UDC 6.16.34-008.87-06:(616.345+616.348+616.351)]-006.6(477.8)

Colorectal cancer associated changes in gut microbiome of patients from western region of Ukraine

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The emergence, progression, and treatment of colorectal cancer (CRC) both influence and are influenced by the gut microbiome. In this study, we aimed to elucidate differences in the gut microbial composition between patients with colorectal cancer and healthy individuals from the western region of Ukraine. To achieve this, 16S rRNA amplicon metagenomic sequencing was employed. As a result, patients diagnosed with CRC exhibited a significant reduction in gut microbial diversity. This decrease was reflected in a reduced relative abundance of health-promoting bacteria (by 6- to 170-fold), along with an increased abundance of Escherichia coli and Bacteroides thetaiotaomicron (5- and 7-fold, respectively) in the CRC group. At the same time, we were unable to detect typical CRC-associated biomarker bacteria, such as Fusobacterium nucleatum. To evaluate the potential of E. coli abundance as a biomarker, quantitative PCR (qPCR) was applied to quantify E. coli cells in stool samples from healthy individuals and patients with CRC. However, substantial variability in E. coli cell numbers was observed in both groups, rendering this parameter unsuitable for CRC diagnosis. Overall, our data provide an initial insight into CRC-associated alterations of the gut microbiome in patients from the western part of Ukraine.
Key words: microbiome; colorectal cancer; intestine; metagenomic sequencing.

INTRODUCTION

Colorectal cancer (CRC) accounts for nearly 10% of all cancer cases worldwide [1]. It ranks third among cancer types by incidence, following breast and lung cancer. In 2018, 1.8 million new CRC cases were reported globally, and this number has continued to increase, reaching 1.93 million cases in 2022 [2]. At the same time, CRC represents the second leading cause of cancer-related mortality, with 903,000 deaths reported in the same year. A similar trend is observed in Ukraine, where 15,022 new CRC cases and 6,395 confirmed lethal cases were registered in 2024, according to the National Cancer Registry of Ukraine [3]. These data underscore the importance of identifying CRC risk factors and biomarkers suitable for early diagnosis. At the same time, hereditary factors account for only a small proportion of CRC cases, highlighting the substantial contribution of environmental factors to the disease development [4, 5]

One of the major factors shaping the gut environment is the bacterial communities that inhabit it. It is estimated that approximately 10^{11}–10^{13} bacterial cells reside in the human intestine [6]. This large microbial community constitutes an essential component of human physiology, biochemistry, and overall health. The gut microbiome is involved in numerous processes, including nutrition and energy metabolism, production of bioactive molecules, and maturation of the immune system [7].

Consequently, alterations in the intestinal microbiota can have profound effects on colorectal homeostasis and overall human health. Fluctuations in microbial composition and in the abundance of specific bacterial groups in the

human intestine are frequently associated with various diseases [8]. The colorectal microbiota has therefore attracted considerable attention as a potential contributor to CRC development [9]. There is a growing number of evidence indicating that gut bacteria are directly involved in disease incidence, as well as in the modulation of therapeutic responses [10]. At the same time, changes in the relative abundance of specific bacterial species within the intestinal microbiota are being explored as potential biomarkers for the early diagnosis of CRC [11].

CRC-associated microbiota is commonly studied using either shotgun metagenomic sequencing or amplicon-based metagenomic sequencing [12]. These approaches allow comparison of the microbial community composition between healthy individuals and patients with CRC. Metagenomic studies have led to the identification of a pro-carcinogenic core of the CRC-associated microbiome [13]. Across studies, regardless of the age and sex of patients with CRC, an increased abundance of four bacterial species has been consistently reported: Bacteroides fragilis, Escherichia coli, Enterococcus faecalis, and Streptococcus gallolyticus [13, 14].

In addition, several bacterial species have been reported to be specifically associated with CRC, including *Fusobacterium nucleatum, Parvimonas micra, Porphyromonas asaccharolytica, Prevotella intermedia, Alistipes finegoldii*, and T*hermanaerovibrio acidaminovorans* [14–16]. *F. nucleatum*, an oral commensal, is generally considered as a potential biomarker of CRC and a contributing factor in colon carcinogenesis [15]. It is assumed to be directly involved in CRC development by producing the virulence factor protein FadA, inducing a microenvironment that promotes tumour development, and suppressing antitumor immunity. There is also strong evidence that E. coli and B. fragilis contribute to CRC incidence.

Thus, a growing body of data supports the idea that the gut microbiome plays a significant role in cancer emergence, progression, prevention, and treatment. However, it remains unclear whether changes in microbiome composition can be used for cancer prediction and diagnosis using simple and efficient PCR-based methods. In addition, there is increasing evidence that the microbiome composition of healthy individuals and patients with CRC exhibits geographic-specific features related to regional dietary peculiarities [17]. In this study, we aimed to compare the gut microbiome composition of patients with CRC and healthy individuals in order to identify characteristics typical of the population of the western region of Ukraine, with the goal of identifying specific bacterial species that could serve as potential CRC biomarkers.

METHODS

Patients' selection, sample collection, and ethical approval. Six patients with CRC and six age- and sex-matched healthy individuals were selected for metagenomic analysis. Patients from the western region of Ukraine were referred to the Proctology Department of Lviv Regional Hospital (Lviv, Ukraine) during 2024. The CRC group (P-samples) included four men and two women, with ages ranging from 55 to 80 years (median 64.5 years). In all cases, CRC was diagnosed by colonoscopy and confirmed by biopsy followed by histological examination, establishing colorectal adenocarcinoma. All CRC patients were treatment-naïve at the time of sampling.

The control group (C-samples) consisted of three men and three women, aged 48 to 64 years (median 55.6 years). Control participants were selected following a general medical examination and were considered generally healthy, with no symptoms related to gastrointestinal tract disorders. Individuals from both the CRC and control groups were excluded from the study if, within the previous three months, they had consumed commercial probiotics, undergone antibiotic therapy, taken medications potentially affecting the microbiome, suffered from chronic

metabolic diseases (primarily diabetes), or experienced major dietary changes.

Sample collection was performed between January and March 2024. Stool samples were collected at least 14 days after colonoscopy, which is considered the minimum period required for microbiome recovery.

The study was conducted in accordance with the principles of the Helsinki WMA Declaration. All participants provided written informed consent for the collection of biological material for research purposes. Ethical approval was obtained from the Ethics Committee of the Institute of Hereditary Pathology of the National Academy of Medical Sciences of Ukraine (approval No. 78).

Metagenomic DNA isolation, amplicon sequencing and data processing. Metagenomic DNA was extracted using the DNeasy PowerSoil Pro Kit (“Qiagen”, Germany). For each sample, 100 mg of stool material was used. DNA quality was assessed by gel electrophoresis using the MIDI-1 system (“Carl Roth”, Germany) and quantified with the dsDNA BR fluorescent quantification kit (“Denovix”, USA). DNA samples were stored at −80 °C until further use.

The V3-V4 region of the 16S rRNA gene was amplified using the primer pair 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) [18]. PCR amplification was performed on a MasterCycler Gradient thermocycler (“Eppendorf”, Germany) using Q5 DNA polymerase (“NEB”, USA). PCR products were purified with the QIAquick Gel Extraction Kit (“Qiagen”, Germany). Illumina sequencing adapters and barcodes were added by ligation using the NEBNext Ultra II Ligation Module (“NEB”, USA).

Amplicon libraries were sequenced on an Illumina MiSeq platform by Novogene Europe (Germany) in paired-end 2 · 250 bp mode, with approximately 100,000 reads per sample. Raw reads were end-trimmed with Cutadapt v.3.3 and merged using FLASH v.1.2.11, with parameters set to a minimum overlap of 50 bp and a maximum mismatch rate of 5%; sequences exceeding this mismatch threshold were discarded. Chimeric sequences and singletons (sequences present in a single copy) were removed using vsearch v.2.16.0 [19].

Clustering into operational taxonomic units (OTUs) was performed using UPARSE v.7, and taxonomic annotation of OTUs was carried out with QIIME v.1.9.1 [20]. Data visualization was performed in the R environment (v.4.0.3) using the MegaR package [21].

Quantitative real time PCR quantification of E. coli. For E. coli quantification, a larger cohort comprising 25 patients with confirmed CRC and 25 healthy individuals from the western region of Ukraine were selected. All patients with CRC were recruited at the Proctology Department of Lviv Regional Hospital, Lviv, Ukraine. The CRC group included 14 men and 11 women aged 39 to 87 years (median age 66.5 years). In all cases, the diagnosis of colorectal adenocarcinoma was confirmed.

The control group consisted of 10 women and 15 men aged 28 to 78 years (median age 58.1 years). The selection and exclusion criteria described above were applied to both groups. The study was conducted with written informed consent from all participants and was approved by the Ethics Committee of the Institute of Hereditary Pathology of the National Academy of Medical Sciences of Ukraine (approval No. 78).

Stool samples were collected as described for the metagenomic studies. Metagenomic DNA was isolated using the DNeasy PowerSoil Pro Kit (“Qiagen”, Germany) and quantified with the dsDNA BR fluorescent quantification kit (“Denovix”, USA). DNA concentrations were normalized to that of the sample with the lowest concentration (10 ng/µl). Quantitative real-time PCR was performed on a Rotor-Gene RG-6000 2-plex system (“Qiagen”, Germany). Detection and quantification of Escherichia coli were carried out using the YSL-qP-EC-E. coli-100 kit («YouSeq», United Kingdom).

PCR conditions and detection parameters were set according to the manufacturer’s

recommendations. The reaction volume was 25 µl. Data analysis was performed using Rotor-Gene 6000 software ("Qiagen", Germany). Fluorescence signal curves were slope-corrected, and the threshold was set at 0.032, corresponding to the exponential phase of the amplification curve. Threshold cycle (Ct) values were determined automatically for all samples. All collected data passed the outlier removal procedure. The quantification was performed using serial 10-fold dilutions of the E. coli positive control template provided with the YSL-qP-EC-E. coli-100 kit.

RESULTS

Metagenomic amplicon sequencing based analysis of microbial consortia of healthy individuals and patients with colorectal cancer. Fecal samples from 6 healthy individuals (control, C) and 6 patients with diagnosed CRC (patients, P) were subjected to 16S rRNA amplicon sequencing with a depth of approximately 100,000 reads per sample. The characteristics of the obtained sequencing data are presented in Table.

For all samples, at least 100,000 effective reads were obtained after read assembly and removal of chimeric sequences. These reads were suitable for bacterial identification. Two samples, P5 and C5, were distinguished by a higher sequencing depth, with nearly 200,000 effective tags each. The length of assembled sequences exceeded 400 bp in all cases, supporting accurate phylogenetic assignment of operational taxonomic units (OTUs).

Effective tags were further clustered into taxon tags at different taxonomic levels using a 97% sequence identity threshold. As expected, genus level tags were identified with the highest efficiency. Notably, samples P5 and C5 exhibited the lowest number of identified OTUs despite having the highest number of effective reads.

For each sample, a Rank abundance curve was constructed to illustrate the dependence between the number of reads and the identified OTUs. This analysis allows estimation of whether the sequencing depth is sufficient to uncover the majority of bacterial species present in each sample. For all samples, the curves changed from a steep to a more gradual slope at

The number of taxon tags identified for each sample and efficiency of OTUs assignment (control group (C) and patients with CRC (P))

Sample name	Taxon tags	Genus level tags	Species level tags	Unclassified Tag	OTUs	Family OTUs	Genus OTUs	Species OTUs
P1	106478	100626	34690	4	303	44	107	49
P2	142709	139538	52617	0	230	37	88	49
P3	168289	167873	70763	0	183	38	83	41
P4	144874	142693	37060	0	206	37	88	38
P5	211109	211063	115423	12	28	20	25	21
P6	102830	102706	85965	0	141	37	75	45
P group	876289	864499	396518	16	505	54	155	91
C1	155903	153883	83470	0	223	38	96	59
C2	149152	144877	105072	0	256	42	101	52
C3	150243	148249	120939	0	203	34	73	51
C4	139045	134063	59151	0	274	46	115	63
C5	183007	171393	163496	11	42	10	10	9
C6	108793	134520	57772	0	325	40	92	56
C group	886143	886985	589900	11	605	61	169	127

approximately 20,000-30,000 reads, indicating that this number of reads was sufficient to identify most bacteria in the studied samples.

At the same time, samples P5 and C5 exhibited the lowest species diversity even at the maximum sequencing depth. Furthermore, these two samples were dominated by representatives of the genus Enterococcus, accounting for 99% of reads in sample P5 and 87% in sample C5 (Fig. 1). Such a high titer of a single genus may indicate contamination, most likely occurring during sample collection. Therefore, these two samples were excluded from further analysis.

All samples from both study groups (excluding P5 and C5) were pooled and analyzed to identify common features and differences in

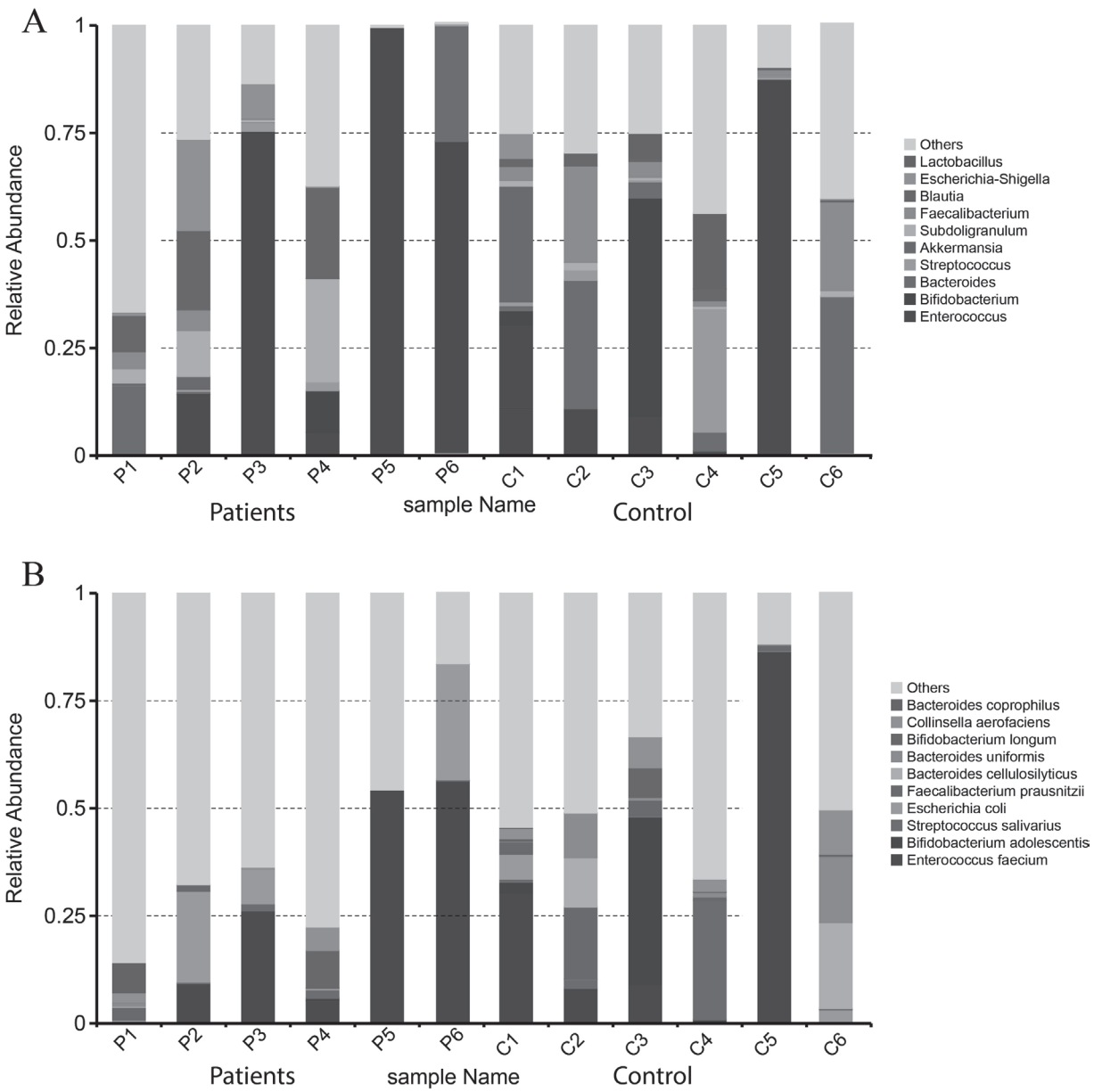

Fig. 1. The barplot showing the relative distribution of 10 most abundant genera (A) and species (B) in the studied microbiome samples

the bacterial community composition of healthy individuals (C group) and patients with CRC (P group). Bacterial diversity within the two groups was assessed using the Shannon and Simpson alpha indices. As shown in Fig. 2A, the microbiome of the control group exhibited greater richness and uniformity, reflected by higher overall alpha indices and their narrower distribution. This indicates greater complexity and stability of gut microbial communities in healthy individuals compared with CRC patients.

In contrast, the distribution of alpha diversity indices was much broader in the CRC group, indicating substantial interindividual variation in microbial composition. Analysis of similarity (ANOSIM) yielded an R value of 0.104, further supporting this observation. The positive R value indicates that overall microbial community composition differed significantly between samples from CRC patients and the control group [22].

Taxonomic analysis of the fecal microbiota of healthy individuals and CRC patients revealed the ten most abundant genera common to both groups: Enterococcus, Bifidobacterium, Bacteroides, Streptococcus, Akkermansia, Subdoligranulum, Faecalibacterium, Blautia, Escherichia, and Lactobacillus (Figure 2B). Bacteria belonging to these genera accounted for more than 65% of all identified taxa. However, their abundances differed significantly between the microbiomes of healthy individuals and CRC patients.

In both groups, Enterococcus was among the dominant genera, with a threefold higher abundance in the P group (33% vs. 10% in the C group). Enterococcus was highly represented in 3 of 5 CRC samples (P2 - 14%, P3 - 74%, and P6 - 72%) (Fig. 2B). In contrast, in the C group, the most abundant genus was Bacteroides (15%), followed by Bifidobacterium (11%) and Faecalibacterium (10%). In CRC patients, the relative abundances of these genera were significantly lower; for example, Bifidobacterium accounted for only 2% of the microbial community in the P group.

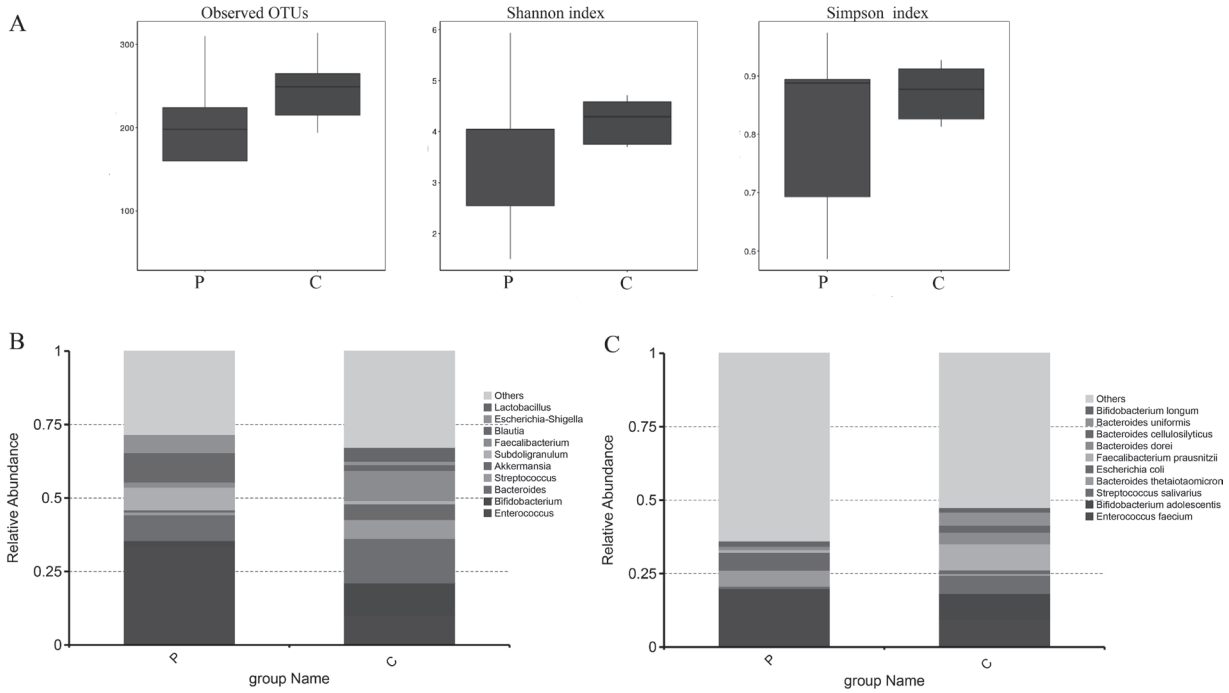

Fig. 2. A. Comparison of the faecal microbiome alpha diversity indices of healthy individuals (Hc) and patients with CRC (Ic). The barplot showing the relative distribution of the 10 most abundant genera (A) and species (B) in the studied microbiome of CRC patients (Ic) and healthy individuals (Hc)

The most dramatic changes were observed for Lactobacillus, whose relative abundance in CRC patients was approximately 170-fold lower than in healthy individuals. Overall, lactic acid-producing bacteria were markedly reduced in the P group (Streptococcus – 6.5-fold decrease; Akkermansia – 7.5-fold decrease). On the other hand, in CRC patients, the abundance of bacteria classified as Subdoligranulum (7.1-fold increase), Blautia (5.2-fold increase) (both belonging to the class Clostridia), and the Escherichia-Shigella group (5.0-fold increase) was higher compared with the microbiome of healthy individuals.

Similar trends were observed at the species level. In all cases, the fecal microbiome of CRC patients was enriched in E. coli (5-fold) and B. thetaiotaomicron (7-fold) (Fig. 2C). In contrast, the abundance of B. adolescentis decreased significantly (44-fold).

The Linear Discriminant Effect Size (LEfSe) analysis was used to identify species and genera that differ significantly between microbiomes and could potentially serve as biomarker taxa [23]. The LEfSe analysis clearly identified E. coli as a potential biomarker species for the P group (Fig. 3). At the same time, none of the CRC biomarker bacteria commonly reported in the literature, including F. nucleatum, were detected in the P samples. This absence may reflect limitations of the applied approach or may indicate CRC-associated microbiota differences related to regional dietary and cultural characteristics [15, 17].

qPCR based quantification of E. coli in the gut microbial consortia of healthy individuals and patients with colorectal cancer. The metagenomic data clearly demonstrated an increased relative abundance of B. thetaiotaomicron and E. coli in fecal samples from CRC patients and highlighted the latter as a potential biomarker species (Fig. 3). E. coli is a common commensal and pathogenic bacteria of the human intestine. The observed differences in its abundance between healthy individuals and patients with CRC make this bacterium an attractive target for testing as a CRC prognostic marker.

To evaluate this idea, fecal samples from 25 healthy individuals and 25 patients with CRC were collected, and metagenomic DNA was isolated. E. coli abundance was quantified using a TaqMan probe specific for the E. coli dnaA gene. As expected from the metagenomic data, the overall absolute abundance of E. coli in samples from CRC patients was higher, with a median of $4.4 \cdot 10^5$ cells per 100 mg of feces in the CRC group compared with $2.3 \cdot 10^5$ cells in the control group (Fig. 4). In 15 of 25 samples from the CRC group, the number of E. coli cells exceeded 10^6 per 100 mg of feces. However, among healthy individuals, E. coli abundance was also relatively high, reaching 10^5-10^6 cells in 14 of the 25 samples analyzed. Moreover, E. coli cell counts were highly heterogeneous in both groups, which precludes drawing a clear conclusion regarding a direct association between fecal E. coli abundance and CRC.

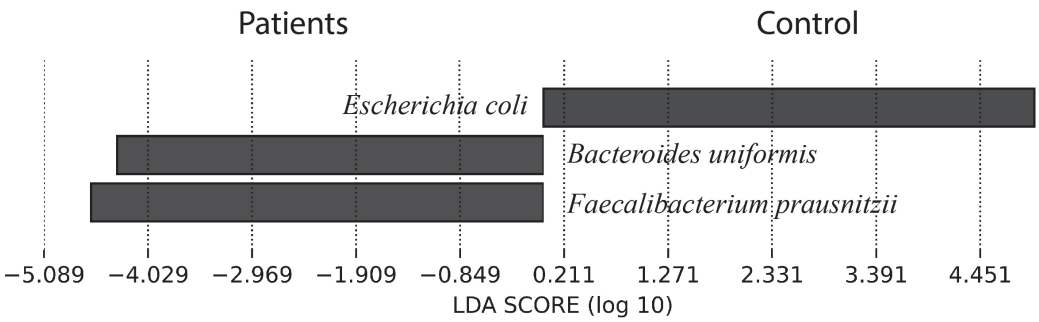

Fig. 3. Histogram of LDA scores between Hc (red) and Ic (green) groups of samples. The LDA score distinguishes biomarker species whose abundance shows significant differences between analysed groups

DISCUSSION

The geographic scope of human microbiome studies is largely limited to Western Europe and North America [24]. At the same time, it is well recognized that local conditions shape the characteristics of intestinal microbial communities in both healthy individuals and patients with CRC, depending on cultural and dietary traditions as well as environmental factors. This limits the direct applicability of data obtained for other populations to Ukraine.

In this study, we present an initial analysis of the gut microbial community composition in healthy individuals and CRC patients from the western region of Ukraine. Despite the relatively small sample size, our data provide preliminary insights into the characteristics of the intestinal microbiome in the studied groups. The results allowed us to identify a group of bacterial taxa whose altered occurrence may be associated with CRC. This group includes representatives of the genera Enterococcus, Subdoligranulum, Blautia, and Escherichia, whose relative abundance was significantly higher in the CRC patients' feces.

While Enterococcus and Escherichia are commonly reported to be associated with CRC, the other two genera, Subdoligranulum and Blautia, appear to have a more geographically specific distribution [16]. Subdoligranulum has been frequently detected in CRC patients from Japan and China [16]. The genus Blautia, which includes several former members of Clostridium and is often considered to have probiotic properties, has not previously been reported to be associated with CRC.

Consistent with other studies, we also observed a significant reduction in lactic acid-producing bacteria in samples from CRC patients, with the most pronounced decrease observed for Lactobacillus species [13]. At the same time, we were unable to detect typical CRC biomarker bacteria such as F. nucleatum, P. micra, and T. acidaminovorans in the analyzed samples [15, 25]. Three additional species commonly reported in CRC patients, P. asaccharolytica, P. intermedia, and A. finegoldii, were present only in negligible amounts.

The identified changes in the composition of microbial consortia associated with CRC could potentially be used as biomarkers of this disease [13]. However, in our study the CRC core microbiome was highly variable from patient to patient, both in taxonomic composition and

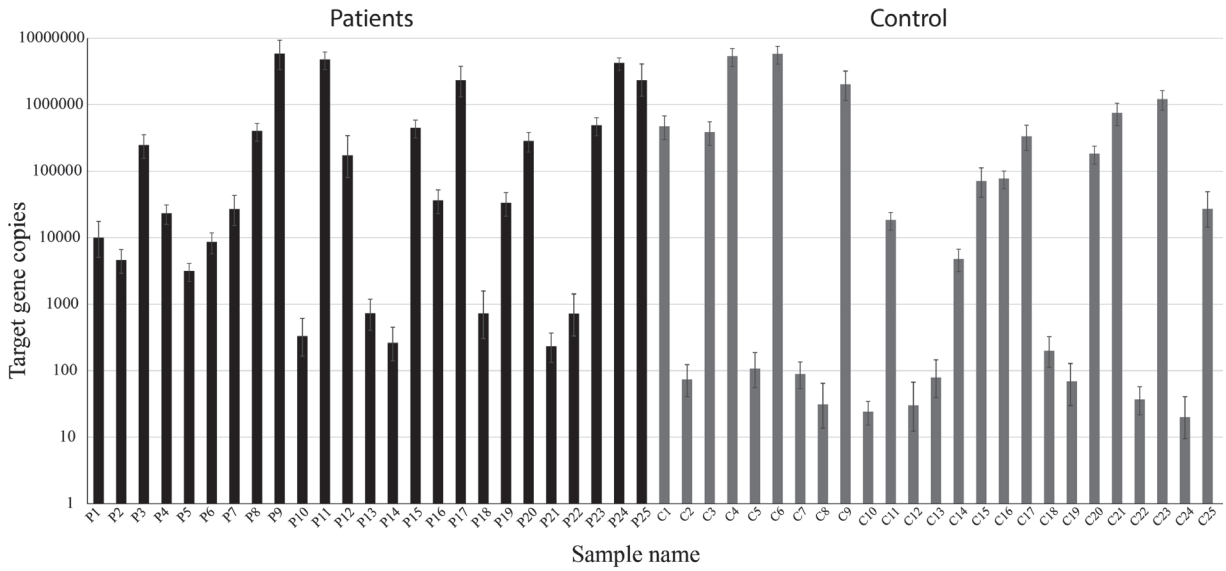

Fig. 4. Absolute quantities of E. coli cells per 100 mg of faeces determined by qPCR in the samples from patients with diagnosed CRC (I) and healthy individuals (logarithmic scale)

in the relative abundance of different taxa. This variability makes monitoring of one or a few species impractical for diagnostic purposes.

Our attempt to use E. coli as a CRC biomarker was unsuccessful due to the large fluctuations in the abundance of this bacterium in samples from different patients. Bacteria of the genera Enterococcus, Subdoligranulum, Blautia and the Escherichia-Shigella group were found to be significantly overrepresented in the microbiome of patients with CRC. At the same time, the relative abundance of health-promoting bacteria, primarily the lactic acid bacteria group, was dramatically reduced in patients with CRC. Complex studies of colorectal microbial consortia that take into account data from different countries and regions may lead to improved CRC diagnosis and prognosis [11].

For this reason, even relatively small datasets that contribute to the global effort to identify CRC-associated microbial biomarkers are of high importance. Further investigations of intestinal microbiome alterations in larger cohorts of CRC patients from across Ukraine may ultimately lead to the identification of biomarker bacteria specific to the Ukrainian population, thereby simplifying the diagnosis of this form of cancer.

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ЗМІНИ МІКРОБІОМУ КИШЕЧНИКА, ПОВ'ЯЗАНІ З КОЛОРЕКТАЛЬНИМ РАКОМ, У ПАЦІЄНТІВ ІЗ ЗАХІДНОГО РЕГІОНУ УКРАЇНИ

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Виникнення, прогресування та лікування колоректального раку (КРР) часто залежать від мікробіому кишечника. Мета нашого дослідження з›ясувати відмінності в мікробному складі мікробіому кишечника пацієнтів з КРР та здорових осіб із західного регіону України. Було використано метагеномне секвенування амплікону 16S рРНК. У пацієнтів з діагнозом КРР спостерігалося значне зниження мікробного різноманіття мікробіому. Це відображалося у зменшенні відносної кількості корисних для здоров'я бактерій (від 6 до 170 разів), також збільшувалася кількості Escherichia coli та Bacteroides thetaiotaomicron (у 5 та 7 разів відповідно). Водночас нам не вдалося ідентифікувати у досліджуваних зразках типові бактерії-біомаркери КРР, такі як Fusobacterium nucleatum. Метод полімеразної реакції у реальному часі застосовували для визначення клітин E. coli (як біомаркера КРР) у зразках калу хворих і здорових осіб. Однак в обох групах спостерігалася значна варіабельність кількості клітин E. coli, що робить цей критерій непридатним для діагностики КРР. Наші результати дають початкове уявлення про зміни в кишковому мікробіомі, що пов'язані з КРР, у пацієнтів із західної частини України.

Ключові слова: колоректальний рак, метагеномний аналіз, мікробом.

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Received 01.12.2025