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Original article

Gut microbiota analysis in colorectal diseased patients in Menoufia University Hospitals, Egypt

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ABSTRACT

Background: Gut microbiota is a diverse group of bacteria living in digestive tract of human. Imbalance of this community (dysbiosis) was linked to several gastrointestinal diseases. Our objective was to assess the alterations in gut microbiota among patients with colorectal disorders. **Methodology:** This study enrolled 70 patients with colorectal diseases and 30 controls. All participants were subjected to total colonoscopy and biopsy taking for histopathology investigation. Stool samples were collected, homogenized and divided to four portions for aerobic, anaerobic culture and 16S rRNA PCR based sequencing analysis. **Results:** This study included 30 patients with ulcerative colitis (UC), 20 patients with colorectal adenoma (CRA) and 20 patients with colorectal carcinoma (CRC). Regarding microbiota analysis in controls, Firmicutes, Actinobacteria, Proteobacteria and Bacteroidetes represented 72.7%, 15.1%, 9.1% and 3.0% respectively. None of the potential pathogens *H. pylori* and *Pseudomonas* spp. were isolated. For UC patients, Firmicutes, Proteobacteria and Actinobacteria represented 51.4%, 32.4% and 14.3% respectively. None of *Bifidobacterium* spp. was isolated from UC patients. For CRA and CRC patients, Proteobacteria was the most frequently isolated (38.7%, 56.7%) followed by Firmicutes (29.0%, 17.8%) and then the Bacteroidetes (20.9%, 13.4%) respectively. Isolated *H. pylori* and *Pseudomonas* spp. represented (9.6% &16.4%) and (8.1% & 14.9%) from CRA and CRC patients respectively. The totally isolated Firmicutes in controls, UC, CRA and CRC patients were 24, 3.6, 1.4 and 2 times the isolated Bacteroidetes respectively. **Conclusion:** Gut microbiota differs between patients and controls. Future studies can assess modifying gut microbiota in high-risk CRC patients as a preventative intervention.

Introduction

The gut microbiota is regarded as a "super organ" that has several functions in human body including digestion, nutrients` absorption, immunity development and functioning particularly innate immunity, as well as production of anti-inflammatory responses. Additionally, it influences microbiota-gut-brain axis and maintains the intestinal epithelial barrier [1].

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The term gut microbiota refers to the microbial species that live in the gastrointestinal tract and are almost equivalent to the total number of cells in the body while gut microbiome is the collective microbiota's genomes that are 100 times more than that of the human genome [2].

Genetics, age, environment and lifestyle habits as diet, smoking and alcohol intake are factors that could cause variance in microbiota environments. Gut microbiota dysbiosis or intestinal dysbiosis refers to imbalance in microorganisms' composition and their metabolic functions in the intestines [3].

Normal intestinal microflora includes multiple major groups of bacteria, the two most dominant of which are Bacteroidetes and Firmicute. Dysbiosis has been linked to a variety of diseases as colorectal cancers, inflammatory bowel disease (Crohn's disease and ulcerative colitis), allergic disorders, obesity, Type 1 diabetes mellitus and autism [4].

As reported by GLOBOCAN global cancer statistics 2020, colorectal cancer is the second most deadly tumour and the third most prevalent malignancy in185 countries [5]. Consequently, investigating the gut microbiota of various colorectal illnesses may provide light on the microbial process underlying colorectal carcinogenesis [6]. Apart from the standard culture techniques, 16S rRNA gene amplicon sequencing could detect the diversity of microbial communities down to the genus level [7].

In this work, 16S rRNA gene amplicon sequencing and conventional culture techniques were used to shed light on the gut microbiota of patients with colorectal disorders.

Subjects and methods

Study design and study population

This study enrolled 70 patients with colorectal diseases (study group) and 30 healthy individuals (control group), recruited from Tropical Medicine and Internal Medicine Departments at Menoufia University Hospitals during the period from July 2022 to November, 2023. A full history was taken from all participants. The study was approved by the Ethical Committee of Research, Faculty of Medicine, Menoufia University (IRB approval number: 12/2023BIO12-2). Written informed consent was signed by all participants. Individuals who received antimicrobial therapy during last 6 months were excluded.

All participants were subjected to

1. Total colonoscopy and biopsy taking

Total colonoscopy with biopsy sampling using (Olympus Evis CV 100 videoscop) for histopathological examination to confirm the diagnosis.

a. Bowel preparation

Polyethylene glycol which is inert and non-absorbable solution, induced cathartic effect prior to colonoscopy. It was administered in split dosing (half the preparations taken the night before colonoscopy and the other half six hours before it) [8].

b. Sedation and patient monitoring

Midazolam was IV administered for sedation (starting dose was 0.5-2mg).

c. Biopsy taking

Colonoscopic biopsies were taken every 10 cm from 4 quadrants and from any strictures, polyp or mass lesion with surrounding flat mucosa. Samples were preserved using formalin and sent for histopathological examination and diagnosis **[9].**

Stool samples collection, processing and culturing techniques

Stool specimens were homogenized and processed as soon as possible at Research Laboratory of Medical Microbiology & Immunology Department, and Central Laboratory of Faculty of Medicine, Menoufia University. The stool samples were divided into 4 portions:

The 1st portion was transferred in cooked meat media (Oxoid® Limited, Basingstoke, UK) and was innoculated immediately on Columbia blood agar (Oxoid® Limited, Basingstoke, UK) plate supplemented with vitamin K and hemin (Sigma Aldrich, USA) at 37°C in an anaerobic chamber (Oxoid® Limited, Basingstoke, UK) with anaerogen Gas Pack and resazurine indicator strip (Oxoid® Limited, Basingstoke, Hampshire, England) then examined within 7 days using different media as described by **El Menofy et al.** [10].

The 2nd portion was cultured aerobically on MacConkey agar and eosin methylene blue (EMB) agar for aerobic Gram-negative bacteria and identified as described by MacFaddin [11]. The 3rd portion was cultivated on blood agar and nutrient agar (Lab M, UK) for aerobic Gram-positive bacteria that were identified as described by **Koneman et al.** [12].

The 4th portion was stored at -80º C for 16S rRNA sequencing analysis [10].

Bacterial 16S rRNA PCR based sequencing

Stool was thawed gently and DNA extraction was done using QIA (Qiagen GmbH) extraction kits according to the Manufacturer's instructions. PCR was performed in a thermo cycler (Biometra, Germany) using 2 primers targeting the V4–V5 regions for bacterial 16S rRNA. The 1st primer sequence was as follow: Forward 5`-AATGATACGGCGACCACCGAGATCTACAC-i5a and Reverse 5`-CAAGCAGAAGACGGCATACGAGAT-i7a where a i5 and i7 represent 8nt index sequences that allow identification of sequences originated from each pre-specified DNA sample. The 2nd primer sequence was Forward 5`-TATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3` and Reverse 5`-AGTCAGTCAGGCCCCGTCAATTCMTTTRAGT-3`. The cycle steps were as follow: initial denaturation at 98°C for 4 min, 25 cycles at 98°C for 20 s, 65°C for 20 s and 72°C for 35 s, and a final extension at 72°C for 10 min. PCR products were separated by gel electrophoresis, then purified with thermos scientific Gene JET PCR Purification (Kit K0701, K0702) [13].

The aforementioned forward and reverse PCR primers, Taq DyeDeoxy™ and ABI Prism™ terminator cycle sequencing kits (Applied Biosystems, Foster City, CA, USA) were used to sequence the purified PCR products. The nucleotide Basic Local Alignment Search Tool (BLAST) was used to assess the sequencing data and identify the closest relatives on the NCBI website (http://www.ncbi.nlm.nih.gov) [13].

Fecal occult blood testing

Was performed at the local laboratory of the Menoufia University Hospitals and results were taken from patient records.

Statistical analysis

SPSS (Social Science Software Statistical Package), version 22, was used. Descriptive statistics included number, percentage, mean, SD, and range. Analytical statistics included Student's t test for comparison of two groups with quantitative variables, chi squared test for comparison of qualitative data while Z test was used to compare each two percentages. *P* value ≤0.05 was considered to be significant.

Results

This study included 30 patients with ulcerative colitis (UC), 20 patients with colorectal adenoma (CRA) and 20 patients with colorectal carcinoma (CRC) according to the histopathology investigation. In comparison to control group: UC, CRA and CRC were significantly (p <0.05) higher among patients with age group >51 year; UC was significantly (p <0.05) higher among patients with high socioeconomic level; the majority of CRA, and CRC patients were smokers (p <0.05); alcohol consumption, low fibres diet, positive family history of colorectal diseases were significant (p <0.05) risk factors for UC, CRA and CRC; 43.3%, 65.0% and 50% of UC, CRA and CRC patients respectively had BMI >30. Positive faecal occult blood testing was significantly (p <0.05) more frequent among UC, CRA and CRC patients in relation to the control group as shown in **table (1)**.

The 30 UC patients included in the study are classified according to the location of the inflammation into 10 patients with Proctitis, 8 with left sided colon involvement and 12 with pancolitis. Regarding microbiota analysis for UC patients in relation to the healthy controls: total Proteobacteria were more predominant in all types of UC with statistically significant difference (p =0.01); *Escherichia coli* was significantly more predominant in proctitis patients; *Shigella dysenteriae* and *Klebsiella pneumonia* were higher in all cases of UC but with non-statistically significant difference (p > 0.05); there was no statistical significant difference between UC patients and controls regarding distribution of *Helicobacter pylori* and *Acinetobacter* spp. Except for coagulase negative *Staphylococci* and *Lactobacillus* spp. which were significantly (p< 0.05) more dominant in the control group, other isolated Firmicutes species were nearly similar between UC groups and control group (p > 0.05). The isolated Bacteroidetes species are more predominant in all UC classes than controls with no statistically significant difference except in the left sided UC patients (p =0.04). The totally isolated Firmicutes in UC patients are 3.6 times the isolated Bacteroidetes while in the control group, they represent 24 times the isolated Bacteroidetes. None of *Bifidobacterium* spp. was isolated from UC patients while single *Fusobacterium* spp. was isolated from a case of pancolitis as provided in **table (2)**.

Gut microbiota analysis of adenomas and CRC patients revealed that Proteobacteria was the most frequently isolated (38.7%, 56.7%) followed by Firmicutes (29.0%, 17.8%) and then the Bacteroidetes (20.9%, 13.4%) respectively. In healthy controls, Firmicutes have the upper hand by 72.7% followed by Actinobacteria (15.1%), Proteobacteria (9.1%) and then Bacteroidetes (3.0%). None of the potential pathogens *Helicobacter pylori* and *Pseudomonas* spp. were isolated from healthy controls compared to (9.6% &16.4%) and (8.1% & 14.9%) from adenomas and CRC patients respectively. Also, *Shigella dysenteriae* and *Klebsiella pneumonia* are more abundant in adenomas (6.4% & 3.2%) and CRC patients (10.4% for both) than controls (3.0%& 0.0%) with non-statistically significant difference (p>0.05). Coagulase negative *Staphylococci* were not isolated from any of adenomas or CRC patients compared to (15.1%) from controls (p <0.05). *Clostridium* spp. isolates are more dominant in adenomas patients (8.1%) than controls (6.1%) and carcinomas (0.0%). In our study the totally isolated Bacteroidetes are more abundant in adenomas (20.9%) and CRC patients (13.4%) compared to controls (3.0%) with only statistically significant difference between adenomas patients and controls (p = 0.04). Regarding isolated Actinobacteria, the *Bifidobacterium* spp. were significantly higher among healthy controls (15.1%) compared to adenomas (1.6%) and CRC patients (0.0%). The *Fusobacterium* spp. was not isolated from controls compared to 9.6% from adenomas and 10.4% from CRC patients with non-statistically significant difference as shown in **table (3)**.

Our results showed that 97.9% of cases with identified microbiota isolates by conventional culture method had positive gene by PCR. Also, the PCR detected the gene of 100% of the samples that gave unidentified isolates by the conventional method with fair agreement (Kappa = 0.37).

In the assessment of PCR validity, its sensitivity was 97.9% but specificity couldn't be calculated as all participants showed positive isolates in 100% of stool specimens by conventional culture method from which 96% was identified and 4% was not identified. There were no negative culture results of any stool specimen especially we selected patient who didn't take any antibiotics during last 6 months as shown in **table (4).**

The PCR based 16S rRNA sequencing, the confirmatory test for microbiota detection and identification was done for stool samples of 30 colorectal diseased patients (UC, CRC, and adenoma) who chosen randomly by stratified random sampling technique to ensure sharing of cases from different stages of the studied colorectal diseased groups including the 4 cases that gave 6 unidentified isolates by the conventional culture methods. The selected 30 cases showed positive gene detection by PCR. The PCR based sequencing showed nearly similar species that detected by the conventional culture testing with detection of the strain subspecies and those isolates that were not identified by the culture technique. The *Helicobacter pylori* was the mostly abundant Proteobacteria in UC (13.9%) and CRC (10.7%) patients. Regarding Firmicutes, *Streptococcus gallolyticus* was more abundant in adenomas (13.6%) and carcinomas (16.1%) followed by *Peptostreptococcus stomatis* in adenomas (11.4%) and carcinomas (12.5%). *Fusobacterium nucleatum* was detected in 5.6%, 11.4% and 12.5% of UC, adenomas and CRC patients respectively. The percentage of *Bacteroides fragilis* and *Prevotella copri* in UC, adenomas and carcinomas patients were as follow (11.1% for both) (9.1%, 6.8%) and (10.7%, 7.1%) respectively as shown in **figure (1, 2, & 3**).

Table 1. Demographic data and risk factors among the studied colorectal diseased groups in relation to control group.

Demographic data & risk factors	**Ulcerative colitis (N=30)%**	**Colorectal Adenoma (N=20)%**	**Colorectal carcinoma (N=20)%**	**Total (N)%**	**Healthy control group (N=30)%**	**P value**
Sex						0.54[1]
Male	13 (43.3)	14 (70.0)	7 (35.0)	(34)48.6	12 (40.0)	**0.04[2]**
female	17(56.7)	6 (30.0)	13 (65.0)	(36)51.4	18 (60.0)	0.72[3]

Age						
25-35	3(10.0)	3 (15.0)	1 (5.0)	12 (17.2)	6 (20.0)	**0.01[1]**
36-50	10 (33.3)	6 (30.0)	6 (30.0)	29 (41.4)	18 (60.0)	**0.03[2]**
>51	17 (56.7)	11 (55.0)	13 (65.0)	29 (41.4)	6 (20.0)	**0.005[3]**
Age						**0.001[1]**
Mean ± SD	41.53±7.41	38.88±9.13	48.22±11.28	45.81±8.55	33.01±9.56	**0.03[2]**
Range	25 – 61	26 – 63	30 - 66	25 – 63	28 – 68	**<0.001[3]**
Residence						0.61[1]
Rural	14 (46.7)	7 (35.0)	8 (40.0)	26 (37.2)	16 (53.4)	0.20[2]
urban	16 (53.3)	13 (65.0)	12 (60.0)	44 (62.8)	14 (46.6)	0.36[3]
Socioeconomic standard						
Low	13 (43.3)	8 (40.0)	7 (35.0)	25 (35.7)	10 (33.3)	**0.008[1]**
Moderate	2 (6.7)	5 (25.0)	3 (15.0)	20 (28.6)	12 (40.0)	0.54[2]
High	15 (50.0)	7 (35.0)	10 (50.0)	25 (35.7)	8 (26.7)	0.11[3]
Smoking						
Non smoker	17 (56.7)	3 (15.0)	3 (15.0)	13 (18.6)	15 (50.0)	0.69[1]
Smoker	6 (20.0)	12 (60.0)	11 (55.0)	40 (57.1)	5 (16.6)	**0.004[2]**
Ex- smoker	7 (23.3)	5 (25.0)	6 (30.0)	17 (24.3)	10 (33.4)	**0.008[3]**
Alcohol consumption						
+ve	5 (16.7)	8 (40.0)	8 (40.0)	17 (24.3)	0 (0.0)	**0.02[1]** **<0.001[2]**
-ve	25 (83.3)	12 (60.0)	12 (60.0)	53 (75.7)	30 (100)	**<0.001[3]**
Diet type						**0.001[1]**
Fiber rich	7 (23.3)	4 (20.0)	5 (25.0)	16 (22.8)	20 (66.7)	**0.001[2]**
Fiber non rich	23 (76.7)	16 (80.0)	15 (75.0)	54 (77.2)	10 (33.3)	**0.004[3]**
BMI: kg/m^2						0.06[1]
18.5-24.9	5 (16.7)	2 (10.0)	8 (40.0)	12 (17.2)	10 (33.4)	**0.002[2]**
25- 29.9	12 (40.0)	5 (25.0)	2 (10.0)	18 (25.7)	15 (50.0)	**0.006[3]**
>30	13 (43.3)	13 (65.0)	10 (50.0)	40 (57.1)	5 (16.6)	
Family history						**0.007[1]**
+ve	12 (40.0)	13 (65.0)	13 (65.0)	41(58.6)	3 (10.0)	**<0.001[2]**
-ve	18 (60.0)	7 (35.0)	7 (35.0)	29 (41.4)	27 (90.0)	**<0.001[3]**
FOBT:						**<0.001[1]**
+ve	16 (53.3)	3 (15.0)	18 (90.0)	27 (38.6)	0 (0.0)	**0.03[2]**
-ve	14 (46.7)	17 (85.0)	2 (10.0)	43 (61.4)	30 (100)	**<0.001[3]**

FOBT: faecal occult blood test **the used test is Chi squared test (X^2)**

1. Comparing ulcerative colitis and healthy control
2. Comparing colorectal Adenoma and healthy control
3. Comparing colorectal carcinoma and healthy control

Table2. Analysis of gut microbiota isolated from different stages of ulcerative colitis patients in relation to healthy controls.

Phylum	Bacterial species	Ulcerative proctitis (N=10)%	Left sided UC (N=8)%	Pancolitis (N=12)%	Total (N=30)%	Control (N=30) %	(P value)
Aerobic bacteria							
Proteobacteria	**Enterobacteriaceae**						
	E. coli	4 (19.04)	1 (2.85)	2 (4.1)	7 (6.7)	2 (6.1)	**(0.01)** [1], (0.77)[2] (0.54)[3], (0.20)[4]
	S. dysenteriae	4 (19.04)	2 (5.7)	5 (10.2)	11 (10.5)	1 (3.0)	
	K. pneumonia	1 (4.75)	2 (5.7)	1(2.05)	4 (3.8)	0 (0.0)	
	Total	9(42.8)	5(14.3)	8(16.3)	22 (20.9)	3 (9.1)	
	H. pylori	0 (0.0)	5 (14.3)	6 (12.2)	11(10.5)	0 (0.0)	------[1], (0.07) [2], (0.39) [3], (0.11)[4]
	***Acinetobacter* spp.**	0 (0.0)	0 (0.0)	1 (2.05)	1 (0.95)	0 (0.0)	----[1], -----[2] (0.84) [3], (0.54)[4]
Total		**9 (42.8)**	**10 (28.6)**	**15 (30.0)**	**34 (32.4)**	**3 (9.1)**	**(0.01) [1], (0.08)[2], (0.04) [3], (0.01) [4]**
Firmicutes	***E. faecalis***	3 (14.3)	5 (14.3)	7 (14.3)	15 (14.3)	5 (15.1)	(0.76) [1], (0.81)[2]. (0.83) [3], (0.87)[4]
	***Bacillus* spp.**	3 (14.3)	5 (14.3)	8 (16.3)	16 (15.2)	4 (12.1)	(0.85)[1], (0.92)[2], (0.83) [3], (0.87) [4]
	Coagulase negative staphylococci (CoNS)	1 (4.7)	1 (2.85)	1 (2.05)	3 (2.8)	5 (15.1)	(0.76) [1], (0.17) [2], (0.07) [3], **(0.03)** [4]
	S. aureus.	0 (0.0)	0 (0.0)	3 (6.1)	3 (2.8)	0 (0.0)	-----[1], -----[2], (0.39) [3], (0.77) [4]
	***Streptococcus* spp.**	0 (0.0)	1 (2.85)	2 (4.1)	3 (2.8)	2 (6.1)	(0.68) [1], (0.96) [2], (0.91) [3], (0.74) [4]
Total		**7 (33.3)**	**12 (34.3)**	**21 (42.0)**	**40 (38.1)**	**16 (48.5)**	(0.42) [1], (0.34) [2], (0.78) [3], (0.39) [4]
Total aerobic bacteria		**16 (76.2)**	**22 (62.8)**	**36 (73.5)**	**74 (70.5)**	**19 (57.6)**	(0.27)[1], (0.84)[2], (0.21)[3], (0.24) [4]
Anaerobic bacteria							
Firmicutes	***Clostridium spp.***	2 (9.5)	2 (5.7)	1 (2.05)	5 (4.7)	2 (6.1)	(0.95)[1], (0.65)[2], (0.72)[3], (0.87) [4]
	***Eubacterium* spp.**	0 (0.0)	1 (2.85)	1 (2.05)	2 (1.9)	1 (3.0)	(0.82) [1], (0.50)[2], (0.65)[3], (0.77)[4]
	***Peptostreptococcus* spp.**	0 (0.0)	0 (0.0)	1 (2.05)	1 (0.95)	0 (0.0)	-----[1], ------[2], (0.84) [3], (0.54) [4]
	***Lactobacillus* spp.**	0 (0.0)	0 (0.0)	1 (2.05)	1 (0.95)	5 (15.1)	(0.16) [1], (0.05)[2], (0.07)[3], **(0.003)**[4]

	Unidentified spp.	1 (4.7)	2 (5.7)	2 (4.1)	5 (4.7)	0 (0.0)	(0.82) [1], (0.50) [2] (0.66) [3], **(0.46)** [4]
Total		**3 (14.3)**	**5 (14.3)**	**6 (12.2)**	**14 (13.3)**	**8 (24.2)**	(0.59) [1], (0.46)[2], (0.26)[3], (0.22) [4]
Bacteroidetes	***Bacteriodes* spp.**	1 (4.7)	3 (8.6)	3 (6.1)	7 (6.7)	0 (0.0)	(0.82) [1], (0.26) [2], (0.40) [3], (0.28)[4]
	***Prevotella* spp.**	1 (4.7)	5 (14.3)	2 (4.1)	8 (7.6)	1(3.0)	(0.68) [1], (0.23) [2], (0.73) [3], (0.60)[4]
Total		**2 (9.5)**	**8(22.85)**	**5 (10.2)**	**15 (14.3)**	**1 (3.0)**	(0.68) [1], **(0.04)**[2], (0.43)[3], (0.15)[4]
Actinobacteria	***Bifidobacterium* spp.**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (15.1)	(0.16)[1],(0.05)[2] **(0.02)**[3],**(<0.001)**[4]
	***Actinomycetes* spp.**	0 (0.0)	0 (0.0)	1 (2.05)	1 (0.95)	0(0.0)	-----[1], ------[2], (0.84) [3], (0.54) [4]
Total		**0 (0.0)**	**0 (0.0)**	**1 (2.05)**	**1 (0.95)**	**5 (15.1)**	(0.16)[1], (0.05) [2], (0.07) [3], **(0.003)** [4]
Fusobacteria	***Fusobacterium* spp.**	0 (0.0)	0 (0.0)	1 (2.05)	1 (0.95)	0 (0.0)	------[1], ------[2], (0.84) [3], (0.54) [4]
Total		(0) (0.0)	(0) (0.0)	1 (2.05)	1 (0.95)	0 (0.0)	------[1], ------[2], (0.84) [3], (0.54) [4]
Total anaerobic bacteria		**5 (23.8)**	**13(37.2)**	**13 (26.5)**	**31 (29.5)**	**14 (42.3)**	(0.27)[1], (0.84)[2], (0.21)[3], (0.24)[4]
Total isolated bacteria		**21 (20)**	**35(33.3)**	**49 (46.7)**	**105**	**33**	
Firmicutes/ Bacteroidetes ratio (F/B)		**10/2 (5:1)**	**17/8 (2.1:1)**	**27/5 (5.4: 1)**	**54/15 (3.6:1)**	**24/1 (24:1)**	

The test is comparing the gut microbiota isolated from UC cases in relation to the control group, the used test is Z test.
P1. Comparing Ulcerative proctitis and healthy controls P2. Comparing left sided UC and healthy controls
P3. Comparing Pancolitis and healthy controls **P4.** Comparing total cases and healthy controls

Table 3. Analysis of gut microbiota isolated from adenomas and CRC patients in relation to healthy controls.

Phylum	Bacterial species	Adenoma (N=20) %	CRC (N=20) %	Control (N=30) %	(P value)
Aerobic bacteria					
Proteobacteria	**Enterobacteriaceae**				
	E. coli	6 (9.6)	2 (2.9)	2 (6.1)	$(0.31)^1$, $(0.13)^2$, $(0.68)^3$
	S. dysenteriae	4 (6.4)	7 (10.4)	1 (3.0)	
	K. pneumonia	2 (3.2)	7 (10.4)	0 (0.0)	
	Total	12(19.3)	16 (23.8)	3 (9.1)	
	***Helicobacter* spp.**	6 (9.6)	11 (16.4)	0 (0.0)	$(0.16)^1$, **$(0.03)^2$**, $(0.90)^3$
	***Acidovorax* spp.**	1 (1.6)	1 (1.5)	0 (0.0)	$(0.75)^1$, $(0.72)^2$, $(0.51)^3$
	***Pseudomonas* spp.**	5 (8.1)	10 (14.9)	0 (0.0)	$(0.33)^1$, $(0.047)^2$, xc $(0.35)^3$
Total		**24 (38.7)**	**38 (56.7)**	**3 (9.1)**	**$(0.005)^1$, $(<0.001)^2$**, $(0.39)^3$
Firmicutes	**Coagulase negative staphylococci (CoNS)**	0 (0.0)	0 (0.0)	5 (15.1)	**$(0.007)^1$, $(0.005)^2$**, --------^{3}
	Enterococcus faecalis	4 (6.4)	3(4.5)	5 (15.1)	$(0.31)^1$, $(0.14)^2$, $(0.92)^3$
	***Bacillus* spp.**	1 (1.6)	2 (2.9)	4 (12.1)	$(0.09)^1$, $(0.17)^2$, $(0.95)^3$
	***Streptococcus* spp.**	5 (8.1)	3 (4.5)	2 (6.1)	$(0.95)^1$, $(0.88)^2$, $(0.63)^3$
Total		**10 (16.1)**	**8 (11.9)**	**16 (48.5)**	**$(0.002)^1$, $(<0.001)^2$,** $(0.66)^3$
Total aerobic		**34 (54.8)**	**46 (68.6)**	**19 (57.6)**	$(0.97)^1$, $(0.38)^2$, $(0.15)^3$
Anaerobic bacteria					
Firmicutes	***Clostridium* spp.**	5 (8.1)	0 (0.0)	2 (6.1)	$(0.87)^1$, $(0.20)^2$, $(0.06)^3$
	***Eubacterium* spp.**	0 (0.0)	1 (1.5)	1 (3.0)	$(0.75)^1$, $(0.81)^2$, $(0.97)^3$
	***Peptostreptococcus* spp.**	1 (1.6)	1 (1.5)	0 (0.0)	$(0.75)^1$, $(0.72)^2$, $(0.51)^3$
	***Lactobacillus* spp.**	2 (3.2)	1 (1.5)	5 (15.1)	$(0.09)^1$, **$(0.02)^2$**, $(0.95)^3$
	Unidentified spp.	0 (0.0)	1 (1.5)	0 (0.0)	--------^{1}, $(0.72)^2$, $(0.97)^3$
Total		**8 (12.9)**	**4 (5.9)**	**8 (24.2)**	$(0.26)^1$, **$(0.02)^2$**, $(0.29)^3$
	***Bacteriodes* spp.**	6 (9.6)	1 (1.5)	0 (0.0)	$(0.16)^1$, $(0.72)^2$, $(0.10)^3$

Bacteroidetes	***Prevotella* spp.**	7 (11.3)	8(11.9)	1(3.0)	(0.32)[1], (0.27)[2], (0.87)[3]
Total		**13 (20.9)**	**9 (13.4)**	**1 (3.0)**	**(0.04)[1]**, (0.20)[2], (0.67)[3]
Actinobacteria	***Bifidobacterium* spp.**	1 (1.6)	0 (0.0)	5 (15.1)	**(0.03)[1], (0.005)[2],** (0.97)[3]
	***Actinomycetes* spp.**	0 (0.0)	1 (1.5)	0 (0.0)	-------[1], (0.72)[2], (0.97)[3]
Total		**1 (1.6)**	**1 (1.5)**	**5 (15.1)**	**(0.03)[1], (0.02)[2],** (0.51)[3]
Fusobacteria Total	***Fusobacterium* spp.**	**6 (9.6)**	**7 (10.4)**	**0 (0.0)**	(0.16)[1], (0.13)[2], (0.88)[3]
Total anaerobic		**28 (45.2)**	**15 (22.4)**	**14 (42.3)**	(0.97)[1], (0.07)[2], **(0.01)[3]**
Total isolated bacteria		**62 (48.1)**	**67 (51.9)**	**(33)**	
Firmicutes/ Bacteroidetes ratio (F/B)		**18/13 (1.4: 1)**	**18/9 (2:1)**	**24/1 (24:1)**	

Table 4: Agreement and correlation of PCR in relation to the conventional culture method as the gold standard for microbiota identification.

	The studied participants (N=100)			**Kappa agreement**
	Conventional Culture			
	Identified	Un identified	**Total**	
PCR				
detected gene	94 (97.9)	4 (100)	98 (98.0)	0.37
undetected gene	2 (2.1)	0 (0.0)	2 (2.0)	
Total	96	4		

- Kappa < 0: No agreement
- Kappa between 0.00 and 0.20: Slight agreement
- Kappa between 0.21 and 0.40: Fair agreement
- Kappa between 0.41 and 0.60: Moderate agreement
- Kappa between 0.61 and 0.80: Substantial agreement
- Kappa between 0.81 and 1.00: Almost perfect agreement.

Figure 1. Microbiota detected by 16S rRNA sequencing of V4- V5 region from stool samples of 30 colorectal diseased patients (UC, CRA and CRC).

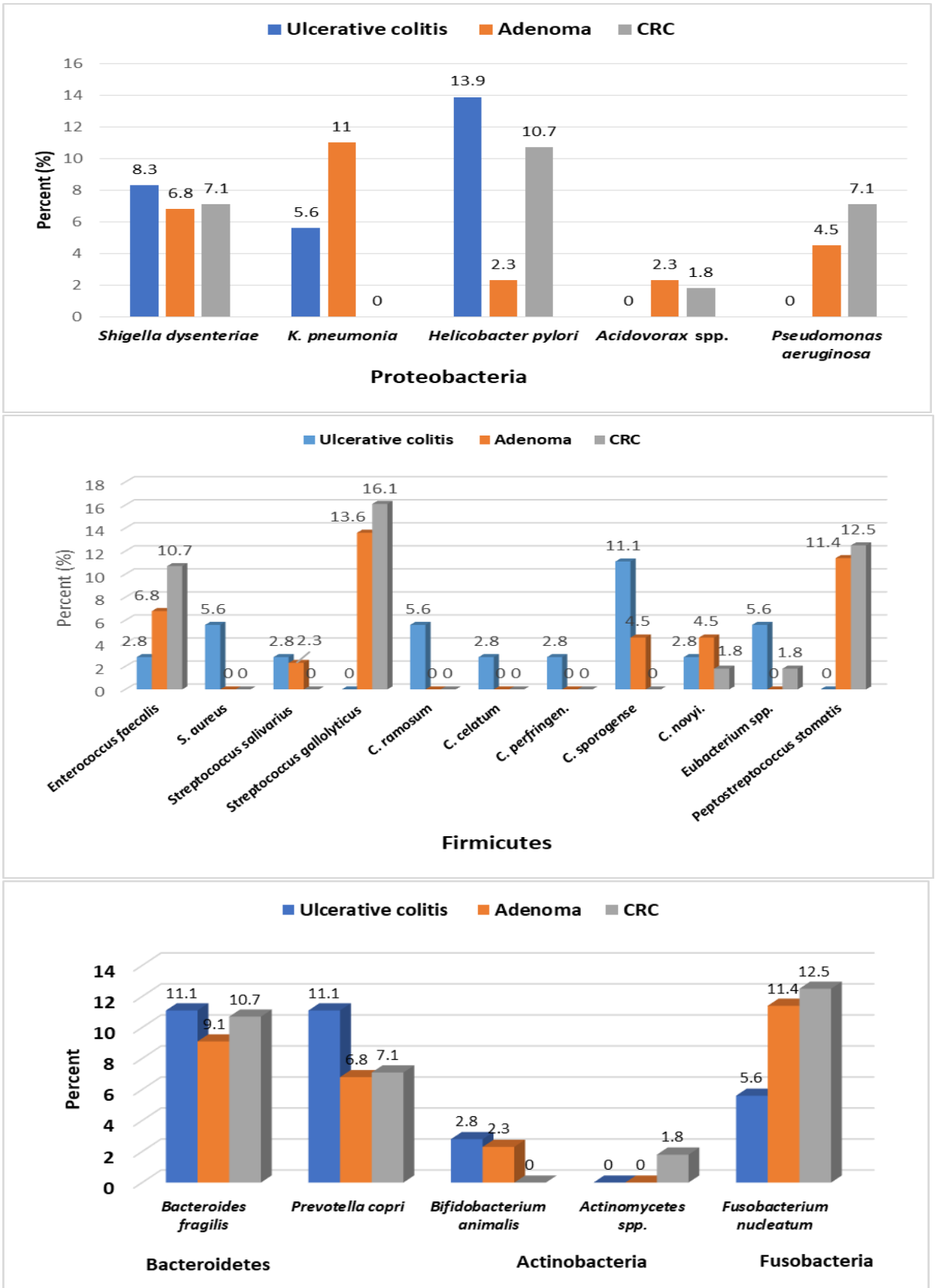
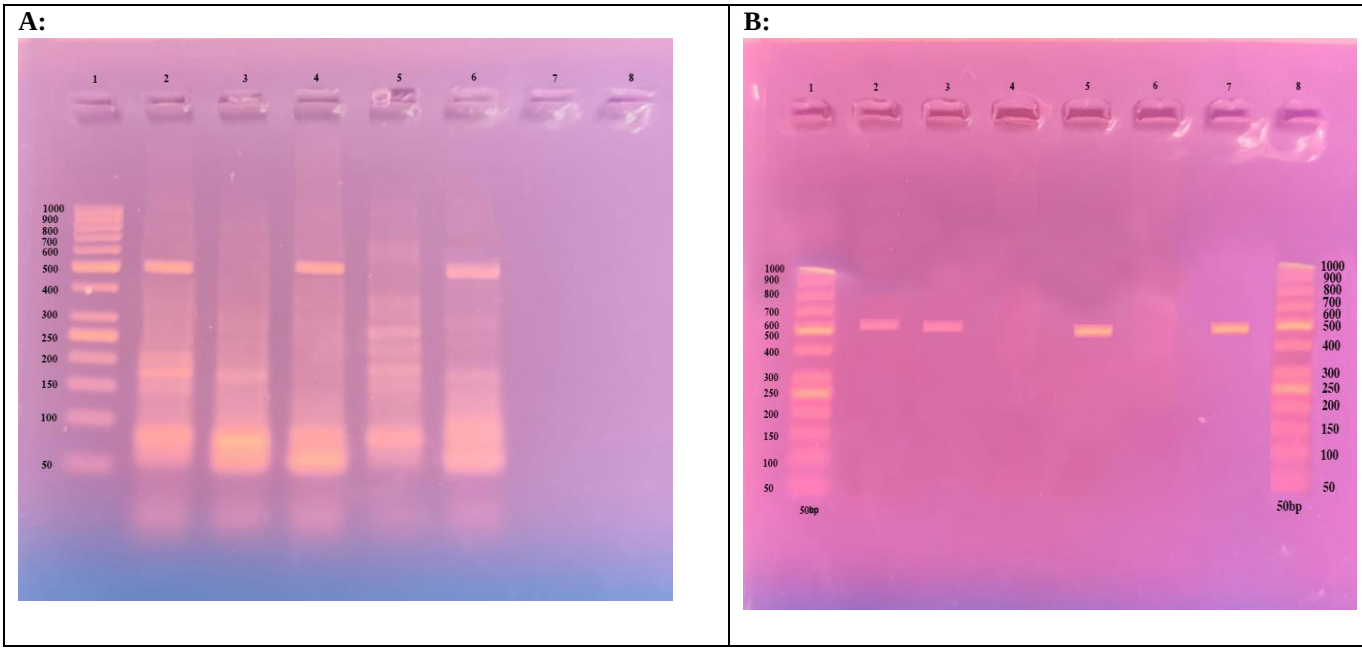


Figure 2. Agarose gel electrophoresis for the PCR amplified products of the V4–V5 regions for bacterial 16S rRNA.



A: Before purification:
Lane 1: DNA 50bp ladder (50bp – 1000 bp).
Lane 2, 4 and 6 were positive for the V4–V5 regions for bacterial 16S rRNA.
Lane 3 and 5 were negative for the V4–V5 regions for bacterial 16S rRNA.
B: After purification:
Lane 1 and 8: DNA 50bp ladder (50bp – 1000 bp).
Lane 2, 3, 5 and 7 were purified PCR amplified products.

Figure 3. Nucleotide sequence of V4-V5 region of 16S rRNA of bacteroidetes bacterium (*prevotella copri*).

Discussion

The gut microbiota plays a major role both in health and disease. External disturbances including antibiotics, infections and dietary modifications are constant threats to the gut flora. This ecosystem is able to respond to this disturbance by reaching a limited number of "stable equilibrium states" which are advantageous to the microbiota and the host. Yet some of these conditions have the potential to interfere with the symbiosis between bacterial community and the host [14].

Our study reported that old age is a significant risk factor for colorectal diseases. This findings run in accordance with **Dulal et al.** [15] and **Zackular et al.** [16], who documented that the old age is significantly associated with colorectal adenoma and carcinoma patients.

. Our study revealed that socioeconomic standard had significant effect on UC but not on CRA or CRC that may differ from **Dulal et al.** [15] who found that the socioeconomic lifestyle significantly affect the colorectal adenoma and carcinoma patients and **Sicilia et al.** [17] in Spain, who reported that the socioeconomic standards in the form of number of bathrooms and number of persons living in the house is not significant risk factor ulcerative colitis.

The current study documented most adenomas and carcinomas patients are active smokers which is not detected in UC this is in agreement with **Dulal et al.** [15], study. **Feng et al.** [18] reported that smoking is a factor for colon microbial dysbiosis that triggers CRC while **Sicilia et al.** [17] mentioned that cigarette smoking behaved as a protector factor for development of ulcerative colitis.

The present study documented significant difference between the studied patient groups and the control group regarding alcohol consumption, dietary lifestyle, BMI and family history of colorectal diseases that meet agreement with **Dulal et al.** [15], **Zackular et al.** [16] and **Feng et al.** [18] studies however, **Sicilia et al.** [17] mentioned that there was non-statistically significant difference between ulcerative colitis patients and controls regarding the family history of colorectal diseases.

Our study reported that the *Proteobacteria* are more abundant in UC patients than controls especially *Escherichia coli*. *Shigella dysenteriae*. *Klebsiella pneumonia* and *Helicobacter pylori* that is nearly coincides with **Campirei et al.** [19] who documented that the *Helicobacter* spp. and *Listeria monocytogenes* have been linked to UC. Also, inflammatory relapse in UC was triggered by *Campylobacter*, *Shigella*, *Salmonella* and *Yersinia*. In addition, Bacteroidetes were predominantly isolated from our UC patients. **Tsai et al.** [20] explained how members of the *Bacteroidetes* contribute colitis by generation of mucin degrading sulphatases. Patients with active UC have been shown to have elevated amounts of bacterial mucin desulphating sulphatases. **Pisani et al.** [14] stated in their study that *Firmicutes* and *Actinobacteria* were more abundant in UC patients compared to healthy controls that disagreed with our study where both were nearly similar among UC and control groups that is may be caused by the small UC patients sample as they are just one group of the studied colorectal diseased groups in relation to the healthy control group.

In general, the exact mechanisms via which the gut microbiota affects the development of adenoma and colorectal cancer are yet unknown. DNA damage, the generation of bioactive carcinogenic metabolites, and development of chronic inflammation are some possible causes [15].

In our study, the most frequently isolated bacteria from CRA and CRC patients was *Proteobacteria* followed by *Firmicutes* and then the *Bacteroidetes*. While in the healthy control group, the *Firmicutes* were mostly isolated followed by *Actinobacteria*, *Proteobacteria* and then *Bacteroidetes*. Additionally, *Shigella dysenteriae* and *Klebsiella pneumonia* as well as the potential pathogens like *Helicobacter pylori* and *Pseudomonas* spp. were more abundant in CRA and CRC patients than in controls. This is in accordance with **Dulal et al.** [15], who documented a higher proportion of Proteobacteria and lower abundance of Bacteroidetes in adenoma cases. Also in rectal biopsies of adenoma, **Sanapareddy et al.** [21] found overabundance of *Pseudomonas*, and *Helicobacter* and other genera of the phylum *Proteobacteria*. These findings suggest that changes in the gut adherent microbial community composition may play a role in the development of adenomas. Similarly, **Wu et al.** [22] documented increased potential harmful microorganisms in CRC patients. **Takakura et al.** [23], and **Sanapareddy et al.** [21] stated that the *Acidovorax* spp. an acid degrading member of phylum Proteobacteria, increases the metabolism of nitro-aromatic chemicals in the gut and causes local inflammation

through its flagellar proteins leading to increased risk of colon neoplasia and adenomas.

In the present study there was no significant difference between CRA, CRC and controls regarding the isolated *Escherichia coli*. In disagreement, **Feng et al.** [18] reported that carcinoma samples were enriched with *Escherichia coli* while **Liu et al**. [7] reported high percentages of *Escherichia coli* in adenoma and carcinoma groups. Furthermore, **Cuevas-Ramos et al.** [24] demonstrated that the activation of DNA double strand breaks is a mechanism by which Genotoxic *Escherichia coli* strains having polyketide synthase (pks) Genotoxic Island promote CRC.

Our results showed some variation from **Zackular et al.** [16] study in which the most dominant phyla among adenomas and CRC patients were *Bacteroidetes* followed by *Firmicutes*, and then *Proteobacteria*. Where adenomas patients had higher relative abundances of *Pseudomonas* with lower relative abundances of *Bacteroidetes*. And carcinomas patients had higher abundances of *Fusobacterium* and *Enterobacteriaceae* with lower relative abundances of *Bacteroidetes*.

Regarding our Firmicutes isolates, the *Streptococcus* spp. were slightly higher in CRA than CRC patients and controls that runs in accordance with **Abdulamir et al.** [25].

In this study, *E. faecalis* was slightly higher in controls (15.1%) than adenomas (6.4%) and CRC patients (4.5%) which deviates from **Huycke et al.** [26] results on experimental models, where some strains of *Enterococcus faecalis* have been linked to CRC as well as colitis- associated CRC through extracellular release of superoxide in host cells that could produce DNA damage.

Our *Clostridium* spp. isolates were more dominant in adenomas (8,1%) than controls (6.1%) and carcinomas patients (0.0%). However, **Barrasa et al.** [27] documented that a few members of the *Clostridium* cluster IX, XI, and XVIa could metabolize primary bile acids to secondary bile acids as deoxycholic acid which might induce CRC progression via interaction with host metabolism and immunity.

In our study, the *Peptostreptococcus* spp. was not isolated from controls compared to 1.6% in adenomas and 1.5% in CRC patients. **Feng et al.** [18] reported that *Peptostreptococcus* stomatis was elevated in adenoma and CRC compared with control samples.

In this study, the totally isolated *Bacteroidetes* were more abundant in adenomas (20.9%) and CRC patients (13.4%) than controls (3.0%) that meets agreement with **Feng et al.** [18]. Also, **Ding et al.** [28] found a *Prevotella*-dominated enterotype common in adenoma and CRC patients. Moreover, **Butt et al.** [29] reported that *Bacillus fragilis* percentages in adenoma and carcinoma groups were 3.96, and 0.17, respectively and some *B. fragilis* strains may act as driving species in colorectal cancer. In the study presented by **Cuevas-Ramos et al**. [24] *Enterotoxigenic Bacteroides fragilis* produced a toxin called fragilysin (*B. fragilis* toxin; BFT) which causes an increase in cell proliferation by activating the Wnt/β-catenin signaling pathway. Also **Sobhani et al.** [30] documented an increased Prevotella in fecal samples from CRC**.**

Our results revealed that the *Fusobacterium* spp. were more isolated from adenomas (9.6%) and CRC patients (10.4%) compared to zero isolates among controls. This coincides with previous studies **[16**, **18, 22, 31, 32-34].** According to **Rubinstein et al.** [35], when *F. nucleatum* binds to E-cadherin by its FadA adhesion protein, β-catenin signaling is activated, causing pro-inflammatory and pro-oncogenic pathways.

Regarding our isolated Actinobacteria, the *Bifidobacterium* spp. was commonly isolated from healthy controls than adenomas and CRC patients that agrees with **Feng et al.** [18] who reported that the gut commensals such as *Bifidobactium animalis*, decreased in faeces from adenoma or carcinoma patients.

In the present study, results of the 16S rRNA sequencing of bacterial V4-V5 region from stool samples of 30 colorectal diseased patients meet agreement with **Abdulamir et al.** [25] who found that DNA from *S. gallolyticus* is present in about 20–50% of colon tumors compared to less than 5% in the normal colon and *Prevotellaceae* and *Peptostreptococcaceae* were significantly enriched in CRC patients than healthy controls. While **Liu et al.** [7] found that *Bacteroides* and *Prevotella* were the most dominant genera in adenoma and carcinoma groups. In **Lucke et al.** [36] study, the 16S rRNA microbiome analysis did not contain sequences from bacterial potential pathogens.

Conclusion

Gut microbiota analysis shows difference between colorectal diseased patients and controls

regarding the type of isolated bacteria. More future studies are needed to assess the early gut microbiota modifications not only by analyzing the bacterial type variation but also by measuring its relative abundance in high-risk patients as an early preventative intervention and for safe and effective treatment

Declaration of conflict of interest

No conflicts of interest.

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