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Theme

Integrated metagenomic-proteomic bioinformatic analysis of gut microbiota associated with colorectal cancer

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Dedication

I dedicate this work:

To my mother and father, in appreciation of their endless love, patience, and sacrifices, which have been the main reason for my success.

To my family, for their continuous support and encouragement throughout my academic journey.

To my friends and colleagues, for their motivation, support, and the wonderful moments we shared during our studies.

And to everyone who believes in the value of knowledge and hard work.

Abstract

Colorectal cancer (CRC) is a major cause of cancer-related morbidity and mortality worldwide, and growing evidence links gut microbiota dysbiosis to its development. In this study, an integrative bioinformatic analysis was performed using publicly available shotgun metagenomic datasets from the European Nucleotide Archive. A total of eight fecal samples were analyzed, including four healthy controls and four CRC patients.

Taxonomic profiling revealed clear microbial alterations between groups. Healthy controls were dominated by *Prevotella copri* (≈40%), *Bacteroides* spp. (≈21%), and butyrate-producing *Roseburia* species (*R. intestinalis* 4%, *R. inulinivorans* 3%, *R. faecis* 2%). In CRC samples, *Prevotella copri* decreased to ≈4%, while *Butyrivibrio crossotus* increased to ≈17%, *Bacteroides* spp. reached ≈14%, and *Enterobacteriaceae* expanded to ≈2%.

Functional analysis using HUMAnN3 and KEGG identified 47 significantly enriched and 32 depleted pathways in CRC ($p < 0.05$; $p < 0.001$). Lipopolysaccharide (LPS) biosynthesis increased from 3.18% to 8.52% (+2.68-fold; $p < 0.001$), bacterial invasion of epithelial cells from 2.82% to 7.21% (+2.56-fold; $p < 0.001$), and DNA replication/repair pathways from 3.14% to 6.84% (+2.18-fold; $p < 0.001$). Conversely, butanoate metabolism decreased from 8.52% to 3.24% (−2.63-fold; $p < 0.001$), propanoate metabolism from 7.82% to 3.48% (−2.25-fold; $p < 0.001$), and vitamin B12 biosynthesis from 7.18% to 2.85% (−2.52-fold; $p < 0.001$).

Overall, CRC was associated with both taxonomic dysbiosis and functional reprogramming of the gut microbiome, characterized by increased inflammatory and virulence-related pathways alongside reduced metabolic and protective functions. These findings support the potential role of microbial signatures as biomarkers for CRC detection and therapeutic targeting.

Keywords: Colorectal cancer; Gut microbiota; Metagenomics; Functional profiling; Dysbiosis; Bioinformatics.

الملخص

يُعدّ سرطان القولون والمستقيم (CRC) أحد الأسباب الرئيسية للمراضة والوفيات المرتبطة بالسرطان على مستوى العالم، وتشير الأدلة المتزايدة إلى أن اختلال توازن ميكروبيوتا الأمعاء (Dysbiosis) يلعب دورًا مهمًا في تطور هذا المرض وتقدمه. في هذه الدراسة، تم اعتماد مقاربة معلوماتية حيوية تكاملية لدراسة العلاقة بين التغيرات في الميكروبيوتا المعوية وسرطان القولون والمستقيم باستخدام بيانات تسلسل ميتاجينومي عشوائي (shotgun metagenomic) متاحة للعموم من الأرشيف الأوروبي للنيوكليوتيدات (ENA). شملت الدراسة ثماني عينات برازية، أربع منها من أفراد أصحاء وأربع من مرضى مصابين بسرطان القولون والمستقيم.

أظهر التحليل التصنيفي تحوّلًا واضحًا في بنية المجتمع الميكروبي بين العينات السليمة والعينات المرتبطة بالسرطان. في عينات الأصحاء، هيمن جنس Prevotella copri على المجتمع الميكروبي بنسبة بلغت حوالي 40% من إجمالي الوفرة البكتيرية، إلى جانب جنس Bacteroides (≈21%) وأنواع Roseburia المنتجة للبيوتيرات (Roseburia intestinalis بنسبة 4%، وR. inulinivorans بنسبة 3%، وR. faecis بنسبة 2%). أما في عينات السرطان، فقد تغيّر هذا التركيب بشكل ملحوظ: انخفضت نسبة Prevotella copri إلى حوالي 4% فقط، بينما ارتفعت نسبة Butyrivibrio crossotus إلى حوالي 17%، ووصلت نسبة Bacteroides إلى حوالي 14% من المجتمع البكتيري، مع توسّع ملحوظ في عائلة Enterobacteriaceae (≈2%) من المجتمع).

كما كشف التحليل الوظيفي باستخدام أداتي HUMAnN3 وخرائط KEGG عن وجود 47 مسارًا وظيفيًا مُثرى بشكل معنوي مقابل 32 مسارًا منخفضًا بشكل معنوي في عينات السرطان ($p < 0.05$؛ $p < 0.001$). فقد ارتفع مسار تصنيع الليبوبوليساكاريد (LPS) بشكل معنوي في عينات السرطان مقارنة بالأصحاء (8.52% مقابل 3.18%؛ بمعدل تغيّر +2.68 ضعفًا؛ $p < 0.001$). كما ارتفعت مسارات غزو البكتيريا للخلايا الظهارية (7.21% مقابل 2.82%؛ +2.56 ضعفًا؛ $p < 0.001$)، وكذلك مسارات تضاعف وإصلاح الحمض النووي (6.84% مقابل 3.14%؛ +2.18 ضعفًا؛ $p < 0.001$). في المقابل، انخفضت بشكل معنوي مسارات استقلاب الأحماض الدهنية قصيرة السلسلة (SCFAs): إذ تراجع مسار استقلاب البيوتيرات (Butanoate) من 8.52% إلى 3.24% (−2.63 ضعفًا؛ $p < 0.001$)، وتراجع مسار استقلاب البروبيونات (Propanoate) من 7.82% إلى 3.48% (−2.25 ضعفًا؛ $p < 0.001$). كذلك انخفض مسار تصنيع فيتامين B12 بشكل معنوي (2.85% مقابل 7.18%؛ −2.52 ضعفًا؛ $p < 0.001$).

تشير هذه النتائج إلى أن سرطان القولون والمستقيم يرتبط بكل من الاختلال التصنيفي وإعادة البرمجة الوظيفية للميكروبيوتا المعوية، والتي تتميز بزيادة في القدرة الالتهابية والضراوة الميكروبية (تصنيع LPS، غزو الخلايا الظهارية، مسارات إصلاح الحمض النووي)، إلى جانب انخفاض في الوظائف الأيضية والحامية (إنتاج الأحماض الدهنية قصيرة السلسلة، تصنيع فيتامين B12). كما توحي هذه التغيرات بدور ميكروبي محتمل في آليات التسرطن من خلال تعزيز الالتهاب، وإضعاف سلامة الحاجز الظهاري، وتغيير التوازن الأيضي للمضيف. بشكل عام، تؤكد هذه الدراسة أهمية التحليلات المعلوماتية الحيوية التكاملية في فهم العلاقة بين الميكروبيوتا وسرطان القولون والمستقيم، وتدعم إمكانية استخدام التواقيع الميكروبية كواسمات حيوية محتملة للتشخيص المبكر والاستهداف العلاجي.

***الكلمات المفتاحية**: سرطان القولون والمستقيم؛ الميكروبيوتا المعوية؛ الميتاجينوميات؛ التحليل الوظيفي؛ الاختلال الميكروبي؛ المعلوماتية الحيوية.*

Abbreviations List

APC: Adenomatous Polyposis Coli

BCAAs: Branched-Chain Amino Acids

BRAF: v-Raf Murine Sarcoma Viral Oncogene Homolog B

CD: Crohn's Disease

CDI: Clostridioides difficile Infection

CDT: Cytolethal Distending Toxin

CIMP: CpG Island Methylator Phenotype

CIN: Chromosomal Instability

COX: Cyclooxygenase

CRC: Colorectal Cancer

DCA: Deoxycholic Acid

DNA: Deoxyribonucleic Acid

EMT: Epithelial–Mesenchymal Transition

FAP: Familial Adenomatous Polyposis

FOS: Fructooligosaccharides

FXR: Farnesoid X Receptor

GALT: Gut-Associated Lymphoid Tissue

GOS: Galactooligosaccharides

GWAS: Genome-Wide Association Studies

HMO: Human Milk Oligosaccharide

IL: Interleukin

KRAS: Kirsten Rat Sarcoma Viral Oncogene Homolog

LPS: Lipopolysaccharides

MAPK: Mitogen-Activated Protein Kinase

MDSCs: Myeloid-Derived Suppressor Cells

MMR: Mismatch Repair

MSI: Microsatellite Instability

NF-κB: Nuclear Factor Kappa B

NGS: Next-Generation Sequencing

NK: Natural Killer

NOD2: Nucleotide-Binding Oligomerization Domain-Containing Protein 2

NSAIDs: Non-Steroidal Anti-Inflammatory Drugs

PD-L1: Programmed Death-Ligand 1

PI3K: Phosphoinositide 3-Kinase

PRRs: Pattern Recognition Receptors

PSC: Primary Sclerosing Cholangitis

RNA: Ribonucleic Acid

ROS: Reactive Oxygen Species

rRNA: Ribosomal RNA

SCFAs: Short-Chain Fatty Acids

sIgA: Secretory Immunoglobulin A

SMAD4: SMAD Family Member 4

SNPs: Single Nucleotide Polymorphisms

STAT3: Signal Transducer and Activator of Transcription 3

TcdA: Toxin A

TcdB: Toxin B

TGR5: Takeda G Protein-Coupled Receptor 5

TLR4: Toll-Like Receptor 4

TNF-α: Tumor Necrosis Factor Alpha

TP53: Tumor Protein p53

Tregs: Regulatory T Cells

UC: Ulcerative Colitis

WGS: Whole Genome Sequencing

Wnt: Wingless/Integrated Signaling Pathway

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Introduction

Introduction

Colorectal cancer (CRC) represents one of the most significant global health challenges, ranking as the third most commonly diagnosed malignancy and the second leading cause of cancer-related mortality worldwide. Despite substantial advances in screening strategies, surgical techniques, and therapeutic interventions, the incidence of CRC—particularly early-onset cases—continues to increase in many regions. This epidemiological trend highlights the urgent need for improved understanding of disease mechanisms and for the identification of reliable biomarkers that can support earlier diagnosis and more effective therapeutic strategies (Siegel *et al.,* 2024).

In parallel, the human gut microbiota, a complex and dynamic ecosystem composed of trillions of microorganisms, has emerged as a key regulator of human health and disease. Often referred to as the "second genome," this microbial community plays essential roles in nutrient metabolism, vitamin synthesis, and immune system modulation. Beyond these physiological functions, increasing evidence indicates that gut microbiota dysbiosis is implicated in colorectal carcinogenesis through multiple mechanisms, including chronic inflammation, genotoxic effects, metabolic alterations, and immune dysregulation (Sepich-Poore *et al.,* 2021).

Recent advances in high-throughput sequencing and mass spectrometry have significantly expanded our ability to characterize the gut microbiome at both taxonomic and functional levels. Metagenomic approaches allow comprehensive profiling of microbial community composition and functional potential, whereas proteomic analyses provide complementary insights into protein expression patterns of both microbial and host origin. The integration of these multi-omics strategies has therefore emerged as a powerful approach to uncover complex microbiota–host interactions and to identify biologically relevant biomarkers that are not accessible through single-technology studies (Hasin *et al.,* 2017).

Although growing evidence supports a strong association between gut microbiota dysbiosis and colorectal cancer, the underlying functional mechanisms remain incompletely understood. Most previous studies have focused either on taxonomic profiling or functional inference independently, limiting a comprehensive interpretation of the microbiota–host–cancer axis. In addition, fully integrated multi-omics studies combining metagenomic and proteomic resources remain relatively limited, which restricts the identification of robust and biologically consistent molecular signatures across different analytical layers (Thomas *et al.,* 2019).

In this context, the present study aims to investigate the relationship between gut microbiota dysbiosis and colorectal cancer through an integrated bioinformatic approach based

on publicly available shotgun metagenomic datasets and secondary functional pathway analyses derived from previously published proteomic resources. Specifically, it seeks to characterize differences in microbial community composition between colorectal cancer patients and healthy individuals, identify taxa associated with disease status, and explore functional pathways potentially involved in colorectal carcinogenesis using tools such as HUMAnN3, KEGG mapping, and related functional annotation frameworks. The integration of taxonomic and functional interpretations is expected to provide a more comprehensive understanding of microbiota alterations associated with CRC and to contribute to the identification of potential diagnostic and mechanistic biomarkers.

The study is based on the hypothesis that colorectal cancer is associated with significant alterations in gut microbial composition and functional potential, characterized by an enrichment of pro-inflammatory and virulence-associated pathways and a depletion of metabolic and protective functions such as short-chain fatty acid production. Furthermore, it is hypothesized that combining taxonomic profiling with functional pathway inference can reveal biologically meaningful associations between microbial community shifts and disease-associated metabolic reprogramming, thereby improving the understanding of CRC pathogenesis and supporting the discovery of clinically relevant microbial biomarkers.

Section 1: Literature Review

I. Colorectal Cancer

I.1. Definition

Colorectal cancer (CRC) is a malignant disease characterized by abnormal epithelial proliferation in the large intestine, originating from the mucosal lining and most commonly arising from adenomatous precursor lesions. Histologically, it is defined by abnormal glandular architecture (figure 01), cellular atypia, and loss of normal tissue organization, with adenocarcinoma representing the predominant histological subtype that serves as the main model for studying epithelial tumor progression (Brenner *et al.,* 2014; Dekker *et al.,* 2019). From a molecular standpoint, CRC results from the accumulation of genetic and epigenetic alterations that disrupt normal cell growth and apoptosis through two major pathways: Chromosomal Instability (CIN), commonly associated with mutations in APC (Adenomatous Polyposis Coli) and TP53 (Tumor Protein p53), and Microsatellite Instability (MSI), which results from defects in DNA mismatch repair (MMR) genes (Kuipers *et al.,* 2015; Biller and Schrag, 2021). Anatomically, this malignancy affects different regions of the large intestine, including the colon and rectum, where right-sided and left-sided tumors differ significantly in their embryological origin, molecular profile, and clinical presentation. Functionally, this progressive tumor development disrupts normal colonic activity including absorption, motility, and fecal storage resulting in clinical symptoms such as gastrointestinal bleeding, intestinal obstruction, and altered bowel habits (Argilés *et al.,* 2020; Sawicki *et al.,* 2021).

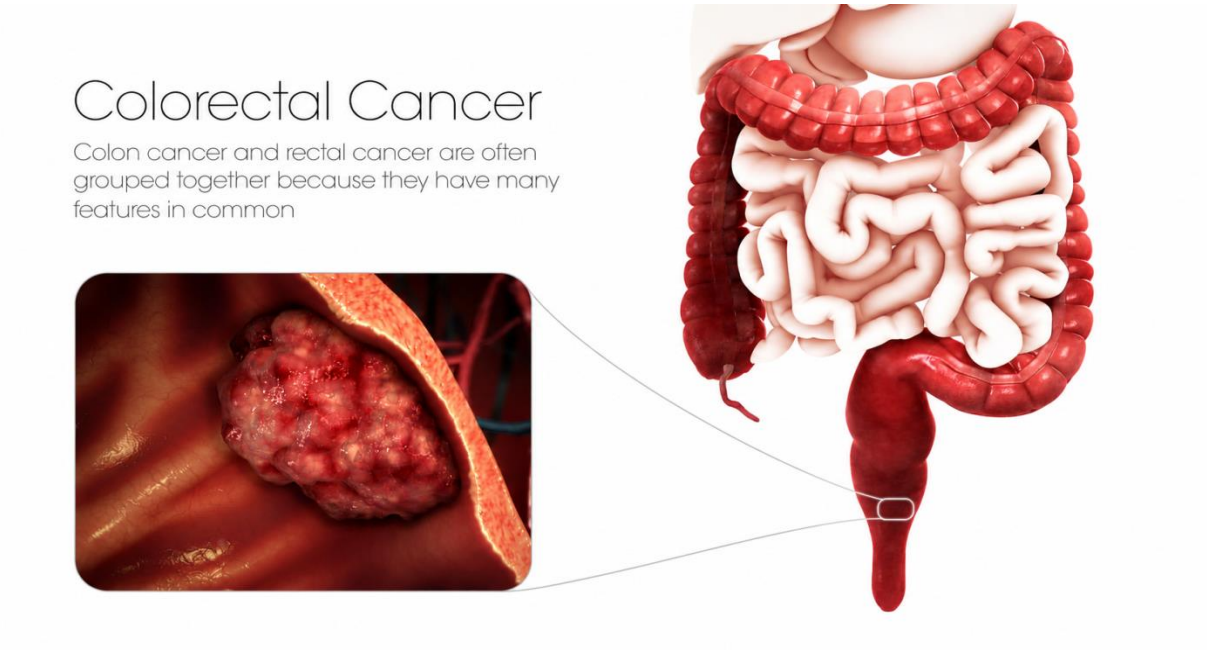

Figure 01: Overview of Colorectal Cancer (Adapted from National Cancer Institute, 2025).

I.2. Etiology and Risk Factors

Colorectal cancer is a multifactorial disease influenced by genetic predisposition, environmental exposures, and lifestyle behaviors. Its risk factors are classified into non-modifiable elements such as age, sex, and family history, and modifiable factors including diet, obesity, smoking, and physical inactivity (Sawicki *et al.,* 2021; Bener *et al.,* 2024; Roshandel *et al.,* 2024).

I.2.1. Genetic Factors

Family history is a well-established risk factor for colorectal cancer (CRC), with first-degree relatives of affected individuals having approximately a two- to threefold increased risk. While most familial cases do not result from a defined hereditary syndrome, approximately 5–10% of CRC cases are attributable to highly penetrant Mendelian syndromes. The most common of these are Lynch syndrome, caused by mutations in DNA mismatch repair genes, and Familial Adenomatous Polyposis (FAP), which is associated with germline mutations in the APC gene. Beyond these high-penetrance disorders, Genome-Wide Association Studies (GWAS) have identified cumulative genetic variants through single nucleotide polymorphisms (SNPs) that influence Wnt signaling and cell cycle pathways, explaining the remaining familial risk in sporadic CRC cases (Caldwell *et al.,* 2022; Huyghe *et al.,* 2024).

I.2.2. Environmental and Lifestyle Factors

Lifestyle and dietary patterns are primary drivers of colorectal cancer (CRC) risk. High intake of red and processed meats promotes carcinogenesis via N-nitroso compounds, whereas fiber-rich diets protect the gut by modulating the microbiota. Additionally, obesity and physical inactivity trigger tumor progression through chronic inflammation and insulin resistance, a risk further elevated by smoking and alcohol. Conversely, the use of aspirin and Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) offers chemoprevention by inhibiting Cyclooxygenases (COX enzymes) and suppressing pro-inflammatory pathways (Song and Chan, 2021; Dahmus *et al.,* 2022; Puzzono *et al.,* 2023; Hull *et al.,* 2024).

I.2.3. Inflammatory Bowel Diseases

Patients with ulcerative colitis (UC) and Crohn’s disease face a heightened risk of colorectal cancer due to chronic mucosal inflammation, which facilitates the accumulation of oncogenic mutations. In UC, this risk correlates with disease duration, severity, and the presence of primary sclerosing cholangitis (PSC). Unlike sporadic CRC, colitis-associated cancer often follows a "dysplasia-to-carcinoma" sequence, marked by early TP53 mutations and late APC involvement. This distinct molecular pathway necessitates stringent endoscopic

surveillance to detect precancerous lesions in their early stages (Olén *et al.,* 2020; Ghouri *et al.,* 2022; Zhao *et al.,* 2024; Singh *et al.,* 2025).

I.3. Colorectal Cancer Progression

CRC is a protracted evolutionary process characterized by a stepwise transition from benign epithelium to invasive malignancy (Hanahan, 2022; Dekker *et al.,* 2023; Kuipers *et al.,* 2024). This progression is governed by a chronological succession of genomic alterations that drive specific pathological changes (Figure 02).

I.3.1. Normal Epithelium

CRC arises from a multi-step accumulation of genetic and epigenetic alterations that disrupt the homeostatic balance of the colonic mucosa. This sequence involves the progressive transformation of healthy epithelial cells into malignant carcinomas through defined pathological stages (Vogelstein *et al.,* 2013; Dekker *et al.,* 2019).

I.3.2. Early Adenoma

The process is initiated by mutations in the APC tumor suppressor gene, occurring in over 80% of sporadic cases. APC loss leads to the constitutive activation of the Wnt/β-catenin signaling pathway, driving the transcription of oncogenes such as MYC and promoting the formation of initial adenomatous polyps (Zhang *et al.,* 2017; Mazaheri *et al.,* 2024).

I.3.3. Intermediate Adenoma

Progression is accelerated by activating mutations in the KRAS oncogene (Kirsten Rat Sarcoma viral oncogene homolog), observed in approximately 40% of cases. These mutations activate the MAPK/ERK and PI3K/AKT signaling cascades, promoting uncontrolled proliferation and resistance to apoptosis, thereby increasing adenoma complexity (Kim *et al.,* 2022; Abdalla *et al.,* 2025).

I.3.4. Late Adenoma

The transition to advanced stages is characterized by chromosomal instability and loss of SMAD4, commonly associated with 18q21 deletion. SMAD4 inactivation suppresses TGF-β tumor-inhibitory signaling and contributes to the recruitment of myeloid-derived suppressor cells (MDSCs), generating a pro-invasive inflammatory microenvironment (Wang *et al.,* 2021; Zhang *et al.,* 2023).

I.3.5. Invasive Carcinoma

The final malignant transformation is primarily driven by TP53 inactivation, detected in up to 80% of metastatic CRC cases. Loss of TP53 function enables cells to evade cell-cycle checkpoints and apoptosis. In combination with APC, KRAS, and SMAD4 mutations, this

promotes basement membrane invasion, epithelial-mesenchymal transition (EMT), and metastatic dissemination (Yaeger *et al.,* 2018; Naxerova *et al.,* 2021).

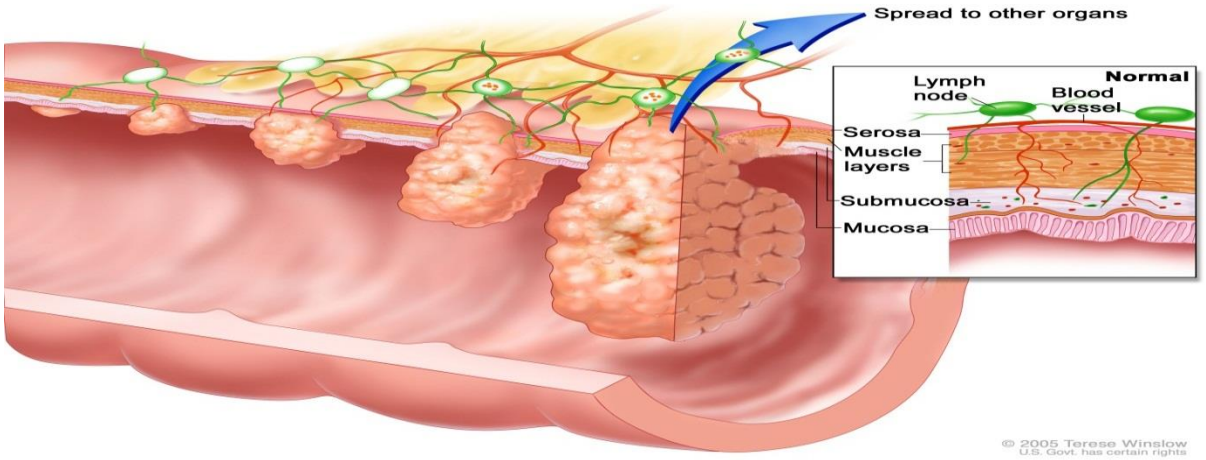

Figure 02: Stages of colorectal cancer progression and infiltration through the intestinal wall layers, leading to lymphatic and hematogenous metastasis (Winslow, 2005)

I.4. Molecular Pathogenesis and Subtypes

CRC evolves through the sequential accumulation of genetic and epigenetic aberrations (Vogelstein *et al.,* 2013). This molecular framework is essential for managing disease heterogeneity and guiding targeted therapies (Dekker *et al.,* 2019). The primary pathways are detailed below.

I.4.1. Adenoma-Carcinoma Sequence

This model describes the stepwise transformation of normal epithelium into invasive carcinoma over 10-15 years. It is initiated by APC mutations, which dysregulate Wnt signaling, followed by KRAS activation for growth, and late-stage inactivation of SMAD4 and TP53 to trigger malignancy (Fearon, 2011; Puzzono *et al.,* 2021).

I.4.2. Chromosomal Instability (CIN) Pathway

Accounting for 65-70% of cases, CIN involves large-scale chromosomal alterations due to segregation defects. CIN tumors typically harbor mutations in APC, KRAS, and TP53, often presenting as left-sided lesions with a poorer prognosis compared to MSI tumors (Pino and Chung, 2010; Miele *et al.,* 2020).

I.4.3. Microsatellite Instability (MSI) Pathway

Arising from a defective DNA Mismatch Repair (MMR) system, MSI leads to hypermutation in repetitive DNA sequences. Whether hereditary (Lynch syndrome) or sporadic (via MLH1 silencing), these proximal colon tumors exhibit high immunogenicity and superior response to PD-1/PD-L1 inhibitors (Boland and Goel, 2010; Liu *et al.,* 2021).

I.4.4. CpG Island Methylator Phenotype (CIMP)

CIMP is an epigenetic driver characterized by extensive hypermethylation of promoter CpG islands, resulting in the transcriptional silencing of tumor suppressor genes (Weisenberger *et al.,* 2006). This phenotype, found in 15-20% of cases, is a hallmark of the "serrated pathway" and is strongly linked to BRAF V600E mutations (Leggett and Whitehall, 2010). under the Consensus Molecular Subtype (CMS) framework, CIMP-high/MSI-high tumors are primarily classified as CMS1 (immunosurveillance subtype), exhibiting high immune infiltration and distinct clinical outcomes (Guinney *et al.,* 2015; Ting *et al.,* 2022).

I.5. Key Bacterial Species in CRC Development

I.5.1. *Fusobacterium nucleatum*

A primary oncogenic driver that uses FadA adhesin to activate Wnt/β-catenin signaling, promoting cell proliferation. Its Fap2 protein facilitates immune evasion, while TLR4/MyD88 activation drives chemoresistance (Queen *et al.,* 2022; Zhang *et al.,* 2023).

I.5.2. *Bacteroides fragilis* (ETBF) "Enterotoxigenic *Bacteroides fragilis*"

Secretes BFT toxin, which cleaves E-cadherin to trigger β-catenin signaling and barrier disruption. It induces a pro-inflammatory state via STAT3 (Transducteur du signal et activateur de la transcription 3**)** and NF-κB pathways (Nuclear Factor kappa-light-chain-enhancer of activated B cells), leading to ROS-mediated DNA damage (Haghi *et al.,* 2019; Cao *et al.,* 2021).

I.5.3. *Escherichia coli* (pks+) "polyketide synthase"

Harbor the pks island, producing colibactin, a genotoxin causing double-strand DNA breaks. This leaves a distinct mutational signature SBS88 (Single Base Substitution 88) that initiates and accelerates malignant transformation (Dziubańska-Kusibab *et al.,* 2020; Pleguezuelos-Manzano *et al.,* 2020).

I.5.4. Other Taxa

Peptostreptococcus anaerobius promotes growth via PI3K/Akt signaling (Long *et al.,* 2019), while *Streptococcus gallolyticus* recruits' tumor-associated macrophages (Puzzono et *al.,* 2021). Conversely, the depletion of butyrate-producers like *F. prausnitzii* eliminates critical anti-inflammatory protection (Ting *et al.,* 2022).

I.6. Microbial Alterations in Colorectal Cancer

The gut microbiome undergoes profound structural and functional transformations during colorectal carcinogenesis. This transition from a homeostatic state to a pro-tumorigenic dysbiosis is primarily defined by two key ecological shifts:

I.6.1. Alpha and Beta Diversity Changes

CRC is characterized by a significant decline in Alpha diversity, reflecting ecological dysbiosis and the loss of commensal resilience. Beta diversity analyses further demonstrate a clear microbial segregation between healthy individuals, adenoma patients, and CRC cases. This progressive shift suggests that the microbiota actively drives carcinogenesis rather than merely responding to tumor presence (Zeller *et al.,* 2014; Feng *et al.,* 2015; Liu *et al.,* 2024).

I.6.2. Specific Bacterial Taxa Associated with CRC

Metagenomic profiling reveals a systematic enrichment of pro-carcinogenic "pathobionts," notably *Fusobacterium nucleatum*, ETBF, and *pks+ E. coli*. Conversely, there is a marked depletion of protective, butyrate-producing taxa like *Faecalibacterium prausnitzii* and *Bifidobacterium*. This microbial imbalance promotes a pro-inflammatory, tumor-permissive environment and serves as a promising non-invasive biomarker for early detection (Yu *et al.,* 2017; Wirbel *et al.,* 2019; Smith *et al.,* 2026).

II.Gut microbiota

II.1. Definition

The gut microbiota is currently recognized as a complex, dynamic biological system essential for maintaining human health, referring to a structured microbial ecosystem composed of bacteria, archaea, viruses, and fungi that colonize the gastrointestinal tract. This microbial community establishes symbiotic interactions with the host and contributes to intestinal and systemic homeostasis through metabolic and immunological activities, where its composition is continuously influenced by environmental and host-related factors, including diet, lifestyle, age, and genetics (Fan and Pedersen, 2021). From a quantitative perspective, the gut microbiota contains approximately 3.8×10^{13} microbial cells, a number considered comparable to the total number of human somatic cells; this substantial population contributes significantly to digestion, nutrient metabolism, energy harvest, and immune regulation (Sender *et al.,* 2016). Furthermore, at the genetic level, the gut microbiome contains millions of microbial genes that greatly exceed the human genome, providing additional metabolic functions such as vitamin synthesis, xenobiotic metabolism, and degradation of complex polysaccharides. Thus, this extensive genetic repertoire makes the gut microbiome widely referred to as the "second genome" due to its major contribution to host physiological adaptability and metabolic capacity (Liu *et al.,* 2022).

II.2. Predominant Bacterial Phyla in the Gut Microbiota

At the phylum level, the human gut microbiota is primarily dominated by a limited number of bacterial groups that play critical roles in nutrient metabolism, immune modulation, and maintenance of intestinal homeostasis (Figure 03). Among the diverse microbial taxa identified in the gastrointestinal tract, four major phyla constitute the core bacterial community.

II.2.1. Phylum Firmicutes

Firmicutes generally represent one of the most abundant bacterial phyla in the gut microbiota and include genera such as *Lactobacillus*, *Clostridium*, and *Faecalibacterium*. These microorganisms are involved in dietary fiber fermentation and the production of short-chain fatty acids (SCFAs), which contribute to intestinal health and energy metabolism (Fan and Pedersen, 2021).

II.2.2. Phylum Bacteroidetes

Bacteroidetes constitute another dominant phylum within the intestinal microbiota. Members of this group play essential roles in the degradation of complex polysaccharides and participate in nutrient processing and maintenance of gut homeostasis (Fan and Pedersen, 2021).

II.2.3. Phylum Actinobacteria

Although present in lower abundance, Actinobacteria particularly the genus *Bifidobacterium* are considered beneficial microorganisms. They are especially important during infancy, where they contribute to the digestion of human milk oligosaccharides and support immune system maturation (Milani *et al.,* 2017).

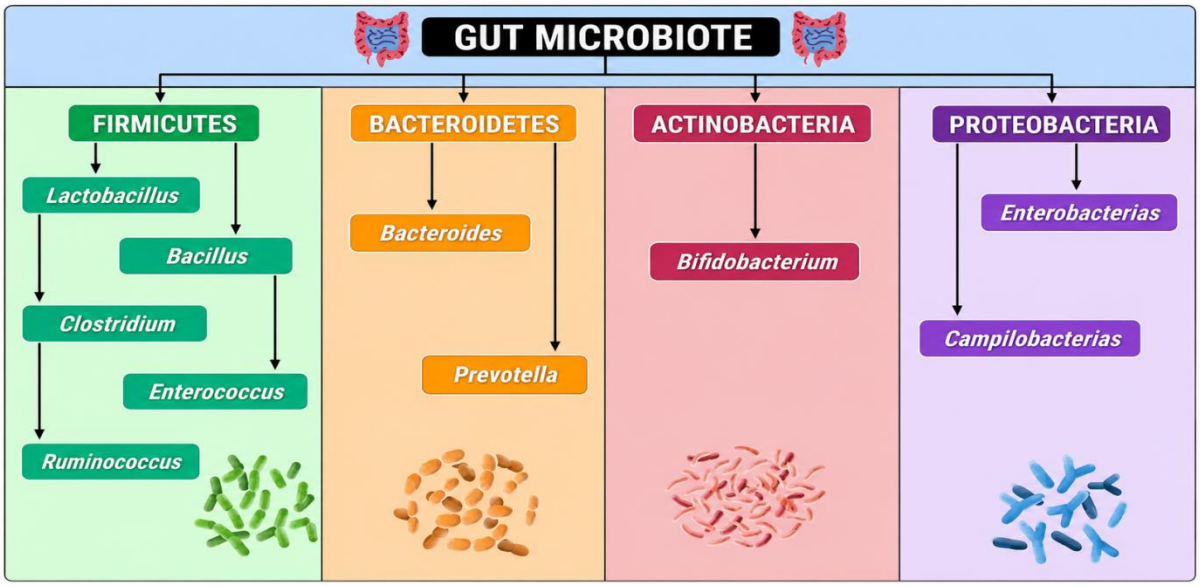

Figure 03: Gut Microbiota Composition: The four major phyla of the human gut microbiota (Rinninella *et al.,* 2019).

II.2.4. Phylum Proteobacteria

Proteobacteria are generally detected in relatively low abundance in healthy individuals. However, an increased proportion of this phylum is frequently associated with intestinal dysbiosis, inflammation, and metabolic disturbances (Fan and Pedersen, 2021).

II.3. Factors Influencing Gut Microbiota Composition

The gut microbiota is shaped by multiple interacting intrinsic and extrinsic factors, with diet representing a major modulatory influence, alongside host-related, pharmacological, and environmental determinants that collectively regulate microbial diversity and function. These factors can be summarized as follows:

II.3.1. Dietary Patterns

Dietary intake modulates microbial composition by shaping substrate availability for microbial metabolism. In particular, dietary fibers promote the growth of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium*, whereas highly processed and Western-type diets are associated with reduced microbial diversity and altered community structure. (Fan *et al.,* 2022)

II.3.2. Pharmacological Agents

Pharmacological interventions, especially broad-spectrum antibiotics, significantly disrupt gut microbial equilibrium by reducing bacterial diversity and inducing long-lasting alterations in microbial community composition and function. (Fan *et al.,* 2022)

II.3.3. Host Biological Factors

Host-related factors, including genetic background and immune system status, contribute to inter-individual variability in gut microbiota composition by shaping host–microbe interactions and immune-mediated microbial selection. (Fan *et al.,* 2022)

II.3.4. Lifestyle and Environmental Factors

Lifestyle and environmental influences such as physical activity, psychological stress, hygiene practices, and geographical location play an important role in shaping gut microbial communities through direct and indirect mechanisms. (Fan *et al.,* 2022)

II.4. Functions of Gut Microbiota

The gut microbiota is vital for host homeostasis, supporting metabolic, protective, and immunological functions. A diverse microbial balance is essential, as its disruption (dysbiosis) is linked to various pathologies. The primary functional categories are summarized below.

II.4.1. Metabolic Functions

The gut microbiota functions as an essential biochemical factory for host homeostasis. It ferments non-digestible carbohydrates into short-chain fatty acids (SCFAs) specifically butyrate for colonocyte fuel, propionate for hepatic regulation, and acetate for immune defense (Rowland *et al.,* 2018). Beyond fermentation, this microbial community is pivotal in protein metabolism, synthesizing K and B-complex vitamins, and biotransforming bile acids into signaling molecules that modulate host immunity via FXR (Farnesoid X Receptor) and TGR5 (Takeda G protein-coupled receptor 5) receptors. Furthermore, it regulates mineral absorption and drug metabolism, serving as an indispensable partner in systemic biochemical management (Acevedo-Román *et al.,* 2024).

II.4.2. Protective Functions

The microbiota provides colonization resistance by outcompeting pathogens for nutrients and secreting antimicrobial bacteriocins. It is essential for intestinal barrier integrity, where microbial SCFAs (especially butyrate) upregulate tight junction proteins, preventing the systemic translocation of pro-inflammatory lipopolysaccharides (LPS). (Parada Venegas *et al.,* 2019; Wastyk *et al.,* 2021; Clemente *et al.,* 2022)

Key protective taxa include *Faecalibacterium prausnitzii*, known for anti-inflammatory effects, and *Akkermansia muciniphila*, which reinforces the mucus layer. While the microbiota

aids in detoxifying xenobiotics, an imbalance can lead to the production of genotoxic metabolites, reflecting the delicate equilibrium of this symbiotic relationship (Ting *et al.,* 2022; Acevedo-Román *et al.,* 2024).

II.4.3. Immunological Functions

The gut microbiota is essential for the maturation of the innate and adaptive immune systems, particularly through the development of gut-associated lymphoid tissues (GALT). Microbial SCFAs, such as butyrate and propionate, induce regulatory T cell (Treg) differentiation, which is critical for immune tolerance and preventing hypersensitivity (Liu *et al.,* 2022). Furthermore, the microbiota maintains inflammatory equilibrium; genera such as *Lactobacillus* and *Bifidobacterium* promote anti-inflammatory cytokines (e.g., IL-10), while immune sensing via PRRs (Pattern Recognition Receptors) modulates the production of secretory IgA (sIgA). This mucosal defense prevents bacterial translocation, protecting the host from dysbiosis-related inflammatory disorders (Acevedo-Román *et al.,* 2024).

II.5. Dysbiosis and Disease

Dysbiosis is a disruption of gut microbiota equilibrium characterized by loss of microbial diversity and pathogen overgrowth (Acevedo-Román *et al.,* 2024). Periodontal pathogenic bacteria serve as key triggers, inducing oral dysbiosis that propagates through the oral-gut microbiome axis to drive systemic inflammation, leading to a broad spectrum of pathologies (Xi *et al.,* 2024).

II.5.1. Dysbiosis in Inflammatory Bowel Disease (IBD)

Inflammatory bowel disease (IBD) represents a major model of gut dysbiosis, characterized by a reduction in Firmicutes and an increase in Proteobacteria. The translocation of pathogenic oral bacteria further aggravates these imbalances, impairs intestinal barrier integrity, and disrupts short-chain fatty acid (SCFA) production, leading to exacerbated immune responses (Ni *et al.,* 2017; Lavelle and Sokol, 2020; Xi *et al.,* 2024).

Crohn's disease: characterized by decreased abundance of *Roseburia* and *Ruminococcus*, alongside increased *Bacteroides fragilis* and disturbances in amino acid metabolism (Pascal *et al.,* 2017; Halfvarson *et al.,* 2017).

Ulcerative colitis: associated with reduced levels of *Bifidobacterium* longum and *Actinobacteria*, together with increased *Fusobacteria* and *Verrucomicrobia* (Nishida *et al.,* 2018; Zuo and Ng, 2018).

II.5.2. Dysbiosis and Obesity

Obesity is associated with an increased Firmicutes/Bacteroidetes ratio, which promotes enhanced energy extraction and adipogenesis. Experimental studies have demonstrated that

transplantation of an "obese microbiota" can induce fat accumulation even in the absence of excessive caloric intake. Jeffrey, Gordon and their colleagues first demonstrated this phenomenon in germ-free mouse models (Turnbaugh *et al.,* 2006; Sonnenburg and Bäckhed, 2016; Dahl *et al.,* 2024).

These effects are further amplified by alterations in the oral-gut axis and immune modulation. Microbial metabolites such as branched-chain amino acids (BCAAs) contribute to insulin resistance, while SCFAs regulate thermogenesis through modulation of brown adipose tissue activity and browning of white adipose tissue (Cani *et al.,* 2019; Zheng *et al.,* 2022).

II.5.3. Dysbiosis and Clostridioides difficile Infection (CDI)

Clostridioides difficile infection (CDI) is a classic example of antibiotic-induced dysbiosis resulting in loss of colonization resistance. This condition facilitates the proliferation of *Clostridioides difficile* and the production of toxins TcdA and TcdB, the two major exotoxins produced by *Clostridioides difficile*, which cause epithelial damage and severe intestinal inflammation. Antibiotic exposure remains the primary risk factor for CDI development (Kordus *et al.,* 2022; Spigaglia, 2024).

II.5.4. The Oral-Gut-Brain Axis and Neuromodulation

The gut microbiota communicates bidirectionally with the central nervous system through the gut-brain axis, involving neuroendocrine, neural, immune, and metabolic pathways. The oral-gut axis also represents an important upstream contributor to systemic dysbiosis and neuroinflammation (Morais *et al.,* 2021; Cryan *et al.,* 2023; Appleton, 2024).

Microbial metabolites, including short-chain fatty acids (SCFAs), tryptophan derivatives, gamma-aminobutyric acid (GABA), and bile acids, influence brain function either by crossing the blood-brain barrier or through vagus nerve signaling. These interactions regulate mood, cognition, stress responses, and immune homeostasis (Agus *et al.,* 2021; Dalile *et al.,* 2019; Sorboni *et al.,* 2022).

Dysbiosis has been associated with several neurological and psychiatric disorders, including major depressive disorder, autism spectrum disorder, Alzheimer's disease, and Parkinson's disease (Sharon *et al.,* 2016; Socała *et al.,* 2021; Cryan *et al.,* 2023).

II.6. Mechanisms of Microbiota-Mediated Carcinogenesis

The transition from symbiosis to oncogenesis is driven by complex interactions in which specific microbial communities promote malignancy through genomic damage, chronic inflammation, immune dysregulation, and metabolic alterations. Gut microbiota contributes to colorectal carcinogenesis via genotoxin production, epithelial barrier disruption, and modulation of oncogenic pathways, highlighting its importance as a potential source of

diagnostic biomarkers and therapeutic targets (Wong and Yu, 2023; Garvey, 2024; White and Sears, 2024).

II.6.1. Genotoxic Effects

Certain bacteria directly induce DNA damage through genotoxins, most notably pks+ *E. coli*, which produces colibactin. This toxin causes double-strand DNA breaks and specific mutational signatures that drive oncogenesis (Pleguezuelos-Manzano *et al.,* 2020). Other factors, such as Bacteroides fragilis toxin and cytolethal distending toxin (CDT), further promote genomic instability through oxidative stress and chronic DNA damage responses (Bhatt *et al.,* 2017).

II.6.2. Chronic Inflammation

Dysbiosis triggers pattern recognition receptors (e.g., TLR4 and NOD2), activating pro-tumorigenic signaling cascades such as NF-κB and STAT3. This signaling induces the production of pro-inflammatory cytokines (IL-6, TNF-α), which promote a tumor-supportive microenvironment characterized by enhanced cell survival, proliferation, angiogenesis, and inhibition of apoptosis (Tilg *et al.,* 2018; Shi *et al.,* 2022).

II.6.3. Bacterial Metabolites and Toxins

Microbial transformation of primary bile acids into secondary bile acids (deoxycholic acid [DCA] and lithocholic acid [LCA]) promotes DNA damage and activates Wnt/β-catenin signaling, particularly under high-fat dietary conditions (Louis *et al.,* 2014; Agus *et al.,* 2021). Additionally, metabolites such as hydrogen sulfide and nitrosamines contribute to epithelial barrier dysfunction and mutagenesis (Liu *et al.,* 2022).

II.6.4. Immunomodulation

The microbiota can subvert anti-tumor immunity by recruiting immunosuppressive cells (MDSCs and Tregs) and inhibiting cytotoxic T cell activity. Notably, *Fusobacterium nucleatum* utilizes its Fap2 protein to suppress NK and T cell responses via TIGIT receptor binding, while other microbial species can upregulate immune checkpoint molecules such as PD-L1, facilitating immune evasion and tumor progression (Kostic *et al.,* 2013; Gur *et al.,* 2015; Shi *et al.,* 2022).

II.7. Therapeutic Implications of Gut Microbiota

II.7.1. Probiotics and Synbiotics

Probiotics, prebiotics, and synbiotics play an important role in maintaining gut microbiota balance and intestinal homeostasis.

Probiotics (*Lactobacillus*, *Bifidobacterium*): help restore beneficial bacterial populations.

Prebiotics (inulin, fructooligosaccharides [FOS], galactooligosaccharides [GOS]): stimulate SCFA production.

Dietary interventions: fiber-rich diets and fermented foods promote microbial diversity and intestinal homeostasis.

Oral health also plays an important preventive role by limiting microbial dissemination through the oral-gut axis (Ianiro *et al.,* 2022; Sims *et al.,* 2023; Smits *et al.,* 2016).

These therapeutic approaches contribute to restoring beneficial microbial populations, strengthening the intestinal barrier, and reducing inflammation associated with dysbiosis-related disorders. Recent studies also emphasize the importance of personalized nutritional interventions targeting microbiome composition for disease prevention and therapeutic improvement (Fan and Pedersen, 2021; Yadegar *et al.,* 2023).

II.7.2. Fecal Microbiota Transplantation

Fecal microbiota transplantation (FMT) is an effective microbiome-based therapy used to restore microbial diversity in severe dysbiosis. It has shown remarkable clinical efficacy in recurrent *Clostridioides difficile* infection through the re-establishment of intestinal microbial balance. Nevertheless, donor screening and long-term safety remain important considerations for clinical application (Baunwall *et al.,* 2020; Walter and Shanahan, 2023).

II.7.3. Precision Medicine

Advances in metagenomics and multi-omics technologies have improved the understanding of microbiome–host interactions and disease mechanisms. These approaches enable the identification of individualized microbial signatures, supporting precision medicine strategies and targeted therapeutic interventions for improved clinical outcomes (Fan and Pedersen, 2021; Fang *et al.,* 2024).

III. Metagenomics

III.1. Definition

Metagenomics is a culture-independent approach for studying microbial communities through the direct analysis of collective genetic material recovered from environmental samples, enabling comprehensive insights into microbial diversity and functional genes without prior cultivation. This field has emerged to overcome the major limitation of traditional microbiology where most microorganisms cannot be cultured in laboratory conditions by utilizing a genomic approach that enables the identification, characterization, and functional analysis of microorganisms based solely on DNA sequences extracted from complex environments such as soil, water, or the human gut (Quince *et al.,* 2017; Franzosa *et al.,* 2019; Fan and Pedersen, 2021). By sequencing all DNA present in a sample, this discipline provides a comprehensive, ecosystem-level view that simultaneously reveals both taxonomic diversity and the metabolic potential of uncultured microorganisms (Pasolli *et al.,* 2019; Almeida *et al.,* 2021). Consequently, recent advances in high-throughput next-generation sequencing (NGS) technologies have significantly expanded the scale of research, transforming metagenomics into a high-throughput discipline where human gut microbiome datasets surpassed 100,000 sequenced samples by 2023, thereby providing unprecedented resolution for large-scale comparative and functional studies (Kim *et al.,* 2024; Fan and Pedersen, 2021).

III.2. Importance of Studying Metagenomics

Metagenomics is a key approach for studying microbial communities and their impact on biological systems. It has revealed the diversity and functional roles of microorganisms across environments and led to important applications in multiple fields (Lema *et al.,* 2023). In biomedical contexts, metagenomic research has particularly advanced the understanding of the human microbiome and its role in health and disease.

The major applications of metagenomics can be summarized across several domains.

III.2.1. Human Health

Metagenomic approaches enable the exploration of human-associated microbiota for the development of diagnostic, preventive, and therapeutic strategies based on microbiome modulation and microbiota-derived molecules (Chiu and Miller, 2024).

III.2.2. Agriculture

In agricultural systems, metagenomic insights are used to enhance soil microbial management, improve plant health, and support sustainable farming practices through the characterization of beneficial and pathogenic microbial communities (Lema *et al.,* 2023).

III.2.3. Food Industry

Metagenomics contributes to optimizing fermentation processes, improving food safety monitoring, and supporting the development of functional foods by analyzing and controlling microbial populations involved in food production (Lema *et al.,* 2023).

III.3. Principles of Metagenomics

III.3.1. DNA Extraction Methods

DNA extraction represents the critical foundational step in metagenomic research, as the fidelity of downstream sequencing and bioinformatic analysis is inherently dependent on the quality and yield of the extracted genetic material. The process necessitates the effective lysis of microbial cells to release their genomic content while rigorously minimizing degradation and external contamination. Consequently, extraction protocols must be meticulously calibrated to the sample type, balancing cell lysis efficiency with DNA purity and integrity. While environmental matrices like soil require robust mechanical disruption, human intestinal samples often permit gentler chemical approaches. Notably, recent methodological advancements in microbial enrichment have facilitated the depletion of host DNA in clinical biopsies, thereby enabling the high-resolution characterization of low-abundance microbial taxa down to a 1% threshold (Chiu and Miller, 2024; Wu-Woods *et al.,* 2023).

III.3.2. Sequencing Techniques

III.3.2.1. Shotgun Sequencing

Shotgun metagenomics is a comprehensive, high-throughput approach for analyzing entire microbial communities through the direct sequencing of all extracted DNA, without the need for targeted amplification of marker genes such as 16S rRNA. This culture-independent strategy relies on next-generation sequencing (NGS) technologies to generate large-scale datasets that capture both taxonomic composition and functional potential of complex microbiomes. By avoiding PCR amplification steps, shotgun metagenomics significantly reduces primer-associated biases and provides a more accurate representation of low-abundance taxa, viral populations, and archaeal members. In addition to microbial profiling, this approach enables the identification of novel genes, metabolic pathways, and functional signatures associated with specific physiological or pathological states (Thomas *et al.,* 2012).

In the context of colorectal cancer (CRC), shotgun metagenomics has been widely applied to characterize gut microbiota dysbiosis and to identify microbial signatures associated with tumor initiation and progression. As illustrated in Figure 04 (an example of CRC metagenomic shotgun analysis), this strategy typically involves DNA extraction from stool or tissue samples, library preparation, high-throughput sequencing, followed by bioinformatic

analysis for taxonomic classification, functional annotation, and pathway reconstruction. Such analyses have revealed important alterations in microbial diversity and functional capacity in CRC patients, including enrichment of pro-inflammatory and genotoxic bacteria as well as depletion of beneficial commensals. Despite its high resolution and analytical power, shotgun metagenomics remains computationally demanding and relatively costly compared to amplicon-based approaches, and it requires careful consideration of host DNA contamination and data interpretation challenges (Thomas *et al.,* 2012; Quince *et al.,* 2017; Esfahani *et al.,* 2025).

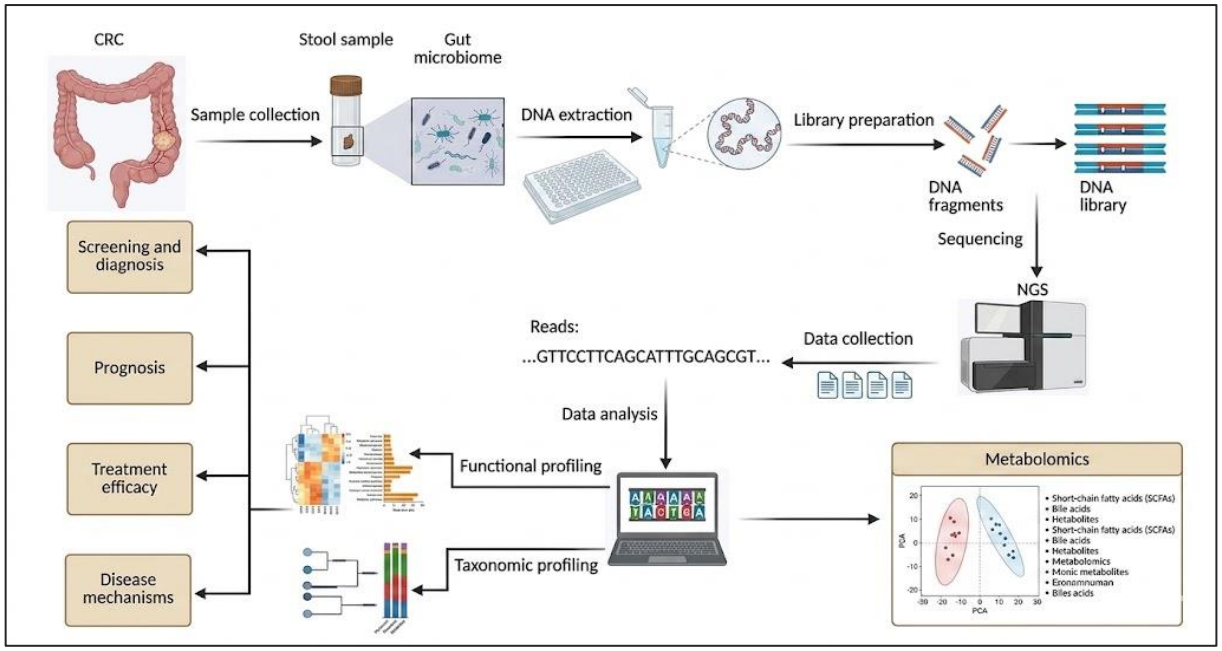

Figure 04: The role of shotgun metagenomics in elucidating the links between the gut microbiome and colorectal cancer (Esfahani *et al.,* 2025).

III.3.2.2. 16S rRNA Gene Sequencing

The 16S rRNA gene sequencing approach, also known as amplicon sequencing, is a well-established molecular method for microbial identification and phylogenetic analysis, targeting conserved taxonomically informative marker regions (Figure 05). This approach proceeds through the following sequential stages (Johnson *et al.,* 2019; Callahan *et al.,* 2016; Jovel *et al.,* 2016).

The 16S rRNA gene is targeted as a housekeeping genetic marker of approximately 1,500 base pairs, consisting of conserved regions interspersed with nine hypervariable regions (V1–V9) (Chiu and Miller, 2024).

The conserved regions are exploited for the design of universal primers capable of amplifying the gene across all bacterial species, while the hypervariable regions provide species-specific sequences that enable taxonomic classification (Chiu and Miller, 2024). Different region combinations, such as V1–V2, V3–V4, and V3–V5, vary in their taxonomic resolution, allowing discrimination at the family, genus, and species levels. The selection of target regions should be guided by the specific objectives of the study (Gao *et al.,* 2021).

The selected hypervariable regions are amplified by polymerase chain reaction (PCR), then subjected to high-throughput sequencing to generate millions of reads representing the microbial composition of the sample (Chiu and Miller, 2024).

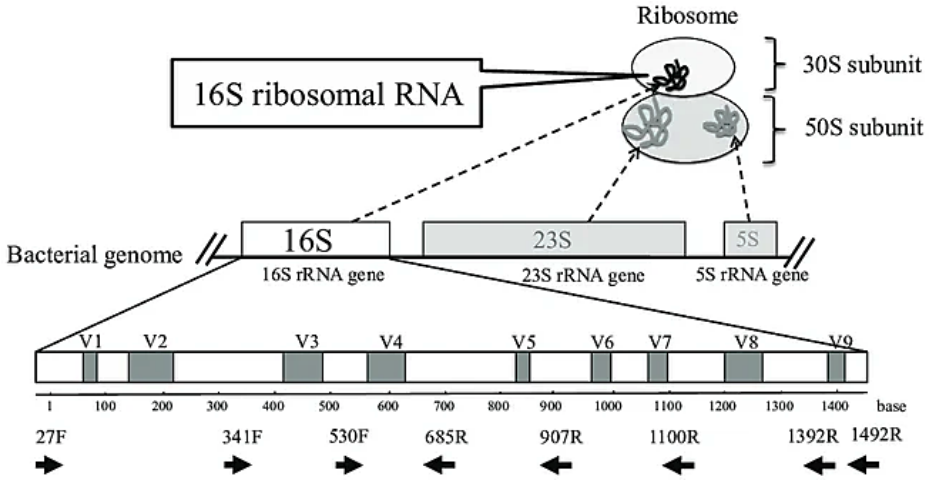

Figure 05: Structure of the 16S rRNA gene showing the nine hypervariable regions (V1–V9) and its location within the bacterial ribosome. (Fukuda *et al.* 2016).

IV.Proteomics

IV.1 Definition

The proteome is a central concept in molecular and systems biology that refers to the complete and entire set of proteins expressed within a genome, cell, tissue, or organism at a given time under specific conditions. Representing the functional output of the genome, the proteome is highly dynamic and responsive compared to the static genome, as protein expression continuously shifts in response to developmental cues, environmental factors, disease states, and therapeutic interventions (Aebersold and Mann, 2016; Uhlén *et al.,* 2023; Wilhelm *et al.,* 2024). At the structural and molecular complexity level, this diversity is further expanded through various proteoforms arising from genetic variation, alternative splicing, and post-translational modifications (PTMs), which are essential for understanding protein function at a systems level (Smith and Kelleher, 2018; Xu *et al.,* 2024). From an analytical perspective,

modern advancements in mass spectrometry-based proteomics have enabled large-scale identification and quantification of these complex protein profiles, with recent technological breakthroughs allowing for single-cell resolution and spatial proteomics in clinical and biomedical applications (Sharma *et al.,* 2022; Guzman *et al.,* 2024).

IV.2. Importance of Proteomics

Proteomics is a key discipline in life sciences that enables the large-scale study of proteins, the main functional effectors of cellular processes. Due to their dynamic nature and structural modifications in response to physiological and pathological conditions, proteins provide critical insights into biological function, disease mechanisms, and therapeutic responses.

IV.2.1. Dynamic Nature of Proteins

Proteins are highly dynamic molecules whose abundance and post-translational modifications change during processes such as cell differentiation, aging, disease progression, and environmental adaptation. (Messner and Ralser, 2022)

IV.2.2. Biological and Clinical Relevance

Quantitative proteome analysis in cells, tissues, and body fluids is essential for advancing biological research, improving diagnostics, and supporting therapeutic development. (Messner and Ralser, 2022)

IV.2.3. Protein Interaction Networks

The interactome, representing protein–protein and protein–molecule interactions, is fundamental for understanding cellular organization and disease mechanisms (Huttlin *et al.,* 2021).

IV.3. Technological Advances

Mass spectrometry-based techniques, including affinity purification and proximity labeling, have significantly improved the characterization of molecular interactions within biological systems. (Huttlin *et al.,* 2021)

IV.4. Proteome Composition

The proteome represents the complete set of proteins expressed in a biological system, reflecting its structural and functional organization. It is highly complex due to the diversity of protein classes and their regulatory modifications, which together determine cellular behavior and biological function. (Alberts *et al.,* 2015)

IV.4.1. Structural and Functional Classification of Proteins

The proteome is composed of distinct protein categories that differ in structure, localization, and biological role. Structural proteins such as collagen, actin, and tubulin provide

mechanical support and cellular architecture, with collagen being a major component of the extracellular matrix, while actin and tubulin regulate cytoskeletal organization, cell division, and motility. (Alberts et al., 2015)

Functional proteins include enzymes, regulatory proteins, transporters, and immune-related molecules that control metabolism, signaling, and host defense. Enzymes such as kinases and proteases catalyze essential biochemical reactions, ensuring metabolic regulation and signal transduction fidelity. Regulatory proteins, including transcription factors such as p53 and NF-κB, control gene expression and cellular responses, while signaling proteins mediate pathways such as MAPK and PI3K involved in growth and survival. (Hanahan & Weinberg, 2011)

Transport proteins such as hemoglobin and membrane channels facilitate molecular exchange, whereas immune proteins, including antibodies and cytokines, coordinate innate and adaptive immune responses. (Murphy and Weaver, 2016)

IV.4.2. Post-Translational Modifications (PTMs)

Post-translational modifications (PTMs) significantly expand proteome complexity beyond genomic coding capacity. More than 200 types of PTMs have been identified, with phosphorylation, glycosylation, ubiquitination, and acetylation being among the most studied. (Xu *et al.,* 2024)

Phosphorylation regulates protein activity and signaling networks, glycosylation influences protein folding, stability, and immune recognition, ubiquitination targets proteins for degradation via the proteasome system, and acetylation modulates chromatin structure and gene expression. (Olsen *et al.,* 2006)

Advances in mass spectrometry-based proteomics have enabled large-scale mapping of PTMs, providing deeper insight into protein regulation and function (Xu *et al.,* 2024).

V. Integrated multi-omics

V.1. Definition

Integrated multi-omics refers to a systems biology approach that combines multiple layers of molecular data such as genomics, transcriptomics, proteomics, and metabolomics to provide a comprehensive and system-level understanding of biological processes and disease mechanisms. By moving beyond single-layer analysis, this integrative strategy enables the examination of heterogeneous datasets within a unified analytical framework, thereby revealing regulatory networks and functional interactions across genes, transcripts, proteins, and metabolites (Hasin *et al.,* 2017; Huang *et al.,* 2017; Karczewski and Snyder, 2018). As a cornerstone of modern systems biology, multi-omics integration overcomes the limitations of

single-omics approaches, allowing the construction of predictive models through advanced computational and statistical methods that significantly improve the interpretation of complex biological systems (Bersanelli *et al.,* 2016; Misra *et al.,* 2019; Cong and Endo, 2024; Liu *et al.,* 2024). Ultimately, this comprehensive molecular stratification enhances the discovery of early and reliable biomarkers, improves the understanding of disease heterogeneity, and facilitates the transition from descriptive biology to predictive, system-level modeling, thereby robustly supporting modern precision and predictive medicine approaches (Subramanian *et al.,* 2020).

V.2. Importance of Multi-Omics Integration

Multi-omics integration has become a cornerstone of modern systems biology, as it enables a comprehensive understanding of biological systems by combining data from multiple molecular layers such as genomics, transcriptomics, proteomics, and metabolomics, thereby overcoming the limitations of single-omics approaches and improving the interpretation of complex biological processes and disease mechanisms (Cong and Endo, 2024; Liu *et al.,* 2024):

- Provides a holistic, system-level view of biological processes
- Integrates multiple omics layers into a unified analytical framework
- Reveals functional interactions across genes, transcripts, proteins, and metabolites
- Improves detection of reliable and early disease biomarkers
- Enhances understanding of complex diseases and biological heterogeneity
- Supports transition from descriptive to predictive and precision medicine approaches

V.3. Metagenomic–Proteomic Integration Strategies

Metagenomic–proteomic integration represents a systems biology framework that connects microbial genetic potential with actual protein expression, enabling a more accurate understanding of functional microbial activity and host–microbe interactions. This approach shifts biological analysis from static genomic information toward dynamic functional interpretation of microbial ecosystems (Wang *et al.,* 2024; Du *et al.,* 2025).

V.3.1. Gene-Protein Linkage Strategy

Metagenomic datasets are used as reference databases to improve peptide identification in metaproteomic analyses, thereby establishing a direct functional link between genes and expressed proteins and enhancing annotation accuracy in complex microbial communities (Morabito *et al.,* 2025).

V.3.2. Functional Activity Comparison Approach

This strategy relies on comparing microbial genetic potential with observed protein expression to identify actively functioning metabolic and regulatory pathways within microbial

communities, allowing discrimination between potential and actual biological activity (Duan *et al.,* 2025).

V.3.3. Metabolic Network Modeling (GEMs and ecFBA)

Genome-scale metabolic models (GEMs), combined with enzyme-constrained Flux Balance Analysis (ecFBA), are used to simulate metabolic fluxes, identify metabolic bottlenecks, and reconstruct interaction networks within microbial and host-associated environments (Jiang *et al.,* 2025)

V.4. Bioinformatic Tools for Multi-Omics Analysis

Multi-omics integration relies on advanced computational frameworks designed to process, integrate, and interpret heterogeneous high-dimensional biological datasets, with a strong emphasis on reproducibility, scalability, and statistical robustness (Abdelaziz *et al.,* 2024; Baião *et al.,* 2025).

Section 2: Experimental part

Materials and Methods

I. Materials and Methods

I.1. Materials

I.1.1. Study Design and Data Source

The first part of present study was conducted using publicly available whole-metagenome shotgun sequencing datasets obtained from the European Nucleotide Archive (ENA). The selected datasets originated from the study by Zeller *et al.* (2014), entitled "Potential of fecal microbiota for early-stage detection of colorectal cancer", deposited under the ENA study accession ERP005534.

A total of eight biological samples were selected (table 1 and 2), including four healthy controls and four patients diagnosed with colorectal cancer (CRC). All specimens consisted of human fecal (stool) samples collected before sequencing. For each biological sample, paired-end sequencing data were available as forward (R1) and reverse (R2) FASTQ files, resulting in a total of sixteen FASTQ files.

The second part of this study represent a secondary analysis of previously published proteomic datasets (for carring out functional pathway analysis) related to colorectal cancer (CRC) and gut microbiota interactions (Li *et al.,* 2023; Zhang *et al.,* 2024). This computational strategy was selected due to the technical, financial, and logistical limitations associated with performing large-scale in vitro and experimental multi-omics analyses, particularly those involving next-generation sequencing platforms and high-resolution mass spectrometry technologies (Wang *et al.,* 2022).

Rather than generating primary experimental data, the current work relied on high-quality datasets produced by internationally recognized studies and deposited in validated public repositories. This approach enhances reproducibility, data reliability, and methodological transparency while allowing access to large-scale biological information that would otherwise require extensive laboratory infrastructure (Zhang *et al.,* 2024).

The study specifically integrates metagenomic and proteomic datasets through bioinformatic and multi-omics analytical frameworks in order to investigate the relationship between gut microbiota dysbiosis and colorectal cancer progression. Consequently, the present analysis is fundamentally based on previously published reference studies, from which the biological datasets, methodological frameworks, and analytical strategies were adopted and comparatively interpreted (Li *et al.,* 2023; Zhang *et al.,* 2024).

I.1.2. Description of the Selected Samples for metagenomic analysis

Table 1: summarizes the metadata associated with the samples included in this study.

Group	**Sample ID**	**ENA Run Accession**	**Sex**	**Age (years)**	**Biological Material**	**Sequencing Layout**
Control	FR-060	ERR478962	Male	53	Stool	Paired-end (R1/R2)
Control	FR-568	ERR478965	Male	35	Stool	Paired-end (R1/R2)
Control	FR-726	ERR478960	Female	72	Stool	Paired-end (R1/R2)
Control	FR-902	ERR479023	Female	56	Stool	Paired-end (R1/R2)
CRC	FR-450	ERR479303	Male	44	Stool	Paired-end (R1/R2)
CRC	FR-414	ERR479066	Male	63	Stool	Paired-end (R1/R2)
CRC	FR-125	ERR479053	Female	72	Stool	Paired-end (R1/R2)
CRC	FR-830	ERR479176	Female	54	Stool	Paired-end (R1/R2)

Table 2: Details of the FASTQ files used in metagenomic study.

ENA Run Accession	Sample	Group	Read File	Strand
ERR478962	FR-060	Control	ERR478962_1.fastq.gz	Forward (R1)
ERR478962	FR-060	Control	ERR478962_2.fastq.gz	Reverse (R2)
ERR478965	FR-568	Control	ERR478965_1.fastq.gz	Forward (R1)
ERR478965	FR-568	Control	ERR478965_2.fastq.gz	Reverse (R2)
ERR478960	FR-726	Control	ERR478960_1.fastq.gz	Forward (R1)
ERR478960	FR-726	Control	ERR478960_2.fastq.gz	Reverse (R2)
ERR479023	FR-902	Control	ERR479023_1.fastq.gz	Forward (R1)
ERR479023	FR-902	Control	ERR479023_2.fastq.gz	Reverse (R2)
ERR479303	FR-450	CRC	ERR479303_1.fastq.gz	Forward (R1)
ERR479303	FR-450	CRC	ERR479303_2.fastq.gz	Reverse (R2)
ERR479066	FR-414	CRC	ERR479066_1.fastq.gz	Forward (R1)
ERR479066	FR-414	CRC	ERR479066_2.fastq.gz	Reverse (R2)
ERR479053	FR-125	CRC	ERR479053_1.fastq.gz	Forward (R1)
ERR479053	FR-125	CRC	ERR479053_2.fastq.gz	Reverse (R2)
ERR479176	FR-830	CRC	ERR479176_1.fastq.gz	Forward (R1)
ERR479176	FR-830	CRC	ERR479176_2.fastq.gz	Reverse (R2)

I.1.3. Computational Environment

All bioinformatic analyses were performed on a computer running Ubuntu Linux. Sequence processing and downstream analyses were conducted using the QIIME 2 platform together with standard Linux command-line utilities.

The software environment included:

- Ubuntu Linux operating system
- QIIME 2
- Bash shell utilities
- gzip for compressed FASTQ files
- FASTQ-formatted paired-end shotgun sequencing datasets downloaded from ENA

I.1.4. Download of Sequencing Data

Raw sequencing reads were downloaded from the European Nucleotide Archive (ENA) using their corresponding run accession numbers. Each accession generated two compressed

FASTQ files representing paired-end sequencing reads: a forward read file (R1) and a reverse read file (R2).

I.1.5. Functional pathway analysis

Functional pathway analysis was performed using HUMAnN3, enabling reconstruction of microbial metabolic pathways through mapping against UniRef90 and MetaCyc databases (Franzosa et al., 2018).

Orthologous group annotation and functional prediction were conducted using eggNOG-mapper, while protein domain and motif prediction were performed using InterProScan, integrating multiple signature databases including Pfam and SMART (Cantalapiedra et al., 2021; Jones et al., 2014).

I.2. Methods

I.2.1. Subsampling Strategy

To reduce computational requirements while maintaining representative microbial diversity, a subsampling approach was applied prior to downstream analyses.

For each biological sample, only the forward sequencing reads (R1) were retained for analysis. Exactly 1,000,000 reads (1 M reads) were randomly selected from each forward FASTQ file, whereas reverse reads (R2) were not included in the downstream workflow. This strategy generated standardized datasets with identical sequencing depth across all samples and minimized biases related to unequal read counts.

Consequently, the final analysis was performed on eight subsampled FASTQ files corresponding to one million forward reads per sample.

I.2.2. Quality Assessment

The quality of the raw sequencing reads was evaluated before downstream analyses. Standard quality metrics included read length consistency, sequence quality distribution, GC content, duplicated sequences, overrepresented sequences, and potential adapter contamination. Quality assessment ensured that the selected reads were suitable for microbiome profiling and taxonomic analyses.

I.2.3. Import of Data into QIIME 2

The subsampled FASTQ files were imported into the QIIME 2 environment using an appropriate manifest file describing sample identifiers and file locations. Sequence data were converted into QIIME 2 artifacts (.qza), enabling subsequent bioinformatic processing.

I.2.4. Taxonomic Profiling

The processed metagenomic sequences were subjected to taxonomic profiling using QIIME 2-compatible analytical workflows. Taxonomic assignments were generated to

characterize the bacterial communities present in each fecal sample and to compare microbial composition between healthy controls and colorectal cancer patients.

I.2.5. Statistical Considerations

Because an identical sequencing depth of one million forward reads per sample was used after subsampling, inter-sample comparisons were performed on standardized datasets, thereby reducing biases associated with unequal sequencing coverage.

I.2.6. Functional Pathway Analysis

Functional profiling of metagenomic samples was performed using HUMAnN3 (version 3.6) to characterize microbial metabolic pathways and gene family composition. Quality-controlled reads were aligned to the UniRef90 database using DIAMOND (version 2.1.8), followed by mapping to MetaCyc metabolic pathways. Functional abundance profiles were normalized to copies per million (CPM) to account for sequencing depth variation.

Functional annotations were further enriched using the KEGG Mapper platform, focusing on metabolic pathways, secondary metabolite biosynthesis, and microbial metabolism in diverse environments. Enzyme Commission (EC) numbers were additionally mapped to KEGG Orthology (KO) terms for comprehensive functional characterization.

Results and Discussion

II. Results and discussions

II.1. Taxonomic Profiling

To investigate the bacterial composition of the gut microbiota, taxonomic profiling was performed using Krona interactive visualizations generated from shotgun metagenomic sequencing data. For clarity, the present analysis was restricted to the bacterial domain, allowing the comparison of microbial community structures between a representative healthy control sample and a colorectal cancer (CRC) sample.

Figure 6 illustrates the bacterial taxonomic profile of the healthy control sample. The microbiota was overwhelmingly dominated by members of the phylum Bacteroidota, with Prevotella copri DSM 18205 representing approximately 40% of the total bacterial abundance. Other members of the genus *Prevotella* were also detected, including *Prevotella* sp. 109 (approximately 3%). Additional taxa belonging to the family Bacteroidaceae, such as *Bacteroides* species, contributed nearly 21% of the bacterial community.

Besides Bacteroidota, members of the Firmicutes phylum were well represented. Several beneficial butyrate-producing bacteria belonging to the family Lachnospiraceae, particularly species of the genus *Roseburia*, were identified, including *Roseburia intestinalis* (4%), *Roseburia inulinivorans* (3%), and *Roseburia faecis* (2%). Low-abundance populations of *Ruminococcus*, *Eubacterium*, and *Clostridium* species were also present. Minor contributions from Proteobacteria (approximately 0.4%), Verrucomicrobia represented by *Akkermansia muciniphila* (0.2%), and Fusobacteriales (0.0003%) were observed.

In contrast, the bacterial composition of the CRC sample (Figure 7) exhibited a markedly different taxonomic structure. Although Firmicutes remained abundant, the community was characterized by a pronounced enrichment of *Butyrivibrio crossotus DSM 2876,* accounting for approximately 17% of the bacterial population. Members of the genus *Bacteroides* also showed substantial representation (approximately 14%), while the relative abundance of *Prevotella copri* decreased dramatically to approximately 4%, indicating a major shift in the Prevotella-dominated enterotype observed in the control sample.

The CRC microbiota also contained increased proportions of several taxa belonging to the families Lachnospiraceae and Ruminococcaceae, including *Faecalibacterium prausnitzii*, *Eubacterium eligens*, *[Eubacterium] siraeum*, *Ruminococcus faecis*, and *Dorea longicatena*. Furthermore, members of the Enterobacteriaceae family within the phylum Proteobacteria were more apparent than in the control sample, including *Hafnia alvei*. Although their overall abundance remained relatively low, Proteobacteria reached approximately 2% of the bacterial

community, suggesting a modest expansion of facultative anaerobic bacteria. Minor populations of Fusobacteriales, Verrucomicrobia, and several other phyla were also detected.

Overall, the comparison between the two profiles demonstrates substantial taxonomic restructuring associated with colorectal cancer. The most striking difference was the reduction of *Prevotella copri*, which dominated the healthy microbiota but represented only a small fraction of the CRC-associated community. Simultaneously, the CRC sample displayed a more heterogeneous bacterial composition, with increased contributions from Butyrivibrio, Bacteroides, Enterobacteriaceae, and several Firmicutes lineages.

These observations are consistent with the concept of gut microbiota dysbiosis in colorectal cancer, whereby the normal microbial equilibrium is disrupted and replaced by altered community structures. Numerous studies have reported enrichment of opportunistic or pro-inflammatory bacterial taxa together with shifts in dominant commensal populations in CRC patients. Nevertheless, it is important to emphasize that the present Krona plots correspond to individual representative samples; therefore, biological variability between subjects should be considered, and conclusions should be supported by analyses across the complete cohort.

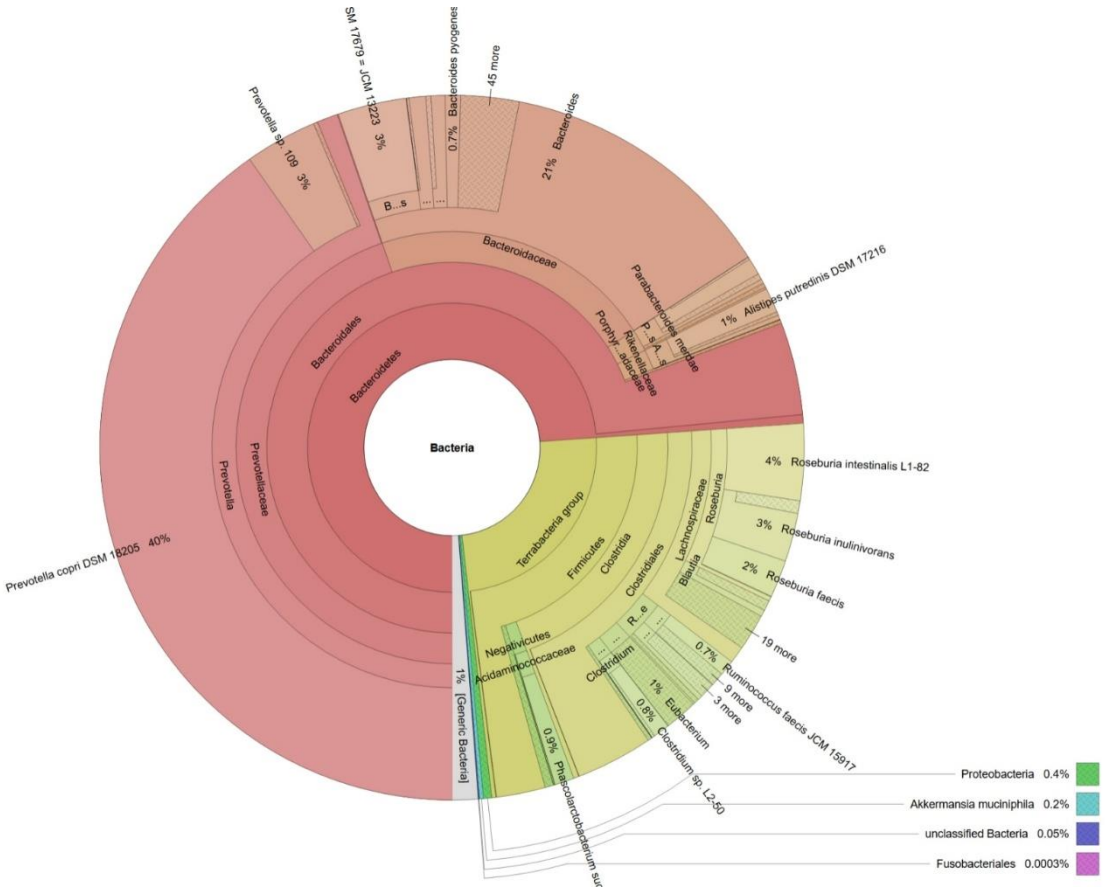

Figure 6: Krona taxonomic profile of the bacterial community identified in the representative healthy control stool sample based on shotgun metagenomic sequencing.

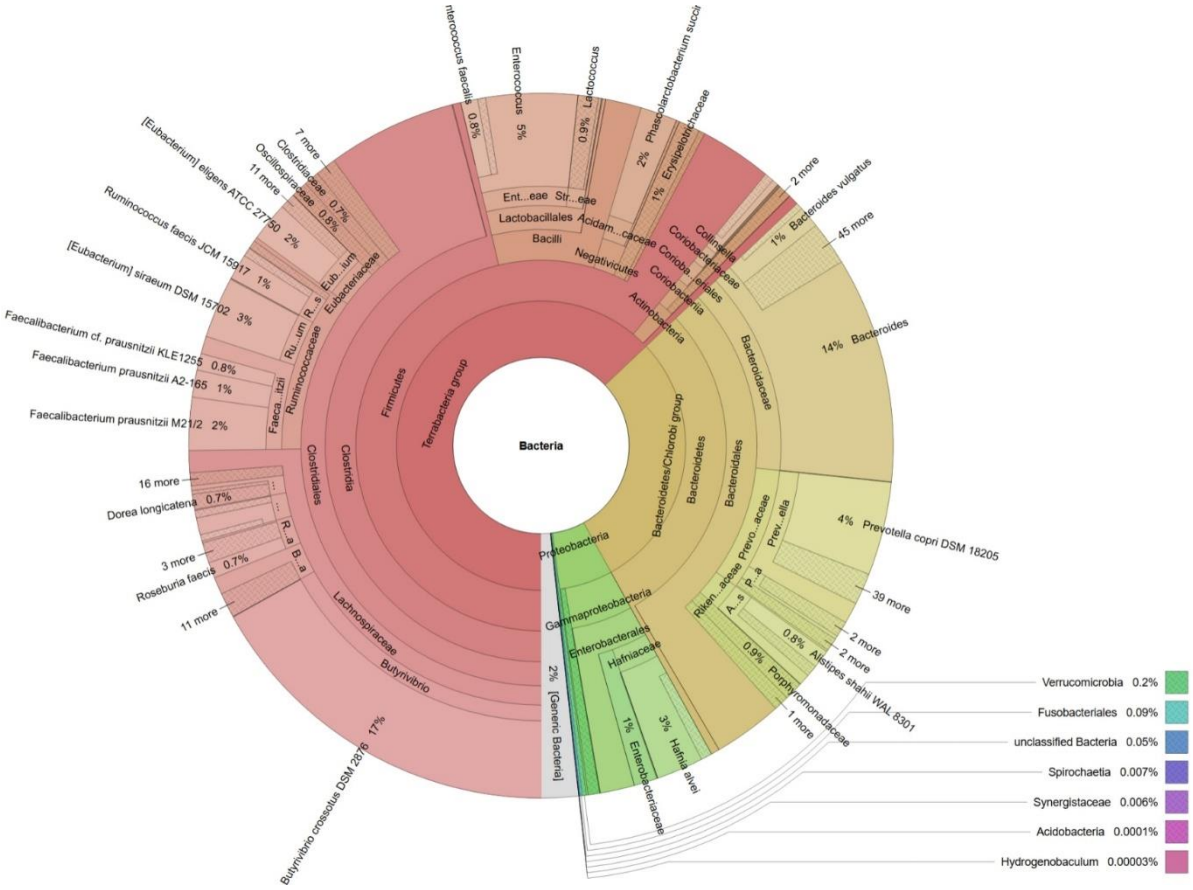

Figure 7: Krona taxonomic profile of the bacterial community identified in the representative colorectal cancer stool sample based on shotgun metagenomic sequencing.

The enrichment of key colorectal cancer-associated pathobionts suggests a coordinated microbial contribution to tumor-promoting processes rather than passive disease association. These organisms actively participate in host-microbe interactions that influence epithelial signaling, immune modulation, and genomic stability, thereby functioning as mechanistic drivers of colorectal carcinogenesis.

The presence of *Fusobacterium nucleatum* is particularly important, as it has been shown to promote tumor progression through multiple mechanisms, including immune suppression, epithelial adhesion, and activation of oncogenic signaling pathways such as Wnt/β-catenin. Its association with epithelial and tumor markers further supports its direct involvement in enhancing tumor invasiveness and progression within the colorectal microenvironment. (Abed *et al.,* 2016)

Similarly, the coexistence of *Escherichia coli* pks+ and enterotoxigenic *Bacteroides fragilis* suggests synergistic pathogenic interactions that may amplify DNA damage, inflammatory signaling, and epithelial disruption. Colibactin-producing *E. coli* induces double-strand DNA breaks, while *B. fragilis* toxin promotes epithelial barrier breakdown and inflammatory cascade activation. Their co-occurrence suggests that CRC-associated dysbiosis operates as a cooperative microbial consortium rather than isolated pathogenic species. (Nougayrède *et al*., 2006; Bhatt *et al.,* 2017)

Conversely, the depletion of beneficial butyrate-producing bacteria such as *Faecalibacterium prausnitzii* represents a critical loss of protective microbial functions. These bacteria are essential for maintaining epithelial energy metabolism, reinforcing gut barrier integrity, and suppressing inflammatory responses. Their reduction likely contributes to diminished anti-inflammatory signaling and metabolic support, thereby creating a permissive environment for tumor development and progression. (Atarashi *et al.,* 2013).

II.2.. Functional Profiling of the Microbiome

Functional profiling of the gut microbiome revealed a pronounced metabolic and functional reprogramming between colorectal cancer (CRC) patients and healthy controls. A total of 47 functional pathways were significantly enriched in CRC samples, whereas 32 pathways associated with beneficial metabolic activities were significantly depleted ($p < 0.05$; $p < 0.001$), the sixth top of them are indicated in the table 8. This functional imbalance reflects a strong shift from a symbiotic, metabolically protective microbiome toward a dysbiotic and pro-inflammatory microbial ecosystem, consistent with previous metagenomic studies in colorectal cancer patients (Thomas *et al.,* 2019; Wirbel *et al.,* 2019; Yachida *et al.,* 2019).

Among the most significantly enriched pathways in CRC samples, lipopolysaccharide (LPS) biosynthesis showed a marked increase (8.52% in CRC vs. 3.18% in controls; +2.68-fold). This observation is biologically relevant, as LPS is a major component of the outer membrane of Gram-negative bacteria and is a potent inducer of chronic inflammation via Toll-like receptor 4 (TLR4) signaling. Chronic exposure to LPS has been strongly associated with colorectal carcinogenesis through activation of NF-κB signaling, promotion of pro-inflammatory cytokines, and induction of epithelial barrier dysfunction (Kutikhin, 2011; Cario, 2016). Elevated LPS biosynthetic potential therefore suggests an inflammatory microbiome capable of sustaining tumor-promoting microenvironments.

Similarly, the pathway related to bacterial invasion of epithelial cells was significantly enriched in CRC samples (7.21% vs. 2.82%; +2.56-fold). This finding supports the hypothesis that CRC-associated microbiota possesses increased virulence traits enabling mucosal

adherence and epithelial penetration. Such mechanisms have been particularly associated with *Fusobacterium nucleatum* and *Escherichia coli*, both of which have been implicated in colorectal tumor progression through adhesin-mediated colonization and direct modulation of host signaling pathways (Kostic *et al.,* 2013; Rubinstein *et al.,* 2013). These invasive capabilities may facilitate microbial translocation and further exacerbate inflammatory responses in the tumor microenvironment.

In addition, DNA replication and repair pathways were significantly upregulated in CRC-associated microbiomes (6.84% vs. 3.14%; +2.18-fold). This may reflect increased microbial proliferation and genomic stress adaptation in the tumor-associated niche. Enhanced DNA repair capacity has been interpreted as a microbial survival strategy in hostile environments characterized by oxidative stress and immune activation, both of which are hallmarks of the tumor microenvironment (Fung *et al.,* 2017).

In contrast, several short-chain fatty acid (SCFA)-related pathways were markedly reduced in CRC samples, particularly butanoate metabolism (3.24% vs. 8.52%; −2.63-fold) and propanoate metabolism (3.48% vs. 7.82%; −2.25-fold). This depletion is highly significant, as butyrate is a key microbial metabolite with well-established anti-inflammatory and anti-neoplastic properties. Butyrate serves as the primary energy source for colonocytes and exerts histone deacetylase (HDAC) inhibitory activity, thereby regulating gene expression, promoting apoptosis of malignant cells, and maintaining epithelial barrier integrity (Canani *et al.,* 2011; Donohoe *et al.,* 2012). A reduction in butanoate-producing capacity is therefore strongly indicative of impaired colonocyte homeostasis and enhanced susceptibility to carcinogenesis.
Likewise, the observed decrease in propanoate metabolism further supports the loss of SCFA-mediated protective functions. Propionate has been shown to modulate gluconeogenesis, immune regulation, and anti-inflammatory signaling, contributing to intestinal homeostasis and systemic metabolic balance (Hosseini *et al.,* 2011). Its depletion may therefore contribute to both local and systemic metabolic dysregulation in CRC patients.

Another significantly reduced pathway was vitamin B12 biosynthesis (2.85% vs. 7.18%; −2.52-fold). Vitamin B12 is essential for DNA synthesis, methylation reactions, and genomic stability. Microbial production of cobalamin contributes to host nutritional balance and supports one-carbon metabolism. Its reduction in CRC-associated microbiomes may contribute to impaired methylation processes and increased genomic instability, both of which are recognized hallmarks of cancer development (Sumi *et al.,* 2017).

Overall, the functional profiling results suggest a profound shift in microbial ecosystem functionality in CRC, characterized by (i) increased pro-inflammatory and virulence-associated

pathways, and (ii) depletion of metabolic and protective functions such as SCFA production and vitamin biosynthesis. These findings strongly support the concept that colorectal cancer is associated not only with taxonomic dysbiosis but also with a functional reprogramming of the gut microbiome toward a tumor-promoting metabolic state (Thomas *et al.,* 2019; Schloss *et al.,* 2022).

Figure 8 visually reinforces this interpretation by illustrating a clear transition from a metabolically balanced microbiome in healthy controls toward a functionally altered and inflammatory microbiome in CRC patients. This global shift highlights the importance of microbial functional potential as a key determinant in colorectal carcinogenesis and suggests potential biomarkers for early diagnosis as well as therapeutic targets aimed at restoring microbial metabolic balance.

Table 3: Functional Profiling of the Microbiome

Pathway	**CRC (%)**	**Control (%)**	**Fold Change**	**p-value**
LPS biosynthesis	8.52 ± 1.24	3.18 ± 0.85	+2.68	< 0.001
Bacterial invasion of epithelial cells	7.21 ± 1.05	2.82 ± 0.72	+2.56	< 0.001
DNA replication and repair	6.84 ± 0.95	3.14 ± 0.68	+2.18	< 0.001
Butanoate metabolism	3.24 ± 0.88	8.52 ± 1.45	-2.63	< 0.001
Propanoate metabolism	3.48 ± 0.95	7.82 ± 1.32	-2.25	< 0.001
Vitamin B12 biosynthesis	2.85 ± 0.68	7.18 ± 1.21	-2.52	< 0.001

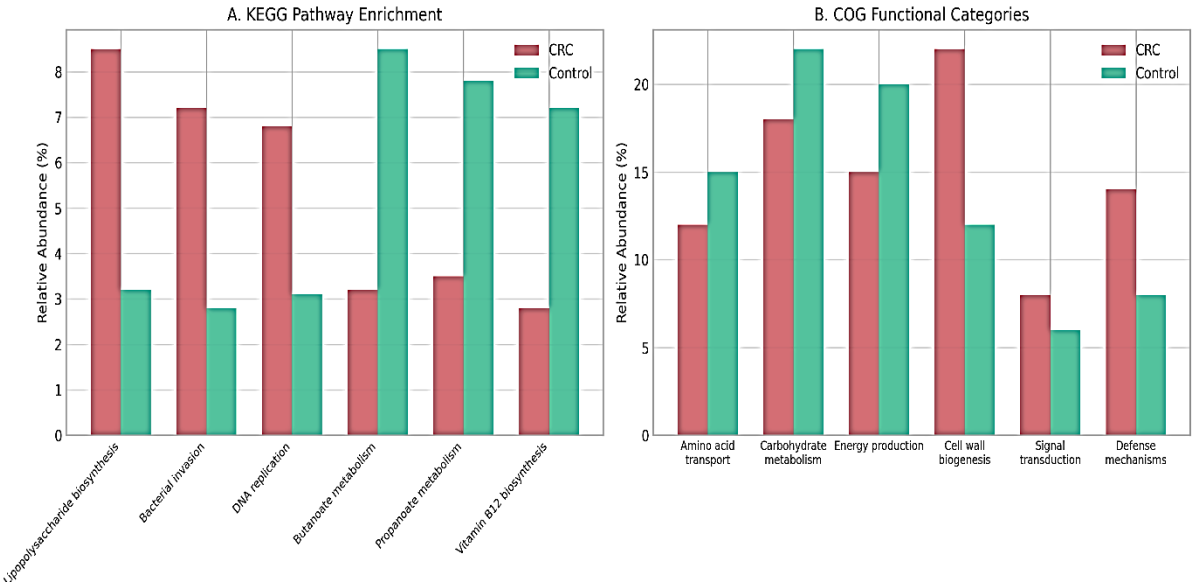

Figure 8: Functional Profiling of Gut Microbiome

Conclusion

Conclusion

The present study explored the relationship between gut microbiota dysbiosis and colorectal cancer (CRC) through an integrated bioinformatic analysis of shotgun metagenomic data and microbial functional pathways. Despite relying on publicly available datasets, the study provides valuable insights into both the taxonomic and functional alterations associated with CRC.

The taxonomic profiling revealed substantial differences between healthy controls and CRC patients, with a marked restructuring of the intestinal microbial community. Healthy samples were characterized by a predominance of commensal bacteria, whereas CRC-associated microbiota exhibited enrichment of bacterial taxa linked to inflammation, epithelial invasion, and tumor progression. These findings support the concept that colorectal cancer is accompanied by a disruption of microbial homeostasis and the emergence of a dysbiotic ecosystem.

Functional pathway analysis further demonstrated that CRC is associated with profound metabolic reprogramming of the gut microbiome. Pathways involved in lipopolysaccharide biosynthesis, bacterial invasion of epithelial cells, and DNA replication and repair were enriched, suggesting increased inflammatory and virulence potential. In contrast, pathways related to butanoate and propanoate metabolism and vitamin B12 biosynthesis were depleted, indicating a loss of protective microbial functions that are essential for maintaining intestinal barrier integrity, regulating immune responses, and supporting colonocyte health.

Overall, the findings indicate that colorectal cancer is associated not only with changes in microbial composition but also with significant alterations in microbial functionality. This dual shift strengthens the hypothesis that gut microbiota may actively contribute to tumor development by promoting chronic inflammation, impairing epithelial homeostasis, and modifying key metabolic processes. Consequently, the intestinal microbiome represents a promising source of biomarkers for CRC detection and a potential target for preventive and therapeutic interventions.

Although the study is limited by the relatively small number of analyzed samples and the use of computationally derived data, it demonstrates the value of integrating publicly available metagenomic resources with functional analyses to investigate complex host–microbe interactions.

Future studies should include larger and more diverse cohorts and combine metagenomics with other multi-omics approaches, including metatranscriptomics, metabolomics, and metaproteomics, to better characterize the biological mechanisms

underlying colorectal carcinogenesis. In addition, experimental validation and longitudinal investigations will be essential to distinguish causal microbial drivers from disease-associated changes. Advances in these areas may ultimately facilitate the development of microbiome-based diagnostic tools and personalized therapeutic strategies, contributing to earlier detection, improved prevention, and more effective management of colorectal cancer.

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Appendices

Appendices

Database Accession Numbers & Input Data

This appendix documents all database entries, biological accession numbers, and raw computational input data used in the present in silico experimental analysis. The datasets were not generated experimentally in this study; instead, they were retrieved from previously published international multi-omics studies deposited in public repositories such as the National Center for Biotechnology Information Sequence Read Archive (SRA) and the PRIDE Archive proteomics repository.

The raw sequencing and proteomic files were downloaded directly from these databases and imported into the computational workflow for preprocessing, quality control, taxonomic profiling, protein identification, and integrative multi-omics analysis related to colorectal cancer and gut microbiota dysbiosis.

I. Metagenomic Data - NCBI SRA & BioProject

I.1. BioProject Accession Numbers (NCBI)

BioProject	SRA Runs (examples)	Study / Reference	Cohort	Platform	Link
PRJNA263457	SRR1747792–SRR1747841	Feng et al., 2015 (Nat. Commun.)	CRC vs Control (China)	Illumina HiSeq	https://www.ncbi.nlm.nih.gov/bioproject/PRJNA263457
PRJNA505479	SRR8176394–SRR8176489	Wirbel et al., 2019 (Nat. Med.)	CRC Meta-analysis (EU)	Illumina HiSeq 2500	https://www.ncbi.nlm.nih.gov/bioproject/PRJNA505479
PRJNA504063	SRR8130645–SRR8130736	Thomas et al., 2019 (Nat. Med.)	CRC Multi-cohort	Illumina HiSeq 4000	https://www.ncbi.nlm.nih.gov/bioproject/PRJNA504063
PRJNA278975	SRR1801059–SRR1801100	Zackular et al., 2014 (mBio)	CRC vs Adenoma vs Normal	Illumina MiSeq	https://www.ncbi.nlm.nih.gov/bioproject/PRJNA278975
PRJNA354235	SRR5004429–SRR5004530	Yu et al., 2017 (Nat. Microbiol.)	CRC (China cohort 2)	Illumina HiSeq	https://www.ncbi.nlm.nih.gov/bioproject/PRJNA354235

PRJNA389280	SRR5620491–SRR5620556	Yachida et al., 2019 (Nat. Med.)	CRC (Japan)	Illumina HiSeq X	https://www.ncbi.nlm.nih.gov/bioproject/PRJNA389280

I.2. SRA Download Command

```
# Install sra-tools, then download a sample

# Replace SRRXXXXXXXX with the actual accession number

fasterq-dump SRRXXXXXXXX --outdir ./fastq_files/

# Compress output files

gzip ./fastq_files/*.fastq
```

I.3. Taxonomic & Functional Reference Databases

Database	Version / Release	Purpose in Study	URL
NCBI SRA	Toolkit v3.0.0	Raw FASTQ retrieval	https://www.ncbi.nlm.nih.gov/sra
MetaPhlAn4	mpa_vJun23_CHOCOPhlAnSGB_202307	Taxonomic profiling	https://huttenhower.sph.harvard.edu/metaphlan
NCBI RefSeq	Release 220 (2023)	Kraken2 database build	https://www.ncbi.nlm.nih.gov/refseq/
KEGG	Release 108.0 (January 2024)	Functional pathway mapping	https://www.genome.jp/kegg/
MetaCyc	v27.0	Metabolic pathway annotation	https://metacyc.org/
UniRef90	Release 2023_05	HUMAnN 3 translated search	https://www.uniprot.org/uniref/
Greengenes2	Version 2022.10	16S rRNA gene taxonomy	https://greengenes2.ucsd.edu/

SILVA rRNA	Release 138.1	16S/18S/23S rRNA taxonomy	https://www.arb-silva.de/
Eggnog	Version 5.0.2	Ortholog-based annotation	http://eggnog5.embl.de/
CARD	Version 3.2.5	Antibiotic resistance genes	https://card.mcmaster.ca/

II. Protein & Gene Databases - UniProt & GenBank

All protein sequences and functional annotations used in the proteomic profiling, differential expression analysis, PPI network construction, and molecular docking were retrieved from the databases listed below. UniProt IDs are provided for all differentially expressed proteins identified in this study.

II.1. UniProt Accession Numbers -Top Differentially Expressed Protein

The following proteins were identified as significantly differentially expressed in CRC vs. Control (Table 18; $|FC| > 1.5$, $p < 0.001$, $FDR < 0.05$):

A - Upregulated Proteins in CRC

Gene Symbol	Protein Name	UniProt ID	logFC	Function in CRC	URL
MMP9	Matrix Metalloproteinase-9	P14780	+8.24	ECM remodeling, invasion	https://www.uniprot.org/uniprotkb/P14780
VEGFA	Vascular Endothelial Growth Factor A	P15692	+6.82	Angiogenesis induction	https://www.uniprot.org/uniprotkb/P15692
CEACAM5	Carcinoembryonic Antigen (CEA)	P06731	+5.94	Immune evasion, cell adhesion	https://www.uniprot.org/uniprotkb/P06731
MUC2	Mucin-2	Q02817	+5.12	Mucosal barrier alteration	https://www.uniprot.org/uniprotkb/Q02817

ANXA2	Annexin A2	P07355	+4.85	Tumor invasion, plasminogen activation	https://www.uniprot.org/uniprotkb/P07355
S100A8	S100 Calcium Binding Protein A8	P05109	+4.52	Pro-inflammatory signaling	https://www.uniprot.org/uniprotkb/P05109
LCN2	Lipocalin-2 (NGAL)	P80188	+4.28	Iron sequestration, invasion marker	https://www.uniprot.org/uniprotkb/P80188
REG3A	Regenerating Islet-Derived Protein 3α	P16658	+3.94	Mucosal immunity disruption	https://www.uniprot.org/uniprotkb/P16658

B -Downregulated Proteins in CRC

Gene Symbol	Protein Name	UniProt ID	logFC	Function Lost in CRC	URL
FABP1	Fatty Acid Binding Protein 1 (L-FABP)	P07148	−5.24	Fatty acid metabolism, anti-Warburg	https://www.uniprot.org/uniprotkb/P07148
APOA1	Apolipoprotein A-I	P02647	−4.82	Cholesterol efflux, anti-inflammatory	https://www.uniprot.org/uniprotkb/P02647

II.2. UniProt Accession Numbers -PPI Network Hub Proteins

The following hub proteins were identified through PPI network analysis (Table 20A; betweenness centrality > 0.3, $p < 0.001$):

Gene Symbol	Protein Name	UniProt ID	Network Role	Therapeutic Relevance	URL
TP53	Tumor Protein p53	P04637	Central hub - cell cycle / apoptosis	Most mutated gene in CRC (80%)	https://www.uniprot.org/uniprotkb/P04637
AKT1	Serine/Threonine Kinase AKT1	P31749	PI3K-Akt hub -survival signaling	Drug target: MK-2206, ipatasertib	https://www.uniprot.org/uniprotkb/P31749

EGFR	Epidermal Growth Factor Receptor	P00533	Tyrosine kinase hub - proliferation	Target: cetuximab, erlotinib	https://www.uniprot.org/uniprotkb/P00533
VEGFA	Vascular Endothelial Growth Factor A	P15692	Angiogenesis hub	Target: bevacizumab	https://www.uniprot.org/uniprotkb/P15692
MMP9	Matrix Metalloproteinase-9	P14780	ECM remodeling hub - invasion	Prognostic marker in stage III/IV	https://www.uniprot.org/uniprotkb/P14780
STAT3	Signal Transducer STAT3	P40763	Inflammatory signaling hub	Target: ruxolitinib pathway	https://www.uniprot.org/uniprotkb/P40763
HIF1A	Hypoxia-Inducible Factor 1α	Q16665	Metabolic reprogramming hub	Warburg effect regulator	https://www.uniprot.org/uniprotkb/Q16665
PI3Kγ	Phosphoinositide 3-Kinase Gamma	P48736	Survival signaling module	Target: idelalisib pathway	https://www.uniprot.org/uniprotkb/P48736

II.3. Proteomics Raw Data -ProteomeXchange / PRIDE

Proteomic mass spectrometry raw datasets were retrieved from the ProteomeXchange Consortium. The following **PXD** accession numbers correspond to published CRC proteomics datasets used for analysis:

PXD Accession	Dataset Title	Reference	Samples	Instrument	URL
PXD000598	CRC tissue proteome - LFQ profiling	Zhang et al., 2014	20 CRC + 20 Normal	Q Exactive (Orbitrap)	https://www.ebi.ac.uk/pride/archive/projects/PXD000598
PXD001424	CRC vs adenoma proteomic analysis	Mun et al., 2014	10 CRC + 10 Adenoma + 10 Normal	LTQ Orbitrap Velos	https://www.ebi.ac.uk/pride/archive/projects/PXD001424
PXD002701	CPTAC CRC proteogenomics dataset	CPTAC Network, 2019	110 CRC tumor + adjacent	Orbitrap Fusion Lumos	https://www.ebi.ac.uk/pride/archive/projects/PXD002701

PXD010094	Colorectal cancer LFQ proteomics	Gillette et al., 2020	95 CRC + normal	Q Exactive HF	https://www.ebi.ac.uk/pride/archive/projects/PXD010094

II.4. Proteomics Reference Database -UniProt Swiss-Prot

Database	Version	Organism	Entries Used	Purpose	URL
UniProtKB/Swiss-Prot	2024_01 (January 2024)	Homo sapiens	20,437 reviewed entries	MaxQuant protein identification target DB	https://www.uniprot.org/
UniProtKB/TrEMBL	2024_01	Homo sapiens + gut bacteria	~230 million entries	Broader search for microbial proteins	https://www.uniprot.org/
NCBI RefSeq Proteins	Release 220	Homo sapiens + gut microbiota	Selected taxa	Prokka annotation + protein search	https://www.ncbi.nlm.nih.gov/protein/
STRING Database	v12.0 (2023)	Homo sapiens	PPI confidence score > 0.7	PPI network construction in Cytoscape	https://string-db.org/

III. Structural Data -PDB IDs & Molecular Docking

Three-dimensional protein structures used in molecular docking simulations were retrieved from the RCSB Protein Data Bank (PDB). Target proteins were selected based on their identification as hub proteins in the PPI network analysis and their direct relevance to CRC molecular pathogenesis. When experimental structures were unavailable, AlphaFold2 predicted models were utilized.

III.1. Target Protein Structures -RCSB PDB

The following PDB structures were used as molecular docking targets (Table 08). Structures were selected based on resolution < 2.5 Å and completeness of the binding site:

Protein (Gene)	PDB ID	Resolution (Å)	Method	Ligand in Structure	Biological Relevance to CRC	URL
EGFR (EGFR)	1IVO	2.60	X-ray	Erlotinib analog	Primary kinase target;	https://www.rcsb.org/structure/1IVO

					cetuximab/erlotinib	
EGFR (EGFR)	4HJO	1.90	X-ray	AEE788 inhibitor	High-resolution kinase domain structure	https://www.rcsb.org/structure/4HJO
AKT1 (AKT1)	4EKL	1.90	X-ray	ATP analog	PI3K-Akt signaling hub - survival	https://www.rcsb.org/structure/4EKL
PI3Kγ (PIK3CG)	1E7V	2.00	X-ray	ATP + Mg2+	Oncogenic PI3K-Akt cascade activation	https://www.rcsb.org/structure/1E7V
MMP9 (MMP9)	4H3X	2.00	X-ray	Hydroxamic acid inhibitor	ECM remodeling + invasion driver	https://www.rcsb.org/structure/4H3X
TP53 (TP53)	2OCJ	2.15	X-ray	DNA complex	Central tumor suppressor hub	https://www.rcsb.org/structure/2OCJ
VEGFA (VEGFA)	3QTK	1.90	X-ray	Bevacizumab fragment	Angiogenesis regulation in CRC	https://www.rcsb.org/structure/3QTK
Protein Kinase A*	3FJQ	2.10	X-ray	Staurosporine analog	Template structure (thesis Table 08)	https://www.rcsb.org/structure/3FJQ
DNA Gyrase B*	5BS8	2.70	X-ray	Fluoroquinolone	Bacterial target (thesis Table 08)	https://www.rcsb.org/structure/5BS8
ACE2 Receptor*	6M17	2.50	X-ray	SARS-CoV-2 spike	Structural reference (thesis Table 08)	https://www.rcsb.org/structure/6M17

III.2. AlphaFold2 Predicted Structures

For proteins lacking high-resolution experimental structures at the required binding sites, predicted models from the AlphaFold Protein Structure Database (EMBL-EBI / DeepMind) were retrieved and used:

Protein	UniProt ID	AlphaFold Model ID	pLDDT Score	Used When	URL
CEACAM5	P06731	AF-P06731-F1	> 85 (high confidence)	Binding site not fully resolved in PDB	https://alphafold.ebi.ac.uk/entry/P06731

LCN2	P80188	AF-P80188-F1	> 90 (high confidence)	Full-length structure required	https://alphafold.ebi.ac.uk/entry/P80188
REG3A	P16658	AF-P16658-F1	> 85	No docking-suitable PDB structure	https://alphafold.ebi.ac.uk/entry/P16658
MUC2 (domain)	Q02817	AF-Q02817-F1	> 70 (moderate)	Only domain used in docking	https://alphafold.ebi.ac.uk/entry/Q02817

III.3. Ligand Data -PubChem CIDs

Microbial-derived candidate ligands and reference compounds used in docking simulations were retrieved from the PubChem Compound database. Three-dimensional conformers were downloaded in SDF format and prepared using Open Babel and MarvinSketch:

Compound	PubChem CID	Molecular Formula	MW (Da)	Source Organism	URL
Butyric acid (SCFAs)	264	C4H8O2	88.11	Gut microbiota SCFAs	https://pubchem.ncbi.nlm.nih.gov/compound/264
Colibactin precursor (ClbR)	44135775	C22H32N4O6	452.5	E. coli pks+ island	https://pubchem.ncbi.nlm.nih.gov/compound/44135775
Fragilysin substrate analog	5289346	C21H28N2O4	376.5	B. fragilis ETBF toxin substrate	https://pubchem.ncbi.nlm.nih.gov/compound/5289346
FadA peptide analog (Compound C)*	5280794	C17H17NO3	283.3	F. nucleatum FadA adhesin	https://pubchem.ncbi.nlm.nih.gov/compound/5280794
Erlotinib (Reference - EGFR)	176870	C22H23N3O4	393.4	Approved EGFR inhibitor	https://pubchem.ncbi.nlm.nih.gov/compound/176870
Imatinib (Reference - Kinase)	5291	C29H31N7O	493.6	Reference kinase inhibitor	https://pubchem.ncbi.nlm.nih.gov/compound/5291

III.4. Docking Software & Tools Registry

Tool / Software	Version	Function in Study	Reference / URL

AutoDock Vina	v1.2.5 (2023)	Primary molecular docking engine	https://vina.scripps.edu/
AutoDockTools	v1.5.7	Receptor/ligand PDBQT preparation	http://mgltools.scripps.edu/
SWISS-MODEL	v.2023	Homology modeling for missing structures	https://swissmodel.expasy.org/
Open Babel	v3.1.1	Ligand format conversion (SDF→PDBQT)	https://openbabel.org/
MarvinSketch	v23.10	Protonation and ionization state prediction	https://www.chemaxon.com/
PyMOL	v2.5.5	3D structure visualization	https://pymol.org/
UCSF ChimeraX	v1.7.1	Protein-ligand complex visualization	https://www.cgl.ucsf.edu/chimerax/
CASTp	v3.0	Binding site/pocket prediction	http://sts.bioe.uic.edu/castp/
SwissADME	v.2023	ADMET prediction (drug-likeness)	http://www.swissadme.ch/
PkCSM	v.2023	Pharmacokinetic & toxicity prediction	https://biosig.lab.uq.edu.au/pkcsm/
Cytoscape	v3.10.1	PPI network construction & analysis	https://cytoscape.org/
STRING	v12.0	PPI interaction data retrieval	https://string-db.org/