

Mucosa-associated Cultivable Aerobic Gut Bacterial Microbiota among Colorectal Cancer Patients Attending at the Referral Hospitals of Amhara Regional State, Ethiopia

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Abstract

Background: Colorectal cancer (CRC) is one of the top ten causes of cancer deaths in the world. Despite an increased prevalence of colorectal cancer has been documented from developing countries, there is no any report regarding gut microbiota among colorectal cancer patients in Ethiopia. Therefore, the current study evaluated cultivable aerobic gut bacterial distributions among malignant and its adjacent normal biopsies of CRC patients.

Methods: CRC patients who were under colorectal cancer resection surgery during April 2017 to February 2018 at Felege Hiwot Referral and University of Gondar Teaching Hospitals enrolled in the study. Biopsy specimens were taken from malignant and its adjacent normal-appearing tissues. Bacterial cultivation, quantification and characterization of saline washed biopsies were performed under aerobic and candle jar conditions. Differences in bacterial microbiota compositions between malignant and normal tissue biopsies were evaluated and analyzed using Microsoft excel 2010 and GraphPad Prism5 statistical software.

Results: Fifteen CRC patients were participated with a mean age of 53.8 ± 10.8 years old and majorities (73.3%) of patients were in between the age groups of 40 and 60 years old. The mean \pm SD bacterial microbiota of malignant biopsies ($3.2 \times 10^5 \pm 1.6 \times 10^5$ CFU/ml) was significantly fewer than that of adjacent normal tissue biopsies ($4.0 \times 10^5 \pm 2.2 \times 10^5$ CFU/ml). This dysbacteriosis is positively correlated with the occurrence of CRC ($p=0.019$). Proteobacteria (55.6%), Firmicutes (33.3%) and Fusobacteria (11.1%) were the most frequently isolated phyla from non-malignant biopsies while only Proteobacteria (58.8%) and Firmicutes (41.2%) from malignant ones. Family level differences were observed among phyla (Firmicutes and Proteobacteria) isolated from the study participants. For instance, the relative abundance of family Bacillaceae from malignant (26%) was lower than the normal biopsies (39%). On other hand, family Enterobacteriaceae was twice more abundant in malignant tissues (45%) than in its matched normal tissues (23%). Furthermore, the family Enterococcaceae (14%) of phylum Firmicutes was solely isolated from malignant tissue biopsies.

Conclusion: The overall microbial composition of normal and malignant tissues was considerably different among the study participants. Further studies with culture independent analysis of mucosal microbiota will provide detail pictures of microbial composition and pathogenesis of CRC in Ethiopian settings.

Background

Colorectal cancer (CRC) is the fourth most common causes of cancer deaths in the world with about 900,000 deaths annually (1) next to lung cancer (2). It accounts for approximately 10% of cancer-related mortality in western countries (3). Although a population based data is unavailable from Ethiopia, colorectal cancer is a problem of significant magnitude with unresectable tumors (4). Based on a single

cancer registry data of Addis Ababa City, the Global cancer statistics center reported 4,716 (7%) new CRC cases in 2018 (2) which makes CRC ranked at the third of cancer cases in Ethiopia.

Various non-modifiable and environmental factors are casually associated with the incidence of colorectal cancer. Age and hereditary factors are among non-modifiable factors that cannot be controlled by an individual while environmental factors including dietary change, urban residence, smoking habit, heavy alcohol consumption, and physical inactivity and obesity are considered as modifiable factors (5).

Inflammatory bowel diseases (ulcerative colitis and Crohn's disease) are keenly associated with the development of colorectal cancer in which various immune regulatory pathways have been identified in ulcerative colitis associated CRC (5)(6). Among these, chronic intestinal inflammation, defective mucosal barrier and host-microbe dynamics promote tumorigenesis of colorectal regions of gastrointestinal tract (6). Moreover, microbial-induced chronic colitis drives the progression of adenoma to invasive carcinoma (7).

In spite of microbial composition of the human intestine is obviously correlated to the health conditions, human gut microbiota have emerged as a major environmental factor that modulate the risk of colorectal cancer. Dysbiosis of gut microbiota (8)(9) is now assumed to be an underlying factor in the development of colorectal cancer. Currently, several researches are trying to associate the change in the composition of human intestinal microbiota with colorectal cancer occurrence. However, most studies might not show strong association due to different constraints including use of non-intestinal biopsy investigations and convenience of specimen (10). Mucosa-associated microbiota potentially affects CRC risk primarily through direct interaction with the host (11) and it is significantly varied organization in CRC patients and healthy individuals (12).

Granting an increase in prevalence of colorectal cancer has been documented from developing countries (2)(13), reports on gut microbiota in relation to colorectal cancer are not yet issued particularly in Ethiopia. Phenotypic, genotypic and toxin gene analyses of gut microbiota composition have not yet been done among colorectal cancer patients in the study area and in Ethiopia at large. Therefore, this study was aimed at determining the microbial distribution and characterizing cultivable aerobic gut mucosal associated bacteriobiota among cancerous and adjacent apparently normal tissues of colorectal cancer patients.

Result

Fifteen colorectal cancer patients were recruited from two referral hospitals: Felege Hiwot Referral Hospital (n=8) and University of Gondar Teaching Hospital (n=7). Nine (60%) were males with a male to female ratio of 1.5:1. The cumulative mean age \pm SD of the study participants were 53.8 ± 10.8 years with a range of 38 to 79 years old. Eleven (73.3%) of the study participants were between the age groups of 38 and 60 years of the first two quartiles while elders with ≥ 60 years old were only 4 (26.7%) (Table 1).

Table 1: Socio-demographic characteristics of study participants with colorectal cancer

		1 st Quartile	2 nd Quartile	3 rd Quartile	Total
		N(%)	N(%)	N(%)	N(%)
		4(26.7)	7(46.6)	4(26.7)	15(100)
	Mean ± SD	40.8 ± 3.6	54.6 ± 4.7	65.5 ± 9.1	53.8 ± 10.8
	Median	39.5	56	61.5	56
Age	Minimum	38	48	60	38
[in years]	Maximum	46	59	79	79
	Male N (%)	3(75)	4(57)	2(50)	9(60)
Sex	Female N (%)	1(25)	3(43)	2(50)	6(40)
	Male : Female ratio		1.5 : 1		

The overall abundance of cultivable aerobic bacteria was recovered from triplicate culture plates and compared with types of biopsies. The mean ± SD population of aerobic bacteria cultivated from normal-featuring biopsies was approximately $4.0 \times 10^5 \pm 2.2 \times 10^5$ CFU/ml while it was $3.2 \times 10^5 \pm 1.6 \times 10^5$ CFU/ml from malignant tissues. According to the Pearson r test, significant correlation was observed between a reduced bacterial microbiota (dysbacteriosis) of washed malignant tissue suspensions and the occurrence of colorectal cancer ($p=0.019$, Pearson $r=0.596$, 95% CI=0.120–0.849) (Figure 1).

As the Box-Whiskers appearance indicates, the mean bacterial population of malignant tissue biopsies was significantly different from the adjacent normal tissues biopsies at $p<0.05$ (Figure 1). The relative abundance of bacteria at family or genus level in each cancerous specimen was much smaller compared to the other equivalent normal tissue biopsies. The upper range value of bacterial abundance of malignant tissues [6.8×10^5 CFU/ml] was reduced at a minimum by 2.0×10^5 CFU/ml of washed biopsy suspension from its matched normal tissue biopsies count [8.6×10^5 CFU/ml]. Similarly, the lower range value of washed malignant tissue biopsies [1.7×10^5 CFU/ml] was also 2.0×10^4 CFU/ml fewer than its equivalent counts of adjacent normal tissues [1.9×10^5 CFU/ml] (Figure 1).

Comparing the mucosal microbiota of malignant niche to its matched adjacent normal tissues indicated varied bacterial compositions over those two groups of samples of CRC patients. Three bacterial phyla; Proteobacteria (55.6%), Firmicutes (33.3%) and Fusobacteria (11.1%) were over represented in non-malignant tissues of CRC patients (Figure 2) while only two phyla; Firmicutes (41.2%) and Proteobacteria (58.8%) were recovered from malignant biopsies of CRC patients (Figure 3). In addition, more bacterial diversity has been observed from apparently healthy tissue specimens group than its equivalent, particularly among the age groups of 55 to 65 years (Figure 2 and 3).

Though most members of phylum Fusobacteria are obligate anaerobic bacteria, a genus *Streptobacillus* (Figure 4) under a family Leptotrichiaceae (20%) with a microaerophilic nature was recovered only from the normal tissue biopsies using CO₂ enriched cultivation. Phyla Firmicutes and Proteobacteria recovered from both groups of tissues showed no difference while family level differences between biopsy groups were observed. The relative abundance of family Bacillaceae isolated from non-malignant tissue biopsies was at (39%) of the total isolated bacterial families while it was much lower proportion (26%) from malignant tissue biopsies. On the other hand, the relative abundance of family Enterobacteriaceae (45%) isolated from malignant tissue was twice higher than from the matched control biopsies (23%). Furthermore, the family Enterococcaceae (14%) was isolated only from malignant biopsies of CRC patients (Figure 4).

Discussion

The variability of microbial population of gastrointestinal tract is currently correlated to the occurrence of different disorders including colorectal cancer. Though several advanced researches have been conducted in developed countries by using genomic and metagenomic approaches to characterize microbial cells in fecal or mucosal specimens, there is no any published data related to the overall microbiota profile of mucosal or fecal specimens of CRC patients in Ethiopia. Therefore, the current study was aimed at determining the distribution of at least cultivable aerobic bacterial microbiota of cancerous and normal-featuring tissues of CRC patients.

The dysbiosis of bacterial microbiota abundance and distribution in malignant tissues from adjacent normal biopsies is currently become an indicative in the diagnosis and prognosis of CRC patients. These alterations are also demonstrated in our study by the presence of abundant bacterial microbiota in normal biopsies [$\bar{x}=4.0 \times 10^5$ CFU/ml] while much smaller bacterial population [approximately 2.0×10^5 CFU/ml less] from malignant tissue biopsies of CRC patients (Figure 1).

In this study, we found higher abundance of bacterial composition of phyla; Proteobacteria (55.6%), Firmicutes (33.3%) and Fusobacteria (11.1%) in normal biopsies of CRC patients (Figure 2). However, it is much different from a study reported by Eckburg *et al.* (14), in which 90% of bacterial composition of normal luminal microbiota belongs to the phyla; Firmicutes and Bacteriodes, the remaining minor constituents were Proteobacteria and Actinobacteria. On the other hand, a review study by Villegger and colleagues (15) presented a clear increase in both Proteobacteria and Bacteroidetes in tissues from patients with colorectal adenoma compared with tissues from healthy volunteers. Among members of phylum Fusobacteria (16), only a genus *Streptobacillus* in the family Leptotrichiaceae was isolated from normal tissues of CRC patients (Figure 2). It could be due to the alternative method we employed, candle jar for fastidious bacterial cultivation, probably supported the growth of microaerophilic bacteria. Other most fusobacterial members strictly require anaerobic environment to grow (17) and are associated greatly with cancer tissues than in normal tissues (18). Despite the genera Bacteriodes (19), Leptotrichia species (20)(21) and Fusobacteria (19)(22) were the most frequently identified and reported bacteria from

malignant tissues of colorectal cancer, our study didn't show any of the above mentioned species while we employed candle jar cultivation.

According to the author Lau *et al.* (23), *Streptobacillus hongkongensis* is a novel bacterial species that permanently found in human oropharynx and there might be more other *Streptobacillus* species probably also residing in human oropharynx. This genus might get easy access to the lumen of the colorectal regions (24).

The microbial abundance of family Bacillaceae in malignant biopsies (26%) was lower than the abundance in non-malignant tissues (39%) while the family Enterobacteriaceae, a member of phylum Proteobacteria (25) was over-represented (45%) from malignant group of tissue (Figure 4). This observation could be supported by the fact that family Enterobacteriaceae is considerably a member of the carcinogenic bacteria that constitute Lipopolysaccharides (LPS), D-Lactate and other bacterial components which positively correlated with the incidence and progression of inflammatory bowel diseases (IBD) as well as colorectal cancer (26)(27)(28).

More recently, studies (15)(29) indicated a drastic difference in the microbial composition of colitis-associated CRC patients, with an increase in the family Enterobacteriaceae compared with the mucosa of sporadic CRC patients. For instance, colibactin-producing *E. coli* were more frequently identified in microsatellite stable CRC and higher colonization by negative-colibactin *E. coli* bacteria were detected in patients with microsatellite instability CRC (15).

Our study also revealed that significant abundance of family Enterococcaceae was identified only from malignant biopsies (Figure 4). This finding supports previously reported evidences that patients with ulcerative colitis and Crohn's disease have larger members of family Enterococcaceae than healthy controls (30)(31)(32). Furthermore, genus Enterococcus is among certain bacterial species that has been identified to play a key role in the incidence and development of colorectal cancer (15)(29). In this genus, *Enterococcus faecalis* can even trigger macrophages and other immune cells to produce procarcinogenic enzymes capable of damaging target cell DNA that contribute to CRC carcinogenesis (33)(34).

The imbalance of these bacteria and their gene products (35)(36) that underlies mucosal surface of intestinal microvilli would facilitate the replication of opportunistic pathogens which might have direct contribution in the onset and progression CRC. Hence, these findings could be a base line information for future investigations focusing on potential pro-oncogenic pathogens of gastrointestinal cancers in the study area.

Strengths:

The current study used intact biopsies of both malignant and adjacent normal-appearing tissues of study participants where most faecal specimen microbiome studies might not show typical characteristics of adherent gut microbiota of the colorectal cancer patients.

Limitations:

Since our study employed a culture-based aerobic cultivation, huge segment of mucosal-associated microbiota; obligate anaerobes, fungal agents and uncultivable microbes were not addressed. Microbial distributions in relation to anatomic positions of colorectal biopsies, cancer stage, anticancer or antibiotic use, comorbid diseases and long term dietary habit were not well considered. However, with these limitations, the study will provide base line information for future development of culture independent studies of gut microbiota in the study area.

Conclusion

The findings presented in the current study suggested a relative abundance and distributions of cultivable aerobic bacterial microbiota of malignant tissues were significantly different from its adjacent normal tissue biopsies. Our study also showed that families of Enterobacteriaceae and Enterococcaceae were the most frequently recovered bacterial family from malignant tissues while detail considerations of these bacteria in the initiation and progression of colorectal cancer remains unclear. Therefore, large scale and deep metagenomic analysis of gut microbiota differences in Ethiopian population play key roles in the future development of advanced diagnostic, prognostic and therapeutic strategies of colorectal cancer patients.

Methods

Patient recruitment and mucosal biopsy

At Gastroenterology and Digestive Clinics of Felege Hiwot Referral and University of Gondar Teaching Hospitals, 15 confirmed CRC patients who underwent surgical resections of cancerous tissues were enrolled in the study during the study period of April, 2017 to February, 2018. Patients who were confirmed solely for colorectal cancer and appointed for open colorectal surgery were included in the study. These patients were instructed for bowel preparation before surgery carried out. However, patients who had been administered with antibiotics in the last two weeks prior to surgery, who exhibited either metastases or other cancers (liver, pancreatic and lung cancers) and who presented insufficient tissue biopsies were excluded from the study. The protocol was reviewed and approved by the Institutional Review Board (IRB) of College of Medicine and Health Sciences, Bahir Dar University and Amhara National Regional Health Bureau. Informed consent from each study participant was obtained and information was kept confidential. Two biopsies (with 5-7 x 5-7mm dimensions) were collected from malignant and adjacent normal-appearing tissues of the colorectal lumen of CRC patients during open resection surgery. Each biopsy specimen was aseptically collected using sterile falcon tube containing sterile normal saline and immediately processed in the bacteriology laboratory. Saline washed biopsy suspensions were used for aerobic cultivation. Biopsy specimens were preserved at 4°C where delayed analysis was unavoidable. Findings were analyzed and interpreted accordingly using statistical software.

Bacterial count and identification

All collected biopsies were intensively washed with 5 ml of normal saline. Twenty μ l suspension of each saline-washed specimen was suspended on to each three plates of meat peptone agar. MacConkey agar, a selective media, was also employed to isolate common pathogenic bacteria like *Salmonella* and *Shigella* species. Colony forming unit (CFU) count, morphological characteristics of bacterial isolates at average logarithmic growth phase and identification of bacterial species using a series of biochemical tests were aseptically performed. Sterility and performance of the prepared media were checked by parallel inoculation of locally available control strains of American Type Culture Collection: *S. aureus* (ATCC[®]-25923), *P. aeruginosa* (ATCC[®]-27853) and *E. coli* (ATCC[®]-25922).

Statistical Analysis

Statistical data analysis and plotting were performed by means of Microsoft excel 2010 and GraphPad Prism5 software. Statistically significant level was considered at $p \leq 0.05$.

List Of Abbreviations

CAC – Colitis-associated cancer

CFU – Colony forming unit

CRC – Colorectal cancer

IBD – Inflammatory bowel disease

IQR – Interquartile range

IRB – Institutional Review Board

Declarations

Ethics approval and consent to participate

Ethical clearance was obtained from Institutional Review Board (IRB) of Amhara Regional Health Bureau, Bahir Dar. Then permission letter was obtained from Felege Hiwot Referral Hospital and University of Gondar Teaching Hospital. Informed consent was obtained from study participants after explaining the objective of the study. Any study participant who was not willing to participate in the study was not forced to participate. Data obtained from the study participants were kept confidential using only codes.

Data Availability Statement

The datasets collected and analyzed in the present study are available with the first author up on request.

Competing interests

We authors declare that we have no competing interest.

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Authors' contributions

YA contributed to study conception and design, laboratory investigations, acquisition of data, data analysis and interpretation and wrote the first draft and final version of manuscript; MG and MO contributed to study participant enrolment, demonstrated the quality of biopsies, final manuscript preparation. YZ contributed to study conception and design, laboratory investigations, acquisition of data, final manuscript preparation. All authors read and approved the final manuscript.

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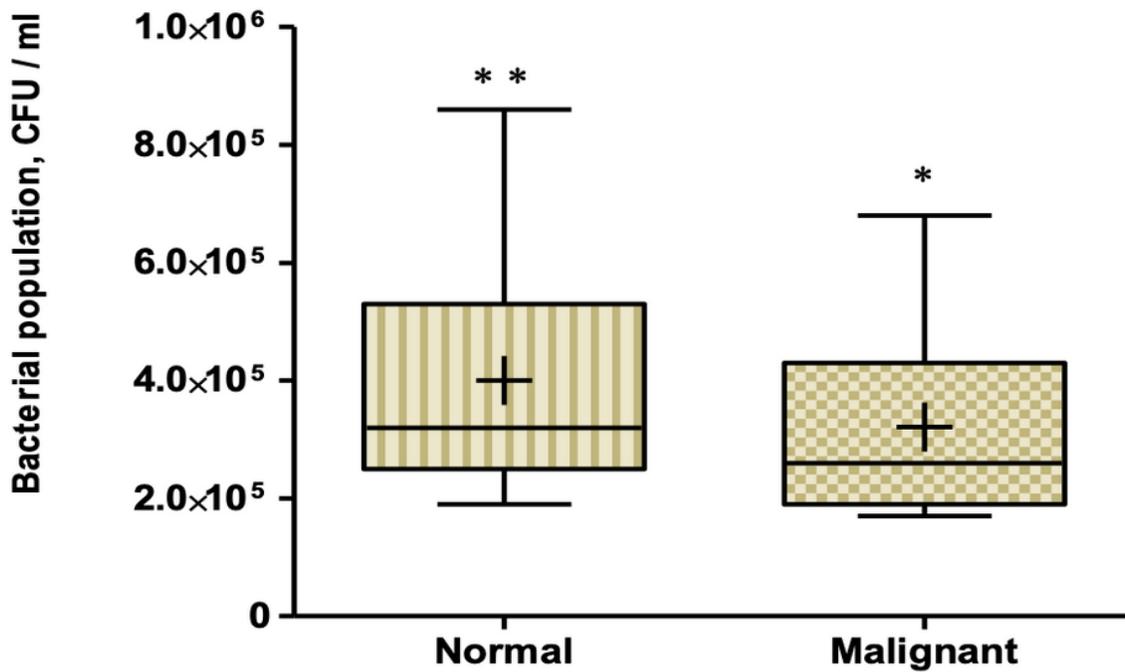
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Figures



Bacterial Population CFU/ml	Minimum	Maximum	Mean \pm SD	95% CI	P-value [Pearson r test]
Normal Biopsies	1.9×10^5	8.6×10^5	$4.0 \times 10^5 \pm 2.2 \times 10^5$	$(3.1 \times 10^5, 5.1 \times 10^5)$	0.009**
Malignant Biopsies	1.7×10^5	6.8×10^5	$3.2 \times 10^5 \pm 1.6 \times 10^5$	$(2.5 \times 10^5, 4.0 \times 10^5)$	0.019*

Figure 1

Box – Whisker plot of bacterial microbiota abundance in normal and malignant tissue biopsies of CRC patients. The plot shows median values, means (+ sign in boxes), interquartile ranges (IQR) (boxes) and 1.5 x IQR (whiskers). Bacterial population isolated from paired biopsies of CRC patients was significantly associated with the occurrence of tumor at (* $p < 0.05$) or being normal tissue at (** $p < 0.01$).

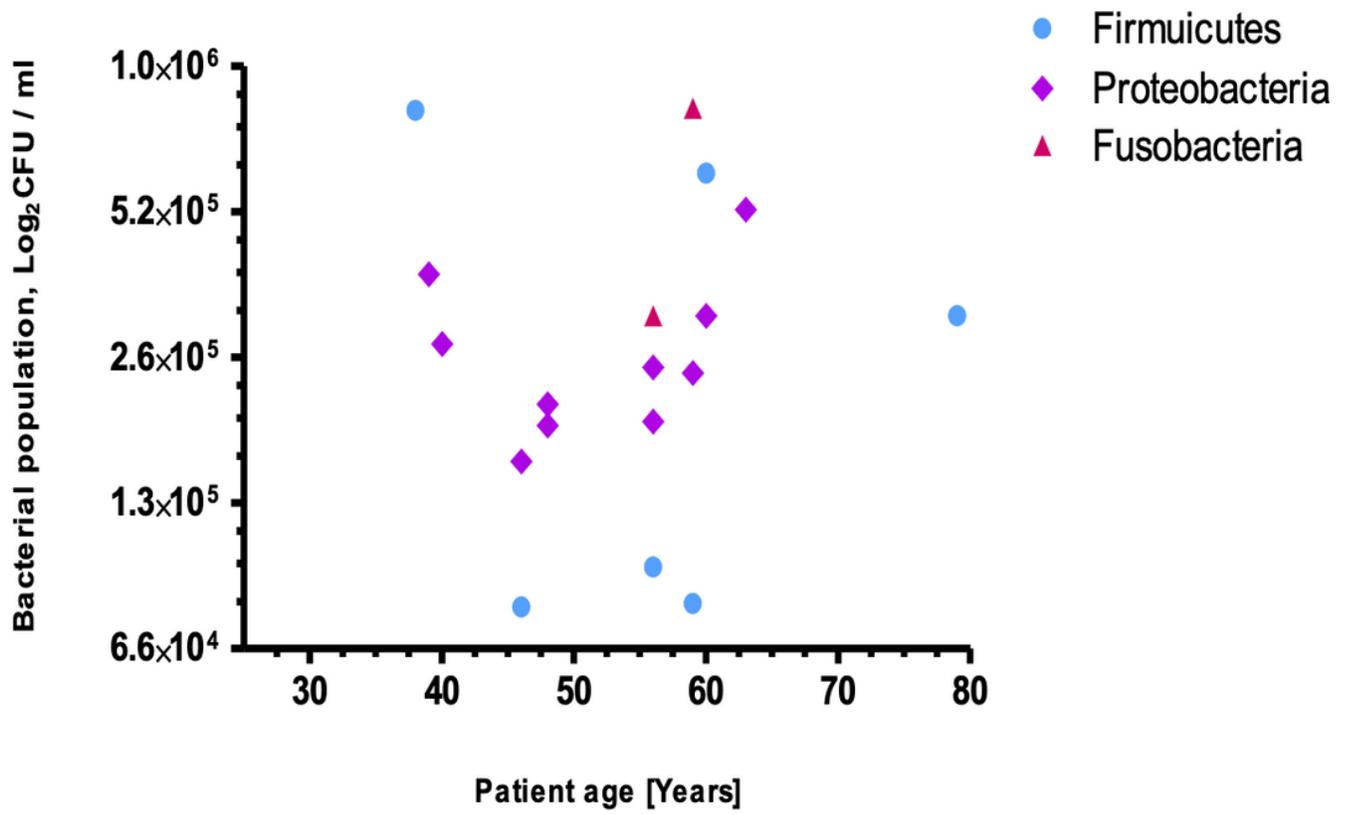


Figure 2

Age-specific bacterial phylum distribution isolated from normal tissue biopsies of CRC patients

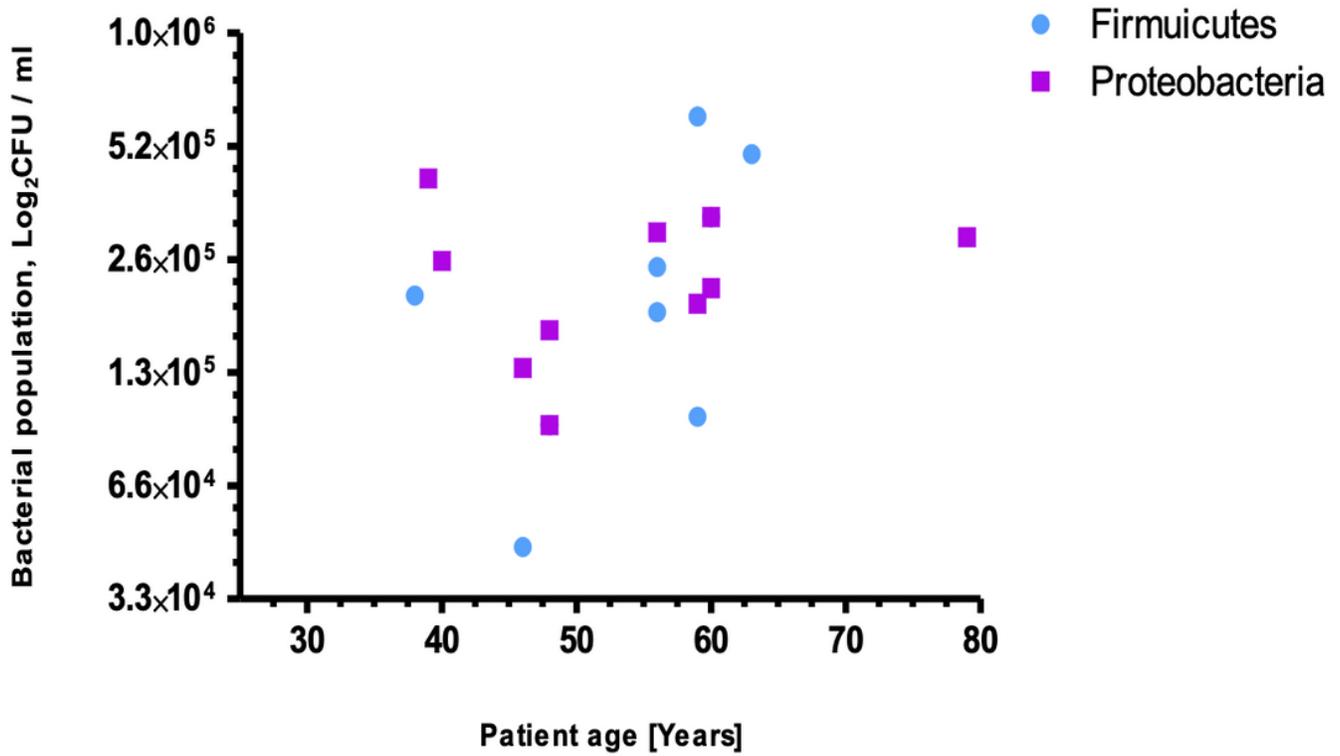


Figure 3

Age-specific bacterial phylum distribution isolated from malignant tissue of biopsies of CRC patients

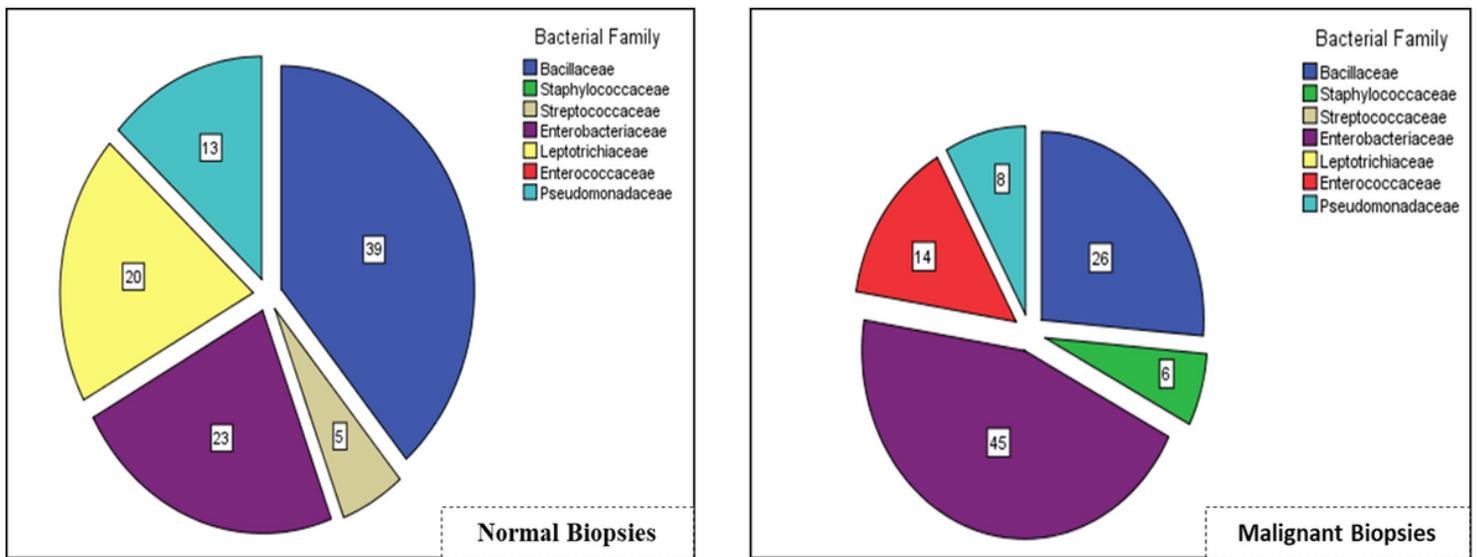


Figure 4

Bacterial family distribution in Normal and Malignant biopsies of CRC patients. Numbers are in percentage of the total family coverage.