

Differences in gut microbiota and serum Trimethylamine N-oxide (TMAO) levels in patients with colorectal cancer with a small, nested case-control study

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Abstract

The dysbiosis of the gut microbiota caused by drug metabolism and diets may influence the gastrointestinal (GI) barrier and their ability for normal attachment and further immunity system, which all could be associated with the medical efficacy of colorectal cancer (CRC) during chemotherapy.

This study aims to investigate Trimethylamine N-oxide (TMAO), a gastrointestinal product readily entering the bloodstream, as a potential risk factor for various diseases, including CRC.

To investigate the relationship between gut microbiota dysbiosis and carcinogenesis in CRC patients, we analyzed taxonomic alterations in the gut microbiota of 77 subjects, including 36 CRC patients and 41 normal controls. We collected samples of the participants' microbiome from their fecal material and utilized 16S rRNA sequencing to identify the microbial composition. Additionally, to predict the functions of the GI microbiota, Phylogenetic Investigation of Communities by Reconstructing Unobserved States (PICRUSt) was employed. This could serve as a promising biomarker for colorectal cancer. Moreover, the serum level of TMAO between the CRC and healthy controls was also compared, and it was observed that the intestinal microbiome was changed in CRC patients; however, the serum level of TMAO was not correlated with the progression of CRC.

In conclusion, in the present study, instead of relying solely on TMAO, which is a convenient clinical test, we focused on the treatment of CRC by emphasizing the modification of the intestinal microbiome.

Keywords: CRC, Intestinal microbiome, TMAO, 16s rRNA, PICRUSt

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Introduction

This study investigates the gut microbiota and Trimethylamine N-oxide (TMAO) levels in Colorectal cancer (CRC) patients, probing their roles in CRC pathogenesis. CRC, the second most deadly malignancy, will be even more common in the coming decades in developed countries. This is associated with an increasingly sedentary lifestyle, industry-processed food, smoking, heavy alcohol consumption, high-fat diets, low-fiber, overweight, and other environmental factors (Guo et al., 2021; Leng et al., 2021). Despite recent monoclonal antibody therapy advances, the overall 5-year relative survival rate of CRC is less than 10% (Chang et al., 2021; Chen and Alexanderson, 2021; He et al., 2021; Hozhabri et al., 2021). Hence, obtaining novel and helpful approaches for the medication for CRC patients is imperative. Previous studies have proposed that one of the etiologies of CRC is associated with enterobacteria (Dohlman et al., 2021; Martins et al., 2021; Zhang et al., 2019; Zhang et al., 2021a; Zheng et al., 2020; Zhou et al., 2020). Intestinal dysbacteriosis brings about abnormal changes in the gut microbiota and may induce aberrant immune cell function and metabolism in the colorectal tissues, which may further trigger the chronic inflammatory condition and consequentially lead to the epithelial-mesenchymal transition (EMT), and finally, tumors and metastasis (Liu et al., 2020; Wirth et al., 2020; Yi et al., 2021). Therefore, alleviating the intestinal dysbacteriosis condition may be a new approach to improve the treatment of CRC, especially in the early stages.

In recent years, phosphatidylcholine, betaine, and carnitine were found to be metabolized to TMAO by GI microbiota and hepatic FMO3: flavin-containing monooxygenase-3 (Brown and Hazen, 2018; Wu et al., 2020; Zhang et al., 2021b). TMAO is a gut bacterial metabolite that easily circulates into the bloodstream. The TMAO level in plasma depends on liver FMO3 activity and gut microbial flora, which is influenced by drug administration. The correlation between elevated plasma levels of TMAO and an increased risk for adverse cardiovascular events was reported (Brown and Hazen, 2018). TMAO is involved in cholesterol accumulation and the conversion of foam cells from macrophages, which may potentially build up plaques on the blood vessel's endothelium, thus contributing to atherosclerosis, stroke, infarction, and other

cardiovascular diseases (Restini et al., 2021; Wu et al., 2020). Some studies have already suggested a link between TMAO and the risk of several cancers (Govindarajulu et al., 2020; Khan and Nayeem, 2020; Liu et al., 2017; Oellgaard et al., 2017). TMAO has been reported to be implicated in CRC in recent years, even though the mechanisms or pathways involved in TMAO-induced CRC are unknown. TMAO has been found to play a direct role in the progression of liver fibrosis and impaired liver function in certain animal models. Given the current knowledge about the connection between the gut microbiota and the development of cancer, specifically colorectal cancer (CRC), researchers have been concentrating their efforts on investigating whether metabolites produced by the gut microbiota, such as TMAO, can alter cell properties and lead to cancer development (Bae et al., 2014). Current evidence showed that TMAO increased the expression of vascular endothelial growth factor (VEGF) A in CRC HCT-116 cells and promoted tumor growth (Yang et al., 2022), indicating cell proliferation through inflammatory factors. Since TMAO is directly released into the circulatory system, it may eventually lead to renal elimination (Janeiro et al., 2018), so TMAO could be a diagnosis biomarker. However, there are also opposite studies showing TMAO is not associated with CRC. Guertin KA et al. reported that the serum TMAO, carnitine, or betaine were not associated with colorectal cancer risk; at least, it was not statistically significant through a nested study by comparison of 644 CRC and 644 health control (Guertin et al., 2017).

TMAO can be absorbed by extrahepatic tissues or excreted in urine in normal conditions. However, this can be changed when excessive TMAO enters serum, which can significantly lead to oxygen-antioxygen imbalance. There is more and more evidence that TMAO, under certain inflammatory conditions, may increase reactive oxygen species (ROS), oxidative stress, DNA damage, and protein misfolding in the intestines. Thus, TMAO could be regarded as a perpetrator rather than a protector, at least most likely in CRC patients.

The primary objective of this study is to investigate the roles of gut microbiota and TMAO in the pathogenesis of CRC. We hypothesized that alterations in the gut microbiota and elevated levels of TMAO are significant contributors to CRC development. This study explored the relationship between GI microbiome and TMAO in the intestinal



dysbacteriosis of patients with CRC. Its clinical significance within the Chinese ethnic population was explored, which may provide a novel mechanism and alternative therapeutic strategy for patients with CRC.

Material and Methods

Patients' demographics

This study was based on a nested case-control clinical design and approved by the local Ethics Committee of The Eighth Affiliated Hospital (Shenzhen, China), Sun Yat-sen University (No. R3101/2018) and strictly followed by the Declaration of Helsinki (1964). All patients hospitalized for gastric diseases were introduced to participate in the TAMO/CRC research project and agreed to sign the informed consent forms from 01.2018 – 12.2020. The clinical trial registration number ChiCRT200003245 was submitted at <http://www.chictr.org.cn>. Serum samples of 36 patients with colorectal cancer and 41 healthy controls who fit the study's criteria were collected with the routine protocols in our hospital. Patients with abnormal liver, pancreas, and kidney function, non-CRC malignant tumors and systemic infections were excluded from the control group. The plasma samples were frozen in liquid nitrogen within half an hour after collection and then stored at -80 °C for TMAO and other laboratory analyses. All participants did not take other drugs (such as metformin) that could potentially affect the microbiota.

Feces Sample collection

The stool samples were collected based on routine clinic laboratory tests but with research-orientated instruction. The sterile stool collection kits were used for fecal sampling and transport standardized in our hospital throughout the procedure for years. The method ensured that fresh feces were collected appropriately from the subjects and sent for inspection within half an hour. The specimens were collected 3 days before the operation and the first natural defecation after an operation. Briefly, about 15 - 30 g of freshly passed feces by a sterile feces catcher was collected and then transferred into a small, autoclaved, or completely clean (sterile) container without contamination of urine or toilet tissue. The samples were then instantly frozen in liquid nitrogen and kept at -80 °C for subsequent laboratory analysis.

DNA extraction and 16S rRNA sequencing

Amplification and sequencing of 16S amplicons are widely used for profiling intestinal microbiota structure. To extract DNA from fecal material, the QIAamp DNA Stool Mini Kit from Qiagen, Germany, was utilized, following the instructions of the manufacturer. Briefly, 250 µL of the fecal sample was transferred to a 2-mL tube with 1.2 mL ASL (adenylosuccinate lyase) lysis buffer and 0.3 g sterile zirconia beads (0.1 mm in diameter) after treatment with InhibitEX tablets (Qiagen). The samples were heated at 95 °C for 15 min and homogenized using the Qiagen Tissue Lyser II. The supernatant was subsequently subjected to a QIAcube automated DNA/RNA purification system. The purity and concentration of the DNA were analyzed by employing a NanoDrop2000 UV spectrophotometer., and DNA integrity was analyzed using 1% agarose gel electrophoresis.

The purified DNA was analyzed with the Qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA). PCR amplification of the 16S rRNA gene's variable region 6 (V6) was performed using a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems) with universal primers (1048F, 5'-GTGSTGCAYGGYYGTCGTCA -3'; 1194R, 5'-ACGTCRTCCMCNCCTTCCTC-3') for subsequent sequencing. PCR was initiated at 94 °C for 3 min, followed by 25 cycles of 94 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s, and a final extension at 72 °C for 10 min. Finally, amplicon libraries from all samples were pooled in equal molar concentrations and subsequently subjected to sequencing. The sequencing was conducted using a 500-cycle MiSeq reagent kit, following the paired-end method on the Illumina HiSeq 4000 platform. The identification of generated sequences was based on similarity to reference 16S rRNA gene sequences available in NCBI 16s ribosomal RNA sequences (Bacteria and Archaea) database by crossing an individual strain carrying a density-based K-nearest neighbor clustering with the similarity score at <https://blast.ncbi.nlm.nih.gov/Blast.cgi>.

Analysis of plasma TMAO

The anticoagulant was added to the BD Vacutainer® Rapid Serum Tube before blood collection. Each participant's blood samples were collected after 12 h of fasting in our hospital. The TMAO level in plasma was measured as previously described (Janeiro et al., 2018). In the pyrogen-free tubes, peripheral venous



blood is in the final concentration of 10mM ethylenediaminetetraacetic acid (EDTA) anticoagulant. The samples were centrifuged at 2,000 g for 20 minutes within 30 minutes of collection to separate the plasma, which was then stored at -80°C. TMAO levels were quantified using HPLC-APCIMS/MS with d9-TMAO as the internal control (Caymanchem Ltd., Item No. 17354).

Statistical analysis

All experiments (n=3) were paired with their controls. All data were analyzed using SPSS 19.0 software. The values were presented as mean $\pm$ SD. The analysis of sequencing data was carried out using the Quantitative Insights into Microbial Ecology (QIIME) pipeline, version 1.9.0, which can be assessed at <http://qiime.org>. Operational taxonomic units (OTUs) were compared to the Greengenes database (version 13.8) and the 16S ribosome RNA sequence database, encompassing both Bacteria and Archaea, accessible at <https://blast.ncbi.nlm.nih.gov>. PICRUSt was applied to predict microbial functional profiles. Charts were generated using ggplot2 and RColorBrewer (v1.1-2) packages. GI bacterial diversity (alpha-diversity) assessment utilized the phyloseq package (Luo et al., 2011). Statistical significance was defined as a P-value below 0.05.

Results

No statistical difference between control and CRC groups in biomatrix

In the initial phase of our nested case-control clinical investigation, we observed that the participants exhibited distinct characteristics. The age of individuals in the colorectal cancer (CRC) group had a mean value of 58.7, whereas in the control group, it was 51.58 (Table 1). A significant majority of the participants (>95%) were married. It's noteworthy that these subjects, who commenced drinking at the age of 19, consumed an average of 100 mL of alcohol daily and had a consistent smoking habit for 36 years. Our analysis revealed a slight difference in body mass index (BMI) between CRC patients and control subjects, with the mean values being 28.54 for cases and 25.9 for controls (P=0.03). Alcohol consumption also exhibited some variation between the two groups (P=0.05). However, there were no substantial differences observed regarding meat consumption (P=0.79), regular dietary habits (P=0.30), or vegetarianism. Additionally, marital status did not appear to be a significant differentiating factor either. (Table 1). Using redundancy analyses, we chose 56 core genera that responded to BMI and QDI (Quality of Diet Index) with a recommended dietary allowance of energy (Figure 1).

Table-1. Baselines of colorectal cancer cases and controls in a nested case-control clinical study during 2018-2020.

	Colorectal cancer cases (N=36)		Controls (N=41)		P-value
	N	Value	N	Value	
Age at baseline	36	58.75	41	51.58	0.39
College Graduate	36	29		28	0.33
Height (cm)	36	164.0	41	163.7	0.39
Weight (kg)	36	58.75	41	78.9	0.13
Body mass index* (kg/m ²)	36	28.54	41	25.9	0.03 ^a
Meat/normal/vegetarian	36	3/28/5	41	5/30/6	0.4/0.3/0.7
Male/female	36	26/10	41	25/16	0.78/0.62
Alcohol (g)	36	18.8	41	16.5	0.05 ^a
Serum biomarkers (μmol/L)					
Marriage status	36	35	41	40	0.88
Trimethylamine N-oxide (TMAO)	36	4.8 (3.9 ^b)	41	4.7	0.51



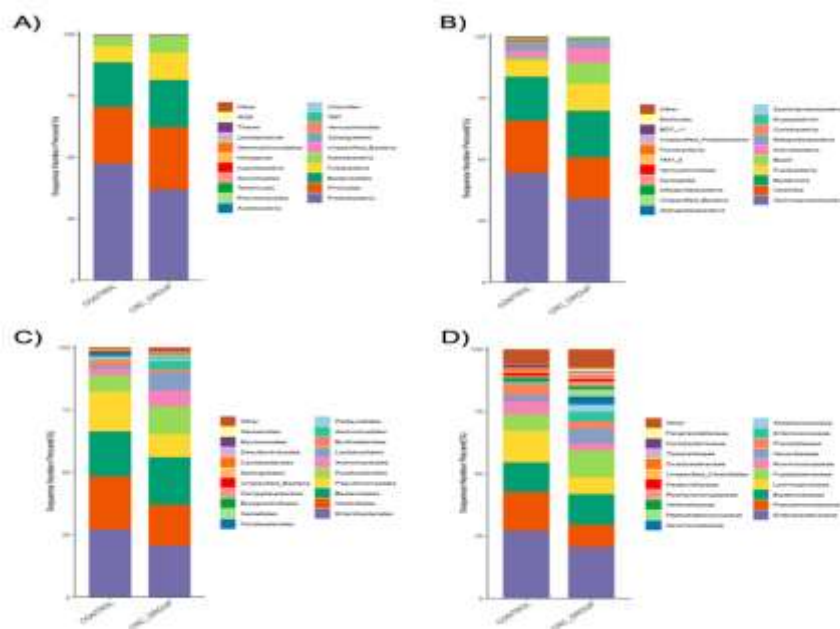


Figure-3. Taxonomic classification and comparison (percentage) of the 16S rRNA gene sequences at the (A) phylum ($p < 0.05$), (B) Class level ($p < 0.05$), (C) order level ($p < 0.09$), (D) family level ($p < 0.07$) for control and CRC groups. Phylum level, classification or taxonomic rank below kingdom and above class. Genus level Shannon diversity and Relative Abundance between control and CRC groups.

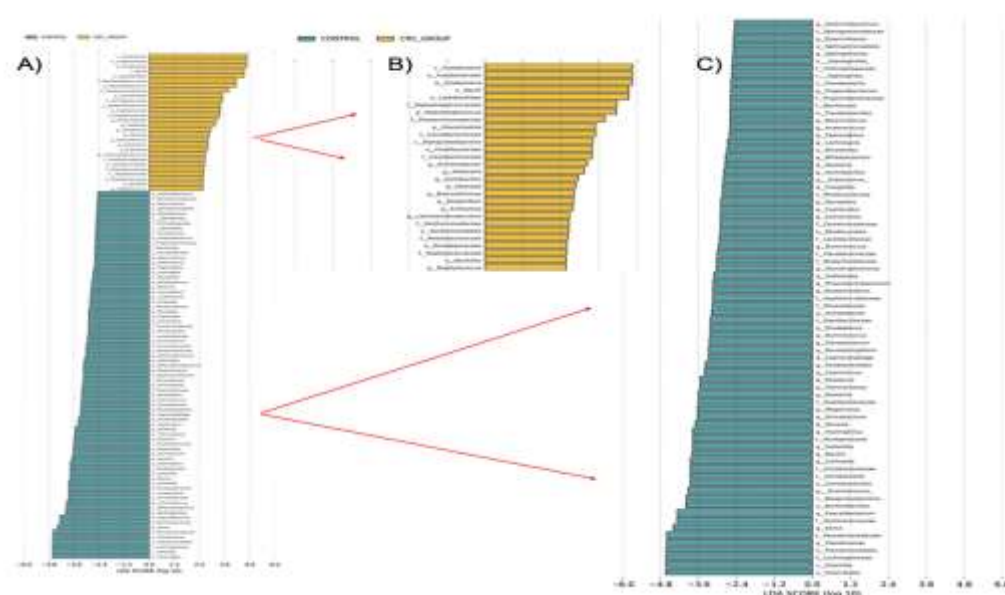


Figure-4. The graphics of Linear discriminant analysis (LDA) exhibit the comparison of the levels of TMAO between the control and CRC groups by PICRUSt, which gives the functional prediction for rumen microorganisms. Bar graph showing the relative abundance of the most abundant genera (>the threshold ± 2.4 score) in CRC and control groups. A. Combined CRC and control groups in both groups. B. CRC group with 28 genera, C Control group with 75 genera. LDA scores of enriched bacterial taxa (LDA > 2 of LEfSe). Bacterial taxa and genes enriched in control are in blue, and the CRC group is in yellow.

Among large quantities and variants of gut microbiota-derived metabolites, Increasing TMAO in CRC has been particularly paid attention to since the over-expression of cyclooxygenase -2. Nuclear factor erythroid-2-related factor-2 (Nrf2) is a pivotal factor in conferring protection against oxidative stress. Moreover, electrophilic oxo-derivative (EFOX) molecules, which are COX-2 dependent, serve as anti-inflammatory mediators by triggering the Nrf2-dependent antioxidant response element (Luo et al., 2011). These studies also intrigue the TMAO functionality.

Key factors for structural segregation of the groups LefSe software (https://huttenhower.sph.harvard.edu/lefse/) identified high-dimensional biomarkers and revealed genomic signatures by means of statistically significant differences in biological characteristics. This study used LefSe to identify specific bacterial phylotypes to determine abundances that differed significantly between groups (Fig. 5).

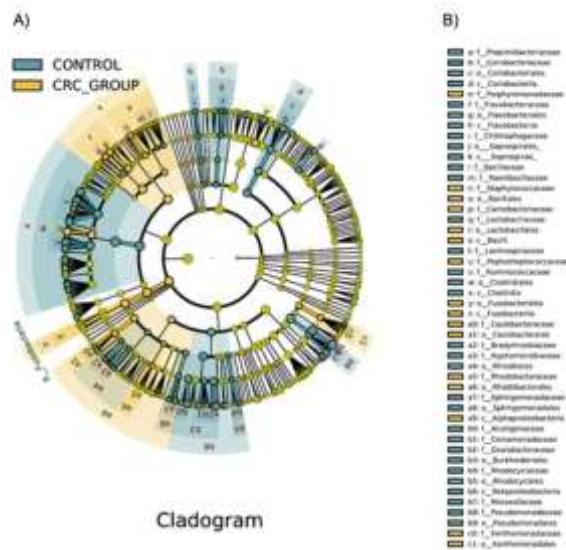


Figure-5. The cladogram displays phylotype differences in the gut microbiota community between healthy individuals and CRC, with key contributors to the structural segregation identified (A) using LefSe and listed (B).

The link between serum TMAO levels and colorectal cancer

The TMAO, produced by gastrointestinal microbiota from dietary TMA precursors, has been investigated

for its potential link to certain cancers. In this study, serum TMAO levels were assessed in a cohort of 108 colorectal cancer patients (including 36 subjects providing fecal samples) and 30 healthy controls (with fecal samples from 41 individuals). Notably, no significant gender or age differences were observed between colorectal cancer patients and the control group.

In colorectal cancer patients, serum TMAO levels ranged from 0.05 to 17.24 $\mu\text{mol/l}$. In contrast, in healthy controls, this range was broader, varying from 0.24 to 20.58 $\mu\text{mol/l}$ (as depicted in Fig. 6). Interestingly, the mean serum TMAO level in colorectal cancer patients did not significantly differ from that of the healthy control group ($p>0.05$).

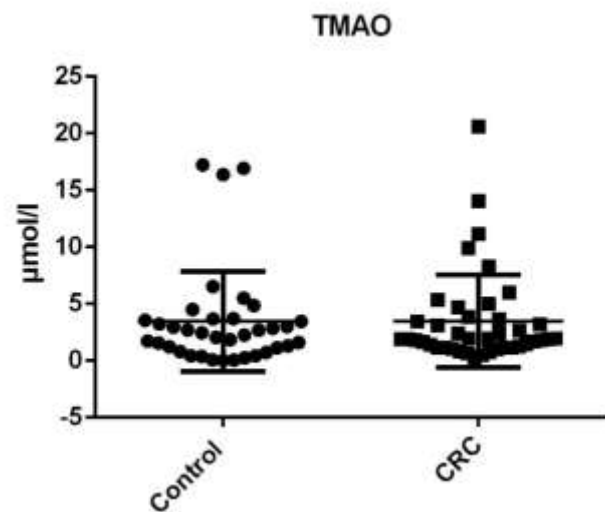


Figure-6. Absolute independent analysis of TMAO concentration in 108 CRC patients compared with 30 health subjects, no statistical significance. $p>0.05$

Discussion

Recent studies have suggested that TMAO, a gut microbiota-derived metabolite, might be a potential biomarker for a range of health conditions, including colorectal cancer. TMAO is produced from dietary nutrients and has been linked to various health outcomes. Specifically, an umbrella review and meta-analysis have indicated that circulating TMAO concentrations are associated with multiple health outcomes, including all-cause mortality, cardiovascular diseases, diabetes mellitus, cancer, and renal function (Li et al., 2022).



These findings suggest that TMAO might play a role in these diseases, including colorectal cancer.

Furthermore, the development of sensitive and reproducible methods for quantifying TMAO at lower quantities has been a focus of recent research. This includes the use of molecularly imprinted polymer (MIP) based sensors, which offer a promising approach for detecting TMAO in body fluids like urine (Lakshmi et al., 2021). Such advancements in detection methods contribute to a deeper understanding of the role of TMAO in health and disease, including its potential implications in colorectal cancer.

Moreover, the enterobacteria have been observed under scanning electronic microscopy; they are present layer by layer on the intestinal endothelium. Their physiology and pathology roles also have been studied from many perspectives. In particular, the gut-associated lymphoid tissues (GALT) are in symbiosis with these microbiotas; most are probiotics, which secrete vitamins and other molecules, including TMAO. Most of these metabolites are beneficial for human health and longevity. Although patients with CRC have benefited from different kinds of chemotherapy with improved overall survival, current chemotherapeutic agents have serious side effects. CRC has been speculated to be associated with diets based on studies of food cultures and demographics (McMurdie and Holmes, 2013; Wang et al., 2019; Xu et al., 2015). Therefore, the discovery and development of the specific molecular targets necessitate certain modulation, especially concerning microbiota in anti-cancer therapy, which is deemed to understand the intestinal microbiome.

In this study, the human microbiome was found to contain over 1,000 microbial species, but the GI tract may contain 100 trillion bacteria (Abdellateif et al., 2020; Lin et al., 2020; Shi et al., 2020). While the gut hosts various bacterial types, the quantities of each species exhibit significant variation. Notably, more than 99% of the gut microbiome is predominantly composed of 30 to 40 specific bacterial species, and the colon harbors the highest microbial population (Zhang et al., 2020). The bacteria present in the gut can be categorized into three broad groups based on their diverse physiological roles within the gastrointestinal environment: pathogenic bacteria, conditional pathogens, and commensal bacteria. Commensal bacteria, which make up over 99% of the gut microbiota, are responsible for generating

beneficial substances, including vitamins and hormones, among others.

In contrast, pathogenic bacteria, such as *Shigella*, *Salmonella*, *Escherichia coli* and *Proteus*, have the potential to directly cause diseases (Huang et al., 2020; Liao et al., 2021). In the current work, *Salmonella* was the most abundant genus in CRC, which presented higher amounts in CRC than in control, thus indicating that the endotoxins and exotoxins from the pathogens of intestinal flora cause severe harmful disorders. In both groups, the intestinal microbiome comprised *Proteobacteria*, *Firmicutes*, and *Bacteroidetes*, followed by far less abundant *Actinobacteria* and *Fusobacteria*. On average, individuals within the CRC group exhibited elevated levels of *Fusobacteria* and *Proteobacteria*, while displaying reduced amounts of *Firmicutes* and *Bacteroidetes* compared to those in the group of healthy individuals. These findings align with prior research conducted on both human specimens and animal models studying CRC (Ekine-Afolabi et al., 2020; Malla, 2020). However, the *Lactobacillus*-related probiotics, such as *Lactobacillus jensenii*, and the pathogen, such as *Helicobacter pylori*, were not determined; it may be more important to determine the population rather than phylum in taxonomy. However, we believe such analysis is needed, which needs to be narrowed down to the species. *Fusobacterium* is a small group of anaerobic gram-negative bacteria commonly found in the digestive tract and is known to cause certain diseases. Many *Fusobacteria* are associated with CRC, but their role in the progression of the disease remains unclear (Chang et al., 2021; Yi et al., 2021). While our current study indicated a higher average count of *Fusobacterium* in individuals with CRC when compared to the control group, it is important to note that further investigations with more extensive sample sizes are essential to validate this trend. Previous research has indicated the specificity of *C. coccoides* and *C. leptum* subgroups to CRC, yet neither of these species was identified in our study. Nevertheless, our findings suggested a tendency for a decrease in *C. perfringens* in CRC, which is consistent with earlier research (Hayase and Jenq, 2020; Sun et al., 2020).

Regarding high levels of free molecules in the blood, higher serum choline concentrations (but not TMAO, carnitine, or betaine) are associated with an increased risk of CRC (Janeiro et al., 2018). Previous studies have linked circulating TMAO concentrations to



elevated inflammation in cancer etiology (Abdellateif et al., 2020). Our finding was that TMAO levels were not significantly different between colorectal cancer patients and healthy controls. Our results may conflict with some studies, but in the Finnish cohort, there was no clear association between serum TMAO levels and rectal cancer risk (Guertin et al., 2017). However, the current work only includes a small sample size (36 CRC vs. 41 healthy controls). In our wild speculation, one of the reasons might be the conversion of TMAO and TMA, both of which can be metabolized by gut bacteria. In contrast, TMAO is an oxidation product of TMA and, therefore, depends on the antioxidant and oxidative environment (Papandreou et al., 2020). To further clarify the mechanism of action of TMAO, a larger sample size, more targeting factors, and better control subjects are needed to determine the role of TMAO in the diagnosis and treatment of CRC.

Our research on gut microbiota and CRC can be contextually situated within the extensive body of existing scientific literature. The role of gut microbiota in influencing CRC was initially suggested in studies like those by Reddy et al. in 1975, highlighting the microbiota's impact on carcinogens in the colon (Reddy et al., 1975). Later studies expanded on this concept, demonstrating how microbial communities from CRC patients could promote carcinogenesis (Wong and Yu, 2019).

The association between specific bacterial species, such as *Fusobacterium nucleatum*, and CRC progression has been highlighted in research by Castellarin et al. in 2012 (Castellarin et al., 2012). Another study focused on the diversity and co-occurrence of various bacteria in relation to colorectal carcinogenesis (Wong and Yu, 2023).

Diet's influence on gut microbiota composition and its subsequent impact on CRC risk has also been a significant area of study. Dietary patterns, especially the intake of dietary fiber, can profoundly influence the gut microbiome, thus affecting CRC risk (Song and Chan, 2017).

The therapeutic potential of targeting gut microbiota in CRC treatment and prevention is an emerging area of interest. Studies have delved into molecular pathways and the interactions between microbial species, inflammation, and CRC, suggesting new approaches for CRC management (Sun and Kato, 2016).

The development of CRC involves genetic and epigenetic aberrations: Chromosomal instability,

methylation of CpG island methylator phenotype, and instability of microsatellite DNA regions. However, environmental factors, including diets and lifestyle habits, influence CRC. Studies have revealed that fish, particularly shallow-water teleosts and seafood, exhibit elevated levels of TMAO. Consumption of seafood led to a rise in the urinary excretion of TMA and TMAO in the postprandial period, as observed 24 hours later. Still, there is no statistical evidence for fish consumption linked to CRC yet (Samerotte et al., 2007). So TMAO is a cause for CRC or effect, which needs further research.

However, we acknowledge certain limitations in our study. The research, conducted on a small, specific sample of hospitalized gastric disease patients, primarily in a Chinese population, may not be broadly generalizable. This selection bias, along with potential unmeasured confounding factors like diet and medication, could influence the association observed between gut microbiota, serum TMAO levels, and colorectal cancer.

Conclusion

In conclusion, this investigation reveals notable alterations in the gut microbiota of CRC patients, alongside distinctive plasma TMAO levels when compared to healthy controls. While these variations are not directly associated with CRC progression, they nonetheless contribute to our understanding of the disease's pathophysiology. This research underscores the intricate relationship between gut microbiota and CRC, providing a foundation for future studies. It suggests the potential of gut microbiota as a therapeutic target or diagnostic marker in CRC management. The findings, although limited by the study's specific demographic and geographical focus, highlight the necessity for larger-scale, more diverse studies to further elucidate the role of gut microbiota and TMAO in CRC.

Ethics approval and consent to participate

Ethical approval was granted by the ethics committee of Sun Yat-sen University (Shenzhen, China) (No. R3101/2018) on behalf of all participating units with Clinical Trial Number ChiCRT2000032475, and written informed consent was obtained from all subjects.



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Conflict of Interest: None.

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Contribution of Authors

Huang J: Designed this study, wrote the manuscript and performed the experimental work

Che X: Provided most of the statistical analysis, figures, and tables for the manuscript

Zhong Q: Designed this study and wrote the manuscript and supervised the overall research

All authors read and approved the final manuscript.

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