Study of the 16s rRNA gene sequences of Gut Microbiome in some Egyptian Patients with Colorectal Cancer

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Abstract

Background: Colorectal cancer (CRC) is a heterogeneous disease of the gut epithelium up to 90% of disease risk is attributed to environmental factors like food and oncogenic bacteria.

Objectives: The current study's objectives are to determine the gut microbiota that is probably linked to CRC and to evaluate the degree of variation between patients and healthy people.

Patients and methods: The gut bacterial microbiome of 40 CRC patients and 40 healthy controls were analyzed using quantitative SYBR Green real-time PCR technique targeting 16S rRNA of selected bacteria.

Results: The gut microbiome of the patients contained a higher relative abundance of *Bacteroides* as well as *Clostridium difficile (C.difficile)* and lower relative abundance of *Firmicutes , Prevotella , Ruminococcus , Bifidobacteria* and *F. prausnitzii* than controls. Shannon diversity index and Bray-Curtis dissimilarity index are statistically significant low in the CRC group compared to the control group.

Conclusion: The current study showed evidence of changes in the gut Microbiome composition of CRC patients considering them less diverse and more dysbiotic compared to the healthy controls.

Keywords : Gut microbiome ; Colorectal cancer ; Dysbiosis ; Real time PCR

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Introduction

Microbiome includes trillions of microorganisms, such as bacteria , viruses , fungi and protozoan distributed all over the human body **. (Garrett ,2015)** The microbiome resides mainly on the gastrointestinal, vaginal and respiratory tract's mucosal surfaces with different concentrations and relative abundance among individuals. **(Nasr et al., 2020)** Changes in the microbiota composition occur due to different factors such as geographical locations, lifestyle , age and genetics, leading to significant individual specific variations as a finger print. **(Thaiss et al., 2016)**

Dysbiosis of the gut microbiota can alter the host's physiology, which results in the pathogenic processes of various diseases, according to emerging research gut dysbiosis may trigger many diseases. **(Holmes et al., 2011; Tang and Hazen 2014)**

Through a variety of mechanisms, such as the initiation of a chronic inflammatory state or immunological response, alteration of stem cell dynamics, production of toxic and genotoxic compounds, and alteration of the host metabolism, the gut microbiota can contribute to the development and progression of CRC. **(Cani et al., 2016; Tsilimigras et al., 2017)** The findings verified significant changes in the gut microbiota either prior to or during the development of colorectal cancer. **(Gagnière et al., 2016)**

Since the middle of the 20th century, theories on bacteria-driven carcinogenesis in CRC have been proposed, when **McCoy and Mason 3rd (1951)** initially proposed a link between sigmoid cancer and *Enterococcus.* **Cani et al. (2016)** formulated the 'alpha-bug' hypothesis, which postulates that species like *Bacteroides fragilis* play a central pro-oncogenic, enterotoxigenic role in the development of CRC. Additionally *B. fragilis* lead to multistep development of colorectal carcinogenesis. Given the link between microbial dysbiosis and CRC, researchers are actively looking into the gut microbiome as a potential source of diagnostic and prognostic markers. **(Zackular et al., 2014; Zeller et al., 2014)**

As patients with inflammatory bowel diseases (IBD) constantly have a higher risk of developing CRC than the general population, chronic inflammation is a recognized risk factor for CRC.**(Beaugerie and Itzkowitz 2015; Nadeem et al., 2020)** Correspondingly, pro-inflammatory bacterial species are more prevalent in CRC patients. *Fusobacterium nucleatum* is the most abundant and well-reported bacterium in the faecal and mucosa CRC associated microbiota. **(Yang et al., 2019a)**

All these data highlights the potential role of gut microbiome in pathogenesis , diagnosis , prevention and treatment of CRC.

Patients and methods

Subjects

Forty patient (22 males, 18 females) diagnosed with CRC, who presented to the gastroenterology clinic of Alexandria main university hospital, were enrolled in the study. CRC patients who age less than 75 years old were included in the study. The group included forty healthy individuals (19 males, 21 females) in good mental and physical health.

Exclusion criteria include people with diabetes mellitus, inflammatory bowel disease, or hepatic diseases, as well as those with known immunodeficiencies, dietary intolerances, age greater than 75, or BMI greater than 30 kg/m2. Probiotics, antibiotics, nonsteroidal anti-inflammatory drugs, or proton pump inhibitors were not taken by any of the study participants.

Ethical approval code : 0201356

Clinical Procedures

CRC was diagnosed by colonoscopy and appropriate biopsies from the found lesion , full data is reported about the lesion including endoscopic appearance , histopathological results , site of lesion (rectal or colonic) and side of colonic lesions (right or left) , staging was done by CT assessment of the presence of distant metastasis. History taking including patient complaints , family history , and surgical history in addition to physical examination findings and routine laboratory investigations were also reported.

Sample collection, preservation, and transport

Stool samples from patients and controls were collected in sterile containers, transferred to the Main Gut Microbiome Laboratory at Alexandria University, and stored there at 80 °C for later analysis.

DNA Extraction

Using the QIAamp® Fast DNA Stool Mini Kit (Qiagen, Germany), DNA was extracted from 180–220 mg stool samples following the manufacturer's instructions. Before PCR testing, DNA extracts were kept at -80°C. The PCR procedure required two l of DNA extract.

1) **Sybr green real-time PCR:**
2) Oligonucleotide primers targeted the 16S ribosomal RNA (rRNA) gene sequences of selected genera , phyla or species constituting the gut microbiome were used (*Bacteroids, Ruminococcus, Prevotella, Clostridium difficile F. prausnitzii and A. muciniphila, Bacteroidetes, Firmicutes, Bifidobacterium, Lactobacillus, B.fragilis, Fusobacterium nucleatum and Peptostreptococcus*). The amplification of the conserved 16SrRNA sequence of all bacteria served as the denominator against which the amplification of other bacteria was evaluated, in addition to a broad-range primer. The descriptions for each primer (Invitrogen, USA) were from previously released research. (**Riggio and Lennon, 2002; Bundgaard-Nielsen et al., 2019; Ahmed et al., 2020, El-Zawawy et al., 2021)**

Amplification process was performed in real-time PCR cycler, the Rotor-Gene Q (Qiagen, Germany) using a SensiFASTTM SYBR® No-ROX PCR kit (Bioline Co., UK). Using a 20 µl reaction volume containing 4 picomole of each primer the reaction was initiated. The reaction sequence consisted of 10 minutes of initial denaturation at 95˚C, followed by 40 denaturation cycles for 30 seconds at 95 ˚C then annealing for 30 seconds at 60˚C, and extension for 30 seconds at 72˚C. To check the specificity of the amplified products melting curve analysis was performed (**Fig.1**) . Quantitation of selected bacterial DNA was expressed as relative quantitation (the cycle threshold (Ct) at which DNA for a specific target was detected relative to the cycle threshold (Ct) at which universal bacterial DNA was detected). The Rotor Gene software automatically calculates this relative quantification and displays it as a relative fold difference. **(El-Zawawy et al., 2021).**

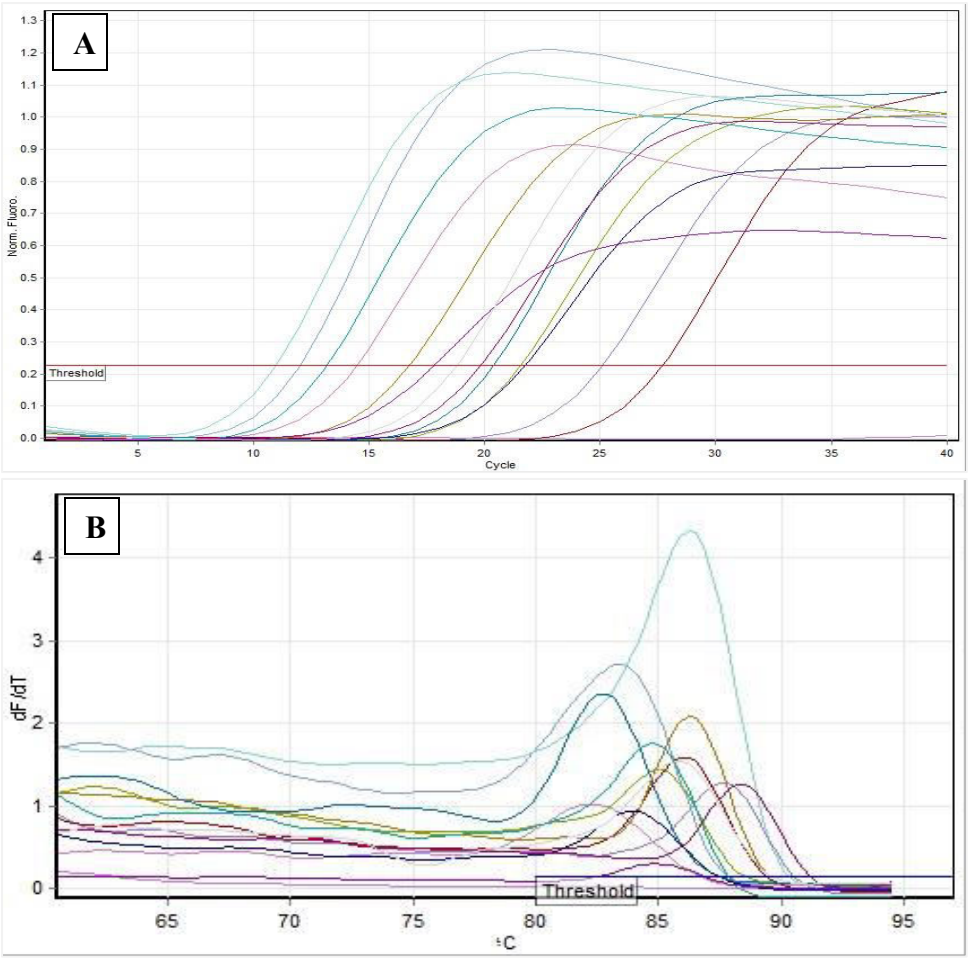

Fig. 1 .PCR amplification plot (A), and melting curve (B) of colon cancer case

Statistical analysis

Data were entered and analysed using "Statistical Package for Social Sciences" version 20 (SPSS PASW Statistics, Chicago). For quantitative variables, the terms range, mean, median, and standard deviation were employed (Normality test : Kolmogorov–Smirnov test)."Mann-Whitney" and "Kruskal Wallis" tests were applied to compare them. To compare qualitative variables, the "Chi-Square," "Fisher's Exact," and "Monte Carlo" tests were applied. To evaluate correlations between various quantitative variables, the "Spearman Correlation Coefficient" was utilised. P 0.05 is considered significant at the 5% level of significance. Using "the Shannon diversity index" (Shannon 1948), the variance of the microbial community was represented as alpha diversity, and "the Bray-Curtis similarity index" was used to measure similarity between the microbiota in the two groups. **(Bray and Curtis, 1957)**

Results

Out of the 40 CRC patients, 22 (55%) were males and 18 (45%) were females, with male to female ratio of 1.2:1. Their mean age ± SD was 50.70 ± 14.33 years, and their age ranged from 23-74 years. Their mean weight ± SD was 79.38 ± 7.39 Kg, and their weight ranged from 59-91 Kg. Their mean height ± SD was 172.3 ± 5.86 cm, and their height ranged from 161-187 cm. The BMI ranged between 21-30 kg/m2 with mean ± SD 26.74 ± 2.06.

Out of the 40 control subjects, 19 (47.5%) were males and 21 (52.5%) were females, with male to female ratio of 0.9:1. The mean age ± SD of the cases was 48.60 ± 15.38, and their age ranged from 23-75 years. . Their mean weight ± SD was 81.35 ± 14.58 Kg, and their weight ranged from 59-95 Kg. Their mean height ± SD was 168.9 ± 7.93 cm, and their height ranged from 154-185 cm . The BMI ranged between 25-34 kg/m^2 with mean ± SD 28.48 ± 4.41.

In the CRC group their mean height was statistically higher than the control group (P = 0.034) , also their mean BMI was statistically lower than the control group (P = 0.028) (**Table.1**).

Table 1. Demographic data of the study groups

Variables	CRC Cases (n = 40)		Control (n = 40)		Test of Sig.	p
	No.	%	No.	%		
Sex						
Male	22	55.0	19	47.5	$\chi2$= 0.450	0.502
Female	18	45.0	21	52.5		
Age (years)						
≤40	14	35.0	17	42.5	$\chi2$= 0.474	0.491
>40	26	65.0	23	57.5		
Min. – Max.	23.0 – 74.0		23.0 – 75.0		U= 709.50	0.383
Mean ± SD.	50.70 ± 14.33		48.60 ± 15.38			
Median (IQR)	57.0 (39.5 – 62.0)		55.50 (33.0 – 60.0)			
Weight (kg)						
Min. – Max.	59.0 – 91.0		58.0 – 142.0		U= 768.0	0.758
Mean ± SD.	79.38 ± 7.39		81.35 ± 14.58			
Median (IQR)	80.50 (76.5 – 85.0)		80.0 (72.5 – 89.0)			
Height (cm)						
Min. – Max.	161.0 – 187.0		154.0 – 185.0		t= 2.166*	**0.034***
Mean ± SD.	172.3 ± 5.86		168.9 ± 7.93			
Median (IQR)	172.5 (168.0 – 177.0)		170.5 (161.0 – 175.0)			
BMI (kg/m2)						
Min. – Max.	21.38 – 30.11		21.83 – 46.90		t= 2.257*	**0.028***
Mean ± SD.	26.74 ± 2.06		28.48 ± 4.41			
Median (IQR)	27.09 (25.6 – 28.1)		27.33 (25.9 – 30.4)			

IQR: Inter quartile range; SD: Standard deviation; t: Student t-test ; U: Mann Whitney test
p: p value for comparing between the studied groups; *: Statistically significant at p ≤ 0.05

The 40 CRC patients were categorized according to the clinical data collected, as regards: the site of the mass, side of colonic involvement, distant metastasis, histopathological examination and clinical presentation. Out of 40 CRC patients, 13 (32.5%) had rectal cancer 27 (67.5%) had colonic cancer, where 18 (66.7%) were left-side colon cancer and 9 (33.3%) were right-side colon cancer. Out of 40 CRC patients , 29 (72.5%) had no distant metastasis (M0) and 11 (27.5%) and distant metastasis (M1). Out of 40 CRC patients , 10 (25%) were well differentiated adenocarcinoma , 28 (70%) were moderately differentiated adenocarcinoma and 2 (5%) were mucinous adenocarcinoma.

Out of 40 CRC patients , 31 (77.5%) presented with abdominal pain, 14 (35%) presented with bleeding per rectum, 30 (75%) presented with alteration of bowel habits , 3 (7.5%) presented with change in stool caliber, 18 (45%) presented with iron deficiency anemia, 1 (2.5%) presented with pain during defecation and 1 (2.5%) presented with weight loss. **(Table.2**).

Table 2. Distribution of the CRC patients according to clinical data (n = 40)

Disease profile	**No.**	**%**
Site		
Colonic	27	67.5
Rectal	13	32.5
Side of Colonic (n = 27)		
Right	9	33.3
Left	18	66.7
Distant metastasis		
M0	29	72.5
M1	11	27.5
Histopathology		
Well differentiated adenocarcinoma	10	25.0
Moderately differentiated adenocarcinoma	28	70.0
Mucinous adenocarcinoma	2	5.0
Clinical presentation		
Abdominal pain	31	77.5
Bleeding per rectum	14	35.0
Alteration of bowel habits	30	75.0
Change in stool caliber	3	7.5
Iron deficiency anemia	18	45.0
Pain during defecation	1	2.5
Weight loss	1	2.5

Gut microbiome Analysis

Quantitation of specific bacteria DNA is not expressed as an absolute number but is expressed relative to the total bacteria DNA present in the stool sample. The relative abundance values of the various bacteria are shown in the following manner (4.75 x 10-5 is shown as 4.75E-05).

Phylum Level Analysis

Bacterial phylum analysis shows that patients with CRC have a statistically significant low relative abundance in *Firmicutes* ($p<0.001$). However, there is no statistically significant in relative abundance between cases and control as regards *Bacteroidetes.*

Genus Level Analysis

There is a statistically significant increase in *Bacteroides* relative abundance ($p=0.004$) and decrease in *Prevotella* ($p=0.007$) and *Ruminococcus* ($p<0.001$) relative abundance in CRC group compared to control group.

Species Level Analysis

There is a statistically significant decrease in *Bifidobacteria* ($p<0.001$) and *F. prausnitzii* ($p<0.001$) relative abundance in the CRC group compared to the control group.

Clostridium difficile (*C.difficile*) relative abundance is statistically significantly higher in CRC group compared to the control group ($p=0.003$). On the other hand, there is no statistically significant difference between CRC and control group as regard s

Lactobacilli, A. mucinophilia, Peptostreptococcus anaerobius, Fusobacteria nucleatum, Bacteroides fragilis and *Desulfovibrio* relative abundance. (**Table.3, Fig.2).**

Table 3. Comparison between CRC and control groups gut microbiome

Variables	CRC Cases (n = 40)	Control (n = 40)	U	P
Firmicutes Median IQR	2.89E-1 1.84E-1 – 3.70E-1	4.83E-1 3.06E-1 – 6.0E-1	454.50*	**0.001***
Bacteroidetes Median IQR	4.43E-1 2.47E-1 – 7.03E-1	4.18E-1 2.70E-1 – 5.54E-1	764.0	0.729
Prevotella Median IQR	6.44E-3 1.64E-3 – 4.59E-2	3.98E-2 9.44E-3 – 1.08E-1	521.50*	**0.007***
Bacteroides Median IQR	4.43E-1 1.70E-1 – 6.22E-1	1.31E-1 6.92E-2 – 2.83E-1	497.50*	**0.004***
Ruminococcus Median IQR	8.29E-3 7.70E-4 – 2.47E-2	4.19E-2 2.53E-2 – 6.19E-2	303.0*	**<0.001***
Bifidobacteria Median IQR	2.39E-3 6.32E-4 – 8.61E-3	2.08E-2 7.69E-3 – 3.50E-2	338.50*	**<0.001***
Lactobacilli Median IQR	1.94E-3 3.17E-4 – 8.04E-3	5.57E-3 7.35E-4 – 3.42E-2	629.50	0.101
A.muciniphila *Median IQR*	1.28E-3 1.40E-4 – 1.17E-2	2.73E-3 6.12E-4 – 1.50E-2	655.50	0.164
F. prausnitzii Median IQR	6.34E-2 7.64E-3 – 1.22E-1	1.10E-1 6.32E-2 – 2.60E-1	433.00*	**<0.001***
C. difficile Median IQR	0.0E+0 (8 positive cases) 0.0E+0 – 0.0E+0	0.0E+0 0.0E+0 – 0.0E+0	640.00*	**0.003***
Peptostreptococcus anaerobius Median IQR	9.88E-4 3.94E-4 – 2.96E-3	1.36E-3 5.74E-4 – 4.68E-3	703.50	0.353
Fusobacteria				

nucleatum Median IQR	1.74E-2 5.92E-3 – 3.71E-2	2.49E-2 1.78E-2 – 3.46E-2	617.00	0.078
Bacteroides fragilis Median IQR	1.14E-2 3.72E-4 – 1.10E-1	2.07E-3 1.50E-4 – 2.10E-2	612.00	0.070
Desulfovibrio Median IQR	2.19E-1 1.11E-1 – 4.12E-1	2.45E-1 1.58E-1 – 3.14E-1	756.50	0.676

IQR: Inter quartile range; SD: Standard deviation; U: Mann Whitney test ; p: p value for comparing between the studied groups; *: Statistically significant at $p \leq 0.05$

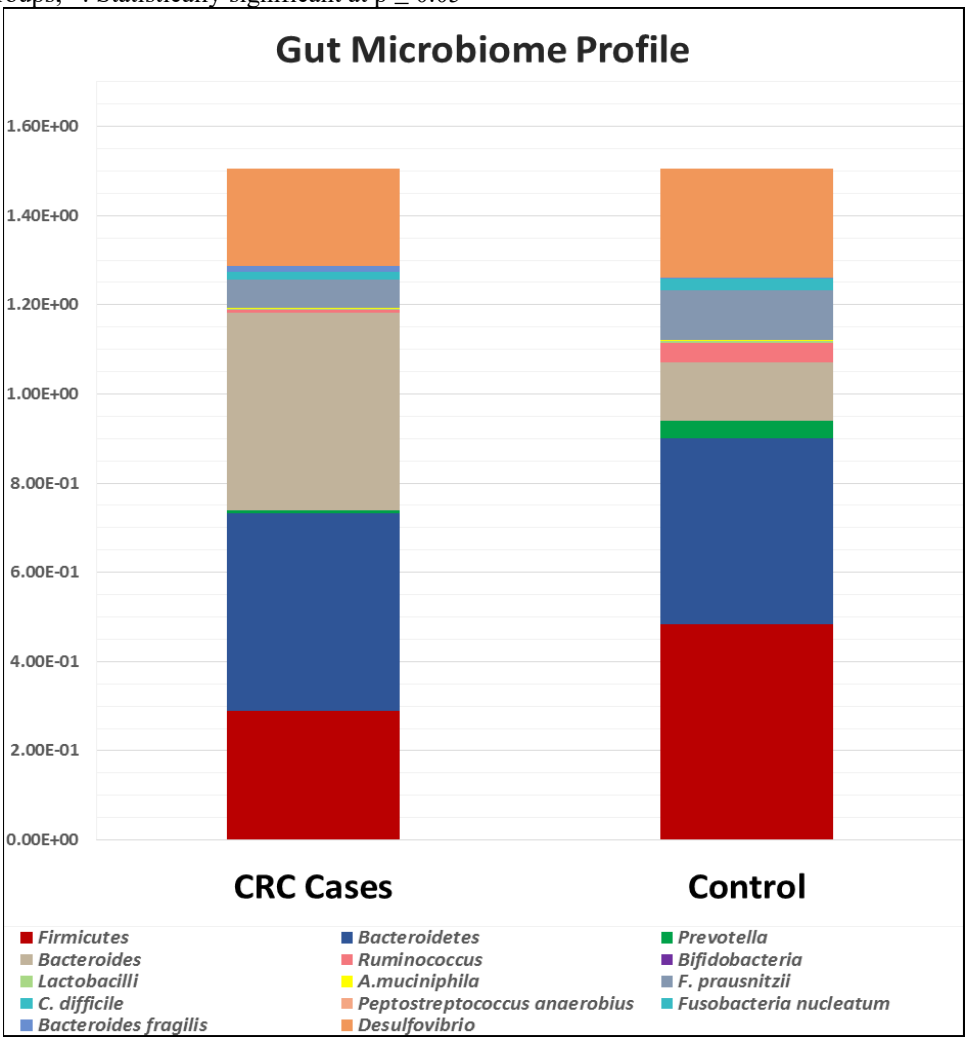

Fig.2. The gut microbiome profile of the study groups

Alpha Diversity

Shannon diversity index is statistically significantly low in the CRC group (1.55) compared to the control group (1.81). ($p<0.001$)

Bray-Curtis dissimilarity index (DSI)

The Bray-Curtis dissimilarity index was performed to study the degree of dissimilarity between the microbiota in the CRC group relative to the control group. The mean DSI was 33.80 % ranging from 8% to 82%. (**Table.4**)

Table 4. Comparison between CRC and control groups gut microbiome diversity index and dissimilarity index (%)

Variables	CRC Cases (n = 40)	Control (n = 40)	U	p
DI				
Min. – Max.	0.20 – 2.01	1.44 – 2.05	421.50*	**<0.001***
Mean ± SD.	1.55 ± 0.43	1.81 ± 0.12		
Median IQR	1.72 1.43 – 1.79	1.81 1.76 – 1.90		
DSI (%)				
Min. – Max.	8.0 – 81.93		-	-
Mean ± SD.	33.80 ± 20.08	-		
Median (IQR)	26.42 (21.31 – 37.62)			

IQR: Inter quartile range; SD: Standard deviation; U: Mann Whitney test ; p: p value for comparing between the studied groups; *: Statistically significant at p ≤ 0.05

Discussion

The risk of colorectal cancer can rise as a result of changes in the intestinal microbiome, which can initiate chronic inflammatory conditions and produce carcinogenic chemicals. Different stages of colorectal cancer are associated with particular microbiome changes. **(Shen et al., 2010 ; Kasai et al., 2016)**

In the present study, 55 % of the CRC patients were males and 45 % were females, This is in agreement with a previous study by **Sinha et al. (2016)** who studied gut microbiome in 42 CRC patients 25 (59.2%) of them were males

CRC incidence rates are 30% higher in men than in women, with rectal cancer incidence rates being 60% higher in males than in women . Women also have a lower prevalence of adenomas overall and advanced adenomas, as would be predicted.**(Ferlitsch et al., 2011; Lieberman et al., 2014)**

Differences in risk factor exposures such as cigarette smoking and sex hormone, as well as complicated interactions between these variables, are likely to be the cause of gender disparities.. **(Murphy et al., 2011)**

40 CRC patients from Alexandria Main University Hospital (AMUH) and 40 healthy controls of similar age and sex participated in the study. All individuals had their stools sampled. The identification and quantitation of particular bacterial phyla, genera, and/or species were carried out using the quantitative SYBR Green Real-Time PCR technique targeting 16S rRNA.

The current study showed dysbiosis in CRC patients; low microbial diversity, decreased *Firmicutes* (p<0.001), *Prevotella* (p=0.007), *F. prausnitzii* (p<0.001), *Cl. Difficile* (p<0.001), *Bifidobacterium* (p<0.001) ,*Ruminococcus* (p<0.001) and increased *Bacteroides* (p<0.001) relative abundance as compared to controls.

As regards *Firmicutes,* **Flemer et al. (2017)** stated that its level in CRC patients was low as compared to normal individuals.

Mori et al. (2018) revealed that *Firmicutes* was the most prevalent phylum and contributed with the lowest relative abundance in CRC samples, according to an examination of the bacteria present. *Firmicutes* were more relative abundance in both healthy subjects and patients with adenomas and hyperplastic polyps.

Regarding *Prevotella*, **Allali et al. (2018)** evaluated the microbiome composition of stool samples from CRC patients and healthy subjects using 16S rRNA amplicon sequencing, and found that Prevotella was the most substantially over represented species in normal samples compared to CRC samples. Also , **Yang et al. (2019b)** concluded that *Prevotella* was considerably lower in the CRC group than in the healthy control group in a study of the gut microbiomes of 161 healthy individuals (76 males and 85 females) and 89 CRC patients (52 males and 37 females). ($p < 0.05$).

Yang et al. (2019b) also showed statistically significant abundance of *Bacteroides* in the CRC group as compared to the control group.

As regards *Bifidobacteria*, **Saus et al. (2019)** noted that *Bifidobacteria* is low in CRC patient as compared to normal individuals

Faghfoori et al. (2021) who used flow cytometry to examine the impact of five different Bifidobacteria species' secreted metabolites on the level of anti- or pro-apoptotic gene expression , discovered that colon cancer cell survival rates were significantly lower in all studied Bifidobacteria than in control groups when exposed to cell-free supernatants. Apoptosis is promoted by Bifidobacteria secreted metabolites, according to studies from flow cytometry and RT-PCR. Bifidobacteria species may prevent CRC by up- and down-regulating anti- and pro-apoptotic genes, respectively

Regarding *F. prausnitzii,* **He et al. (2021)** used 16SrDNA sequencing to identify the microbial diversity and communities in the faeces of healthy and cancer patients, the results revealed that the presence of *F. prausnitzii* is considerably lower in the cancer group than it is in the normal group. ($P< 0.05$).

Fukugaiti et al. (2015) examined the gut microbiome of CRC patients who were in the Sao Paulo state cancer institute. The results showed significantly higher levels of F. nucleatum and C. difficile in the cancer group patients compared to healthy controls, suggesting a potential role for these bacteria in colon carcinogenesis and promising role in CRC screening. Although all of the tested bacteria were detected in the majority of the faecal samples, quantitative differences between the cancer group and healthy controls were only detected for F. nucleatum and C. difficile. The current study found that the CRC group (8 positive cases) had a statistically significant higher relative abundance of *C. difficile* than the control group.

Despite its strong relation to CRC , the present study showed insignificant results as regards *F. nucleatum* this may be due to different sample size between different studies , different dietary habits and different stages of CRC cases included in the present study. In addition , **Tahara et al. (2014)** found *F. nucleatum* enrichment is associated with specific molecular subsets of CRCs including TP53 wild type, CIMP positivity, MSI ,CHD7/8 mutation positivity and hMLH1 methylation positivity,. Many studies stated that *F. nucleatum* may be associated with inflammatory bowel diseases (IBD) which is a risk factors for CRC. **(Strauss et al., 2011)**

The complexity of the microbiome poses many difficulties, as it is impossible to identify a single bacterium as a universal microbial marker due to the high population-specific variation in the composition of the intestinal microbiota caused by sex, age, diet, drug use, genetic background, and geographic location. So, .In assessing dysbiosis, dealing with gut bacteria as ratios or indices may be more effective. **(Cheng and Ling, 2020)**

Diversity and evenness were studied between CRC and control groups as alpha diversity by the shannon diversity index which is statistically significantly lower in the CRC group indicating less diverse , more dysbiotic gut microbiome in CRC patients. **Gao et al. (2015)** and **Wang et al. (2021)** reported similar results.

As regards the CRC group in the current study, Dissimilarity index is 26.42% , reflecting the degree of difference compared to control group , **Cronin et al. (2022)** performed a genus-level calculation of the Bray-Curtis dissimilarity index, which demonstrated statistically significant microbiome separation between the several examined groups. (patients with newly diagnosed CRC, non-CRC, patients after resection for CRC and patients with polyps) , highlighting the importance of DSI in the detection of microbiome changes in early stages of CRC and early after surgical intervention.

Conclusions

The current study showed evidence of changes in the gut microbiome of CRC patients compared to healthy controls. The findings of the present study could potentially guide implementation of gut microbiome in prevention, early detection, diagnosis, post-surgical assessment and selection of the most suitable treatment protocol. Different methods can detect gut microbiome diversity and dissimilarity which can be used as an early marker of microbiota changes and for follow up of microbiota modification.

List of abbreviations:

CRC : Colorectal cancer
IBD : Inflammatory bowel disease
CT : Computed tomography
BMI : Body mass index

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Author Contributions: All authors contributed to the study conception and design. All authors performed material preparation, data collection, and analysis. All authors wrote the draft of the manuscript, read, and approved the final manuscript.

Compliance with Ethical Standards

Competing interests : The authors declare that they have no competing interest.

Funding : No funding received to perform the study.

Availability of data and material: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request

Ethical Approval and consent to participate

All procedures performed in the study involving human participants were following the ethical standards of the institutional research committee (Medical Research Ethics Committee
of Alexandria Faculty of Medicine, Egypt) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

An informed written consent was obtained from each subject prior to inclusion in the study

Consent to publication: Not applicable

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