunit II
TNF-a	Tumor necrosis factor a
MIP3a	Macrophage Inflammatory Protein-3
CCL20	C-C motif chemokine ligand 20
IL22	Interleukin 22
IL21	Interleukin 21
IL17F	Interleukin 17F
AOM	Azoxymethane
DSS	Dextran sodium sulfate
RASA1	RAS p21 protein activator 1
MAPK	Mitogen-activated protein kinase
PAK1	P21-activated kinase 1
CXCL1	C-X-C motif chemokine ligand 1
CXCL16	C-X-C motif chemokine ligand 16
CXCR6	C-X-Cmotif chemokine receptor 6
KRT7	Keratin 7
CARD3	Caspase activation and recruitment domain 3
EMT	Epithelialmesenchymaltransition
METTL3	N6-adenosinemethyltransferase complex catalytic subunit
ICAM1	Intercellular adhesion molecule 1
AhR	Aryl hydrocarbon receptor
CSC	Cancer stem cell
MDM2	Mouse double minute 2 homolog
ALDH	Aldehyde dehydrogenase
OCT4	Octamer-binding transcription factor 4
ATP	Adenosine triphosphate
ENO1	Enolase 1
H3K27Ac	Acetylation of histone H3 lysine 27
ANGPTL4	Angiopoietin-like 4
CYP2J2	Cytochrome P450 2J2
EpOME	Epoxyoctadecaenoic acids
TME	Tumor microenvironment
TAMs	Tumor-associated macrophages
MDSCs	Myeloid-derived suppressor cells;
CCL3	C-C motif chemokine ligand 3
CXCL2	C-X-C motif chemokine ligand 2
CCL5	C-C motif chemokine ligand 5
MMP9	Matrix metallopeptidase 9
FOXP3	Forkhead box P3
CEACAM1	CEA cell adhesion molecule 1
TIGIT	T cell immunoglobulin and ITIM domain
PD-1	Programmed cell death protein 1
PD-L1	PD-1 ligand 1

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 09 January 2023
DOI 10.3389/fcimb.2022.1064737

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Intestinal Microbiome, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 08 October 2022
ACCEPTED 05 December 2022
PUBLISHED 09 January 2023

CITATION
Gai X, Qian P, Guo B, Zheng Y, Fu Z, Yang D, Zhu C, Cao Y, Niu J, Ling J, Zhao J, Shi H and Liu G (2023) Heptadecanoic acid and pentadecanoic acid crosstalk with fecal-derived gut microbiota are potential non-invasive biomarkers for chronic atrophic gastritis. *Front. Cell. Infect. Microbiol.* 12:1064737. doi: 10.3389/fcimb.2022.1064737

Heptadecanoic acid and pentadecanoic acid crosstalk with fecal-derived gut microbiota are potential non-invasive biomarkers for chronic atrophic gastritis

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Background: Chronic atrophic gastritis (CAG), premalignant lesions of gastric cancer (GC), greatly increases the risk of GC. Gastroscopy with tissue biopsy is the most commonly used technology for CAG diagnosis. However, due to the invasive nature, both ordinary gastroscope and painless gastroscope result in a certain degree of injury to the esophagus as well as inducing psychological pressure on patients. In addition, patients need fast for at least half a day and take laxatives.

Methods: In this study, fecal metabolites and microbiota profiles were detected by metabolomics and 16S rRNA V4-V5 region sequencing.

Results: Alteration of fecal metabolites and microbiota profiles was found in CAG patients, compared with healthy volunteers. To identify the most relevant features, 7 fecal metabolites and 4 microbiota were selected by random forest (RF), from A and B sample sets, respectively. Furthermore, we constructed support vector machines (SVM) classification model using 7 fecal metabolites or 4 gut microbes, or 7 fecal metabolites with 4 gut microbes, respectively, on C sample set. The accuracy of classification model was 0.714, 0.857, 0.857, respectively, and the AUC was 0.71, 0.88, 0.9, respectively. In C sample set, Spearman's rank correlation analysis demonstrated heptadecanoic acid and pentadecanoic acid were significantly negatively correlated to *Erysipelotrichaceae_UCG-003* and *Haemophilus*, respectively. We constructed SVM classification model using 2 correlated fecal

metabolites and 2 correlated gut microbes on C sample set. The accuracy of classification model was 0.857, and the AUC was 0.88.

Conclusion: Therefore, heptadecanoic acid and pentadecanoic acid, crosstalk with fecal-derived gut microbiota namely Erysipelotrichaceae_UCG-003 and Haemophilus, are potential non-invasive biomarkers for CAG diagnosis.

KEYWORDS

chronic atrophic gastritis, gut microbiota, metabonomics, random forest, support vector machine

Introduction

Chronic atrophic gastritis (CAG) is the final consequence of an inflammatory process which finally results in loss of appropriate mucosal glands (Rodriguez-Castro et al., 2018). CAG is usually considered as premalignant lesions of gastric cancer (GC), and greatly increases the risk of GC (Park and Kim, 2015).

Gastroscopy with tissue biopsy is the most commonly used technology for CAG diagnosis in clinic (Yu et al., 2011; Chooi et al., 2012; Rodriguez-Castro et al., 2018). However, there are several limitations: 1) Gastroscope, including both ordinary gastroscope and painless gastroscope, is invasive, and need at least half a day fasting and even need eat Laxatives, and results in a certain degree of injury to the esophagus (Yu et al., 2011); 2) Ordinary gastroscope often induces nausea and vomiting, which brings psychological pressure to patients; 3) Painless gastroscope needs anesthesia which will be a certain risk, especially for the elderly patients with basic diseases (Schaub and Kern, 2004; Choi et al., 2018; Hao et al., 2020). Therefore, new non-invasive technology for CAG diagnosis in clinic is urgently expected.

Researchers paid more and more attention to dysfunction of metabolites in gastrointestinal diseases especially in GC of rats or patients (Yu et al., 2011; Xu et al., 2017; Zu et al., 2020; Coker et al., 2022; Wang et al., 2022). Metabolites in plasma, such as azelaic acid, glutamate, 2-hydroxybutyrate, urate, creatinine and threonate characterized progressive stages from chronic superficial gastritis (CSG) to GC and might be the potential markers to indicate a risk of GC. (Yu et al., 2011). Many intervention methods in traditional Chinese medicine (TCM) such as Huangqi Jianzhong Tang (Liu et al., 2020), electro-acupuncture and moxibustion (Liu et al., 2017; Xu et al., 2017; He et al., 2018), as well as berberine (Tong et al., 2021) and palmatine (Chen et al., 2020), could modulate metabolites in CAG rats, indicating the potential role of metabolites in pathological process of CAG. However, metabolite profiles for CAG patients has not been well-clarified yet.

The gastrointestinal tract is the site that the gut microbiota interacts with the host. Gut microbiota produces functional molecules like short-chain fatty acids and various metabolites (Morrison and Preston, 2016). Gut microbiota even modulates host metabolism (Morrison and Preston, 2016; Zhang et al., 2018). Gut microbiota disturbance has been also proved to involve in inflammatory bowel diseases which could be recovered by healthy gut microbiota transplantation (Tung et al., 2011; Li et al., 2017). Also, gut microbiota homeostasis benefits the regulation of gastrointestinal function (Cani et al., 2019). Gut microbiota is also proved to involve in the process of CAG in rats (Sgambato et al., 2017). The abundance of bacteria in patients with CAG increased with the reduced secretion of gastric acid and that the changes in intestinal microbiota contribute to the progression from intestinal metaplasia (IM) to gastric cancer (Sharma et al., 1984; Park et al., 2019; Zhang et al., 2019; Zhou et al., 2021). Similar results were also found in CAG rats (Zhou et al., 2021). Therefore, the metabolites-microbiota crosstalk might involve in the pathological process of CAG.

There is a crosstalk between gut microbiota and metabolites (Wang and Zhao, 2018; Jia et al., 2021; Yang and Cong, 2021). However, up to nowadays, there is no research demonstrating the crosstalk between gut microbiota and metabolites in the feces of CAG patients. Therefore, in present study, the microbiota profiles, metabolites profiles and the possible crosstalk between gut microbiota and metabolites in the feces of CAG patients were clarified, and finally the potential non-invasive biomarkers including gut microbiota and metabolites in the feces of CAG patients were also investigated.

Materials and methods

Study design and population

As shown in Figure 1, we consecutively recruited 66 healthy volunteers and 110 CAG patients who received an endoscopic examination in Shanghai University of TCM affiliated Shuguang Hospital, Yueyang Hospital and Longhua Hospital. The fecal metabolites of 78 participants (A sample set) including healthy

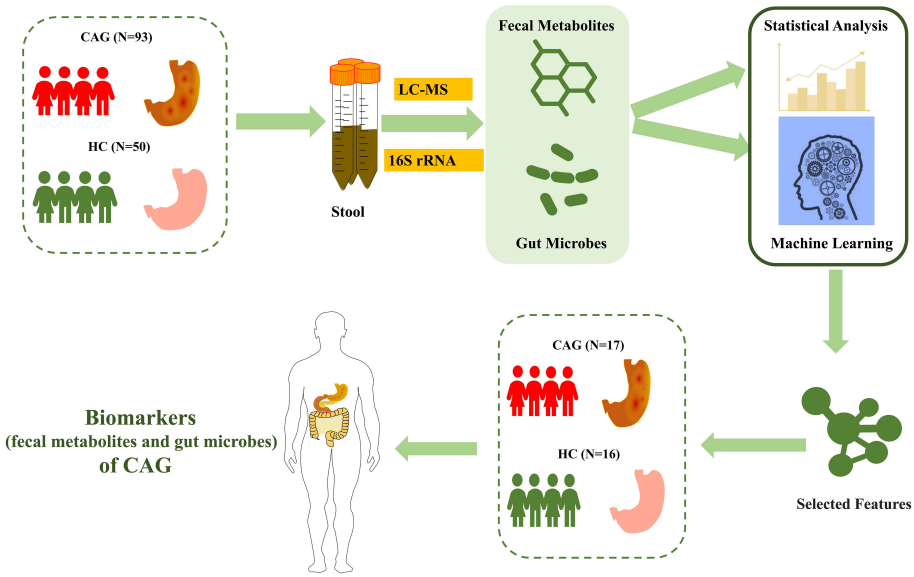

FIGURE 1
Study design and population. After pathological diagnosis and exclusion process, a total of 176 fecal samples (110 patients with CAG and 66 healthy controls) from Shanghai University of TCM affiliated Shuguang Hospital were prospectively collected. We divided into the discovery phase and validation phase. In the discovery phase, we characterized fecal metabolites among A sample set (30 healthy controls and 48 CAG patients) and gut microbiome among B sample set (20 healthy controls and 45 CAG patients). Furthermore, we identified the markers of gut microbiome and fecal metabolites to construct CAG classifier by random forest model from A sample set and B sample set, respectively. In validation phase, we constructed CAG classification model using 7 fecal metabolites or 4 gut microbes, or 7 fecal metabolites with 4 gut microbes, respectively, on the C sample set (16 healthy controls, 17 CAG patients) to validate diagnosis efficacy.

control group (N=30) and CAG group (N=48) were detected by using ultraperformance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS) system (ACQUITY UPLC-Xevo TQ-S, Waters Corp., Milford, MA, USA). The gut microbes of 65 participants (B sample set) including healthy control group (N=20) and CAG group (N=45) were detected by using 16S rRNA sequencing. In addition, both the profiles of gut microbes and metabolites in feces of 33 participants (C sample set) including healthy control group (N=16) and CAG group (N=17) were detected by using UPLC-MS/MS and 16S rRNA sequencing as a small verification cohort (Figure 1). The characteristics of the study population were showed in Table 1. There was no significant difference among the CAG group and HC group (A, B and C sample sets) in the gender (p=0.103, P=0.068, P=1.000), mean age (p=0.055, P=0.140, P=0.163), and body mass index (BMI) (p=0.147, P=0.277, P=0.688). The histological assessment was done by the experienced pathologists following clinical guidelines according to "the updated Sydney System" (Dixon et al., 1996). The inclusion

TABLE 1 The characteristics of the study population.

Groups	Gender, male, n (%)	Age, years,median (min-max)	BMI, kg/m^2, median (min-max)
CAG_a (n=48)	18 (37.50%)	53.67 (35-72)	22.72 (16.33-29.30)
HC_a (n=30)	6 (20.00%)	51.93 (40-72)	22.44 (16.02-28.52)
P values	P=0.103	P=0.055	P=0.147
CAG_b (n=45)	14 (31.11%)	59.20 (38-80)	21.92 (16.33-30.30)
HC_b (n=20)	11 (55.00%)	56.60 (47-80)	22.63 (18.59-27.34)
P values	P=0.068	P=0.140	P=0.277
CAG_c (n=17)	5 (29.41%)	53.47 (40-64)	24.08 (22.49-26.67)
HC_c (n=16)	5 (31.25%)	48.44 (41-56)	24.67 (23.12-27.34)
P values	P=1.000	P=0.163	P=0.688

criteria were a confirmed diagnosis of CAG according to pathological examination. Patients with gastric polyps, gastric bleeding, gastric tumors, gastrointestinal resection and special gastritis were excluded. This study was approved by the Medical Ethical Committee of Shuguang Hospital (2020-834-41-01). All participants signed the informed consent.

All clinical information was recorded using the questionnaire made by our study team. Participants were given a fecal sampler and provided detailed illustrated instructions for sample collection. Fecal samples freshly collected from each participant were immediately transported to the laboratory and frozen at -80° C immediately. The biochemical reports of serum were provided by the above hospitals.

Targeted fecal metabolomics profiling and data processing

All fecal-derived metabolites in this study, were detected by using UPLC-MS/MS with Q300 assay kits for a targeted approach (Metabo-profile Biotechnology, Shanghai, China). All samples were stored at -80°C prior to analysis. The fecal samples were prepared as described previously (Xie et al., 2021). Briefly, the fecal samples were lyophilized, and about 5 mg of each sample was weighed and transferred into a safety lock tube. Homogenization with 25 µl of ultrapure water was followed by extraction with 120 µL of methanol containing internal standards, followed by homogenated for another 3 min and centrifugation at 18 000 g for 20 min. Then the supernatant was transferred to a 96-well plate for derivatization. The following procedures were then performed on an Biomek 4000 workstation (Biomek 4000, Beckman Coulter, Inc., Brea, CA, USA). 20 µL of freshly prepared derivatization reagent was added to each well, and after derivatization at 30°C for 60 min, 330 µL of ice-cold 50% methanol solution was added to dilute the sample, then stored at -20°C for 20 minutes. This was followed by centrifugation at 4 000 g for 30 min at 4°C, and 135 µL of the supernatant from each well was transferred to a new 96-well plate with 10 µL internal standards in each well. All of the standards were obtained from Sigma-Aldrich (St. Louis, MO, USA), Steraloids Inc. (Newport, RI, USA) and TRC Chemicals (Toronto, ON, Canada). A series of standard calibration solutions were diluted for the calibration curve. The calibration curve and the corresponding regression coefficients were obtained by internal standard adjustment. Then, the absolute concentrations of 146 metabolites in fecal samples were detected by UPLC-MS/MS by using Q300 assay kits (Metabo-profile Biotechnology, Shanghai, China).

For mass spectrometer, capillary: 1.5 (ESI+), 2.0 (ESI-) Kv, source temp.: 150°C, desolvation temp.: 550°C, and desolation gas flow: 1 000 L h^{-1}. The raw data were deposited into the MetaboLights database (Accession number: MTBLS5990).

For data processing, the raw data files generated by UPLC-MS/MS were processed by using the MassLynx software (v 4.1, Waters Corp., Milford, MA, USA) to perform peak integration, calibration, and quantitation for each metabolite. The calculated absolute concentrations of metabolites were used for univariate analyses and multivariate analyses. Statistical analysis, and pathway analysis were processed on iMAP platform (v1.0; Metabo-Profile, Shanghai, China). A standardized z-score transformation was applied to convert the concentration values to z-scores before analysis in heatmap. Potential biomarkers of differential fecal metabolites were characterized by $P<0.05$ using student t test or Wilcoxon test based on whether the data were normally distributed between the two groups. Partial least squares-discriminant analysis (PLS-DA) was performed using metaX to discriminate different variables between groups. The logarithmic change (FC) value calculated by comparing the average of the peak area metabolites of both groups. Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/) was used to search and identify important metabolic pathways.

DNA extraction, 16S rRNA V4-V5 region sequencing and data processing

Microbial community genomic DNA was extracted from fecal samples using the QIAamp DNA Stool Mini Kit according to manufacturer's instructions. DNA concentration and purity were checked by running the samples on 1.2% agarose gels. Polymerase chain reaction PCR) amplification of 16S rRNA genes was performed by using general bacterial primers (515F 5'-GTGCCAGCMGCCGCGGTAA-3' and 926R 5'-CCGTCAATTCMTTTGAGTTT-3'). The primers also contained the Illumina 5'overhang adapter sequences for two-step amplicon library building, following manufacturer's instructions for the overhang sequences. The initial PCR reactions were carried out in 50 µL reaction volumes with 1-2 µL DNA templates, 200 µM dNTPs, 0.2 µM of each primer, 5X reaction buffer 10 µL and 1U Phusion DNA Polymerase (New England Biolabs, USA). PCR conditions consisted of initial denaturation at 94°C for 2 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 30 s, with a final extension of 72°C for 5 min. The second step PCR with dual 8-base barcodes were used for multiplexing. Eight cycle PCR reactions were used to incorporate two unique barcodes to either end of the 16S amplicons. Cycling conditions consisted of one cycle of 94°C for 3 min, followed by eight cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 30 s, followed by a final extension cycle of 72°C for 5 min. Prior to library pooling, the barcoded PCR products were purified by using a DNA gel extraction kit (Axygen, China) and quantified by using the FTC -3000 TM real-time PCR. The

libraries were sequenced by 2*300 bp paired-end sequencing on the MiSeq platform using MiSeq v3 Reagent Kit (Illumina) at Tiny Gene Bio-Tech (Shanghai) Co., Ltd. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession number: SRP350700).

The raw fastq files were demultiplexed based on the barcode. PE reads for all samples were run through Trimmomatic (version 0.35) to remove low quality base pairs using these parameters (SLIDINGWINDOW: 50:20 MINLEN: 50). Trimmed reads were then cut primer and adaptors by using cutadapt (version:1.16). And then further merged using FLASH program (version 1.2.11) with default parameters. The low quality contigs were removed based on screen. seqs command using the following filtering parameters, maxambig= 0, minlength = 200, maxlength = 485, maxhomop= 8. The 16S sequences were analyzed using a combination of software mothur (version 1.33.3), UPARSE (usearch version v8.1.1756, http://drive5.com/uparse/), and R (version 3.6.3). The demultiplexed reads were clustered at 97% sequence identity into operational taxonomic units (OTUs) by using the UPARSE pipeline (https://drive5.com/usearch/manual8.1/uparse_pipeline.html). The OTU representative sequences were assignment for taxonomy against Silva 128 database with confidence score ≧ 0.7 by the classify.seqs command in mothur.

The data were analyzed on the online platform of Majorbio Cloud Platform (www.majorbio.com) (Ren et al., 2022). For the alpha-diversity analysis, Shannon and Sobs index were calculated. The linear discriminant analysis (LDA) effect size (LEFSe) method was used to analyze significant differences between two groups of bacterial genera on the basis of log10 LDA>2.0. We conducted the Spearman's rank correlation analysis to predict the correlation between fecal metabolites and gut microbes. We used PICRUSt2 to perform the functional prediction of gut microbiota. First, the OTU abundance was standardized by PICRUSt. Each OTU has its own Greengene ID, then the KEGG Ortholog (KO) information of each OTU was obtained by Greengene ID of each OTU, finally, the abundance of KO was also calculated. According to the KEGG database, PICRUSt can be used to obtain the level information of metabolic pathways, and the abundance table of each level can be obtained respectively.

Feature selection using the random forest and evaluation using the receiver operator characteristic curves

Feature selection was conducted by using Python version 3.6.12 and machine learning library scikit-learn version 0.23.2. We used random forest (RF) to calculate the importance of 35 fecal metabolites and 27 gut microbes in CAG diagnosis, and sort them in descending order. Then we trained an (support vector machine) SVM classification model circularly with a step size of one, and determine the significant features (biomarkers) of fecal metabolites and gut microbes that make the best performance of classification model on the A and B sample sets. In order to improve the generalization ability and accuracy of the model, we used 5-fold cross-validation and grid search. The discrimination ability of the model was evaluated by using ROC curve, ROC space defines the false positive rate (FPR) as the X axis and the true positive rate (TPR) as the Y axis. a coordinate point (x = FPR, y = TPR) can be calculated by given a binary classification model and a threshold, and all coordinate points of each threshold of a model are drawn in space, which is called the ROC curve of a specific model. The evaluation index is the area under the ROC curve (AUC), The AUC>0.7 indicates that the model has predictive value, the closer the AUC to 1, the better the model performance. In order to verify whether the biomarker of fecal metabolites and gut microbes can well identify the new data set to achieve the purpose of diagnosing CAG, the selected biomarkers were used in the C sample set and established SVM model, The ROC curve was also drawn for evaluation. In the process of selecting significance features based on random forest algorithm and establishing the SVM model, the sample set is divided into 4/5 training set and 1/5 testing set. The code had been deposited in GitHub (https://github.com/fuzh97/SHUTCM-FDU).

Statistical analysis

The data in text were expressed as mean±standard deviation (m±SD), M (min -max) or M (Q25, Q75). Differences between two groups were analyzed by student *t*-test or Mann-Whitney (U-test) using SPSS25.0, based on whether the data were normally distributed between the two groups. P<0.05 were considered statistically significant.

Results

Bile acid, total cholesterol and low-density lipoprotein were higher in serum of CAG patients

As demonstrated in Table 2, the levels of bile acid, total cholesterol and low-density lipoprotein in CAG patients were higher than those in healthy volunteers (P< 0.05). However, there was no significant difference in the levels of high-density lipoprotein cholesterol, triglyceride and total bilirubin between healthy and CAG patients.

Alteration of fecal-derived metabolites profiles in CAG patients

As shown in Figure S1, in A sample set, the composition of fecal metabolites in CAG group and HC group was analyzed by

TABLE 2 Comparison of HDL-C, TG, BA, T-bil, CHOL and LDL-C between CAG_a group and HC_a group [M (Q25, Q75)].

Pathological indexes	CAG_a (n=48)	HC_a (n=30)	P values
High density lipoprotein-cholesterol (HDL-C) (mmol/L)	1.46 (1.41, 1.54)	1.41 (1.25, 1.60)	P=0.550
Triglycerides (TG)(μmol/L)	1.33 (0.90, 1.85)	1.26 (0.83, 1.65)	P=0.590
Bile acid (BA)(mmol/L)	3.10 (1.75, 4.40)	1.85 (1.27, 2.70)	P=0.002**
Total bilirubin (T-bil) (μmol/L)	14.20 (11.63, 16.60)	13.05(9.93, 15.53)	P=0.130
Total cholesterol (CHOL)(mmol/L)	5.67 (5.22, 6.48)	5.10 (4.85, 5.70)	P=0.002**
Low density lipoprotein-cholesterol (LDL-C) (mmol/L)	3.63 (3.41, 4.12)	3.25 (3.00, 3.64)	P=0.004**

Values were expressed as M(Q25, Q75) (n = 5/group, feces; n = 10/group, cecum contents). Data were analyzed by t-test. **P < 0.01 vs. control group.

metabolomics based on UPLC-MS/MS. A total of 146 metabolites belonging to 16 categories, were identified in fecal samples from CAG_a group and HC_a group, including 31 amino acids, 27 bile acids, 24 fatty acids, 16 organic acids, 10 carbohydrates, 9 SCFAs, 6 benzonic acids, 6 indoles, 5 phenylpropanoic acids, 3 phenols, 2 phenylpropanoids, 2 benzenoids, 2 carnitines, 1 pyridine, 1 DHA and 1 steroids and steroid derivatives.

PLS-DA is a versatile algorithm that can be used for predictive and descriptive modelling as well as for discriminative variable selection. In this present study, PLS-DA method was used to reflect the difference of metabolites between HC_a group and CAG_a group, and to investigate the aggregation tendency of the same group and the separation tendency of the different groups. The results demonstrated that there was a separation tendency between HC_a group and CAG_a group. The metabolites in CAG_a group were mainly distributed in the left quadrant, and metabolites in HC_a group were mainly distributed in the right quadrant. The score plots of PLS-DA are shown in Figure 2A.

As shown in Figure 2B and Table S1, there were 35 fecal metabolites in CAG_a group significantly different from that in HC_a group, namely 7 fatty acids (azelaic acid, heptadecanoic acid, palmitoleic acid, pentadecanoic acid, myristic acid, oleic acid, citramalic acid); 6 amino Acids (gamma_aminobutyric acid (GABA), alanine, valine, sarcosine, arginine, asparagine); 4 SCFAs (ethylmethylacetic acid, isobutyric acid, propionic acid, isovaleric acid); 3 phenylpropanoic acids (2-phenylpropionate, phenyllactic acid, hydrocinnamic acid); 3 organic acids (alpha-ketoisovaleric acid, ketoleucine, 3-methyl-2-oxopentanoic acid); 2 benzoic acids (3-aminosalicylic acid, gallic acid); 2 bile acids (lithocholic acid 3 sulfate (LCA-3S), apocholic acid (apoCA)); 2 indoles (indoleacrylic acid, indole-3-propionic acid); 2 phenols (4-hydroxyphenylpyruvic acid, p-hydroxyphenylacetic acid); 1 benzenoids (phenylpyruvic acid); 1 carbohydrates (gluconolactone); 1 carnitines (carnitine); 1 phenylpropanoids (cinnamic acid). Comparing with the fecal metabolites of healthy people in HC_a group, 11 fecal-derived metabolites (cinnamic acid, indoleacrylic acid, 2-phenylpropionate, ketoleucine, azelaic acid, 3-methyl-2-oxopentanoic acid, indole-3-propionic acid, phenylpyruvic acid, 4-hydroxyphenylpyruvic acid, alpha-ketoisovaleric acid, hydrocinnamic acid) were down regulated, and 24 fecal-derived metabolites (alanine, isobutyric acid, valine, isovaleric acid, gallic acid, palmitoleic acid, apoCA, oleic acid, p-hydroxyphenylacetic acid, arginine, ethylmethylacetic acid, heptadecanoic acid, citramalic acid, phenyllactic acid, 3-aminosalicylic acid, propionic acid, GABA, asparagine, carnitine, pentadecanoic acid, sarcosine, LCA-3S, gluconolactone, myristic acid) were up regulated. (P< 0.05).

As shown in Figure 2C, KEGG analysis indicated that the differentiated metabolites were mainly focused in Valine, leucine and isoleucine biosynthesis; Valine, leucine and isoleucine degradation; Alanine, aspartate and glutamate metabolism; Aminoacyl-tRNA biosynthesis; Phenylalanine metabolism; Pantothenate and coenzyme A (CoA) biosynthesis; Phenylalanine, tyrosine and tryptophan biosynthesis; Arginine and proline metabolism; Propanoate metabolism; D-Arginine and D-ornithine metabolism; Cyanoamino acid metabolism; Taurine and hypotaurine metabolism; Tyrosine metabolism; Pentose phosphate pathway; Ubiquinone and other terpenoid-quinone biosynthesis; Nitrogen metabolism; Butanoate metabolism; Glycine, serine and threonine metabolism.

As demonstrated in Figure 3, PLS-DA assay results demonstrated that there was a separation tendency between HC_c group and CAG_c group. The metabolites in CAG_c group were mainly distributed in the left quadrant, and metabolites in HC_c group were mainly distributed in the right quadrant. The score plots of PLS-DA are shown in Figure 3A.

In C samples set, from feces of CAG_c group and HC_c group, there were 29 fecal metabolites belonging to 13 catergories in CAG_c group significantly different from that in

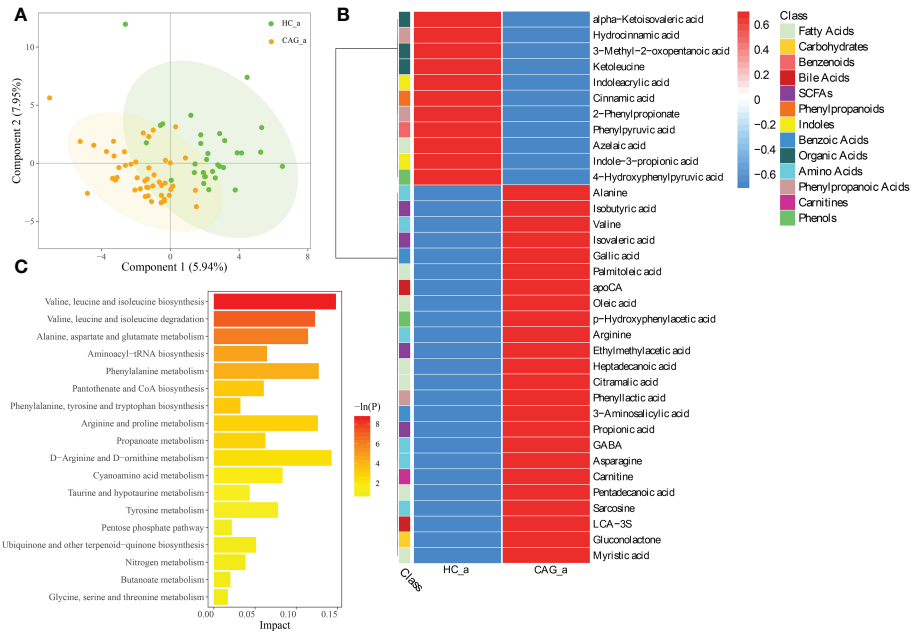

FIGURE 2
Alteration of fecal-derived metabolites profiles in CAG_a patients. **(A)** PLS-DA indicated the difference of fecal metabolites between CAG_a and HC_a healthy volunteers. **(B)** Alteration of fecal-derived metabolites profiles in CAG_a patients. **(C)** All differential metabolites enriched for metabolic pathways based on KEGG analysis. Values were expressed as mean ± SD (n=30/HC_a, n=48/CAG_a) Data were analyzed by t-test or Wilcoxon test based on whether the data were normally distributed between CAG_a group and HC_a group.

HC_c group, namely 5 fatty acids (pentadecanoic acid, dihomo - gamma - linolenic acid, (docosapentaenoic acid) DPA, adrenic acid, heptadecanoic acid); 5 amino acids (glutamic acid, dimethylglycine, 2-phenylglycine, aspartic acid, pyroglutamic acid); 4 organic acids (lactic acid, 3-hydroxybutyric acid, 2-hydroxybutyric acid, oxalic acid); 4 bile acids (7-DHCA (7-dehydrocholic acid), 3-DHCA (3-dehydrocholic acid), bHDCA (beta_hyodeoxycholic acid), 7-KetoLCA (7-ketolithocholic acid)); 2 benzoic acids (gallic acid, phthalic acid); 2 phenylpropanoic acids (hydroxyphenyllactic acid, 3-hydroxyphenylhydracrylic acid); 1 SCFAs (propionic acid); 1 phenylpropanoids (3,4-dihydroxyhydrocinnamic acid); 1 indoles (1H-indole-3-acetamide); 1 phenols (homovanillic acid); 1 benzenoids (mandelic acid); 1 carbohydrates (gluconolactone); 1 steroids and steroid derivatives (murocholic acid). Comparing with the fecal metabolites of healthy people in HC_c group, only 2 fecal-derived metabolites (aspartic acid, pyroglutamic acid) were downregulated, and 27 fecal-derived metabolites (propionic acid, bHDCA, phthalic acid, oxalic acid, mandelic acid, 3-hydroxybutyric acid, glutamic acid, homovanillic acid, 3-hydroxyphenylhydracrylic acid, 1H-indole-3-acetamide, gallic acid, dimethylglycine, 7-ketoLCA, heptadecanoic acid, DPA, pentadecanoic acid, murocholic acid, 3-DHCA, 7-DHCA, 2-phenylglycine, 2-hydroxybutyric acid, lactic acid, hydroxyphenyllactic acid, adrenic acid, dihomo-gamma-linolenic acid, 3,4-dihydroxyhydrocinnamic acid, gluconolactone) were upregulated. ($P < 0.05$) (Figure 3B, Table S2).

As shown in Figure 3C, KEGG analysis indicated that the differentiated metabolites were mainly focused in Propanoate metabolism; Alanine, aspartate and glutamate metabolism; Tyrosine metabolism; Glutathione metabolism; Nitrogen metabolism; Butanoate metabolism; Synthesis and degradation of ketone bodies; Histidine metabolism; Nicotinate and nicotinamide metabolism; Glycine, serine and threonine metabolism; D-Glutamine and D-Glutamate metabolism; Cyanoamino acid metabolism; Aminoacyl–tRNA biosynthesis; Arginine and proline metabolism; Pantothenate and CoA biosynthesis; beta-Alanine metabolism; Glycolysis or Gluconeogenesis; Pyruvate metabolism; Lysine biosynthesis; Pentose phosphate pathway; Ubiquinone and other terpenoid-quinone biosynthesis; Glyoxylate and dicarboxylate metabolism; Cysteine and methionine metabolism; Tryptophan metabolism; Porphyrin and chlorophyll metabolism.

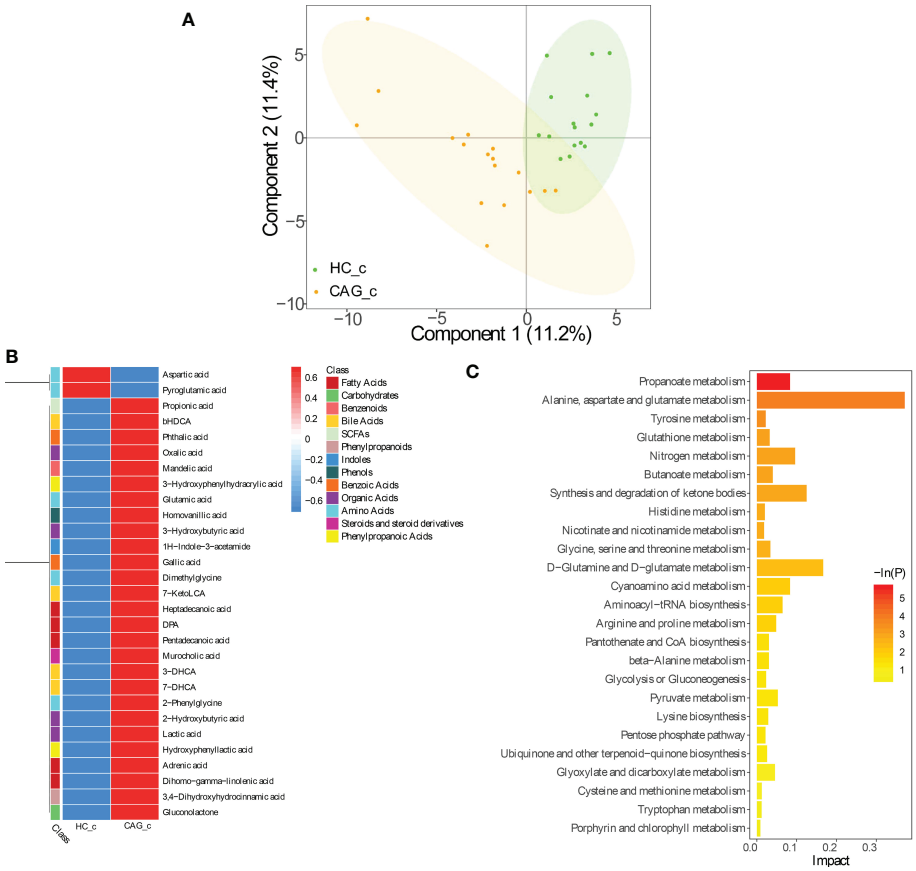

FIGURE 3
Alteration of fecal-derived metabolites profiles in CAG_c patients. **(A)** PLS-DA indicated the difference of fecal metabolites between CAG_c and HC_c healthy volunteers. **(B)** Alteration of fecal-derived metabolites profiles in CAG_c patients. **(C)**. All differential metabolites enriched for metabolic pathways based on KEGG analysis. Values were expressed as mean ± SD (n=16/HC_c, n = 17/CAG_c). Data were analyzed by t-test or Wilcoxon test based on whether the data were normally distributed between CAG_c group and HC_c group.

Alteration of fecal-derived gut microbiota profiles in CAG patients

Therefore, to clarify the change of fecal gut microbiota of CAG patients, the diversity and composition of fecal-derived gut microbiota were analyzed by Miseq sequencing. The Sobs index and Shannon index were used to estimate α -diversity. Sequencing of 16S rRNA gene V4-V5 region of gut microbiota showed that there were no difference of Sobs index and Shannon index in feces between HC_b group and CAG_b group (Figure 4A, B).

Linear discriminant analysis Effect Size (LEfSe) determines the features (organisms, clades, operational taxonomic units, genes, or functions) most likely to explain differences between classes by coupling standard tests for statistical significance with additional tests encoding biological consistency and effect relevance. (Segata et al., 2011)

Furthermore, in order to further distinguish the difference of intestinal flora between HC_b group and CAG_b group, we used the LEfSe to further analyze the bacterial flora markers with significant difference between the CAG_b group and HC_b group. The level of bacterial taxonomy chosen ranged from phylum to genus, with the threshold value of LDA set at 2, and linear discriminant analysis (LDA) was used to determine the most likely explanation for the difference between the CAG_b group and HC_b group. As demonstrated in Figure 4C and Figure 5, there were 1 phylum (*Tenericutes*) and 27 genera with significant difference between the CAG_b group and HC_b group (Figure 5A).

In 27 genera, 2 genera namely *Eggerthella* and *Scardovia* belonged to the phylum of *Actinobacteria*, 3 genera namely *Paraprevotella, norank_f: Bacteroidales_S24-7_group and Odoribacter* belonged to the phylum of *Bacteroidetes*; 17 genera namely *[Eubacterium]_rectale_group, Phascolarctobacterium, Subdoligranulum, Ruminococcaceae_UCG-002,*

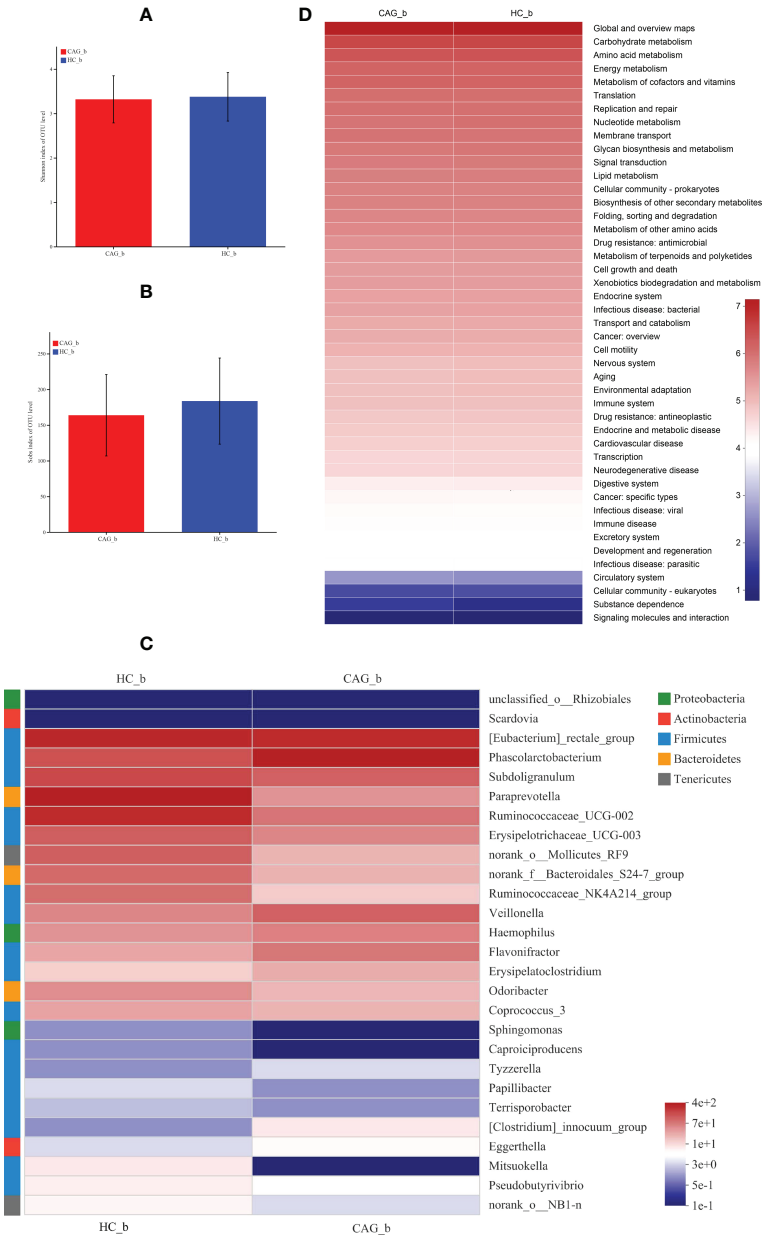

FIGURE 4
Alteration of fecal-derived gut microbiota profiles in CAG_b patients. **(A)** Shannon index of OTU level indicated there is no difference of α-diversity of gut microbiota in feces of CAG patients, compared with HC_b healthy volunteers. **(B)** Sobs index of OTU level indicated there is no difference of α-diversity of gut microbiota in feces of CAG patients, compared with HC_b healthy volunteers. **(C)** Alteration of fecal-derived gut microbiota profiles in CAG_b patients. **(D)** KEGG analysis indicated the pathways mediated by the differentiated fecal microbiota.

Erysipelotrichaceae_UCG-003, Ruminococcaceae_NK4A214_group, Veillonella, Flavonifractor, Erysipelatoclostridium, Coprococcus_3, Caproiciproducens, Tyzzerella, Papillibacter, Terrisporobacter, [Clostridium]_innocuum_group, Mitsuokella and Pseudobutyrivibrio belonged to the phylum of *Firmicutes*, 3 genera namely *Haemophilus, unclassified_o:Rhizobiales and Sphingomonas* belonging to the phylum of *Proteobacteria*, 2 genera namely *norank_o:Mollicutes_RF9 and norank_o:NB1-n* belonged to the phylum of *Tenericutes* (Figure 4C). In genus level, we found that 8 genera namely *Phascolarctobacterium*, *Veillonella*, *Haemophilus*, *Flavonifractor*, *Erysipelatoclostridium*, *Clostridium_innocuum_group*, *Eggerthella* and *Tyzzerella* were significantly increased in feces of CAG_b patients, compared with that of HC_b people; however, there were 19 genera namely *Papillibacter*, *Pseudobutyrivibrio*, *Terrisporobacter*, *norank_o_NB1_n*, *Sphingomonas*,

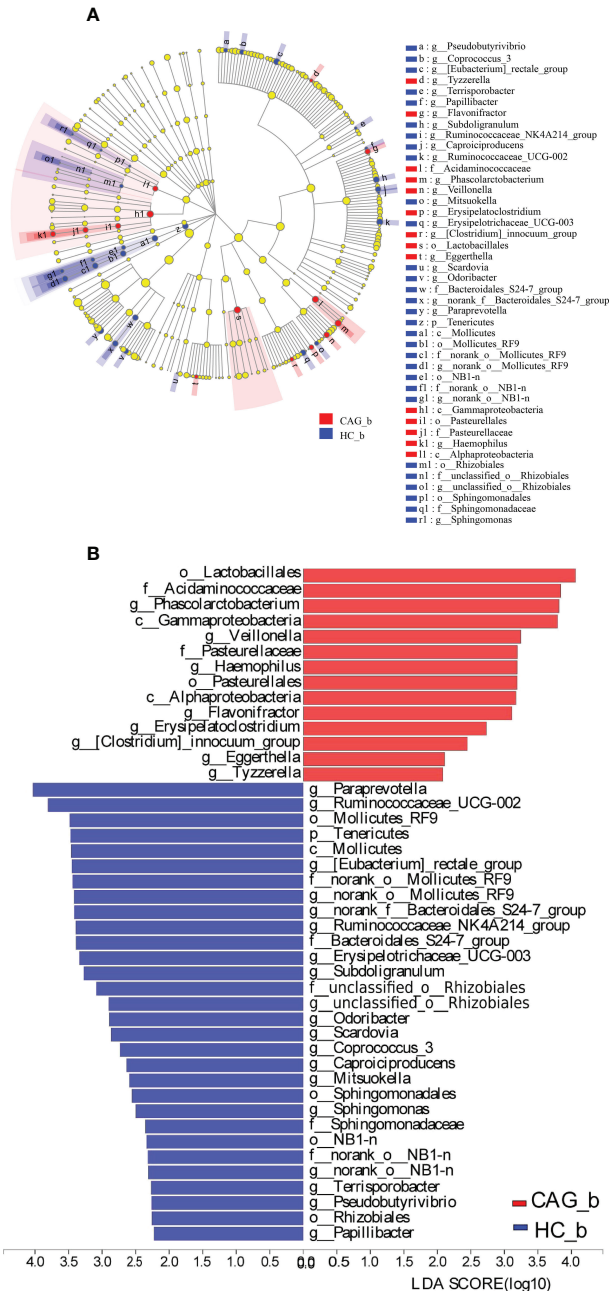

FIGURE 5
Enriched gut microbiota profiles in feces of CAG_b patients and/or HC_b healthy volunteers. **(A)** Cladogram plot: red nodes indicated significantly enriched bacterial colony with significant impact in CAG patients, and blue nodes indicated significantly enriched bacterial colony with significant impact in HC_b healthy volunteers. Light yellow nodes indicated bacterial colony without significant difference in both CAG patients and HC_b healthy volunteers. **(B)** LDA discriminant analysis histogram: red bar represented the bacterial colony enriched in CAG patients; blue bar represented the bacterial colony enriched in the HC_b healthy volunteers.

Mitsuokella, *Caproiciproducens*, *Coprococcus_3*, *Scardovia*, *Odoribacter*, *unclassified_o_Rhizobiales*, *Subdoligranulum*, *Erysipelotrichaceae_UCG_003*, *Ruminococcaceae_NK4A214_group*, *norank_f_Bacteroidales_S24_7_group*, *norank_o_Mollicutes_RF9*, *Eubacterium_rectale_group*, *Ruminococcaceae_UCG_002*, *Paraprevotella* were significantly decreased in feces of CAG_b patients (Figure 5B).

Furthermore, as shown in Figure 4D, we further analyzed the function of fecal-derived gut microbiota in CAG_b patients

by PICRUSt2 analysis, the results indicated that gut microbiota in CAG_b patients mainly involved in carbohydrate metabolism, amino acid metabolism, energy metabolism, metabolism of cofactors and vitamins, translation, replication and repair, nucleotide metabolism, membrane transport, glycan biosynthesis and metabolism, signal transduction, lipid metabolism, cellular community-prokaryotes, biosynthesis of other secondary metabolites, folding, sorting and degradation, metabolism of other amino acids, drug resistance: antimicrobial, metabolism of terpenoids and polyketides, cell growth and death, etc.

Similar results were obtained in the feces of CAG_c and HC_c samples. In order to further distinguish the difference of intestinal flora between HC_c group and CAG_c group, LEfSe software was used to further analyze the bacterial flora markers with significant difference between the CAG_c group and HC_c group. The level of bacterial taxonomy chosen ranged from phylum to genus, with the threshold value of LDA set at 2, and linear discriminant analysis was used to determine the most likely explanation for the difference between the CAG_c group and HC_c group (Figure 6). The results showed that there were 2 phylum (*Fusobacteria*, *Lentisphaerae*) and 29 genera with significant difference between the the CAG_c group and HC_c group (Figure 6B). In 29 genera, 1 genus of *Atopobium* belonged to the phylum of *Actinobacteria*, 2 genera namely *Paraprevotella*, *norank_f_ Bacteroidales_ S24-7_group* belonged to the phylum of *Bacteroidetes*, 1 genera of *Fusobacterium* belonged to

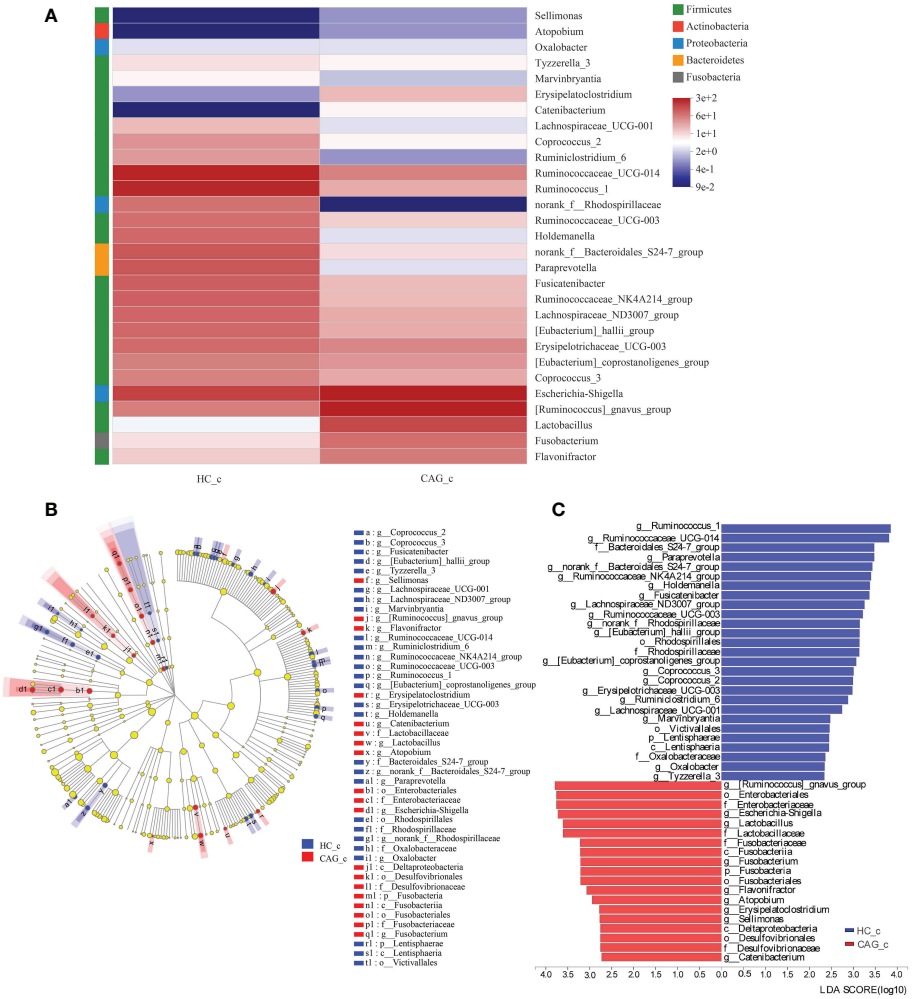

FIGURE 6
Alteration of fecal-derived gut microbiota profiles in CAG_c patients. **(A)** Alteration of fecal-derived gut microbiota profiles in CAG_c patients. **(B)** Cladogram plot: red nodes indicated significantly enriched bacterial colony with significant impact in CAG_c patients, and blue nodes indicated significantly enriched bacterial colony with significant impact in healthy volunteers (HC_c). Light yellow nodes indicated bacterial colony without significant difference in both CAG patients and healthy volunteers (HC_c). **(C)** LDA discriminant analysis histogram: red bar represented the bacterial colony enriched in CAG_c patients; blue bar represented the bacterial colony enriched in the healthy volunteers (HC_c).

the phylum of *Fusobacteria*; 3 genera namely *Escherichia-Shigella*, *Oxalobacter*, *norank_f_Rhodospirillaceae* belonged to the phylum of *Proteobacteria*; 22 genera namely *Sellimonas*, *Tyzzerella_3*, *Marvinbryantia*, *Erysipelatoclostridium*, *Catenibacterium*, *Lachnospiraceae_UCG-001*, *Coprococcus_2*, *Ruminiclostridium_6*, *Ruminococcaceae_UCG-014*, *Ruminococcus_1*, *Ruminococcaceae_UCG-003*, *Holdemanella*, *Fusicatenibacter*, *Ruminococcaceae_NK4A214_group*, *Lachnospiraceae_ND3007_group*, *[Eubacterium]_hallii_group*, *Erysipelotrichaceae_UCG-003*, *[Eubacterium]_coprostanoligenes_group*, *Coprococcus_3*, *[Ruminococcus]_gnavus_group*, *Lactobacillus*, *Flavonifractor* belonged to the phylum of *Firmicutes* (Figure 6A). There were 9 genera including *Catenibacterium*, *Sellimonas*, *Erysipelatoclostridium*, *Atopobium*, *Flavonifractor*, *Fusobacterium*, *Lactobacillus*, *Escherichia-Shigella*, *[Ruminococcus]_gnavus_group* were significantly enriched in the feces of CAG_c samples; and 20 genera namely *Tyzzerella_3*, *Oxalobacter*, *Marvinbryantia*, *Lachnospiraceae_UCG-001*, *Ruminiclostridium_6*, *Erysipelotrichaceae_UCG-003*, *Coprococcus_2*, *Coprococcus_3*, *[Eubacterium]_coprostanoligenes_group*, *[Eubacterium]_hallii_group*, *norank_f:Rhodospirillaceae*, *Ruminococcaceae_UCG-003*, *Lachnospiraceae_ND3007_group*, *Fusicatenibacter*, *Holdemanella*, *Ruminococcaceae_NK4A214_group*, *norank_f_Bacteroidales _S24-7_group*, *Paraprevotella*, *Ruminococcaceae_UCG-014*, *Ruminococcus_1* were significantly enriched in the feces of HC_c samples (Figure 6C).

Feature selection using the RF and evaluation using the ROC curves

Feature selection of 35 fecal metabolites on the A sample set

As demonstrated in Figure 7, we used RF to calculate the importance of 35 fecal metabolites and trained an SVM classification model on the A sample set. We determined the biomarkers of fecal metabolites according to the best accuracy of classification model (details are shown in the " Materials and methods" section). When the features were 7 fecal metabolites, the best accuracy of classification was 0.938 (Figure 7A). The importance of 7 fecal metabolites in descending order was heptadecanoic acid (0.079), azelaic acid (0.077), indoleacrylic acid (0.071), indole-3-propionic acid (0.067), pentadecanoic acid (0.055), palmitoleic acid (0.047), 2-phenylpropionate (0.043) (Figure 7B). Then ROC curves were used to evaluate the classification ability of the model. The results have shown that

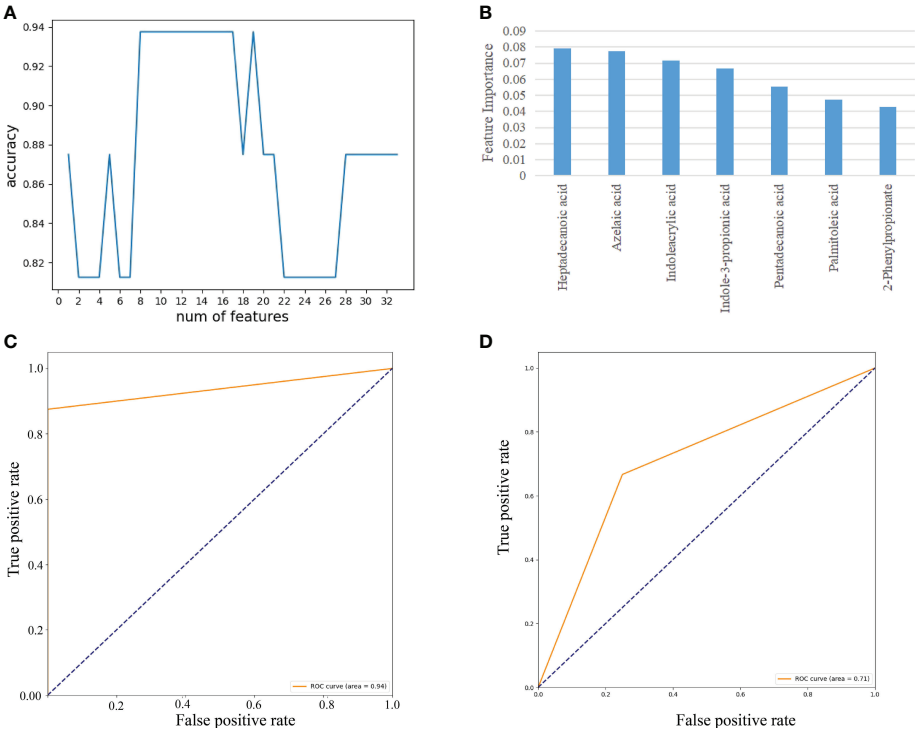

FIGURE 7
Feature selection of fecal metabolites by using the random forest (RF) and evaluation using the ROC curves. **(A)** The accuracy changes of classification model on the A sample set. **(B)** The importance in descending order (7 fecal metabolites). **(C)** Evaluation using ROC curve on the A sample set. **(D)** Evaluation using ROC curve on the C sample set.

7 fecal metabolites could distinguish CAG patients from healthy controls, as indicated by the AUC, which had a value up to 0.94 on the A set (Figure 7C). Moreover, we constructed SVM classification model using 7 fecal metabolites on the C sample set. The accuracy of classification model was 0.714. The AUC was 0.71 (Figure 7D).

Feature selection of 27 gut microbes on the B sample set

As demonstrated in Figure 8, we used RF to calculate the importance of 27 gut microbes and trained a SVM classification model on the B sample set. We determined the biomarkers of gut microbes according to the best accuracy of classification model (details are shown in the " Materials and methods" section). When the features were 4 gut microbes, the best accuracy of classification was 0.923 (Figure 8A). The importance of 4 gut microbes in descending order was *g:Phascolarctobacterium* (0.115), *g:Erysipelotrichaceae_UCG-003*(0.077), *g:Veillonella* (0.070), *g:Haemophilus*(0.064)(Figure 8B). Then ROC curves were used to evaluate the classification ability of the model. The results have shown that 4 gut microbes could distinguish CAG patients from healthy controls, which had a value up to 0.95 on the A set (Figure 8C). Moreover, we constructed SVM classification model using 4 gut microbes on the C sample set. The accuracy of classification model was 0.857. The AUC was 0.88 (Figure 8D).

Classification model based on fecal metabolites and gut microbes on the C sample set

As shown in Figure 9, we used RF and SVM to calculate the features importance (7 fecal metabolites and 4 gut microbes) and trained a classification model on the C sample set. The importance of 7 fecal metabolites and 4 gut microbes in descending order was *Heptadecanoic acid* (0.152), *g: Erysipelotrichaceae_UCG-003*(0.146), *3-Indolepropionic acid* (0.104), *g:Veillonella*(0.102), *Pentadecanoic acid*(0.100), *Azelaic acid*(0.078), *g:Phascolarctobacterium*(0.070), *2-Phenylpropionate*

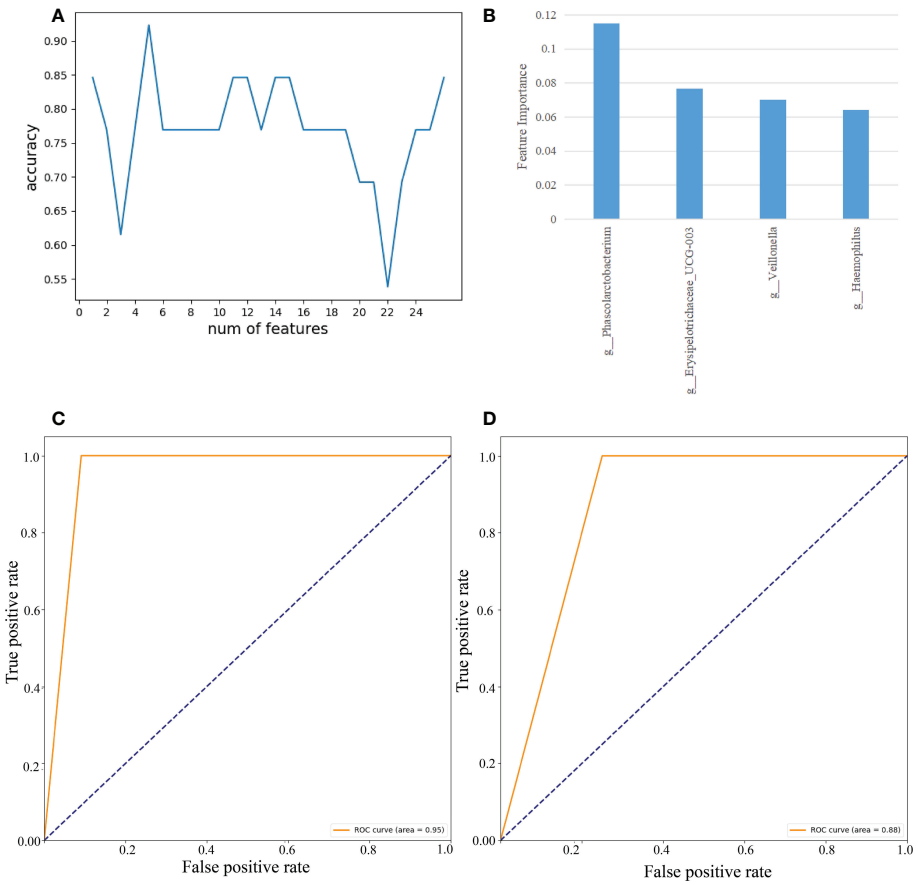

FIGURE 8
Feature selection of fecal gut microbes on the B sample set by using the random forest (RF) and evaluation using the ROC curves. **(A)** The accuracy changes of classification model on the B sample set. **(B)** The importance in descending order(4 gut microbes). **(C)** Evaluation using ROC curve on the A sample set. **(D)** Evaluation using ROC curve on the C sample set.

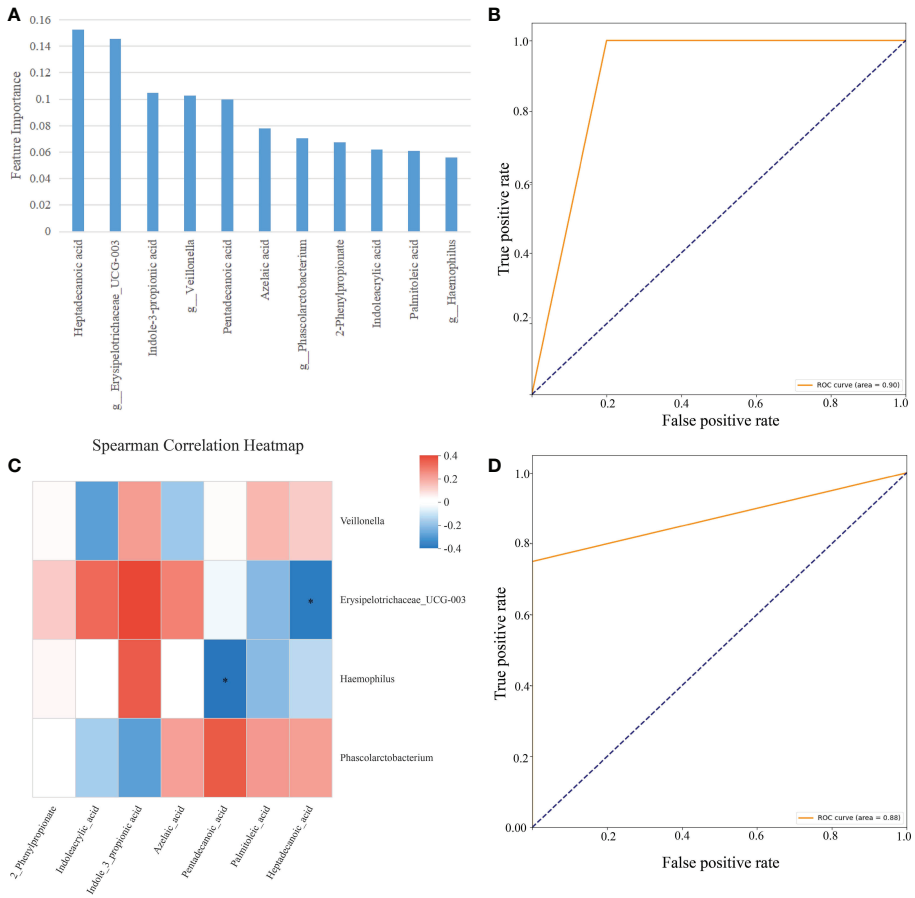

FIGURE 9
Classification model based on fecal metabolites and gut microbes on the C sample set. **(A)** The importance in descending order (7 fecal metabolites and 4 gut microbes). **(B)** Evaluation using ROC curve on the C sample set. **(C)** Spearman correlation heatmap, Spearman's correlation between 7 fecal metabolites and 4 gut microbes, the abscissa is 7 metabolites and the ordinate is 4 intestinal flora, the color scale represents the spearman r value, with red and blue indicating positive and negative correlations, respectively, and *p< 0.05. **(D)** Evaluation using ROC curve on the C sample set (2 fecal metabolites and 2 gut microbes).

(0.068), *Indoleacrylic acid*(0.061), *Palmitoleic acid*(0.061), *g: Haemophilus*(0.056)(Figure 9A). The accuracy of classification model was 0.857. And the AUC was 0.90 (Figure 9B). The results have shown that 7 fecal metabolites and 4 gut microbes could distinguish CAG patients from healthy controls.

Then we conducted the Spearman' s rank correlation analyses to discovery the correlation between 7 metabolites and 4 gut microbiotas on the C sample set. Interestingly, Heptadecanoic acid was significantly negatively correlated to *Erysipelotrichaceae_UCG-003* (Figure 9C, R=-0.347, P=0.048, $p < 0.05$); Pentadecanoic acid was significantly negatively correlated to *Haemophilus* (Figure 9C, R=-0.364, P=0.037, $P < 0.05$);

The above result showed that 2 correlated fecal metabolites and 2 correlated gut microbes maybe more imported for CAG diagnosis. So, we constructed SVM classification model using 2 correlated fecal metabolites and 2 correlated gut microbes on the C sample set. The accuracy of classification model was 0.857. The AUC was 0.88 (Figure 9D).

Discussion

Although gastroscopy with tissue biopsy is the most used technology for CAG diagnosis (Chooi et al., 2012; Rodriguez-Castro et al., 2018), it is always an uncomfortable experience for CAG patients because of its invasive process, fasting, eating laxatives, esophagus injury, nausea and vomiting as well as psychological pressure. Therefore, new non-invasive effective methods for CAG diagnosis in clinic is very urgent.

Disturbed metabolites in blood are often associated with different diseases (Yu et al., 2011; Xu et al., 2017; Zu et al., 2020;

Coker et al., 2022; Wang et al., 2022). Previous researches indicated that in blood plasma, fifteen identified metabolites contributed most to the differentiating between CSG and GC, and characterized different stages of GC. 2-hydroxybutyrate, pyroglutamate, glutamate, asparagine, azelaic acid, ornithine, urate, 11-eicosenoic acid, 1-monohexadecanoylglycerol and g-tocopherol were increased, while creatinine, threonate were decreased in GC patients, indicating that oxidative stress and perturbed metabolism of amino acids and fatty acids might be invovled in the pathological process of GC (Yu et al., 2011). However, as to CAG, in CAG rats, 19 plasma metabolites and 18 urine metabolites were enrolled to construct the circulatory and excretory metabolome of CAG rats, which was in response to alterations of energy metabolism, inflammation, immune dysfunction, as well as oxidative stress. Seven plasma biomarkers and 7 urine biomarkers were screened to elucidate the pathogenesis of CAG based on the further correlation analysis with biochemical indexes. Finally, 3 plasma biomarkers (arginine, succinate and 3-hydroxybutyrate) and 2 urine biomarkers (α-ketoglutarate and valine) highlighted the potential to indicate risks of CAG in virtue of correlation with pepsin activity and ROC analysis (Cui et al., 2017). However, characteristic metabolites profiles of CAG in patients has not been well-clarified yet. Moreover, metabolites profiles, gut microbiota profiles as well as crosstalk between bacteria and metabolites in feces of CAG patients has not been clarified yet. In our present study, metabolites profiles, gut microbiota profiles as well as the possible crosstalk between bacteria and metabolites in feces of CAG patients were clarified, moreover, the biomarkers including metabolites and gut microbiota for CAG patients were also identified.

RF is a classifier containing multiple decision trees, each of trees is a classifier. For an input sample, N trees will have N classification results (Reynolds et al., 2019). RF integrates the results of all the classification votes, designating the category with the highest number of votes as the file output, which is equivalent to sampling both the sample and the features, thus enhancing generalization. The main advantages of the RF algorithm are: the small variance of the trained module, its generalization ability and its insensitivity to partially missing features due to the use of random sampling (Noble, 2006). Then ROC curves were used to evaluate the classification ability of the model.

In the present study, we demonstrated that there were 35 metabolites significantly changed in the feces of CAG patients in A sample set, compared with healthy volunteers. Using RF, 7 fecal metabolites (heptadecanoic acid, azelaic acid, indoleacrylic acid, indole-3-propionic acid, pentadecanoic acid, palmitoleic acid, 2-phenylpropionate) were selected from A sample set, to classify CAG from healthy people, as indicated by AUC on the A set. SVM is very powerful classifiers in complex datasets compared to the other many machine methods (Reynolds et al., 2019). It aims to create a decision boundary between two classes that enables the prediction of labels from one or more feature vectors (Noble, 2006). SVM as a classifier has been used in cancer classification (Reynolds et al., 2019; Huang et al., 2018) and biomarker selection (Zhang et al., 2021), since the high throughput microarray gene expression data was available in the early 2000's. In our present study, after constructing SVM classification model using 7 fecal metabolites, the accuracy of classification model was 0.71, and the AUC was 0.71 on the C sample set. Therefore, metabolites disturbance indeed involves in the process of CAG, and could clarify CAG from healthy volunteers.

Gut microbiota lives in the gastrointestinal tract, and involving in modulating gastrointestinal function through producing functional molecules and metabolites, and interacting with the host metabolism (Morrison and Preston, 2016; Zhang et al., 2018). Healthy gut microbiota transplantation could recover inflammatory bowel diseases induced by gut microbiota disturbance (Tung et al., 2011; Li et al., 2017). Intestinal microbiota alteration in patients with CAG resulted in the reduced secretion of gastric acid and also contributed to the progression from IM to gastric cancer (Sharma et al., 1984; Park et al., 2019; Zhang et al., 2019; Zhou et al., 2021). However, the detailed relation of intestinal microbiota and CAG has been poorly investigated. In present study, the abundance of many fecal bacteria was significantly altered in CAG patients, compared with healthy volunteers. Then we used RF to select features of fecal bacteria for CAG patients. By using RF, 4 gut microbes (*g_Phascolarctobacterium*, *g_Erysipelotrichaceae_UCG-003*, *g_Veillonella*, *g_Haemophilus*) were selected as the features to classify CAG from healthy volunteers in B sample set. After constructing SVM classification model using 4 gut microbes, and the accuracy of classification model was 0.857 and the AUC was 0.88 on the C sample sets. Thus, fecal microbiota alteration especially *g_Phascolarctobacterium*, *g_Erysipelotrichaceae_UCG-003*, *g_Veillonella*, *g_Haemophilus*, could be as biomarkers for CAG patients.

There is a crosstalk between gut microbiota and metabolites (Wang and Zhao, 2018; Jia et al., 2021; Yang and Cong, 2021). However, up to nowadays, there is no research demonstrating the crosstalk between gut microbiota and metabolites in feces of CAG patients. In present study, RF and SVM were used to calculate the features importance (including the above 7 fecal metabolites and the above 4 gut microbes) and trained a classification model on the C sample sets. The accuracy of classification model was 0.857, and the AUC was 0.90. The results have shown that 7 fecal metabolites and 4 gut microbes could distinguish CAG patients from healthy volunteers. And it also indicated that it might be better to use features including fecal gut microbiota and fecal metabolites, than that of only using gut microbiota or metabolites to clarify CAG from healthy people, indicating there might be a crosstalk between fecal-derived microbiota and metabolites.

Therefore, we further used Spearman's rank correlation analysis to predict the possible fecal-derived gut microbiota-metabolites crosstalk in CAG patients in the C sample set. Interestingly, in the selected above 7 fecal metabolites and the above 4 gut microbes, heptadecanoic acid was significantly negatively correlated to *Erysipelotrichaceae_UCG-003*; and pentadecanoic acid was significantly negatively correlated to *Haemophilus*, indicating a possible intricate relationship between fecal microbiota and fecal metabolites, such as heptadecanoic acid, *Erysipelotrichaceae_UCG-003*, pentadecanoic acid, *Haemophilus*. We further constructed SVM classification model using 2 correlated fecal metabolites and 2 correlated gut microbes on the C sample sets. The accuracy of classification model was 0.857, and the AUC was 0.88. The accuracy of classification model and AUC with heptadecanoic acid, *Erysipelotrichaceae_UCG-003*, pentadecanoic acid, *Haemophilus*, was similar with that with 4 gut microbiota and 7 metabolites, indicating there is possibly a crosstalk between heptadecanoic acid and *Erysipelotrichaceae_UCG-003*, as well as pentadecanoic acid and *Haemophilus* in the feces of CAG patients, and fecal-derived microbiome-metabolites crosstalk possibly involves in the pathological process of CAG, which should be further clarified and confirmed with a microbiome-based study based on shotgun metagenomics and metatranscriptomics. Therefore, the microbiota and the microbial-associated metabolites are possily potential diagnostic biomarkers and therapeutic targets for CAG.

Erysipelotrichi belongs to the Firmicutes phylum, and the bacterial family *Erysipelotrichaceae* which are immunogenic and possibly inter-host variation, and highly increased in mouse models of inflammatory bowel diseases (IBD) (Zhao et al., 2013; Palm et al., 2014; Dinh et al., 2015; Kaakoush, 2015), but significantly lowered in IBD patients (Dey et al., 2013; Gevers et al., 2014). Interestingly, and in the lumen of gastrointestinal tract of patients with colorectal cancer, the abundance level of *Erysipelotrichaceae* was significantly enriched (Chen et al., 2012; Zhu et al., 2014). *Erysipelotrichi* also appear to affect cholesterol and lipid metabolism in the GI tract (Parmentier-Decrucq et al., 2009). Distinct functional roles for the UCG-003 subtype have not been reported (Singh et al., 2019). The FUT2 loss-of-function mutations are very common and related with inflammatory bowel disease (IBD). Researchers further found that FUT2 loss-of-function mutations also increased $CD8^+$ inducing *Alistipe* and *Phascolarctobacterium* and Th17 inducing *Erysipelotrichaceae UCG-003* in IBD patients (Cheng et al., 2021). In present study, interestingly, compared with healthy volunteers, the abundance of *Erysipelotrichaceae UCG-003* was significantly lowered in feces of CAG patients, indicating CAG might be a compensation condition against GC progress, and *Erysipelotrichaceae UCG-003* might be closely related with CAG. However, the detailed underlying molecular mechanism of *Erysipelotrichaceae UCG-003* on CAG still needed to be clarified.

Previous researches demonstrated that lipid metabolism involving in GC progress (Yu et al., 2011). Both pentadecanoic acid and heptadecanoic acid are multifaceted odd-chain fatty acids (OCFA) (Pfeuffer and Jaudszus, 2016), pentadecanoic acid and heptadecanoic acid can also be synthesized endogenously, for example, from gut-derived propionic acid (3:0) (Pfeuffer and Jaudszus, 2016), although most gut microbial propionic acid is absorbed and mostly metabolized by the liver (Al-Lahham et al., 2010). A number of studies have shown an inverse association between OCFA concentrations in human plasma phospholipids or RBCs and risk of type 2 diabetes and cardiovascular disease (Hodge et al., 2007; Patel et al., 2010; Mozaffarian et al., 2013; Santaren et al., 2014; Pfeuffer and Jaudszus, 2016). Heptadecanoic acid was proved to inhibit cell proliferation in PC-9 non-small-cell lung cancer cells with acquired gefitinib resistance *in vitro* (Xu et al., 2019). Therefore, the increased heptadecanoic acid in the feces of CAG patients might be associated with the decreased absorption into host or helping host to defeat against the pathological changes of stomach in CAG patients. However, up to nowadays, the relation of heptadecanoic acid and *Erysipelotrichaceae_UCG-003*, and pentadecanoic acid and *haemophilus*, has not been clarified yet. And we will further clarify the crosstalk between heptadecanoic acid and *Erysipelotrichaceae_UCG-003*, and pentadecanoic acid and *haemophilus*, and how to modulate the pathological process of CAG in the next study.

In conclusion, heptadecanoic acid, *Erysipelotrichaceae_UCG-003*, pentadecanoic acid, *haemophilus* were the potential biomarkers for CAG diagnosis in clinic. And heptadecanoic acid is the most potential biomarker for CAG diagnosis, and possibly involving in the pathological process of CAG. Furthermore, microbiome-metabolites crosstalk possibly involves in the pathological process of CAG, which should be further clarified and confirmed.

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

The studies involving human participants were reviewed and approved by Medical Ethical Committee of Shuguang Hospital.

The patients/participants provided their written informed consent to participate in this study. This study was approved by the Medical Ethical Committee of Shuguang Hospital (2020-834-41-01). All participants signed the informed consent.

Author contributions

XG, PQ, and BG did most of experiments and wrote the original draft. YZ and ZF did data analysis, diagnostic marker acquisition and ROC evaluation. DY, CZ, YC, JN, collected fecal samples and diagnostic information from subjects. JL guided on collection of stool samples. DY and CZ took part in the fecal microbial and metabolic data analysis, respectively. JZ, HS, and GL designed experiments and wrote the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the National Natural Science Foundation of China (81873236); Shanghai Municipal Natural Science Foundation (21ZR1462800); Shanghai Key Laboratory of Health Identification and Assessment (21DZ2271000); the Opening Project of Shanghai Key Laboratory of Compound Chinese Medicines (17DZ2273300).

Acknowledgments

We thanked Metabo-profile Biotechnology (Shanghai, China) for targeted fecal metabolomics profiling, data processing and metabolomic analysis.

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/fcimb.2022.1064737/full#supplementary-material

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 20 January 2023
DOI 10.3389/fcimb.2023.1091825

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 07 November 2022
ACCEPTED 03 January 2023
PUBLISHED 20 January 2023

CITATION
Ni B, Kong X, Yan Y, Fu B, Zhou F and Xu S (2023) Combined analysis of gut microbiome and serum metabolomics reveals novel biomarkers in patients with early-stage non-small cell lung cancer. *Front. Cell. Infect. Microbiol.* 13:1091825. doi: 10.3389/fcimb.2023.1091825

Combined analysis of gut microbiome and serum metabolomics reveals novel biomarkers in patients with early-stage non-small cell lung cancer

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Non-small cell lung cancer (NSCLC) is the predominant form of lung cancer and is one of the most fatal cancers worldwide. Recently, the International Association for the Study of Lung Cancer (IASLC) proposed a novel grading system based on the predominant and high-grade histological patterns for invasive pulmonary adenocarcinoma (IPA). To improve outcomes for NSCLC patients, we combined serum metabolomics and fecal microbiology to screen biomarkers in patients with early-stage NSCLC and identified characteristic microbial profiles in patients with different grades of IPA. 26 genera and 123 metabolites were significantly altered in the early-stage NSCLC patients. *Agathobacter*, *Blautia*, *Clostridium*, and *Muribaculacea* were more abundant in the early-stage NSCLC patients compared with healthy controls. For the different grades of IPA, the characteristic microorganisms are as follows: *Blautia* and *Marinobacter* in IPA grade type 1; *Dorea* in IPA grade type 2; and *Agathobacter* in IPA grade type 3. In the metabolome results, the early-stage NSCLC group mainly included higher levels of sphingolipids (D-erythro-sphingosine 1-phosphate, palmitoyl sphingomyelin), fatty acyl (Avocadyne 1-acetate, 12(S)-HETE, 20-Carboxy-Leukotriene B4, Thromboxane B3, 6-Keto-prostaglandin f1alpha, Sebacic acid, Tetradecanedioic acid) and glycerophospholipids (LPC 20:2, LPC 18:0, LPC 18:4, LPE 20:2, LPC 20:1, LPC 16:1, LPC 20:0, LPA 18:2, LPC 17:1, LPC 17:2, LPC 19:0). Dysregulation of pathways, such as sphingolipid metabolism and sphingolipid signaling pathway may become an emerging therapeutic strategy for early-NSCLC. Correlation analysis showed that gut microbiota and serum metabolic profiles were closely related, while *Muribaculacea* and *Clostridium* were the core genera. These findings provide new biomarkers for the diagnosis of early-stage NSCLC and the precise grading assessment of prognostic-related IPAs, which are of clinical importance and warrant further investigation of the underlying molecular mechanisms.

KEYWORDS

early-stage NSCLC, IPA, gut microbiome, serum metabolite, biomarkers

Introduction

Lung cancer, one of the deadliest malignancies, poses a huge threat to human health with increasing morbidity and mortality worldwide (Siegel et al., 2018). It consists of non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), with NSCLC being the most common form, accounting for more than 80% of lung cancers (Herbst et al., 2018; Duma et al., 2019). Lung adenocarcinoma (LADC), the most common pathologic type of NSCLC, is an important factor that discriminates patient prognosis (Travis et al., 2015). Recently, a novel grading system based on the predominant and high-grade histological patterns for invasive pulmonary adenocarcinoma (IPA) has been proposed by the International Association for the Study of Lung Cancer (IASLC) (Moreira et al., 2020). The model has consistently been found to correlate with prognosis and consists of: Grade 1: lepidic predominant tumor; Grade 2: acinar or papillary predominant tumor, both with no or less than 20% of high-grade patterns; and Grade 3: any tumor with 20% or more of high-grade patterns (solid, micropapillary and or complex gland). The established grading system is based on prognostic-related histological criteria and has utility and prognostic significance for IPA (Hou et al., 2022). Importantly, most lung cancer patients are initially diagnosed at an advanced stage of the disease, often with a poor prognosis. Therefore, developing biomarkers with high sensitivity and specificity to assess lung cancer progression and treatment effects will greatly improve disease management and patient survival.

The gut microbiome, recognized as the second genome of humans (Qin et al., 2010), has attracted considerable attention in recent decades. It contains more than 100 times genes than the human genome and performs key roles on human health. Dysregulation of the gut microbiota has been found to be associated with many cancers (Matson et al., 2018; Santoni et al., 2018), and disruption of metabolite balance caused by altered microbiome homeostasis may promote tumorigenesis. Recent studies have shown that the occurrence and development of NSCLC are also related to the human gut microbiota, and the interactions between these microbes can affect the function of multiple pathways including metabolism, inflammation, and immunity (Zhuang et al., 2019; Zheng et al., 2020; Lu et al., 2021; Zhao et al., 2021). These studies suggest that gut microbiota signatures have the potential to diagnose and assess the development and progression of non-small cell lung cancer.

Despite extensive progress in linking the gut microbiome to lung disease (the 'gut-lung axis') (Keely et al., 2012; Dumas et al., 2018; Zhang et al., 2020a), so far, the interactions between the gut microbiome and metabolome in patients with early-stage NSCLC have not been reported. Here, we recruited 43 patients with early-stage non-small cell lung cancer and 35 healthy individuals, and their stool and serum samples were tested and analyzed accordingly. Comparing the composition of gut microbiota and serum metabolites by bioinformatics analysis to search for early pathogenesis and potential biomarkers in patients with non-small cell lung cancer. On the other hand, we sought to link gut microbiota changes with a novel grading system for pulmonary adenocarcinoma, thereby providing a rationale for accurate diagnosis and typing of early-stage lung cancer.

Materials and methods

Study design and samples

A total of 78 participants who came to the Department of Thoracic Surgery of the Third Affiliated Hospital of Harbin Medical University were recruited, including 43 patients with early-stage non-small cell lung cancer and 35 healthy relatives of these patients (Table 1). Sixty-three serum (35 NSCLC and 28 healthy) and seventy-eight stool (43 NSCLC and 35 healthy) samples were collected. Fecal and serum samples were collected according to protocols approved by the local ethics committee, and written informed consent was obtained from all participants.

Collate clinical parameter information (including age, gender, body mass index (BMI), tumor stage, novel adenocarcinoma grade, smoking, family history, etc.), and exclude any unhealthy conditions by electrocardiogram and chest X-ray results. The main exclusion criteria were as follows: (1) ≤ 18 years old or > 80 years old, (2) individuals who had received antibiotics or probiotics in the past 3 months, (3) underlying diseases such as diabetes and hypertension.

Sample collection

Fecal and serum samples were collected in the morning after an overnight fast (≥8 h). The stool samples were divided into 3 equal parts (200 mg each), placed in sterile cryovials, and immediately transported to the laboratory for storage at -80°C. Blood samples were collected in coagulation tubes. After the blood was collected, the blood was gently mixed up and down for about ten times, and then centrifuged at 1800g for 10 minutes. The supernatant (serum) was collected in a 1.5 ml centrifuge tube, centrifuged at 13,000 g for 2 min, and the supernatant was transferred to a cryovial and stored at -80°C for further analysis.

DNA extraction

DNA from different samples was extracted using the CTAB according to manufacturer 's instructions. Analyze the integrity and fragment size of the extracted DNA using 1% agarose gel electrophoresis. And NanoDrop 2000 (Thermo Scientific, USA) was used to measure the extracted DNA quality.

16S rDNA sequencing

PCR amplification was performed using the following primers: 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC -3'). The 5' ends of the primers were tagged with specific barcodes per sample and sequencing universal primers. And then the PCR products were purified by AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) and quantified by Qubit (Invitrogen, USA). The amplicon pools were prepared for sequencing and the size and quantity of the amplicon library were assessed on Agilent 2100 Bioanalyzer (Agilent, USA) and with the Library Quantification Kit for Illumina

TABLE 1 Characteristics of health people and early-stage NSCLC patients.

Characteristics	Early-stage NSCLC (n = 43)	Healthy control (n = 35)	P value
Age (mean ± SD)	58.63 ± 9.92	55.8 ± 8.44	0.178
Male/female (No.)	18/25	18/17	0.399
BMI (kg/m^2) (mean ± SD)	24.99 ± 3.42	24.29 ± 2.82	0.323
Tumor type, n (%)			—
ADC	38 (88.37)	—	
SCC	5 (11.63)	—	
Disease stage, n (%)			—
0	1 (2.33)	—	
I	34 (79.07)	—	
II	8 (18.6)	—	
Novel IASLC grading of IPA, n (%)			—
I	7 (16.28)	—	
II	12 (27.91)	—	
III	10 (23.26)	—	
Smoking status, n (%)			0.172
Smoker	16	8	
Non-smoker	27	27	
Tumor metastasis, n (%)			—
Non-metastasis	43 (100)	—	
Metastasis	0	—	
Family history, n (%)			0.132
Yes	27	16	
No	16	19	

(Kapa Biosciences, Woburn, MA, USA), respectively. The libraries were sequenced on NovaSeq PE250 platform.

Microbiome data analysis

Paired-end reads were assigned to samples based on their unique barcodes and truncated by cutting off the barcode and primer sequence. Merge paired-end reads using FLASH (Reyon et al., 2012). Raw reads were quality filtered according to fqtrim (v0.94) under specific filter conditions to obtain high quality clean labels. Chimeric sequences were filtered using Vsearch software (v2.3.4) (Caporaso et al., 2010). After dereplication using DADA2, the representative sequence with single-base accuracy is obtained, that is, the ASV (Amplicon Sequence Variants) feature table and feature sequence. Alpha diversity and beta diversity were calculated by QIIME2 after random normalization to the same sequences, and the graphs were drawn by R package. Blast was used for sequence alignment, and the characteristic sequences of each representative sequence were annotated with the SILVA database. LEfSe (Segata et al., 2011) analysis and Wilcoxon rank sum tests were used to identify genera that were differentially abundant between groups of subjects. Other diagrams were implemented using the R package (v3.5.2) and GraphPad Prism software.

Analysis of serum samples

Metabolites in serum samples were extracted using 80% methanol buffer. 400 μL of pre-chilled 80% methanol was added to 100 μL of the sample, vortex for 1 min, incubated for 5 min at room temperature, then overnight at -20°C. After centrifugation at 4000 g for 20 min, the supernatant was transferred to a new 96-well plate. QC samples were prepared by pooling together 10 μL of each extract. Metabolites were stored at -80°C prior to liquid chromatography-mass spectrometry (LC-MS) analysis (Want et al., 2006; Barri and Dragsted, 2013).

Non-targeted metabolomics analysis

UHPLC-MS/MS analyses were performed using a Vanquish UHPLC system (Thermo Fisher, Germany) coupled with an

Orbitrap Q ExactiveTMHF-X mass spectrometer (Thermo Fisher, Germany). The sample was injected onto a Hypesil Gold column (100 × 2.1 mm, 1.9 μm) using a 12-minute linear gradient at a flow rate of 0.2 mL/min. The eluents for positive polarity mode were 0.1% formic acid–water (A) and methanol (B). The eluents used for negative polarity mode were 5 mM pH 9.0 ammonium acetate (A) and methanol (B). The Q ExactiveTM HF-X mass spectrometer was operated in positive/negative mode with a spray voltage of 3.5 kV, a capillary temperature of 320°C, a sheath gas flow of 35 psi, an auxiliary gas flow of 10 L/min, S-lens RF class 60, Auxiliary gas heater temperature 350°C.

Metabolomic data analysis

Statistical analysis was performed using statistical software R (R version R-3.4.3), Python (Python 2.7.6 version) and CentOS (CentOS 6.6 version). When the data were not normally distributed, area normalization was used for positive state transformation method.

These metabolites were annotated using the following databases: the KEGG database (https://www.genome.jp/kegg/pathway.html), the HMDB database (https://hmdb.ca/metabolites) and the LIPIDMaps database (http://www.lipidmaps.org/). Partial least squares discriminant analysis (PLS-DA) was performed in metaX (Wen et al., 2017). Metabolites with VIP > 1 and P value < 0.05 and fold change (FC) ≥ 1.2 or FC ≤ 0.833 were considered differential metabolites. Volcano plots were used to filter metabolites of interest based on log2 (fold change) ≥ 0.263 or log2 (fold change) ≤ -0.263, and -log10 (P-Value) metabolites from ggplot2 in R language. For cluster heatmaps, data were normalized using z-scores of regions of differential metabolite intensity and plotted in R by the heatmap package. The functions of these metabolites and metabolic pathways were investigated using the KEGG database. Metabolic pathway enrichment of differential metabolites was carried out.

Statistical analysis

Patient characteristics were expressed as mean ± standard deviation (SD), differences between groups were compared using the χ^2 test or independent samples *t*-test. Wilcoxon rank-sum test (for two groups) and Kruskal-Wallis test (for more than two groups) were used to compare differences among microbial groups. Student's *t*-test and fold change analysis were used to compare metabolites between groups. The relationship between microorganisms and metabolites was assessed using Spearman rank correlation analysis. Values of $P < 0.05$ were considered as statistically significant.

Results

Gut microbial profile of early-stage NSCLC patients

To determine whether gut microbial changes were associated with early-stage NSCLC, we examined different groups of fecal microbiome samples, including 43 NSCLC patients and 35 healthy individuals, by 16S rRNA gene sequencing. All patients with non-small cell lung cancer are in the early stage and have not developed distant metastasis, including stage 0 (adenocarcinoma in situ, AIS) (2.33%), stage I (79.07%), and stage II (18.6%). The detailed clinical characteristics of all participants are shown in Table 1. There were no significant differences in age, gender, smoking status body mass index (BMI) and family history between the two groups ($P > 0.05$).

Using amplicon sequence variants (ASVs) to track the dynamics of bacterial abundance in feces from different groups, Venn plots visualized the number of ASVs shared and unique between the healthy control (HC) group and the early-stage NSCLC group (Figure 1A). We found the two groups shared 1821 ASVs, and the early-stage NSCLC group had more unique ASVs than HC group (Supplementary Table 1). And then we analyzed the community structure of gut microbes (Supplementary Table 2). At the phylum level, *Firmicutes*, *Bacteroidota*, *Proteobacteria*, and *Actinobacteriota* were the main components in both the HC group and early-stage NSCLC group, with the abundance of *Firmicutes* and *Proteobacteria* being higher in the NSCLC group (Figure 1B). At the family level, compared to the HC group, the abundance of *Lachnospiraceae*, *Bacteroidaceae*, and *Enterobacteriaceae* were higher in early-stage NSCLC group, while the abundance of *Bifidobacteriaceae*, *Prevotellaceae* and *Veillonellaceae* were lower (Figure 1C). At the genus level, apart from the similar abundance of *Faecalibacterium* in both HC group and NSCLC group, *Bacteroides* and *Escherichia-Shigella* were slightly more abundant in early-stage NSCLC group, while *Bifidobacterium*, *Megamonas*, *Prevotella_9* and *Dialister* were relatively lower (Figure 1D).

Next, statistical analysis of microbial abundance was performed. Both early-stage NSCLC group and HC group showed comparable numbers of observed OTUs (operational taxonomic units). The Shannon and Simpson indexes both showed that community diversity was similar among the two groups. The Chao1 index showed no significant differences in community richness between early-stage NSCLC group and HC group. These data suggest that global community alpha diversity is similar between early-stage NSCLC group and HC group (Figure 2A). When comparing microbial community structure, beta diversity showed differences between the two groups (Figure 2B).

Specific gut microbiome signatures in early-stage NSCLC patients

We next compared gut microbes with significant differences in expression abundance between HC and early-stage NSCLC groups at the phylum and genus levels. In total, 1 phylum and 12 genera were significantly decreased in the abundance of early-stage NSCLC patients (Figure 3B), while 14 genera were conversely enriched (Figure 3A). *Desulfobacterota*, the only phylum with significant differences between these two groups, was more abundant in the HC group. At genus level, *Agathobacter*, *Blautia*, *Clostridium*, an uncharacterized genus of family *Muribaculacea*, *Cetobacterium*, an uncharacterized genus of family *Pasteurellaceae* and eight other genera were significant abundant in early-stage NSCLC than in HC group, whereas *Lanchnoclostridium*, *Prevotella*, *Lachnospia*, *Catenibacterium*, *Oscillospira*, *UGG-003*, *Lachnospiaceae_UGG-010*,

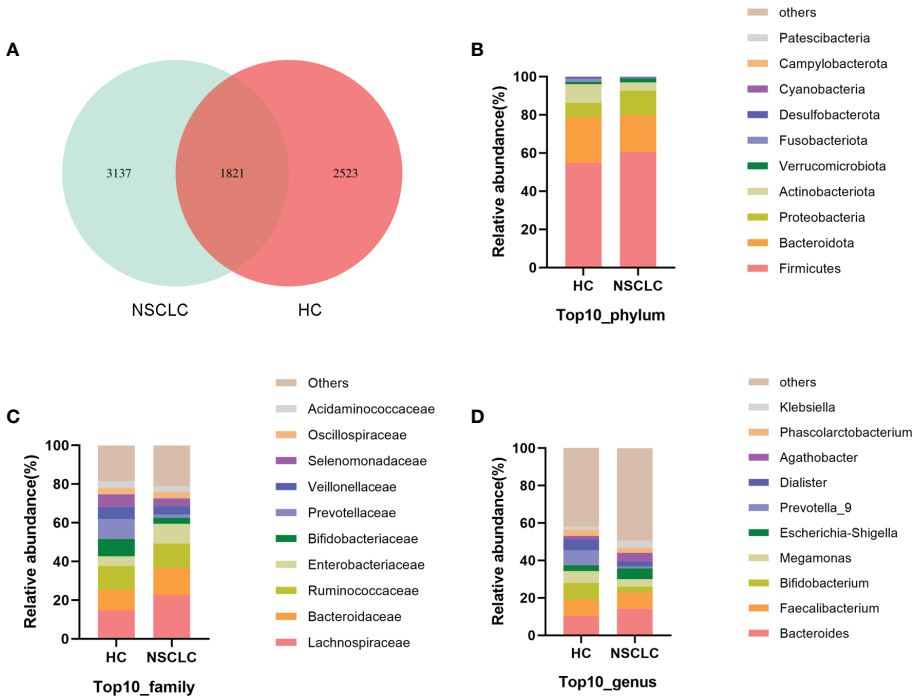

FIGURE 1
The characteristics of gut microbiota community structure **(A)** The Venn diagram shows unique and common ASVs in early-stage NSCLC and HC. **(B–D)** The top 10 representative species and their proportions in the two groups at the level of phylum **(B)**, species **(C)**, and genus **(D)**.

an uncharacterized genus of family *vandinBe97*, *Acidaminococcus*, *Prevotellaceae_NK3B31*_group, *Oxalobacter* were significantly enriched in the HC group.

Linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al., 2011) was then used to generate cladograms to reveal differences in taxa abundance between early-stage NSCLC and HC (Figure 4A). There were 25 and 8 bacterial taxonomic clades that were significantly different in HC and early-stage NSCLC groups, respectively [log10 (LDA score) > 3] (Figure 4B). We found that *Clostridia* class was significantly higher in the early-stage NSCLC group. *Agathobacter* and *Blautia* were the prominent gene level biomarkers for early-stage NSCLC group. For healthy controls, the *Desulfovibrionia* and *Negativicutes* were the abundant class, and *Prevotella_9*, *Prevotella*, *Lachnospira* and *Catenibacterium* were the most prominent genus level biomarkers. Overall, these findings demonstrated that the early-stage NSCLC group had relatively lower microbial abundance than the HC group and was sufficient enough to distinguish healthy individuals from early-stage NSCLC patients.

Gut microbial compositions correlate with a novel grading system of IPA

Adenocarcinoma was the main type of pathology in NSCLC (38/43, 88.37%). In this study, after ruled out adenocarcinoma *in situ* (1 case) and minimally invasive adenocarcinoma (8 cases), we classified invasive pulmonary adenocarcinoma (IPA) into three groups (grade 1, n =7; grade 2, n =12; grade 3, n =10) according to the new grading

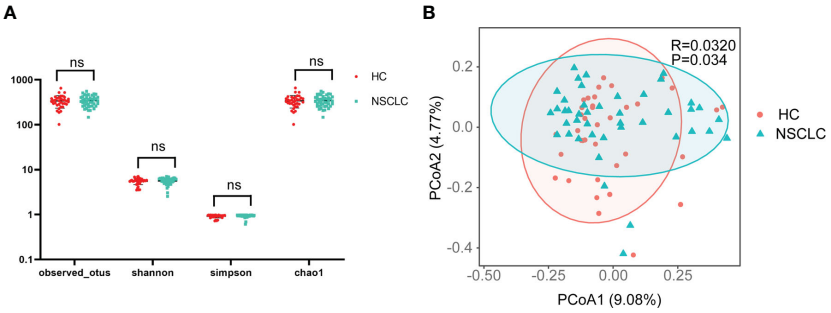

FIGURE 2
Comparison of α- and β-diversity of gut microbiota in HC and early-stage NSCLC groups **(A)** Differences in α diversity between early-stage NSCLC and HC based on the observed outs, shannon, simpson and chao1. **(B)** PCoA shows β diversity differences between the two groups (Bray-Curtis, R = 0.032, P< 0.05).

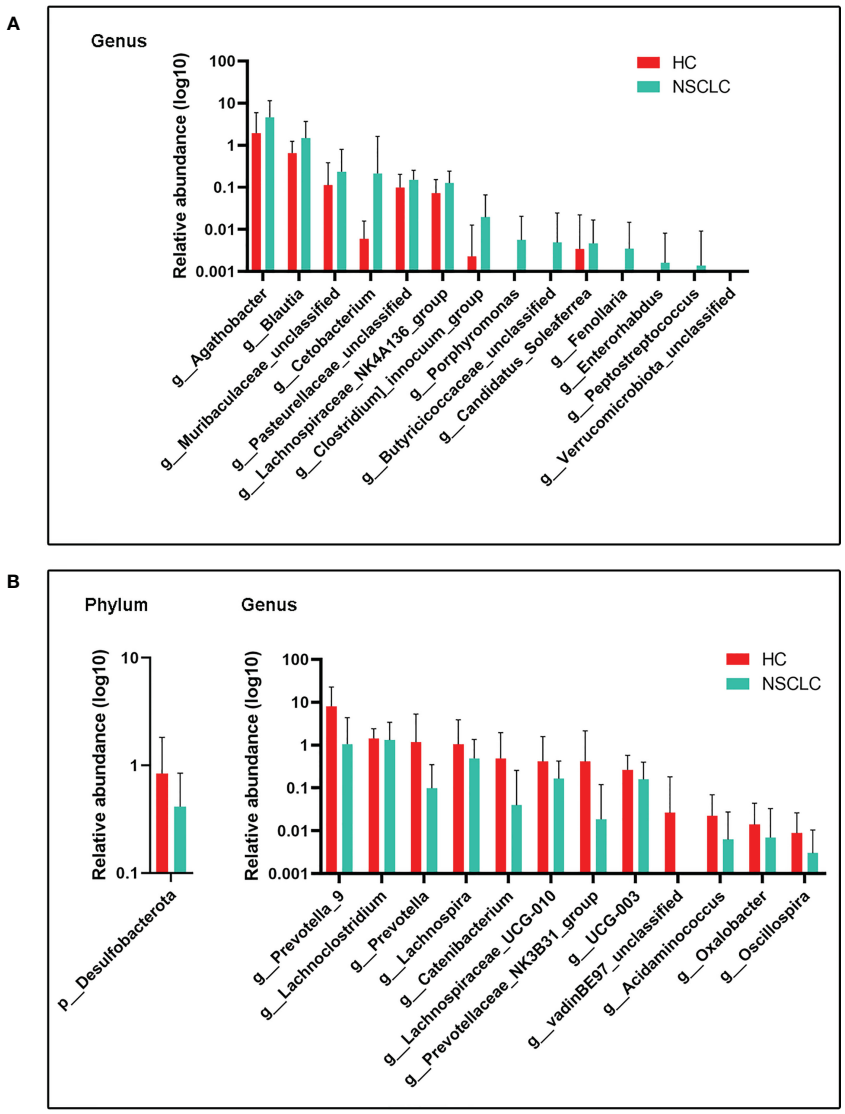

FIGURE 3
Differences in gut microbiota abundance between early-stage NSCLC and HC **(A)** Increased microbiota abundance in early-stage NSCLC at the genus level (P< 0.05). **(B)** Decreased microbiota abundance in early-stage NSCLC at the phylum and genus levels (P< 0.05). P values were calculated using the two-tailed Wilcoxon rank-sum test.

system proposed by the IASLC (Moreira et al., 2020; Deng et al., 2021). Differences in microbial composition between healthy patients and IPA patients with different grades under the new grading system were analyzed at the phylum and species levels (Supplementary Figure 2). Relative abundance analysis showed clear distinctions between high and low abundance taxa, and used color gradients to reflect similarities and differences in the composition of multiple samples at each taxonomic level. As shown in Supplementary Figure 2, according to the change of the color gradient, the differences between the four groups of samples can be seen intuitively. The data showed that the dominant flora of IPA patients in each group was different from that of healthy people, suggesting a correlation between the florae features and the histopathological process of invasive lung adenocarcinoma.

Next, we analyzed biomarkers between IPA patients with different grades and healthy controls by multi-level LEfSe (Figure 4C). There were significant differences in 7, 7, 3, and 4 bacterial taxonomic clades in the healthy group and in the invasive pulmonary adenocarcinoma grades type 1, type 2, and type 3 group, respectively [log10 (LDA score) > 3] (Figure 4D). The key species were *Erysipelatoclostridium* in HC; *Blautia* and *Marinobacter* in IPA grade type 1; *Dorea* in IPA grade type 2; and *Agathobacter* in IPA grade type 3. These results showed that the fecal gut microbiota was specific for a novel graded type of invasive lung adenocarcinoma.

General overview of the serum metabolome

Previous studies have revealed that gut microbiota has a significant impact on blood metabolite profiles (Wikoff et al., 2009; Wilmanski et al., 2019). To further explore changes in gut microbe-host interactions, we performed LC-MS/MS-based non-targeted metabolomic analysis of serum from healthy individuals and patients with early-stage NSCLC. A total of 866 metabolites were

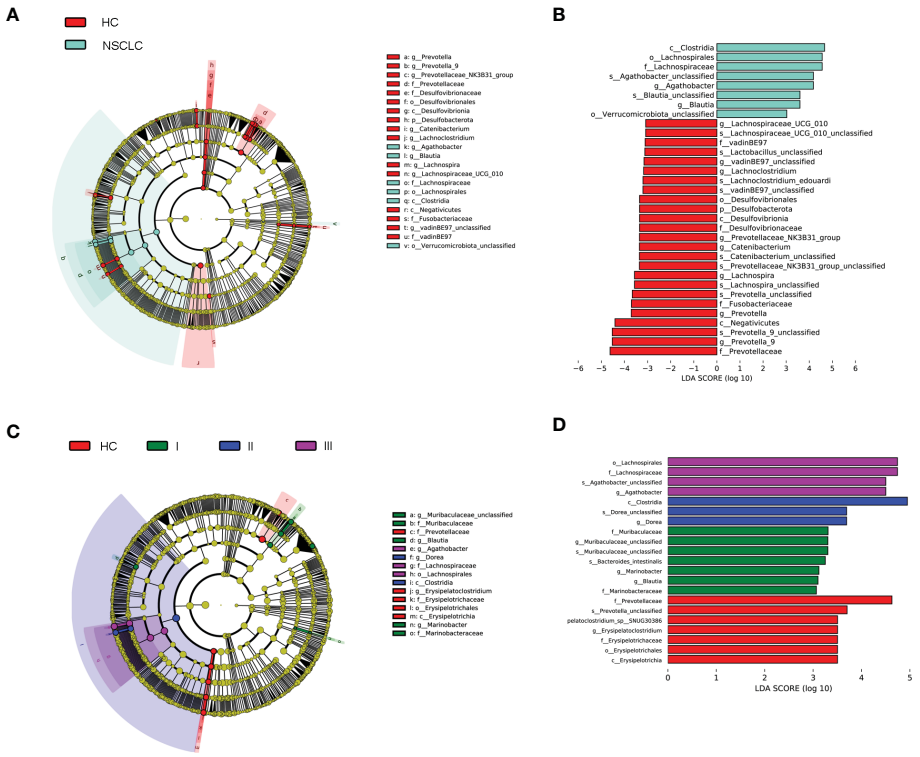

FIGURE 4
Linear discriminant analysis (LDA) combined with effect size (LEfSe). **(A)** Cladograms of the phylogenetic distribution of the microbiota with significant differences between early-stage NSCLC and HC analyzed by LefSe. **(B)** Histogram of the distribution of LDA values for LEfSe analysis of intestinal flora in the two groups (LAD score ≥ 3). **(C)** Cladograms of the phylogenetic distribution of the microbiota with significant differences across IPA grade I, grad II, grade III and HC analyzed by LefSe. **(D)** Histogram of the distribution of LDA values for LEfSe analysis of intestinal flora in four groups of samples (LAD score ≥ 3). The listed bacterial floras are significantly gathered for their respective groups ($P < 0.05$, Kruskal-Wallis test).

identified and quantified, including 553 positive ions and 313 negative ions (Supplementary Table 3).

Differentially abundant metabolites between HC and early-stage NSCLC groups

Supervised multivariate statistical analysis using partial least squares discriminant analysis (PLS-DA) to maximize screening for differential metabolites across groups. The PLS-DA score plot showed a clear separation between the HC group and the early-stage NSCLC group (Figure 5A). Permutation tests indicated that the data were not overfit, the R2Y and Q2 values were 0.87 and -0.4, respectively, validating the OPLS-DA model (Figure 5B). The differentially expressed metabolic ions are screened by p value of the t-test and variable difference contribution (VIP), where VIP≥1.0, P< 0.05 as the filter condition. A total of 123 different metabolites were identified in serum between the HC and early-stage NSCLC groups (Supplementary Table 4), most of which were upregulated. Figures 6 revealed the changes in these metabolites. In the HC group, the abundant

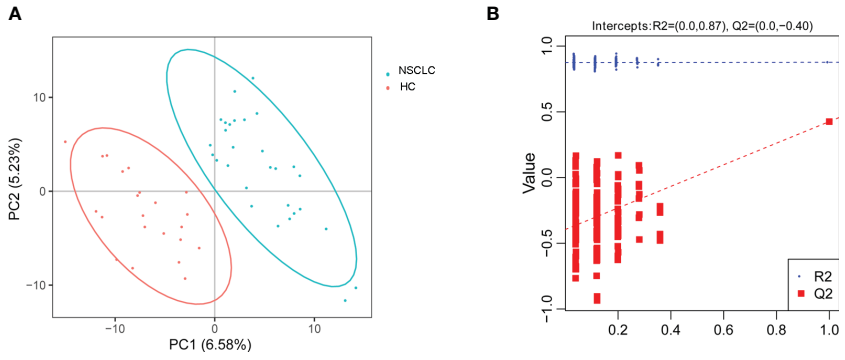

FIGURE 5
Principal component analysis. **(A)** PLS-DA score plot shows the difference in metabolites between groups. **(B)** Comparison of real and permuted model parameters in validation tests.

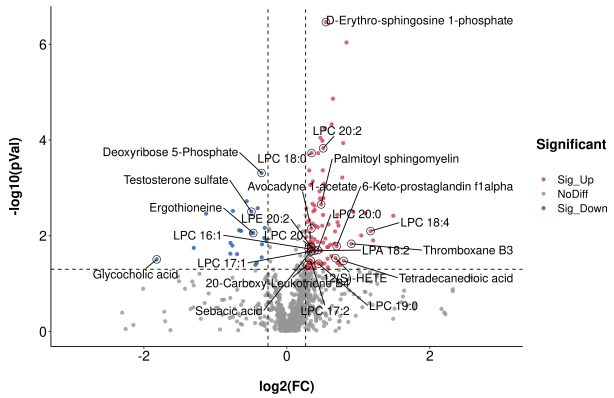

FIGURE 6
The significant different metabolites between early-stage NSCLC and HC group by Volcano plot Blue dots on the left are metabolites down-regulated in NSCLC vs HC, red dots on the right represent metabolites up-regulated in NSCLC vs HC, grey dots are metabolites that are not significantly different.

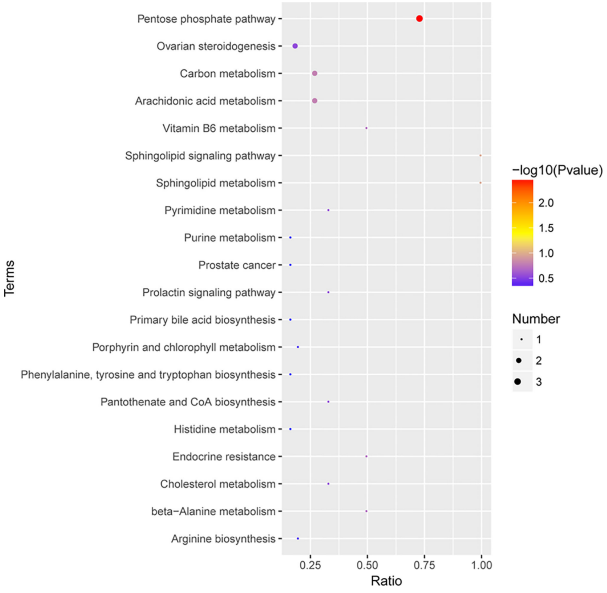

FIGURE 7
The KEGG pathway enrichment scatter plot displays important discriminatory metabolic processes of early-stage NSCLC vs HC patients.

metabolites mainly included carbohydrates and carbohydrate conjugates (Deoxyribose 5-phosphate), steroids and steroid derivatives (Testosterone sulfate, Glycocholic acid, D-erythrose 4-phosphate) and amino acids, peptides and analogs (Ergothioneine). In contrast, metabolites with higher levels in the early-stage NSCLC group mainly included sphingolipids (D-erythro-sphingosine 1-phosphate, palmitoyl sphingomyelin), fatty acyl (Avocadyne 1-acetate, 12(S)-HETE, 20-Carboxy-Leukotriene B4, Thromboxane B3, 6-Keto-prostaglandin f1alpha, Sebacic acid, Tetradecanedioic acid) and glycerophospholipids (LPC 20:2, LPC 18:0, LPC 18:4, LPE 20:2, LPC 20:1, LPC 16:1, LPC 20:0, LPA 18:2, LPC 17:1, LPC 17:2, LPC 19:0). In order to show the relationship between samples and the expression differences of metabolites in the two groups more intuitively, we performed hierarchical clustering analysis, and the results for the top 50 metabolites of p values with significant differential expression were shown in Supplementary Figure 3.

The KEGG pathway enrichment analysis was then performed on the differentially abundant metabolites (Supplementary Table 5). The results showed that the differential metabolites of early-stage NSCLC and HC were mainly involved 20 pathways (Figure 7), including sphingolipid metabolism, sphingolipid signaling pathway, primary bile acid biosynthesis, the pentose phosphate pathway, carbon metabolism, arginine biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis, etc.

Multi-omics analysis revealed differences between HC and early-stage NSCLC groups

To further investigate microbiota-metabolite interactions associated with early-stage NSCLC, we assessed correlations between 27 genera and 32 metabolites (Figure 8A; Supplementary Table 6). The results showed that the abundance of several microbial genera in the early-stage NSCLC group were positively correlated with serum metabolite levels (Sperman's correlation analysis, $P<0.05$, Figure 8A). Then, based on the above microbiome data, a co-occurrence network was constructed to elucidate the major interactions between the early-stage NSCLC associated microbiome and metabolites (Figure 8B). The results showed correlations between *Muribaculaceae*, *Clostridium*, *Blautia*, *Agathobacter* and the related metabolites. From the graph, *Muribaculacea* and *Clostridium* seemed to be the core genera given that they were positively correlated with metabolites enriched in early-stage NSCLC and negatively correlated with certain metabolites enriched in HC (eg, Deoxyribose 5-Phosphate and Testosterone sulfate).

Discussion

Lung cancer is the malignant tumor with the highest morbidity and mortality worldwide. NSCLC, the most common form of lung cancer, has a poor prognosis mainly because it is diagnosed at an advanced stage. One way to improve outcomes for patients with NSCLC is early diagnosis. With the development of imaging technologies such as CT imaging, positron emission tomography-computed tomography (PET-CT), and magnetic resonance imaging (MRI), the detection rate of early-stage NSCLC has increased significantly. However, no effective early-stage NSCLC biomarkers are currently available. In this study, we explored the changes in gut microbiota and serum metabolic profiles of patients with early-stage NSCLC, and combined these two omics to search for possible pathogenesis and potential biomarkers. At the same time, we also identified for the first time the characteristics of the intestinal flora of lung adenocarcinoma patients with different grades under the new grading system (Moreira et al., 2020; Deng et al., 2021), which is of great significance for precise treatment and control of prognosis.

Changes in gut flora abundance are closely related to the occurrence and development of cancer (Schwabe and Jobin, 2013; Garrett, 2015). In the present study, we provided evidence that early-stage NSCLC patients have lower abundances of *Bacteroidota* and

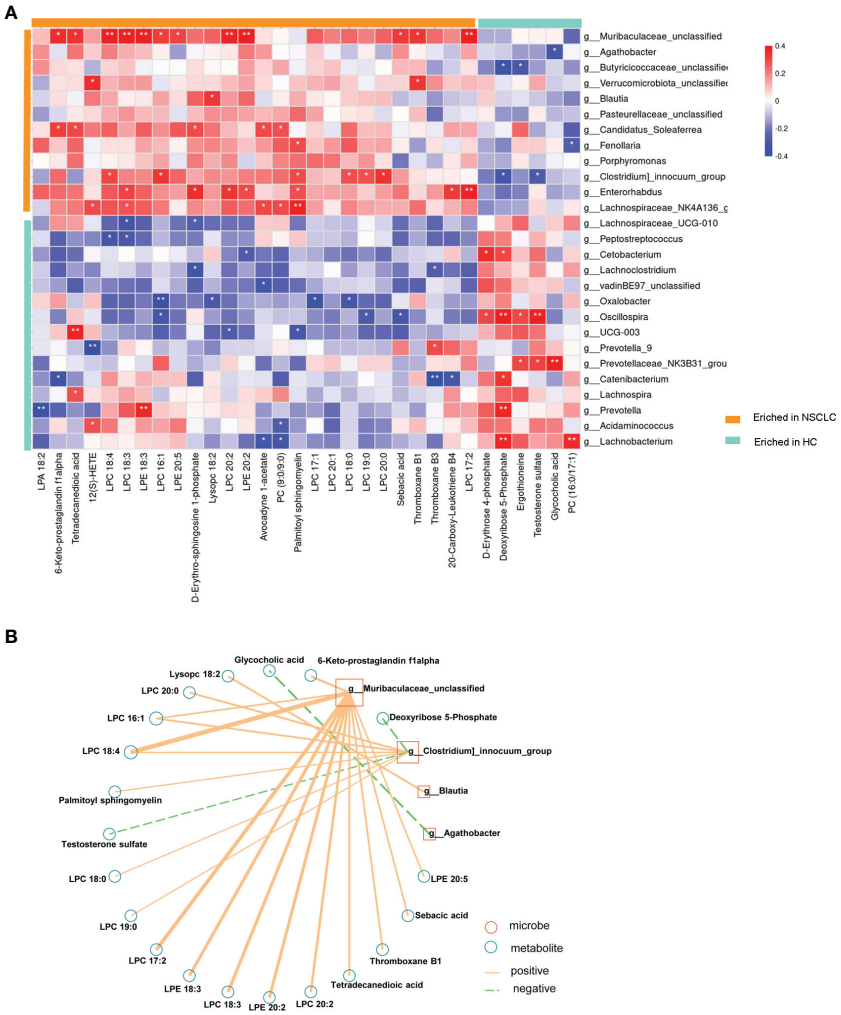

FIGURE 8
Multi-omics approaches revealed microbiota-metabolite interactions in early-stage NSCLC patients Heatmap demonstrates the correlations between 27 differentially abundant genera and 32 differentially abundant metabolites (Spearman's correlation analysis). P-value, *p<0.05; **p<0.01; ***p<0.001 **(B)** Early-stage NSCLC associated networks based on integrated fecal microbiome and serum metabolome.

Actinobacteriota, while relatively higher abundances of *Firmicutes* and *Proteobacteria* (Figure 1B), suggesting the potential links between gut bacteria and early-stage NSCLC. In general, dysregulation of gastrointestinal metabolism is repeatedly associated with a decreased *Firmicutes*/*Bacteroidota* ratio (Etxeberria et al., 2015; Li et al., 2018), which was found opposite in our study. We attempted to identify the reasons for the differences from the populations included and the experimental design. Since our subjects were all newly diagnosed and treated patients and their relatives represented healthy controls, the influence of genetic factors was excluded. We consider that the differences may be due to subject selection criteria or patient heterogeneity. On the other hand, previous studies included lung cancer patients with multiple pathological types (such as small cell lung cancer, NSCLC, etc.) or with different stages (such as advanced lung cancer, etc.). While in this study, we focused on exploring the gut microbiota of patients with early-stage NSCLC.

In the search for key discriminating microorganisms, it was found that *Agathobacter* and *Blautia* were the prominent differential genera of early-stage NSCLC (Figure 4B). Previous clinical studies have shown that two butyrate-producing gut bacteria (*Agathobacter* and *Blautia*) can favorably modulate the host immune response, were enriched in advanced NSCLC patients with better prognosis, and could become potential biomarkers for metastatic NSCLC patients treated with immune checkpoint inhibitors (Hakozaki et al., 2020; Martini et al., 2022). Consistently, *Clostridia*, another significantly genus increased in early-stage NSCLC, is thought to produce short-chain fatty acids (SCFAs) that provide essential nutrients and energy to colonic epithelial cells, induce regulatory T cells, and have anti-inflammatory effects by enhancing epithelial barrier integrity (Scaldaferri et al., 2013). All these indicated that the gut microbiota of patients with early-stage NSCLC is closely related to host immunity. Since multiple gut microbiota can disrupt host homeostasis by affecting the level of the host immune system, changes in this balance will lead to chronic inflammation and immune-related diseases, thereby promoting or attenuating the carcinogenic process (Kau et al., 2011; Zeng et al., 2016; Khan et al., 2020). In our study, the subjects were all early-stage NSCLC patients, and the abundance of these immune-related gut microbiota was significantly increased, which may slow down the further development of NSCLC by modulating the host immune system.

Correspondingly, the key differential genus in HC were *Prevotella_9*, *Prevotella*, *Lachnospira* and *Catenibacterium* (Figure 4B). *Prevotella* belongs to the *Prevotaceae* family of the genus *Bacteroides*, which has a variety of bacterial species and is the dominant genus in the human gut. Furthermore, studies have shown that *Prevotella* decreases with lung cancer progression (Qin et al., 2022) and that changes in NSCLC patients are associated with response to immunotherapy (Jin et al., 2019b). *Lachnospira* was reported as a "favorable" gut microbiome that protects the host from cancer by producing butyrate, which plays an important role in suppressing tumor growth, regulating immunity, and participating in anti-inflammatory responses (Daniel et al., 2017). Previous studies found that both *Prevotella* and *Lachnospira* were decreased in lung cancer patients (Liu et al., 2019; Zhang et al., 2019; Qin et al., 2022), which is consistent with our findings (Figure 3B). These results suggested that tumor development is intricately linked to the immune system (Hanahan and Weinberg, 2011), and that carcinogenesis is often caused by dysbiosis rather than by the activity of specific pathogenic microorganisms (Matson et al., 2018; Jin et al., 2019a).

Adenocarcinoma is the most common pathological type in NSCLC. A novel grading system for lung adenocarcinoma proposed by IASLC will help identify prognostic groups and provide a common approach to prognostic stratification of lung adenocarcinoma patients who may benefit from emerging management and treatment options. This study was the first to identify specific gut microbiota in patients with different grades of invasive lung adenocarcinoma (Figures 4C, D), which is of great help in understanding cancer progression and prognosis in patients with lung adenocarcinoma more accurately.

Differences in the microbiome may not be used to clearly explain the role of the microbiome in health and disease (Integrative, 2014). Therefore, the use of a prospective multi-omics approach combined with comprehensive analysis of microbes and metabolites may be a way to reveal disease pathogenesis. In this study, compared with healthy people, serum glycerophospholipids (eg: LPC 20:2, LPC 18:0, LPC 18:4, LPE 20:2, LPC 20:1, LPC 16:1, LPC 20:0, LPA 18:2, etc.) were significantly higher in early-stage NSCLC patients (Figure 6). Glycerophospholipids are one of the main components of cell membranes, and are involved in many important life processes such as cell transmembrane transport, energy metabolism, signal transduction and cancer development (Lee et al., 2012; Santos and Schulze, 2012). High serum phospholipids and fatty acids in lung cancer patients have been previously reported (Ros-Mazurczyk et al., 2017; Zhang et al., 2020b), and our findings were consistent with previous studies (Zhao et al., 2021). Analysis of pathway enrichment using differential metabolites found that sphingolipid metabolism and sphingolipid signaling pathways were enriched in early-stage NSCLC vs HC. Sphingolipid metabolism has been shown to be the most dysregulated pathway in NSCLC patients (Petrache and Berdyshev, 2016), and alterations in gene expression patterns in this metabolic pathway were found to be strongly associated with poor prognosis in NSCLC patients (Meng et al., 2021). Sphingolipids (D-erythrosphingosine 1-phosphate and palmitoyl sphingomyelin), the metabolites significantly upregulated in early-stage NSCLC (Figure 7), can regulate various biological processes by controlling the signaling functions in cancer cell signaling networks, such as growth, proliferation, migration, invasion and/or metastasis (Hannun and Bell, 1987; Dressler et al., 1992). Currently, emerging therapeutic strategies targeting enzymes involved in sphingolipid metabolism and/or signaling for cancer therapy are presented. Furthermore, our results found primary bile acid biosynthesis and bile secretion pathways enriched in early-stage NSCLC vs HC (Figure 7). Disorders of bile acid metabolism have been shown to be associated with poor prognosis and promote the further development of aggressive lung adenocarcinoma (Nie et al., 2021). In this study, a multi-omics analysis of changes in the microbiome and metabolome was performed, and it was found that the abundance of gut microbiota was closely related to serum metabolic activity. For example, *Clostricium*, one of the genera with significant higher abundance in the early-stage NSCLC group in the LEfSe analysis (Figure 4B), was positively correlated with multiple glycerophospholipid (LPC 20:0, LPC 16:1, LPC 18:4, LPC 18:0, LPC 19:0). Moreover, *Muribaculaceae*, another characteristic microorganism of early-stage NSCLC (Figure 3A), was found to be associated with various phospholipids (LPC 18:4, LPC 17:2, LPE 20:2, LPC 20:2, etc.) and faty acyl (Sebacic acid, Tetradecanedioic acid). Previous studies found that *Muribaculaceae* is an important predictor of intestinal short-chain fatty acid concentration and that its acetate products regulate animal fat metabolism (Ormerod et al., 2016; Smith et al., 2019). These findings have potential clinical implications for patients with early-stage NSCLC.

Conclusion

In summary, our results suggested that abnormalities in gut microbiota and metabolomics are closely related to the occurrence and development of early-stage NSCLC. Our multi-omics analysis further discovered the possible relationship between certain gut microbiota and serum phospholipids and fatty acids in early-stage NSCLC patients, and provided a basis for future research on the pathogenesis and treatment of NSCLC. It is worth mentioning that this study has the following limitations. First of all, our sample size for IPA grading is relatively small, and more sample data is needed to support it. On the other hand, most of the patients in this study were from northeastern China, which may have a certain impact on the progression of lung disease due to the colder regions and poor air quality in winter. In addition, the microbiome of this study was based on 16sRNA gene sequencing, which may be less comprehensive than metagenomic sequencing. Importantly, more later functional experiments are needed to further verify the possible targets screened in this study, so as to provide a stronger theoretical basis for the screening of early-stage NSCLC targets.

Data availability statement

The datasets presented in this study are deposited in online repositories. The names of the repositories and accession numbers can be found below: https://data.mendeley.com/datasets/hw576fjg9s; https://data.mendeley.com/datasets/fpnmx3f5tp.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of the Third Affiliated Hospital of Harbin Medical University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

BN and SX contributed to conception and design of the study. BN, XK, and YY performed the experiment and statistical analysis. BN wrote the draft of the manuscript. SX helped revise the manuscript. BF and FZ helped perform the analysis with constructive discussions. All authors contributed to manuscript revision and approved the submitted version.

Acknowledgments

We thank all the participating patients and healthy volunteers in this study. We thank LC-Bio Technology Co., Ltd, Hangzhou, Zhejiang Province, China for their technical support for this study.

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/fcimb.2023.1091825/full#supplementary-material

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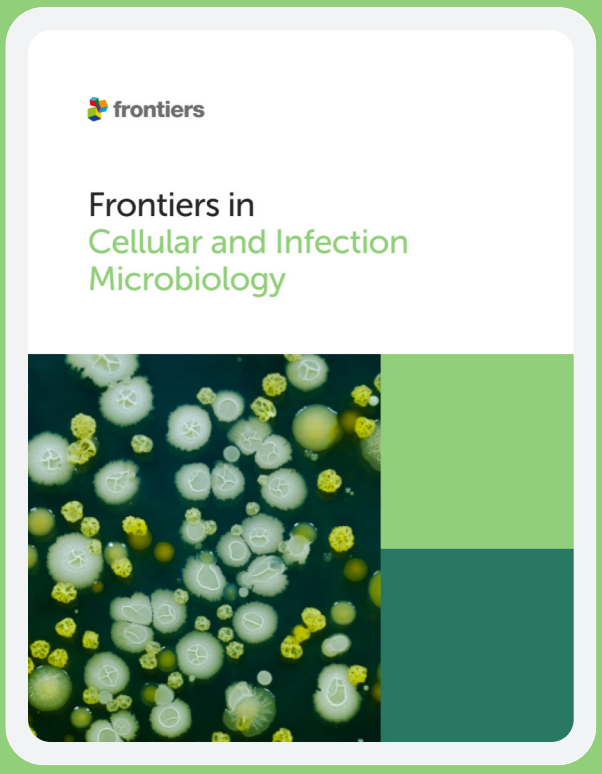

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