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Colorectal Cancer and *Lactobacillus plantarum*: A qPCR-Based Study of FFPE Tissues

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A B S T R A C T

Introduction: Colorectal cancer (CRC) is a major global health issue. The gut microbiota has become an important factor in CRC development, with increased focus on microbes such as *Lactobacillus plantarum*. This study aimed to examine the presence and relative abundance (*GAPDH*-normalised copy number) of *L. plantarum* in colorectal tissues using a qPCR approach.

Materials & Methods: DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissues from three groups: tumour tissues from CRC patients (n = 50), matched adjacent normal tissues (n = 50), and normal tissues from individuals without CRC (n = 30). Species-specific primers targeting the *groEL* gene were used to measure *L. plantarum*, normalised against human *GAPDH*. Statistical analyses compared occurrence and levels across groups and assessed associations with age and sex.

Results: Although bacterial load did not vary significantly across tumour, Adjacent, and Normal tissues, detection frequency was higher in tumour tissues ($p = 0.0459$). *L. plantarum* was also detected more often in individuals aged ≥60 ($p = 0.0024$). Subgroup analysis revealed higher loads in Normal tissues of older adults and females and lower abundance in Adjacent tissues of females.

Conclusion: *L. plantarum* was detected more frequently in tumour tissues despite consistent bacterial loads, indicating possible persistence rather than growth. These findings highlight age-, sex-, and tissue-specific colonisation patterns and emphasise the need for further research on the tumour-associated ecology of beneficial microbes.

Keywords: Colorectal Neoplasm; Gastrointestinal Microbiome; *Lactobacillus plantarum*; Real-Time Polymerase Chain Reaction; Paraffin Embedding

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Introduction

Colorectal cancer (CRC) remains one of the leading public health challenges worldwide, and its incidence continues to rise in both developed and developing nations. The disease arises from a multifactorial process involving genetic predispositions, environmental exposures, and microbial influences. Only a minority of cases are explained by hereditary syndromes, whereas the majority are sporadic and associated with modifiable risk factors such as diet, obesity, metabolic disorders, and physical inactivity. In recent years, attention has increasingly turned toward the intestinal microbiome as both a modifiable risk factor and a possible target for prevention in CRC development (1, 2). The human gut harbours a vast and diverse microbial ecosystem that plays a central role in maintaining intestinal and systemic health. Among these microorganisms, species of Lactobacillus are widely recognised for their probiotic effects. They help preserve mucosal barrier integrity, regulate immune functions, generate short-chain fatty acids, and restrict the growth of pathogenic microbes. Experimental data suggest that *Lactobacillus plantarum* may exert anti-tumour activity by dampening pro-inflammatory signalling and limiting oxidative stress, which points to its possible protective role in CRC (3, 4). Nevertheless, human studies have produced mixed results. For example, a large U.S. cohort did not find a significant reduction in colorectal tumour recurrence following dietary supplementation with *L. plantarum* (5). Similarly, several clinical trials testing mixed Lactobacillus formulations—including *L. plantarum*—have reported inconclusive findings in relation to CRC progression (6). These inconsistencies underline the complexity of host–microbe interactions and the need for more strain-specific and mechanistic investigations.

The situation is further complicated by the phenomenon of dysbiosis, in which the normal microbial balance is disturbed. Dysbiosis, increasingly linked with CRC, can result from ageing-related immune decline, chronic inflammation, or changes in diet. Such alterations often lead to a reduction in beneficial taxa like Lactobacillus spp., compromising mucosal defences, weakening immune surveillance, and promoting inflammation, all of which may contribute to tumorigenesis (7, 8). Given that microbiome composition varies by geography, lifestyle, and genetics, findings from one population cannot always be generalised to another. In Iran, where diet and genetic background differ substantially from Western populations, data on the relationship between probiotics and CRC remain scarce. To address this gap, the present study measured the gene copy numbers of *Lactobacillus plantarum* in formalin-fixed paraffin-embedded (FFPE) colonic tissue from patients with CRC and from non-cancer controls. This species was selected because of its well-documented probiotic attributes, its contribution to barrier protection and immune regulation, and its suggested anti-carcinogenic activity in preclinical studies.

Materials and methods

Collection of FFPE Tissue Samples and Clinical Data

A total of 130 formalin-fixed paraffin-embedded (FFPE) colorectal tissue samples were included in this study. The sample size was chosen to be larger than that of several earlier reports, which typically analysed 40–50 cases (8). The material was classified into three groups: (1) tumour tissue from 50 patients with histopathologically confirmed CRC [CRC], (2) 50 paired non-cancerous tissues taken from the margin of the same resections [Adjacent], and (3) 30 normal colorectal tissues from individuals without any histological signs of malignancy [Normal].

All blocks were obtained from the pathology archive of Imam Reza Hospital, Kermanshah, Iran. Haematoxylin and eosin (H&E)-stained slides were re-examined by an experienced pathologist to confirm diagnoses and verify sample classification. Demographic and clinical information, including

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patient age, sex, and disease-related characteristics, was retrieved from hospital records.

Sectioning and DNA Extraction from FFPE Samples

Sections 3 μm in thickness were cut from each FFPE block under sterile conditions using a microtome and placed into sterile 1.5 mL tubes. DNA extraction was carried out with the SinaPure DNA–FFPE Tissue Kit (Sinaclon, Iran) according to the manufacturer's instructions. DNA purity and concentration were measured on a NanoDrop ND-1000 spectrophotometer. Extracted DNA was kept at –80°C until PCR analysis.

Preparation of Standard Bacterial DNA

Genomic DNA from a reference strain of *Lactobacillus plantarum* was kindly supplied by the Research Center of Shahed University, Tehran, Iran. Its quality and concentration were re-checked using NanoDrop spectrophotometry to ensure accuracy before use in standard curve construction.

Standard Curve Construction for Absolute Quantification

The *groEL* gene of *L. plantarum* was chosen as the diagnostic target. Species-specific primers (Table 1) were used to amplify the gene. PCR products were serially diluted tenfold (seven dilutions, from undiluted to 10^{-6}) to prepare standard curves. Real-time PCR was performed on the Applied Biosystems StepOnePlus Real-Time PCR System using the conditions in Tables 2 and 3. Cycle threshold (Ct) values were plotted against log-transformed dilution factors to generate the curve.

For normalisation, the same strategy was applied to the human housekeeping gene *GAPDH*. This step controlled for variation in DNA input and extraction efficiency.

Primer Selection and Validation

Although 16S rRNA is commonly used for bacterial detection, it does not always provide species-level discrimination within Lactobacillus. Therefore, *groEL*, which shows higher interspecies variability, was selected. Primers were designed to amplify a 144 bp fragment of *groEL* using Primer-BLAST (NCBI). Design parameters were adjusted for product size, melting temperature, and specificity. BLAST analysis confirmed that the primers were highly specific to *L. plantarum*, with no relevant cross-reactivity.

Experimental validation included melt curve analysis after each run. A single sharp peak at 84.79°C was consistently observed (Figure 1). No primer-dimers or non-specific products were detected. Controls without template DNA and reactions with unrelated bacterial DNA were also negative. These results confirmed the specificity of the primers.

To experimentally verify specificity, melt curve analysis was conducted after each qPCR run. The resulting melt curves consistently showed a single sharp peak with a melting temperature (Tm) of 84.79°C (Figure 1), confirming specific amplification of the *L. plantarum groEL* target. No evidence of non-specific products or primer-dimer formation was observed. Additionally, no amplification occurred in no-template controls or in reactions using DNA from unrelated bacterial strains. These findings confirm the high specificity of the *groEL* primers for *L. plantarum*.

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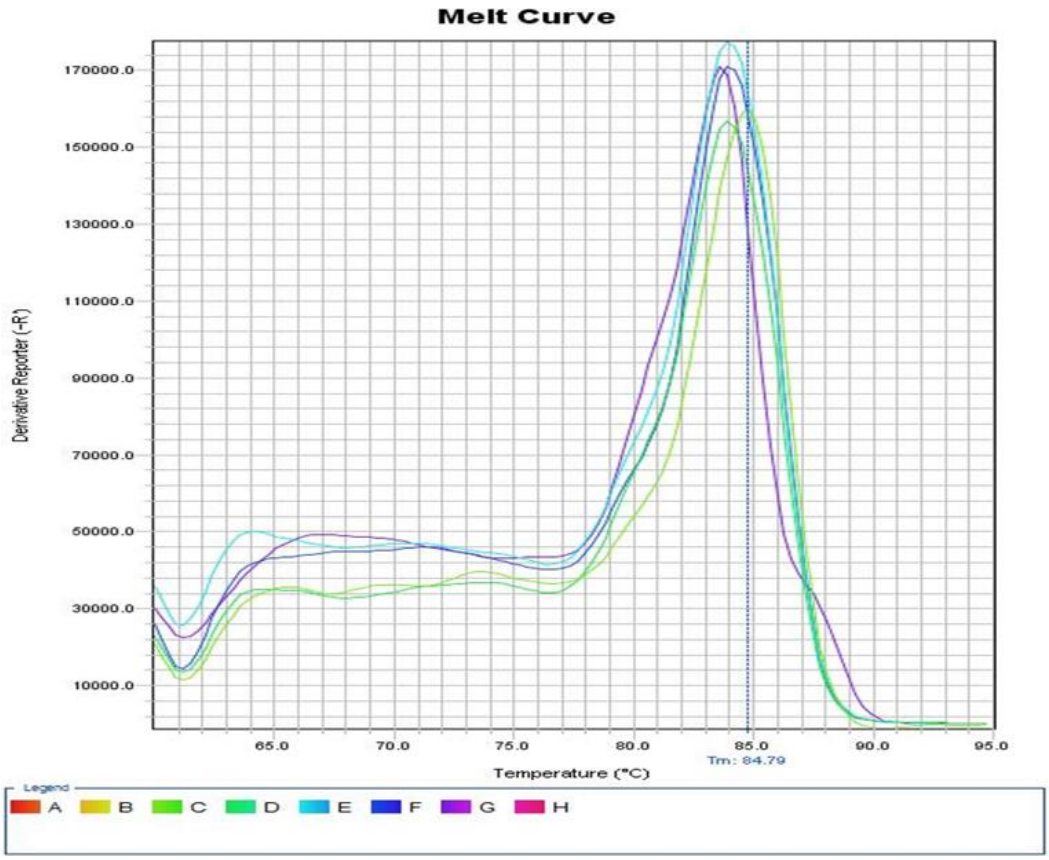

Figure 1. Melt curve from qPCR with *Lactobacillus plantarum groEL* primers. A single clear peak was seen at 84.79 °C, which suggests the reaction amplified only the intended target. No extra peaks were visible. Negative controls, including no-template samples, produced no signal.

Table 1. The primer sequences used in this study.

Gene	Bacteria detected	Primer sequence 5' to 3' (Forward/Reverse)	Product size (bp)	Tm (ºC)	Reference
groEL	*Lactobacillus plantarum*	5'- GTTTTTCAGCGACAACGGCT -3'	144	59.97	The Present study
		5'- GTTTCAACGCCGCAACTGAT -3'		60.04	
GAPDH	*Homosapiens*	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]	*groEL* (*Lactobacillus plantarum*)	*GAPDH* (Homosapiens)
2xqPCR BIOSyGreen Mix (SYBRGreen High Rox)	10	10
Forward primer (10µM)	0.8	0.8
Reverse primer (10µM)	0.8	0.8
DNA	1	2
Water	7.4	6.4

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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.	50 for *L. plantarum** 60 for *GAPDH*	40

* The annealing temperatures for *L. plantarum* (50 °C) and *GAPDH* (60 °C) were determined through primer-specific optimization using gradient PCR and confirmed via melt curve analysis to ensure target-specific amplification without non-specific products.

Quantitative Real-Time PCR

DNA from FFPE samples was analysed by quantitative PCR to determine *L. plantarum* load. Primer sequences, reaction volumes, and thermal cycling conditions were identical to those used for standard curve construction (Tables 1–3). Bacterial copy numbers were calculated from the equation of the standard curve (Y = mX + C). Copy numbers were then normalised to *GAPDH* to adjust for sample-to-sample differences in DNA quantity.

Negative controls included no-template controls (NTCs) and paraffin-only sections, while positive controls were prepared from known bacterial DNA.

DNA Integrity and Internal Controls

Given the fragmentation typical of FFPE-derived DNA, primers were designed to produce amplicons shorter than 200 bp for both bacterial (*groEL*) and human (*GAPDH*) targets. *GAPDH* amplification was successful in all 130 cases, with an average Ct around 24, confirming adequate DNA quality. In samples where *GAPDH* was amplified but *L. plantarum* was not, the result was interpreted as a true absence of the bacterium rather than DNA degradation.

Statistical and Data Analysis

Data were analysed to assess possible links between bacterial load and clinical or pathological variables. Initial analyses included ANOVA, independent t-tests, and χ^2 tests. Multiple linear regression was then used to model variations in bacterial copy number, and logistic regression was applied to evaluate the likelihood of *L. plantarum* presence.

Results

A total of 130 colorectal tissue samples preserved as FFPE blocks were included in this study, comprising three distinct groups: 50 tumor, 50 Adjacent, and 30 Normal tissues. Demographic and clinical characteristics of CRC patients and normal controls are presented in Table 4. *Lactobacillus plantarum* was detected in 63 individuals, comprising 61.9% males (39 cases) and 38.1% females (24 cases). Among these positive cases, 60.32% (38 individuals) were aged ≥60 years, while 39.68% (25 individuals) were under 60.

Table 4. Demographic and Clinical Data of Patients and Controls.

Variables		CRC[1] (n=50)	Adjacent[2] (n=50)	Normal[3] (n=30)	P value
Age		57 ± 15.1 (32 to 85)	57 ± 15.1 (32 to 85)	64.7 ± 15.9 (27 to 88)	¶ 0.055
Sex	Female	22 (44)	22 (44)	10 (33.3)	#0.582
	Male	28 (56)	28 (56)	20 (66.7)	
Cancer type	Adenocarcinoma	48 (96)	48 (96)	0 (0)	#*<0.001
	Gastrointestinal stromal tumor	1 (2)	0 (0)	0 (0)	

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	Mucinous adenocarcinoma	1 (2)	1 (4)	0 (0)	

[1] FFPE samples from CRC patients, 2 FFPE samples near tumor acquired from the same CRC patients, 3 FFPE samples from non-CRC controls, #Chi-squared test (to compare categorical variables among the three groups), ¶ ANOVA test (to compare continuous variables among the three groups), *Significant at the 5% level ($p < 0.05$)

As previously described, bacterial gene quantification was normalised to the human housekeeping gene *GAPDH* to account for variations in DNA input across samples. Accordingly, all subsequent references to "copy number" pertain to values normalised against *GAPDH* expression [i.e., log (copy number of bacteria/copy number of *GAPDH*)]. The quantity of positive samples along with the bacterial copy numbers in both normal and tumour tissues are presented in Table 5. The assay's sensitivity, established through serial dilutions of purified bacterial DNA, yielded a limit of detection (LOD) of approximately 10^2 copies for *L. plantarum*. Samples producing Ct values greater than 35 were deemed unreliable and were excluded from downstream statistical evaluation. In all cases, the NTC and other negative control samples either showed no amplification up to cycle 40 or, when a signal was observed, it appeared between cycles 38 and 40, most likely indicating non-specific background amplification rather than true positives. Representative amplification plot for the *L. plantarum groEL* gene is shown in Figure 2.

Table 5. Distribution, Relative Copy Numbers, and mean Ct Values of *Lactobacillus plantarum* Across three Tissue Groups.

	CRC [2] (n=50)	Normal adjacent to CRC tissue [3] (n=50)	Normal [4] (n=30)	P value
Number (%) of patients with positive[1] test results Yes No	 20 (40) 30 (60)	 39 (78) 11 (22)	 24 (80) 6 (20)	[#]<0.001*
Range of Ct values	31.97±2.02	31.04±1.95	30.45±2.06	[¶]0.011*
Mean Ct values	27.70 to 36.30	25.30 to 36.50	26.20 to 35.80	[¶]0.011*
Log (Bacterial copy no. /copy no. of *GAPDH*)	1.88 ± 0.46 (2.75 to 0.42)	2.14 ± 0.85 (3.6 to 1.45)	1.89 ± 0.99 (3.41 to 1.27)	[¶]0.313

[1]"Positive" denotes detectable amplification of *L. plantarum* with Ct ≤ 35, regardless of bacterial load magnitude. [2]Samples from CRC patients, [3]Acquired from the same CRC patients, [4] Samples from non-CRC controls, [#]Chi-squared test (to compare categorical variables among the three groups), [¶] ANOVA test (to compare continuous variables among the three groups), [*]Significant at the 5% level ($p < 0.05$)

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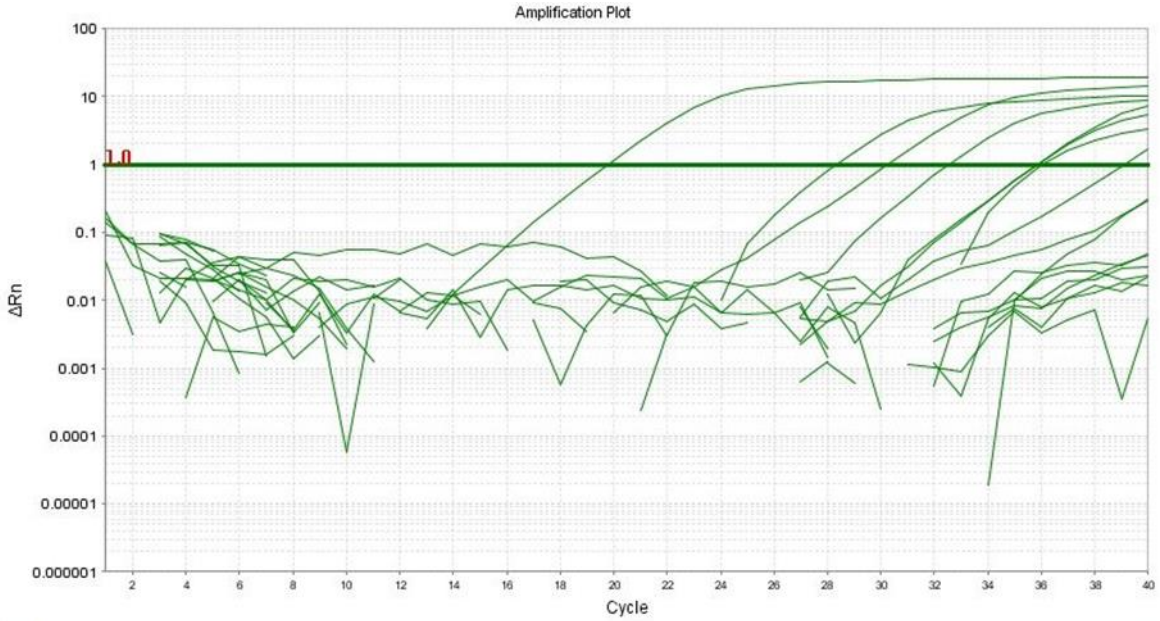

Figure 2. qPCR amplification curve for *Lactobacillus plantarum*. Amplification profiles of clinical samples show characteristic exponential increases in fluorescence intensity starting around cycle 15, consistent with efficient and specific target amplification.

An integrated analysis of both load and presence patterns revealed nuanced associations between *Lactobacillus plantarum* and colorectal tissue status. One-way ANOVA and multiple linear regression analyses revealed no statistically significant differences in *L. plantarum* load across tumour, Adjacent, and Normal tissue groups ($p > 0.1$) (Figure 3). Likewise, bacterial abundance was not significantly associated with patient age or sex ($p > 0.05$) (Table 6).

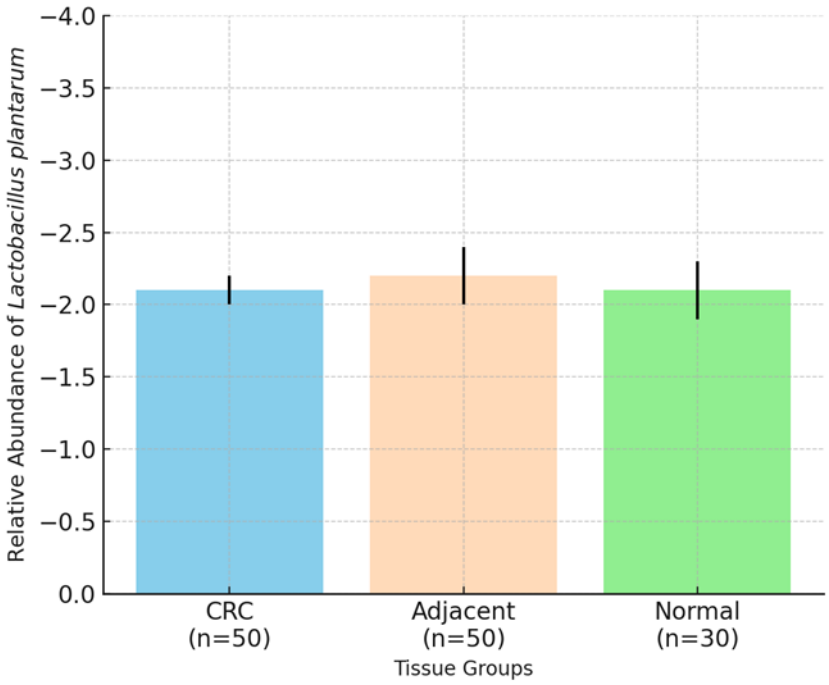

Figure 3. Relative levels of *Lactobacillus plantarum* in Tumor, Adjacent, and Normal colorectal tissues. Values were normalized to *GAPDH* and log-transformed. To make the bars point upward while keeping the scale accurate, the Y-axis was flipped. No meaningful difference in bacterial load was found among the three groups (ANOVA, $p > 0.1$). Error bars show ±SEM.

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Table 6. Distribution of Positive Cases for *Lactobacillus plantarum* Stratified by Tissue Type and Age Group (<60 Years vs. ≥60 Years).

Tissue type / Bacteria	Number of positive cases (%)						P value[4]
93 (100)	CRC Tumors[1] (n=50)		Adjacent-Normal[2] (n=50)		Non-CRC Normal[3] (n=30)		> 0.05
	<60 yrs	>=60 yrs	<60 yrs	>=60 yrs	<60 yrs	>=60 yrs	
	18 (19.35)	21 (22.58)	10 (10.75)	20 (21.51)	7 (7.53)	17 (18.28)	

[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

Presence-based analyses revealed more informative trends. Logistic regression showed a significant association between tissue type and detection of *L. plantarum*, with detection rates significantly higher in tumour tissues compared to Adjacent tissues (p = 0.0459; Figure 4a). However, the difference between Normal and Adjacent tissues did not reach statistical significance (p = 0.2007). These findings suggest a preferential presence of *L. plantarum* in cancerous tissue environments, even in the absence of marked differences in bacterial load. Patient age also emerged as a significant predictor of bacterial detection (p = 0.0024), with individuals aged ≥60 years more likely to test positive for *L. plantarum* than those under 60 (Figure 4b). In contrast, no statistically significant association was found between bacterial presence and patient sex (p = 0.687). Subgroup analyses provided additional insight into demographic interactions. Among females, *L. plantarum* load was significantly higher in Normal tissues compared to Adjacent tissues (p = 0.031), suggesting that proximity to tumours may reduce bacterial abundance in this subgroup. Furthermore, within the Normal tissue group, both females and individuals aged ≥60 years exhibited significantly higher bacterial abundance compared to males (p = 0.016) and individuals aged <60 (p = 0.0194), respectively. These findings highlight potential sex- and age-related influences on bacterial colonisation patterns, particularly in non-cancerous tissues.

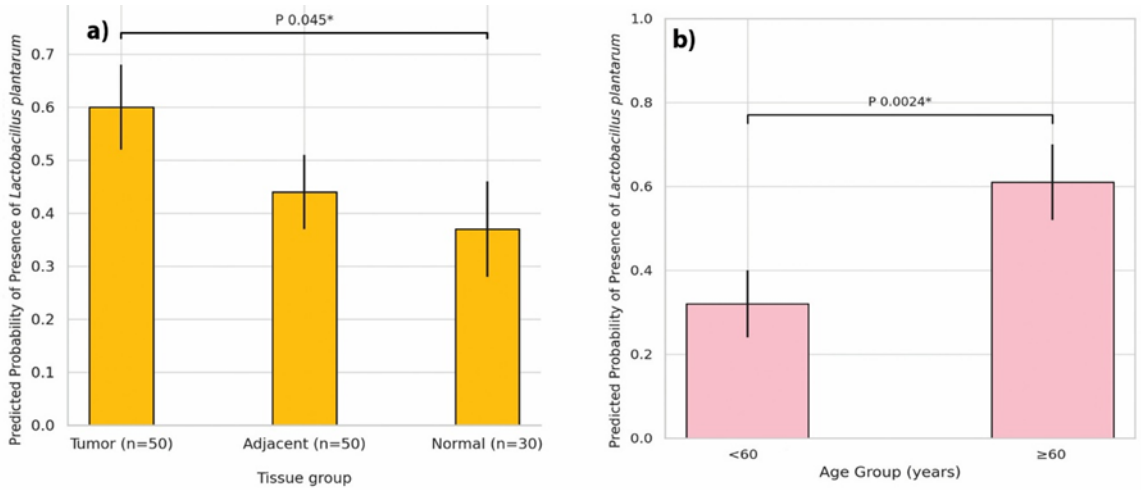

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Figure 4. Predicted Probability of Detection of *Lactobacillus plantarum*. a). By tissue type: The probability of detection was highest in Normal tissues and lowest in Tumor tissues. A statistically significant difference was observed between Tumor and Normal tissues (p 0.0459 *), **b)** By age group: Individuals aged ≥60 years had a significantly higher predicted probability of harboring the bacterium compared to those <60 years (p 0.0024 **). Error bars in both panels represent the standard error of the predicted probabilities.

Discussion

This study set out to examine the distribution of *Lactobacillus plantarum* in colorectal tissues from patients with cancer and from non-cancer controls. In discussing our findings, we consider tissue-specific microbial patterns, demographic influences, and possible clinical implications.

The contribution of *L. plantarum* to colorectal cancer (CRC) has long been debated. Experimental work has often emphasised its anti-inflammatory and anti-tumorigenic activity, but clinical studies have not been consistent, with some showing no protective association. Such variation may be due to strain-level differences, host–microbiota interactions, or tissue-related factors influencing colonisation. By analysing both presence and abundance in different tissue types, our study adds detail to this discussion. Interestingly, we found no major differences in bacterial load between tumour, Adjacent, and Normal tissues. However, presence-based analysis revealed an unexpected result: *L. plantarum* was more frequently detected in tumour samples, despite stable copy number levels across groups. This was surprising, given the widely held view of *L. plantarum* as a probiotic organism. One possible explanation is that the tumour environment supports persistence rather than growth. Tumours are characterized by hypoxia, altered nutrient availability, and localised immune suppression (9, 10). These conditions may allow bacteria to survive without proliferation. *L. plantarum* is also known to form biofilm-like structures and withstand oxidative or inflammatory stress (11, 12), which may enable its retention in malignant tissue. Such persistence could explain the paradoxical enrichment in detection without a rise in bacterial load (13). This pattern contrasts with previous FFPE-based microbiome studies, which have reported increased abundance of pathobionts such as Fusobacterium nucleatum in CRC (14). The distinct behaviour of *L. plantarum* highlights that not all microbes follow the same ecological dynamics in the tumour microenvironment. Another possibility is that tumour hypoxia simply favours survival of facultative anaerobes, including *L. plantarum*, without necessarily implying active adaptation or host interaction.

Age also appeared to influence detection patterns. Individuals aged 60 and above were more likely to harbour *L. plantarum*. This may reflect age-related shifts in the gut microbiome or immune function that allow certain species to persist (15). Sex-specific effects were also observed. In women, *L. plantarum* load was higher in Normal tissues compared with Adjacent tissues. Within the Normal group, both females and older individuals showed higher abundance. Hormonal influences, differences in mucin composition, or other mucosal factors may help explain these patterns (16). However, sample sizes were small in some subgroups, especially the Normal female category (n = 10), so these results should be interpreted with caution. Overall, our findings suggest that presence and abundance capture different aspects of host–microbe interaction. From a clinical perspective, the preferential detection of *L. plantarum* in tumour tissues points to a role as a potential biomarker of tumour-related changes, rather than as an actively proliferating bacterium.

Preclinical studies have shown that *L. plantarum* can reduce inflammation and oxidative stress by mechanisms such as NF-κB downregulation (17, 18). Yet, clinical data remain mixed. For example, one trial found no effect of *L. plantarum* supplementation on CRC recurrence (5), while another study of combined Lactobacillus strains showed minimal impact on tumour markers (19). Such inconsistencies

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may arise from strain variation, dosage, or patient-related factors.

Our results do not suggest that *L. plantarum* proliferates in CRC, but they do indicate selective retention in tumour tissues and in older patients. This supports the view that its clinical significance is context-dependent. Further work is needed to examine its strain-level functions, interactions with host immunity, and possible value in prevention or therapy.

Limitations and Strengths

This study had several strengths. It used qPCR on clinically relevant tissue types (tumour, Adjacent, and Normal), providing targeted insights into the tumour microenvironment. However, limitations should also be noted. DNA degradation from FFPE samples is always a concern, subgroup sizes were limited, and metadata on diet, probiotics, or antibiotic use were not available. These factors restrict interpretation. Larger cohorts and more detailed clinical data are needed to build on our findings.

Conclusion

In conclusion, *L. plantarum* was identified more frequently in tumour tissues compared to adjacent tissues, despite no significant variations in bacterial load. This contradictory pattern implies persistence within the tumour microenvironment rather than active proliferation. Possible mechanisms include immune modulation, hypoxia tolerance, or biofilm formation. These findings emphasise the complex spatial dynamics of gut microbes in CRC. From a public health perspective, monitoring microbial markers such as *L. plantarum* could add value to current screening approaches. Future research should include strain-level analysis, immune profiling, and multi-omics methods, along with prospective cohorts that capture dietary and lifestyle information, to clarify the role of *L. plantarum* in CRC development and progression.

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Ethical Considerations

The study protocol was reviewed and approved by the Ethics Committee of Ilam University of Medical Sciences (IR.MEDILAM.REC.1403.063).

Financial Disclosure

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Competing Interests' Disclosure

The authors declare no conflicts of interest.

Authors' contributions

Conceptualization: PA, Methodology: FK, PA, NAR, BSK, Validation, Software: PA, FK, NAR, BSK, Resources, Formal Analysis: RP, PA, Investigation & Data Curation: FK, Writing – Original Draft Preparation: PA, Writing – Review & Editing: All authors, Visualization: RP, FK, Supervision and Project Administration: PA.

Writing Disclosure

No AI tools were used for writing the main sections of this article.

Data Availability Statement

The data that support the findings of this study will be made available to readers upon reasonable request from the corresponding author.

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