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Metagenomic and Functional Analysis of Gut Microbiome in Colorectal Cancer Patients in Misan Province/Southerin of Iraq

A Thesis

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بسم الله الرحمن الرحيم

(يرفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنْكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ).

صدق الله العلي العظيم

سورة المجادلة الاية (**١١**)

Summary:

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths and the third most commonly diagnosed cancer worldwide. The microbiome plays a role in influencing the development and progression of CRC, both positively and negatively. In this study, stool samples were collected from 21 individuals divided into two groups: 12 with early-stage CRC and 9 healthy controls. 16S Amplicon-based next-generation sequencing (NGS) on Illumina NovaSeq (PE250-Seq) was performed on the samples and the results showed that there was a difference in the fecal bacterial microbiome between the two groups. Firmicutes, Bacteroidota, Actinobacteriota, Proteobacteria and Verrucomicrobiota were dominant in the gut microbiome in both groups, Firmicutes was the predominant phylum, representing 55.68% in healthy individuals and 62.09% in patients with CRC. The less abundant phylum were Verrucomicrobiota 0.00% in healthy individuals and 3.89% in CRC patients. Statistically, the Bacteroidota phylum showed a decrease in CRC compared to healthy people (p=0.002), while the Desulfobacterota and Verrucomicrobiota phylum showed a significant increase in CRC compared to healthy people p=0.04, 0.01, respectively. At the genera level, statistically significant differences were observed where *Actinomyces* (p=0.02), *Desulfovibrio* (p=0.02), *Bacteroides* (p=0.03), *Monoglobus* (p=0.01), *Eggerthella* (p=0.02) and *Akkermansia* (p=0.005) were significantly enriched in colorectal cancer patients compared to healthy controls, while *Mitsuokella* (p=0.001), *Faecalibacterium* (p=0.01), *Roseburia* (p=0.04), *Lachnospiraceae*_UCG_004 (p=0.01), *Lachnospira* (p=0.006), *Eubacterium* (p=0.01), *Prevotella* (p=0.001), *Barnesiella* (p=0.008) and *sutterella* (p=0.004) were less Enriched in colorectal cancer patients compared to healthy controls. On the other hand,to study the role of gut bacterial metabolites

in the development of colorectal cancer. A set of metabolites were identified by untargeted analysis using analyzed using Statistical Analysis of Metagenomic Profiles (STAMP version 2.1.3). The results showed the following pathways were downregulated in CRC compared to healthy controls: adenosylcobalamin salvage from nicotinamide, Calvin-Benson-Bassham cycle, fatty acid &beta, fatty acid elongation – saturated, methylerythritol phosphate pathway, Reductive acetyl coenzyme A pathway, super pathway of polyamine biosynthesis and peptidoglycan biosynthesis. Others were upregulated in CRC compared to healthy controls: D-galactarate degradation, D-glucarate degradation, enterobactin biosynthesis, fucose degradation, super pathway of chorismate metabolism, super pathway of glycolysis, pyruvate dehydrogenase, TCA, and glyoxylate bypass, L-arginine degradation, heme biosynthesis, super pathway of hexanol degradation, and enterobactin biosynthesis. Based on the results of the microbiome present in stool samples of colorectal cancer patients and compared with stool samples of healthy people, it can be concluded that the gut microbiome plays a significant role in carcinogenesis.

Contents

List of Figures

List of Tables

List of Abbreviations

Abbreviations	Key
NGS	Next Generation Sequencing
CVDs	CardioVascular Diseases
CRC	ColoRectal Cancer
SCFAs	Short-Chain Fatty Acids
PD-1	Programmed Death 1
IBD	Inflammatory Bowel Diseases
TLR	Toll like Receptor
ECA	Enterobacterial Common Antigen
IBS	Irritable Bowel Syndrome
F/B	Firmicutes/Bacteroidetes
BPH	Benign prostatic Hyperplasia
NAFLD	Non-Alcoholic Fatty Liver Disease
NASH	Non-Alcoholic SteatoHepatitis
GDM	Gestational Diabetes Mellitus
GI	GastroIntestinal
LPS	LipoPolySaccharide
CNS	Central Nervous System
ANS	Autonomic Nervous System
ENS	Enteric Nervous System
rRNA	Ribosomal Ribonucleic Acid
EGFR	Epidermal Growth Factor Receptor
COX-2	CycloOxygenase-2
DC	Dendritic Cells
RONS	Reactive Oxygen and Nitrogen Species
ROS	Reactive Oxygen Species
H2S	Hydrogen Sulphide
ETBF	Enterotoxigenic *Bacteroides Fragilis*

TH17	T Helper 17
BFT	Bacteroides Fragilis Toxin
BMI	Body Mass Index
NF-κB	Nuclear Factor kappa B
TNF-α	Tumor Necrosis Factor
M	Male
F	Female
Con	Control

References

Chapter Four:

Result and Discussion

Chapter Three:

Meterials and Methods

Chapter Tow:

Literature Review

Chapter one:

Introduction

Introduction:

The human microbiome refers to the collection of microorganisms, including bacteria, viruses, fungus, and protozoa, and their genetic material, that inhabit or are present on the human body. (Rahman *et al*., 2022)

The gut microbiome consists of a diverse and abundant population of bacteria (Saus *et al*., 2019), maturing into a stable microbiome over 2 to 3 years (Loke *et al*., 2020). It is estimated that the human gut harbors around 40 trillion different types of microorganisms (Pandey *et al*., 2023). The colon being the most densely populated area of the digestive system, housing approximately 70% of the human microbiota (Sekirov *et al*., 2010)

The eubiosis (balanced state) plays a crucial role in several physiological activities, including bolstering the host's immune system, aiding in nutrition absorption, and defending the body from harmful microbes, Multiple studies have shown a reciprocal relationship between gut microbiota and many organs in the human body, including the intestines, lungs, brain, and skin, Depending on the localized regions, microbiota can be classified into gut, oral, respiratory ,and skin microbiota, the microbial communities are in symbiosis with the host, contributing to homeostasis and regulating immune function, microbiota animbalance (dysbiosis) lead to dysregulation of bodily functions and diseases including cardiovascular diseases (CVDs), respiratory diseases, cancers ,etc (Hou *et al*., 2022; Gebrayel *et al*., 2022).

The cancer is characterized by the dysfunction and dysregulation of cell division in body fluids and specific tissues, leading to uncontrolled cellular proliferation that can invade neighboring tissues (Pelizzer *et al*., 2016). Colorectal cancer (CRC) is the third most diagnosed cancer, accounting for 6.1% of diagnoses, and is the second leading cause of cancer-related deaths, responsible for 9.2% of mortality (Sung *et*

al., 2021). Risk factors for CRC include a family and genetic history of cancer or related issues such as colon polyps, inflammatory bowel diseases, diabetes mellitus , undergoing cholecystectomy, and various lifestyle factors, evidence suggests that CRC risk is also heightened by overweight and obesity, lack of physical activity, cigarette smoking, alcohol consumption, and poor dietary habits (low in fiber, fruits, vegetables, calcium, and dairy products, and high in processed and red meats), Recent studies reported that the gut microbiome and their metabolites have a role influence colorectal cancer risk (Song *et al*., 2020; Sawicki *et al*., 2021; Li *et al*., 2022).

Dysbiosis refers to a condition when the gut loses beneficial bacteria and becomes populated with harmful bacteria that promote cancer-related behaviourssuch as angiogenesis, reduced apoptosis, and increased cell proliferation Thus, the composition of the microbiome has an impact on the formation of tumours in the colon (Lucas *et al*., 2017; Sherafat *et al*., 2018). According to investigations, some bacteria are shown to be more abundant in individuals with CRC, while others are observed to decrease (O'keefe, 2016; Temraz *et al*., 2019). The colon microbiota ,of a CRC patient showed an increase in the abundance of bacteria such as *Alistipes Akkermansia* spp., *Fusobacteria, Porphyromonadaceae*, *Coriobacteridae*, and *Methanobacteriales* (O'keefe, 2016; Borges-Canha *et al*., 2015; Siddiqui *et al*., 2022). enrichment of *Lachnospiraceae*, *Eubacterium* ,*Blautia Subdoligranulum Agathobacter*,*Romboutsia* and *Faecalibacterium* decreased (Dikeocha *et al*., 2022; Du *et al*., 2022). The particular bacteria associated with colorectal cancer (CRC) ,include *Bacteroides fragilis*, *Escherichia coli*, *Fusobacterium nucleatum Enterococcus faecalis*, and *Streptococcus gallolyticus* (Alhinai *et al*., 2019).

The gut microbiome produces many biologically active compounds such as bile acids, short-chain fatty acids, endotoxins, ammonia, polyamine, phenols, etc., and

they play a role in forming a connection between the intestinal microbiome and the host (Schroeder and Bäckhed, 2016; Wu *et al*., 2021). These metabolites play a role in carcinogenesis or cancer prevention and reducing the risk of cancer (Cho and Blaser, 2012; Zhang *et al*., 2019). The production of some amino acids such as tryptophan develops cancer and tumor formation (Johnson *et al*., 2016). Some amino acid compounds such as ammonia and hydrogen sulfide increase the risk of DNA damage, intestinal inflammation and the development of CRC (Windey *et al*., 2012). Hydrogen sulfide is produced in the intestine by the bacteria *Desulfovibrio*, which is a harmful sulfur-reducing bacteria (Marquet *et al*., 2009). Cysteine and methionine are toxic and contribute to the spread of malignant cells and the development of cancer (Marchesi *et al*., 2016). Polyamine production is associated with CRC and is considered a toxic metabolite (Di Martino *et al*., 2013). Bile acids induce toxic effects and increase the invasion of cancer cells (Kahouli *et al*., 2013)

This study aimed to:

A-The study the impact of differences in gut bacterial microbiome on CRC patients as compared with healthy which was achieved by the following objectives

B- Genetic identification of bacteria from stool samples from patients with colorectal cancer and healthy subjects.

C- DNA extraction from stool specimens of CRC patients and compared with healthy control individuals.

D- Performing meta-analysis using next-generation sequencing and software packages concerning assembly, processing, clustering, alpha, and beta diversity.

E- Functional analysis of some bacterial metabolites and study their roles in Colorectal Cancer.

2- Literature Review

2.1 Microbiome.

Humans are basically interdependent creatures. Since they are essentially sterile, at birth the humans encounter microbes and form a microbiota at the same time as their immune systems. All multicellular creatures, including plants, include an assemblage of microorganisms known as a **microbiota** (Malard *et al*., 2021).That live in a specific location within the human body is referred to as the human microbiome. Numerous anatomical body locations, including the skin, mucosa, gastrointestinal system, respiratory tract, urogenital tract, and mammary gland, are colonised by microorganisms (figure 2-2). Together, they create a distinct and sophisticated ecosystem that adjusts to the environmental requirements of every niche (Whiteside *et al*., 2015; Hou *et al*., 2022), as show in figure (2-1)

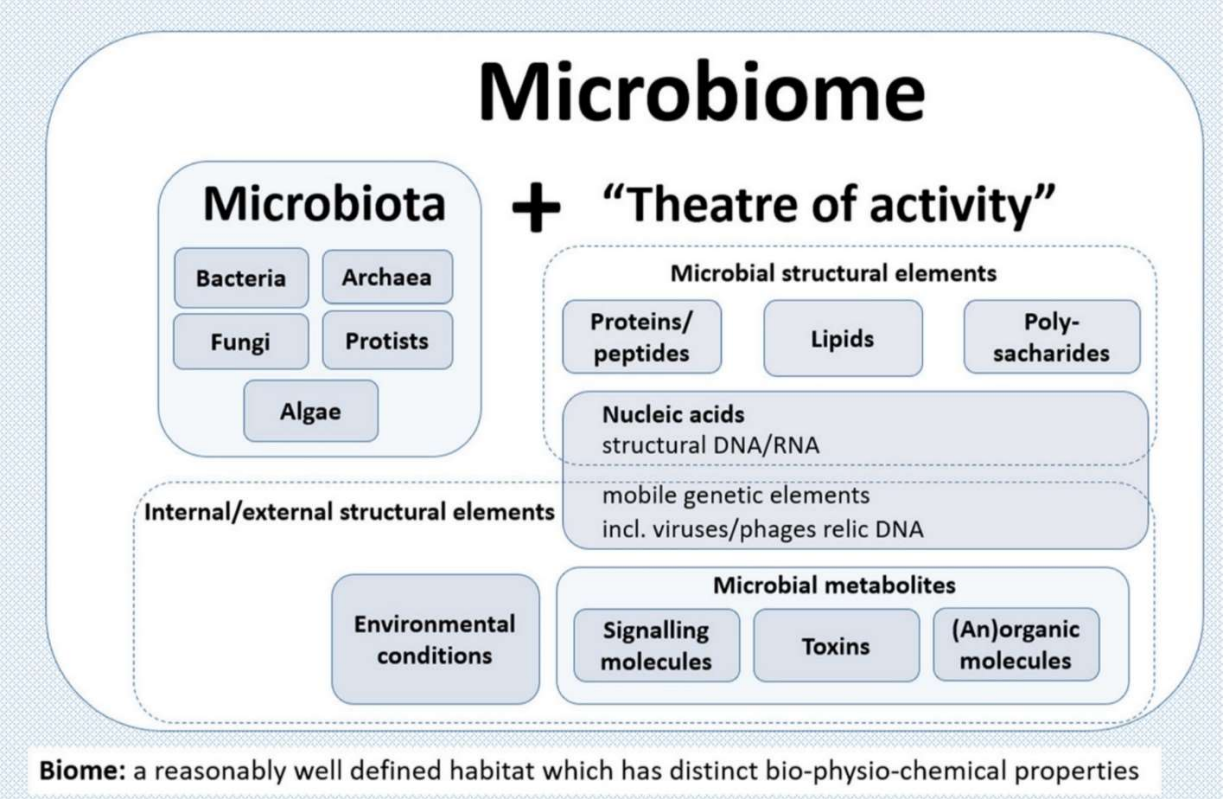

Figure (2-1): diagram illustrates the makeup of the microbiome (Berg *et al*., 2020).

Human Microbiota is a collection of microorganisms living within and interacting with the human body (Grice and Segre, 2011; Berg et al., 2020). The many relationships might be harmful, mutualistic, or commensalistic,the genetic material of organisms (microbiota) (Whiteside *et al*., 2015)

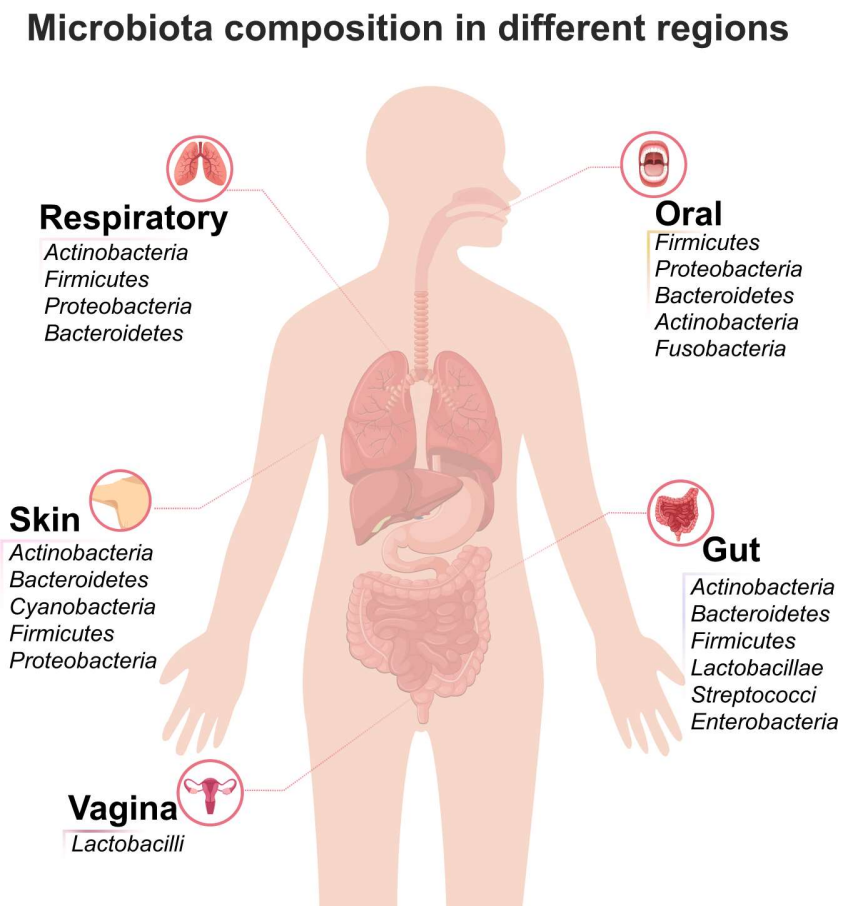

Figure (2-2): Distribution of human microbiota at various anatomical sites. The primary bacterial genera found in the mouth cavity, respiratory system, skin, stomach, and vagina are emphasized (Hou *et al*., 2022).

2.2-The gut microbiome

The "gut microbiota" is the group of bacteria, archaea, and eukarya that inhabit the gastrointestinal system, over thousands of years, they have coevolved with the host to develop a complex and mutually beneficial interaction (Symbiosis) (Backhed *et al*., 2005; Neish, 2009; Rosenberg and Zilber-Rosenbreg, 2018; Donovan, 2020; Suárez and Triviño, 2020).

Each person has a unique microbiome that develops from birth as a result of exposure to a complex bacteria in their gut that differs based on how it was delivered, vaginal birth is preferred because it exposes the baby's gut to the diverse microbiota of the mother, creating a maternal signature in the baby's early microbiome (Grenham *et al*., 2011; Wong and Yu, 2019).

The gut microbiota in adult human's is an immensely varied and ever-changing ecosystem, estimated to consist of around 39 trillion microbial cells, which is almost similar to the amount of human cells in our body (Sender *et al*., 2016). Furthermore, it contains over 1,000 times more genes than the human genome (Sender *et al*., 2016; Tierney *et al*., 2019; Koh and Bäckhed, 2020).

2.3.The composition and significance of the gut microbiome:

The gastrointestinal system is home to a community of microorganisms known as the gut microbiota, which is present in greater quantity than human body cells (Breban *et al.,* 2016; Al-Rashidi, 2022).

Firmicutes*,* Bacteroidota*,* Verrucomicrobia*,* Actinobacteria, and Proteobacteria are the five phyla that makeup approximately half of the faecal bulk, with the first two accounting for 90% of the total (Wu *et al.,* 2020).

Firmicutes phylum are gram-positive bacteria characterised by their rod or spherical morphologies and reproduction by binary fission. This phylum comprises microorganisms with varied features that are well-suited to a wide range of biological niches, the Firmicutes phylum is classified into seven subphyla: *Bacilli*, *Clostridia*, *Erysipelotrichia*, *Limnochordia*, *Negativicutes*, *Thermolithobacteria*, and *Tissierellia* (Seong *et al*., 2018; Padayachee *et al*., 2020)

Bacteroidota genes of this phylum is Bacteroides, can inhabit the colon and make up a significant portion of the bacteria in the gut. These bacteria, which cannot survive in the presence of oxygen and belong to the Gram-negative group, have various functions in collecting bacteria that reside in the human gut (Kim and Pamer, 2017). They are essential contributors to the complex network of microorganisms that support the digestion process in the gut as established commensals, mutualists, and beneficial organisms; This genus includes many species, the most important of which is *Bacteroides fragilis* and *Bacteroides vulgatus* (Zafar and saier, 2021).

Verrucomicrobia This phylum consists mostly of environmental microorganisms (Jakobsson *et al*., 2010) and is closely connected to the *Planctomycetes* and *Chlamydiae* phyla in terms of phylogeny. *Akkermansia muciniphila* is a Gram-negative bacteria that belongs to this particular phylum (Glover *et al*., 2022). It has the ability to degrade mucin and was first discovered in human stools (Jernberg *et al*., 2010).

Actinobacteria: The Gram-positive bacteria known as Actinobacteria is one of the four main phyla of the gut microbiota. Even they being a very small percentage, they are essential for maintaining intestinal homeostasis. The classes within this phylum, especially *Bifidobacteria*, are widely utilized as probiotics, showcasing advantageous benefits in a number of clinical diseases (Binda *et al*., 2018)

Proteobacteria: Gram-negative bacteria, that are potentially dangerous and often found in low abundance, which made the majority of this phylum.

Some examples of these bacteria are *Escherichia coli*, *Vibrio cholerae*, *Salmonella typhi*, *Pseudomonas aeruginosa* and *Helicobacter pylori* (Shreiner *et al*., 2015).

The gut microbiota have three main functions are: metabolic, structural and protective, and (Grenham *et al*., 2011; Wong and Yu, 2019). The gut microbiota facilitates the absorption of nutrients and minerals, the synthesis of various enzymes, vitamins, and amino acids, and the synthesis of short-chain fatty acids (SCFAs). Acetate, propionate, and butyrate are among the fermentation byproducts of gut microbiota that are crucial for gut health because they give epithelial cells energy, improve the integrity of the epithelial barrier, offer immunomodulation, and ward against infections (Wong and Yu, 2019).

Numerous studies show a clear correlation between gut microbiota and certain illnesses as well as host health (Thakur *et al*., 2016; Ohadian Moghadam and Momeni, 2021). Age, nutrition, stress, and illnesses all affect how many different types and amounts of bacteria there are in the gastrointestinal tract and other body areas. Research on both human and animal models has demonstrated a link between dysbiosis a permanent imbalance of the gut's microbial community and conditions including diabetes, obesity, cancer, irritable bowel syndrome (IBS), inflammatory bowel diseases (IBD), and problems of the cardiovascular and central neurological systems (Belizário and Faintuch, 2020).

2.4 Firmicutes/Bacteroidetes (F/B) Ratio:

The Firmicutes/Bacteroidetes ratio is considered a vital biomarker (Mange et al., 2020). The F/B ratio indicates the equilibrium of symbiotic bacteria in the intestines (An *et al*., 2023). The F/B ratio experiences a substantial rise in healthy individuals

as they age (Vaiserman *et al*., 2020), and it is notably greater in women compared to males (Koliada *et al*., 2021). Imbalances in the F/B ratio may lead to the development of illnesses such as inflammation, autoimmune disease, and cancer. For instance, obesity has a significant F/B ratio, but inflammatory bowel disease (IBD) shows a low F/B ratio (Stojanov *et al*., 2020). The F/B ratio exhibited a greater magnitude in the benign prostatic hyperplasia (BPH) population compared to the group without BPH (Takezawa *et al*., 2021). Non-alcoholic fatty liver disease (NAFLD)/nonalcoholic steatohepatitis (NASH) patients had a decreased F/B ratio compared to healthy controls (Vallianou *et al*., 2021). Furthermore, there was a positive correlation between a greater F/B ratio and a lower level of the genus *Bacteroides* with an increased left atrial diameter (Tsai *et al*., 2021). The F/B ratio was elevated in pregnant women with gestational diabetes mellitus (GDM) compared to those without GDM (Sililas *et al*., 2021).

2.5. The human gastrointestinal microbiome's development:

The prevailing belief is that the microbiota develops from birth, yet a few studies have shown that microorganisms can be found in womb tissues like the placenta, casting doubt on this belief (Aagaard *et al.,* 2014; Rodriguez *et al.,* 2015). The GI tract becomes colonised quickly after birth, and erratic alterations in the microbiota are caused by illnesses, antibiotic use, and dietary modifications (Koenig *et al.,* 2011; Rodriguez *et al.,* 2015). *Lactobacilli* are abundant in the microbiota during the initial few days, which is indicative of the high *Lactobacilli* load in the vaginal flora (Aagaard *et al.,* 2012; Avershina *et al.,* 2014). On the other hand, facultative anaerobes such as *Clostridium species* colonise the microbiota of newborns delivered via cesarean section, depleting it and delaying the colonisation of the *Bacteroides* genus (Jakobsson *et al*., 2014; Amabebe et al., 2020).

While 72% of newborns delivered vaginally have faecal microbiota that is similar to their mothers', this ratio drops to 41% in babies delivered via cesarean section (Bäckhed *et al*., 2015). Actinobacteria and Proteobacteria are the two primary phyla that dominate the microbiota during the early phases of development (Bäckhed, 2011; Rodriguez *et al*., 2015).

The makeup, variety, and functional capacities of the baby microbiota approximate those of the adult microbiota by the time the child reaches the age of 2.5 years (Koenig *et al.,* 2011; Rodriguez *et al.,* 2015). Even while the gut microbiota's composition is generally stable throughout maturity, life experiences can nonetheless disturb it (Dethlefsen *et al.,* 2011). In contrast to younger participants where *Clostridium* cluster XIVa is more common, the microbial community changes in adults over 65, with an increased number of Bacteroidetes phyla and *Clostridium cluster* IV (Claesson *et al.,* 2011).

On the other hand, a different research found that although the microbiota of a cohort of centenarians was much less diverse, the microbiota of a young cohort and an older population (70 years old) were reasonably comparable (Biagi, 2010; Rinninella *et al*., 2019).

Additionally, group specific alterations in the centenarian microbiota were observed, including a shift in the profile of butyrate makers (*Faecalibacterium prausnitzii* declining, for example) and an increase in the number of facultative anaerobes (*Escherichia coli*, for example) (Biagi, 2010). Diversity and living circumstances, such as communal living or long-term residential care, have been found to be significantly correlated in the senior population (Claesson *et al*., 2012)

In general, older people have less ability of their microbiota to perform metabolic functions such amylolysis and the synthesis of short-chain fatty acids (SCFAs), they

have more proteolytic activity (Woodmansey *et al*., 2004). As the body of research supporting the importance of SCFAs as important immunological and metabolic mediators grows, it was hypothesised that a decline in SCFAs would promote the inflammatory ageing process in the elderly's gut (Biagi *et al*., 2013).

2.6.The microbiota's function in human health:

Due to its extensive genetic makeup and metabolic complement, the gut microbiota offers several advantageous characteristics to the host. Among these bacteria' most significant functions are their assistance in preserving the mucosal barrier's integrity, their provision of nutrients including vitamins, and their defence against infections. Furthermore, for optimal immune function, commensal microbiota and the mucosal immune system must interact (Thursby and Juge, 2017).

2.6.1 Short chain fatty acid production:

The expression of carbohydrate active enzymes by colonic bacteria allows them to digest complex carbohydrates and produce metabolites such SCFAs (Musso *et al*., 2010). In the GI tract, the three main SCFAs propionate, butyrate, and acetate are normally present in a 1:1:3 ratio (Louis *et al*., 2014). In the gastrointestinal tract, these SCFAs are quickly absorbed by epithelial cells and have a role in the control of several biological processes, including gene expression, chemotaxis, differentiation, proliferation, and apoptosis (Corrêa-Oliveira *et al*., 2016). While butyrate and propionate are generated by distinct gut bacterial subsets via various biochemical pathways, acetate is produced by the majority of gut anaerobes (Louis and Flint, 2017). Glycolysis and acetoacetyl-CoA convert carbohydrates into

butyrate, while the synthesis of propionate can occur by either the succinate or propanediol route, depending on the kind of sugar (Louis and Flint, 2017). Bacteroidetes is the primary producer of propionate in the human gut, while Firmicutes is mostly responsible for butyrate synthesis (Morrison and Preston, 2016; Louis and Flint, 2017).

For instance, it is believed that both directly and through metabolic cross feeding, the fermentation of starch by specialized Actinobacteria and Firmicutes, such as *Eubacterium rectale* or *Eubacterium hallii,* greatly contributes to the formation of butyrate in the colon (Louis and Flint, 2017). One important propionate producer with a focus on mucin breakdown is *Akkermansia muciniphila* (Derrien *et al*., 2004). Acetate is discharged into peripheral tissues, whereas propionate is mostly absorbed by the liver (Guarner and Malagelada, 2003). A recent study examined the impact of SCFAs on human metabolism, anti-inflammatory and anti-cancer properties of butyrate are well known (Morrison and Preston, 2016; Lin and Zhang, 2017). For colonocytes, butyrate is a very crucial energy source (Corrêa-Oliveira *et al*., 2016).

2.6.2. Regulation of bile acid levels

Bile acids, synthesised in the liver, facilitate the digestion of fats and the absorption of fat soluble vitamins when released into the small intestine, although primary bile acids are initially secreted in a conjugated form, certain commensal bacteria, such as Firmicutes (e.g., *Clostridium*, *Lactobacillus*, and *Enterococcus* species), Bacteroidetes (e.g., *Bacteroides* species), and Actinobacteria (e.g., *Bifidobacterium* species) (Winston and Theriot, 2016). Possess a bile salt hydrolase enzyme that converts the acids back to an unconjugated form this facilitates the enterohepatic recirculation of the majority of the bile acid pool (Urdaneta and Casadesús, 2017)

Commensals convert a tiny portion of primary bile acids that reach the large intestine into secondary bile acids, this process is carried out by a specific group of microorganisms (<0.025% of the bacteria in the gut) that produce alpha dehydroxylase enzymes (Winston and Theriot, 2016). *Clostridium scindens* is the most extensively studied species in this particular function (Winston and Theriot, 2016; Qian *et al*., 2020). Both primary and secondary bile acids play a role in glucose metabolism and possess antibacterial detergent characteristics that help prevent colonization by harmful microorganisms (Shah *et al*., 2021). Secondary bile acids have a significant impact in suppressing the development of *Clostridioides difficile*, specifically (Shen, 2015). Microbiota have a crucial function in maintaining the balance of bile acids in the body, therefore, it is becoming more important to use microbiota as a biomarker to differentiate between a "healthy" microbiome, where secondary bile acids are more prevalent than primary bile acids in the colon, and a "unhealthy" microbiome (Qian *et al*., 2020).

2.6.3 Biosynthesis of vitamins

The intestinal bacteria play a crucial role in the production of some vitamins, gut microbiota, including strains of Firmicutes, Actinobacteria, and Proteobacteria, synthesise endogenous B vitamins like cyanocobalamin (B12) and thiamine (B1) (Wexler and Gooman, 2017; oot and Werneke, 2020; Wan *et al*., 2022). These vitamins play a crucial role in several metabolic processes, such as DNA replication and repair, that are important for maintaining good health (Dattola et al., 2020). Various symbiotic bacteria, such as *Bacteroides fragilis*, *Eubacterium lentum*, *Enterobacter agglomerans*, *Serratia marcescens*, and *Enterococcus faecium*, also synthesise vitamin K (Kho *et al*., 2018). Changes in the quantities of these bacteria may therefore impact the availability of these naturally occurring vitamins (Bidell *et al*., 2022).

2.6.4. Protective role

The gut microbiota primarily serves as a mechanism for pathogen displacement or "colonisation prevention", the gut microbiota inhibits the colonisation of pathogens by competing for attachment sites and nutrition, The gut bacteria play a role in the development of both innate and adaptive immunity by sending signals that assist maintain a balance between proinflammatory and anti-inflammatory responses (Min and Rhee, 2015; Scott, 2020). In addition to the native gut microbiota, microbial associated components also elicit an immune response, an instance of this is capsular polysaccharide A, which is generated by *Bacteroides fragilis* and has shown an anti-inflammatory impact in the gastrointestinal tract, microbial metabolites such as butyrate have immunomodulatory effects via inhibiting the activation of nuclear factor-kb and/or by interacting with G protein coupled receptors, hence, the symbiotic microorganisms residing in the gastrointestinal tract actively contribute to the formation and maintenance of immune system reactions (Khan *et al*., 2021).

2.7. The microbiota's function in human disease

the microbiota provides several advantages to the host, nevertheless, dysbiosis a changed microbial composition has the potential to upset these processes, the microbiota has been implicated in several intestinal and extra-intestinal disorders (Chang and Lin, 2016; Schroeder and Bäckhed, 2016; Hou *et al*., 2022), as show in figure (2-3).

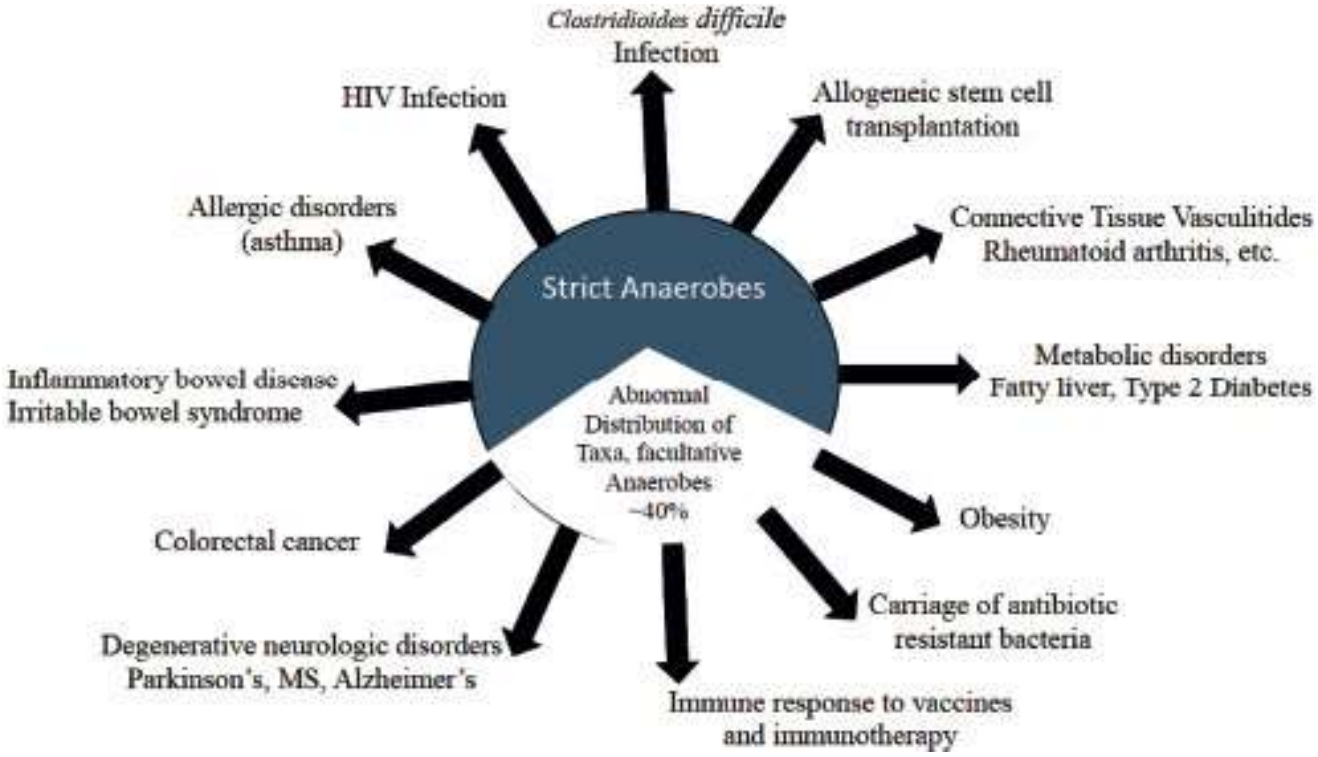

Figure (2-3): Diseases linked to dysbiosis (Dupont et al., 2020)

2.7.1. Diabetes and Obesity (Metabolic syndrome)

Dysbiosis is linked to diseases including obesity, diabetes mellitus type 2, and the metabolic syndrome overall (Larsen *et al.,* 2010; Qin *et al.,* 2012; Relman, 2015). There are three processes linked to the development of these illnesses and the microbiota, a carbon source for energy production is one of them. the second relates to the modification of certain human genes and proteins that control how much energy is used, the regulation of bacterially derived lipopolysaccharide (LPS) levels in plasma, which can cause persistent subclinical inflammation, is the last mechanism, through the activation of Toll like receptor 4 (TLR4), the last one causes the development of insulin resistance (Blaut, 2015; Saad *et al*., 2016)

The majority of research on humans suggests that a rise in the Firmicutes/Bacteroidota ratio is associated with an increase in low-grade inflammation, also linked to increased insulin resistance, inflammation, and obesity is the gut microbiota's low diversity (Cornejo-Pareja *et al.*, 2019; Pascale *et al.*, 2019).

Studies on the metagenomic composition of the human intestinal microbiota have provided evidence to support the link between obesity, insulin resistance, and elevated levels of many proinflammatory cytokines including TNF-α and interleukin 6 (Turnbaugh *et al.,*2009; Le Chatelier *et al*., 2013).

2.7.2 gastrointestinal disorders

The gut microbiota's microorganisms and humans have a mutualistic connection, however certain bacteria can become virulent and alter their symbiotic characteristics as a result of nutritional, environmental, and genetic variables (Nagao-Kitamoto *et al.,* 2016; Caruso *et al.,* 2020). A number of studies indicate that changes in the gut microbiota and its metabolic processes are associated with the onset and development of gastrointestinal disorders, including but not limited to severe diarrhoea, celiac disease, and irritable bowel syndrome (Manichanh *et al.,* 2012; Palm *et al*., 2014).

Therefore, intricate interactions between a number of variables, including host genetics, environmental hazards, and the status of the gut microbiota, influence the formation of disorders such intestinal inflammation (Fakhoury *et al.,* 2014 ;Ananthakrishnan *et al*., 2015). As the number of Firmicutes decreases, the gut microbiota is the one that is directly impacted (Caruso *et al.,* 2020). Clinical research has provided ample evidence for this claim, demonstrating that individuals with

intestinal inflammation have far less variety and richness in their microbiota (Manichanh *et al*., 2012; Shah *et al*., 2018; Caruso *et al*., 2020). In addition, the disease's pathophysiology is marked by the build up of certain pathobionts, including *Ruminococcus gnavus* and *Escherichia coli* (Frank *et al*., 2007; Kamada *et al*., 2013).

Microbial species including *Bacteroides fragilis* and *Escherichia coli* have been shown to adapt to ulcerative colitis, a condition marked by inflammation and ulceration of the colon's lining, the ileum's mucosa has developed to get adhered to by these microbes, which then crumble the wall (Darfeuille-Michaud *et al*., 2004; Pickard *et al*., 2017), It has also been shown that individuals with IBS have harmful bacteria adherent to their intestinal wall (Caballero *et al*., 2015; Ahmed *et al*., 2016; Hall *et al*., 2017). In addition, there is a higher ratio of Firmicutes to Bacteroidetes than in healthy individuals (Ahmed *et al*., 2016; Patel *et al*., 2016). More precisely, there are more species in the *Clostridium* cluster XIVa and *Ruminococcaceae* families and fewer in the *Bacteroides* family (Jeffery *et al*., 2012).

2.7.3. The nervous system and psychiatric disorders

the microbiome-intestine-brain axis is a two-way communication network that comprises the gut microbiome, autonomic nervous system (ANS), central nervous system (CNS), enteric nervous system (ENS), the endocrine system and the immunological system, (Cryan and Dinan, 2012).

Numerous neurological and psychiatric disorders, such as multiple sclerosis, anxiety, depression, Parkinson's disease, autism, and Alzheimer's disease, have been linked to dysbiosis of the gut microbiome (Sampson and Mazmanian, 2015; Sharon *et al*., 2016). Furthermore, some studies demonstrate that the intestinal microbiota

influences the gut-brain link, which may result in stress and anxiety symptoms (Frankiensztajn *et al*., 2020).

Also, there seem to be a connection between pain tolerance mechanisms and the gut microbiome (Cryan and O'Mahony, 2011; Iebba *et al*., 2012). Similary, the circadian cycle, host behavior, mood, and neurological functions have all been shown to be closely linked to the gut microbiome (Sampson and Mazmanian, 2015). Though the exact processes underlying this relationship's generation stay unclear, the "microbiota-gut-brain axis" theory has been suggested as a possible explanation (Cryan and Dinan, 2012). The following are some of these mechanisms:

Involvement of the vagus nerve, there is a connection between the ENS and the CNS that provides a direct communication pathway between gut microbiota and the CNS (Bravo *et al*.,2011; Forsythe *et al*., 2014). Involvement of the cardiovascular system, this system controls the influence of several metabolites generated by gut bacteria on CNS activities, including hormones, neurotransmitters, and SCFA (Sampson and Mazmanian, 2015). Regulation of signals and the synthesis of neurotransmitters, gut microbiota apparently modulates the expression of central neurotransmitters and related receptors, and some species produce neurotransmitters, such as acetylcholine, dopamine, and adrenaline, or induce their synthesis (Gershon, 2013; Yano *et al*., 2015). By producing SCFA, the gut microbiota can influence how quickly microglia mature and how permeable the blood brain barrier (Braniste *et al*., 2014; Erny *et al*., 2015; Reigstad *et al*., 2015). Gut microbiota modulates the activation of peripheral immune cells that regulate CNS immune reactions (Dantzer *et al*., 2000; Fung *et al*., 2017). Research has shown variations between neurological disease patients and healthy individuals (Du *et al*., 2020). An examination of 16s rRNA from faecal microbiota in healthy persons revealed that the percentage of bacteria belonging to the phyla Firmicutes and Bacteroidota is larger than that of the

phyla Proteobacteria, Actinobacteria, Fusobacteria, and Verrucomicrobia (Dash *et al*., 2015). Furthermore, patients with anxiety and depression have much lower microbial variety and abundance (Du *et al*., 2020). As a result, there is less concentration of the Ruminococcus and Lactobacillus genera, as well as the Lachnospiraceae and Ruminococcaceae families (Kelly *et al*., 2017).

2.7.4 Cancer

Recent studies have reported on the significance of the gut microbiota in the development of many cancer types (Loo *et al*., 2017; Wong *et al*., 2019). Pathogenesis has been shown to be caused by a variety of processes, including aberrant microbial translocation, molecular mimicry, and dysregulation of both local and systemic immunity (De Martel *et al*., 2012). Genetic susceptibilities are only one of these pathways, according to reports, some bacteria found in the gut microbiota have the ability to cause cancer or inhibit the growth of tumour cells, about 20% of cancer cases are caused by infectious organisms, such as bacteria (Zhang *et al*., 2020)

Furthermore, a difference has been noted between cancer patients and healthy persons with regards to the population and microbial diversity that exist at the gut level (Xuan *et al*., 2014). There has been written on the connection between gut microbiota and carcinogenesis, with a focus on bacterial metabolites. Thus, the impacts of certain toxins or virulence factors produced serve as the primary basis for the processes of bacterial-mediated carcinogenesis (Arthur *et al*., 2012; Bultman, 2014)

Moreover, microbial metabolites, such polyamines and secondary bile acids, also contribute to the development of cancer cells and the creation of tumours via the β-catenin signalling pathway (Pai et al., 2004), the transactivation of the epidermal growth factor receptor (EGFR) (Cheng *et al*., 2005), and elevated COX-2 activity (Brown and DuBois, 2005). Because of this, the immune system of the intestinal mucosa is linked to cancer, and its relationship with the gut microbiota is thought to be crucial for maintaining homeostasis (Pickard *et al*., 2017; Wang *et al*., 2020). This interaction has an impact on the stimulation of cell differentiation and the prevention of bacterial adhesion and colonization (Shui *et al*., 2020). Accordingly, microorganisms like *Bacteroides fragilis* cause T cells that are CD4+ to differentiate into regulatory T cells (Round and Mazmanian, 2010), which have the ability to identify antigenic compounds linked to the bacterial genera *Clostridium and Bacteroides,* as well as secrete significant quantities of anti-inflammatory cytokines, such as interleukin-10 (IL-10) (Cebula *et al*., 2013; Yi *et al*., 2019). The gut microbiota also affects immunity at the systemic level in addition to the local one (Wang *et al*., 2020). Because microorganism produced metabolites penetrate the circulation, they interact with toll like receptors (TLRs) to influence the immune response in distant organs (Shui *et al*., 2020). The gut microbiota's bacteria may interact with the immune system to indirectly encourage the growth of cancer cells, even if they do not directly cause carcinogenesis (Gagliani *et al*., 2014). As a result, a compromised immune system promotes the growth of certain bacterial taxa and initiates signalling cascades that result in oncogene transcription (Gagliani *et al*.,2014; Wang *et al*., 2020). Furthermore, by causing inflammation or immunosuppression via the generation of cytokines, gut microbiota may indirectly support cancer (Yu and Schwabe, 2017; Li *et al*., 2019). Finally, changes in the makeup of the gut microbiota are now linked to the development of a number of

malignant neoplasms, including certain cancers such as brain, gastric, colorectal, pancreatic, and breast cancer (Chen *et al*., 2019).

2.7.4.1 Ductal Adenocarcinoma of the Pancreas

The overall survival rate for this illness is less than five years, making it one of the most dangerous malignant neoplasms (Zhang *et al*., 2020). Since surgical excision is often not feasible, chemotherapy is the mainstay of treatment, pancreatitis and pancreatic ductal adenocarcinoma are significantly impacted by gut microbiota, which may cause chemoresistance in some individuals (Amrutkar and Gladhaug, 2017; Akshintala *et al*., 2019). In the events leading to pancreatic carcinogenesis, *Fusobacterium nucleatum* causes autophagy, inflammation, and chemoresistance (Yu and Schwabe, 2019; Zhang *et al*., 2019). Moreover, it has been shown that in patients with this disorder, there is a reduction in Firmicutes and Bacteroidota and an increase in Proteobacteria and Verrucomicrobia. These changes are followed by the activation of inflammatory pathways in tumour tissues (Panebianco *et al*., 2018). Additionally, it has been shown that a greater risk of pancreatic ductal adenocarcinoma presentation is linked to the presence of intratumoral pathogens and bacteria, including Rahnella, *Acinetobacter, Aquabacterium*, and *Oceanobacillus* (Heikkilä *et al*., 2018; Mei *et al*., 2018). Development of this disease involves the intestinal mucosa, epithelial and dendritic cells (DC), and different cells from the immune system (Niess and Reinecker, 2006). The above mentioned microorganisms are part of the gut microbiota and promote the development of adenocarcinoma through the release of a large number of metabolites (Li et al., 2019), which interact with TLRs and also induce systemic inflammation and

immune responses associated with pancreatic carcinogenesis and therapeutic resistance (Zhang *et al*., 2020).

2.7.4.2 Breast Cancer

Breast cancer is the most prevalent kind of cancer that affects women globally, despite considerable advancements in diagnosis and therapy, about 40,000 fatalities occur annually (Viale, 2020). Research has shown a robust correlation between dysbiosis and the development of neoplasms, including breast cancer (Bultman, 2014). Therefore, beyond genetic, environmental, and lifestyle variables, current research has concentrated on the role of gut microbiota in the development of b+reast cancer (Van *et al*., 2015). Variations in *Bifidobacterium, Faecalibacterium prausnitzii,* and *Blautia* numbers have been used as biomarkers linked to the clinical stage of breast cancers (Luu *et al*., 2017). Furthermore, there is a correlation between these variations and the patient's BMI, compared to patients of average weight, it has been shown that obese and overweight women with breast tumours exhibit lower quantities of Firmicutes*, Faecalibacterium prausnitzii,* and *Blautia* spp., as well as a lower prevalence of *Akkermansia muciniphila* (Luu *et al*., 2017; Fruge *et al*., 2020). Similarly, there is a change in the faecal microbiota's composition and a decrease in microbial diversity in postmenopausal women who have breast cancer (Laborda-Illanes *et al*., 2020). Particularly, there have been reports of decreased Dorea and *Lachnospiraceae* proportions and increased amounts of *Ruminococcaceae, Faecalibacterium*, and *Clostridiaceae* (Goedert *et al*., 2015). Comparably, it has been noted that the populations of many species, including *Salmonella enterica*, *Fusobacterium nucleatum*, *Acinetobacter radioresistens, Citrobacter koseri,* and *E. coli,* have increased (Zhu *et al*., 2018). However, various considerations, such age, ethnicity, and geographic location, must be made when

determining the features of the gut microbiota of patients with breast cancer (Laborda-Illanes *et al*., 2020).

2.7.4.3 Stomach Cancer

Gastric cancer is a prevalent kind of tumour that is distinguished by both sudden and long-lasting inflammation (Engstrand and Graham, 2020). Similarly to other forms of cancer, the gut microbiota is associated with the onset of this illness, and *Helicobacter pylori* is the primary carcinogenic factor (Graham, 2015). The inflammatory response caused by *H. pylori* is linked to the level of virulence shown by each strain (Miftahussurur *et al*., 2017). The process of carcinogenesis starts with genetic instability resulting from the disruption of the host's DNA chain (Rakoff-Nahoum and Medzhitov, 2009)

According to the latest research, it has been shown that *H. pylori* infection causes damage to genetic material via two potential processes (Kidane, 2018). Initially, there is an increase in the presence of immune cells, neutrophils and macrophages, which results in the generation of reactive oxygen and nitrogen species (RONS) (Suzuki *et al*., 1994). RONS, induce harm to the DNA by causing single strand breaks and promoting the upregulation of oncogenes (Feig *et al*., 1994). On the other hand, RONS activates the transcription factor NF-κB, which leads to the production of oncogenes and cell cycle regulators (D'Angio and Finkelstein, 2000). Furthermore, this factor migrates to the nucleus and combines with a Nucleotide excision repair protein complex (endonucleases XPG and XPF) that cuts the promoter regions of genes, affecting gene expression due to double-strand breaks (Hartung *et al*., 2015)

While gastric acidity acts as a crucial barrier that prevents microbes from entering the gastrointestinal tract (Engstrand and Graham, 2020). *H. pylori* is able to survive in the stomach despite the harsh conditions. However, its ability to establish colonies in gastric glands is limited due to the excessive production of acid in these cavities (Hanada and Graham, 2014). Nevertheless, the simultaneous occurrence of inflammation and the presence of *H. pylori* exacerbates harm in different areas of the stomach. The consequence of this damage is the degeneration caused by the proliferation of this microbe, which is more prevalent in gastric cancer compared to gastritis and intestinal metaplasia (Eun *et al*., 2014; Wang *et al*., 2015).

Furthermore, it has been discovered that individuals with *H. pylori* infection and precancerous gastric lesions have differences in the relative abundance of the main phyla, Bacteroidota, Firmicutes, and Proteobacteria, in their faecal microbiota (Li et al., 2017). Several other bacteria, including *Peptostreptococcus stomatis*, Slackia exigua, *Parvimonas micra*, *Streptococcus anginosus*, and Dialister *pneumosintes*, have been examined in relation to gastric cancer. However, *Helicobacter pylori* is the most widely linked bacterium to gastric carcinogenesis (Coker *et al*., 2018)

2.7.4.4 Brain cancer

The correlation between gut microbiota and brain cancer has been a subject of increasing study in recent years (Dehhaghi *et al*., 2020). The association between the microbiota-gut-brain axis and brain tumours has been attributed to the mechanisms play in this axis since studies have shown that these processes may either promote or inhibit the development of brain tumours (Fung *et al*., 2017). Intestinal microbes use tryptophan as a substrate to generate indoles. These molecules participate in the signalling pathways between the gastrointestinal tract

and the immune system (Agus *et al*., 2018). The metabolism of particular Tryptophan occurs in the kynurenine pathway, producing of nicotinamide adenine dinucleotide and other neuroactive compounds (Dehhaghi *et al*., 2020). According to reports, an imbalance in the kynurenine pathway may have a role in the development of brain cancer by disrupting the immune response to tumours (Adams *et al*., 2012; Platten *et al*., 2019). In a similar manner, the gut microbiota has the potential to impact the microenvironment of brain tumours through various mechanisms. These include: Regulating the growth and activation of T cells (Jin *et al*., 2019), influencing microglia (Schalper *et al*., 2017; Roesch *et al*., 2018), affecting the production of cytokines, arginine, and tryptophan through kynurenine (Martin-Gallausiaux *et al*., 2018; Kaur *et al*., 2019), generating reactive oxygen species (ROS) and antioxidants (Roesch *et al*., 2018; Mehrian-shai *et al*., 2019).

2.7.4.5 Colorectal Cancer

Several bacterial species, including *Fusobacterium nucleatum*, *Peptostreptococcus anaerobius*, and *enterotoxigenic Bacteroides fragilis*, have been found as being associated with the development of colorectal cancer (Fong *et al*., 2020) In addition, a higher abundance of *F. nucleatum* has been linked to a decreased likelihood of survival in individuals with colon cancer (Mima *et al*., 2016). This is because *F. nucleatum* promotes chemoresistance, which triggers autophagy (Yu *et al*., 2017), ultimately resulting in treatment failure or the return of the illness (Wang *et al*., 2020). The aforementioned species stimulate tumour growth (Yang *et al*., 2017), facilitate inflammation (Chung *et al*., 2018), shield the tumour from immune system responses (Long *et al*., 2019), and inflict harm on the DNA of host cells (Rubinstein *et al*., 2013). Each of these elements has a role in the development of cancer. In colorectal cancer, there has been significant focus on protein toxins generated by the

intestinal microbiota, similar to other forms of cancer (Fiorentini *et al*., 2020). The carcinogenic impact of these toxins may be attributed to their direct assault on DNA, resulting in genomic instability or increased cell proliferation and resistance to programmed cell death in cancer cells owing to abnormalities in cellular signalling pathways (Candela *et al*., 2014).

2.7.4.5.1. Some bacteria contributing in colorectal cancer

Sulfidogenic Bacteria

Sulfidogenic bacteria such as *Desulfovibrio, Fusobacterium,* and *Bilophila wadsworthia* have been implicated in the development of colorectal cancer (CRC) due to their ability to produce hydrogen sulphide (H2S). H2S, a genotoxin, has been detected in more than 80% of sporadic CRC cases, it is recognised for its potential to harm DNA, resulting in genomic instability and a significant occurrence of mutations, the presence of hydrogen sulphide (H2S) may impede the functioning of mitochondria, leading to excessive cell formation via the Ras/MAPK pathway, which is a well-known mechanism of carcinogenesis, a diet that is rich in meat and fat is associated with an increased presence of *sulfidogenic* bacteria (Flemer *et al*., 2017; Dahmus *et al*., 2018).

Streptococcus bovis :

Streptococcus bovis is a kind of bacteria that naturally resides in the gastrointestinal tract of around 16% of individuals. Proteins produced by *S. bovis* induce the excessive production of COX-2 and inflammation, which is often seen in colorectal

cancers (CRCs) and may impede programmed cell death and promote the growth of new blood vessels. The relate between *S. bovis* and colorectal cancer was first notarized in 1966 (Dahmus *et al*., 2018). Research has shown that between 6% and 71% of occurrences of *S. bovis* bacteremia are associated with colon neoplasia. As a result, colonoscopy is suggested for individuals in this category, a research discovered that 55% of instances of endocarditis caused by *S. bovis* were associated with colorectal neoplasia. Streptococcus bovis has a cell wall antigen that is specifically attracted to the collagen IV found in the mucosa of the colon. Additionally, it stimulates the production of pro-inflammatory cytokines such as interleukin (IL)-8, IL-1, and COX-2. These cytokines, in turn, promote the growth of new blood vessels (angiogenesis) and cell division (proliferation), while simultaneously reducing the programmed cell death (apoptosis) of cancerous cells, *Streptococcus gallolyticus* (*S. bovis biotype* I) was seen in 71% of CRC cases, while other subtypes of *S. bovis* had an occurrence rate of 17%. Out of all the tumour specimens of CRC , 49% had the DNA of *S. gallolyticus*, but only 8% of healthy tissues had this DNA, the presence of *S. gallolyticus* IgG antibodies was associated with increased risks of CRC. The study revealed that serum antibodies against *S. gallolyticus* were detected in 68% of patients with colorectal cancer (CRC), 78% of patients with adenomas, and only 17% of control individuals (Burnett-Hartman et al., 2008; Dahmus et al., 2018).

Bacteroides fragilis :

Bacteroides fragilis is a prevalent anaerobic bacteria found in the human body. The *Enterotoxigenic Bacteroides fragilis* (ETBF) strain contains the *B. fragilis* toxin (BFT) (Dahmus *et al*., 2018). The underlying biological mechanisms of benign familial tumours (BFT) leading to the development of colorectal cancer have been

acknowledged (Figure 2-4). BFT stimulates the Wnt/β-catenin pathway, leading to an augmentation in cell proliferation. Additionally, it stimulates NFkB, which encourages the production of inflammatory mediators, resulting in inflammation and the development of cancer (Dulal *et al*., 2014; Flemer *et al*., 2017). ETBF binds to colonic epithelial cells and induces the splitting of the tumor-suppressor E-cadherin, leading to enhanced cell permeability, studies have also shown that ETBF stimulates the development of cancer via the T helper 17 (TH17) dependent pathway. Ulger-Toprak *et al*. detected *Bacteroides fragilis* in the faecal samples of 56 out of 73 individuals with CRC, and among them, 21 patients had the BFT gene associated with *Bacteroides fragilis.* This is much higher in comparison to five out of the 40 healthy controls (Ulger-Toprak *et al*., 2006). A research conducted by Boleij *et al*. revealed that the mucosa of the patients had a considerably higher frequency of BFT positive results compared to the control biopsies. The presence of BFT was seen in 100% of tissues from late-stage CRC, compared to 72.7% positive in early-stage CRC (Boleij *et al*., 2015). The presence of *B. fragilis* in colorectal cancer mucosa is a predictor of overall survival over a period of three years (Flemer *et al*., 2017; Dahmus *et al*., 2018).

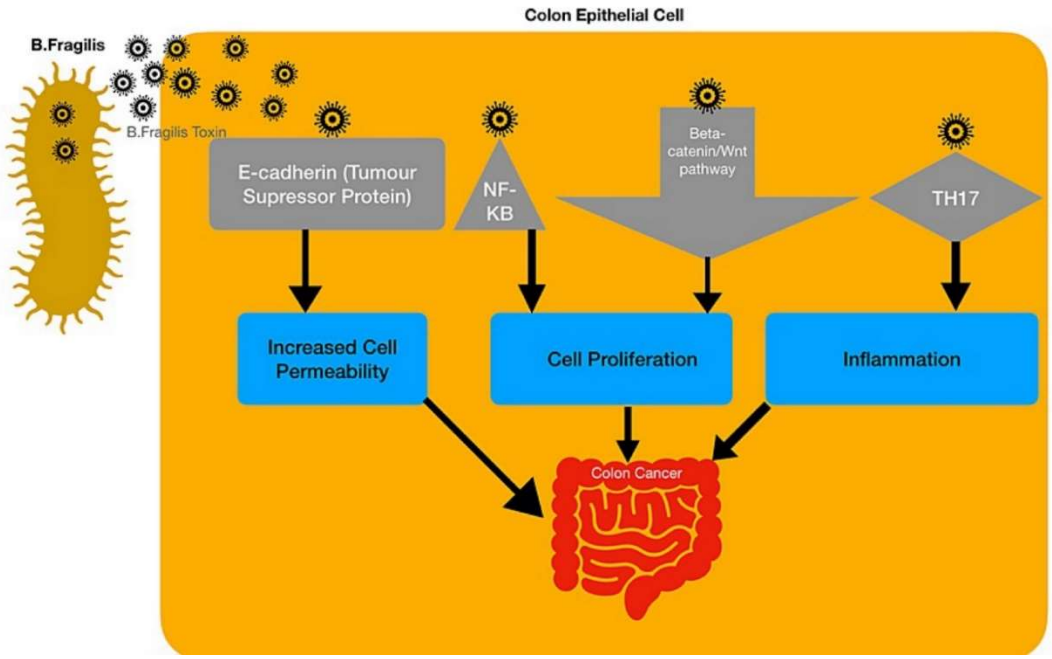

Figure (2-4): The pathophysiology of *Bacteroides fragilis* toxin leading to the development of colorectal cancer. (Artemev *et al*., 2022)

Acidovorax spp:

Acidovorax spp. belongs to the phylum *Proteobacteria*. It is strongly associated with a high risk for colorectal cancer (CRC). The organism breaks down nitro-aromatic chemicals, the flagellar proteins that cause inflammation in the immediate area (Dulal *et al*., 2014).

Other bacteria:

Peptostreptococcus bacteria act in a carcinogenic manner through their products, which contribute to their increased production and the formation of an anaerobic tumor environment, in addition to the proliferation of bacteria. Some types of bacteria, such as *E.coli*, are genotoxic, which means they damage DNA (Wong and Yu, 2019; Ternes *et al*., 2020). Some bacteria may be involved in cancer

development through their interaction with host immune receptors and cancer cells by secreting metabolites, and proteins known as secretomes, secretomes include growth factors, proteases, cytokines, and other proteins, metabolites include a variety of metabolic products resulting from gut microbiota metabolism, as well as cancer-associated metabolites that play a role in carcinogenesis (Wong and Yu, 2019; Ternes *et al*., 2020). Cancer-associated metabolites include substances such as L-2-hydroxyglutarate, succinate, fumarate, D-2-hydroxyglutarate, and lactate, which accumulate in cancer cells as a result of metabolism, Some of these metabolites, such as lactic acid, have the ability to provide energy to cancer cells and promote cancer growth, while others, such as butyrate, contribute to decreased expression of genes involved in inflammation and tumor growth (Wong and Yu, 2019; Ternes *et al*., 2020).

2.8. Microbiome Diagnosis:

The understanding of the makeup, dynamics, and function of human-associated microbiota has significantly advanced with the introduction of Next-Generation Sequencing (NGS), particularly metagenomic NGS, around fifteen years ago. Metagenomic NGS (also referred to as mNGS) offers a comprehensive methodology for examining microbial genomes within complex mixtures of microbial and host cells, in metagenomic uses varying software (Such as: QIIME/ QIIME2, Mothur, BaseSpace, RDP Classifier) (Walker-Daniels, 2020; Schlaberg, 2020).

There are many platforms used in NGS such as: Roche 454, ion torrent, oxford nanopore, IIIumina (MiSeq, HiSeq, NextSeq, NovaSeq) (Walker-Daniels, 2020).

Walker-Daniels, 2020 reported that Illumina is less expensive than other platforms and is used to generate short reads with high throughput and lower error rates, the reads are pooled to create a more accurate assembly.

Microbiome research fundamentally revolves on two options: one focusses on structural features, such as the identification and abundance of bacterial species, while the other examines functional elements, namely the activities of the microbial community, marker gene analysis, commonly referred to as amplicon- based analysis, is the conventional method for structural microbiome investigations, this targeted sequencing technique encompasses 16S rRNA sequencing for bacteria (Baruch *et al*., 2021; Silva *et al*., 2021).

2.8.1. Diagnostic Methods

Amplicon Sequencing: rRNA

The high-resolution characterization of bacterial communities has been primarily achieved through PCR amplification of bacterial marker genes, often the mitochondrial-encoded 16S rRNA gene, with NGS of the resulting amplification products, known as amplicon sequencing, much of the current microbiome research is based on 16S rRNA profiling. The use of the 16S rRNA gene for sequence-based bacterial identification began in the mid-1980s (Schlaberg, 2020). Initially, diagnostic laboratories adopted 16S rRNA gene sequencing to identify isolated bacterial colonies with Sanger sequencing as the conventional method that replaced Targeted Next-Generation Sequencing (Sikkema-Raddatz *et al*., 2012). The 16S rRNA gene, which is approximately 1500 bp long, serves as a dominant taxonomic marker for bacterial classification because of its ubiquity in prokaryotes, its relatively slow evolutionary rate, and its structural characteristics. The 16S rRNA

gene contains nine highly variable sections interspersed with conserved regions essential for maintaining the secondary structure of 16S rRNA (Noller and Woese, 1981) (figure 2-5). The alternating pattern makes the 16S rRNA gene an ideal target for amplification using PCR primers, facilitating the acquisition of conserved sections for sequencing by NGS techniques, while the hypervariable regions serve as taxonomic markers(Soergel *et al*., 2012), In most diagnostic methods only a portion of the 16S rRNA gene is amplified. Sequencing strategies may often be based on the V3′ and/or V4′ hypervariable regions (Soergel *et al*., 2012).

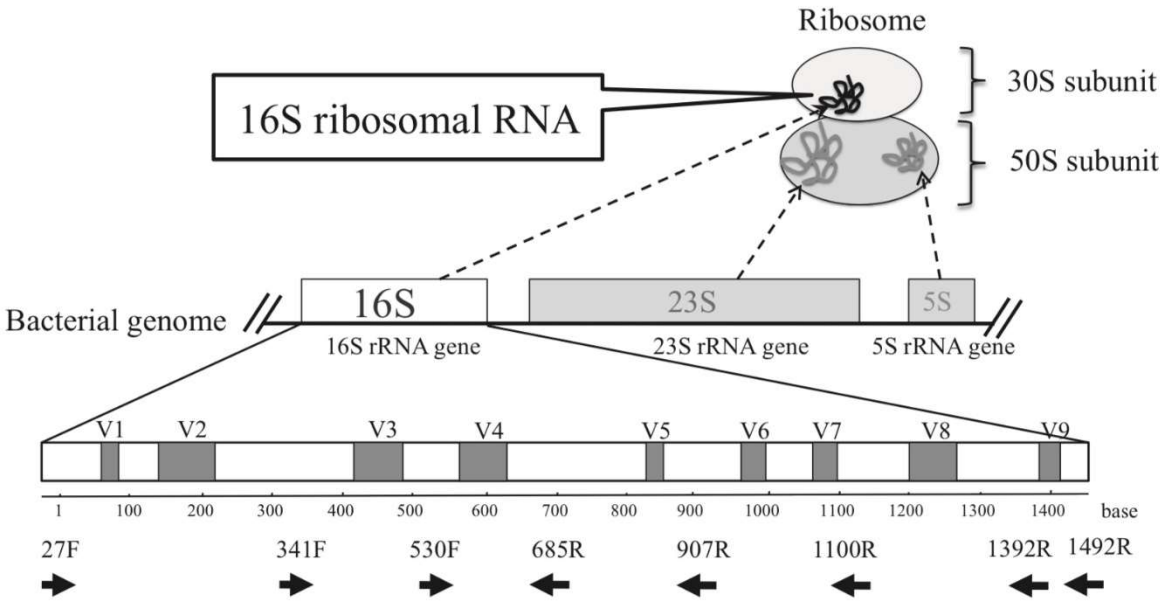

Figure (2-5): Diagram of bacterial rRNA genes (hypervariable regions and conserved regions) (Fukuda *et al*., 2016)

Metagenomics

16s rRNA sequencing is a prevalent method capable of determining the taxonomy of many bacterial taxa in a sample, and specifically identifying genus and species under certain conditions. Shotgun metagenomics techniques likewise assess DNA sequences but use more specific sequencing methods (Walker-Daniels, 2020). And compared to gene-based approaches, the shotgun has the advantage of detecting all classes of microorganisms (viruses, bacteria, fungi, etc.), with greater taxonomic accuracy overall, the ability to type and genotype strains, identify antimicrobial resistance genes and pathogens, and predict the functional potential of microbial communities (Quince *et al*., 2017). By comparing sequence data to complete gene libraries and/or functionally annotated genes, it yields many complex results (Schlaberg, 2020).

Amplicon-based sequencing and shotgun metagenomic approaches to microbiome research both require sample preparation, amplicon-based sequencing requires intact DNA, while shotgun metagenomic approaches use DNA that has been sheared into small fragments that can be sampled from any site of the body's microbiome, the DNA must then be isolated, using a special DNA isolation kit (Douglas *et al*., 2020).

Metatranscriptomic

Metatranscriptomic methods utilise analogous analytical concepts to shotgun metagenomics but specifically focus on the RNA transcribed from microbial cells, enabling evaluations of the expression activities of these organisms. (Bashiardes *et al*., 2016). Shotgun metagenomics and metatranscriptomics predominantly utilise Illumina sequencing technologies, particularly the HiSeq or NovaSeq platforms, owing to their high throughput and economical cost per base. Nonetheless, there has

been a shift towards PacBio and Oxford Nanopore sequencing technologies to utilise longer read lengths that facilitate gene calling and genetic mapping to reference genomes. Total RNA extraction from the microbiome sample, RNA enrichment, fragmentation, cDNA synthesis, and the synthesis of transcriptome libraries for sequencing are common methods (Bikel *et al*., 2015). RNA sequence readings correspond to various genomes and pathways (Kanehisa *et al*., 2000).

Metabolomics and Metaproteomics

The metabolites produced by bacteria and their interactions with the host's metabolism and microbiota are described by metabolomics investigations (Lamichhane *et al*., 2018; Zierer *et al*., 2018). Antibiotics, antibiotic byproducts, and intermediates of bacterial and host metabolism are among the small molecules that are often measured using these methods.

To find known compounds, metabolomics often use mass spectrometry (Zierer *et al*., 2018). To identify and measure the proteins in a microbiome, metaproteomics employs mass spectrometry. (Blakeley-Ruiz *et al*., 2019; Lai *et al*., 2019). Metaproteomics and metabolomics are rapidly developing approaches for studying the microbiome (Galloway-Peña and Hanson, 2020).

2.8.2. measurements diversity of Alpha and Beta:

Measures of Alpha and Beta Diversity: According to Kuczynski et al. (2012), alpha diversity measures the intra-sample variability and make group comparisons easier. Species richness estimators such observed OTUs, the Chao 1 index, and the Shannon and Inverse Simpson indices are often used alpha diversity measures that evaluate species richness and evenness (Chao, 1987; Kim *et al*., 2017).

Because they are less sensitive to the quantity of sequences in a sample, richness and evenness estimators like Shannon and Inverse Simpson are thought to be more reliable (Knight et al., 2018). While dominant or abundant operational taxonomic units (OTUs) predominantly influence the Inverse Simpson index, rare OTUs mostly affect the Shannon index (Bent and Forney, 2008).

Beta diversity evaluates the differences in diversity between samples, typically computed throug evaluating feature dissimilarity, yielding a distance matrix for all sample pairings (Barwell *et al*., 2015). As a conventional measure of beta diversity, the Bray-Curtis dissimilarity offers a numerical evaluation that takes taxonomic abundance into account when comparing two communities (Lozupone and Knight, 2005). It is possible to investigate the extent of variation in community compositions between samples by evaluating taxonomic presence, absence, and abundances (Galloway-Peña and Hanson, 2020).

3. Meterials and methods:

3.1.Meterials:

3.1.1: Tools and apparatuses

Table (3-1): summarises the tools and apparatuses utilized in this study :

No	Tools and Apparatus	Company/ origin
1	Vortex mixture	Medilab/ South Korea
2	Microcentrifuges	Hettich / Germany
3	Micropipettes (10, 100 and 1000 µl)	Dragon MED/ China
4	Sensitive Balance	Sartorius/ Germany
5	Tips	Sterling/Ltd./UK
6	Gloves	Broche/ Malaysia
7	Eppendorf tubes	Bioneer/ South Korea
8	Nano drop 2000	Thermo Scientific / USA

3.1.2. The Kit.

Table (3-2): Kit used in study.

No	Kit	Objective	Company/ origin
1	QIAamp	DNA extraction from stool	QIAGEN / Germany

3.2. Methods:

3.2.1. Sample Collection and Standards

In this study, twenty-one indoor stool samples were collected from participants aged 40 and 60 years in Governorate, Southeastern city of Iraq, during a period from September 2023 to January 2024. Twelve stool samples collected after first dose of chemotherapy from patients diagnosed with Colorectal Cancer (CRC) by oncologist

who attended to the Oncology Department of Al-Sader Teaching Hospital in Maysan Governorate and another nine samples from healthy volunteers in the same city for comparison.

3.2.2. Criteria for diagnosis

For patients with CRC involved in this study, the following criteria were excluded: a history of chronic diseases such as hypertension, diabetes, renal failure, irritable bowel syndrome, familial history of CRC, or recent use of antibiotics, anti inflammatory drugs, probiotics, prebiotics, radiotherapy, and chemotherapy within the past month prior to stool sample collection. (Appendix 1)

3.2.3. Ethics Approval

The approval for conducting this study was provided by the Ethical Committee of Missan Health Research Ethics at Missan Health Directorate Training and Human Development Center (No.: 3369, date 20/09/2023) (Appendix 2). This study was conducted in accordance with the local legislation and institutional requirements of a research Protocol, Ministry of Health Republic of Iraq (Form number 02/2021) . Written informed consent for each participant was requested in accordance with the local and national legislations and institutional requirements.

3.2.4.Bacterial DNA Extraction and 16S rRNA sequencing

During a short time at early morning and under cold conditions indoor stool samples were collected. the bacterial DNA was immediately isolated from roughly 250g human faecal samples using the QIAamp® PowerFecal® Pro DNA Kit following the manufacturer's protocol :

1. Tow hundred fifty milligrams of stool or biosolid was added to the Bead Tube provided.

2. Four hundred µl of CD1 was added, followed by vortexing for 5 minutes. The mixture was then placed on ice for a minute. Another 400 µl of CD1 was added, vortexed for 5 minutes.

3. The PowerBead Pro Tube was centrifuged at 15,000 x g for 1 minute.

4. The supernatant was transferred to a clean 2 ml Microcentrifuge Tube (provided), and this step was repeated.

5. Tow hundred µl of Solution CD2 was added and vortexed for 5 seconds.

6. The solution was centrifuged at 15,000 x g for 1 minute at room temperature. Avoiding the pellet, up to 700 µl of supernatant was transferred to a clean 2 ml Microcentrifuge Tube (provided).

7. Six hundred µl of Solution CD3 was added and vortexed for 5 seconds.

8. Six hundred fifty µl of the lysate was loaded onto an MB Spin Column and centrifuged at 15,000 x g for 1 minute.

9. The flow-through was discarded, and step 8 was repeated to ensure all lysate passed through the MB Spin Column.

10. The MB Spin Column was carefully placed into a clean 2 ml Collection Tube (provided), avoiding any splashing of the flow-through onto the MB Spin Column.

11. Five hundred µl of Solution EA was added to the MB Spin Column and centrifuged at 15,000 x g for 1 minute.

12. The flow-through was discarded, and the MB Spin Column was placed back into the same 2 ml Collection Tube.

13. Five hundred µl of Solution C5 was added to the MB Spin Column and centrifuged at 15,000 x g for 1 minute.

14. The flow-through was discarded, and the MB Spin Column was placed into a new 2 ml Collection Tube (provided).

15. The column was centrifuged at up to 16,000 x g for 2 minutes. The MB Spin Column was carefully placed into a new 1.5 ml Elution Tube (provided).

16. Fifty µl of Solution C6 was added to the center of the white filter membrane, and this step was repeated.

17. The column was centrifuged at 15,000 x g for 1 minute. The MB Spin Column was discarded, and the DNA was ready for downstream applications.

. The extracted DNA was stored at −30°C. The concentration and quality of the extracted DNA were determined using a NanoDrop

Purified bacterial DNA for Next Generation Sequencing (NGS)-based 16S Amplicon Sequencing on an Illumina NovaSeq (PE250-Seq) instrument with 2 x 300 base paired-end reads at BMKGENE Biomarker Technologies (Hongkong) Company Limited China www.bmkgene.com. The universal primers of the 16S rRNA genes were amplified the V3-V4 hypervariable regions (5′-ACTCCTACGGGAGGCAGCAG-3′, 5′-GGACTACHVGGGTWTCTAAT-3′). The raw sequences (Fastq file) were processed with QIIME2 as described previously (and Ibrahim *et al*., 2020; Cao *et al*., 2023), with exception for using 242 as the truncation length and both trims 20 in DADA2 (Callahan *et al*., 2016). The stand-alone version of PICRUSt2 was used for the evaluating the potential, functional metagenome (Douglas *et al*., 2020).

3.2.5. Statistical analysis

The data were normalized for each sample and the relative abundance is presented as mean ± SD and differences within and between groups were assessed using the SRplot tool (https://www.bioinformatics.com.cn/srplot); Stack bars with fill was used to visualized taxa Relative Abundance and Wilcox test of two groups' comparison of ratio between 2 taxa was used. The alpha and beta diversities and Heat tree analysis of the microbial communities were assessed using several methods available in the Microbiome Analyst. In addition (https://www.microbiomeanalyst.com), the taxa relative abundance and the MetaCyc pathways predict functional analysis in PICRUSt for metagenomic function imputation also analyzed using statistical analysis of Metagenomic Profiles (STAMP version 2.1.3) by Welch's test no correction for checking the extend the error. A p value of <0.05 was considered significant.

4.1. Results:

4.1.1. Nanodrop results

Table (4-1): Nanodrop results

				Concentration

Sample ID	Gender	Age	Health Condition	ng/ul	260/280	260/230
CRC1	M	54	CRC	113.4	1.72	0.83
CRC 2	F	52	CRC	297.6	1.9	2.2
CRC 3	M	49	CRC	297.9	1.73	1.21
CRC 4	F	60	CRC	257.1	1.87	1.75
CRC 5	F	45	CRC	356.1	1.88	1.83
CRC 6	M	48	CRC	622	1.82	1.79
CRC 7	F	60	CRC	589.1	1.79	0.82
CRC 8	M	53	CRC	213.6	1.94	2.7
CRC 9	M	55	CRC	155.5	1.96	2.13
CRC 10	F	57	CRC	60.8	2	2.8
CRC 11	M	60	CRC	422.1	1.9	1.92
CRC 12	M	60	CRC	245.7	1.75	1.4
Con1	M	43	Con	334.6	1.87	1.94
Con2	M	49	Con	392	1.83	0.84
Con3	M	48	Con	419.4	1.84	1.6
Con4	M	45	Con	568.2	1.8	0.81
Con5	M	43	Con	503.8	1.83	1.98
Con6	M	40	Con	465.7	1.84	1.47
Con7	M	38	Con	256	1.84	1.16
Con8	M	39	Con	145	1.84	0.85
Con9	M	41	Con	1326.8	1.83	1.63

4.1.2 Basic statistics of Sequence Reads in Stool Samples:

Basic statistics for the number of reads and clusters of similar sequences from stool samples of twenty-two participants; 9 healthy and 12 colorectal cancer (CRC) patients (Table 4-2) ,were analyzed using QIIME II software. Initially, the total number of reads was 1,439,888 for the healthy group and 1,920,681 for the CRC group. Good quality reads were overlapped to merge paired reads of each individual samples. Then, these sequences were assigned and filtered to remove un-combinable read-pairs, resulting in a total of 1,708,083 combined reads: 1,082,877 from CRC patients and a total of 1,139,200 combined reads from healthy volunteers (Table 4-3). Using these combined readings, the final analysis of each individual sample was performed.

Table (4-2): BMI of the samples

Sample ID	Gender	Age	Health Condition	BW (kg)	Tall (cm)	BMI (kg/m2) BMI = Wight (kg) / Tall (m2)
CRC1	M	54	CRC	74	181	22.6
CRC 2	F	52	CRC	66	170	22.84
CRC 3	M	49	CRC	61	160	23.83
CRC 4	F	60	CRC	52	153	22.21
CRC 5	F	45	CRC	61	157	24.75
CRC 6	M	48	CRC	73	179	22.78
CRC 7	F	62	CRC	50	152	21.64
CRC 8	M	53	CRC	64	170	22.15
CRC 9	M	55	CRC	75	170	25.95
CRC10	F	57	CRC	61	164	22.68
CRC11	M	60	CRC	65	172	22 .0
CRC12	M	60	CRC	63	170	21.8
Con1	M	43	Con	76	172	25.69

Con2	M	49	Con	73	164	27.14
Con3	M	52	Con	71	176	22.92
Con4	M	49	Con	75	164	27.89
Con5	M	43	Con	78	173	26.06
Con6	M	40	Con	72	164	26.77
Con 7	M	38	Con	71	170	24.57
Con 8	M	39	Con	72	171	24.62
Con 9	M	41	Con	68	172	22.99

Table (4-3): Basic statistics reads of quality control of sequence data processing of healthy control (A) and CRC participants (B)

A								
Sample-ID	Input	Filtered	% of input passed filter	Denoised	Merged	% of input merged	non-chimeric	percentage of input non-chimeric
Healthy1	160127	145446	90.83	143589	130521	81.51	99439	62.1
Healthy2	159925	143015	89.43	141324	130089	81.34	97141	60.74
Healthy3	159830	144506	90.41	141962	124122	77.66	88011	55.07
Healthy4	159977	144579	90.37	142989	131898	82.45	91449	57.16
Healthy5	160226	144453	90.16	142594	128286	80.07	95735	59.75
Healthy6	160040	143104	89.42	1q41416	131093	Q1	96820	60.5
Healthy7	159885	145328	90.9	142114	120900	75.62	77079	48.21
Healthy8	159937	145544	91	142793	121541	75.99	67078	41.94
Healthy9	159941	145084	90.71	142123	120750	75.5	79407	49.65
Total	1439888	1301059	813.23	1280904	1139200	712.05	792159	495.12
B								

Sample-ID	Input	Filtered	% of input passed filter	Denoised	Merged	% of input merged	non-chimeric	percentage of input non-chimeric
CRC1	160084	145194	90.7	142723	124425	77.72	79034	49.37
CRC2	160113	144421	90.2	142331	127580	79.68	88626	55.35
CRC3	160083	144893	90.51	143028	129896	81.14	91283	57.02
CRC4	159890	144458	90.35	141600	121880	76.23	91015	56.92
CRC5	160315	146013	91.08	143905	128387	80.08	92752	57.86
CRC6	159862	140863	88.12	138053	119340	74.65	93431	58.44
CRC7	159763	143335	89.72	140420	120133	75.19	87588	54.82
CRC8	160123	144814	90.44	142761	129111	80.63	82967	51.81
CRC9	160027	146159	91.33	145015	136773	85.47	99958	62.46
CRC10	160289	145110	90.53	143694	135141	84.31	88555	55.25
CRC11	159998	144410	90.26	143141	137022	85.64	104387	65.24
CRC12	160134	145168	90.65	141412	117442	73.34	83281	52.01
Total	1920681	1734838	1083.89	1708083	1527130	954.08	1082877	676.55

4.1.3. Phylogenetic Composition and relative abundance

A total of 451 taxa from 11 phyla (**Actinobacteriota**, **Bacteroidota**, **Cyanobacteria**, **Desulfobacterota**, **Elusimicrobiota**, **Firmicutes**, **Fusobacteriota**, **Patescibacteria**, **Proteobacteria**, **Synergistota** and **Verrucomicrobiota**), encompassing 182 genera, are illustrated in table (4-4). The composition and abundance of the gut microbiome differ significantly between healthy individuals and those with colorectal cancer (CRC), as showing in Figure (4-2)

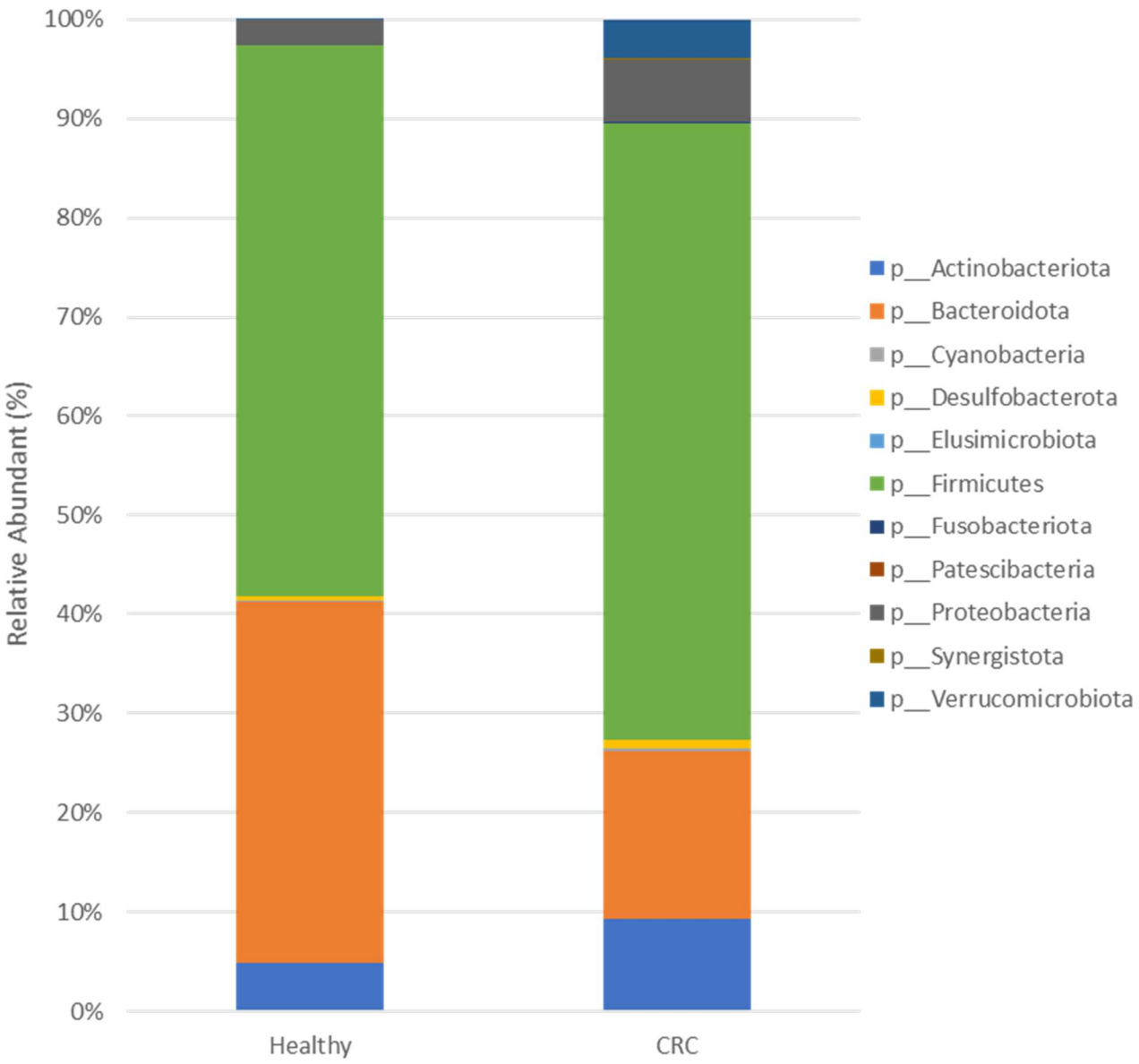

Figure (4-1): Bacterial taxonomy composition at Phyla level of Healthy and CRC faecal samples

The analysis of gut microbiota at the phylum level is illustrated in Figure (4-3) and table (4-3). Figure (4-3a) shows **Firmicutes** was the predominant phylum, representing 55.68% in healthy individuals and 62.09% in patients with CRC. This was followed by **Bacteroidota**, which comprised 36.25% in healthy participants and only 16.94% in CRC patients. The less abundant phyla were **Actinobacteriota**

(4.89% in healthy individuals and 9.29% in CRC patients), **Proteobacteria** (2.55% in healthy individuals and 6.33% in CRC patients), and **Verrucomicrobiota** (0.00% in healthy individuals and 3.89% in CRC patients). Additionally, less than 1% of other phyla were observed in both groups. The proportion of **Bacteroidota** was significantly reduced in CRC patients compared to healthy controls, while the abundance of **Verrucomicrobiota** was significantly increased (Figure 4-3b). Figure (4-3c) illustrates that the ratio of **Firmicutes** to **Bacteroidota** was higher in CRC patients compared to healthy individuals, whereas the ratio of **Bacteroidota** to both **Actinobacteriota** and **Proteobacteria** was lower in CRC patients.

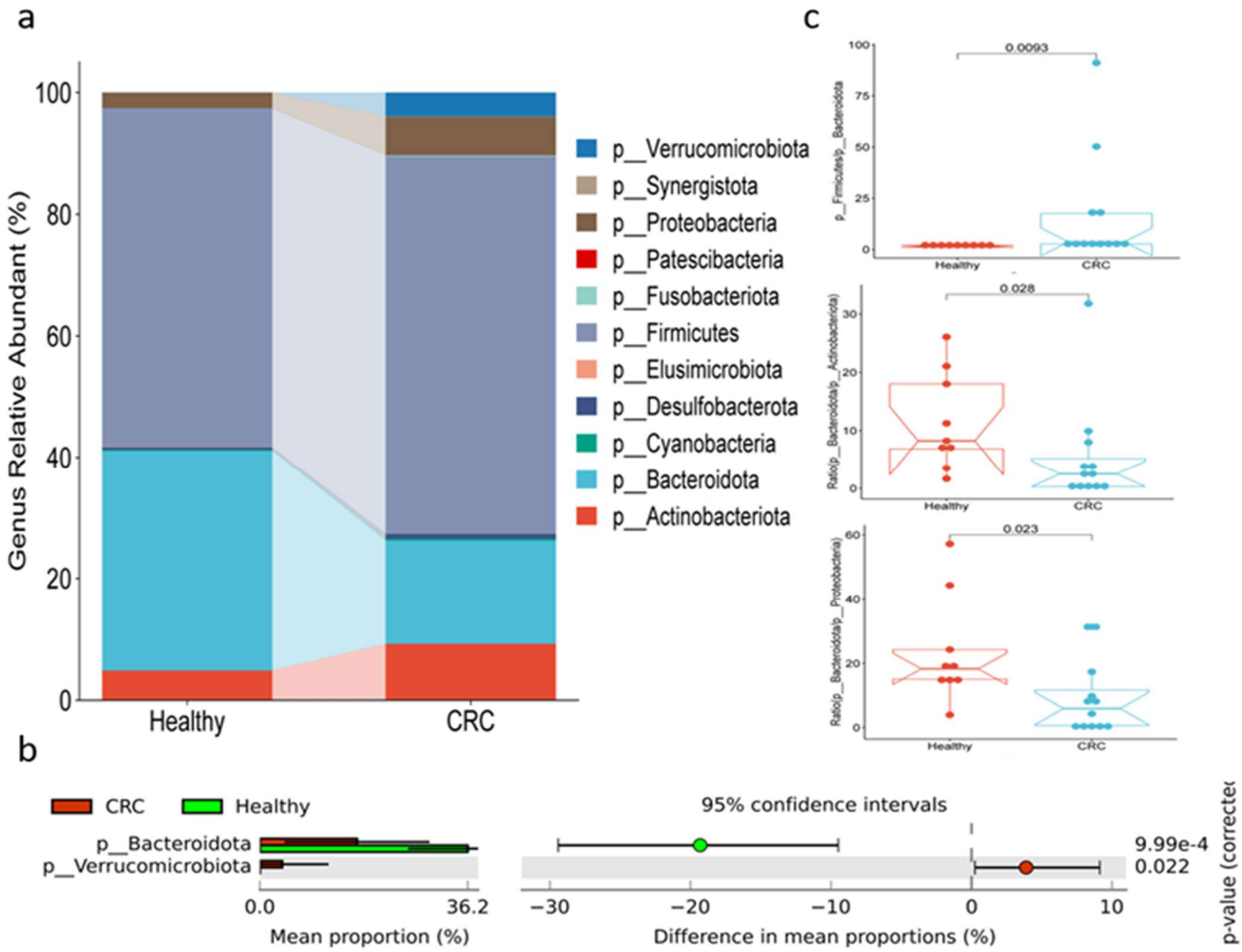

Figure (4-2): Bacterial compositions at phylum level in fecal samples of healthy and CRC cases (a) the relative abundant (b) differences in abundance and (c) the ratios among phyla

Table (4-4) A: Bacterial Phyla Relative Abundance of in Healthy Control (A) and CRC Participant (B)

A | Control	Con1	Con2	Con3	Con4	Con5	Con6	Con7	Con8	Con9				Mean	Standard Deviation
p__Actinobacteriota	6.11	1.89	12.14	4.32	1.85	5.52	7.35	2.05	2.81				4.89	3.38
p__Bacteroidota	21.50	49.40	20.26	31.54	39.00	45.48	50.33	37.03	31.69				36.25	11.07
p__Cyanobacteria	0.11	0.08	0.00	0.00	0.00	0.00	0.00	1.08	0.18				0.16	0.35
p__Desulfobacterota	0.00	0.00	0.00	0.71	0.04	0.08	0.02	2.14	0.61				0.40	0.71
p__Elusimicrobiota	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.35	0.06				0.05	0.12
p__Firmicutes	71.77	47.76	66.21	61.76	56.97	45.91	40.22	47.98	62.56				55.68	10.68
p__Fusobacteriota	0.00	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.00				0.01	0.03
p__Patescibacteria	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00				0.00	0.00
p__Proteobacteria	0.49	0.86	1.39	1.57	2.13	3.02	2.07	9.36	2.10				2.55	2.66
p__Synergistota	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00				0.00	0.01
p__Verrucomicrobiota	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.01	0.00				0.00	0.00

B | CRC	CRC-1	CRC-2	CRC-4	CRC-5	CRC-6	CRC-7	CRC-8	CRC-9	CRC-10	CRC-11	CRC-12	CRC-13	Mean	Standard Deviation
p__Actinobacteriota	9.76	15.55	1.34	8.31	2.57	4.15	5.22	13.14	24.32	16.96	6.25	3.95	9.29	6.93
p__Bacteroidota	20.77	4.02	42.45	24.13	25.60	33.22	17.87	4.32	0.65	12.36	1.21	16.74	16.94	13.20
p__Cyanobacteria	0.00	0.01	0.00	1.29	0.99	0.24	0.45	0.18	0.00	0.00	0.17	0.24	0.30	0.42
p__Desulfobacterota	1.13	1.18	0.24	1.96	0.61	2.56	0.05	0.14	0.33	0.00	0.97	0.77	0.83	0.79
p__Elusimicrobiota	0.00	0.01	0.00	0.00	0.15	0.02	0.00	0.00	0.00	0.00	0.00	0.12	0.03	0.05
p__Firmicutes	58.15	68.96	53.53	61.25	69.06	52.88	75.84	81.66	59.03	52.09	60.84	51.77	62.09	9.81
p__Fusobacteriota	0.00	0.00	0.00	0.00	0.00	1.53	0.00	0.00	0.00	0.00	0.00	0.91	0.20	0.49
p__Patescibacteria	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00
p__Proteobacteria	4.81	10.18	2.44	2.74	0.83	3.39	0.56	0.57	4.32	18.59	2.22	25.32	6.33	7.88
p__Synergistota	0.00	0.06	0.00	0.00	0.07	0.94	0.00	0.00	0.00	0.00	0.00	0.06	0.09	0.27
p__Verrucomicrobiota	5.37	0.03	0.00	0.31	0.11	1.06	0.02	0.00	11.35	0.00	28.33	0.11	3.89	8.42

At the family level this study reveals notable differences between healthy individuals and those with colorectal cancer (CRC). The family ***Prevotellaceae*** is significantly more prevalent in healthy individuals (29.43%) compared to CRC patients (8.39%). The family ***Lachnospiraceae*** is relatively consistent in abundance, appearing in both groups at slightly below 20%. For healthy subjects, ***Ruminococcaceae*** is found at 15.38%, while in CRC patients, it is somewhat lower 12.25%. Other families, such as ***Erysipelato clostridiaceae*** and ***Coriobacteriaceae***, are present in lower abundances, 2.3% and 2.25%, respectively, in both groups. However, specific families show notable variations between healthy and CRC conditions. For instance, ***Veillonellaceae*** (4.5%), ***Selenomonadaceae*** (2.9%), ***Erysipelotrichaceae*** (2.5%), and ***Muribaculaceae*** (1.2%) are more prevalent in healthy individuals but are substantially reduced in CRC cases. Conversely, CRC patients have higher proportions of ***Oscillospiraceae***, and ***Akkermansiaceae***, each around 4.5%, compared to lower levels in healthy subjects. Notably, ***Eggerthellaceae*** and ***Christensenellaceae*** are found at 2.1% and 1.1%, respectively, in CRC patients, whereas their levels are 0.23% in healthy individuals as shown in figure (4-4a). Statistical analysis confirms that the abundance of ***Prevotellaceae*** is significantly reduced in CRC patients versus healthy, while ***Eggerthellaceae***, ***Oscillospiraceae***, and ***Akkermansiaceae*** are significantly increased, figure(4-4b). Furthermore, the ratios of ***Prevotellaceae*** and ***Ruminococcaceae*** to ***Eggerthellaceae***, ***Oscillospiraceae***, and ***Akkermansiaceae*** are significantly lower in CRC patients compared to healthy controls, as show in figure (4-4c).

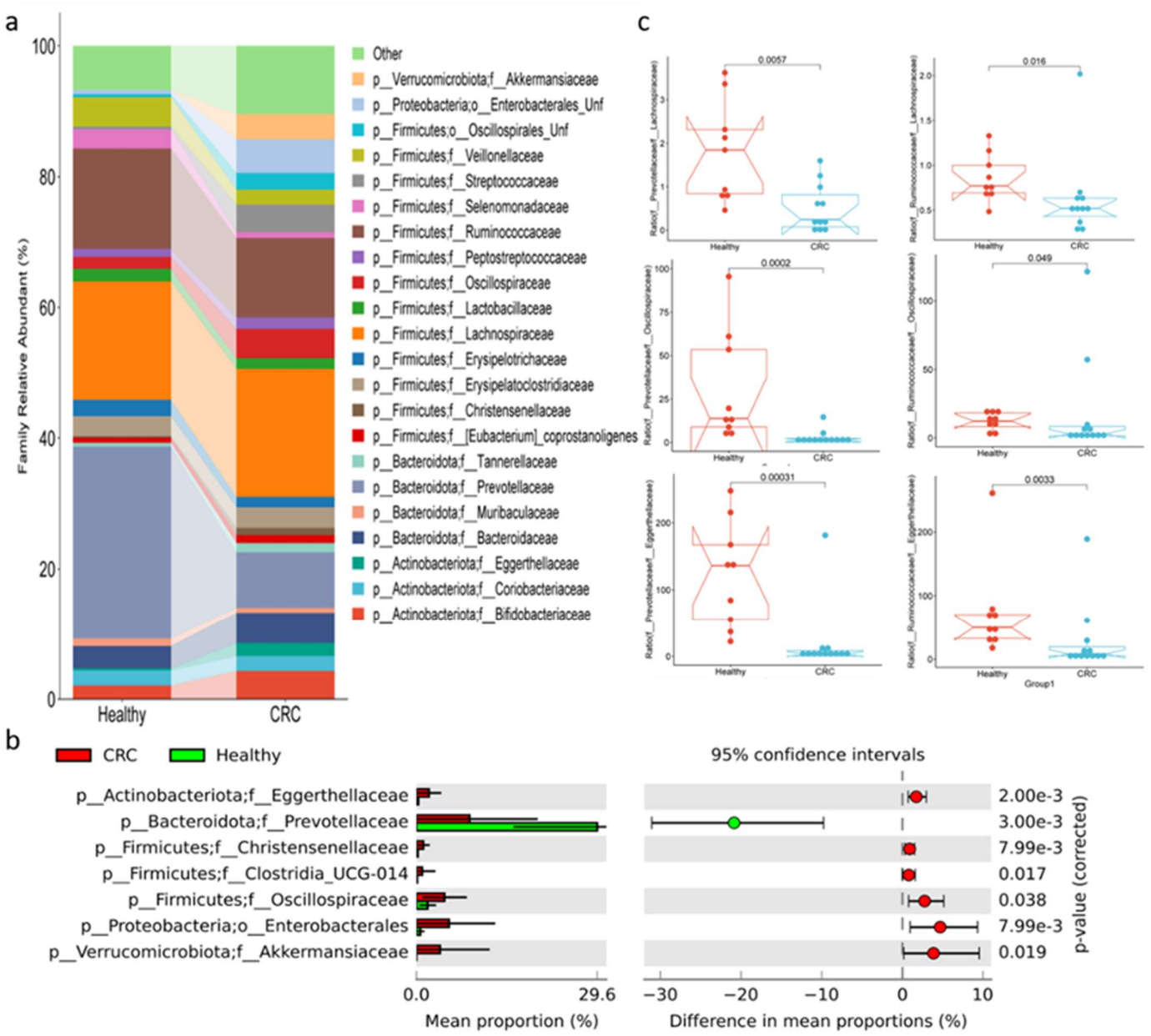

Figure (4-3): Bacterial compositions at family level in fecal samples of healthy and CRC cases (a) the relative abundant (b) differences in abundance and (c) the ratios among families.

At the genus level as illustrates in details in Figure (4-5) , ***Prevotella*** (26.6%) is the most prevalent in healthy individuals, followed by ***Faecalibacterium*** (12.2%). In contrast, these genera are significantly less abundant in individuals with colorectal

cancer (CRC), where their prevalence drops to approximately 7.5%. Other genera, such as ***Bacteroides***, ***Bifidobacterium***, and ***Streptococcus***, are found in lower amounts in CRC patients, around 4.7%, compared to their proportions in healthy individuals (3.4% ***Bacteroides***, 2.1% ***Bifidobacterium***, and 0.4% *Streptococcus*). ***Akkermansia*** is present in 3.8% of CRC cases but absent in healthy subjects. Conversely, in healthy individuals, genera like ***Dialister*** (3.3%), ***Mitsuokella*** (2.5%), ***Agathobacter*** (2.1%), and ***Alloprevotella*** (2.1%) are more abundant compared to CRC patients, where their proportions are reduced by at least half. Genera with less than 1% abundance were not included in (Figure 4-5a). The abundance of ***Prevotella***, ***Faecalibacterium***, and ***Mitsuokella*** was significantly reduced in CRC compared to healthy individuals. On the other hand, the levels of ***Eggerthella***, ***Eubacterium hallii*** group, ***Blautia***, ***Christensenellaceae*** *R-7* group, Clostridia UCG-014, and ***Akkermansia*** are significantly elevated in CRC cases (Figure 4-5b). Figure (4-5c) illustrates that the ratios of ***Prevotella*** to ***Blautia*** and ***Christensenellaceae*** *R-7* group, as well as the ratio of *Prevotella* to *Bacteroides*, are significantly reduced in CRC patients compared to healthy individuals, as showing in Figure (4-6).

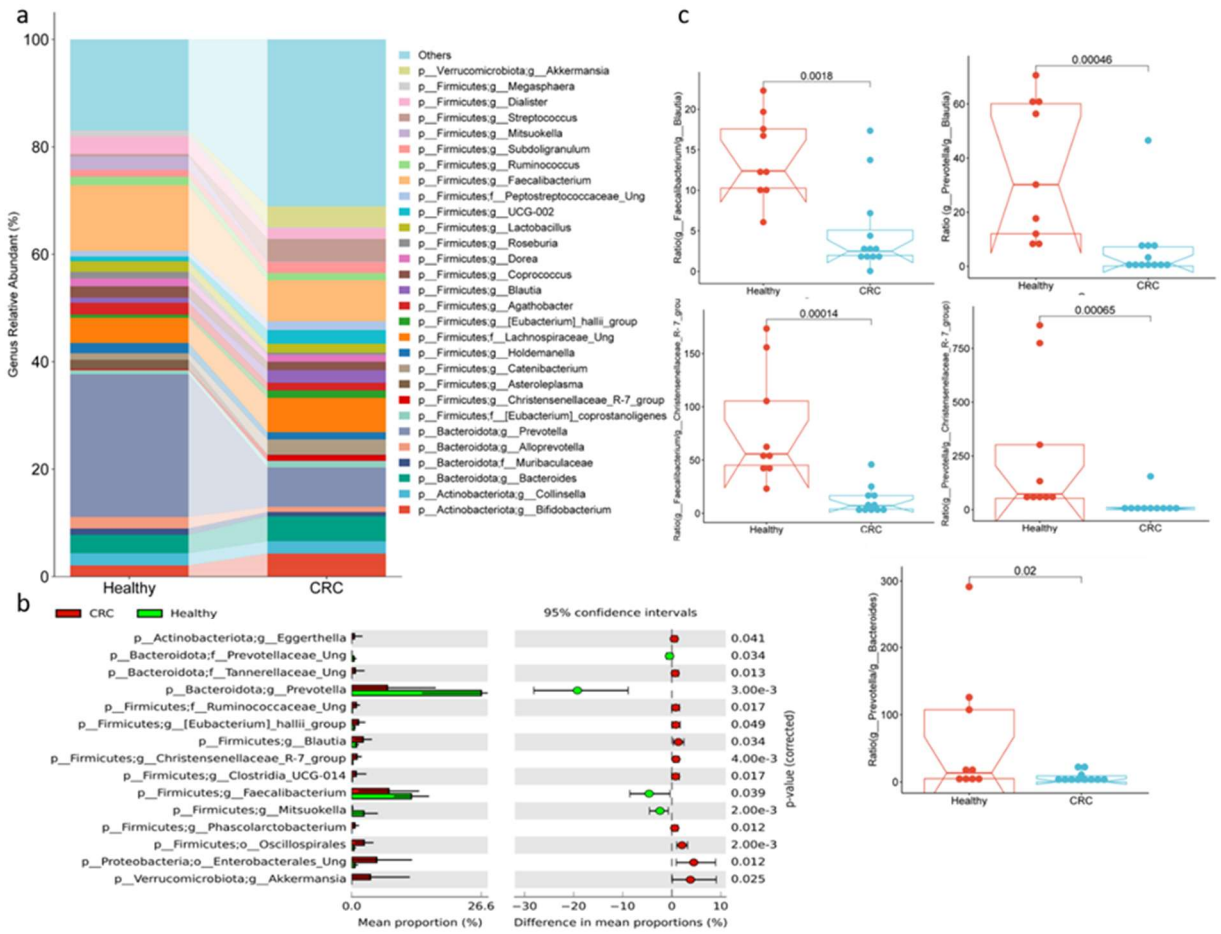

Figure (4-4). Bacterial compositions at genus level in fecal samples of healthy and CRC cases (a) the relative abundant (b) differences in abundance and (c) the ratios among families.

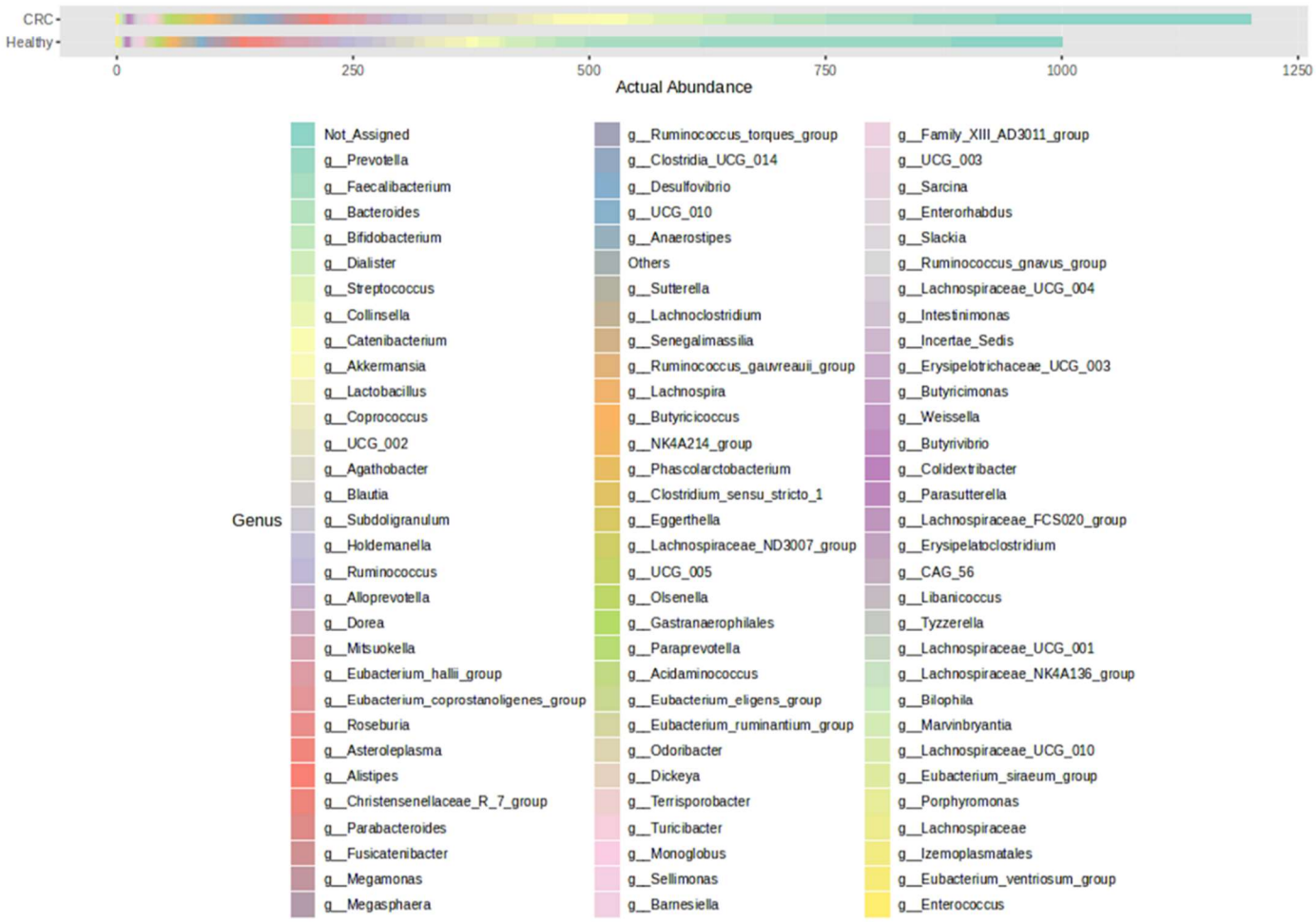

Figure (4-5): Bacterial taxonomy composition at genus level of Healthy and CRC faecal samples

4.2.4.Microbial Shifts Associated with Colorectal Cancer Detected by Metacoder Tree Analysis

The analysis of microbial taxa using Metacoder Tree reveals significant reductions in various taxa between colorectal cancer (CRC) patients and healthy controls, spanning from the phylum to the genus level (Figure 4-7). Within the **Bacteroidota** phylum (p=0.003), remarkably reductions were observed at multiple taxonomic levels: **Bacteroidia** class (p=0.003), **Bacteroidales** order (p=0.002), and the families ***Prevotellaceae*** (p=0.001) and ***Barnesiellaceae*** (p=0.021). Specifically, the genera ***Barnesiella*** (p=0.008) and ***Prevotella*** (p=0.002) within these families showed significant decreases. Although no significant change was detected at the phylum level for **Firmicutes**, several taxa at different levels within this phylum exhibited substantial reductions. These include the **Negativicutes** class (p=0.006), the **Veillonellales_Selenomonadales** order (p=0.005), and the families ***Veillonellaceae*** (p=0.009) and ***Selenomonadaceae*** (p=0.003). Within the ***Selenomonadaceae*** family, the genus ***Mitsuokella*** (p=0.001) was notably reduced. Additionally, in the Clostridia class, five genera showed significant reductions, particularly within the ***Lachnospiraceae*** family of the **Lachnospirales** order and they are including ***Eubacterium eligens*** group (p=0.016), ***Coprococcus*** (p=0.021), ***Lachnospira*** (p=0.006), and ***Lachnospiraceae*** UCG 004 (p=0.013). The genus ***Faecalibacterium*** (p=0.019) from the ***Ruminococcaceae*** family in the **Oscillospirales** order also showed significant reduction. In the **Bacilli** class, a notable decrease was observed in the genus ***Asteroleplasma*** (p=0.049) from the ***Erysipelatoclostridiaceae*** family in the ***Erysipelotrichales*** order. In addition, within the **Proteobacteria** phylum, the genus ***Sutterella*** (p=0.004), from the ***Sutterellaceae*** family (p=0.004) and the **Burkholderiales** order (p=0.002), was significantly reduced.

In contrast, several taxa show significant increases at various levels in patients with CRC compared to healthy controls. Notably, within the **Firmicutes** phylum and *Clostridia* class, three genera exhibit marked increases across different orders. These include ***Monoglobus*** (p=0.018) from the ***Monoglobaceae*** family in the **Monoglobales** order (p=0.019), ***Sporobacter*** (p=0.013) from the ***Oscillospiraceae*** family in the **Oscillospirales** order, and *Family_XIII_AD3011*_group (p=0.011) from the ***Anaerovoracaceae*** family in the **Peptostreptococcales-Tissierellales** order (p=0.017).

Moreover, significant increases are observed in genera from various phyla and classes. ***Bacteroides*** (p=0.032) from the ***Bacteroidaceae*** family (p=0.014), within the **Bacteroidales** order of the **Bacteroidia** class in the **Bacteroidota** phylum, shows an in-crease. Similarly, ***Eggerthella*** (p=0.027) from the ***Eggerthellaceae*** family in the **Coriobacteriales** order of the **Coriobacteriia** class within the **Actinobacteriota** phylum is elevated. Additionally, ***Dickeya*** (p=0.014) from the ***Pectobacteriaceae*** family (p=0.014) in the **Enterobacterales** order (p=0.042) of the **Gammaproteobacteria** class within the **Proteobacteria** phylum is increased. Finally, ***Akkermansia*** (p=0.005) from the ***Akkermansiaceae*** family (p=0.006) in the **Verrucomicrobiales** order (p=0.006) of the **Verrucomicrobiae** class (p=0.003) within the **Verrucomicrobiota** phylum (p=0.017) also demonstrates a significant rise.

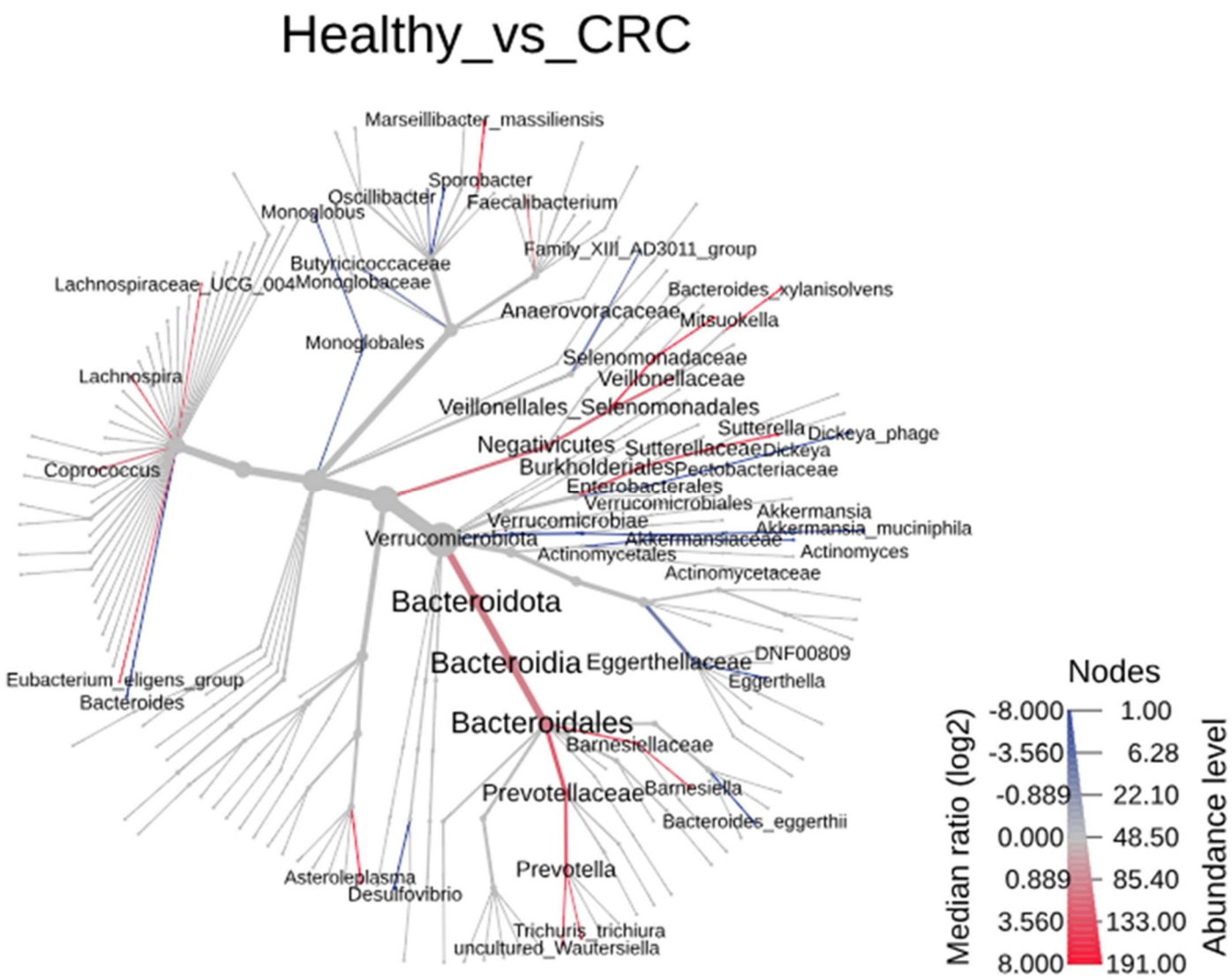

Figure (4-6). The heat tree analysis of microbial taxa showing the taxonomic differences of fecal microbiota between CRC and healthy controls using Metacoder

4.2.5. The Diversity of the bacterial communities

The diversity of taxa between healthy individuals and those with CRC involved were analyzed by both α-diversity and β-diversity metrics. For α-diversity, metrics such as Observed Features, Pielou Evenness, and Doubles showed significant reductions in CRC patients compared to healthy individuals. However, Shannon, Chao1, and Simpson indices did not reveal any notable differences between the two groups (Figure 4-8a). For β-diversity, bacterial community differences between CRC and healthy individuals were assessed using the Bray-Curtis Index, Jaccard Index, and Jensen-Shannon Divergence (Figure 4-8b). In all three β-diversity measures, there was a clear clustering of healthy individuals, with distinct separation from CRC patients in the distance matrices, as show in Figure (4-9).

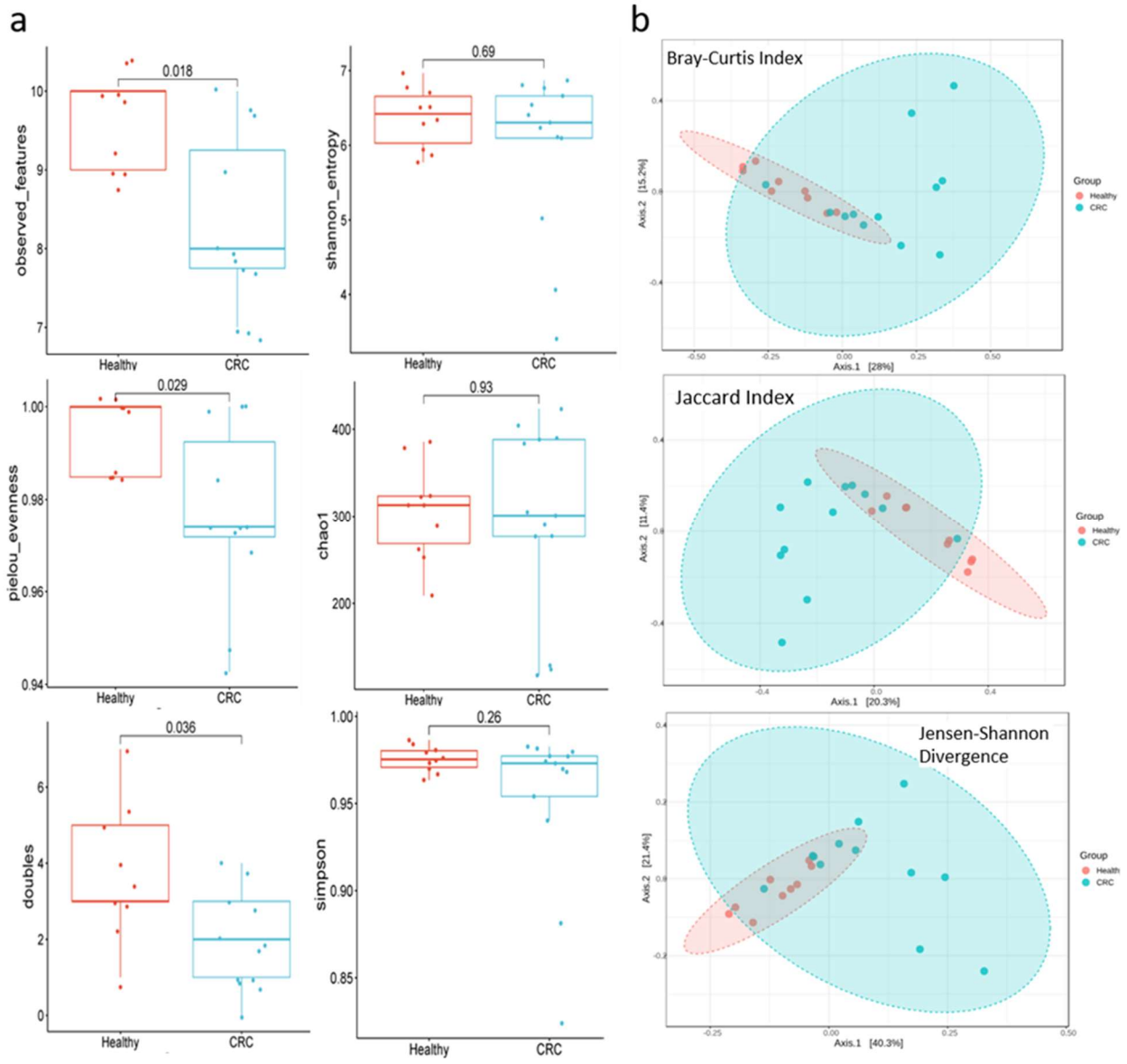

Figure (4-7). Figure(4-8a) Alpha Diversity of Colorectal cancer (CRC) patients exhibit lower species richness (Ob-served Features), evenness (Pielou's Evenness), and "doubles" metrics compared to healthy indi-viduals. However, similar Alpha diversity of the Shannon, Simpson, and Chao1 indices show no significant differences between the two groups. A p value of <0.05 was considered significant. Figure(4-8b) Beta Diversity: PCoA plots illustrate between-sample diversity using three distance metrics (Bray-Curtis, Jaccard Index, and Jensen-Shannon Divergence). Healthy individuals (red) display a distinct, narrower clustering pattern, indicating a more consistent microbial community structure. In contrast, CRC patients (blue) show a clear separation, highlighting the divergent microbiome composition associated with CRC.

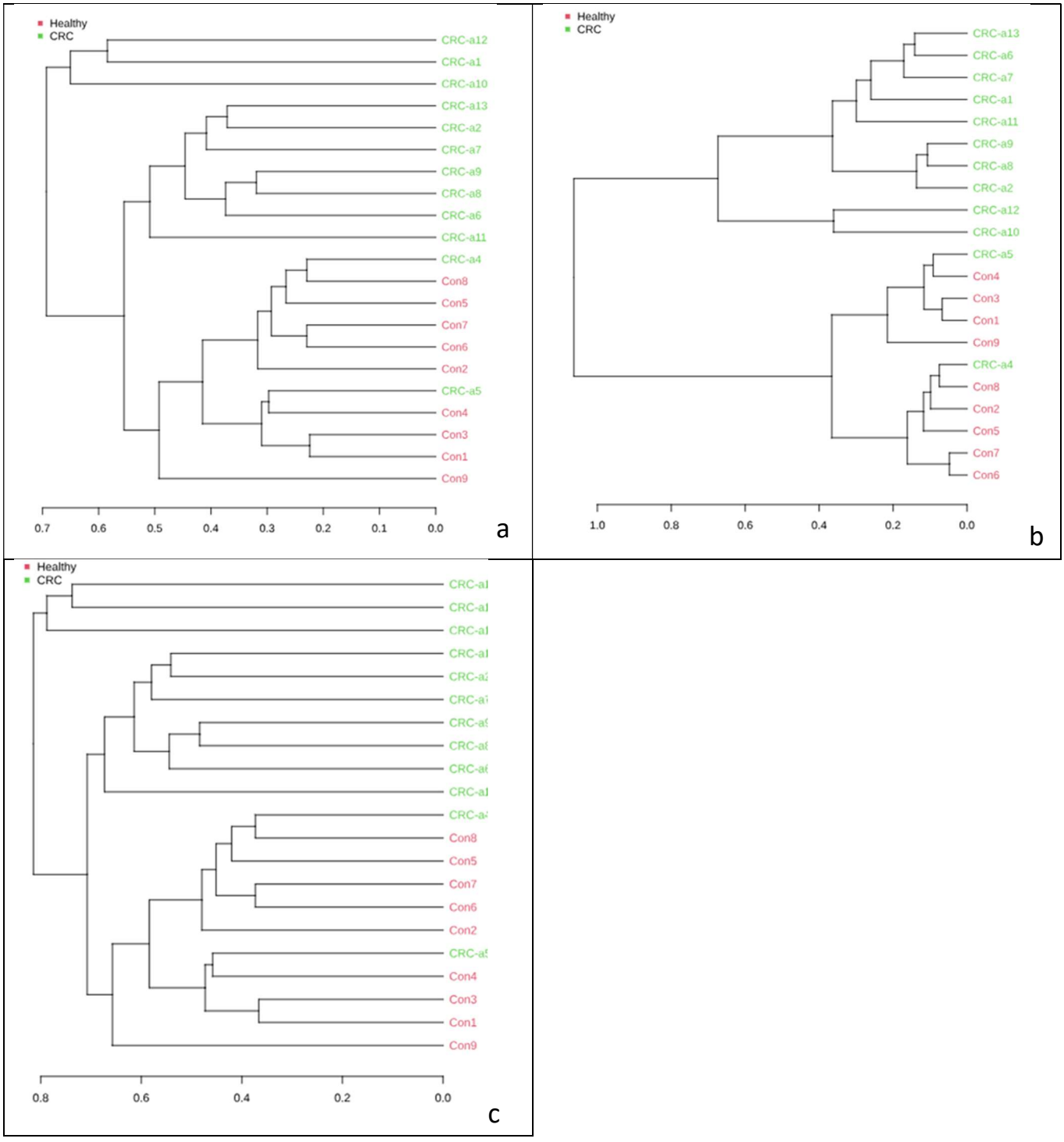

Figure (4-8): Dendrogram Analysis (a) Bray-Curtis Index (b) Jensen-Shannon Divergence (c) Jaccard Index

4.1.6. The MetaCyc pathways

Data were analyzed using Statistical Analysis of Metagenomic Profiles (STAMP version 2.1.3) to identify a number of MetaCyc pathways by untargeted analysis, as show in Table (4-5).

Table (4-5): MetaCyc pathways of CRC patients

MetaCyc pathways Upregulated of CRC	**MetaCyc pathways Downregulated of CRC**
D-galactarate degradation I (p = 0.02)	adenosylcobalamin salvage from cobinamide (p = 0.03)
D-glucarate degradation I (p = 0.02)	Calvin-Benson-Bassham cycle (p = 0.01)
enterobacterial common antigen biosynthesis (p = 0.04)	Reductive acetyl coenzyme A pathway (p= 0.02)
enterobactin biosynthesis (p = 0.01)	superpathway of polyamine biosynthesis (p = 0.007)
Fucose degradation (p= 0.02)	fatty acid &beta (p = 0.03)
superpathway of hexitol degradation (p = 0.03)	fatty acid elongation – saturated (p = 0.03)
heme biosynthesis (anaerobic) (p = 0.004)	methylerythritol phosphate pathway (p =0.03)
L-arginine degradation (p = 0.04)	peptidoglycan biosynthesis (p = 0.04)
superpathway of glycolysis, pyruvate dehydrogenase, TCA, and glyoxylate bypass (p = 0.02)	
superpathway of chorismate metabolism (p = 0.009)	

4.2. Discussion:

It has been previously described that the gut microbiome as the "forgotten organ" of the human body (O'Hara and Shanahan, 2006). Due to lack of knowledge regarding direct impact roles of gut microbiome on health and disease conditions by Iraqi academics and researchers. Therefore, this study for the first time describes the composition and metabolic potential of gut microbiota in healthy and CRC patients in Iraq.

In this study, the Firmicutes was the predominant phylum, followed by Bacteroidota, while other phyla being much less abundant in both participants groups. Although an increase in Firmicutes was observed in CRC patients, this difference was not statistically significant when compared to healthy controls while a significantly reduction of Bacteroidota phylum in CRC patients, and these similar changes have been observed in previous findings (Qin *et al*., 2010; Wang, 2012). Additionally, *Verrucomicrobiota* was found significantly higher in CRC compared to healthy individual and this is consonance with previously studies recorded by (Baxter *et al*., 2014; Weir *et al*., 2014; Wang *et al*., 2023), as shown in table (4-2). Furthermore, the ratio of Firmicutes to Bacteroidota was higher in CRC patients compared to healthy individuals, which aligns with findings from previous studies (Sun and Kato, 2016; Pandey *et al*., 2023). These studies recorded that the bacterial relatives abundant and their ratio are a key indicator of gut health, and with any significant changes indicating a positive biomarker of gut dysbiosis. Indeed, the Firmicutes/Bacteroidetes ratio is closely associated with the development of inflammatory bowel diseases (IBDs) and the progression of CRC (Sun and Kato 2016; Pandey *et al*., 2023). Although there was no significant increase found of both Actinobacteriota and Proteobacteria in CRC patients, the ratio of these phyla to Bacteroidota was significantly reduced in these patients, and these ratios had not

been previously documented in CRC cases. A study indicated that Proteobacteria may promote the risk of metabolic diseases and CRC, and playing a crucial role in tumors with proficient mismatch repair and other phyla were more common in tumors with deficient mismatch repair (Xu *et al*., 2023).

Further analysis revealed significant differences at the genus level between healthy people and CRC patients. Notably, there was a marked decline in the genus of *Roseburia* in CRC compared to healthy, which aligns with findings from previous conducted studies (Tamanai-Shaccori *et al*., 2017; Kang *et al*., 2023). This bacterium is a Gram-positive anaerobic with crucial roles in digestion and production of short-chain fatty acids (Tamanai-Shaccori et al., 2017). Additionally, *Roseburia* contributes to host immune regulation and exhibts anti-inflammatory properties, as it acts as a PD-1 (programmed death 1) antagonist that stimulates T cells to target and kill cancer cells (Tamanai-Shaccori *et al*., 2017). Similarly, a reduction in *Sutterella* was also observed in CRC and this bacterium an anaerobic Gram-negative bacterium and linked to the pathogenesis of inflammatory bowel disease, and metabolic diseases (Hiippala *et al*., 2016). This reduction further underscores the potential role of gut microbiota in CRC pathogenesis. Furthermore, *Barnicella* levels was also reduced in CRC patients and this result is in line with the recent study by Sarhadi *et al*. (2020). This bacterium has been associated with an anti-cancer immune response by enhancing the response of some types of helper T cells (Sarhadi *et al*., 2020). Similarly, *Faecalibacterium* relative abundant was also decreased in CRC patients compared to healthy people, which aligns with findings from previous conducted studies Ma *et al*. (2020). These studies have confirmed that a positive correlation between *Faecalibacterium* and healthy subjects and it has a crucial role as anti-tumor bacteria, which it works to prevent invasion, promote programmed cell death, and limit the spread of various cancer cells in various organ, such as breast,

lung, colorectal, and colon cells (Ma *et al*., 2020). In addition, the abundance of *Prevotella* also reduced in CRC patients and this finding was supported by Yang *et al*. (2019). Yang and his colleagues noted that presence of *Prevotella* is linked to a diet rich in fiber, and its levels increased after the diagnosis of CRC of reduces mortality cases. Other genera such as *Eubacterium*, *Lachnospira*, and *Lachnospiraceae_UCG_004*, also found less abundant in CRC and these finding were identical similar with the recent studies (Liu *et al*., 2020; Ma *et al*., 2020; Du, 2022; Lu *et al*., 2022). A reduced abundance of *Eubacterium* and *Lachnospira* species in intestine and leads to deficit in SCFAs such as valerate and butyrate and other metabolites like folic acid. This deficit may promote intestinal lymphomagenesis (Ma *et al.,* 2020; Lu *et al*., 2022).

In contrast, several genera revealed an increase in CRC patients compared to healthy individuals. Cleary, *Bacteroides* and *Blautia* were more abundant in CRC cases and these finding are similar to previous studies (Cuív PÓ et al., 2017; Yuan *et al*., 2018; Cai *et al*., 2021). Indeed, certain *Bacteroides* species have the ability to invade colonic epithelial cells and induce pro-inflammatory cytokines, which are major contributes to ulcerative colitis and the pathogenesis of IBD (Cuív PÓ *et al*., 2017). Additionally, these bacteria may promote colorectal carcinogens by an increasing aberrant crypt focus by this bacterium nay promotes colorectal carcinogenesis (Yuan *et al*., 2018). Cai *et al*. (2021) reported that the relative abundance of *Blautia* was significantly higher in colorectal neoplasms. This bacterium is positively correlated with the expression of two types of cytokines, which is associated with a poorer prognosis in colorectal cancer treatments. Furthermore, *Actinomyces* levels are also elevated in CRC patients compared to healthy people and this result is in line with the recent study by Xu *et al*. (2022). This bacterium activates the TLR2/NF-κB signaling pathway and diminishing the effectiveness number of CD8+ T cells against

the CRC environment, particular in co-localized with cancer-associated fibroblasts in colorectal cancer (Xu *et al*., 2022). Consequently, this bacterium is considered a tumoral microbiota that facilitates tumor formation. Xu and his colleagues' also further emphased that higher abundant of *Actinomyces* along with other pro-tumor taxa and these changes is considered the key microbiota in the young CRC patients. A significant increase in *Akkermansia* spp. has been observed in CRC patients compared with healthy people, and this result was matched to recent human and mice studeis (Siddiqui et al. 2022; Wang et al., 2022). Wang and his colleagues reported that a high abundance of *Akkermansia* in the gastrointestinal tract of mice elevated early level of inflammation and promoted proliferation of intestinal epithelial cells and this enhances the formation of CRC (Wong *et al*., 2022). However, other studies have shown reduction in *Akkermansia* abundance associated with severe symptoms of CRC. Some studies indicates that *Akkermansia* did not play a role in the development of CRC (Faghfuri and Gholizadeh, 2024). This the role of *Akkermansia* in promoting or inhibiting CRC remains unclear and need further investigation (Faghfuri and Gholizadeh, 2024). Similarly, an increase in *Desulfovibrio* abundance also have been noted in CRC patients compared with healthy people, and this result consistent with the recent finding of research by Li et al. (2024). Yan *et al*. (2020). Kushkevych *et al*. (2021). Species of *Desulfovibrio*, a known intestinal sulfate-reducing bacteria (Kushkevych *et al*., 2021), exhibit high abundance that leads to elevated the production of hydrogen sulfide (Windey *et al*., 2012), an increase of the H2S levels in the intestine have toxic effects on intestinal cells, impacting the mucosal lining, progression of leaky gut, and then cause intestinal cell DNA damage, and potentially contributing to inflammatory bowel diseases and carcinogenesis (Marchesi *et al*., 2016). *Eggerthella* are anaerobic Gram-positive bacteria that are commonly found in the human gastrointestinal tract, they can cause life-threatening infections (Wong and Rubinstein, 2014). They have

been previously reported as biomarkers for CRC (Yu et al., 2017 , Qin et al., 2024). The results showed an increase in Eggerthella in CRC compared to healthy controls, the result is consistent with what was published by Qin et al. (2024)

On the other hand, gut health is linked to metabolic pathways that the microbiome is responsible for. Some of them are beneficial and reduce the disease in general and colorectal cancer in particular, while others increase the severity of the disease. In the results, there were significant differences in metabolic pathways between CRC patients and healthy controls.

Calvin-Benson-Bassham cycle decreased in CRC patients compared to healthy controls. It is considered a beneficial pathway and is associated with improved diagnosis of CRC (Huh *et al*., 2022). The result is consistent with what was published in a study conducted by Kim *et al*. (2024) which confirmed that the Kelvin pathway is beneficial for reducing the risk of CRC (Kim *et al*., 2024).

Fatty acid elongation - saturated decreased in CRC patients compared to healthy controls. Research has shown that fatty acid elongation - saturated is important in promoting bowel movements (Zhao *et al*., 2018).

Peptidoglycan biosynthesis was decreased in CRC patients compared to healthy controls. Peptidoglycan are molecules that make up the bacterial cell wall and have antitumor effects in the host by promoting apoptosis (Chen and Li, 2020). Heme biosynthesis II (anaerobic) was decreased in CRC patients compared to healthy controls. Heme biosynthesis is important in the pathogenesis of disease by pathogenic bacteria (Choby and Skaar, 2016).

The metabolism of adenosylcobalamin salvage from cobinamide was decreased in CRC patients compared to healthy controls, and this result is consistent with what was published by Liu *et al*. (2024).

The superpathway of polyamine biosynthesis II was decreased in CRC patients compared to healthy controls. This is congruent with findings by Kulecka *et al*. 2024

The superpathway of hexitol degradation and fatty acid beta decreased in CRC patients compared to healthy controls. This result is not congruent with what was found by Kulecka *et al*. (2024).

Methyl erythritol phosphate pathway I was decreased in CRC patients compared to healthy controls. This pathway is found in most bacteria and is important in oxidative stress responses and detection and also acts as an antioxidant (Perez-Gil *et al*., 2024).The reductive acetyl coenzyme A pathway was decreased in CRC patients compared to healthy controls, in general This pathway is used in cancer treatment (Guertin and Wellen, 2023).

In a study conducted on stool samples by Arcila-Galvis *et al*., which mapped the microbial biomarkers of patients with gastrointestinal disorders, showed an increase in D-glucarate degradation I and D-galactarate degradation I, which bacteria use as an energy source (Arcila-Galvis *et al*., 2022). In our results, an increase in D-galactarate degradation I and D-glucarate degradation I was observed in CRC patients compared to healthy controls. Enterobacterial common antigen (ECA) biosynthesis was increased in CRC patients compared to healthy controls. ECA is an antigen present on the outer membrane of the *Enterobacteriaceae* family and is considered a virulence factor (Goździewicz *et al*., 2015). ECA a proinflammatory agent, is able to induce the secretion of large amounts of pro-inflammatory cytokines, such as TNF-α, IL-8, and IL-1β (Mirsepasi-Lauridsen et al., 2019; Khorsand et al., 2022).

L-arginine degradation increased in CRC patients compared to healthy controls. L-arginine deficiency causes many intestinal problems as it increases bacterial

resistance, perpetuates chronic inflammation, affects the disruption of beneficial intestinal microbes, and increases pathogenic microbes (Nüse *et al*., 2023).

The results showed an increase in Enterobactin biosynthesis in CRC patients compared to healthy controls and these results were congruent with a study of Mathlouthi *et al*. (2022). Enterobactin is a type of sidrophare and is one of the pathways of virulence factors. Enterobactin plays a role in enhancing the inflammatory response and reducing the generation of essential reactive oxygen species in intestinal epithelial cells (Saha *et al*., 2020). Reducing ROS helps bacteria form colonies and protects them from pyochelin toxicity (Adler *et al*., 2012).

Chorismate biosynthesis was shown to be increased in CRC patients compared to healthy controls. Chorismate is a common precursor of three aromatic amino acids: tryptophan, tyrosine and phenylalanine (Ashniev *et al*., 2022). Tryptophan supports the environment necessary for the formation of colorectal cancer. Yang *et al*. (2019), found in their study increased tryptophan metabolism in CRC patients. Wang *et al*. (2010) confirmed the role of Phenylalanine in CRC as it contributes to the spread and migration of cancer cells. Coker *et al*. (2022) demonstrated an increasing in tyrosine metabolism in CRC patients compared to healthy controls and demonstrated that tyrosine is associated with carcinogenesis.

The superpathway of glycolysis, pyruvate dehydrogenase, TCA, and glyoxylate bypass was increased in CRC patients compared to healthy controls. This finding is consistent with what Khattab et al. found in a study conducted on Egyptian CRC patients (Khattab *et al*., 2023), it was found that increased glycolysis helps in the development of CRC (Wang *et al*., 2019). This is also supported by the decrease in bacteria that produce short-chain fatty acids such as Roseburia.

Fucose degradation increased in CRC compared to healthy controls. Fucose improved colitis and downregulated pro-inflammatory cytokines (He *et al*., 2019;

Sitkin *et al*., 2021), fucose is useful in intestinal inflammation and participates in a range of interactions between the host and the microbiome such as the production of short-chain fatty acids such as butyrate and propionate (Sitkin *et al*., 2021).

5.1 Conclusion:

1- It is noted the observed differences in alpha diversity, with certain indices showing a significant difference of some parameter in CRC patients compared to healthy controls, highlight notable alteration in the gut microbiota. In CRC, there was increased enrichment of *Actinomyces*, *Desulfovibrio*, *Bacteroides*, *Monoglobus*, *Akkermansia* and *Eggerthella* compared to healthy controls.

2- These change, along with the clear differences and separations of β-diversity in bacterial communities between CRC vs Healthy as well as the significant changes whether up or down in these gut microbiotas, suggested marked gut microbiome dysbiosis in those patients with CRC, the gut microbiota of CRC patients remains distinct from that healthy individual.

3- Metabolites resulting from intestinal bacteria of patients with colorectal cancer might affect cancer and its development, such as increased metabolism D-galactarate degradation I, D-glucarate degradation I, enterobactin biosynthesis, fucose degradation and superpathway of chorismate metabolism, in CRC.

4- The limitation of this study is the relatively concerned with sample size which arises from difficulties in obtaining stool samples from both group participants in our region, particularly from CRC patients.

5.2. Recommendations:

1-In the future It is preferred to collect longer samples as possible to draw more clear picture, as this requires a longer study period.

2- Study other types of cancer than CRC.

3- Study the impact of microbiome on other diseases such as obesity, autism, asthma, etc.

4- Study the influence of other type of microbiome such as virobiome , archeabiome or other than bacteriobiome

5- Study the correlation between CRC progression and some types of cytokines.

6- Study the role of the most common tumorigenic bacteria detected in the study.

7- More study details about the impact of pathway which have role in up regulation and down regulation of cancer

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Appendix (1):

Republic of iraq
Ministry of Health
Misan Health Directorate
Training & Human Development Center

جمهورية العراق
وزارة الصحة
دائرة صحة ميسان
مركز التدريب والتنمية البشرية

No :
Date :

العدد: ٢٢٦٩
التاريخ : ٢٠٢٣/ ٩ / ١٠

الى/ مركز الشفاء للاورام
مركز ميسان لعلاج الاورام في الميمونة
مستشفى ميسان للطفل والولادة

م/تسهيل مهمة

يهديكم مركزنا اطيب التحايا ...

استنادا الى كتاب جامعة ميسان / كلية العلوم/ شعبة الشؤون العلمية والدراسات العليا المرقم ٣٢٣ في ٢٠٢٣/٩/١٠ م

يرجى تسهيل مهمه طالبة الماجستير السيدة (فاطمة جواد كاظم) لغرض الحصول على المعلومات والعينات الخاصة ببحثها الموسوم

(Metagenomic and functional Analysis of Microbiome in colo- Rectal cancer Patients Misan Province / southern of Iraq).

للفترة من ٢٠٢٣/٩/٢٠ ولغاية٢٠٢٤/١٢/٣١ وفق الضوابط واصوليا.

مع التقدير...

رقم الوصل ٥٧٨٩٠ التاريخ ٢٠٢٣/٩/٢٠

الدكتورة الصيدلانية
إبتسام عبود جابر
ماجستير صيدلة

الصيدلانية
انعام صدام لفته

الدكتورة الصيدلانية
انعام صدام لفته
مدير المركز
٢٠٢٣/٩/٢٠

نسخة منه الى:
- شعبة ادارة المعرفة / مع الاوليات.

Appendices (2): Questionnaire sheet

Date of completion

/ / 2023

Identification code of participant

Personal details

1.Name :

2.Sex : male female

3. Age:

4. Chronic diseases:

- hypertension
- Renal failure
- Diabetes mellitus: Type 1 Type 2

5. STAGE OF CANCER:

Stage 1 stage 2 stage 3 stage 4

6. DURATION OF DISEASE:

7. Drug (Antibiotic): the patient treatment period

3 7 10 15 30

8. type of antibiotic used :

9. THE WEIGHT:

Participant consent :

Name…….

Signature …….

Data …….

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة ميسان
كلية العلوم
قسم علوم الحياة

التحليل الجيينومي والوظيفي للمايكروبيوم المعوي لمرضى سرطان القولون والمستقيم في محافظة ميسان / جنوب العراق

رسالة مقدمة

الى مجلس كلية العلوم / جامعة ميسان

وهي جزء من متطلبات نيل درجة الماجستير علوم في علوم الحياة

من قبل

1445 هـ 2024م

الخلاصة:

سرطان القولون والمستقيم ثاني أكبر سبب للوفيات المرتبطة بالسرطان وثالث أكثر أنواع السرطان تشخيصًا على مستوى العالم. يلعب الميكروبيوم دورًا في التأثير على تطور وتقدم سرطان القولون والمستقيم، سواء بشكل إيجابي أو سلبي. في هذه الدراسة، تم جمع عينات البراز من 21 فردًا مقسمين إلى مجموعتين: 12 مصابًا بسرطان القولون والمستقيم في مرحلة مبكرة و9 من الضوابط الأصحاء. تم إجراء تسلسل الجيل التالي (NGS) القائم على 16s Amplicon على Illumina NovaSeq (PE250-Seq) على العينات وأظهرت النتائج وجود اختلاف في ميكروبيوم البراز بين المجموعتين. كانت Firmicutes, Bacteroidota, Actinobacteriota , Proteobacteria و Verrucomicrobiota هي السائدة في ميكروبيوم الأمعاء لكلا المجموعتين. مثلت شعبة Firmicutes 55.68% في الافراد الاصحاء و 62.09% في مرضى سرطان القولون والمستقيم, كانت شعبة Verrucomicrobiota الأقل وفرة 0.00% في الافراد الاصحاء و 3.89% في مرضى سرطان القولون والمستقيم. احصائياً أظهرت شعبة Bacteroidota انخفاضا لدى مرضى سرطان القولون والمستقيم مقارنة بالاشخاص الاصحاء(p= 0.002) ,بينما أظهرت شعبة Verrucomicrobiota و Desulfobacterotea زيادة كبيرة لدى مرضى سرطان قولون والمستقيم مقارنة بالاشخاص الاصحاء (p= 0.004,0.01) على التوالي. على مستوى الأجناس لوحظت فروق معنوية حيث كانت البكتيريا *Actinomyces* (p=0.02) , *Desulfovibrio* (p=0.02) , *Bacteroides* *Monoglobus* (p=0.01) , *Eggerthella* (p=0.02),(p=0.03) و *Akkermansia* (p=0.005) غنية بشكل كبير في مرضى سرطان القولون والمستقيم مقارنة بالضوابط الصحية، بينما كانت البكتيريا بينما Roseburia (p=0.04)، Faecalibacterium (p=0.01)، Mitsuokella (p=0.001)، Eubacterium (p=0.01)، Lachnospiraceae_UCG_004 (p=0.01)، Lachnospira (p=0.006)، Prevotella (p=0.001)، Barnesiella (p=0.008) و sutterella (p=0.004)

أقل ثراءً في مرضى سرطان القولون والمستقيم مقارنة بالضوابط الصحية. من ناحية أخرى، يرتبط تطور سرطان القولون والمستقيم بالتمثيل الغذائي وتغيراته. تم التعرف على مجموعة من المستقلبات من خلال التحليل غير المستهدف باستخدام التحليل الإحصائي للملفات التعريفية الميتاجينومية Statistical Analysis of Metagenomic Profiles (STAMP version 2.1.3). أظهرت النتائج أن المسارات التالية تم انخفاض تنظيمها في سرطان القولون والمستقيم مقارنة بالضوابط الصحية: adenosylcobalamin salvage from nicotinamide, Calvin-Benson-Bassham cycle, fatty acid &beta, fatty acid elongation – saturated, methylerythritol phosphate pathway, Reductive acetyl coenzyme A pathway, super pathway of polyamine biosynthesis وpeptidoglycan biosynthesis

وتم ارتفاع تنظيم مسارات أخرى في سرطان القولون والمستقيم مقارنة بالضوابط الصحية: D-galactarate degradation, D-glucarate degradation, enterobactin biosynthesis, fucose

degradation, super pathway of chorismate metabolism, super pathway of glycolysis, pyruvate dehydrogenase, TCA, and glyoxylate bypass, L-arginine degradation, enterobactin و heme biosynthesis, super pathway of hexanol degradation, biosynthesis. بناءً على نتائج الميكروبيوم الموجود في عينات البراز لمرضى سرطان القولون والمستقيم ومقارنتها بعينات البراز للأشخاص الأصحاء، يمكن الاستنتاج أن ميكروبيوم الأمعاء يلعب دورًا مهمًا في التسرطن. يجب أن تركز الأبحاث المستقبلية على توضيح الآليات الأساسية لتأثير الميكروبيوم على تطور السرطان. ويمكن أن تمهد مثل هذه الدراسات الطريق لتطوير التدخلات العلاجية التي تهدف إلى استعادة توازن ميكروبيوم الأمعاء، مما قد يقلل من حدوث السرطان وتطوره