Iranian Journal of Microbiology
Volume 18 Number 1 (February 2026) 55-66
DOI: http://doi.org/10.18502/ijm.v18i1.20907

ORIGINAL ARTICLE

Presence and copy number variations of *H. pylori*, pks$^+$ bacteria, *E. faecalis*, and *B. bifidum* in colorectal cancer: an integrated study using FFPE tissue samples

Farzaneh Korani[1,2], Nayeb Ali Rezvani[3], Noorkhoda Sadeghifard[1,2], Behrooz Sadeghi Kalani[1,2], Reza Pakzad[4], Parisa Asadollahi[1,2*]

[1]Department of Microbiology, Faculty of Medicine, Ilam University of Medical Sciences, Ilam, Iran
[2]Clinical Microbiology Research Center, Ilam University of Medical Sciences, Ilam, Iran
[3]Department of Clinical Biochemistry, Kermanshah University of Medical Sciences, Kermanshah, Iran
[4]Department of Epidemiology, Faculty of Health, Ilam University of Medical Sciences, Ilam, Iran

Received: August 2025**, Accepted:** December 2025

ABSTRACT

Background and Objectives: Colorectal cancer (CRC) is a leading malignancy with multifactorial etiology, including genetic, environmental, and microbial factors. Bacteria such as *Helicobacter pylori*, pks$^+$ bacteria, *Enterococcus faecalis*, and *Bifidobacterium bifidum* have been linked to CRC, though their roles remain controversial. Some may promote inflammation and genotoxicity, while others may confer protective effects. This study assessed the presence and relative abundance of these bacteria in colorectal FFPE tissue samples.

Materials and Methods: This case-control study included three groups of FFPE tissue samples: tumor tissues from CRC patients (Tumor, n=50), normal tissues adjacent to tumors (Adjacent, n=50), and normal tissues from non-CRC individuals (Normal, n=30). Sections were prepared with a microtome, and bacterial gene copy numbers were quantified using species-specific primers and quantitative real-time PCR, normalized to human GAPDH. Associations with age, sex, and neoplastic type were analyzed ($p < 0.05$).

Results: *B. bifidum* was significantly higher in Adjacent tissues compared to Tumor and Normal ($p < 0.0001$). *H. pylori* detection increased progressively from Normal to Adjacent to Tumor tissues ($p = 0.002$). pks$^+$ bacteria were detected only in individuals ≥60 years ($p = 0.014$). *E. faecalis* load was higher in Tumor tissues of females and older adults, though overall presence did not differ significantly among groups.

Conclusion: Enrichment of *B. bifidum* and increased *H. pylori* detection near tumors suggest the tumor microenvironment favors bacterial persistence. Age- and sex-related patterns in pks$^+$ and *E. faecalis* highlight host influences on microbial distribution in CRC, supporting further mechanistic studies.

Keywords: Colorectal neoplasms; Tissues; Polyketide synthases; *Helicobacter pylori*; *Enterococcus faecalis*; *Bifidobacterium bifidum*

*Corresponding author: Parisa Asadollahi, Ph.D, Department of Microbiology, Faculty of Medicine, Ilam University of Medical Sciences, Ilam, Iran; Clinical Microbiology Research Center, Ilam University of Medical Sciences, Ilam, Iran. Tel: +98-08432235714 Fax: +98-2227136-0843 Email: Asadolahi.p@gmail.com

Copyright © 2026 The Authors. Published by Tehran University of Medical Sciences.
This work is licensed under a Creative Commons Attribution-Non Commercial 4.0 International license (https://creativecommons.org/licenses/by-nc/4.0/). Noncommercial uses of the work are permitted, provided the original work is properly cited.

INTRODUCTION

Colorectal cancer (CRC) is one of the most commonly diagnosed malignancies worldwide and ranks as the third most prevalent cancer in many developed countries (1). Although approximately 15% of CRC cases are attributed to hereditary factors, the majority (about 85%) are linked to environmental and lifestyle risk factors such as smoking, alcohol consumption, obesity, physical inactivity, type 2 diabetes, high-fat and red meat diets, and insufficient fiber intake (2). In recent years, the role of gut microbiota has gained increasing attention as a significant contributor to CRC pathogenesis (3).

Certain bacterial species have been implicated in the initiation and progression of CRC by promoting chronic mucosal inflammation, inducing epithelial DNA damage, and disrupting host immune responses. Several studies have confirmed associations between CRC and specific bacteria, including *Streptococcus bovis*, enterotoxigenic *Bacteroides fragilis*, *Clostridium septicum*, *Fusobacterium* species, and pks$^+$ bacteria (1, 4-7).

Among these, pks$^+$ bacteria are of particular interest due to their ability to produce colibactin, a genotoxin encoded by the polyketide synthase (pks) genomic island. Colibactin interferes with the eukaryotic cell cycle, causing DNA damage, cell cycle arrest, and potentially carcinogenic mutations (1, 8, 9). Throughout this manuscript, "pks$^+$ bacteria" refers to organisms carrying the pks island, not exclusively pks$^+$ *E. coli*, as this genomic island can be found in multiple *Enterobacterales*, including *E. coli*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Citrobacter koseri* (10).

The role of other microorganisms such as *Helicobacter pylori* and *Enterococcus faecalis* in CRC development remains controversial. Although a few specific strains of *E. faecalis* have been identified for their probiotic properties, their use is limited to strain-specific preparations, and most reports highlight the organism's potential carcinogenicity (11, 12). These harmful effects are attributed to its production of extracellular superoxide and reactive oxygen species (ROS), which can promote genomic instability and damage colonic epithelial DNA. Additionally, *E. faecalis* has been shown to produce metalloproteinases that may compromise the intestinal epithelial barrier and trigger inflammation (2).

Following the well-established role of *H. pylori* in gastric cancer, research has increasingly explored its potential involvement in colorectal carcinogenesis. Two meta-analyses published in 2006 and 2008 suggested a positive association between *H. pylori* infection and increased CRC risk. Proposed mechanisms include alterations of the colonic microbiota, direct colonization of the colon causing chronic inflammation, and bacterial toxin production (13-15).

Supporting this, some studies have found significantly higher *H. pylori* copy numbers in cancerous colorectal tissues compared to normal controls, suggesting a possible microbial contribution to tumor development (15). While *H. pylori*, pks$^+$ bacteria, and *E. faecalis* are implicated as potential CRC-promoting pathogens, the evidence regarding their roles remains controversial. In contrast, certain commensal species, such as *Bifidobacterium bifidum*, are believed to exert protective effects. As a well-known member of the healthy gut microbiota, *B. bifidum* demonstrates anti-inflammatory and anticancer properties, including immune modulation and suppression of pathogenic bacteria (16). Although some studies have examined the presence of *Bifidobacterium* species within colorectal tumor tissue, the specific distribution and tissue-level behavior of these bacteria in CRC remain insufficiently characterized (17).

Iran exhibits distinct CRC incidence trends and microbial exposure patterns compared with many Western and Asian populations (18), suggesting that regional epidemiology may shape unique microbiome–CRC relationships. Despite this, data on tissue-based microbial patterns in Iranian CRC patients remain scarce. Given these gaps and the scarcity of data from Iranian populations, the present study aimed to investigate the gene copy numbers of *H. pylori*, pks$^+$ bacteria, *E. faecalis*, and *B. bifidum* in formalin-fixed paraffin-embedded (FFPE) colonic tissues from CRC patients and non-CRC controls in western Iran. This investigation also contributes region-specific data from western Iran, where dietary habits, environmental exposures, and CRC epidemiology differ from many previously studied populations, potentially shaping distinct microbial patterns.

MATERIALS AND METHODS

Retrieval of formalin-fixed paraffin-embedded (FFPE) tissue blocks and associated patient data. In accordance with previous studies (19), we analyzed

a total of 130 FFPE colorectal tissue samples. These comprised: tumor tissues from CRC patients (n=50) [Tumor], histologically normal tissues adjacent to the tumor from the same patients (n=50) [Adjacent], and normal colonic tissues from individuals without malignancy (n=30) [Normal]. This third group served as a control to strengthen the comparative analysis.

The FFPE tissue blocks were selected from the pathology archive of Imam Reza Hospital, Kermanshah. Subsequently, microscopic re-evaluation of the corresponding FFPE tissue slides was performed by a pathologist to confirm the previous diagnosis.

In a retrospective review, patients' medical records were examined to extract relevant clinical information, including colorectal cancer characteristics, and demographic data such as age and sex, which were recorded for further analysis.

This study was conducted in accordance with the Declaration of Helsinki and relevant national guidelines and regulations. The Normal FFPE blocks were obtained from individuals who had undergone colonoscopy or surgical procedures for non-malignant conditions, and whose pathology reports confirmed the absence of malignancy. These samples were anonymized archival specimens. In accordance with institutional and national regulations, the Ethics Committee approved the use of these archived materials and waived the requirement for individual informed consent. The ethical approval was obtained from the Ethics Committee of Ilam University of Medical Sciences (Approval Code: IR.MEDILAM.REC.1402.123).

Sectioning of FFPE tissue samples and DNA extraction. From each FFPE tissue block, a 3-micrometer-thick section was cut by a trained technician using a microtome. The sections were then placed into 1.5 mL microtubes for further processing.

DNA was extracted from the FFPE tissue samples using the SinaPure DNA–FFPE Tissue DNA Extraction Kit (Sinaclon, Iran), according to the manufacturer's instructions.

The quality and quantity of the extracted DNA were measured using a NanoDrop spectrophotometer, and the extracted DNA samples were stored at -80°C until further use.

Preparation of standard bacterial strain DNA. DNA of *H. pylori*, *E. coli* ATCC (as a representative of pks^+ bacteria (20-22)), and *E. faecalis* ATCC 29212 were purchased from the Research Center of Shahed University, Tehran. The concentration and purity of the extracted DNAs were subsequently re-measured using a NanoDrop spectrophotometer (ND1000, USA) to confirm their quality.

Standard curve generation using DNA from standard bacterial strains. PCR was performed using primers designed to detect diagnostic genes of the respective bacteria (Table 1). Subsequently, serial 10-fold dilutions (7 dilutions) of the amplified gene fragments were prepared for quantification (1:1, 1:10, 1:100, 1: 1,000, 1: 10,000, 1: 100,000, and 1: 1,000,000).

The qPCR reaction mixtures for all seven dilutions were prepared according to the listed volumes (Table 2). The reactions were then run, and the relative standard curve was generated using the Applied Biosystems StepOnePlus Real-Time PCR System (USA) under the conditions described (Table 3).

Note: The DNA dilution steps and standard curve generation were also performed for the human *GAPDH* gene. This allowed normalization of the bacterial copy number results against this human reference gene, accounting for possible variations in the amount of extracted DNA among samples and preventing interference in the calculations.

Quantification of bacterial copy number in FFPE tissue samples by quantitative real-time PCR (qPCR). In this study, we investigated the association between the copy numbers of *H. pylori*, pks^+ bacteria, and *E. faecalis* and CRC. Quantitative real-time PCR (qPCR) was performed on DNA from patients' FFPE tissues using the primers in Table 1, with reaction volumes and conditions identical to those used for the standard curve (Tables 2 and 3). Ultimately, the bacterial copy numbers in the FFPE tissues were calculated by substituting the obtained Ct values into the standard curve equation $Y= mX + C$, where Y is the Ct value, m is the slope of the best-fit line, X is the logarithm of the concentration/copy number, and C is the Y-intercept (the point where the best-fit line crosses the Y-axis).

Note: The copy number obtained for each bacterium was normalized to the human reference gene GAPDH to account for potential variations in DNA extraction between samples and to prevent interfer-

Table 1. The primer sequences used in this study

Gene	Bacteria detected	Primer sequence 5' to 3' (Forward/Reverse)	Product size (bp)	Tm (°C)	Reference
UreC	*Helicobacter pylori*	5'- TATCACTCCAGGTAGAAGA -3'	103	59	The Present study
		5'- CATCGTTAGAAGCGTTA -3'		59	
Colibactin B	pks+ bacteria	5'- TGACGGTATTGATGCTAT -3'	102	56.8	The Present study
		5'- TTGACTGAATAAGGTAGG -3'		56.1	
gyrB	*Enterococcus faecalis*	5'- GTTGGTTCCTCTGTTGTT -3'	92	61	The Present study
		5'- ACGACGATATTCTTGGTAGTA -3'		61.3	
16S rRNA	*Bifidobacterium bifidum*	5'-CTGGCAGCCGTGACACTACT-3'	102	62.16	(23)
		5'-TGAACTGGCCGTTACGGTCT-3'		61.47	
GAPDH	*Homo sapiens*	5'- CTCTCCCATCCCTTCTC -3'	88	60.9	The Present study
		5'- GTTGCCTGTCCTTCCTA -3'		61.4	

Table 2. The qPCR reaction mixture volumes used for the detection of targeted genes

Materials Used for qPCR (µL)	*ureC* *(Helicobacter pylori)*	*colibactinB* (pks+ bacteria)	*gyrB* *(Enterococcus faecalis)*	*16S rRNA* *(Bifidobacterium bifidum)*	*GAPDH* *(Homo sapiens)*
2xqPCR BIOSyGreen Mix (SYBRGreen High Rox)	10	10	10	10	10
Forward primer (10µM)	0.8	0.8	0.8	0.8	0.8
Reverse primer (10µM)	0.8	0.8	0.8	0.8	0.8
DNA	1	1	1	1	2
Water	7.4	7.4	7.4	7.4	6.4

Table 3. Thermal cycling conditions for qPCR

Steps	Time	Temperature (°C)	Cycles
Polymerase Activation	2 min.	95	1
Denaturation	5 sec.	95	
Annealing/Extension	20-30 sec.	57 for *H. pylori* 60 for pks + bacteria 58 for *E. faecalis* 56 for *B. bifidum* 60 for *GAPDH*	40

ence in the calculations. Hence, the qPCR data reflect relative abundance normalized to a human reference gene (*GAPDH*), and do not represent absolute bacterial load.

During the qPCR, in addition to the no-template control (NTC), negative DNA extraction controls—microtubes containing only kit buffers without tissue samples—were randomly included to monitor potential contamination from the kit reagents or environment. Furthermore, paraffin-only samples separated from the FFPE blocks (without tissue) were also tested to rule out contamination of the paraffin material with any of the three target bacterial species. Bacterial DNA was used as the positive control.

Analysis of the association between bacterial copy number, colorectal cancer, and patient demographic data using statistical methods. To investi-

gate potential associations between bacterial profiles and clinicopathological factors, initial comparisons were conducted using ANOVA, t-tests, and Chi-square analyses. These were subsequently followed by multiple linear regression (to assess bacterial load) and logistic regression (to evaluate presence/absence), offering additional insight into predictive relationships.

Ethics approval and consent to participate. This study was conducted in accordance with the Declaration of Helsinki and relevant national guidelines and regulations. Ethical approval was granted by the Ethics Committee of Ilam University of Medical Sciences (Approval Code: IR.MEDILAM.REC.1402.123).

RESULTS

FFPE tissue blocks and associated patient data. This study analyzed 130 samples: 50 tumor [Tumor] and 50 adjacent [Adjacent] tissues from CRC patients, and 30 normal [Normal] tissues from controls. Their demographic and clinical data are shown in Table 4. Histologically, 95% of CRC cases were adenocarcinomas.

Associations between bacterial markers, demographic factors, and CRC. The copy number obtained for each bacterium was normalized to the human reference gene *GAPDH*; thus, the term "copy number" refers to values normalized against *GAPDH* [i.e., log (copy number of bacteria/copy number of *GAPDH*)].

The number of positive samples, relative copy numbers, and the mean Ct values for each bacterium in normal and tumor tissues are summarized (Table 5).

The limit of detection (LOD) of the assay, determined by serial dilution of bacterial DNA, was approximately 10^2 copies for *H. pylori* and about 10 copies for the other bacterial strains. Samples with cycle threshold (Ct) values above 35 were considered unreliable and excluded from the final statistical analyses. Negative controls, including no-template controls (NTCs), consistently showed no amplification up to cycle 40; in rare instances where late signals emerged (Ct 38-40), they were interpreted as background noise attributable to non-specific amplification rather than true bacterial presence.

Helicobacter pylori. For *H. pylori*, one-way ANOVA (FIG.1a) and multiple linear regression both revealed no significant differences in bacterial load across Tumor, Adjacent, and Normal tissues ($p > 0.05$). In other words, the amount of *H. pylori* remained similar in all tissue types.

However, Chi-square analysis ($p = 0.007$; Table 5) confirmed a significant association between tissue type and *H. pylori* presence, and logistic regression ($p = 0.002$) further demonstrated a progressive increase in detection rates from Normal to Adjacent and finally to Tumor tissues (FIG.2a). This implies that although

Table 4. Baseline demographic and clinical variables among Tumor[1], Adjacent[2], and Normal[3] tissue Groups

Variables		Tumor (n=50)	Adjacent (n=50)	Normal (n=30)	P value
Cancer type	Adenocarcinoma	48 (96)	48 (96)	0 (0)	¶*<0.001
	Gastrointesinal stromal tumor	1 (2)	0 (0)	0 (0)	
	Mucinous adenocarcinoma	1 (2)	1 (4)	0 (0)	
Gender	Female	22 (44)	22 (44)	10 (33.3)	#0.582
	Male	28 (56)	28 (56)	20 (66.7)	
Age		57 ± 15.1 (32 to 85)	57 ± 15.1 (32 to 85)	64.7 ± 15.9 (27 to 88)	#0.055

[1]Tumor tissues from CRC patients

[2]Normal tissues adjacent to CRC tissues from the same patients

[3]Normal tissues from non-CRC individuals

[#]Chi-squared test

[¶] ANOVA test

[*]Significant at $p < 0.05$

Table 5. Distribution, relative copy numbers, and mean Ct values of bacteria across three tissue groups

Qualitative assessment: number (%) of patients testing positive	Tumors[1] (n=50)	Adjacent-Normal[2] (n=50)	Normal 3 (n=30)	P value
Helicobacter pylori				
Yes	39 (78)	24 (48)	10 (33.3)	[#]0.007*
No	11 (22)	26 (52)	20 (66.7)	
Pks+ Bacteria				
Yes	1 (2)	2 (4)	3 (10)	[#]0.274
No	49 (98)	48 (96)	27 (90)	
Enterococcus faecalis				
Yes	10 (20)	18 (36)	6 (20)	
No	40 (80)	32 (64)	24 (80)	[#]0.130
Bifidobacterium bifidum				
Yes	18 (36)	16 (32)	17 (56.7)	
No	32 (64)	34 (68)	13 (43.3)	[#]0.593
Mean and Range values of Ct				
Helicobacter pylori	32.89 ± 0.98 (31.49 to 35)	32 ± 1.3 (31.31 to 34.14)	33.22 ± 1.16 (32.39 to 34.02)	[¶]0.017*
Pks+ Bacteria	34.15 ± - (33.15 to 34.15)	34.24 ± 3.43 (33.81 to 35.66)	32.11 ± 3.73 (28.70 to 34.10)	[¶]0.408
Enterococcus faecalis	34.49 ± 2.19 (30.51 to 35.25)	34.28 ± 3.22 (28.29 to 34.73)	32.90 ± 0.92 (32.01 to 33.88)	[¶]0.185
Bifidobacterium bifidum	33.68 ± 2.98 (29.61 to 35)	34.78 ± 1.5 (33.85 to 34.29)	32.31 ± 2.79 (27.24 to 34.21)	[¶]<0.001*
Log (copy no. of DNA/copy no. of *GAPDH*)[4]				
Helicobacter pylori	-4.26 ± 0.3 (-5.01 to -3.65)	-4.4 ± 0.58 (-5.35 to -3.04)	-4.16 ± 0.63 (-5.11 to -2.35)	[¶]0.267
Pks+ Bacteria	-4.94 ± 0.87 (-5.56 to -4.33)	-3.9 ± 0.1 (-3.9 to -3.9)	-2.8 ± 0.44 (-3.13 to -2.3)	[¶]0.070
Enterococcus faecalis	-4.83 ± 1.08 (-6.6 to -2.89)	-4.61 ± 0.78 (-5.6 to -3.31)	-3.96 ± 0.51 (-4.74 to -3.35)	[¶]0.150
Bifidobacterium bifidum	-5.16 ± 0.71 (-5.87 to -2.24)	-4.08 ± 1.04 (-5.58 to -1.52)	-5.48 ± 0.45 (-6.14 to -4.51)	[¶]<0.001*

[1]Tumor tissues from CRC patients

[2]Normal tissues adjacent to CRC tissues from the same patients

[3]Normal tissues from non-CRC individuals

[4]The negative values indicate a lower copy no. of bacteria compared to *GAPDH*

[#]Chi-squared test

[¶] ANOVA test

*Significant at $p < 0.05$

the overall load does not vary, the likelihood of detecting *H. pylori* increases with proximity to tumor tissue.

Subgroup analysis further revealed that in females, *H. pylori* load was significantly lower in Tumor tissue compared to Normal ($p = 0.03$), and a borderline higher load was found in Normal tissue of females compared to males ($p \approx 0.05$). Gender distribution of positive cases for each bacterial species is summarized in Table 6.

Pks+ bacteria. For pks^+ bacteria, no significant differences in bacterial load were observed across the three tissue groups, as shown by ANOVA and t-tests (Fig. 1b), and further supported by multiple linear

Table 6. Gender distribution of positive cases for each bacterial species

Bacteria	Number of Males positive (%)	Number of Females positive (%)	P value[1]	χ^2
Helicobacter pylori	32 (54.2)	27 (45.8)	0.133	2.26
pks+ Bacteria	4 (80.0)	1 (20.0)	0.637	0.22
Enterococcus faecalis	13 (54.2)	11 (45.8)	0.654	0.2
Bifidobacterium bifidum	33 (67.3)	16 (32.7)	0.146	2.11

[1] P values are based on Chi-square tests for gender distribution among positive cases.

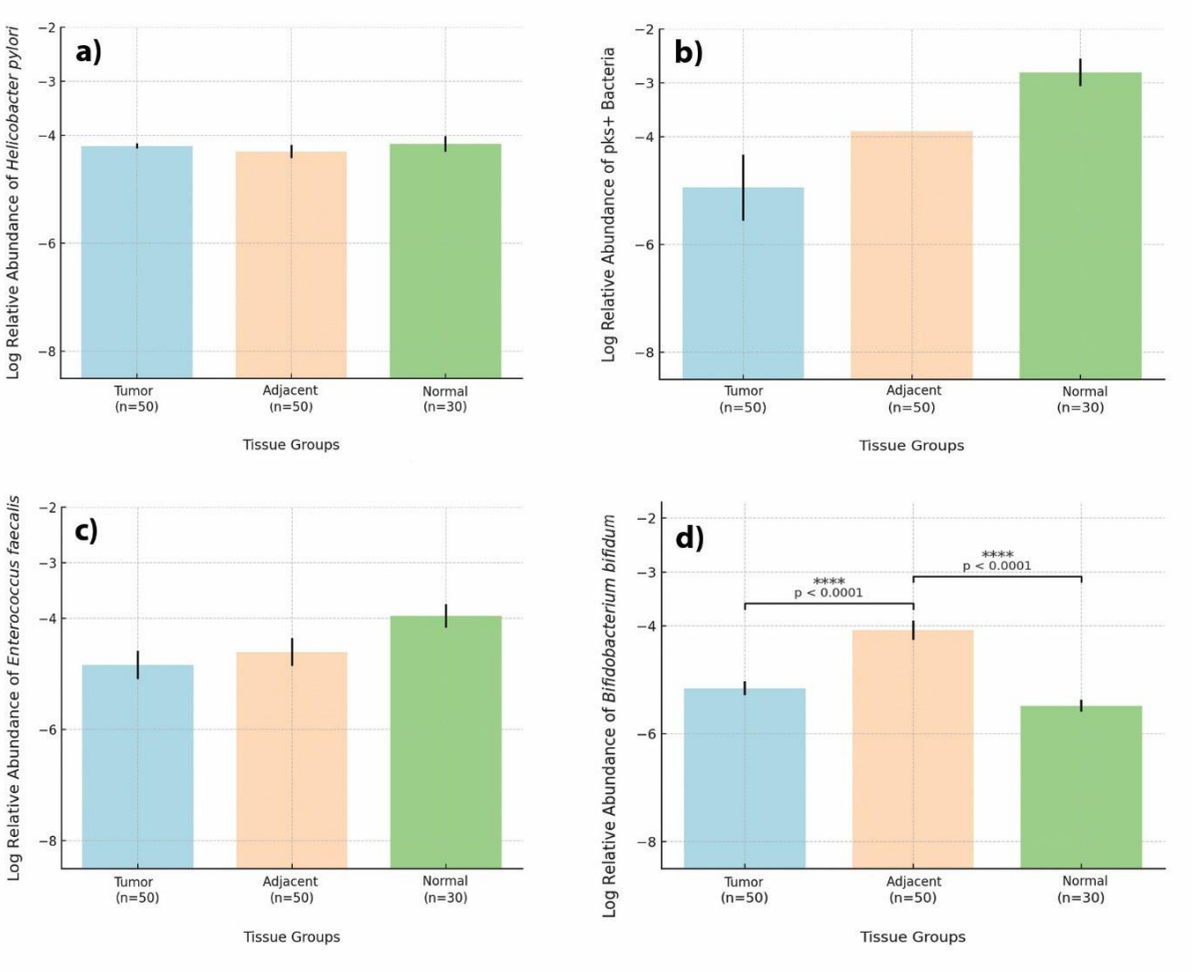

Fig. 1. Relative abundance of four bacterial groups—(a) *Helicobacter pylori*, (b) pks$^+$ bacteria, (c) *Enterococcus faecalis*, and (d) *Bifidobacterium bifidum*—across three tissue groups: Tumor (n=50), Adjacent (n=50), and Normal (n=30). Abundance was calculated as $\log_{10}$ (bacterial copy number / GAPDH copy number); The y-axis (log-transformed values) was inverted to allow upward bars while preserving correct magnitude relationships across tissue groups. Error bars represent mean ± SEM. One-way ANOVA with Tukey's post hoc test was used for statistical analysis. For *B. bifidum*, highly significant differences are indicated with **** ($p < 0.0001$).

regression ($p > 0.05$ for all predictors). This is likely due to the low detection rate—only 6 positive cases (2 Tumor, 1 Adjacent, 3 Normal) were identified (Table 5)—which limited statistical power. Therefore, all findings related to pks$^+$ bacteria should be interpreted descriptively rather than inferentially due to insufficient statistical power for reliable group comparisons.

Nonetheless, logistic regression identified age as a significant predictor of pks$^+$ bacterial presence ($p = 0.014$), with all positive cases found in patients aged 60 or older (Table 7 and Fig. 2b). No associations were found with tissue type or sex.

Enterococcus faecalis. In the case of E. *faecalis*, ANOVA and multiple linear regression (Fig. 1c) showed no significant difference in bacterial load across tissue groups. Similarly, Chi-square analysis showed no difference in bacterial presence across tissues ($p > 0.05$; Table 5), while logistic regression revealed only a borderline trend ($p = 0.065$).

However, subgroup analysis found that bacterial load was significantly higher in Tumor tissues of females ($p < 0.01$) and individuals aged 60 or older ($p = 0.04$). These results suggest that *E. faecalis* may be more abundant—but not necessarily more frequently present—in Tumor tissues of older adults and females.

Bifidobacterium bifidum. *B. bifidum* showed a distinctly localized enrichment pattern. ANOVA indicated that Adjacent tissues had significantly higher bacterial load compared to both Tumor and Normal tissues ($p < 0.0001$), with no significant difference between Tumor and Normal ($p = 0.3947$) (Fig. 1d). This enrichment was consistent across age groups (Table 7) and unaffected by sex. Logistic regression showed no significant association between *B. bifidum* presence and tissue type or age ($p > 0.05$), though a borderline trend with sex was observed ($p \approx 0.078–0.107$). Overall, *B. bifidum* presence rate is not tissue-dependent, but when the bacterium is present, it is significantly enriched in Adjacent tissues. This suggests that the microenvironment near tumors may favor colonization and growth of *B. bifidum.*

DISCUSSION

Colorectal cancer (CRC) is one of the most common malignancies worldwide, including in Iran (18). Its pathogenesis is multifactorial, involving genetic predisposition, environmental exposures, dietary habits, and increasingly, the role of the gut microbiome. In recent years, considerable attention has been devoted to the potential impact of specific bacterial species—particularly *Helicobacter pylori*, pks$^+$ *Escherichia*

Table 7. Distribution of bacterial species across tissue groups stratified by age (<60 Years vs. ≥60 Years)

Tissue type / Bacteria	CRC Tumors[1] (n=50)		Adjacent-Normal[2] (n=50)		Non-CRC Normal[3] (n=30)		P value[4]
	Number of positive cases (%)						
Helicobacter pylori 83 (100)	<60 yrs 18 (21.69)	>=60 yrs 21 (25.30)	<60 yrs 13 (15.66)	>=60 yrs 11 (13.25)	<60 yrs 7 (8.43)	>=60 yrs 13 (15.66)	1.0
pks$^+$ Bacteria 6 (100)	0 (0)	2 (33.33)	0 (0)	1 (16.67)	0 (0)	3 (50)	> 0.05
Enterococcus faecalis 34 (100)	9 (26.47)	9 (26.47)	3 (8.82)	7 (20.59)	1 (2.94)	5 (14.71)	1.0
Bifidobacterium bifidum 83 (100)	17 (20.48)	15 (18.07)	18 (21.69)	16 (19.28)	7 (8.43)	10 (12.05)	> 0.05

[1]Tumor tissues from CRC patients

[2]Normal tissues adjacent to CRC tissues from the same patients

[3]Normal tissues from non-CRC individuals

[4]Chi-squared test

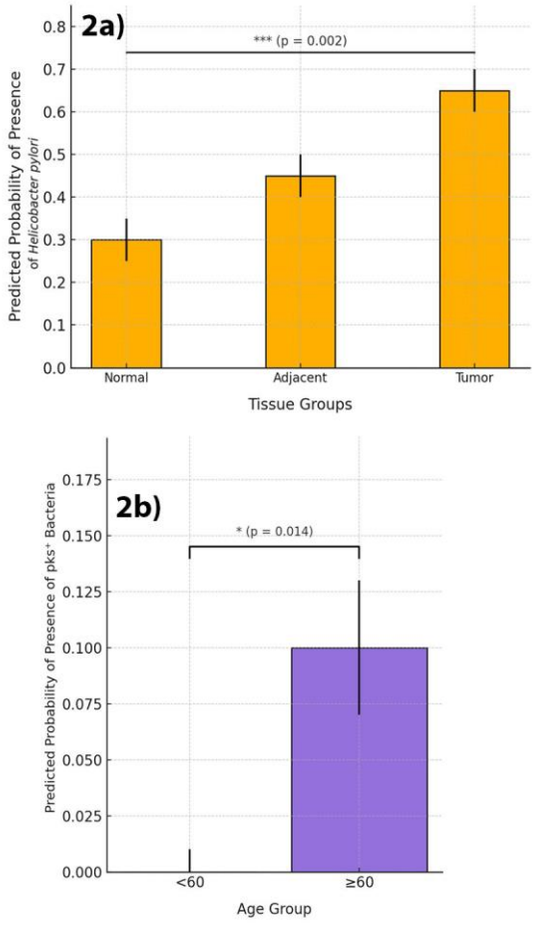

Fig. 2. Predicted probabilities of bacterial presence based on logistic regression models.

(a) Predicted probability of *Helicobacter pylori* detection across tissue groups. The likelihood of presence increases significantly from Normal to Adjacent to Tumor tissues ($p = 0.002$; ***).

(b) Predicted probability of pks$^+$ bacterial presence by age group. A significantly higher detection rate was observed in individuals aged ≥60 years ($p = 0.014$;*).

Error bars in both panels represent the standard error of the predicted probabilities.

coli, *Enterococcus faecalis*, and *Bifidobacterium bifidum*—on CRC initiation and progression. The present study aimed to investigate the association of these four bacterial groups with CRC by examining their relative abundance and detection across CRC tumor tissues (Tumor), normal tissues adjacent to tumor (Adjacent), and non-cancerous control tissues (Normal) in FFPE samples using qPCR. Additionally, demographic variables including patient age and sex were considered to explore possible host-microbe interactions that could modulate these associations. Our results showed that *H. pylori* load remained similar across all three tissue types. However, the likelihood of detecting the bacterium increased progressively from Normal to Adjacent to Tumor tissues. This pattern suggests that while *H. pylori* is present at comparable levels in tissues harboring the bacterium, its detection is more likely in tissues closer to cancerous regions. A possible explanation is that tumor-adjacent and tumor tissues create a more permissive microenvironment for bacterial persistence or detection. Cancer-associated changes—such as mucosal barrier disruption, local immune suppression, tissue remodeling, or altered pH—may reduce bacterial clearance or enhance survival of existing microbes. Clinically, this may imply that *H. pylori* is not necessarily proliferating more in tumors but is more stably retained or less effectively eliminated in these regions, potentially contributing to localized inflammation or immune modulation near neoplastic areas.

These observations may help reconcile conflicting findings in the literature. Large-scale epidemiological and Mendelian randomization studies have generally found no strong causal link between *H. pylori* infection and colorectal cancer (CRC) risk (24, 25), consistent with our finding of stable bacterial load across tissue types. On the other hand, several clinical studies have reported increased *H. pylori* prevalence in CRC patients (25, 26), which aligns with our detection-based results showing higher presence rates near tumor tissue. This discrepancy may reflect *H. pylori*'s primary adaptation to the gastric environment and its potentially transient or opportunistic colonization of the colon.

Additionally, subgroup analyses revealed a significantly lower *H. pylori* load in Tumor tissue compared to Normal tissue among females, and a borderline higher load in Normal tissues of females compared to males. These findings may point to sex-specific host–microbiota interactions, potentially influenced by hormonal or immune factors.

Turning to pks$^+$ bacteria, no significant differences in the load of bacteria were observed across tissue groups, and detection rates were notably low—only 6 positive cases. Nevertheless, logistic regression identified age as a significant predictor of pks$^+$ bacterial presence, with all positive detections occurring in patients aged ≥60 years. This suggests that while these bacteria are not enriched in Tumor tissues, they

may be more prevalent in older individuals, possibly due to age-related shifts in gut microbiota or immune status. However, because only six samples were positive overall, any observed associations, such as the age-related trend, must be considered exploratory rather than conclusive.

Although previous studies have suggested a link between pks$^+$ *E. coli* and CRC through colibactin-induced DNA damage (27, 28), our findings suggest that their presence alone—especially at low abundance—may not be a reliable biomarker in CRC pathogenesis. Moreover, pks$^+$ strains are found in 10–20% of healthy individuals (29), further emphasizing the need for context-dependent interpretation.

As for *Enterococcus faecalis*, analyses revealed no significant difference in overall bacterial load across tissue groups. Logistic regression showed a borderline association in bacterial presence across tissues, suggesting limited discriminatory power across tissue types.

However, subgroup analyses revealed that bacterial load was significantly elevated in Tumor tissues of females and individuals aged ≥60. This pattern suggests that while the overall frequency of detecting *E. faecalis* (presence/absence) does not vary significantly across tissues or demographic groups, when the bacterium is present in Tumor tissues of older individuals or females, it tends to exist at higher concentrations.

This could reflect host-specific or age-related changes that allow *E. faecalis* to proliferate more efficiently once established.

These nuanced findings align with the broader literature describing the dualistic role of *E. faecalis* in CRC. Some studies suggest protective effects, including immune activation or induction of tumor cell apoptosis (11, 12), while others implicate the bacterium in pro-carcinogenic mechanisms such as DNA damage via reactive oxygen species (30). Such opposing effects may depend on strain variability, local immune context, or microbial competition within the tumor microenvironment. Therefore, the observed increase in *E. faecalis* load among specific subgroups may signal a context-dependent role in tumor biology that warrants further functional investigation.

Finally, *B. bifidum* exhibited a distinct spatial distribution. Its abundance was significantly higher in Adjacent tissues compared to both Tumor and Normal tissues, which did not differ from each other. This enrichment pattern was consistent across age groups (<60 and ≥60 years) and was not influenced by sex. Although logistic regression revealed no significant association between *B. bifidum* presence and tissue type or age, a borderline trend toward higher detection in males was observed.

These findings suggest that while *B. bifidum* can be present in all tissue types, it is specifically enriched in the peritumoral microenvironment, likely facilitated by tumor-associated alterations in nutrient availability, immune activity, or local signaling. This localized enrichment raises important questions about its functional role—whether protective, harmful, or neutral—in cancer progression and underscores the need for mechanistic studies.

Overall, although this study did not identify strong associations between total bacterial copy number and colorectal cancer (CRC) at the population level, several biologically meaningful patterns nevertheless emerged. *H. pylori* detection increased with tissue pathology, especially in females; *E. faecalis* showed age- and sex-specific enrichment; pks$^+$ bacteria were detected only in older individuals; and *B. bifidum* was most abundant in Adjacent tissues, independent of age or sex. These patterns differ from some earlier reports that describe reduced *B. bifidum* and increased *E. faecalis* or pks$^+$ *E. coli* directly within tumor tissue. In our cohort, however, the distribution of these bacteria appeared to be influenced more by demographic factors or by the microenvironment surrounding the tumor rather than by the tumor tissue itself. While these trends do not imply causality, they underscore that microbe–host interactions in CRC can vary across populations and may be shaped by both biological context and local tissue conditions.

CONCLUSION

This study reveals distinct and biologically relevant microbial patterns associated with colorectal cancer (CRC). Most notably, *Bifidobacterium bifidum* exhibited a marked enrichment in Adjacent tissues, independent of age or sex, suggesting a localized response within the tumor-associated microenvironment. Additionally, *Helicobacter pylori* was more frequently detected in Tumor and Adjacent tissues, pointing to enhanced persistence near cancerous regions despite stable overall load.

Age- and sex-specific trends were also observed:

pks$^+$ bacteria were found exclusively in individuals aged ≥60, while *Enterococcus faecalis* showed elevated abundance in Tumor tissues of older adults and females. Although bacterial abundance for individual species did not differ significantly across tissue groups for most species, these presence-based and demographic patterns underscore the potential influence of tumor proximity and host factors in shaping microbial distribution.

Collectively, these findings highlight nuanced microbial shifts potentially influenced by tumor proximity and host factors. Notably, such patterns may not be apparent through bulk abundance analyses alone, underscoring the need for further investigation into their roles in CRC pathogenesis and progression.

Limitations and future directions. A key limitation of this study is the use of FFPE samples, which may compromise DNA quality and reduce assay sensitivity. Furthermore, qPCR performed on FFPE-derived DNA cannot distinguish live from dead bacteria, and therefore the detected microbial signals may reflect residual, degraded, or translocated bacterial DNA rather than true active colonization. Relatedly, detection of *H. pylori* DNA in colonic tissue does not establish physiological mucosal colonization, as confirmation would require histological visualization or in situ imaging, data that were not available in this study. Additionally, the very small number of pks$^+$-positive samples (n = 6) precludes robust statistical inference, and therefore all pks$^+$-related findings should be regarded as descriptive and non-definitive. Moreover, clinical metadata such as tumor stage, prior antibiotic or probiotic use, and dietary or lifestyle information were unavailable, precluding more detailed stratified analyses. Furthermore, the bacterial patterns observed in this study were influenced more by host-related factors (age, sex) and by the peri-tumoral microenvironment than by tumor tissue itself, which limits the strength of conclusions regarding CRC-specific microbial associations.

Future studies should:

• Utilize fresh tissue specimens to ensure high DNA integrity.

• Include larger, well-characterized cohorts.

• Perform functional assays to assess the biological effects of specific bacterial strains.

• Incorporate metagenomic or metatranscriptomic profiling.

• Account for clinical variables such as cancer stage, molecular tumor phenotypes, and host immune/hormonal profiles.

• Apply histological or in situ validation of bacterial presence (e.g., for *H. pylori*).

• Employ methods that distinguish live from dead bacteria.

The implementation of these strategies will better elucidate the potential contributions of the gut microbiome to CRC pathogenesis and progression.

ACKNOWLEDGEMENTS

We gratefully acknowledge the financial and institutional support from Ilam University of Medical Sciences for this study.

REFERENCES

1. Boleij A, Tjalsma H. Gut bacteria in health and disease: a survey on the interface between intestinal microbiology and colorectal cancer. *Biol Rev* 2012; 87: 701-730.
2. Wang T, Cai G, Qiu Y, Fei N, Zhang M, Pang X, et al. Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers. *ISME J* 2012; 6: 320-329.
3. Garrett WS. Cancer and the microbiota. *Science* 2015; 348: 80-86.
4. Kostic AD, Gevers D, Pedamallu CS, Michaud M, Duke F, Earl AM, et al. Genomic analysis identifies association of Fusobacterium with colorectal carcinoma. *Genome Res* 2012; 22: 292-298.
5. Rubinstein MR, Wang X, Liu W, Hao Y, Cai G, Han YW. Fusobacterium nucleatum promotes colorectal carcinogenesis by modulating E-cadherin/β-catenin signaling via its FadA adhesin. *Cell Host Microbe* 2013; 14: 195-206.
6. Arthur JC, Perez-Chanona E, Mühlbauer M, Tomkovich S, Uronis JM, Fan TJ, et al. Intestinal inflammation targets cancer-inducing activity of the microbiota. *Science* 2012; 338: 120-123.
7. Castellarin M, Warren RL, Freeman JD, Dreolini L, Krzywinski M, Strauss J, et al. Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. *Genome Res* 2012; 22: 299-306.
8. Dougherty MW, Hoffmann RM, Hernandez MC, Airan Y, Gharaibeh RZ, Herzon SB, et al. Genome-scale CRISPR/Cas9 screening reveals the role of PSMD4 in colibactin-mediated cell cycle arrest. *mSphere* 2025; 10: e00692-24.

9. Dougherty MW, Valdés-Mas R, Wernke KM, Gharaibeh RZ, Yang Y, Brant JO, et al. The microbial genotoxin colibactin exacerbates mismatch repair mutations in colorectal tumors. *Neoplasia* 2023; 43: 100918.
10. Putze J, Hennequin C, Nougayrède JP, Zhang W, Homburg S, Karch H, et al. Genetic structure and distribution of the colibactin genomic island among members of the family *Enterobacteriaceae*. *Infect Immun* 2009; 77: 4696-4703.
11. Chi D, Lin X, Meng Q, Tan J, Gong Q, Tong Z. Real-time induction of macrophage apoptosis, pyroptosis, and necroptosis by *Enterococcus faecalis* OG1RF and two root canal–isolated strains. *Front Cell Infect Microbiol* 2021; 11: 720147.
12. Dadashi M, Sahebi A, Arjmand-Teymouri R, Mirzaii M, Mousavian M, Yaslianifard S. Evaluation of *Enterococcus faecalis*, *Lactobacillus acidophilus*, and *Lactobacillus plantarum* in biopsy samples of colorectal cancer and polyp patients compared to healthy people. *Arch Clin Infect Dis* 2022; 17(1): e116165.
13. Zumkeller N, Brenner H, Zwahlen M, Rothenbacher D. *Helicobacter pylori* infection and colorectal cancer risk: a meta-analysis. *Helicobacter* 2006; 11: 75-80.
14. Zhao YS, Wang F, Chang D, Han B, You DY. Meta-analysis of different test indicators: *Helicobacter pylori* infection and the risk of colorectal cancer. *Int J Colorectal Dis* 2008; 23: 875-882.
15. Gagnière J, Raisch J, Veziant J, Barnich N, Bonnet R, Buc E, et al. Gut microbiota imbalance and colorectal cancer. *World J Gastroenterol* 2016; 22: 501-518.
16. Wei H, Chen L, Lian G, Yang J, Li F, Zou Y, et al. Antitumor mechanisms of bifidobacteria. *Oncol Lett* 2018; 16: 3-8.
17. Kosumi K, Hamada T, Koh H, Borowsky J, Bullman S, Twombly TS, et al. The amount of *Bifidobacterium genus* in colorectal carcinoma tissue in relation to tumor characteristics and clinical outcome. *Am J Pathol* 2018; 188: 2839-2852.
18. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2021; 71: 209–249.
19. Khodaverdi N, Zeighami H, Jalilvand A, Haghi F, Hesami N. High frequency of enterotoxigenic *Bacteroides fragilis* and *Enterococcus faecalis* in the paraffin-embedded tissues of Iranian colorectal cancer patients. *BMC Cancer* 2021; 21: 1353.
20. Kaewkod T, Tobe R, Tragoolpua Y, Mihara H. Medicinal plant extracts protect epithelial cells from infection and DNA damage caused by colibactin-producing *Escherichia coli*, and inhibit the growth of bacteria. *J Appl Microbiol* 2021; 130: 769-785.
21. Arsov A, Petrova P, Gerginova M, Tsigoriyna L, Armenova N, Ignatova I, et al. Bacterial tolerance to 1-butanol and 2-butanol: quantitative assessment and transcriptomic response. *Int J Mol Sci* 2024; 25: 13336.
22. Wallenstein A, Rehm N, Brinkmann M, Selle M, Bossuet-Greif N, Sauer D, et al. ClbR is the key transcriptional activator of colibactin gene expression in *Escherichia coli*. *mSphere* 2020; 5(4): e00591-20.
23. Kim HB, Kim E, Yang SM, Lee S, Kim MJ, Kim HY. Development of real-time PCR assay to specifically detect 22 *Bifidobacterium* species and subspecies using comparative genomics. *Front Microbiol* 2020; 11: 2087.
24. Luo F, Zhou P, Ran X, Gu M, Zhou S. No evident causal association between *Helicobacter pylori* infection and colorectal cancer: a bidirectional Mendelian randomization study. *Sci Rep* 2023; 13: 18544.
25. Zhang Y, Lei X, Yang X, Wang Z. Associations between *Helicobacter pylori* infection and twelve gastrointestinal diseases: a Mendelian randomization analysis. *Asian J Surg* 2024; 47: 4592-4593.
26. Liu Y, Yang DQ, Jiang JN, Jiao Y. Relationship between *Helicobacter pylori* infection and colorectal polyp/colorectal cancer. *World J Gastrointest Surg* 2024; 16: 1008-1016.
27. Pleguezuelos Manzano C, Puschhof J, Rosendahl Huber A, van Hoeck A, Wood HM, Nomburg J, et al. Mutational signature in colorectal cancer caused by genotoxic pks^{+} *Escherichia coli*. *Nature* 2020; 580: 269-273.
28. Huber AR, Pleguezuelos-Manzano C, Puschhof J, Ubels J, Boot C, Saftien A, et al. Improved detection of colibactin-induced mutations by genotoxic *E. coli* in organoids and colorectal cancer. *Cancer Cell* 2024; 42(3): 487-496.e6.
29. Sadeghi M, Mestivier D, Sobhani I. Contribution of pks+ *Escherichia coli* to colon carcinogenesis. *Microorganisms* 2024; 12: 1111.
30. Huycke MM, Abrams V, Moore DR. *Enterococcus faecalis* produces extracellular superoxide and hydrogen peroxide that damages colonic epithelial cell DNA. *Carcinogenesis* 2002; 23: 529-536.