



# Evaluation of *Enterococcus faecalis*, *Lactobacillus acidophilus*, and *Lactobacillus plantarum* in Biopsy Samples of Colorectal Cancer and Polyp Patients Compared to Healthy People

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Received 2021 May 11; Revised 2022 May 25; Accepted 2022 May 31.

## Abstract

**Background:** Colorectal cancer (CRC) is one of the leading causes of death in both men and women worldwide. According to different studies, infectious agents or microbiota dysbiosis can play a role in CRC progression.

**Objective:** This study aimed to evaluate the prevalence of *Enterococcus faecalis*, *Lactobacillus acidophilus*, and *Lactobacillus plantarum* in people with polyps or CRC compared to healthy individuals.

**Methods:** In this study, 60 biopsy samples were collected from three groups, including patients with CRC, polyps, and healthy people. The genomic DNA was extracted from the collected samples and amplified by polymerase chain reaction (PCR) to detect *E. faecalis*, *L. acidophilus*, and *L. plantarum*. In the next step, quantitative real-time PCR was used to evaluate the copy number of the bacteria in the studied groups.

**Results:** There was no statistically significant difference between the studied groups regarding age and gender ( $P > 0.05$ ). The mean number of *E. faecalis* was higher in patients with CRC than in patients with polyps and healthy individuals ( $P < 0.05$ ). Also, the mean numbers of *L. acidophilus* and *L. plantarum* were higher in healthy individuals than in patients with polyps and CRC ( $P < 0.05$ ).

**Conclusions:** Our findings indicate that *L. acidophilus* and *L. plantarum* in people with a family history of CRC and patients with polyps may effectively prevent or reduce CRC progression.

**Keywords:** Colorectal Cancer, Polyps, *Enterococcus faecalis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*

## 1. Background

Colorectal cancer (CRC) is one of the most common cancers worldwide (1). It is the leading cause of cancer deaths worldwide and a severe public health issue (2). The CRC risk among different people increases with age (3). Numerous factors such as environmental, genetic, and gut microbiota dysbiosis also have a significant role in developing CRC (4, 5). In general, germs and infectious agents can play a role in the progression of the disease and the severity of malignancies (6, 7). For example, colibactin is the secondary metabolite of *Escherichia coli* that can disrupt the cell cycle, leading to the growth and spread of CRC (8). Also, oxidative stress, caused by an imbalance between the production of active oxygen radicals and the antioxidant system, can be caused by *Enterococcus faecalis* and con-

tribute significantly to the progression of cancer (9, 10). *Enterococcus faecalis* produces reactive oxygen radicals, damaging colon epithelial DNA and being involved in adenomatous polyp's formation and progression into CRC (11).

Some studies on microbial flora and gastrointestinal cancers, especially CRC, show differences in gut microbiota between CRC patients and healthy individuals (12). The essential factors in this difference are age, gut microbiota characteristics, host genotype, changes in the intestinal microbial environment, and nutritional status of the individual (13). Probiotics are living microorganisms (14) that can affect the normal microbial flora and stabilize it, thus having beneficial effects on health (15). Studies have shown that intestinal microbial flora protects against various diseases (16-18). Researchers have attributed these ben-

eficial effects to intestinal microbial balance. Although *Lactobacillus* and *Bifidobacterium* are the most commonly used probiotics (19), many microorganisms show such properties (20).

## 2. Objectives

This study aimed to evaluate the prevalence of *E. faecalis*, *Lactobacillus acidophilus*, and *Lactobacillus plantarum* in people with polyps and CRC compared to healthy individuals in order to find the relationship between these bacteria and CRC and polyps.

## 3. Methods

### 3.1. Samples Collection

This study was performed on 60 patients, including 20 healthy individuals, 20 patients with CRC, and 20 patients with polyps. Patients who did not use antibiotics in the previous month, lacked probiotics consumption, had no familial history of intestinal polyps, and had no history of inflammatory bowel disease were enrolled. The selection of individuals to participate in this study was based on colonoscopy and positive pathology results for polyps and CRC and negative pathology results for healthy individuals. Patients' information was recorded in questionnaires. Patients' biopsy samples were placed on ice, collected in appropriate conditions, and transferred to the laboratory.

### 3.2. Preparation of Standard Strain

We acquired standard strains of *E. faecalis* (ATCC: 29212), *L. acidophilus* (DSM20079), and *L. plantarum* (DSM20174) from the Iranian Biological Resource Center (IBRC). Then, these strains were made on Blood Agar (BA) and Tryptic Soy Broth (TSB) media. After the colonies of bacteria appeared, they were prepared for DNA extraction.

### 3.3. Genomic DNA Extraction

Genomic DNA was extracted from biopsy specimens and standard strains by the QIAamp DNA biopsy mini kit (Qiagen) and tissue genomic DNA extraction mini kit (Favorgen Biotech Corp). After extraction, NanoDrop 2000 (Thermo Scientific) was used to determine the concentration, and then they were kept at -20°C before the amplification steps.

### 3.4. Assessment of the presence of *Enterococcus faecalis*, *Lactobacillus acidophilus*, and *Lactobacillus plantarum* by Polymerase Chain Reaction

Forward and reverse primers for detecting *E. faecalis*, *L. acidophilus*, and *L. plantarum* strains are shown in Table 1. The PCR temperatures and time for designing our program are shown in Table 2. The PCR products were analyzed by electrophoresis on 2% agarose gel and confirmed by visualizing DNA bands using UV illumination.

**Table 1.** Specific Primers for Detecting *Enterococcus faecalis*, *Lactobacillus acidophilus*, and *Lactobacillus plantarum* by Polymerase Chain Reaction

Microorganism	Primers (5->3)	
<i>E. faecalis</i> (21)	Forward	CGAAGATCCAGCAGATGCGGTT
	Reverse	CACGGITGCGATTACGGTIGTTC
<i>L. acidophilus</i> (21)	Forward	AATTCTCTCTCGGCTCCTCTA
	Reverse	CCTTTCTAAGGAAGCGAAGGAT
<i>L. plantarum</i> (21)	Forward	TTACCTAACGGTAAATGC
	Reverse	GCCGCCTAAGGTGGGACAGAT

**Table 2.** Polymerase Chain Reaction Temperatures and Time for Detecting *Enterococcus faecalis*, *Lactobacillus acidophilus*, and *Lactobacillus plantarum*

Steps	Microorganism	Temperature/Time
Initial denaturation	<i>E. faecalis</i>	94°C/ 4 min
	<i>L. acidophilus</i>	95°C/ 5 min
	<i>L. plantarum</i>	95°C/ 5 min
Denaturation	<i>E. faecalis</i>	95°C/ 1min
	<i>L. acidophilus</i>	95°C/ 1min
	<i>L. plantarum</i>	95°C/ 1min
Annealing	<i>E. faecalis</i>	60°C/ 30s
	<i>L. acidophilus</i>	58°C/ 40s
	<i>L. plantarum</i>	60°C/ 40s
Extension	<i>E. faecalis</i>	72°C/ 40s
	<i>L. acidophilus</i>	72°C/ 30s
	<i>L. plantarum</i>	72°C/ 30s
Final extension	<i>E. faecalis</i>	72°C/ 12 min
	<i>L. acidophilus</i>	72°C/ 7 min
	<i>L. plantarum</i>	72°C/ 7 min

### 3.5. Assessment of the Presence of *Enterococcus faecalis*, *Lactobacillus acidophilus*, and *Lactobacillus plantarum* by Quantitative Real-time PCR (qRT-PCR)

The qRT-PCR was performed in a reaction volume of 20 µL comprising 10 µL SYBR Green PCR master mix, 0.5 µL of each forward and reverse primers, 2 µL of rox, and 2 µL

of DNA extracted from the biopsy specimen. The amount of DNA was determined in duplicate, and the mean values were specified. Amplification and detection of DNA were accomplished by the LightCycler® 96 real-time PCR System (Roche Life Science). The reaction conditions were as follows: 94°C for 3 min, 35 cycles of 95°C for 40 s, 55°C for 25 s and 72°C for 5 min (*E. faecalis*), 94°C for 2 min, 40 cycles of 94°C for 45 s, 60°C for 25 s and 72°C for 5 min (*L. acidophilus*), 94°C for 12 min, 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 5 min (*L. plantarum*). Sequence detection software light cycler 96 was used for data analysis. The purified genomic DNA in the range of 1 ng of standard strains was used for determining the amount of *E. faecalis*, *L. acidophilus*, and *L. plantarum* by qRT-PCR. Three different concentrations of standard strains were chosen in the sample editor section. Then, the amounts of copied bacteria in each gram of biopsies were determined by referring to the standard curves.

### 3.6. Statistical Analysis

Completed questionnaires by patients and the qRT-PCR data were analyzed. Descriptive statistics were conducted using the Statistical Package for Social Sciences (SPSS) software (version 21). Appropriate statistical tests, such as chi-square, Mann-Whitney, and Kruskal-Wallis, were applied depending on the variables. P-values of less than 0.05 were considered statistically significant.

## 4. Results

The mean age was  $50 \pm 12.3$ ,  $54 \pm 17$ , and  $54 \pm 8.4$  years for healthy individuals, CRC patients, and polyp patients, respectively. There were no statistically significant differences between the groups regarding age and gender ( $P > 0.05$ ). Five (25.0%) CRC patients, seven (35.0%) polyp patients, and nine (45.0%) healthy individuals had a high-fat diet. There was no statistically significant difference in diet status (high-fat diet) between the groups ( $P > 0.05$ ). Also, 11 CRC patients, 10 polyp patients, and four healthy people had type O blood. Statistical analysis showed a significant difference in the number of people with type O blood between polyps or CRC groups and healthy individuals ( $P < 0.05$ ).

The mean copy numbers of *E. faecalis*, *L. acidophilus*, and *L. plantarum* showed a significant difference between the three groups ( $P < 0.05$ ). The mean copy number of *E. faecalis* was higher in CRC patients than in polyp patients and healthy individuals ( $P < 0.05$ ) (Table 3). Also, the mean copy numbers of *L. acidophilus* and *L. plantarum* were higher in healthy individuals than in polyp patients and CRC patients ( $P < 0.05$ ) (Table 3).

**Table 3.** Copy Numbers of *Enterococcus faecalis*, *Lactobacillus acidophilus*, and *Lactobacillus plantarum* in Biopsy Samples Taken from Patients with Colorectal Cancer, Polyps, and Healthy People

Microorganism	Type of Sample	Minimum	Maximum	Mean
<i>E. faecalis</i>	Healthy	$1.4 \times 10^2$	$8.4 \times 10^4$	$2.2 \times 10^3$
	Polyp	$9.1 \times 10^3$	$7.9 \times 10^5$	$2.3 \times 10^4$
	CRC	$3.3 \times 10^6$	$1.9 \times 10^{14}$	$9.4 \times 10^{10}$
<i>L. acidophilus</i>	Healthy	$9.4 \times 10^{10}$	$6.9 \times 10^{13}$	$5.0 \times 10^{12}$
	Polyp	$8.6 \times 10^7$	$9.2 \times 10^{10}$	$2.9 \times 10^9$
	CRC	$4.9 \times 10^7$	$3.9 \times 10^{10}$	$9.3 \times 10^8$
<i>L. plantarum</i>	Healthy	$8.9 \times 10^6$	$1.9 \times 10^{14}$	$9.7 \times 10^{10}$
	Polyp	$8.6 \times 10^3$	$8.1 \times 10^8$	$2.4 \times 10^5$
	CRC	$7.7 \times 10^3$	$9.8 \times 10^4$	$1.3 \times 10^4$

## 5. Discussion

Colorectal cancer is one of the most common malignancies in men and women worldwide (22). The prevalence of this cancer is significantly increasing in developing countries (23). Some factors are critical in exacerbating or preventing CRC (24). Among these factors are microorganisms, mainly bacteria (25). Therefore, it is essential to discuss the role of these pathogens in CRC.

Gut bacteria are essential in protecting people or causing disease (26). Some bacterial species exacerbate tumor formation and cause cancer, but others maintain intestinal health (27). Various studies show that bacteria can play a role in apoptosis and inflammation in some types of cancer (28-30). Studies also show that some bacteria are involved in chronic infections or the production of toxic agents and can affect cell growth and lead to tumorigenesis (10, 31). According to studies, some bacteria can escape from the immune system. They can also stimulate the immune response through specific cytokines, including interleukin-8 (IL-8) released by inflammatory cells, reactive oxygen species (ROS), and nitric oxide (NO). These substances and other factors such as smoking can significantly increase carcinogenicity. One of the present study results, which was performed by the qRT-PCR method on biopsy specimens, was the higher number of *E. faecalis* in patients with polyps and CRC than in healthy individuals. Also, CRC patients were found to have a higher number of *E. faecalis* than those with polyps, and in both groups, it was higher than in healthy individuals. Also, there was a significant difference in the numbers of *E. faecalis* between CRC patients and polyp patients ( $P < 0.05$ ). Nevertheless, there was no significant difference in the number of *E. faecalis* between polyp patients and healthy individuals ( $P > 0.05$ ). Animal studies have shown that *E. faecalis* alters the

DNA of colon epithelial cells and increases colitis, dysplasia, and adenocarcinoma in mice (32, 33). These study results regarding this bacterium's role in exacerbating or developing CRC confirm the current study findings. Animal studies also showed that changes in the immune system and inflammatory responses could play an essential role in cancer progression.

The metabolic activity of *E. faecalis* in the gastrointestinal tract causes it to produce extracellular superoxide ( $O_2$ ), leading to colon polyps or CRC. Thus, *E. faecalis* can play an important role in exacerbating gastrointestinal cancer. There is evidence that treatment with probiotics can modulate the gastrointestinal function and reduce gastrointestinal disorders (34, 35). Consumption of probiotics can lead to the production of fermented products such as short-chain fatty acids. Probiotics induce the colon's protective enzyme glutathione transferase II (36). These factors reduce the genetic material load in the gut and increase the production of agents inactivating toxic compounds. Butyrate, for example, is one of these protective compounds slowing cancer cell growth (37). Studies also show that the abundance of bacteria and bifidobacteria reduces the risk of colon polyps (38, 39). However, there is ample evidence that probiotics, especially lactobacilli, play an influential role in fighting cancer. They affect the digestive enzymes of animals and humans, inhibit carcinogens in vitro and in vivo, and suppress compounds that induce cancer and tumors (40).

In this study, the SYBR Green method evaluated the copy number of *L. acidophilus* and *L. plantarum* in biopsy specimens. According to the results, the mean copy number of *L. acidophilus* was higher in healthy individuals than in polyp and CRC patients ( $P < 0.05$ ). Also, this bacterium's mean copy number was higher in polyp patients than in CRC patients. However, there was no significant difference in the mean copy number of *L. plantarum* between the polyp and CRC groups. There are several reasons to explain these findings. For example, *L. acidophilus*, one of the most critical and common bacteria in gut flora, may be altered by changes in the gut due to polyps and CRC. Also, *L. plantarum* is one of the most abundant species of the *Lactobacillus* genus, commonly found in fermented foods. As a result, the extended antimicrobial activity of *L. plantarum* is one of the most important therapeutic factors for preventing infections. The present study results on the copy number of *L. acidophilus* in biopsy specimens obtained from polyp and CRC patients compared to healthy individuals are similar to those obtained from *L. plantarum*. A study determined the role of *Lactobacillus* in inducing or preventing the spread of adenoma or CRC (41). Based on the results, lactobacilli can be essential in inducing or spreading malignancy. The results of this study and our previous study

show the dual role of bacteria in preventing or inducing malignancy. These results could pave the way for extensive study of the microbiome of CRC patients compared to healthy individuals. These studies can also provide insight into how bacteria affect health and disease.

Research has shown that the population of some bacteria, including *Fusobacterium nucleatum*, is higher in patients with CRC than in healthy individuals (42). This increase may be due to a decrease in the population of bacteria such as *L. acidophilus* and *L. plantarum*, which in turn leads to dysbiosis. In 2004, Darfeuille-Michaud et al. (43) showed that CRC increased the population of *E. coli* in the colon mucosa. In 2014, Zackular et al. (44) found that *Fusobacterium* or bacteria of the *Fusobacteriaceae* family were more common in the fecal samples of patients with CRC than in healthy individuals. However, the population of intestinal Proteobacteria decreases in patients with CRC. The researchers showed that the population of bacteria such as *E. coli*, *Citrobacter*, *Shigella*, *Flavobacterium*, *Acinetobacter*, and *Chryseobacterium* was reduced in patients with CRC (45). The findings of Baldwin et al. also suggest that *L. acidophilus* and *L. casei* may increase the induction of apoptosis in the carcinoma cell line and may be used as adjuvant chemotherapy (46). Zhang et al. showed that lactic acid bacteria bind to the Trp-P-1 mutagen and reduce the absorption of this substance in the gut, thus reducing the risk of cancer (26). Goldin et al. showed that *L. casei* has the ability to reduce the activity of three harmful fecal enzymes, beta-glucuronidase, nitroreductase, and azoreductase (47). These enzymes can convert pre-mutagenic and precancerous compounds into mutagenic and carcinogenic compounds. Chiu et al. also showed that soluble compounds secreted by *L. casei* induce apoptosis in monocytic leukemia cells (48). As a result, probiotics as a supplement can effectively prevent polyps from turning into cancer. Ultimately, there was a significant difference in type O blood between polyp and CRC patients and healthy individuals. Therefore, people with type O blood may be more prone to polyps and CRC.

### 5.1. Conclusions

In light of the results obtained in this study and the results acquired from previous studies, physicians can prescribe probiotics to help prevent CRC after adenomatous polyps are detected. Probiotics in the diet of the elderly can prevent the development of polyps or their progression to malignancy. In this study, there was a significant difference in blood types. Type O blood was more prevalent than other blood groups in polyp and CRC patients. Therefore, it can be claimed that CRC is more common in people with blood type O, which can be prevented using a proper diet and probiotics.



## Footnotes

**Authors' Contribution:** Study concept and design: S.Y and R.A; Acquisition of data: M.D, A.S, M.MZ, and M.M; Analysis and interpretation of data: M.D; Drafting of the manuscript: M.D and S.Y; Critical revision of the manuscript for important intellectual content: M.D, S.Y, and R.A; Statistical analysis: M.D and M.MZ; Administrative, technical, and material support: M.M and S.Y; Study supervision: S.Y.

**Conflict of Interests:** The authors declare no conflict of interest.

**Ethical Approval:** The Ethics Committee of the Alborz University of Medical Sciences, Karaj, Iran, approved the entire procedure (IR.ABZUMS.REC.1397.221) (link: [ethics.research.ac.ir/EthicsProposalView.php?id=52685](http://ethics.research.ac.ir/EthicsProposalView.php?id=52685)).

**Funding/Support:** None.

**Informed Consent:** Written informed consent was obtained from the participants.

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